Alzheimer's Disease: Advances in Etiology, Pathogenesis and Therapeutics
Edited by Khalid Iqbal, Sangram S. Sisodia and Bengt Winblad

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Dedications

This book is dedicated to Drs Eva Braak and Henry M. Wisniewski, two distinguished Alzheimer's disease researchers who have died since the publication of our previous book on Alzheimer's disease, two years ago.

EVA BRAAK, 'OUTSTANDING BUT UNASSUMING'

Eva Braak, after several years of bold fight, died of ovarian cancer on 25 August, 2000 in Frankfurt, Germany.

Eva was one of the most outstanding but unassuming neuroanatomists and neurobiologists of her generation, who made major research contributions to Alzheimer's disease and related disorders. In the 1970s, Eva and her husband, Heiko Braak, developed the special histological techniques to study whole human brain 100 μm sections, and implemented their use for immunocytochemistry and advanced silver impregnation methods to study the histopathology of Alzheimer's disease and related disorders. They successfully employed these techniques, not only to correlate distinctive pigmentation patterns of several types of nerve cells with the features of these cells in Golgi and immunostained sections but also, in 1989, to identify a new, not infrequently occurring tauopathy, argyrophilic grain disease or 'Braak's disease', which is often marked by cognitive impairment. In 1991, they developed a now widely used staging system for the evolution of Alzheimer's disease histopathology called the Braak Staging System. For these path-breaking research achievements, Eva was honored at the Sixth International Conference on Alzheimer's Disease and Related Disorders, held in Amsterdam, The Netherlands, in July 1998. Eva was the first female scientist to receive the award.

Eva obtained her PhD in Biology from the Georg August University, Göttingen, Germany, in 1967, and her postdoctoral training at the Vogt Institute for Brain Research, University of Düsseldorf, Germany, in 1967–1971. In 1971, she moved to the Christian Albrecht University of Kiel, Germany, where she and Heiko started their pioneering studies on the cytoarchitecture of the human brain. In 1979, Eva followed Heiko to the J.W.

Goethe University of Frankfurt, to work in the Department of Clinical Neuroanatomy, where she was appointed to the rank of Associate Professor.

Eva was born on 26 November, 1939 in Schönwald, Germany. She is survived by her husband, Heiko, whom she married in 1973 and who is the Chairman of the Department of Clinical Neuroanatomy at the J.W. Goethe University. They had no children.

We had the privilege of both carrying out collaborative studies with Eva and having her serve on the Scientific Program Committee of the Sixth International Conference on Alzheimer's Disease and Related Disorders. Eva was one of the most meticulous and outstanding scientists, but at the same time a reserved and quiet person. Her death is a truly great loss to the Alzheimer's disease field and we miss her.

HENRY M. WISNIEWSKI, 'PIONEER AND VISIBLE'

Henry M. Wisniewski, a pioneer and one of the most visible Alzheimer's disease researchers, died of chronic kidney disease on 5 September, 1999 at his home on Staten Island, New York. He was born in Luszkowko, Poland, on 27 February, 1931. He received both his medical (MD in 1955) and research degrees (PhD in 1960, and Docent in 1965) from the Gdansk Medical School in Poland.

In 1961, Henry left Poland for postdoctoral training in Canada and the USA. He spent 1 year in the laboratory of Jerzy Olszewski at the Department of Anatomy and Pathology of the Burke Research Institute, University of Toronto. During 1962–1963 he worked as a research fellow in the laboratory of Igor Klatzo at the NIH, NINCDS, Bethesda, where he made the seminal discovery of the induction of neurofibrillary changes by injection of aluminum into rabbit brain.

In 1966, Henry returned to the USA to join as a Research Associate, the research group of Robert D. Terry at the Albert Einstein College of Medicine, Bronx, New York. He rose at the Einstein College of Medicine from Research Associate to the positions of Assistant, Associate and then, in 1974, to full Professor. During his stay at Einstein he made several major research contributions in the fields of demyelinating diseases and brain aging and dementia. His major contributions in the neurobiology of aging and dementia included the structural characterization of plaque pathogenesis, ultrastructure of Alzheimer's disease paired helical filaments and the documentation of the neuropathological changes in experimentally induced conditions and in aged animals. He documented the similarities and differences between the changes in animals and those occurring in the brains of normal aged persons and patients with Alzheimer's disease.

In 1974, Henry left Einstein to take over the position of Director, MRC Demyelinating Diseases Unit, in Newcastle upon Tyne, UK. In collaboration

with Harish Narang at the MRC Unit Henry confirmed the ultrastructure of paired helical filaments by tilt stage microcopy of these filaments in Alzheimer's disease brain and X-ray images of the models of PHF made from wires. After 2 years he returned to the USA as Director of the New York State Institute for Basic Research in Developmental Disabilities, in Staten Island, New York. He held this position from 1976 until his death in 1999. During his 23 years' reign, 1976–1999 the Institute expanded several-fold in terms of scientific staff and became world renowned for research in Alzheimer's disease. At the Institute, along with Jerzy Wegiel, he carried out pioneering studies on the role of microglia in senile plaque formation in Alzheimer's disease; and with Eirene Popovitch, Andrzej Vorbrodt and Richard Carp, he demonstrated the occurrence of Alzheimer's-type plaques and tangles in conditions with developmental disabilities, and the distribution of plaques in prion disease and the animal analog, scrapie in mice.

In 1988, Henry joined us as a Founder Convener of the International Conferences on Alzheimer's Disease and Related Disorders. Although he had strong views of his own on the pathobiology of Alzheimer's disease and related conditions, he recognized and accepted the importance of the presentation of views counter to his own at the conferences.

Henry was most known for being full of ideas and one who loved discussing and even debating science at public forums. He was a very warm, friendly, hospitable and optimistic person. He fought and worked hard and collaborated with a large number of scientists. He published over 700 scientific papers and review articles and edited or co-edited several books on Alzheimer's disease and related disorders.

With Henry's death, neuroscience, especially the Alzheimer's disease research community, has lost a pioneer and a provocative discussant of Alzheimer's disease research. He is survived by his wife, Krystyna Wisniewski, and his son, Thomas Wisniewski.

Khalid Iqbal
Bengt Winblad

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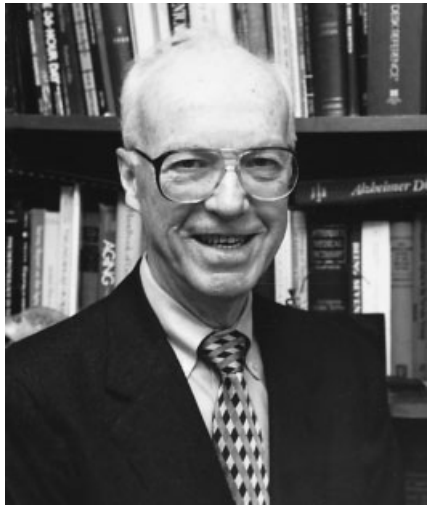
Scientists Honored for Pioneering Research

LIFETIME ACHIEVEMENT AWARDS IN ALZHEIMER'S DISEASE RESEARCH

LEONARD BERG MD

Leonard Berg is Professor Emeritus of Neurology at Washington University School of Medicine, St. Louis, Missouri, USA, where he founded and directed the Alzheimer's Disease Research Center (ADRC) from 1985 to 1997.

A St. Louis native, he completed both BA (1945) and MD (1949) degrees at Washington University. He completed his neurology residency at the Neurological Institute of New York and then held a research appointment at the National Institutes of Health. In 1955 he returned to St. Louis, joined the faculty at the Washington University School of Medicine, and began private practice. Dr Berg was promoted to Clinical Professor in the Department of Neurology in 1972; that same year he initiated the School's Dementia Study Group. He started the University's multidisciplinary Memory and Aging Project with a grant from the National Institute of Mental Health in 1979. In 1984 he was awarded a National Institute on Aging Program Project Grant, 'Healthy Aging and Senile Dementia', and in 1985 was awarded the Alzheimer's Disease Research Center grant, also from the National Institute on Aging.



Dr Berg became a full Professor in the Department of Neurology in 1989, leaving private practice to focus full-time on dementia research until he retired in 1998. He and his colleagues developed the Clinical Dementia Rating (CDR) which is now used world-wide to stage Alzheimer's disease and distinguish between mild Alzheimer's disease and non-demented aging.

Dr Berg has published extensively and given lectures throughout the country and abroad. He served on the Board of Directors of the National Alzheimer's Association for 10 years, chairing its Medical and Scientific Advisory Board during 1991–1995. In 1985 he served as President of the American Board of Psychiatry and Neurology and served on its Board of Directors for 8 years. He was Chairman of the Missouri State Advisory Board on Alzheimer's Disease and Related Disorders during 1988–1995. He had been on the Advisory Panel on Alzheimer's disease for the Department of Health and Human Services since 1993.

Washington University presented Dr Berg with a Distinguished Alumnus Award in 1983, an Alumni/Faculty Award from the Medical Alumni Association in 1989, and the Second Century Award for his long-term commitment and dedication to the medical school in 1999. A Distinguished Alumni Scholarship was established in his honor in 1995. The St. Louis Chapter of the Alzheimer's Association honored him with their 1989 Public Service Award. In 1998, the St. Louis Academy of Science honored Dr Berg with the Peter H. Raven Lifetime Award.

INGE GRUNDKE-IQBAL PHD

Inge Grundke-Iqbal is Head of the Neuroimmunology Laboratory, New York State Institute for Basic Research in Developmental Disabilities, Staten Island, New York, USA. She received her PhD in Biology and Biochemistry from Georg August University, Göttingen, Germany, in 1967 and held postdoctoral fellowships at the Max-Planck Institute for Immunobiology, Freiburg, Germany, the University of Michigan, Ann Arbor, New York University, and Albert Einstein College of Medicine, New York. Dr Grundke-Iqbal was Assistant Professor of Pathology (Neuropathology) in 1974–1977 at Albert Einstein College of Medicine and then in 1977 she took up her present position (equivalent to the rank of full professor) at the Institute for Basic Research in Staten Island.

In 1979, Dr Grundke-Iqbal raised the first antibody to Alzheimer's disease paired helical filaments and discovered the cross-reactivity of these aberrant filaments with brain microtubules. Recognizing the importance of this cross-reactivity, Dr Grundke-Iqbal pursued a systematic analysis of the microtubule

proteins and in 1986 she identified the microtubule-associated protein tau as the major protein subunit of Alzheimer's paired helical filaments. During the same year, she discovered that tau in paired helical filaments and also in brain cytosol from Alzheimer's disease was abnormally hyperphosphorylated. The abnormal hyperphosphorylation of tau is not only one of the most important findings made in the Alzheimer's disease field but also opened a major new area of research. Subsequent studies by Dr Grundke-Iqbal and her colleagues demonstrated that the levels of tau in abnormally hyperphosphorylated form are increased several-fold in Alzheimer's disease, and that the mechanism of the neurofibrillary degeneration, a key lesion in Alzheimer's disease, involves the sequestration of normal microtubule-associated proteins and disassembly of microtubules by the abnormally hyperphosphorylated tau.

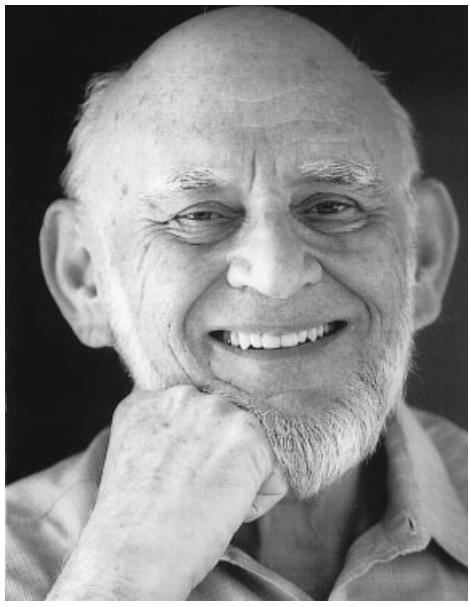
She and her colleagues also discovered: (1) that the abnormal phosphorylation of tau precedes its polymerization into paired helical filaments and incorporation of ubiquitin; (2) that the levels of conjugated ubiquitin are elevated in both the brains and cerebrospinal fluids of patients with Alzheimer's disease; (3) that there is a significant pool of soluble abnormally phosphorylated tau in Alzheimer's disease brain; (4) that the abnormally phosphorylated tau is three- to four-fold more phosphorylated than the normal brain tau; and (5) that by dephosphorylation *in vitro*, paired helical filaments dissociate, the tau released has normal microtubule assembly-promoting activity, and protein phosphatase-2A is a major regulator of the phosphorylation of tau.

Dr Grundke-Iqbal has been a member of the International Scientific Advisory Committee of the International Conferences on Alzheimer's Disease and Related Disorders since their inception. In 1994 she received the New York State Governor's Award.

ROBERT KATZMAN

Robert Katzman, a native of Denver, Colorado, a graduate of the University of Chicago and Harvard Medical School, trained in Neurology at the Neurological Institute, Columbia Presbyterian Medical School. After joining the faculty of the Einstein College of Medicine, he served as Professor and Chair of Neurology there from 1964 to 1984, then as Chair of Neurosciences and founding director of the Alzheimer Research Center at the University of California at San Diego from 1984 to 1990. He continues to work as Research Professor of Neurosciences at the University of California at San Diego.

Although Dr Katzman's initial entry into Alzheimer's disease (AD) work was a neurochemical analysis of the ganglioside loss in the AD brain, carried out with Dr Kinuko Suzuki in 1965, and a recent finding with the late Dr Saitoh was that of an LRP polymorphism that modifies the risk of AD, his major interests have been in the clinical, epidemiological and public health aspects of AD. His experience in the early 1970s as a clinician working with



older individuals with dementia, confirmed in his mind the frequency of AD in the elderly. In 1976 he marshaled evidence that AD was the fourth most common cause of death in the USA. His editorial on this topic led to the first NIH-sponsored symposium on AD in 1977, chaired by Drs Katzman, Terry and Bick. In 1979, as an investigator and as a family member, he helped found the Alzheimer Disease and Related Disorders Association (now the Alzheimer's Association), co-chaired its first Scientific Advisory Board and participated in the 1984 NINCDS/ADRDA consensus conference on diagnostic criteria for AD.

In 1980, Dr Katzman organized a longitudinal study of aging in the Bronx that showed the high incidence of AD in the very elderly. The Shanghai Survey of Dementia documented the high prevalence of dementia in those without education, interpreted as protection against dementia by increased brain reserve.

Dr Katzman is a member of the Institute of Medicine and he was a co-recipient of the Potamkin Prize for Alzheimer's Disease Research in 1992. A recent contribution, together with Dr Karen Bick, is the oral history, *Alzheimer Disease: The Changing View*, a book describing the events between 1960 and 1980 that led to recognition of the public health importance of AD.

GEORGE M. MARTIN

George M. Martin is Professor of Pathology, Adjunct Professor of Genetics and Associate Director of the Alzheimer's Disease Research Center, University of Washington. He received his MD from the University of Washington and has been a member of its faculty since 1957. As the Founding Director of the University of Washington Alzheimer Disease Research Center, he assembled a team of investigators to carry out a linkage analysis of familial Alzheimer's disease, an effort that led to the assignment of the commonest form to chromosome 14 and to the mapping and positional cloning of a related locus on chromosome 1. His laboratory then developed the first presenilin 1 'knockin' mutation in a mouse model and, together with

the Mattson laboratory, elucidated an unusual sensitivity of neuronal cells from such animals to excitotoxic and beta amyloid (Aβ)-induced injury. His group described a strong interaction between the Aβ precursor protein and a human adaptor protein, known as FE65. This was followed by the discovery of a polymorphic form of FE65 that is associated with increased susceptibility to late onset Alzheimer's disease (LOAD). He has published evidence that two isoforms of FE65 are upregulated in relatively unaffected regions of the brain of patients with LOAD, and has emphasized the importance of such investigations as a means of detecting the earliest events in pathogenesis.

Most of his other lines of research have also used genetics to further our understanding of the pathobiology of human aging. Examples include the elucidation of the dominant nature of replicatively senescent cells, the biochemical genetic basis of the Werner syndrome, the first determinations of the rates of accumulation of somatic mutations in aging human epithelial cells, and the first estimates of the number of human genes involved in specific aspects of the senescent phenotype, including adult-onset dementias.

Dr Martin is a Senior Member of the Institute of Medicine of the National Academy of Sciences. He has served on the National Advisory Council and the Board of Scientific Counselors of the National Institute on Aging, and currently serves on the Scientific Advisory Boards of the Ellison Medical Foundation and the Buck Center. He is President-elect of the American Federation for Aging Research.

HENRY M. WISNIEWSKI AWARD FOR LIFETIME ACHIEVEMENT IN ALZHEIMER'S DISEASE RESEARCH

BLAS FRANGIONE MD, PHD

Dr Frangione has contributed to our present-day knowledge of cerebral amyloid and its role in Alzheimer's disease (AD) and aging. Early in 1980, he developed a method for extracting cerebral amyloid from leptomeninges, and he deserves credit for the initial discovery of the role of mutated genes in inherited neurodegenerative disorders associated with cerebrovascular fibrillogenesis.

Working with a vascular variant of familial AD (FAD)-like syndrome in Dutch patients, he showed that the Dutch amyloid was similar to beta amyloid (Aβ) extracted from AD patients. He also showed that the same protein was deposited in asymptomatic elderly people. Dr Frangione then postulated that: (1) in FAD genetic mutations are responsible for different phenotypic expressions; and that (2) the precursor of amyloid (as in different types of systemic and localized amyloidosis) is present in the circulation. Dr Frangione demonstrated in 1990 that in Dutch patients, the gene coding for the amyloid precursor protein (APP) contained a point mutation. This work provided an enormous stimulus to look for mutations in other amyloidosis and established a rationale for examination of mutations of the APP gene in pedigrees of FAD.

Dr Frangione proposed that a conformation change occurs in sA due to point mutations and/or a post-translational modification leading to aggregation and fibrillation. This conformational/aggregational transformation could also result from changes in pH, local concentration, lack of clearance and interaction with other molecules, which he terms 'pathological chaperones', and he identified apolipoprotein E (apoE) as one of these long before linkage analysis demonstrated an association between apoE and late onset FAD and sporadic AD.

Dr Frangione began his research career as a physician in Buenos Aires. He worked at New York University Medical Center before going to Cambridge, UK, where he received his PhD. He also worked at Oxford University and at the Imperial Cancer Research Fund in London before returning to New York University.

He has been recognized by the National Institutes of Health with a 1989 MERIT Award for his studies of systemic amyloidosis, and a 1992 LEAD Award for his work on AD. In 1993, he received the Potamkin Prize for Alzheimer's Disease Research. In 1994, he was awarded the Metropolitan Life Award for Medical Research and, in 1997, the National Institutes of Aging recognized his work with a second MERIT Award. In 1999 he was awarded the Doctor of Science, Honoris Causa, from the University of Buenos Aires in recognition of his contribution to science.

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Preface

Alzheimer's disease has no geographical, racial or economic boundaries. It is the major cause of dementia in middle- to old-age individuals throughout the world. With the increase in human life span, the prevalence of this age-associated disease continues to grow. The importance and the scientific challenge of this disease have been well recognized by the neuroscience research community. New important research findings have been made on the biology, diagnosis and treatment of the disease since the publication of our previous book on Alzheimer's disease, 2 years ago. In the present book the scientists studying the disease have described the advances made by them and others in epidemiology and genetic risk factors, disease mechanisms, early diagnosis and in therapeutic opportunities and new therapeutic drugs. The articles in this book were selected from over 1300 papers presented on the latest findings in the field at the 7th International Conference on Alzheimer's Disease and Related Disorders, held as the major component of the World Alzheimer's Congress 2000 in Washington, DC, in July 2000. We believe this volume will be useful, both to basic scientists and to clinicians interested in Alzheimer's disease and related disorders.

Khalid Iqbal
Sangram S. Sisodia
Bengt Winblad

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Acknowledgments

We are most grateful to the chapter contributors for prompt submission of their articles and to the following members of the Scientific Program Committee for helping us select the contributors from over 1300 papers presented at the 7th International Conference on Alzheimer's Disease and Related Disorders held as a major component of the World Alzheimer Congress 2000, Washington, DC: Professors Marilyn S. Albert, David R. Borchelt, Steven T. DeKosky, Rachelle Doody, Laura Fratiglioni, Bernardino Ghetti, Charlie Glabe, Claudia Kawas, Edward Koo, Eliezer Masliah, Richard Mayeux, Marsel Mesulam, John C. Morris, Ronald C. Petersen, Donald Price, Gerald Schellenberg, Rudolph Tanzi, Leon Thal, John Q. Trojanowski, and Bruce Yankner.

Our special thanks to Janet Biegelson at the New York State Institute for Basic Research, Staten Island, New York, for her secretarial assistance in corresponding with all the authors and in all stages of the work involved with the production of the book in the Senior Editor's office.

Financial support for the book was generously provided by Janssen Pharmaceutica and Research Foundation.

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Note: Page references in *italics* refer to Figures; those in **bold** refer to Tables

Index compiled by Annette Musker

PLATE I

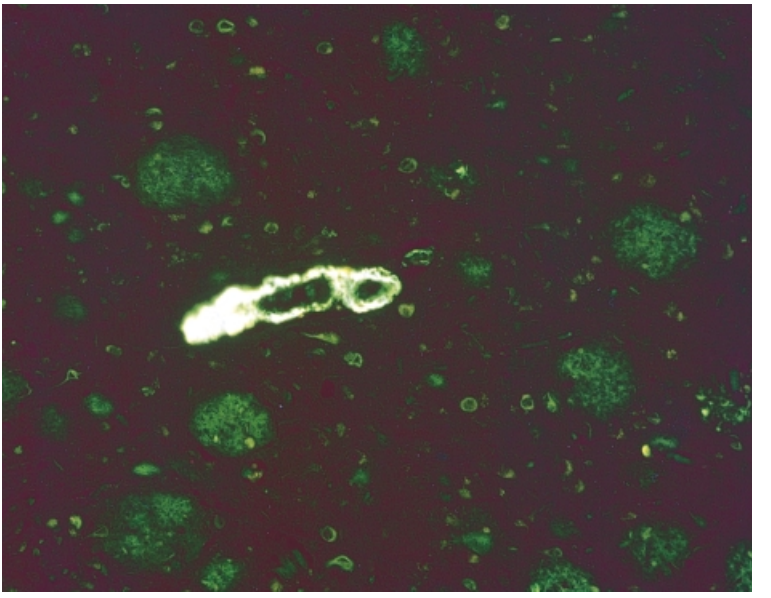

Figure 6.2 Leptomeningeal and parenchymal arteries are brightly fluorescent. Thioflavin S method (x 36)

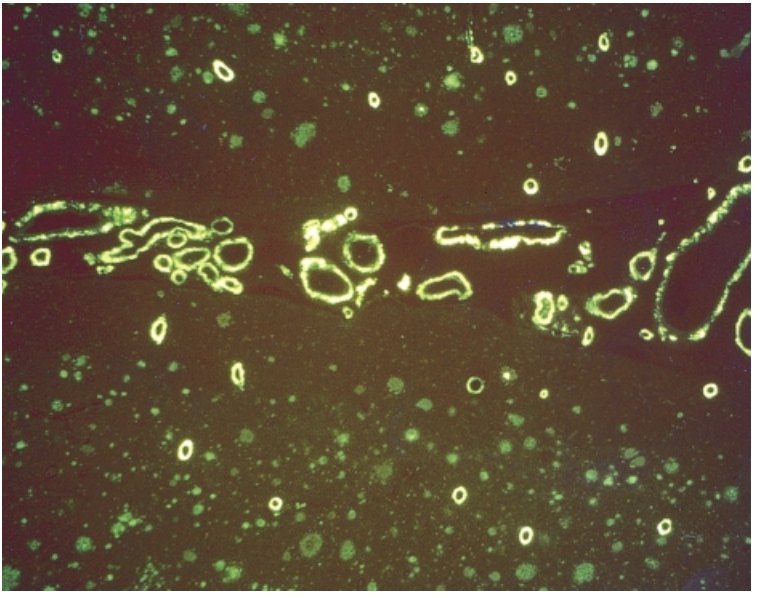

Figure 6.3 'Cotton wool' plaques and neurofibrillary tangles are numerous in the neocortex. Thioflavin S method (x 147)

PLATE II

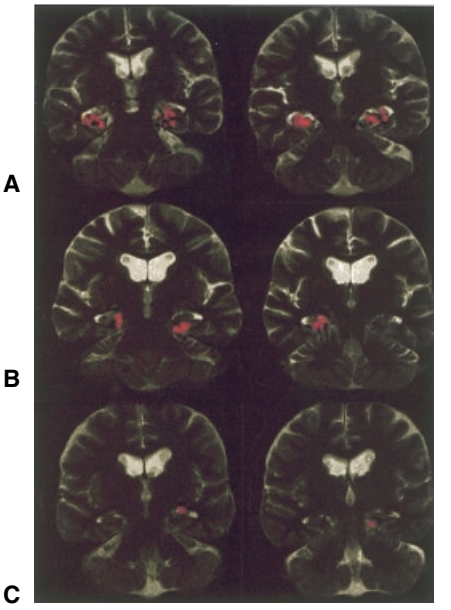

Figure 17.1 Representative examples from fMRI analyses. Two posterior sections acquired from the six section volumes are shown. Only pixels that overlie the hippocampal formation were selected for analysis. Those pixels whose signal intensity significantly increased in association with the presentation of faces are color-coded in red. **A** A 79-year-old man with normal memory. **B** A 74-year-old woman with memory decline and normal entorhinal activation. **C** A 76-year-old man with memory decline and diminished entorhinal activation

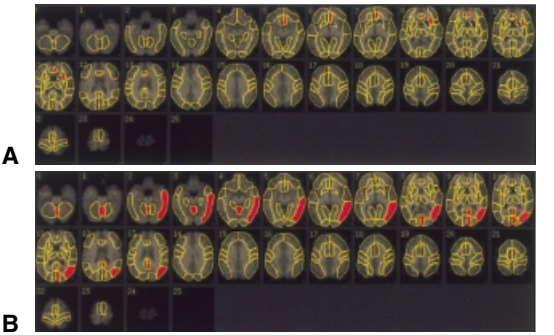

Figure 17.2 Networks expressed by the healthy elderly and Alzheimer's disease subjects. Within each group, differential expression of these networks correlated with study list size on the activation task. Weights for each region's participation in the topography have been overlaid on standard, Tailarach transformed axial MRI sections, with positive weights indicated in red and negative weights indicated in blue. **A** The healthy elders' network. Higher study list size (SLS) was associated with increased activation in left anterior cingulate and anterior insula and decreased activation of the left basal ganglia. **B** Alzheimer's disease network. Higher SLS was associated with the increased activation of the left posterior temporal cortex, calcarine cortex, posterior cingulate, and the vermis

PLATE III

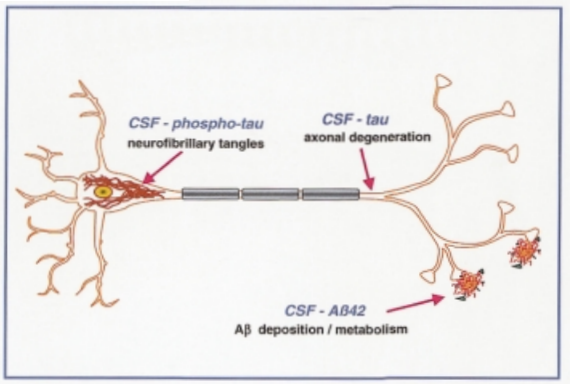

Figure 25.1 Neuron with neurofibrillary tangles in the cytoplasm and two senile (neuritic) plaques by the synapses. Potential CSF markers for Alzheimer's disease, and the pathogenic process they possibly reflect, are given

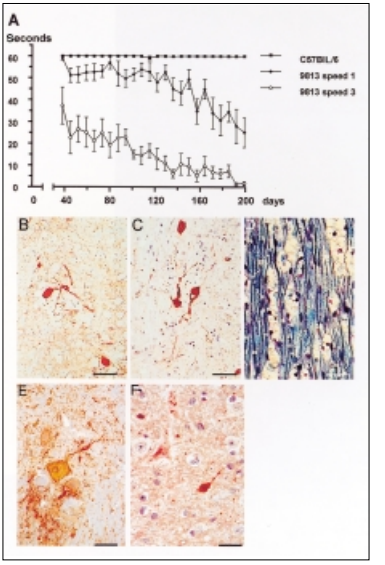

Figure 61.1 Lewy-like pathology and degeneration in the transgenic mouse spinal cord and neuromuscular areas. (A) Rotating rod perfomance of transgenic mice. Starting at age 40 days and up to age 200 days, tansgenic (n = 7) and C57BL/6 non-transgenic littermate male mice (n = 12) were tested for endurance to stay on the rotating rod. The mice were tested weekly, and their performance is shown for two different rotation speeds, speed 1 (12 rpm) and speed 3 (36 rpm). C57BL/6 mice showed maximum endurance performance (60 s) at both speeds. (B, E) α-Synuclein staining of motor neurons in an A53T α-synuclein mutant (B) and wild-type α-synuclein (E) transgenic mouse spinal cord paraffin section with the human selective antibody LB509. (C, F) Anti-Ubiquitin stained motor neurons in spinal cord sections from A53T α-synuclein mutant (C) and wild-type α-synuclein (F) transgenic mice. (D) Holmes-Luxol stained spinal root showing axonal degeneration with breakdown and segmentation of myelin into ellipsoids ('digestive chambers'). Scale bars: (B, C) 50 μm; (D – F) 20 μm

PLATE IV

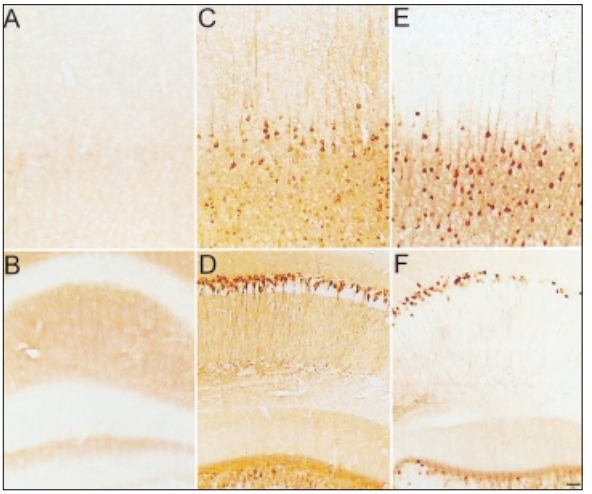

Figure 61.2 α-Synuclein protein expression in the transgenic mouse brain. α-Synuclein immunostaining in the neocortex (A, C, E) and hippocampal CA1 region (B, D, F) on free-floating brain sections of transgenic mice expressing mutated A53T (C, D) and wild-type α-synuclein (E, F), as compared to the respective region in a non-transgenic C57BL/6 mouse (A, B). Scale bar: 50 μm

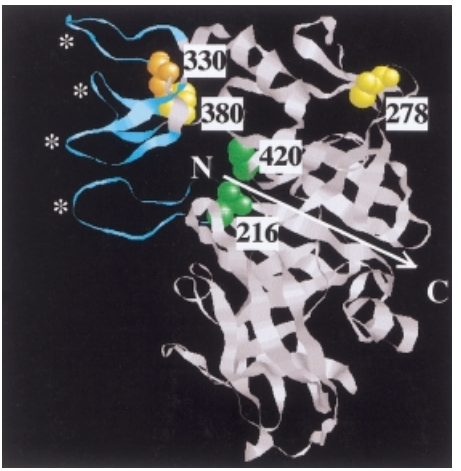

Figure 74.6 Homology modeled structure of ß-secretase. The homology modeled structure for ß-secretase visualized using RasMol software. The position of the active-site cleft is display by an arrow with the positions of the N- and C-termini of the substrate indicated. The inserted sequences in the aspartic acid homology domain of ß-secretase showing no homology to pepsin family members are colored aqua and marked by asterisks. A ribbon model of ß-secretase is shown with cysteine residues displayed as space-filled atoms. Cysteine residues that are homologous to conserved cysteine residues in the pepsin family membes are shown in yellow or orange. Novel cysteines showing no homology to pepsin family members are shown in green. Numerical positions of the cysteine residues in ß-secretase are indicated

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I Epidemiology and Risk Factors

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1 The Transition from Normal Functioning to Dementia in the Aging Population

LAURA FRATIGLIONI, BRENT SMALL, BENGT WINBLAD AND LARS BÄCKMAN

INTRODUCTION

During the last few decades a substantial effort has been made to understand better the etiology and natural history of dementia, which has led to significant results. However, only in recent years has the availability of follow-up data from several community-based studies provided us with the opportunity to explore what happens just before the manifestation of the first dementia symptoms and before the diagnosis of dementia. Most of the studies focused on Alzheimer's disease (AD). The preclinical phase of AD may be considered as the transition from normal aging to dementia.

COGNITIVE FUNCTIONING AND AGING

Bäckman et al. (1999) in a recent review on cognitive functioning in old age concluded that there is a rather global deterioration of cognitive functioning in old age. For some cognitive abilities (e.g. fluid intelligence, episodic memory, and working memory), the onset of decline occurs relatively early and continues into late life; for other abilities (e.g. crystallized intelligence and semantic memory), noticeable decline may not be evident until late adulthood. However, from the mid-70s of life and onward the magnitude of age-related decline appears to be quite similar across different forms of memory and cognition, with only two exceptions: primary memory and implicit memory. For these forms of memory, relative preservation appears to be the empirical rule, even in very old age. Given this scenario, the main challenge is to understand which specific changes in cognition are involved in the initial phases of AD, and to describe the timing and evolution of these changes.

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TIME BEFORE DEMENTIA DIAGNOSIS

Due to the insidious onset of AD, the time of diagnosis is the only definite point in the initial phase of the disease. The time of diagnosis may be defined as the time when diagnostic criteria for dementia and AD are fulfilled. This time may be affected by several factors, including cultural, social, and medical conditions.

The studies conducted so far have focused on early clinical phases, which correspond approximately to 1–2 years before or after diagnosis, and on the preclinical phase, defined as occurring more than 2 years before diagnosis. This review summarizes the results from numerous studies aimed at describing cognitive performance in subjects before AD diagnosis.

COGNITIVE CHANGES IN THE INITIAL PHASES OF DEMENTIA

Even early in the disease process, AD patients typically exhibit deficits across multiple cognitive domains, including attention, memory, verbal ability, visuospatial skill, problem solving, and reasoning (Small et al., 1998). However, it is clear that the largest and most consistent cognitive deficit in early clinical AD is seen within the domain of memory. No form of memory is completely spared from the negative effects of AD (Almqvist, 1996).

Despite the involvement of all forms of memory even in the early phase and mild form of AD, numerous studies have consistently reported the presence of marked deficits in episodic memory in contrast with mild-moderate involvement of other cognitive functions (Almqvist et al., 1996). Episodic memory tasks, which involve conscious retrieval of information acquired in a particular place and a particular time, emerged as the tasks distinguishing most effectively between the normal old person and the patient with mild AD (Herlitz et al., 1995).

CHARACTERIZATION AND EVOLUTION OF COGNITIVE DEFICITS IN PRECLINICAL AD

Most of the studies addressing this topic followed a group of non-demented older persons across a time period of 2–4 years, to detect newly developed AD (incident cases). The cognitive performance at baseline in these incident AD cases was compared to the test results of those subjects who remained non-demented during the risk period. Numerous investigations are based on 2–4 years of follow-up. Few studies have explored a longer period, and even fewer reports tried to characterize the nature of the differences in cognitive performance between preclinical AD and non-demented subjects. Finally, only one study described the course of cognitive deficits in the preclinical

phase of AD. The main findings from these three research lines will be discussed here.

COGNITIVE DEFICITS 2–4 YEARS BEFORE AD DIAGNOSIS

All studies consistently report a clear lowering of cognitive performance among incident AD cases some years before actual diagnosis. Such preclinical deficits in AD have been demonstrated in studies employing global indicators of cognitive performance (Aronson et al., 1990; Yoshitake et al., 1995; Small et al., 1997a). Deficits in specific cognitive abilities have been associated with the subsequent development of AD in one or more studies (Table 1.1). As is true in early clinical AD, the largest and most consistent cognitive deficits are observed within the domain of episodic memory. Small et al. (1997b) found that subjects who would develop AD across three-year follow-up had lower cognitive performance in a variety of tasks, including those assessing not only episodic memory, but also visuoperceptive skill, visuoconstructive skill, letter fluency, and category fluency. However, when all measures were entered into a logistic regression analysis, the tasks assessing episodic memory were found to dominate the prediction model.

The episodic memory deficit in persons who will develop AD appears to be highly generalizable across different materials and testing conditions (Bäckman et al., 1999). However, it may still be the case that some tasks are more effective than others in predicting future development of AD. Bäckman and Small (1998) examined the effect of cognitive support on the results of memory tasks in preclinical AD. Four different tasks that varied systematically with regard to the degree of cognitive support (more study time, organizability, and semantic retrieval cues) were administered. As expected, the incident AD cases showed a clear performance deficit at baseline (three years before diagnosis) but the same qualitative pattern as the controls, with performance gradually increasing across increasing levels of cognitive support. However, at the time of diagnosis, the incident AD cases failed to benefit from more study time and organizability in the free recall test,

Table 1.1. Cognitive deficits in preclinical Alzheimer's disease 2–4 years before diagnosis

Impaired function	Reference
Abstract reasoning	Jacobs et al., 1995; Fabrigoule et al., 1996
Episodic memory	Small et al., 1997b; Hodges et al., 1998; Grober et al., 2000
New learning	Grober and Kawas, 1997
Psychomotor speed	Masur et al., 1994
Verbal ability	Small et al., 1997b; Dartigues et al., 1997; Howieson et al., 1997
Visuospatial skill	Small et al., 1997b

whereas performance gains were observed only in the most supportive condition. The authors suggested that a general impairment of episodic memory may precede reductions in cognitive reserve capacity in the early development of AD.

COGNITIVE DEFICITS 5+ YEARS BEFORE AD DIAGNOSIS

Table 1.2 reports the most recent studies that examined cognitive performance in preclinical AD over a period of more than 5 years before diagnosis. Using the data from the Framingham study, Elias et al. (2000) extended the surveillance period from 13 to 22 years for the subjects initially followed up by Linn et al. (1995). Due to this extension, test results of subjects developing AD at least ten years later were compared with the results from non-demented subjects. Lower scores for measures of new learning, recall, retention, and abstract reasoning obtained during a dementia-free period were associated with the development of AD. Lower scores for measures of abstract reasoning and retention predicted AD after a dementia-free period of ten years.

In the Kungsholmen Project, persons were assessed on three occasions over six years. On the last occasions, some individuals were diagnosed with AD (incident cases), although the entire study sample was non-demented at the first two measurement times. The cognitive assessment of the whole cohort was restricted to the Mini-Mental State Examination (MMSE) (Folstein et al., 1975), which is a global screening instrument for cognitive dysfunction. This instrument assesses multiple cognitive abilities, including orientation as to time and place, immediate and delayed word recall, naming, verbal repetition, reading, writing, and spatial ability. At both preclinical measurement points (six and three years before diagnosis), the incident AD cases showed deficits on one item only, namely delayed recall (Small et al., 2000). This result supports the view that the earliest cognitive deficits in AD are seen within the domain of episodic memory.

Table 1.2. Cognitive deficits in preclinical Alzheimer's disease 5 years or more before diagnosis

	Impaired function	Time before diagnosis
The Framingham Study Linn et al., 1995; Elias et al., 2000	Abstract reasoning Attention Retention of information Verbal memory	At least 5 years
The Kungsholmen Project Small et al., 2000; Bäckman et al., 2001	Delayed recall Free recall Recognition	4–7 years

A more recent study from the same project (Bäckman et al., 2001) reported the results from a comprehensive neuropsychological battery administered to a sample of the entire Kungsholmen Project's cohort. This sample was examined three times during a follow-up period of six years. The incident AD cases already showed clear deficits in the episodic memory tests (free recall and recognition) six years before diagnosis.

COURSE OF THE COGNITIVE IMPAIRMENT IN PRECLINICAL AD

Two studies from the Kungsholmen Project have addressed the issue of cognitive impairment in preclinical AD (Small et al., 2000; Bäckman et al., 2001). When the MMSE subitems were used in the whole cohort, the subjects in a preclinical phase of AD showed deficits in delayed recall function at both preclinical measurement points, but they did not exhibit selective decline between the six-year and the three-year point before diagnosis compared to the non-demented subjects. However during the last three years preceding the diagnosis, a precipitous decline was detected in delayed recall and across most cognitive domains assessed in the MMSE.

When results from the neuropsychological battery were examined during the six years of follow-up (Bäckman et al., 2001), performance in recognition and recall tests of subjects in preclinical AD, although lower than in non-demented subjects, remained pretty stable during the first three years of follow-up. The data suggest that the episodic memory deficit in preclinical AD is characterized by an early onset followed by relative stability, at least until a few years before a diagnosis may be rendered.

IDENTIFICATION OF SUBJECTS WITH PRECLINICAL AD

The results from the Kungsholmen Project suggest that AD is associated with a long preclinical period during which episodic memory deficits are detectable, although accelerated decline in performance may not be seen until the time period preceding diagnosis. Three main questions arise from these data:

1. Are the deficits in episodic memory detected in subjects who later develop AD present throughout their life, or was there a time when these subjects performed as well as people who do not develop AD?
2. What factors determine or initiate the dramatic decline in cognitive performances some years before AD diagnosis? Could a stroke (Snowdon et al., 1997) or a change in social environment (Fratiglioni et al., 2000) be precipitating factors?

Table 1.3. Different markers of subjects in preclinical AD phase

Markers	Significant references
Cognitive assessment	(Discussed in this review)
Self and/or informant reports	Tierney et al., 1996; Schofield et al., 1997; Daly et al., 2000
Non-cognitive symptoms	Berger et al., 1999
Paraclinical examination	Killiany et al., 2000
Precipitating factors	Snowdon et al., 1997; Fratiglioni et al., 2000

3. Is it possible to identify these subjects in the preclinical phase and to delineate a feasible preventive strategy?

To answer these questions further studies are needed. Specifically, the contribution of different markers of preclinical AD needs to be clarified and the information derived from different clinical aspects integrated (Table 1.3).

CONCLUSIONS

- Episodic memory deficits are dominant in both early clinical and preclinical phases of AD.
- These deficits in preclinical AD are generalizable across various dimensions of episodic memory.
- Preclinical episodic memory deficits may be seen several years (six to ten) before diagnosis, but the degree of impairment appears stable up until the period more closely preceding diagnosis.
- In addition to cognitive assessment, other markers such as family reports, depressive symptoms, and paraclinical examinations may help in identifying subjects in the preclinical phase of AD.
- Further studies are necessary to identify possible precipitating factors.

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2 Epidemiology of Alzheimer's Disease and Dementia: Advances and Challenges

ROBERT KATZMAN

INTRODUCTION

A defining event of the twentieth century was the dramatic increase in life expectancy at birth—an increase of over 50%, from under age 50 in 1900 to over age 76 in 2000. With the consequent aging of our populations, age-associated diseases have come to the forefront. Of these, Alzheimer's disease (AD) and related dementing disorders are the most age-dependent. It is anomalous that of all of the risk factors for AD, the biological basis of age is the least understood.

During the past two decades the extent of the public health impact of AD and related dementias has been defined. Jorm et al. demonstrated the consistency of the exponential rise of dementia with age in a quantitative integration of 27 prevalence studies dating back to 1945 (Jorm et al., 1987). Prevalence doubles with each five years of age, as shown by the regression line in Figure 2.1. In this figure, the log of age-specific dementia prevalence is plotted against age. The age-specific prevalence rates obtained in the Jorm meta-analysis, the Eurodem meta-analysis of six later prevalence studies (Hofman et al., 1991) and the Canadian Study of Health and Aging (1994)—whose subjects included 9008 community subjects and 1255 institutional residents in nine provinces—are similar, as shown in Figure 2.1. Katzman and Fox (1999), using the United Nations 1998 population projections (middle series) and prevalence rates based upon the Canadian Study of Health and Aging, adjusted for the changing structure in the age distribution of populations over time, projected that the number of cases of dementia in the developed world would rise from 13.5 million in 2000, to 21.2 million in 2025, and 36.7 million in 2050. Most of the increase is attributable to the aging of the population that will occur in the next 50 years. This extraordinary increase would be mitigated if we learned how to delay the onset of AD. Khatchaturian

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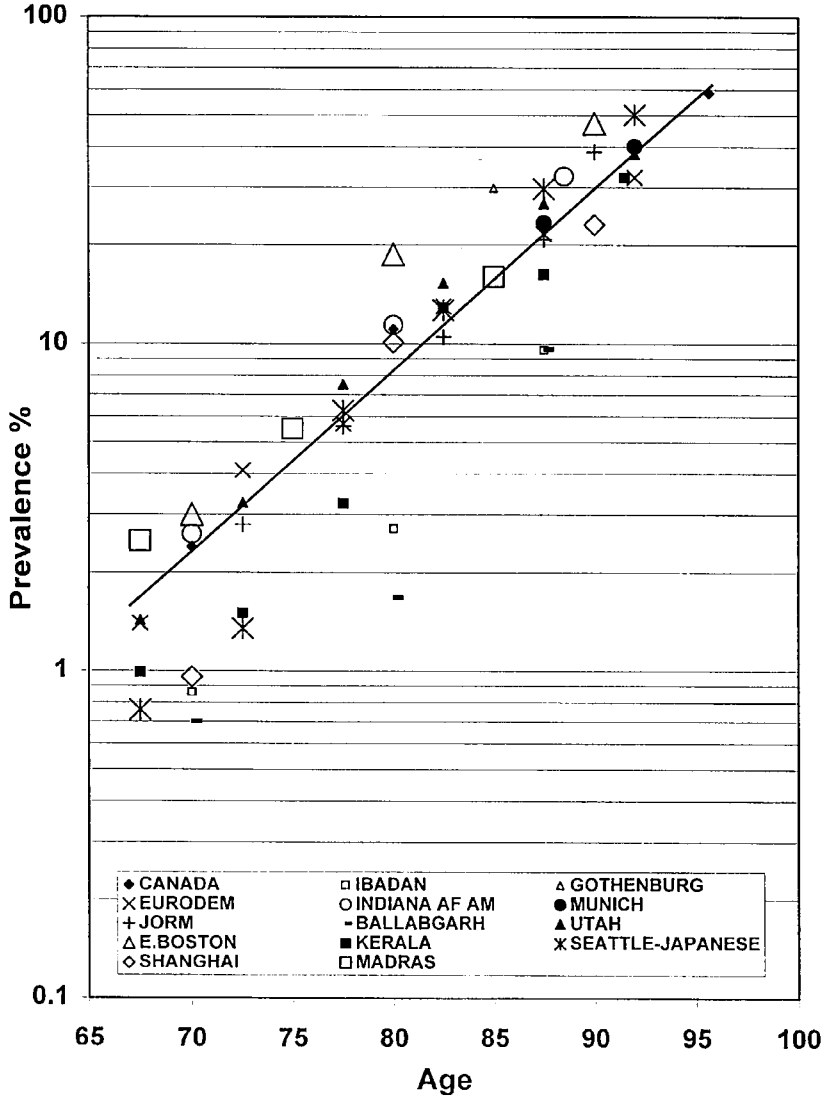

Figure 2.1. Prevalence of age-specific dementia (as log prevalence of age-defined population) plotted against age. The regression line is based upon the meta-analyses of Jorm (Jorm et al., 1987) and Eurodem (Hofman et al., 1991) and the nine-province Canadian study (Canadian Study of Health and Aging, 1994). Additional data points include East Boston (Evans et al., 1989) and Shanghai (Zhang et al., l990); the oldest old in the Canadian study (Ebly et al., 1994); Gothenburg (Skoog et al., 1993); Munich (Fichter et al., 1995); Seattle Japanese Americans (Graves et al., 1999); and Cache County, Utah (Breitner et al., 1999). Pertinent to less developed countries are a comparison of African Americans in Indianapolis and residents of Ibadan, Nigeria (Hendrie et al., 1994), and data from three communities in India: Ballabgarh (Chandra et al., 1998), Kerala (Brenner, 1999), and Madras (Rajkumar et al., 1997)

(1992) pointed out that if dementia prevalence doubles with every five years of age, then prevalence would be halved by delaying the onset of AD by five years. This has become a major research objective.

In the projection of dementia prevalence in the developed world, we used the Canadian prevalence rates since this is the largest population-based study that contains significant numbers of subjects over age 90, the age group that will increase in numbers most rapidly in the next 50 years. What happens to the exponential increase in dementia at advanced ages has been a matter of controversy. Hagnell et al. in 1981, on the basis of data from the Lundby study, suggested that the risk of dementia might decrease after age 85. Unfortunately this does not appear to be true. As shown in Figure 2.1, evidence from recent studies with significant numbers of subjects over the age of 90 [Canada (Ebly et al., 1994), Munich (Fichter et al., 1995), Seattle Japanese Americans (Graves et al., 1996a) and Stockholm (Fratiglioni et al., 1997)] indicates that the exponential increase of the prevalence of dementia continues well past 85, apparently up to age 95. As the prevalence of dementia may exceed 50% after age 95, there must then be a plateau, since there certainly are centenarians with intact cognitive functions. Determining what happens at very advanced ages will be a major objective for future epidemiological studies.

DIAGNOSING VERY MILD CASES OF DEMENTIA IN EPIDEMIOLOGICAL STUDIES

Most of the prevalence studies cited have used either Mini-Mental State Examination (MMSE) scores of 24 or lower or equivalent scores on related tests as a screening tool. East Boston used a sophisticated delayed recall task for screening patients (Evans et al., 1989). This indicated a higher rate of dementia prevalence, a rate considered an 'outlier' by some (see Figure 2.1). Today we often diagnose clinical AD and dementia in less impaired individuals with a fair degree of accuracy. Can this be done in the briefer evaluations required for epidemiological studies? Inclusion of cases of dementia at a very early stage in the course of the illness is now recognized as important, but raises the issue of the accuracy of diagnoses in very mild dementia and also the issue of the accuracy of the differential diagnosis in dementia in the setting of an epidemiological study, where autopsy follow-up is unlikely. This is a circumstance in which the need for readily obtained objective markers is very important.

LESS DEVELOPED COUNTRIES

The Alzheimer Disease International convened a group headed by Dr. Prince of the London School of Hygiene, calling themselves the 10/66 Group,

referring to the fact that only 10% of epidemiological studies have been conducted in the 66% of the world population in developing countries (Prince, 2000).

Dementia prevalence has been intensively studied in developed countries, especially in Japan and in Europe, somewhat less in North America. In Asia, it has also been intensively studied in Taiwan and along the East coast of China, but with few studies in Western China, India, smaller South Asian countries, and Africa, areas with over two billion people. Only a small number of studies have been carried out in South America. One hopes that initiatives by the 10/66 Group to rectify this situation will be successful.

The age-specific prevalence rates in developing countries tend to be somewhat, but not significantly, lower than those in the developed world. However, as shown in Figure 2.1, very low rates have been reported in Ibadan, Nigeria, compared to those in African Americans in Indianapolis, Indiana, in a study carried out jointly by investigators in the two locales. African Americans in New York City have very high rates. In India, Ballabgarh had a very low rate of dementia prevalence, and Kerala a somewhat higher but still quite low rate, whereas the prevalence of dementia in Madras was similar to that in developed countries.

If one were to estimate the number of cases of dementia in the less developed world in the year 2000, using the age-specific prevalence rates from the Shanghai survey, the number of demented individuals would be 8.5 million—about 60% of the number of cases in the developed world, due to the paucity of elderly people. However, by 2025, as the populations in the less developed countries aged (based upon 1998 UN projections), the number of demented individuals would begin to exceed that in the developed world, and by 2050 the demented in the less developed countries would number over 104 million—2.8 times those in the developed countries (Katzman and Fox, 1999).

A particularly difficult problem in some countries is ascertainment of age if there are no pre-existing public or governmental records. Mainland China, Taiwan, Hong Kong, and Singapore have kept such records for years, but India and Nigeria have not. If, despite the great lengths that investigators have gone to to determine age by questioning, there is a systematic error on the side of persons declaring themselves to be older than they are, then part of the variation in prevalence results can be explained.

DEMENTIA AS A MAJOR CAUSE OF MORTALITY

That AD and vascular dementia are as powerful predictors of mortality as cancer has been shown in the Shanghai study (Katzman et al., 1994) and in the Italian longitudinal study (Baldereschi et al., 1999). Mortality data from the East Boston study led Ewbank (1999) to estimate that the number of

deaths from AD is currently similar to the number of deaths from stroke, sharing the listing as the third most common cause of death, even if physicians are still reluctant to include the diagnosis on the death certificate.

INCIDENCE OF DEMENTIA

During the past decade new cases of dementia developing during the follow-up of non-demented individuals from earlier prevalence studies or based on longitudinal studies of normal volunteers, have provided data concerning the incidence of AD and dementia, i.e. the number of new cases per year (Figure 2.2). An exponential increase with age (Jorm and Jolley, 1998) is apparent in this semi-log plot, again with doubling of incidence, prompting the question of what happens to the incidence of dementia after age 95. The regression line in this figure is based upon the values from the Canadian Study of Health and Aging (2000) and values from a Eurodem meta-analysis of four studies (Letenneur et al., 2000). Figure 2.2 also includes data from additional incidence studies (Copeland et al., 1992; Gussekloo et al., 1995; Fichter et al., 1996). There is some variability between sites, which may reflect the smaller numbers of new cases observed in incident studies as compared to prevalent cases. Please note, however, that the incidence in Shanghai is similar to that of East Boston, in contrast to the significant differences in their prevalence rates (Figure 2.1). This reflects the longer life expectancy of demented subjects in Boston as compared to Shanghai at a time when no special dementia care facilities were available in the latter. In 1987, demented individuals in Shanghai had to be cared for almost solely by family or friends.

GENDER

In developed countries, AD appears to be more common in women, clinically and certainly in terms of residents in special care facilities, but whether this simply reflects the greater number of older women as compared to men or represents a true gender difference remains uncertain despite the availability of incidence data. In the Eurodem incidence studies (Letenneur et al., 2000) women were at greater risk, but in the large study carried out in Monongahela, Pennsylvania (Ganguli et al., 2000), a coal mining district, they were not.

EDUCATION AS A PROTECTIVE FACTOR

In the Shanghai survey of dementia, the 27% of the subjects in the sample of 5500 who had received no formal education had approximately twice the

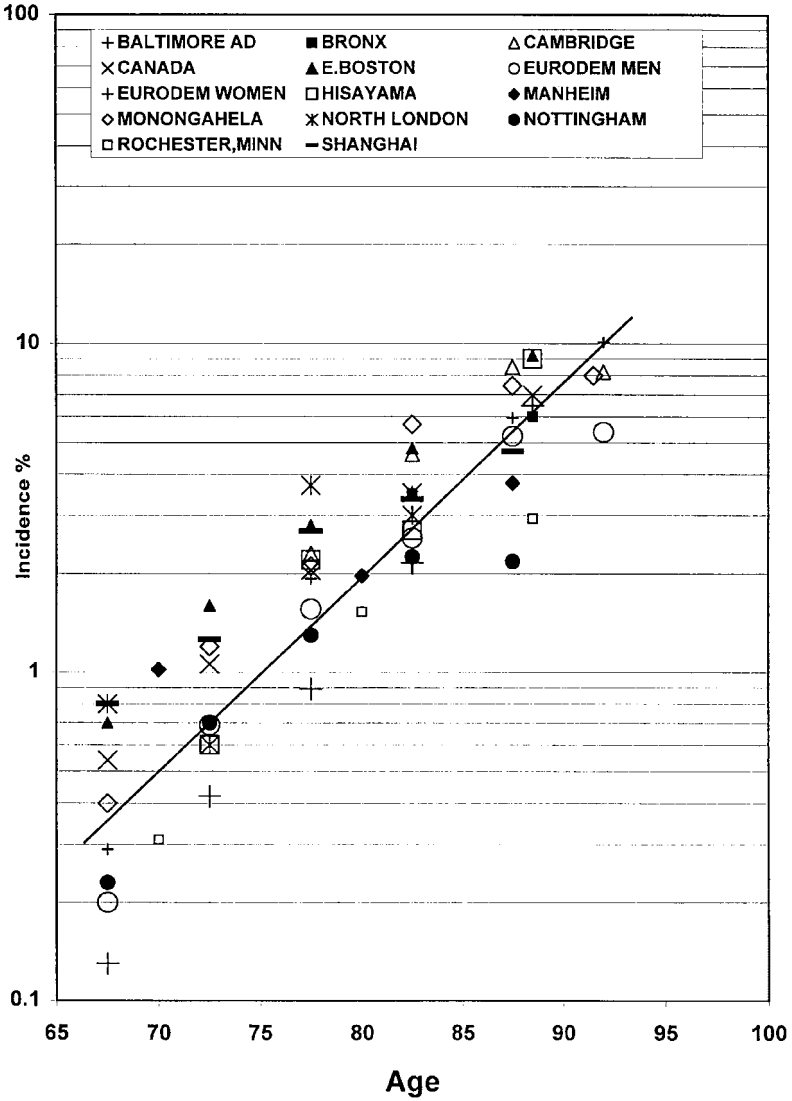

Figure 2.2. Incidence of age-specific dementia (as log of incidence of age-defined population per annum) plotted against age. The regression line is calculated from data from the Eurodem incidence analysis (Launer et al., 1999) and the Canadian Study of Health and Aging (2000). Sites of other studies include Baltimore (Kawas et al., 2000); Bronx (Aronson et al., 1991); Cambridge, England (Paykel et al., 1994); East Boston, Massachusetts (Hebert et al., 1995); Hisayama, Japan (Yoshitake et al., 1995); Mannheim, Germany (Bickel and Cooper, 1994), Monongahela, Pennsylvania (Ganguli et al., 2000); North London (Boothby et al., 1994); Nottingham (Morgan et al., 1992); Rochester, Minnesota (Rocca et al., 1998); and Shanghai, China (E. Yu, in preparation, based upon Zhang et al., 1998). (Updated from Katzman and Kawas, 1999, reproduced by permission of Lippincott, Williams and Wilkins.)

age-specific prevalence of dementia compared with subjects with at least some secondary education (Zhang et al., 1990). Similar results were obtained in the large study carried out in Bordeaux. Incidence data are now available. Again, lack of education is a risk factor for late-life dementia in Shanghai and in Europe (Katzman et al., 1997; Schmand et al., 1997), but in the Eurodem analysis the protective effect of education against incident dementia has been found to be greater in women (Letenneur et al., 2000). An evident explanation of the protective effect of education is that education increases cognitive reserve, thus delaying the onset of the clinical symptoms of dementia (Katzman and Kawas, 1999). This hypothesis has received support from studies of head circumference (Graves et al., 1996b), brain volume (Coffey et al., 1999) and rate of decline once dementia begins (Stern et al., 1999). However, in Indianapolis, Hall et al. (2000) found that lack of education is of greater importance in reducing dementia in African Americans who grew up in rural areas, not in those who grew up in cities. This remains an intriguing area for investigation.

RISK FACTORS: HEAD TRAUMA AND GENETIC PREDISPOSITION

In the early 1980s, case control studies identified family history and head trauma as the important risk factors for AD. Inheritance of one or two copies of the apolipoprotein ε_4 allele (ApoE-ε_4) accounts for a significant portion but not all of the genetic risk in those over the age of 60, and currently polymorphisms on a variety of candidate genes are under investigation, as reported in this volume. It should be noted that the role of ApoE-ε_4 as a risk factor for AD is of uncertain importance in Africa and in African Americans.

Head trauma was established as a risk factor in the Eurodem meta-analysis of case control studies and recently has been found to increase the risk of AD in a large multicenter study of familial AD (Guo et al., 2000), but its role as a risk factor has been challenged on the basis of the possibility of selective recall by the informant for the patient. One can avoid this in incidence studies when exposure information is obtained at baseline from non-demented subjects. To date, results reported from incidence studies have been mixed (Katzman and Kawas, 1999).

PROTECTIVE FACTORS

During the past decade, biological and epidemiological evidence has been adduced that estrogens, NSAIDs, and antioxidants might protect against the onset of AD. These possibilities are of great importance since a drug that is effective in delaying onset of the disease would make a major contribution in

reducing the public health impact of AD (Brookmeyer et al., 1998). Clinical trials of drugs in all three categories are now underway and a number of these studies will be described in this volume.

VASCULAR RISK FACTORS

Many clinicians have been struck by the seeming good health of individuals who develop AD. I was surprised in the 1980s when the Bronx Aging study, which included yearly electrocardiograms in a cohort that was aged 80 years and over, found that silent myocardial infarcts tripled the risk of AD in elderly women (Aronson et al., 1990). Sparks et al. (1990) reported a high correlation between coronary stenosis and cerebral plaques in a medical examiner autopsy series. ApoE-ε_4 predisposes to myocardial infarcts in young adult males and AD later in life. An LRP receptor polymorphism (Kang et al., 1997) may alter both brain amyloid and the risk of AD. The Rotterdam longitudinal study has reported several vascular and cardiovascular risk factors for AD, including diabetes (Ott et al., 1999), but the latter has not been confirmed by others. This is an important area for future investigation.

CHALLENGES

What are the special issues that one can anticipate being addressed by epidemiologists during the next decade? Undoubtedly they include the questions of the prevalence and incidence of dementia in developing countries, now being addressed by the 10/66 initiative. Other major issues include what happens to the incidence of dementia and of AD after age 90 and the question of whether there are cardiovascular risk factors for 'pure' AD. Finally, the testing of putative risk and protective factors in clinical trials will begin to show us how to delay the onset of AD by five years or more, so that the dire predictions of the rapid increase of demented subjects in the world during the next 50 years will not occur.

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3 Epidemiology of Dementia in Down's Syndrome

NICOLE SCHUPF

Neuropathological changes consistent with a diagnosis of Alzheimer's disease (AD) have been found in virtually all individuals with Down's syndrome (DS) over the age of 40, including deposition of β-amyloid (Aβ) in diffuse and neuritic plaques (Mann, 1988; Wisniewski et al., 1994). Most adults with DS will develop dementia by the time they are 70 years of age, and it has been suggested that the identification of processes which contribute to a high risk of AD can serve as a model for the role of genetics in the etiology of AD (Lai and Williams, 1989). The neuropathological manifestations of AD in DS have been attributed to triplication and overexpression of the gene for Aβ precursor protein (βAPP), located on chromosome 21, and the increased risk of AD may be mediated by an increased substrate for cellular production of Aβ peptides.

Before age 50, diffuse plaques are the most prevalent lesion seen in DS, while after age 50, neuritic plaques, containing fibrilized Aβ peptides, predominate (Wisniewski et al., 1994). Examination of the age-specific prevalence of dementia in DS shows that risk of AD begins to increase after 50 years of age (range 38–70 years of age), supporting the hypothesis that dementia in DS is initiated by the transition from diffuse to fibrilized plaques (Lai and Williams, 1989; Visser et al., 1997; Holland et al., 1998; Lai et al., 1999) (Figure 3.1). The wide distribution of age at onset of dementia cannot be accounted for solely on the basis of the triplication of βAPP, since 95% of persons with DS have triplication of the entire chromosome. Rather, other factors must account for individual differences in susceptibility to the development of fibrilized plaques and for the range in age at onset. This paper will review factors that may influence onset of AD by accelerating formation of Aβ, including (1) atypical karyotypes, (2) polymorphisms in apolipoprotein E (apoE), (3) estrogen deficiency, and (4) individual differences in Aβ peptide levels.

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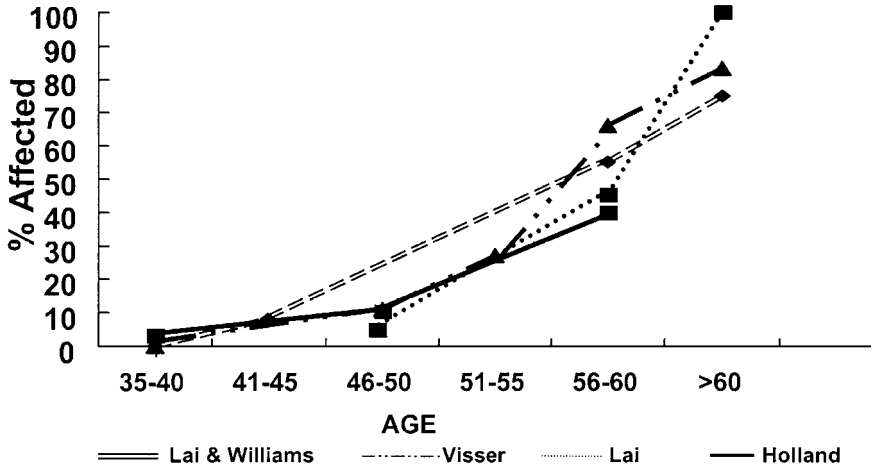

Figure 3.1. Age-specific prevalence of dementia in adults with Down's syndrome

ATYPICAL KARYOTYPES

The high risk of AD in DS is also associated with early mortality, with an average life expectancy at birth of only 56 years (Baird and Sadovnick,1988). Karyotypic analysis of adults aged over 70 years with DS can provide an opportunity to identify genetic factors associated with longevity. While these cases are rare, they are highly informative and suggest that karyotypes that decrease βAPP load are associated with improved survival and reduced risk of AD. Prasher and colleagues determined the clinical and molecular correlates of partial trisomy 21 in a 78-year-old woman with DS [46,XX,rec(21)dup q, inv(21) (p12q22.1)] (Prasher et al., 1998). Although she did not display the full range of stigmata associated with the DS phenotype, she developed several of the characteristic age-related medical conditions, including hypothyroidism, cataracts, hypotonia, and hearing impairment. Analysis of gene sequences on chromosome 21 using fluorescent *in situ* hybridization showed that the partial trisomy excluded the region containing the gene for βAPP, which was present in only two copies. There was no evidence of decline in cognitive or adaptive competence for the five years preceding her death, and no evidence of AD-associated plaque and tangle formation was found at autopsy. Similarly, there have been case reports of two women with DS with respectively 25% and 97% disomy for chromosome 21 (Chicoine and McGuire, 1997; W. B. Zigman, personal communication). Both women had a characteristic DS phenotype and some of the typical age-related medical conditions, including hypothyroidism and cataracts. One woman (with 25% disomy) died without any evidence of dementia at age 83, while

the other woman (with 97% disomy) is still living and shows no evidence of decline in either cognitive or adaptive behavior.

In turn, adults in the general population with mosaicism or translocations involving chromosome 21 may be informative for factors that accelerate the development of AD. There have been several case reports of women without mental retardation and without apparent phenotypic features of DS who developed dementia at very early ages and were shown to be mosaic for DS. One 45-year-old woman who had developed dementia at age 43 was shown on cytogenetic analysis to have a low-grade (1–5%) translocation trisomy 21 in peripheral lymphocytes [mos46,XX/46,XX, −21,+(21q;21q)], and varying degrees of trisomy (ranging from 0 to 100%) in skin fibroblast lines (Shapiro et al., 1990). She had several stigmata of DS, but was never diagnosed with DS. Follow-up neuropsychological assessments over the next two years showed progressive cognitive decline, with radiological evidence of increasing ventricular volumes and significant reductions in regional cerebral glucose metabolism. The cytogenetic findings showed that the region trisomic for chromosome 21 included the locus for βAPP. Another case of presenile dementia with onset at age 41 was observed in a mother of a child with DS who had trisomy 21 in 10% of her peripheral blood lymphocytes (Rowe et al., 1989). The early onset of dementia in these women is consistent with the hypothesis that an increased dose of APP plays a key role in increasing risk.

APOLIPOPROTEIN E IN DOWN'S SYNDROME

In adults with DS, all studies have consistently found that the presence of the apoE-ε2 allele increases longevity and reduces risk of dementia (Hardy et al., 1994; Royston et al., 1994; Schupf et al., 1996; Tyrrell et al., 1998; Sekijima et al., 1998; Lai et al., 1999). In two studies employing survival analysis, the presence of an apoE-ε4 allele was associated with earlier onset of AD (Schupf et al., 1996; Lai et al., 1999) (Figure 3.2). Negative findings have been reported in studies with small sample sizes and, importantly, failure to consider differences in the age at onset of dementia among those with and without an ε4 allele. Since the effect of the ε4 allele is not expressed until midlife, inclusion of sufficient numbers of adults over 50 years of age and analysis using survival methods that can adjust for age and years of follow-up are important methodological considerations. In sum, in DS, as in the general population, the presence of the ε2 allele is protective, while the presence of an ε4 allele is associated with earlier age at onset of dementia. These findings are consistent with the findings of reduced Aβ deposition and less plaque formation (Benjamin et al., 1994; Lippa et al., 1997) in those with an ε2 allele, and with acceleration of Aβ pathology in those with an ε4 allele (Hyman et al., 1995).

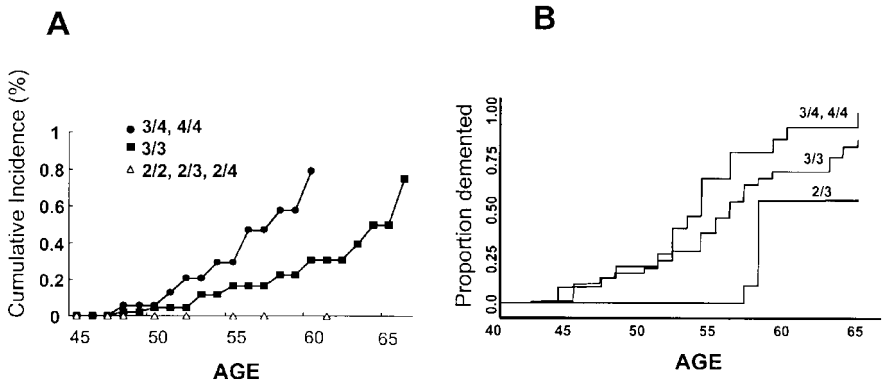

Figure 3.2. Cumulative incidence of dementia by apolipoprotein E genotype. (**A**: Reproduced from Schupf et al., 1996, by permission of Lippincott, Williams and Wilkins; **B** Reproduced from Lai et al., 1999, by permission of Lippincott, Williams and Wilkins)

EFFECT OF ESTROGEN DEFICIENCY

Ovarian steroids are important in the normal maintenance of brain function in basal forebrain regions affected by AD. Estrogen promotes the growth of cholinergic neurons and protects against the formation of Aβ (Simpkins et al., 1997; Jaffe et al., 1994). In guinea pigs, ovariectomy led to increased levels of Aβ-peptides, $A\beta_{1-40}$ and $A\beta_{1-42}$, and this effect was reversible with exogenous estrogen treatment (Petanceska et al., 2000). Studies of postmenopausal women in the general population have shown that estrogen replacement therapy (ERT) may delay or prevent the onset of AD (Tang et al., 1996). Several studies have shown that women with DS experience menopause 4–5 years earlier, on average, than women in the general population, which may contribute to their high risk of AD (Carr and Hollins, 1995; Schupf et al., 1997). Cosgrave et al. (1999) found that earlier menopause is associated with earlier onset of AD, supporting the hypothesis that endogenous estrogen deficiency may accelerate the development of AD (Cosgrave et al., 1999) (Figure 3.3).

β-AMYLOID PROTEIN 1–42

In DS, as in AD, $A\beta_{1-42}$ is the earliest form of Aβ that is deposited (Iwatsubo et al., 1995). $A\beta_{1-42}$ can be observed in diffuse plaques as early as 12 years of age, followed by $A\beta_{1-40}$ more than a decade later. Even in older individuals with DS (>50 years), $A\beta_{1-42}$ is more abundant than $A\beta_{1-40}$ (Teller et al., 1996). Mean plasma levels of both $A\beta_{1-42}$ and $A\beta_{1-40}$ are higher in adults with DS than in age-matched adults from the population (Mehta et al., 1998;

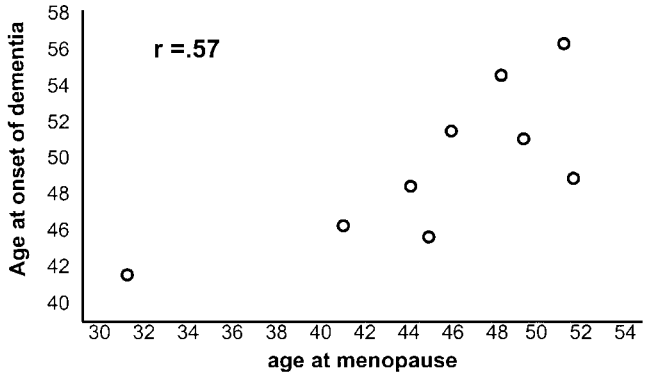

Figure 3.3. Relation of age at onset of menopause to age at onset of dementia. (Reproduced from Cosgrave et al., 1999, by permission of Blackwell Science Ltd.)

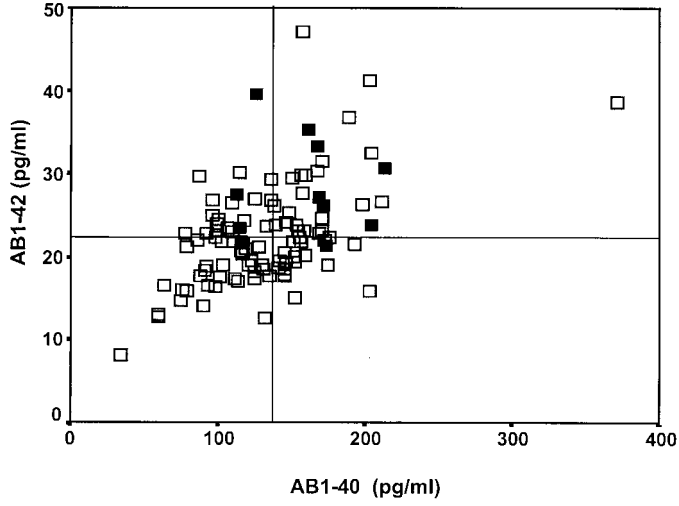

Figure 3.4. Plasma levels of $A\beta_{1-40}$ and $A\beta_{1-42}$ in demented (■) and non-demented (□) adults with Down's syndrome in relation to group medians

Tokuda et al., 1997), but one study found that this increase was not related to dementia status (Tokuda et al., 1997). Our group has found also that $A\beta_{1-42}$ and $A\beta_{1-40}$ levels were significantly higher in demented and non-demented adults with DS than in matched controls from the general population. Aβ peptide levels were highest in demented adults with DS, and mean plasma levels of $A\beta_{1-42}$ increased more than mean plasma levels of $A\beta_{1-40}$ (Figure 3.4). Mean plasma levels of $A\beta_{1-42}$, but not $A\beta_{1-40}$, were elevated both in non-demented and demented adults with the apoE ε4 allele compared to those without the apoE ε4 allele. The selective effect of the apoE ε4 allele on $A\beta_{1-42}$

levels is consistent with two possible mechanisms of action, one involving acceleration of the rate of amyloid fibril formation (Ma et al., 1994) and the other involving diminished clearance of Aβ (McNamara et al., 1998).

SUMMARY AND CONCLUSIONS

Factors that increase the formation of Aβ or accelerate its deposition, such as the presence of apoE ε4 allele, estrogen deficiency, and high levels of $A\beta_{1-42}$ peptides, are associated with earlier onset of dementia in DS, while factors that decrease the formation of Aβ, such as the apoE ε2 allele or atypical karyotypes that reduce APP gene dose, are associated with improved survival and reduced risk of dementia. Since 95% of persons with DS have triplication of βAPP associated with free trisomy, overexpression of APP alone cannot account for the differences in age at onset of dementia within this population. An important task for future work will be to identify the sources of individual variation in premorbid Aβ levels which contribute to risk. These are likely to include the joint effects of factors reviewed here and others not yet identified.

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4 A Genomic Search for Alzheimer's Disease Genes

JONATHAN L. HAINES, L. RENEE BAILEY, JANET M. GRUBBER, DALE HEDGES, JENIFER L. HALL, SANDRA WEST, LEONARD SANTORO, BETH KEMMERER, ANNE M. SAUNDERS, ALLEN D. ROSES, GARY W. SMALL, WILLIAM K. SCOTT, P. MICHAEL CONNEALLY, JEFFERY M. VANCE AND MARGARET A. PERICAK-VANCE

INTRODUCTION

Alzheimer's disease (AD) is the leading cause of dementia in the elderly and the most common form of dementia occurring after the age of 40 (Brookmeyer et al., 1998). AD has a complex genetic etiology with a strong genetic component that likely involves both gene–gene and gene–environment interactions. Pathologically AD is characterized by the deposition of amyloid within senile plaques and cerebral blood vessels, and neurofibrillary tangles found in the neurons of the cerebral cortex and hippocampus (Wisniewski et al., 1993). AD is usually slowly progressive with insidious onset, resulting in memory loss and alterations of higher intellectual function, emotional stability, and cognitive abilities (Guttman et al., 1999).

Definitive clues to the etiology of AD have emerged only begrudgingly even though AD was first described almost a century ago (Alzheimer, 1907). Only in the past 15 years have the powerful tools of genetic analysis been used successfully to identify the four AD genes currently known. Three of these—the amyloid precursor protein (APP) (Goate et al., 1991) and the presenilin 1 and 2 (PS1 and PS2) genes (Levy-Lahad et al., 1995; Rogaev et al., 1995; Sherrington et al., 1995)—were identified using standard positional cloning methods facilitated by simple autosomal dominant inheritance in early-onset (onset generally less than 60 years of age) AD families. These three genes account for the majority of early-onset familial AD but collectively they account for only about 2% of all cases of AD. Studies of these three genes

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have led to new insights into the biology of AD but much remains to be learned.

Compared to that of early-onset AD, the genetic architecture underlying the far more common late-onset AD (Pericak-Vance et al., 1988) is much more complex. Comparison of the recurrence rates in the siblings of AD patients to the general population prevalence (Risch, 1990a,b,c) can be parameterized as a recurrence risk ratio (λs) that is a rough measure of genetic influence. The estimates of λs for AD are surprisingly constant across studies (Breitner et al., 1988; Hirst et al., 1994; Sadovnick et al., 1989) and range from 4 to 5. Power studies (Hauser et al., 1996; Risch, 1990b) show that genes responsible for a λs ratio as low as 1.5 should be locatable using samples of affected sibpairs similar to those in this study.

In 1991 evidence for linkage of late-onset AD to chromosome 19q13 was reported (Pericak-Vance et al., 1991). Apolipoprotein E (apoE), a plasma lipoprotein involved in lipid transport and metabolism, was also known to map to 19q13. However, it was just one of hundreds of possible candidates. Functional polymorphisms in apoE made this a somewhat more interesting candidate, given that the three apoE alleles (-2, -3, -4), when translated, result in different protein isoforms (Menzel et al., 1983; Saunders et al., 1993). When it was realized that apoE was also one component of amyloid plaques (Namba et al., 1991; Wisniewski and Frangione, 1992) the combination of biology and genetic mapping facilitated our identification of the association between the apoE-4 allele in both familial late-onset and sporadic AD patients (Saunders et al., 1993; Strittmatter et al., 1993). Additional analyses (Corder et al., 1993) showed that the apoE-4 allele acts in a dose-dependent manner to increase risk and decrease age of onset in both late-onset familial and sporadic and early-onset sporadic AD (Corder et al., 1993; Okuizumi et al., 1994; Roses and Pericak-Vance, 1997; van Duijn et al., 1994). The apoE-2 allele seems to afford protection against late-onset AD (Corder et al., 1994; Farrer et al., 1997; Locke et al., 1995; Scott et al., 1997; Sorbi et al., 1994). The mechanism by which the specific apoE isoforms uniquely contribute to disease expression is not yet known. ApoE represents the fourth confirmed genetic factor and remains the single most significant biological risk factor identified for AD.

A number of lines of evidence suggest that apoE does not account for all of the genetic variation seen in AD. While the heritability of AD has been estimated at about 80% (Bergen, 1994), more than one-third of late-onset AD cases do not have a single apoE-4 allele. The apoE-4 associated risk of AD appears to differ among ethnic groups, suggesting ethnicity may influence genetic risk of AD (Farrer et al., 1997; Maestre et al., 1995; Tang et al., 1996). This is demonstrated by the studies of an Indiana Amish population (Pericak-Vance et al., 1996) that showed strong familial aggregation of AD despite a virtually non-existent apoE-4 allele frequency. In addition, we have estimated the λs specific for the apoE locus (Roses et al., 1995) to be around

2. Under the assumptions of either additive or multiplicative interactions between genes, and given that the overall λs is between 4 and 5, then apoE can account for at most 50% of the total genetic effect in AD. This suggests that one or more genes with moderately strong effects remain to be identified.

Efforts to identify these additional AD loci have taken two forms: whole-genome screens for genetic linkage using families with multiple sampled affected individuals, and allelic association studies of candidate genes using primarily case-control samples. Two recent genome scans have implicated several chromosomes (1, 4, 6, 9, 10, 12, 19, and 20) as potential locations of additional AD loci (Kehoe et al., 1999a; Pericak-Vance et al., 1997). Perhaps the most promising of these locations is chromosome 12, where the original linkage report (Pericak-Vance et al., 1997) has since been supported by results from two independent samples (Kehoe et al., 1999a; Rogaeva et al., 1998; Wu et al., 1998). Numerous positional candidate genes on chromosome 12 have been examined, including the low-density lipoprotein receptor-related protein (LRP) (Lendon et al., 1997) and α-2-macroglobulin (A2M) (Blacker et al., 1998). These results have also not been consistently replicated (Dow et al., 1999; Rogaeva et al., 1999; Rudrasingham et al., 1999; Scott et al., 1998). There has been some suggestion that there may be more than one gene on chromosome 12, and this may have complicated the identification of either gene (Scott et al., 2000). The broad consensus remains that the chromosome 12 gene(s) have not yet been identified.

In conjunction with using the positional candidate gene approach, several investigators have attempted to identify the remaining AD loci by focusing on candidate genes selected due to their function. These studies have largely used a case-control approach to examine single polymorphisms in candidate genes. These studies have also been uniformly unsuccessful. Over 30 such genes have been reported as being associated with late-onset AD (Table 4.1). At best, the evidence for any of these loci is mixed, and virtually all have had far more negative than positive reports. In fact, some associations have never seen another positive report. These functional candidate genes include, among others, α_1-antichymotrypsin (AACT) (Kamboh et al., 1995), LRP (Lendon et al., 1997), PS1 (Wragg et al., 1996), ubiquitin-B (Vanleeuwen et al., 1998), the HLA complex (Curran et al., 1997; Payami et al., 1997), butyrylcholinesterase K variant (BCHE-K) (Lehmann et al., 1997), non-amyloid component of plaques/α-synuclein (NACP/α-synuclein) (Xia et al., 1996), mitochondrial mutations (Hutchin and Cortopassi, 1995), angiotensin-converting enzyme (DCP1) (Kehoe et al., 1999b), and cathepsin D (Papassotiropoulos et al., 1999). Successful examination of these and other candidate genes requires sufficiently large samples of well-characterized affected individuals to detect modest genetic effects and an even larger sample of appropriately matched cases and controls to avoid spurious associations due to biased sampling. Unfortunately, virtually every study has failed on one or both of these points.

Table 4.1. Proposed candidate genes in late-onset AD

A2M	CYP2D	HTR6	PS1 promoter
AACT	DCP1	IL1	SLC6A4
apoE promoter	DLST	IL6	SNCA
APBB1	ESR1	LBP-1C	TAU
APP promoter	HFE	LRP1	Tf
BCHE	HLA-A	MPO	TGFB
BLMH	HLA-B	Mitochondria	UBB
CTSD	HLA-DR	NOS3	VLDLR

To solve these problems, a strategy of genomic screening, requiring no a priori assumptions about any gene function, has been chosen. To alleviate the problem of sample size, a large dataset of all available families was assembled. We report here the results of our genomic screen on 466 late-onset AD families.

MATERIALS AND METHODS

We performed the genomic screen on 466 families with late-onset AD (Table 4.2, family mean age of onset $\geqslant 60$ years). The families were obtained from the National Institute of Mental Health Alzheimer's disease repository (NIMHR), the Indiana University Alzheimer's Disease Repository (IUADR), the Duke Center for Human Genetics (CHG), the Joseph and Kathleen Bryan Alzheimer's Disease Research Center (Bryan ADRC), the UCLA Neuropsychiatric Institute (UCLA), and the Vanderbilt University Program in Human Genetics. The UCLA, Bryan ADRC, and Vanderbilt families make up the Collaborative Alzheimer Project (CAP). Affected individuals

Table 4.2. Datasets used in genomic screen

Dataset	Families, n	Samples, n (% affected)	ASPs*, n
CAP	62	380 (57)	145
IUADR	118	446 (61)	181
NIMHR	286	816 (69)	413
Total	466	1642 (64)	739

*Affected sibpairs.

were classified in accordance with NINCDS–ADRDA clinical diagnostic criteria (McKhann et al., 1984).

We analyzed 326 microsatellite markers that generated an approximately 10 cM genomic screening grid. Genotyping was performed with 10–30 ng of DNA per reaction, using methodology as previously described (Schuler et al., 1996; Vance and Othmane, 1998). Because a number of families have an extended structure, we used multiple statistical methods to analyze the data. We used both a non-parametric affected sibpair lod score analysis using the computer program ASPEX (MLS) (Risch, 1990a,b,c) as well as a parametric affected relative pair analysis (MLOD) using the computer program FASTLINK. An MLOD represents the maximum lod score over autosomal dominant and autosomal recessive low penetrance (affecteds-only) models, taking the potential for locus heterogeneity into account. The allele frequency used for the disease allele was 0.001. Marker allele frequencies were generated from over 200 unrelated individuals or from published sources. We considered the results for any marker as interesting if the analysis resulted in a two-point lod score (MLS or MLOD) $\geqslant 1.00$. Because of the extensive heterogeneity in AD, we also stratified the data into two subsets of families. One subset contained families that had at least one autopsy-confirmed AD case ($n = 199$) and the other contained all families with an unknown autopsy status ($n = 267$). We present here the preliminary results of these analyses.

RESULTS

The genomic screening results for all regions with either/or an MLS or MLOD $\geqslant 1.00$ are presented in Table 4.3. These 'interesting' regions are found on chromosomes 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, and 19. The region on chromosome 9p22 generates the strongest results with an MLS of 2.97 and an MLOD of 3.10. Not surprisingly, the region on chromosome 19q13 also gives good results, with an MLS of 2.21 and an MLOD of 3.69. The marker on chromosome 19 is approximately 8 cM from the apoE gene and is thus reflecting the genetic effect of apoE on AD.

The results of the subsetting analyses are also shown in Table 4.3. The results on chromosomes 5, 6, 7, 9, 10, 12, and 19 remain interesting in the autopsy-confirmed subset, with new interesting results being observed on chromosome 9q34 and chromosome 18. Two results, those on chromosomes 8 and 11, seem stronger in the autopsy-unknown subgroup. Analysis of the apoE gene in these data demonstrates the connection between autopsy confirmation and apoE genotype. Despite being only 43% of the families, 60% of the overall MLS and 76% of the overall MLOD scores arise from this smaller subset of families.

Table 4.3. Summary of analyses from the genomic screen

Cytogenetic location	MLS			MLOD		
	Confirmed	Unknown	Overall	Confirmed	Unknown	Overall
4q32	0.84	0.47	**1.30**	0.72	0.60	**1.42**
5p15	0.94	0.00	0.42	**2.23**	0.00	**1.59**
6p23	0.00	0.95	0.27	0.00	**1.31**	0.61
6q26	**1.03**	0.00	0.56	**1.20**	0.01	**1.14**
7q31	**1.41**	0.31	**1.56**	**2.18**	0.36	**1.97**
8q13	0.01	0.46	0.37	0.19	**1.28**	**1.23**
8q24	0.13	0.29	0.41	0.61	0.60	**1.19**
9p22	**4.31**	0.08	**2.97**	**3.64**	0.25	**3.10**
9q34	**1.96**	0.00	0.19	**2.05**	0.00	0.44
10p11	0.65	0.02	0.50	**1.22**	0.00	**1.08**
10q22	0.32	0.53	0.77	0.94	0.50	**1.41**
11q25	0.00	0.62	0.24	0.18	**1.11**	**1.10**
12p11	0.17	0.05	0.08	**1.01**	0.18	**1.10**
13q11	0.74	0.13	0.77	0.41	0.39	**1.11**
18q22	0.64	0.00	0.00	**1.14**	0.00	0.01
19q13	**2.09**	0.43	**2.21**	**3.64**	0.63	**3.69**
apoE	**3.42**	**2.18**	**5.68**	**8.98**	**3.64**	**11.85**

*Results MLS or lod score >1.0 in bold.

DISCUSSION

We have identified 16 potential genomic regions of interest in these data using a genomic screening strategy of our large (n = 466) family dataset. Three of these results support the results previously reported for chromosome 12p11 (Pericak-Vance et al., 1997) and chromosome 10q22 (Kehoe et al., 1999a) as well as apoE. The current results should not be considered an independent confirmation, however, since the current dataset includes substantial numbers of families used in those previous analyses.

The most exciting results occur on chromosome 9, where the affected sibpair maximum lod score (MLS) in the combined sample is 2.97. The results for chromosome 9 seemed to come almost entirely from the autopsy-confirmed subset with an MLS of 4.31. This is the highest lod score ever seen in a genomic screen for AD. It is, in fact, larger than the MLS score found for the screening marker on 19q13, the closest screening marker to apoE. This lod score is well above the criteria set by either Lander and Kruglyak (1995) or by Ott (1999) for highly suggestive or significant linkage.

Stratifying the dataset into the smaller subsets, while decreasing the overall number of families in each subset, defines more homogeneous subsets of the data. Some results seem to arise primarily from the autopsy-confirmed set

(e.g. chromosome 9p22), while others seem to arise primarily from the autopsy-unknown subset (chromosomes 8q13 and 11q25). The families in the autopsy-unknown subset may well represent a subset of families consisting of classical AD (both apoE-4 related and unrelated) that have not yet come to autopsy, along with other clinical dementia subtypes. Thus any potential linkages seen in these data may represent genes that influence dementia of a non-Alzheimer's type.

Approaches such as those we have used here, together with new statistical methodologies such as conditional linkage analysis (Scott et al., 2000), generalized family-based association tests such as the Pedigree Disequilibrium Test (Martin et al., 2000), and the use of new molecular technologies such as single nucleotide repeat polymorphisms (SNPs), open the door for additional AD gene discovery. Such strategies are necessary if we are to understand the subtle and complex threads that, woven together, create the intricate tapestry of AD.

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5 Candidate Genes Showing No Evidence of Association with Alzheimer's Disease: Results of the NIMH-AD Genetics Initiative

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INTRODUCTION

The complexity of Alzheimer's disease (AD) genetic research is illustrated by more than 20 genetic risk factors that have been reported for the late-onset form of the disease in the literature to date. However, the great majority of these have not been confirmed in follow-up studies. At present, only one risk factor, a common polymorphism in the apolipoprotein E gene (*APOE*), has been consistently found in several independent samples, using a variety of analytic methodologies (Saunders et al., 1993; Farrer et al., 1997). However, multiple lines of evidence indicate that there are additional genes involved in AD. In fact, a recent study treating AD as a quantitative trait estimated that at least four additional AD genetic susceptibility loci exist beyond *APOE* (Daw et al., 2000). One promising candidate region resides between the short arm and the centromeric region on chromosome 12, a region that has been linked to AD in several independent family-based samples (Pericak-Vance et al., 1998; Rogaeva et al., 1998; Wu et al., 1998), including our own (Blacker et al., 1998). At least two genes that have been suggested to play an important role in AD neuropathology have emerged from this chromosomal region as risk factors for the disease. The first is the gene encoding α-2-macroglobulin (*A2M*), for which two polymorphisms have been associated with AD in

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several family-based and case-control samples (Blacker et al., 1998; Liao et al., 1998; Myllykangas et al., 1999; Dodel et al., 2000). However, various studies failed to find evidence for association of these polymorphisms and AD (e.g. Gibson et al., 2000; Sodeyama *et al.*, 2000). The second candidate is the gene encoding the *A2M*-receptor (or lipoprotein receptor-related protein-1; *LRP1*) which has also been associated with AD in some studies (Kang et al., 1997; Baum et al., 1998) but not in others (Fallin et al., 1997b; Woodward et al., 1998). Besides chromosome 12, associations with other candidate genes have been suggested from chromosomal regions where linkage has not been demonstrated consistently or not at all.

Given the large number of candidates under investigation for AD, it is highly unlikely that all of the reported associations will prove to be genuine AD risk factors. Therefore, testing these genes in independent samples is critical. Because case-control studies are vulnerable to spurious findings due to population admixture, replication using family-based methods, which are not generally vulnerable to this type of bias, is particularly advantageous (Spielman et al., 1993; Horvath and Laird, 1998). We report here the results of family-based association analyses in a large sample of multiplex AD families on five candidate genes that were reported to be significantly associated with AD in previous case-control studies: interleukin-1β (*IL-1B*; Grimaldi et al., 2000), butyrylcholinesterase (*BCHE*; Lehmann et al., 1997), very low density lipoprotein receptor (*VLDL-R*; Okuizumi et al., 1995), cathepsin D (*CTSD*; Papassotiropoulos et al., 1999) and *LRP1* (Kang et al., 1997). For comparison purposes, we also performed similar tests of association for *APOE* in our sample.

METHODS

PATIENTS

Subjects were collected as part of the National Institute of Mental Health (NIMH) Genetics Initiative following a standardized protocol applying NINCDS–ADRDA criteria for the diagnosis of AD (Blacker et al., 1997). This sample presently consists of a total of 1522 subjects in 459 families. The mean age of onset in affecteds was 71.7 ± 8.7 years. In the NIMH study overall, a clinical diagnosis of AD has been confirmed in 94% of the cases that came to autopsy. Approximately half of the sample ($n = 827$; affecteds $n = 460$, unaffecteds $n = 367$) came from discordant sibships ($n = 220$), which were used to test for association. Smaller sample sizes are reported for several of the genes under investigation because they were genotyped while the sample was still being accrued. To avoid examining very early onset AD, which seems to have a distinct genetic etiology, we included only those families in which all examined affected individuals displayed onset ages greater than 50 years.

GENOTYPING OF *APOE* AND CANDIDATE GENES

APOE was genotyped in 827 subjects as described previously (Blacker et al., 1997). A C/T single nucleotide polymorphism (SNP) at bp −511 in the 5′ UTR of *IL-1B* was genotyped in 674 subjects using the same protocol as DiGiovine et al. (1992). The G/A transition at bp 1615 in *BCHE* was genotyped in 337 subjects using the protocol by Lehman et al. (1997). A trinucleotide-repeat at bp −19 in the 5′ UTR of *VLDL-R* was genotyped in 410 subjects using the protocol from Okuizumi et al. (1995). The exon 2 polymorphism of *CTSD* was genotyped in 436 subjects using the same polymerase chain reaction (PCR) conditions as in the original case-control study (Papassotiropoulos et al., 1999) followed by an overnight digest with MwoI and 6% polyacrylamide gel electrophoresis (PAGE). The exon 3 polymorphism in *LRP1* was genotyped in a total of 470 individuals as reported by Kang et al. (1997) using SSCP analysis on a 5% MDE-gel (BMA, Rockland, ME). Each of the above reactions was performed using approximately 30 ng of genomic DNA.

STATISTICAL TECHNIQUES

To test for genetic association between AD and each of the candidate genes, we used the sibship disequilibrium test (SDT; Horvath and Laird, 1998), a family-based association test that compares the average number of candidate alleles between affected and unaffected siblings in each family without the need for parental data. Like the TDT and other family-based association tests, and in contrast to conventional case-control methods, the SDT is not susceptible to bias due to population admixture (Horvath and Laird, 1998). In addition, for each candidate gene we performed conditional logistic regression stratified on family (Witte et al., 1999), with and without controlling for *APOE* ε_4-status.

RESULTS AND DISCUSSION

Our analyses in the NIMH discordant sibship sample revealed no association between AD and any of the five polymorphisms tested (Table 5.1). As would be expected under these circumstances, conditional logistic regression also failed to reveal effects on risk for AD in carriers of the candidate alleles vs. non-carriers, with and without controlling for *APOE* ε_4-status (data not shown). In contrast, we were able to detect a highly significant association of the *APOE* polymorphism using the SDT in the NIMH sample: applied to all 827 discordant siblings both the overrepresentation of the ε_4-allele and the underrepresentation of the ε_2-allele in affecteds were clearly recognized ($p < 1 \times 10^{-7}$ and $p = 0.00024$, respectively).

Table 5.1. SDT results in discordant sibships of the NIMH sample

Candidate gene	Polymorphism	Sample, n	SDT (p value)
IL-1B	SNP (5′ UTR, −511 bp)	674	0.47
BCHE	SNP (Ala539Thr)	337	0.65
VLDL-R	3bp-repeat (5′ UTR, −19 bp)	410	0.96
CTSD⋆	SNP (Ala224Val)	436	0.68
LRP1	SNP (Gln100Gln)	470	0.52

⋆Taken from Bertram et al. (2001).

Although all five of the genes analyzed in this study are plausible candidates for AD on biological grounds, we failed to detect evidence for association between common polymorphisms in these genes and the disease in a large and carefully ascertained family sample. This is in contrast to case-control studies that reported significant associations between these polymorphisms and AD. Given the large number of genetic risk factors reported to be associated with AD today, replication in independent samples is crucial. The use of different analytic approaches, e.g. case-control and family-based, is especially valuable in this regard. Until now, none of the polymorphisms reported here has been investigated using a family-based approach. However, consistent with our negative results, there have been other independent case-control studies that failed to detect significant associations for the polymorphisms in *BCHE* (Brindle et al., 1998; Crawford et al., 1998), *VLDL-R* (Fallin *et al.*, 1997a), *CTSD* (McIlroy et al., 1999) and *LRP1* (Fallin et al., 1997b; Woodward et al., 1998). To our knowledge, no published investigation has yet analyzed the association of the *IL-1B* polymorphism beyond the initial report.

Several possibilities must be discussed when interpreting our negative findings in the light of the initial highly significant results. As pointed out earlier, all previous investigations applied case-control analyses and were thus susceptible to population admixture. Our analyses minimized this potential bias by employing a family-based strategy. Alternatively, the initial findings could be due to a type I statistical error, an interpretation that is rendered more likely by the negative findings in the independent case-control studies described above. Generally, the possibility of type I errors is growing as more and more laboratories around the world report association findings on AD. This problem is likely to be of even greater impact considering the 'publication bias' in favor of positive findings, meaning that more candidate genes have been tested than appear in the literature. On the other hand, if some of the reported associations are in fact genuine, the negative results of this and other studies suggest that the true association is likely to be smaller than initially suggested or might be due to linkage disequilibrium with a

polymorphism nearby. Both scenarios may be easier to detect in case-control studies rather than family-based analyses due to the inherently lower power of the latter, at least under certain disease models. In any case, further analyses on (i) larger samples and (ii) more polymorphisms within each gene analyzed individually and jointly are needed to more clearly elucidate possible effects between the proposed putative susceptibility genes and AD.

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6 Familial Alzheimer's Disease with Spastic Paraparesis Associated with a Mutation at Codon 261 of the Presenilin 1 Gene

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INTRODUCTION

Familial presenile Alzheimer's disease (AD) with symptoms beginning before the age of 55 years occurs in less than 1% of all AD patients. Mutations in the amyloid precursor protein (*APP*) gene on chromosome 21, the presenilin 1 (*PS1*) gene on chromosome 14, and the presenilin 2 (*PS2*) gene on chromosome 1 are known to be the cause of illness in 50% or more of families with presenile AD (Thinakaran, 1999). Mutations in the *PS1* gene are the most common cause with over 70 having been described (Cruts, 2000). Other than the early onset of symptoms, most *PS1* mutations have been associated with dementia with features typical for AD; however, in a small minority of these families there has been some heterogeneity in clinical features. Heterogeneity in clinical and pathologic phenotypes is known to occur in neurodegenerative diseases caused by other mutant genes, as in the cases of the *APP* and prion protein (*PRNP*) genes (Farlow et al., 1994; Ghetti and Gambetti, 1999; Hendriks and Van Broeckhoven, 1996). In addition to dementia, *PS1* mutations have been associated with headaches, myoclonic and/or generalized seizures, and pyramidal tract signs, in particular, spastic paraparesis (Crook et al., 1998; Ezquerra et al., 1999; Kwok et al., 1997; Lopera et al., 1997; van Bogaert et al., 1940; Verkkoniemi et al., 2000). In at least one of these families, a relatively unique neuropathological phenotype has been reported, which is characterized by beta-amyloid (Aβ) angiopathy in meningeal and cortical vessels and so-called 'cotton wool' plaques throughout the neocortex (Crook et al., 1998).

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We report the clinical and neuropathologic features in affected members from a new family with early-onset dementia and spastic paraparesis associated with a novel mutation at codon 261 of the *PS1* gene.

RESULTS

CLINICAL STUDIES

Clinical descriptions of four clinically affected individuals were obtained (patients A, B, C, D). DNA was extracted from blood or tissue from three of these individuals and from one at-risk family member. A detailed clinical and laboratory investigation of this disease was recently completed in patient B at the Indiana University School of Medicine.

Patient A, the father of patients B, C, and D, at age 38 had difficulty walking and attention and concentration deficits. An examination at age 39 revealed an impairment of memory for recent events, inability to handle abstract concepts, and a score of 82 on the Wechsler Adult Intelligence Scale (WAIS). His speech was mildly dysarthric and he had a fine tremor of outstretched arms, hyperreflexia in all extremities, sustained clonus at the ankles, and bilateral Babinski signs; his gait was broad based and spastic. One year later, he was severely demented with little ability to function in any activities of daily living; he was markedly dysarthric and his gait was slow, shuffling, and very spastic as well as having a dystaxic component. There was moderate finger to nose and heel to shin dystaxia. He progressively worsened and died at age 47.

Patient B, a male, began to complain of stiffness in his legs at age 34, and 18 months later he developed memory and cognitive problems that have gradually worsened. At age 37, he is forgetting people's names, forgetting to lock doors at his workplace and verbally perseverating by telling the same stories several times. He cannot find words, but has not been disoriented and still manages his money well.

The general physical examination was within normal limits, but neurological examination revealed poor short-term memory, and difficulty following multistep commands or carrying out complicated tasks and calculations. His speech was mildly dysarthric. A cranial nerve examination revealed only a mild decrease in facial expression. Cerebellar and sensory systems were normal. A motor examination revealed some minimal cogwheel rigidity in his left upper extremity and definite mild spasticity with catches in both of the lower extremities, worse on the left than the right. His muscle stretch reflexes were bilaterally brisk and he had bilateral Babinski signs; his gait was broad based and spastic, but stable.

Neuropsychological testing revealed severe recent memory deficit on list learning, prose recall, and figure recall tasks (1st percentile compared to

same-age peers). Visuospatial and constructional ability were mildly impaired (5th percentile of same age peers). There was mild to moderate dysgraphesthesia but no finger agnosia. Manual motor speed was slightly reduced on the left and dexterity was moderately impaired (< 1st percentile of same age peers). General naming ability and verbal fluency were within normal limits. Self-report of mood was also within normal limits. Mini-Mental State Examination was mildly impaired with a score of 22/30. The neuropsychological results indicated mild dementia with a prominent amnestic component.

CBC, metabolic screen, thyroid stimulating hormone, serum B-12 and serum folic acid levels were all within normal limits. Cerebrospinal fluid analyses revealed a normal level of total protein, glucose and cell count; however, an elevated tau protein level and decreased Aβ protein level consistent with AD were seen.

Visual evoked potential studies were normal. However, sensory evoked potentials (SEP) after stimulation of the left posterior tibial nerve showed delays consistent with lesions or dysfunction in central somatosensory pathways. Nerve conduction velocities and electromyographic studies were normal in the upper and lower extremities. Positron emission tomography with FDG revealed several focal areas of decreased metabolism, both in the cortex and in subcortical nuclei, particularly the right basal ganglia. T2 weighted magnetic resonance imaging (MRI) revealed subtle multifocal areas of increased signal throughout the cerebral white matter. However, FLAIR images showed at least a dozen 0.5–1.5 cm areas of increased signal scattered through the white matter and subcortical nuclei (Figure 6.1).

Patient C initially began having memory and gait problems at age 37. On examination at age 41, he had moderate dementia, increased muscle stretch

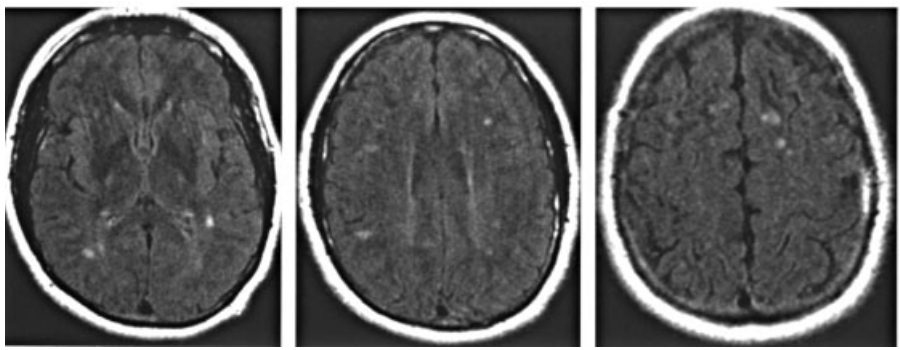

Figure 6.1. MRI scans of 37-year-old male (patient B) with 36-month history of spasticity and 18-month history of amnesia. FLAIR sequence in these three axial cuts reveals multiple subcortical lesions that were also present on T2 images, but less prominently seen

reflexes in all extremities, and bilateral Babinski signs. He had mild intension tremor in his upper extremities, but normal strength. His lower extremities were weak and he used a wheelchair. MRI studies were unremarkable. Over the next three years, he became globally demented and disabled in all activities of daily living. Currently at age 44, all extremities are severely spastic and the lower limbs have contractures. Peripheral blood as well as glucose, protein and cell counts in the cerebrospinal fluid were all normal.

Patient D, a 36-year-old female, initially developed spasticity and memory problems that rapidly progressed over the next four years until she was globally demented with severe spasticity and contractures in all extremities. She was cared for in a nursing home and had a feeding tube for three years until she developed severe pulmonary infection and died.

NEUROPATHOLOGIC STUDIES

Neuropathologic studies were carried out on patient D. The brain was severely atrophic and weighed 963 g. The neocortex was moderately atrophic and the substantia nigra was pale.

For neurohistology we used the hematoxylin and eosin, Heidenhain–Woelcke, Bodian, and thioflavin S methods. For immunohistochemistry we used monoclonal antibodies 10D5 and AT8, recognizing Aβ and abnormally phosphorylated tau, respectively.

Numerous Aβ immunopositive plaques and tau immunopositive neurofibrillary tangles (NFTs) were seen. Most of the neocortical plaques had the so-called 'cotton wool' appearance. Neuritic plaques were rarely seen. Thioflavin S showed the fluorescence associated with leptomeningeal and parenchymal arteries (Figure 6.2; see Plate I) to be brighter than that associated with 'cotton wool' plaques and NFTs (Figure 6.3; see Plate I). NFTs were most numerous in the neocortex and hippocampal formation; they were also present in the amygdala, basal ganglia, thalamus, substantia nigra, and pons. Myelin stain revealed pallor of the lateral pyramidal tracts at the level of the spinal cord and loss of motor neurons in the anterior horns.

Aβ deposits were numerous not only in most gray matter areas but also in the deep white matter of the centrum semiovale. Aβ deposits, most numerous in the neocortex, were also present in the basal ganglia, thalamus, cerebellum, pons and spinal cord. Leptomeningeal and parenchyma vessels showed a severe Aβ angiopathy.

GENETIC STUDIES

Informed consent was obtained from all living participating family members and blood samples were collected. Genomic DNA was obtained from frozen brain tissue from patient D and from whole blood of the three surviving siblings (Madisen et al., 1987; Nichols et al., 1990). The DNA was analyzed

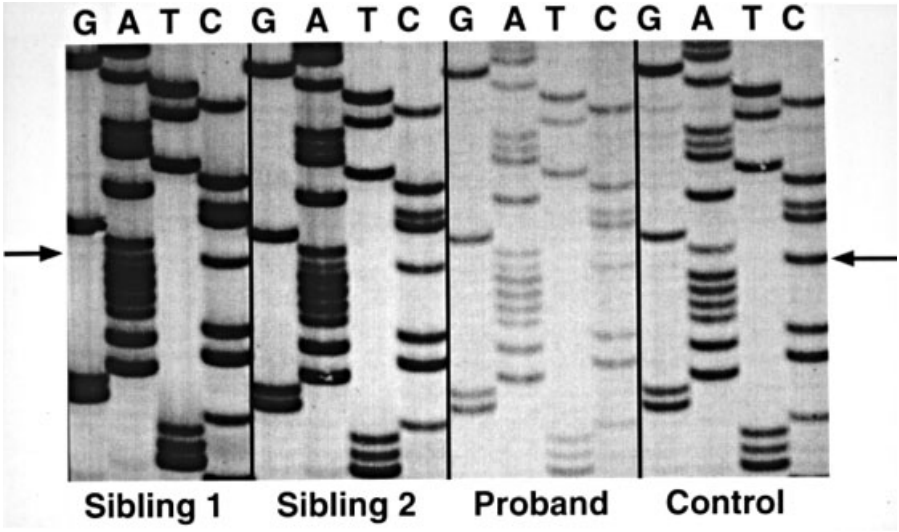

Figure 6.4. Direct sequence of exon 9 of the *PS1* gene. This sequence shows the complement strand. The left arrow points to both an adenosine and a cytosine, which are seen in the proband's and the two siblings' sequences. Only cytosine is observed in the normal control sequence

by direct sequencing of exons 3–13 of the *PS1* gene, and exons 16 and 17 of the *APP* gene (Nichols et al., 1990). Primers from the intronic sequences flanking each exon were used so that the entire exon sequence and the splice signals could be analyzed. Sequences were compared to that of a normal control and to the published gene sequences. Exon 9 of the *PS1* gene was sequenced from DNA samples from 60 unrelated normal controls. The apolipoprotein E (*APOE*) gene was also analyzed by amplification followed by HhaI digestion (Wenham et al., 1991).

The DNA sequence of exons 3–8 and 10–13 of the *PS1* gene and exons 16 and 17 of *APP* were normal in patient D. Sequencing of exon 9 of *PS1* showed a single nucleotide (G to T) substitution in one allele (Figure 6.4). This causes an amino acid change in residue 261 (phenylalanine for valine), which is located in the sixth transmembrane domain of the PS1 protein. This same mutation was also found in two of patient D's affected siblings but not in an at-risk sibling. This mutation was not observed in 60 normal controls (120 normal chromosomes). *APOE* genotyping revealed an E3/E4 genotype for patient D and one sibling and E3/E3 for the other sibling.

DISCUSSION

The clinical phenotype of patients in this family is relatively stereotyped. Onset of symptoms occurs in the late 30s with spastic paraparesis and

dementia. As the illness progresses, a tremor of the upper extremities and ataxia of the lower extremities may develop. In the later stages, there is general paralysis of motor function and profound dementia. Neuropathologically there are 'cotton wool' Aβ deposits throughout the cerebral cortex. Severe amyloid angiopathy is seen in the leptomeningeal and parenchymal vessels of the cerebrum and cerebellum. There is a loss of fibers in the corticospinal tracts consistent with the spastic paraparesis characteristic in these patients.

An observation of particular interest is the fact that SEP, PET, and MRI showed evidence of multifocal, widely distributed abnormalities in patient B.

The disease process appears multifocal, as evidenced by widely distributed abnormalities detected in patient B by SEP, PET, and MRI. The FLAIR images demonstrated multiple subcortical lesions, particularly in the white matter.

An early description of AD with spastic paraparesis was reported in 1940; it was characterized neuropathologically by an amyloid angiopathy, plaques in the neocortex and cerebellum, NFTs, and degeneration of the corticospinal tracts (van Bogaert et al., 1940). No imaging studies were available at that time. More recently, there has been a report of two sisters from a Japanese family developing progressive spastic paralysis and dementia beginning in their mid-50s (Sodeyama et al., 1995). MRI demonstrated frontal atrophy and single-photon emission computerized tomography showed only diffuse hypoperfusion. The patients had plaques and NFTs in the cortex as well as fiber loss in the corticospinal tract. There was particularly severe astrogliosis and neuronal loss in the medial part of the thalamus. Unlike our family, white matter and cerebellum were reported to be normal. Three Australian families with AD and spastic paraparesis have been described with *PS1* mutations; however, details of the clinical and neuropathological features were not reported (Kwok et al., 1997).

The Finnish family with spastic paraparesis and dementia as a result of a deletion of exon 9 of the *PS1* gene is the largest and best-characterized kindred (Verkkoniemi et al., 2000). Based on the data available in 14 patients, the mean age at onset was 51 with clinical features of dementia, dysarthria, spasticity, and in most patients impaired fine motor coordination of the hands. The clinical features of this family are very similar to the family reported here except for the age of onset. MRI studies in three members of the Finnish kindred revealed generalized cortical atrophy with some greater severity in the temporal lobes. Neuropathologically, 'cotton wool' plaques throughout the cortex, NFTs, and amyloid angiopathy were described (Crook et al., 1998); no mention was made of Aβ deposition of the white matter as seen in our family. In the family described here, the 'cotton wool' plaques as well as the amyloid angiopathy were comparable to that described in the Finnish family.

Finally, clinical investigations in a member of this family suggest that MRI using the FLAIR method may be useful for investigating patients with AD

caused by *PS1* mutations. In these families, a detailed neuropathologic examination that includes systems generally not involved in AD should be considered in order to better characterize and define the spectrum of disease.

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7 Genetic Analysis of the Presenilin Pathway in *Drosophila*

IZHAR LIVNE-BAR AND GABRIELLE L. BOULIANNE

INTRODUCTION

Alzheimer's disease (AD) is a degenerative disorder of the central nervous system that causes progressive memory and cognitive decline during mid- to late adult life. Insight into the mechanisms underlying AD has come from the identification of mutations in three genes, amyloid precursor protein (*APP*), presenilin 1 (*PS1*) and presenilin 2 (*PS2*), which have been linked with early-onset familial AD (FAD). However, while mutations in these genes are associated with almost 50% of FAD, they account for less than 5% of all AD cases, which are largely sporadic and late onset. This suggests that other genes must play a role either as causative or susceptibility factors in AD. Recently, increasing evidence has implicated certain alleles of apolipoprotein E (i.e. apoE-4) (Strittmatter and Roses, 1996) and α-2-macroglobulin (A2M; Blacker et al., 1998) as risk factors or susceptibility loci in AD. However, these two loci alone are unlikely to account for all cases of AD and additional loci need to be identified and their role in AD determined.

A powerful method of identifying additional loci implicated in AD is to characterize genetic modifiers of genes such as presenilins or *APP* which have already been linked to AD. A gene whose function modifies the phenotype caused by a gene associated with AD is likely to function in a biochemical pathway with this gene and may therefore also be linked to AD. While it is possible to identify some of these modifiers by a candidate gene approach, as was used to identify *A2M*, it is currently impossible to carry out large-scale genetic screens to identify new modifier loci in mice or humans. In contrast, such modifier screens are common and simple in invertebrates such as *Drosophila*.

Here, we describe how we were able to generate dominant adult phenotypes by ectopic expression of the *Drosophila* presenilin gene (*Dps*) and use these to search for genetic modifiers. Not surprisingly, several of the modifiers we identified corresponded to mutations in members of the Notch

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signaling pathway. This is consistent with previous studies from our laboratory and others (Guo et al., 1999; Levitan and Greenwald, 1995; Ye and Fortini, 1998), which showed that presenilins genetically interact with the Notch pathway. Additionally, we found that mutations in genes such as dynamin and clathrin, which are required for endocytosis, also modify the *Dps* phenotype. Characterization of these and other presenilin genetic modifiers will provide additional insight into the normal function of presenilins, which are a highly conserved, novel family of proteins. More importantly, since genetic modifiers of presenilins could represent additional causative or risk factors in AD they may also provide additional therapeutic targets for this devastating disease.

DROSOPHILA PRESENILIN

We have previously described the cloning and molecular characterization of the presenilin gene in *Drosophila melanogaster* (Boulianne et al., 1997; Guo et al., 1999). Unlike vertebrates, *Drosophila* has a single presenilin gene which is located at position 77B–C on the left arm of the third chromosome. The structure of the *Dps* gene is remarkably similar to that of *Caenorhabditis elegans* and human presenilin genes, and comparison of the predicted amino acid sequence of Dps with that of human presenilins reveals 53% overall sequence identity. Residues predicted to comprise transmembrane domains and the C-terminal portion of the protein show the highest level of sequence identity whereas the large hydrophilic loop between TM6 and TM7 is poorly conserved. Nonetheless, many of the residues mutated in *PS1* or *PS2* and associated with FAD, including several within the loop region, are conserved. In addition, we have found that proteins that bind to this region of *Dps* also bind to human presenilins, suggesting that the residues within the loop form an important functional or structural domain of the presenilins (Guo et al., 2000).

To determine the function of *Dps* we generated and characterized both point mutations and deletions in the *Dps* gene and also created transgenic lines that express either wild-type or FAD-associated mutations in *Dps*. We found that loss-of-function mutations in *Dps* lead to lethality during the pupal stage of development and give rise to underdeveloped eye and wing imaginal discs and defects in neuronal differentiation. We also demonstrated that mutations in *Dps* genetically interact with components of the Notch signaling pathway, including *Notch* and *Delta*, which is required for numerous cell fate decisions in a variety of species. Moreover, we found that *Dps* affects Notch subcellular localization and signaling (Guo et al., 1999). More recently, we have also demonstrated that both the lethality as well as the wing and eye phenotypes of *Dps* mutants could be completely rescued using a 4 kb genomic

construct that encompasses the entire *Dps* locus, indicating that the gene is solely responsible for the phenotype.

We have also generated transgenic lines in which either the wild-type or a mutated (FAD-associated) *Dps* gene is expressed under the transcriptional control of the inducible GAL4/UAS system (Brand and Perrimon, 1993). We find that ectopic expression of a *Dps* transgene in third larval instars gives rise to dominant adult phenotypes in the eye, notum, and wing (Figure 7.1). Interestingly, the phenotypes arising from overexpression of the mutant transgene always appear weaker than those caused by overexpression of the

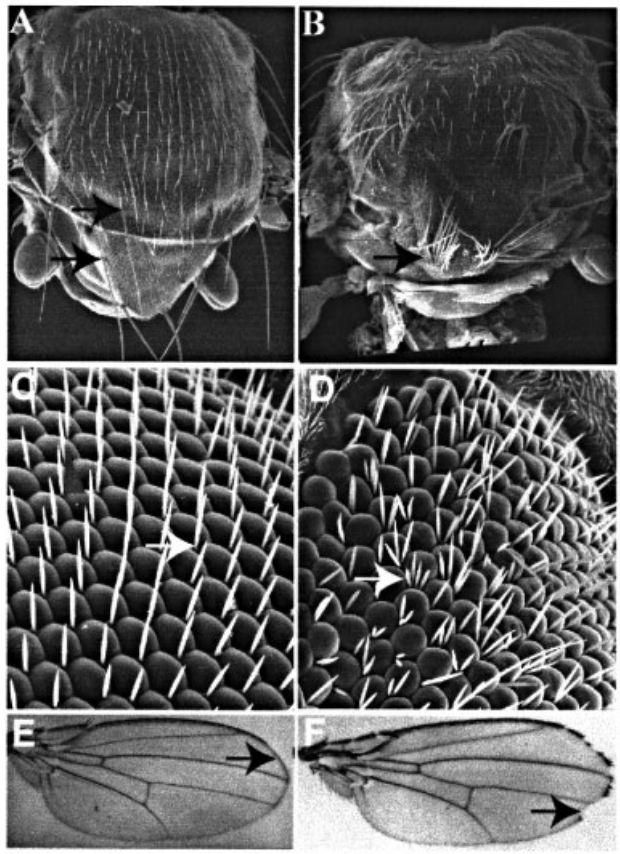

Figure 7.1. Overexpression of *Dps* gives rise to dominant adult phenotypes in the notum, eye, and wing. The wild-type regular pattern of sensory bristles in the notum and eye (arrows in A and C) is replaced by an extraneous-bristle phenotype in *Dps* transgenics (B and D). The smooth margin of the wild-type wing (arrow in E) is replaced by a notched-margin phenotype in *Dps* transgenics (F)

wild-type gene, suggesting that the FAD mutations are behaving as loss-of-function alleles in this assay (data not shown). Specifically, we find that overexpression of wild-type or mutant *Dps* in the thoracic region of the wing imaginal disc, using the *pannier*-GAL4 driver, results in supernumerary sensory bristles that are often misoriented both on the notum and scutellum (Figure 7.1A,B). In the developing eye disc, ectopic expression of *Dps* using the *sevenless*-GAL4 promoter, gives rise to supernumerary interommatidial bristles (Figure 7.1C,D) while the pan-neuronal GAL4 line, C155, gives rise to a rough eye (data not shown). Finally, ectopic expression of *Dps* along the presumptive wing margin using a *cut*-GAL4 driver, gives rise to a notched wing phenotype (Figure 7.1E,F). Although there are slight variations in the severity of the phenotype, all three phenotypes were over 90% penetrant at 29 °C. Not surprisingly, all three phenotypes can be modified by introducing mutations in *Notch* or *Delta*, further demonstrating a key role for presenilins in the Notch pathway.

GENETIC MODIFIERS OF *Dps*

To determine if *Dps* interacts with genes other than those involved in Notch signaling, we first generated recombinant chromosomes that contained both the GAL4 driver (*pannier*-GAL4 or *cut*-GAL4) and our UAS-Dps transgenes. These recombinant *Dps* lines were then crossed to a collection of homozygous lethal P-element mutations obtained from the Berkeley *Drosophila* Genome Project. This collection consists of single P-element insertions that mutate 25% of the vital genes in the fly (Spradling et al., 1999). The progeny of these crosses were classified as modifiers if the transheterozygotes that contained both the recombinant *Dps* chromosome and a single copy of the lethal P-element reproducibly enhanced or suppressed sensory bristle or wing margin phenotypes. To further confirm the interaction between a potential modifier and *Dps*, we also crossed our recombinant *Dps* lines to deficiencies that uncovered the same chromosomal region identified by the P-element lethal line. Examples of both enhancers and suppressors are shown in Figure 7.2. Of the 625 P-element insertion lines tested thus far, six were found to enhance two of the *Dps* phenotypes tested while fourteen were identified as suppressors. A complete analysis of the P-element lines identified is described elsewhere (Livne-Bar et al., in preparation). Several of the interacting P-element lines mapped to known genes including an insertion in *thread*, the *Drosophila* homologue of the apoptosis inhibitor protein, DIAP1 (Hay et al., 1995). Interestingly, we also found that the *Dps* phenotypes could be modified by mutations in genes that have been implicated in endocytosis events within the cell. Since presenilins have been implicated in both protein processing and trafficking events within cells we

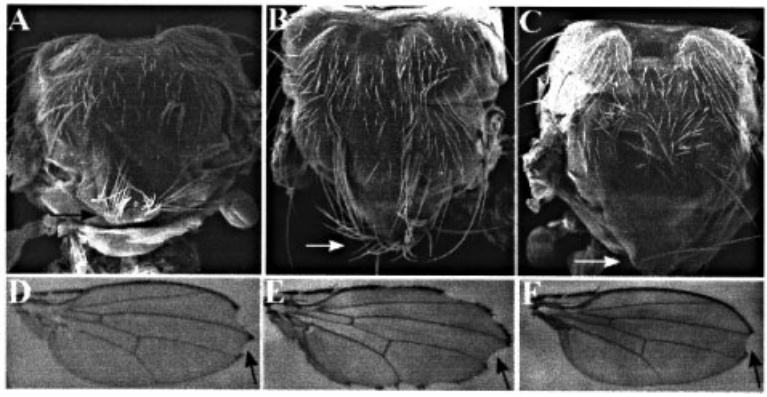

Figure 7.2. Modulation of *Dps* phenotypes by suppressor and enhancer mutations. Overexpression of *Dps* in the notum and the wing leads to extraneous bristle phenotype in the notum (A) and notching phenotype in the wing (D). Mutations that enhance the sensory bristle phenotype (B) or the notching (E) are classified as enhancers while mutations suppressing these phenotypes (C and F) are termed suppressors

decided to further investigate the interaction of *Dps* with various components of the endocytic pathway.

Dps INTERACTS WITH COMPONENTS OF THE ENDOCYTOSIS MACHINERY

The first indication that *Dps* might be interacting with components of the endocytic machinery was that a mutation in *shibire*, the *Drosophila* homologue of dynamin, could suppress the ectopic *Dps* phenotypes. Subsequently, we have also found that mutations in the clathrin heavy chain gene could also suppress *Dps* phenotypes (Figure 7.3). Both dynamin and clathrin are known to be required for recycling of vesicles from the plasma membrane. Clathrin is organized as a complex consisting of three pairs of heavy and light chain proteins that can further polymerize into cages capable of capturing vesicles membranes during coated vesicle formation. Dynamin, a GTPase, is thought to be required for 'pinching off' of vesicles from the plasma membrane (DeCamilli et al., 1995). These vesicles and the proteins contained within them are then recycled through an endosomal compartment where they can undergo further sorting within the cell. Interestingly, previous studies have also shown that shibire/dynamin could also genetically interact with Notch. To determine if *shibire* modified the *Dps* phenotype by affecting Notch, we examined the expression of Notch in transgenic lines that overexpress *Dps* in the presence or absence of *shibire*. As we reported previously (Guo et al.,

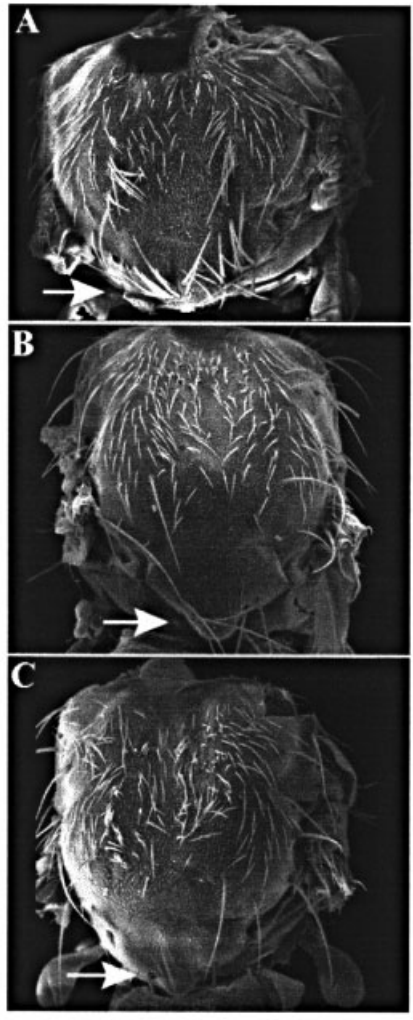

Figure 7.3. *Dps* interacts with components of the endocytic machinery. Scanning electron microscopy showing the dominant sensory bristle phenotype of a *Dps* transgenic fly (arrow in A). This phenotype is suppressed by a shibire mutant (B) and by a clathrin heavy chain mutation (C)

1999), we find that Notch specifically accumulates within cells that overexpress *Dps* (Figure 7.4). However, when a *shibire* temperature sensitive mutation is introduced into this background and the larvae are raised at the restrictive temperature, Notch fails to accumulate within *Dps*-expressing cells even though *Dps* levels are unaffected (Figure 7.4).

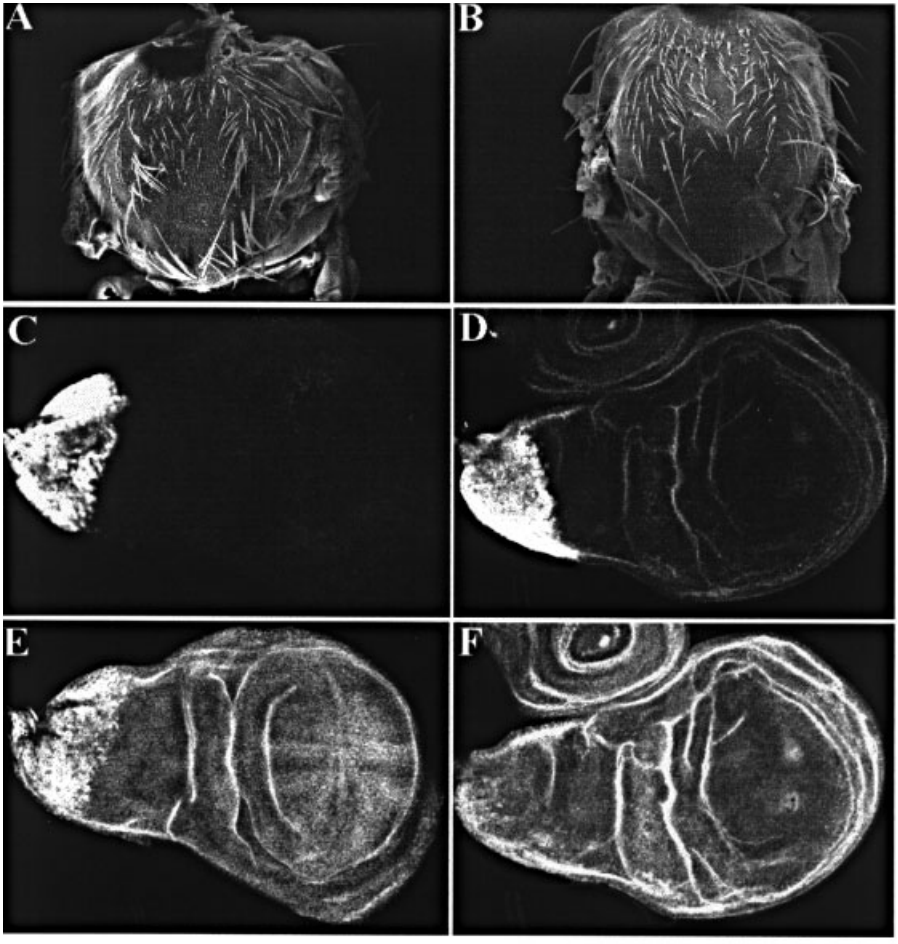

Figure 7.4. Shibire suppresses a dominant *Dps* phenotype by affecting Notch accumulation in *Dps* expressing cells. (A) Scanning electron microscopy (SEM) showing the thorax sensory bristle phenotype of pnrGAL4-Dps. (B) This phenotype is suppressed by a shibire temperature-sensitive mutation when raised at the restrictive temperature. (C) Immunostaining for *Dps* protein reveals the expression of *Dps* in the wing disk of pnrGAL4-*Dps* larvae. (D) The wing disk immunostained with a *Notch* antibody shows *Notch* accumulating in the *Dps* expressing cells. (E) In pnrGAL4-*Dps* larvae with a shibire temperature-sensitive mutation, *Dps* protein levels are unaffected. (F) Notch protein, however, fails to accumulate in the *Dps* expressing cells

CONCLUSIONS

Genetic screens have provided a powerful way of identifying components of various signaling pathways. Here, we describe the use of genetic modifier screens in *Drosophila* to identify components of the presenilin pathway.

Specifically, we have used the GAL4/UAS system to overexpress *Dps* and generate dominant adult phenotypes that could then be used to identify additional *Dps* interacting genes. Not surprisingly, we find that mutations in several members of the Notch signaling pathway, including *Notch* and *Delta*, can modify the *Dps* phenotypes. This is consistent with previous studies demonstrating that presenilins play an integral role in Notch signaling during development.

Interestingly, we also found that mutations in *shibire/dynamin* and the clathrin heavy chain, two genes known to play a role in endocytosis, also suppress the *Dps* phenotypes and abolish *Dps*-dependent Notch accumulation. Previous studies have also shown that *shibire* can interact genetically with *Notch* and *Delta* in sensory bristle specification in the notum (Seugnet et al., 1997). These studies suggest that the interaction of *shibire* with *Dps* reflects its general role in the Notch pathway. Recent studies from our laboratory (Guo et al., 1999) have shown that presenilins are required for trafficking of Notch receptors within the cell. Specifically, we find that Notch accumulates on the plasma membrane in *Dps* mutants. Similar studies in *Drosophila* and mice have also shown that presenilins are required for cleavage of the intracellular domain of Notch (De Strooper et al., 1999; Song et al., 1999; Struhl and Greenwald, 1999; Ye et al., 1999). Whether presenilins are themselves directly responsible for cleaving Notch remains unknown, as is the subcellular location where cleavage occurs. However, several reports demonstrating a tight association between presenilin complexes and cleavage activity strongly suggest that presenilins either encode the γ-secretase responsible for Notch cleavage or act as a co-factor (Li et al., 2000a,b). The fact that presenilins interact with the endocytic machinery and that this interaction affects Notch accumulation raises the interesting possibility that Notch receptors must be endocytosed before cleavage can occur either in an endosomal compartment or elsewhere within the cell.

In addition to identifying components of the Notch pathway, we identified several other modifiers which suggest additional roles for presenilins. One example is *thread*, which encodes a homologue of DIAP1, an apoptosis inhibitor protein (Hay et al., 1995). Thread is not reported to interact with the Notch pathway, but rather with the apoptotic effectors reaper and grim, and it inhibits caspase-mediated apoptosis *in vitro* (Kaiser et al., 1998). The interaction of *Dps* with the apoptotic process is consistent with previous studies where cultured neurons transfected with Alzheimer's related *PS1*, *PS2* or *APP* mutations showed heightened sensitivity to apoptosis (Wolozin et al., 1996; Xu et al., 1999).

In summary, the screen for *Dps* modifiers is a valuable approach to identify *Dps* interacting factors. Some of these factors may be related to known activities, such as interactions with the Notch and apoptotic pathways; others are likely to be novel proteins with roles related to normal or pathogenic activities of the presenilins.

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8 Molecular Genetics and Transgenic Modeling of the Tauopathies

JADA LEWIS, MATT BAKER, MARJON VAN SLEGTENHORST AND MIKE HUTTON

Tau FUNCTION AND DYSFUNCTION

Tau belongs to a family of proteins known as microtubule-associated proteins (MAPs) (Weingarten et al., 1975). Involved in microtubule assembly and stabilization in neurons and probably glia, tau is important for the maintenance of the cytoskeleton (LoPresti et al., 1995). Although tau is normally localized mainly in the axons, where it plays a role in axonal motor-protein transport (Ebneth et al., 1998), somatodendritic localization of tau is thought to occur during tau dysfunction. This altered localization of tau is characteristic of pre-tangle and neurofibrillary tangle inclusions found in neurons and occasionally glia in certain neurodegenerative diseases. Neurofibrillary tangles (NFTs) composed of hyperphosphorylated tau protein are key intracellular lesions in numerous neurodegenerative diseases including Alzheimer's disease (AD), frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17), progressive supranuclear palsy (PSP), and corticobasal degeneration (CBD).

Tau is regulated in part by alternative splicing of exons 2, 3, and 10 to generate six tau mRNAs encoding six different tau isoforms (3R0N, 3R1N, 3R2N, 4R0N, 4R1N, 4R2N) (Figure 8.1). Exons 2 and 3 encode N-terminal tau domains of uncertain function, whereas exon 10 encodes one of four microtubule-binding domains. Thus, alternative splicing of exon 10 gives rise to tau isoforms with 4 (exon 10+) or 3 (exon 10−) microtubule-binding repeats (4R and 3R tau, respectively). An approximate 1:1 ratio of 4R:3R tau protein normally exists in adult human brain as opposed to the near exclusive presence of 4R tau isoforms in the murine adult brain (Goedert et al., 1989; Kosik et al., 1989). Aggregation of different isoforms of tau results in NFTs composed of filaments with various morphologies. In AD, the NFTs are

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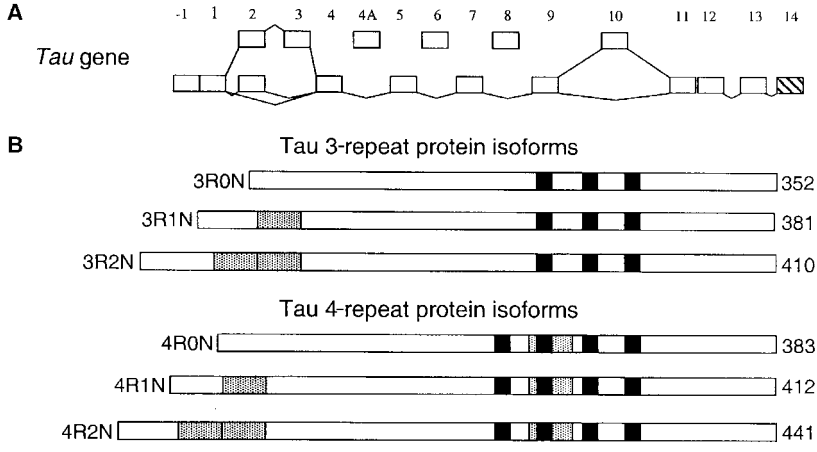

Figure 8.1. Schematic of the *tau* gene, alternative splicing, and protein isoforms. (A) The *tau* gene is encoded on chromosome 17q21. Alternatively spliced exons 2, 3, and 10 are shown above the constitutive exons. Exons 4A, 6, and 8 are generally excluded from human tau mRNA. Most tau transcripts include the intron between exons 13 and 14. (B) Exons 2, 3, and 10 (shaded boxes) are alternatively spliced to yield six tau isoforms (3R0N, 3R1N, 3R2N, 4R0N, 4R1N, 4R2N). The microtubules-binding domains (black boxes) are encoded by exons 9–12

largely composed of paired helical filaments composed of all six tau isoforms with a small number of straight filaments; however, in PSP straight tau filaments composed mainly of four repeat isoforms are the major component of the tau inclusions.

Tau MUTATIONS AND FTDP-17

The association of tau mutations with FTDP-17 provided the first direct evidence that tau dysfunction can lead to neurodegeneration (Hutton et al., 1998; Poorkaj et al., 1998; Spillantini et al., 1998b). Nearly 20 tau mutations have been associated with FTDP-17 (Figure 8.2). These mutations include both intronic and exonic mutations that disrupt the alternative splicing of tau exon 10 as well as missense mutations that alter the function of tau. Tau splicing mutations (i.e. S305N/S, N279K, exon 10 +3, +13, +14, +16) generally increase the ratio of tau isoforms containing four microtubule-binding domains to three microtubule-binding domains (Hutton et al., 1998; Spillantini et al., 1998b; Hasegawa et al., 1999; Stanford et al., 2000). However, one unique deletion mutation, ΔK280, has been shown to inhibit exon 10 splicing *in vitro*. The effect of this mutation (ΔK280) suggests that disruption of the normal 4R:3R ratio, rather than simply an increase in 4R tau, is sufficient for the development of FTDP-17. However, the effect of the

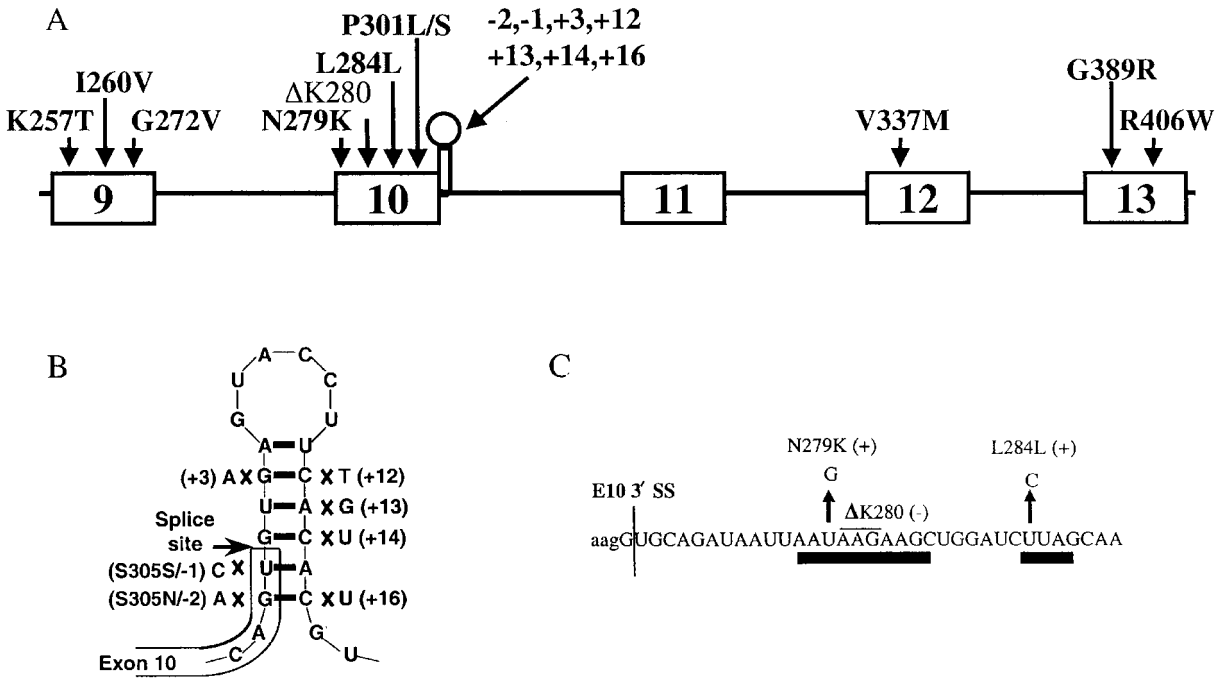

Figure 8.2. Mutations in the *tau* gene associated with FTDP-17. (A) All known pathogenic tau mutations are within exons 9–13, as shown. The majority of the tau mutations are located in exon 10 or near the exon 10-intron splice site. (B) The proposed stem-loop spans the exon 10 downstream splice site. Mutations within this stem-loop, as shown, are predicted to destabilize the structure and allow the U1snRNP greater access to the splice site, thereby increasing exon 10 inclusion. (C) Some mutations (N279K and ΔK280) within exon 10 alter a polypurine positive *cis*-element. The N279K mutation strengthens this element, resulting in increased exon 10+ mRNA, while ΔK280 abolishes this element and reduces exon 10+ mRNA. The L284L eliminates a proposed negative regulatory element, thereby increasing inclusion of exon 10. The (+) and (−) symbols indicate the effect of the mutation on splicing

ΔK280 has yet to be demonstrated *in vivo*. Splicing mutations affect multiple intronic and exonic *cis*-elements that regulate E10 splicing (Figure 8.2). One group of mutations close to the 5′ splice site of E10 has been proposed to disrupt a stem-loop structure that spans the downstream splice site of exon 10 and regulates U1snRNP recognition (Hutton et al., 1998). A mutation within this stem-loop (i.e. exon 10 +13) is predicted to destabilize the secondary structure, increasing U1snRNP access to the exon 10 splice site; therefore, more exon 10+ mRNA encoding four-repeat tau would be produced. A second group of mutations affect *cis*-elements that occur within exon 10. The N279K mutation strengthens a polypurine element and increases exon 10 incorporation while the ΔK280 mutation eliminates this same polypurine element and inhibits splicing (D'Souza et al., 1999).

Most tau missense mutations (i.e. K257T, I260V, G272V, P301L, V337M) are located within exons 9–12 that encode the four microtubule-binding domains. These missense mutations decrease both tau binding to microtubules and tau-induced microtubule assembly *in vitro* (Hasegawa et al., 1998; Hong et al., 1998; Dayanandan et al., 1999; Rizzu et al., 1999). One possible mechanism for these mutations is that reduced microtubule binding increases the tau available for hyperphosphorylation and aggregation. Some tau missense mutations have also been demonstrated to increase tau self-aggregation and polymerization *in vitro*, further suggesting a role for accelerated aggregation in the mechanism of these mutations (Goedert et al., 1999; Nacharaju et al., 1999). Additionally, tau with either the V337M or R406W mutation has been reported to be less susceptible to calpain I degradation, suggesting another possible mechanism by which specific mutations increase the amount of tau available for polymerization into filaments (Yen et al., 1999). For a complete review on the proposed mechanisms of the tau missense and splicing mutations, see Hutton (1999) and van Slegtenhorst et al. (2000).

No tau mutations have been identified in any other neurodegenerative disorder. However, variability in the *tau* gene has also been shown to be a risk factor for two other apparently sporadic tauopathies, PSP and CBD (Conrad et al., 1997; Baker et al., 1999; Higgins et al., 1998; Houlden et al., unpublished work).

Tau POLYMORPHISMS AND PSP AND CBD

PSP is a rare parkinsonian movement disorder that is associated with early postural instability and supranuclear vertical gaze palsy (Litvan and Hutton, 1998). The brains of patients display NFTs that are primarily localized to the subcortical region. The tangles that are observed in PSP contain predominantly four-repeat tau isoforms consisting of straight filaments (Spillantini et al., 1998a).

An initial study of tau variability in PSP demonstrated that the common allele (A0) of a dinucleotide polymorphism, between tau exon 9 and exon 10, was associated with the development of PSP. The A0 allele contains 11 repeats of the TG dinucleotide sequence, whereas A1, A2, A3, and A4 contain 12, 13, 14, and 15 repeats, respectively. An excess of A0/A0 homozygotes was associated with pathology-confirmed PSP cases but not within AD or parkinsonism-dementia complex of Guam cases (Conrad et al., 1997). Subsequent studies by our group have further shown that this tau intronic dinucleotide polymorphism is inherited as part of two extended haplotypes (H1 and H2) that cover the entire *tau* gene (>100 kb) (Baker et al., 1999) (Figure 8.3). A series of tau small nucleotide polymorphisms (SNPs) first identified in FTDP-17 patients was examined for their association with PSP in an effort to define a precise mechanism of genetic susceptibility for this apparently sporadic disorder. The eight SNPs were located in exons 1, 2, 3, 9 (three polymorphisms), 11, and 13 of the *tau* gene. Using these eight polymorphisms, two different haplotypes (H1 and H2) were defined. The polymorphisms were in complete disequilibrium with each other; no recombination was observed between the H1 and H2 haplotypes in approximately 200 Caucasian samples examined. The previously defined A0 allele as well as the A1 and A2 alleles were only inherited with the H1 haplotype, whereas the A3 and A4 alleles were inherited with the H2 haplotype. Inheritance of two copies of the common haplotype (H1) in the *tau* gene is strongly associated with the development of PSP (87.5% in PSP cases, 62.8% in controls; $\chi^2 = 13.85$, $p < 0.001$) (Baker et al., 1999). Additionally, Baker et al. (1999) found no tau mutations in exons 9–13 in 60 PSP patients as well as the entire tau coding in 27 PSP patients. Recent data has provided evidence that the same tau H1 haplotype association is a risk factor for developing another tauopathy, CBD, that has previously been thought to be sporadic (Houlden et al., unpublished work). It is currently unclear, however, which specific polymorphism or combination of polymorphisms within the tau H1 haplotype produces the increased risk for the development of PSP and CBD. One possibility is that exon 10 splicing is altered in PSP and CBD, which is consistent with the selective deposition of 4R tau. However, whatever the precise pathogenic mechanism, the association of a specific tau haplotype with PSP/CBD clearly provides further genetic evidence for a central role for tau in the pathogenesis of PSP and CBD.

ANIMAL MODELS OF NEUROFIBRILLARY TANGLE FORMATION

The role of tau dysfunction in the formation of NFTs, the relationship of tangle formation to other pathogenic events, and factors that modify tangle formation have been difficult to study in humans due to the lack of early-stage

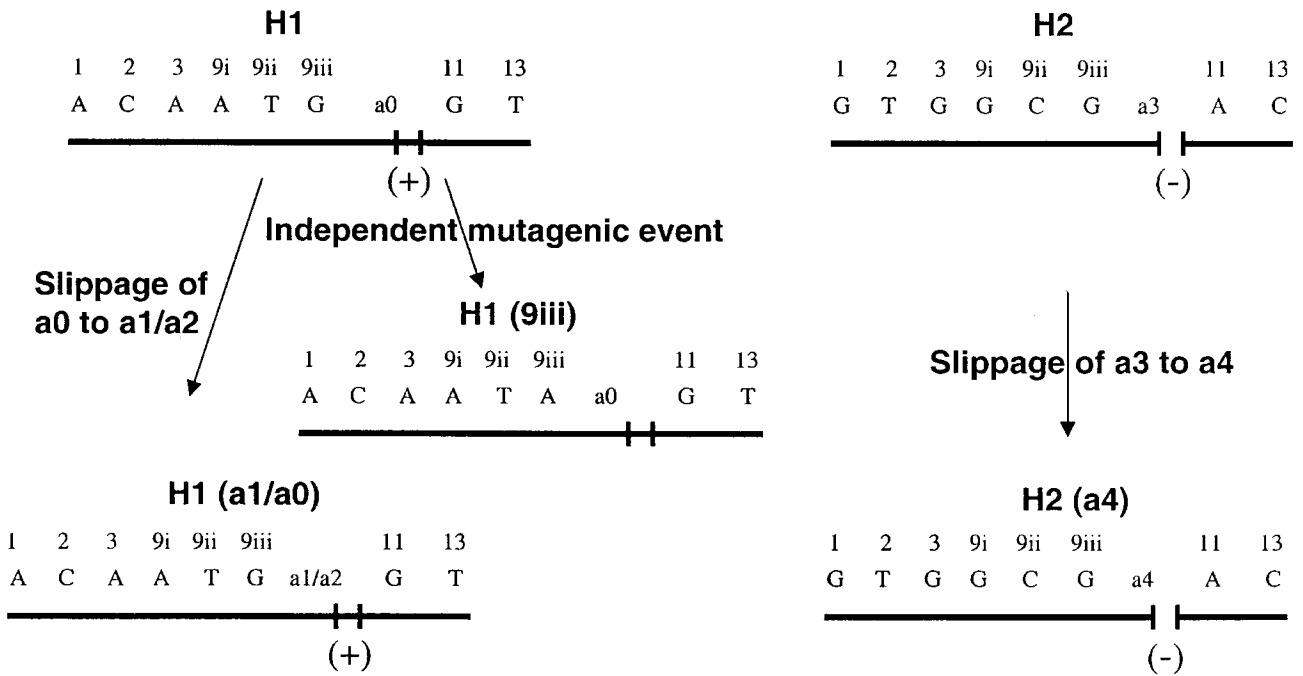

Figure 8.3. Human tau haplotypes and association with PSP and CBD. Schematic representation of human *tau* gene haplotypes. Ancestral haplotypes H1 and H2 are defined by a series of polymorphisms throughout the *tau* gene (>100 kb) that are in complete linkage disequilibrium with each other. H1 and H2 have been modified by subsequent mutational events (examples are shown). However, no recombination is observed between H1 and H2 haplotypes (H1–H1 and H2–H2 recombination may occur). The (+) and (−) symbols indicate a deletion/insertion polymorphism upstream of exon 10; a0–a4 are alleles of the dinucleotide polymorphism between exons 9 and 10. Inheritance of the H1/H1 genotype is a significant genetic risk factor for the development of PSP and CBD. However, the critical polymorphism(s) that influence pathogenesis have not yet been identified

brains from people with tauopathies. Numerous mouse models aimed at modeling the amyloid plaque pathology of AD have been generated by expressing mutant amyloid precursor protein and mutant presenilin-1 protein (reviewed in Duff, 1998). Amyloid plaques accumulate in the brains of many of these models; however, with the exception of tau-positive neurites, tau pathology has been absent in all of these models. In order to model tau dysfunction, multiple groups have now produced tau transgenic mice (Table 8.1).

Three tau transgenic models have been reported which overexpress the longest isoform of wild-type four repeat tau (4R2N). The 4R2N model with the lowest transgene expression (10% of mouse endogenous tau) showed somatodendritic localization of tau in the neurons reminiscent of the 'pre-tangle' state seen in some neurodegenerative conditions, including AD; however, no NFTs were observed (Gotz et al., 1995). Somatodendritic 'pre-tangle' staining of neuronal tau was also seen in 4R2N mice with higher expression levels (up to 6.6× overexpression) (Spittaels et al., 1999; Probst et al., 2000). The 4R2N animals with the highest expression lacked a normal escape extension under tail elevation and showed motor disturbances in rod walking and inverted grid tasks which was consistent with the pathology evident in these animals. These tau transgenic animals were largely characterized by axonopathy with dilated axons containing neurofilament, microtubuli, mitochondria, and vesicles in the brains and spinal cords. Dystrophic neurites that stained with Alz50 and MC-1 were also identified. Astrogliosis (GFAP reactivity) was evident in the line with the highest wild-type transgene expression. However, thioflavin S and silver staining again failed to demonstrate NFTs, and neuronal loss was not evident. Recently, Spittaels et al. (2000) crossed the 4R2N wild-type animals with mice overexpressing a constitutively active glycogen synthase 3 beta (GSK3β). Significantly, the GSK3β-induced tau phosphorylation resulted in a rescue of the characteristic axonopathy and motor phenotype observed in the single wild-type tau 4R2N transgenic animals. Additionally, this forced tau phosphorylation did not result in NFT formation or an increase in insoluble tau protein. These results indicate that GSK3β-promoted tau hyperphosphorylation does not directly result in the abnormal tau aggregation observed in neurodegenerative diseases.

Given the lack of tau neurofibrillary pathology in both the amyloid and the four-repeat tau transgenic models, it was suggested that species differences in tau expression might explain the inability to model tau dysfunction in mice. Since adult mice normally express almost exclusively 4R tau isoforms (Kosik et al., 1989) whereas adult humans normally express a 1:1 ratio of 4R to 3R tau, it seemed possible that this lack of 3R tau in mice could be a crucial difference. Two reports of mouse models overexpressing the shortest isoform of wild-type tau (3R0N) have been made, with expression ranging from below endogenous levels to 15× overexpression (Brion et al., 1999; Ishihara et al.,

Table 8.1. Published tau transgenic models

Reference	Transgene*	Expression	Neuropathology	Behavior
Gotz et al. (1995)	Hu Thy-1 4R2N WT tau	Less than endogenous	Pre-tangles	None
Spittaels et al. (1999)	Mo Thy-1 4R2N WT tau	4×endogenous	Pre-tangles, axonopathy, neurogenic atrophy, gliosis	Motor deficits
Probst et al. (2000)	Mo Thy-1 4R2N WT tau	1.5×endogenous (homozygotes)	Pre-tangles, axonopathy, neurogenic atrophy, insoluble tau	Motor deficits
Brion et al. (1999)	Mo HMG-CR 3R0N WT tau	Less than endogenous	Pre-tangles, neuronal and glial hyperphosphorylated tau	None
Ishihara et al. (1999)	Mo PrP 3R0N WT tau	Up to 15×endogenous	Argyrophilic intraneuronal inclusions, gliosis, reduced axonal transport, insoluble tau	Motor deficits
Duff et al. (2000)	YAC genomic tau	3.7×endogenous	MC-1 immunoreactivity	None
Lewis et al. (2000)	MoPrP 4R0N P301L tau	Endogenous	Neurofibrillary tangles in neurons, cell loss, gliosis, sarkosyl insoluble tau, neurogenic atrophy	Severe motor deficits progressing to death
Gotz et al. (2000)	Mo Thy-1 4R2N P301L tau	Less than endogenous	Neurofibrillary tangles, gliosis, apoptosis, sarkosyl insoluble tau	None

*WT, wild type; Hu, human; Mo, mouse. Expression is based on reported transgenic tau protein compared to endogenous murine tau in hemizygotes unless otherwise stated.

1999). The WT3R model with the lowest expression (14% of total tau protein) showed somatodendritic accumulation of transgenic tau into somatodendritic 'pre-tangles' in both neurons and glia (Brion et al., 1999). WT3R transgenic mice with as much as 5-, 10-, and 15-fold overexpression of the shortest tau isoform (3R0N) driven by the murine prion promoter (MoPrP) were reported by Ishihara et al. (1999). Mice with the highest levels of expression died before three months; however, lines with 10- and 5-fold overexpression were viable. The spinal cords of these tau transgenic animals contained spheroidal intraneuronal inclusions that were tau immunopositive for multiple tau phosphorylation and conformational dependent antibodies. Despite the promising evidence of tau hyperphosphorylation in the intraneuronal inclusions, the inclusions also strongly stained for neurofilament. These inclusions were silver positive; however, they were negative for thioflavin S and ubiquitin. Astrocytosis, axonal degeneration, and motor dysfunction were also reported in this 3R tau model. Additionally, insoluble tau accumulated in the brains and spinal cords of these tau mice with age. The presence of argyrophilic tau represented a significant advance in developing an *in vivo* model of tau dysfunction; however, no NFTs were observed according to published reports of either 3R0N mouse model (Brion et al., 1999; Ishihara et al., 1999).

The tau neurofibrillary pathology in human neurodegenerative disorders occurs in the context of all six tau isoforms, thus it seemed possible that expression of all six tau isoforms would be required to stimulate neurofibrillary pathology in transgenic mouse systems. Duff et al. (2000) reported tau transgenic mice that produced all six wild-type tau isoforms by alternative splicing; however, the exon 10- isoforms, particularly 2−3−10−, were more abundant in the transgenic mice compared to normal human ratios. Neuronal processes and synaptic terminals were positive for transgenic tau in the genomic tau mice, but somatodendritic staining reported in previous tau transgenic mice was absent. Genomic tau transgenic mice had up to 3.7-fold more total tau than non-transgenic mice; however, with the exception of MC-1 tau immunoreactivity, these mice lacked any evidence of neuropathology or behavioral changes up to 8 months of age.

Since overexpression of wild-type tau in mice failed to mimic the neurofibrillary tau pathology that is observed in AD, FTDP-17, and related disorders, several groups have expressed tau containing various FTDP-17 associated mutations that would be expected to accelerate neurofibrillary tangle formation in mice. In August 2000, we reported a tau (P301L) transgenic mouse that develops NFTs, neuronal loss, gliosis, neurogenic atrophy, and profound motor and behavioral deficits (Lewis et al., 2000).

These transgenic mice were generated by expressing the 4R0N tau isoform with the P301L mutation in exon 10 driven by the MoPrP promoter. Expression analyses showed that the level of transgenic tau

(4R0N) in the hemizygotes was approximately equivalent to the endogenous mouse tau level. Despite this relatively low expression level, hemizygous and homozygous animals starting at 6.5 and 4.5 months, respectively, developed NFTs, neuronal loss, and motor and behavioral deficits (Figure 8.4). The P301L mice initially presented with hind-limb dysfunction similar to the lack of escape reflex and muscle weakness previously reported in other 4R tau transgenic mice; however, the extent of the deficits was more profound than those previously reported and the dysfunction was rapidly progressive. Dystonic posturing and immobility developed in the P301L animals within a month of initial motor dysfunction. The P301L phenotype also included docility, reduced weight and vocalization, and eye irritations. Some of the behavioral and motor features of the P301L mice can be correlated not only to symptoms of various human tauopathies but also to the distribution of neuropathology.

As previously reported in other tau models, neurogenic atrophy was observed in the P301L tau transgenic mice. Significantly, the P301L animals showed almost a 50% neuronal loss in the spinal cord, which, along with the neurogenic atrophy, could explain much of the motor dysfunction in these mice. Additionally, unlike previous tau transgenic mouse models, the P301L tau mice developed NFTs in neuronal cell bodies composed mainly of straight tau filaments. The NFTs were concentrated in the spinal cord, brain stem, and some regions of the midbrain of the P301L animals (Table 8.2); however, pre-tangles similar to those described in several other tau transgenic animals had a much wider brain distribution. The NFTs, similar to those found in human tauopathies, were positive with thioflavin S, Congo red, and Gallyas, Bielschowsky, and Bodian silver stains. Tau hyperphosphorylation was evident with immunostaining with numerous antibodies, including AT8, AT100, AT180, CP13, and PHF-1. Additionally, the neurofibrillary tangles were negative for neurofilament and positive for ubiquitin. Consistent with the neuropathology, sarkosyl insoluble, hyperphosphorylated tau including a prominent 64 kD band was extracted from the brains and spinal cords of the P301L 4R0N tau mice. This 64 kD insoluble tau band from the P301L mice co-migrated with pathologic tau from FTDP-17 and AD patients. Dephosphorylation of the insoluble tau from the P301L mice (Lewis et al., 2000) and FTDP-17 and AD patients demonstrated that the 64 kD band contained hyperphosphorylated 4R0N tau (Spillantini and Goedert, 1998), the human isoform expressed in the 2−3−10+ P301L mice. The insoluble tau data demonstrated that similar hyperphosphorylation occurs in both the P301L mice and the human tauopathies. In addition to the 4R0N P301L mice, we have also generated a second P301L (4R2N tau) line that develops similar neuropathology and motor deficits (Lewis et al., 2000).

Recently there has been an additional report of NFTs in P301L tau transgenic animals expressing the 4R2N isoform under the mouse Thy-1

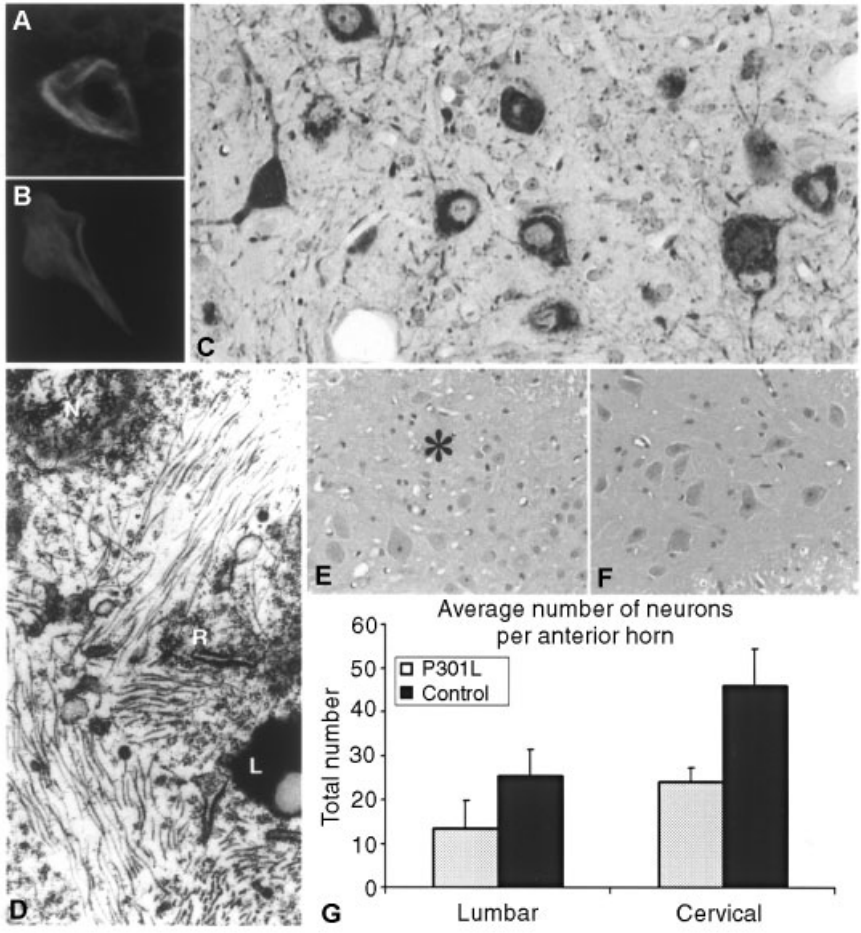

Figure 8.4. Neurofibrillary tangles and neuronal loss in the P301L 4R0N tau transgenic mice. (A) Spinal cord sections from P301L mice show neurofibrillary tangles stained with thioflavin-S and (B) Congo red (confocal microscope). (C) Tau hyperphosphorylation was evident from staining with numerous tau antibodies that recognize specific phospho-epitopes, including CP13. Section is from the cerebellar dentate nucleus. (D) Electron microscopy of the neurofibrillary tangles shows tau filaments in longitudinal and cross-sections (bar = 1 μm). N, nucleus; R, polyribosome; L, lipofuscin granule. (E) Hematoxylin and eosin staining shows a region of neuronal loss (*) in the anterior horn of a P301L mouse compared to (F) non-transgenic littermate. (G) Neuronal counts from both the lumbar and cervical enlargements demonstrated a 48% reduction in motor neurons in the P301L 4R0N tau mice (Lewis et al., 2000)

Table 8.2. Distribution of neurofibrillary tangles in P301L (4R0N) tau transgenic mice

Telencephalon	
Piriform cortex	+
Hippocampus	+
Medial orbital	+ +
Septal nuclei—medial and lateral	+ + +
Bed nucleus of stria terminalis	+ + +
Amygdala—central, cortical, and medial (not basolateral)	+ + +
Preoptic nuclei	+ + + +
Diencephalon	
Thalamus—anterior nuclear group	+ + +
Lateral habenular nucleus	+ + +
Hypothalamus—anterior, lateral, and posterior	+ + + +
Midbrain	
Oculomotor	+ + +
Substantia nigra	+ + +
Subthalamic nucleus	+ + +
Periaqueductal gray	+ + + +
Pretectal region	+ + + +
Red nucleus	+ + + +
Edinger–Westphal	+ + + +
Midbrain tegmentum	+ + + +
Dorsal raphe	+ + + +
Pons	
Locus ceruleus	+ + + +
Trigeminal motor	+ + + +
Pontine tegmentum—reticular nuclei	+ + + + +
Pontine nuclei	+ + + + +
Medulla	
Hypoglossal	+ + + +
Vestibular	+ + + +
Nucleus solitarius	+ + + +
Medullary reticular nuclei	+ + + + +
Cerebellum	
Dentate, interpositus, and fastigial nuclei	+ + + +

+, rare; + +, very few; + + +, few; + + + +, many; + + + + +, very many.

promoter (Gotz et al., 2000). NFTs were identified in the cortex, brain stem, and spinal cord of these P301L animals using thioflavin S and Gallyas silver staining. Filaments with straight and twisted ribbon morphologies that were immunopositive for multiple phosphorylated tau epitopes were observed in insoluble tau extracted from these P301L animals. Additionally, astrocytosis and neuronal apoptosis accompanied the tangle formation in these 4R2N P301L animals. This 4R2N P301L mouse line provides another valuable model of NFT formation and further confirms that tau dysfunction can lead to neurodegeneration.

CONCLUSION

Significant advances have been made in both identifying numerous tau mutations responsible for FTDP-17 and determining the likely mechanisms through which these mutations cause tau dysfunction. The role and timing of tau dysfunction has yet to be determined in many other tauopathies, including AD, PSP, and CBD. Recently, identification of a conserved haplotype (H1) that extends over the entire *tau* gene and its association with both PSP and CBD provides a possible genetic link to tau in diseases that were previously thought to be sporadic tauopathies. Efforts should now focus on defining the exact role of the tau polymorphisms on the development of PSP and CBD and how this relates to the predominance of four-repeat tau isoforms in the NFTs observed in these two diseases. Additionally, the recent development of tau (P301L) mouse models of NFTs by our group and others has provided animal models in which the relationship of tau dysfunction to other key pathologic events such as amyloid production and neurodegeneration can be examined. Crossing mice which develop NFTs with mice that develop amyloid plaques may finally define the role of tau in the amyloid cascade hypothesis and thus define the role of tau in AD. Furthermore, these P301L mice are valuable resources in which potential therapies against tau accumulation can be tested.

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9 Regulation of Four-Repeat tau Expression: Interactions between Exon and Intron Splicing Regulatory Sequences

IAN D'SOUZA AND GERARD D. SCHELLENBERG

The microtubule-associated protein (MAP) family including the phosphoprotein tau are involved in microtubule assembly and stability (Schoenfeld and Obar, 1994; Mandelkow and Mandelkow, 1995). The neuropathological aggregation of tau is manifest in several progressive dementing disorders such as Alzheimer's disease (AD), Pick's disease (PiD), corticobasal degeneration (CBD), progressive supranuclear palsy (PSP), and frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17) (reviewed in Buee et al., 2000). FTDP-17 represents a phenotypically heterogeneous group of autosomal dominantly-inherited dementias caused by recently identified missense mutations in the *tau* gene (Hutton et al., 1998; Poorkaj et al., 1998; Spillantini et al., 1998). Since most of these mutations are concentrated within or in close proximity to the microtubule-binding domains located at the C-terminus of tau, the pathological consequence of tau mutations in FTDP-17 suggests that tau dysfunction or gain of function is toxic in affected neurons and perhaps glial as well. Both the phosphorylation and expression patterns of tau are both under developmental control (Goedert et al., 1989; Lovestone and Reynolds 1997). In fetal human brain a single tau isoform is expressed containing exons 1, 4, 5, 7, 9 and 11–13. In adult human brain five additional variants are expressed by alternative splicing of exons 2, 3, and 10. The conditional inclusion of the fourth microtubule-binding domain encoded by exon 10 (E10) generates *tau* isoforms with four repeats (4R) as opposed to three repeats (3R) in which E10 is absent.

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Tau GENE MUTATIONS AFFECT MULTIPLE SPLICING ENHANCER AND SILENCER SEQUENCES

In vitro functional analyses reveal two classes of *tau* mutations. One class includes mutations $^{G}272^{V}$ (E9), $^{P}301^{L}$, $^{P}301^{S}$, $\Delta 280^{K}$ (E10), $^{V}337^{M}$ (E12), and $^{R}406^{W}$ (E13) that alter tau function by compromising tau's affinity for microtubules and/or tau's ability to polymerize microtubules (Hasegawa et al., 1998; Hong et al., 1998; Dayanandan et al., 1999; D'Souza et al., 1999) as well as by promoting self-assembly of tau as paired helical filaments (PHF) (Goedert et al., 1999; Nacharaju et al., 1999; Barghorn et al., 2000). Mutations within constitutively spliced exons E9, E12, and E13 affect all *tau* isoforms, whereas E10 mutations $^{P}301^{L}$ and $^{P}301^{S}$ affect only 4R tau function. The second class of mutations alter the relative levels of 4RT versus 3RT by affecting potential splicing regulatory sequences in E10 and intron 10 (I10) (Clark et al., 1998; Hutton et al., 1998; Spillantini et al., 1998; D'Souza et al., 1999; Grover et al., 1999; Hasegawa et al., 1999; Rizzu et al., 1999; Yasuda et al., 2000). These include missense ($^{N}279^{K}$ and $^{S}305^{N}$), silent ($^{L}284^{L}$), and deletion ($\Delta 280^{K}$) mutations in E10 as well as mutations E10+12, E10+13, E10+14, and E10+16 in I10. All the above splicing mutations except $\Delta 280^{K}$ greatly enhance E10 inclusion. $\Delta 280^{K}$ is unique in that it not only alters tau protein function, but also almost completely abolishes E10 inclusion (D'Souza et al., 1999). The effect of $\Delta 280^{K}$ on splicing is presumed to be the predominant disease causative mechanism since in the absence of E10 no $\Delta 280^{K}$ protein would be expressed. The varied clinical and phenotypes associated with different splicing mutations suggest that FTDP-17 splicing mutations deregulate E10 inclusion by affecting multiple splicing regulatory sequences. These mutations are predicted to affect at least four *cis*-acting regulatory sequences that include the weak E10 5′ splice site ($^{S}305^{N}$ and E10+3), a PPE or polypurine enhancer sequence ($^{N}279^{K}$ and $\Delta 280^{K}$), an exon splicing silencer (ESS) sequence ($^{L}284^{L}$) in E10 and an intron splicing silencer (ISS) within I10 (E10+12, E10+13, E10+14, and E10+16). Mutational analyses (Figure 9.1) of E10 sequences using in-frame deletions and substitutions further characterize these regulatory sequences affected by *tau* mutations and also reveal additional elements (D'Souza and Schellenberg, 2000). Thus, the first 45 bases of E10 contain two additional exon splicing enhancer (ESE) sequences, an SC35-like enhancer sequence TGCAGAT that resembles the degenerate binding consensus TGCNGYY of the SR protein SC35 (Schaal and Maniatis, 1999), and an A/C-rich enhancer or ACE (Coulter et al., 1997) 15 bases in length. Most ESEs are known to interact with a conserved family of serine/argenine-rich (SR) splicing factors known as SR proteins (Fu, 1995). Purine-rich enhancers containing a GAR (R is a purine) or AAG repeat motif similar to the PPE sequence AAGAAGCTG are known to interact with SR splicing factors (Xu et al., 1993). Mutation $\Delta 280^{K}$ (EΔ5)

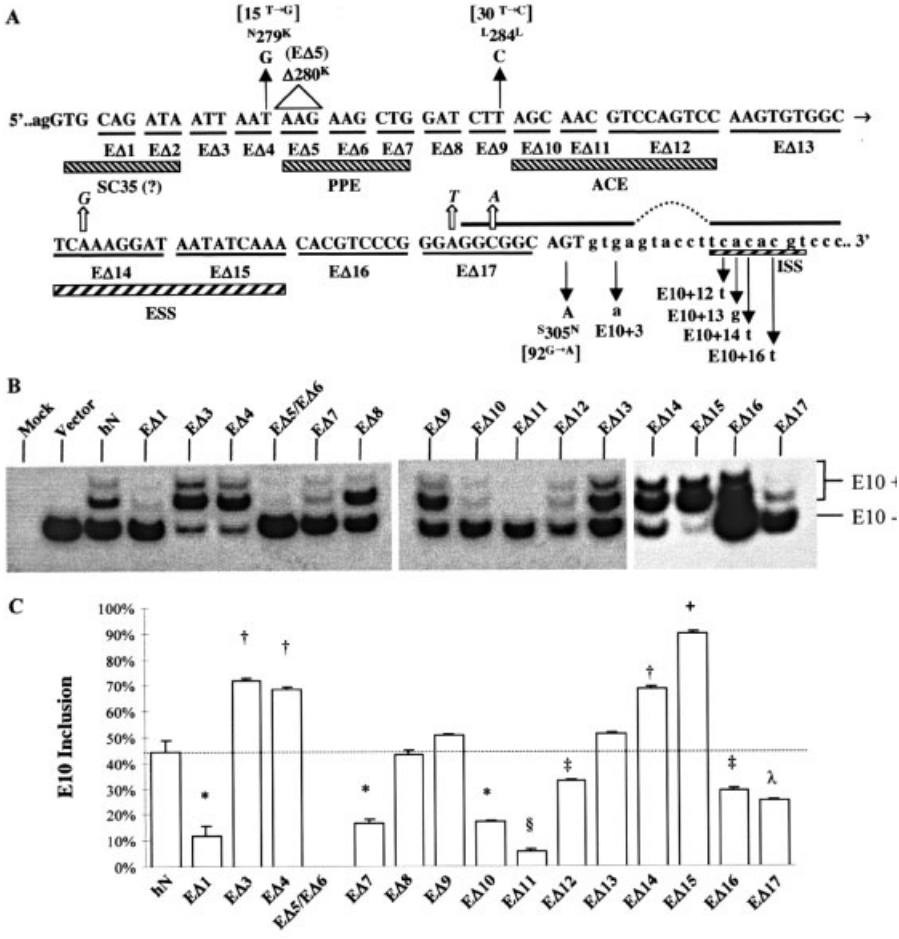


Figure 9.1. Deletion analysis of *tau* E10. (A) Nucleotide sequence showing *tau* E10 in uppercase letters and intron nucleotides in lowercase letters. FTDP-17 mutations are represented by solid arrows except for $\Delta 280^{K}$ which is shown as a triangle. Mouse divergent nucleotides (italics) within E10 are shown by open arrows. Deletions EΔ1–EΔ17 are shown by bars below the sequence. Nucleotides involved in a potential stem-loop are shown by a bar above the sequence with the loop region as a dotted line. Hatched boxes below the sequence represent *cis*-acting regulatory sequences. (B) Autoradiography of E10 splicing by RT-PCR from transiently transfected COS-7 cells. (C) Quantitation of E10 inclusion. Each bar represents the mean of at least three separate transfection experiments. Error bars are standard deviations. A corrected significance criteria of $p < 0.003$ was used and p values for comparison of each construct to normal human E10 are indicated with the following symbols: ‡, $p < 1 \times 10^{-3}$; λ, $p < 1 \times 10^{-4}$; †, $p < 1 \times 10^{-5}$; ⋆, $p < 1 \times 10^{-6}$; §, $p < 1 \times 10^{-7}$ and +, $p < 1 \times 10^{-8}$ (D'Souza and Schellenberg, 2000)

weakens the PPE by removing a single GAR motif or one of two AAG motifs. Additional substitution analyses indicate that mutations $^{N}279^{K}$ ($15^{T\rightarrow G}$) and $^{L}284^{L}$ ($E30^{T\rightarrow C}$) lie immediately 5′ to and strengthen the existing PPE and ACE elements, respectively. Thus, $^{N}279^{K}$ functionally extends the 5′ end of the PPE sequence by three bases and provides an extra GAR or AAG motif. $^{L}284^{L}$ does not compromise an ESS as previously predicted, but increases the overall AC content of the ACE element by extending its 5′ boundary. Human *tau* E10 does, however, contain a bona fide ESS sequence nine bases downstream of the ACE. Purine residues seem critical for ESS function as their substitution causes a dramatic increase in E10 inclusion. Additional *cis*-regulatory sequences represented by deletions EΔ3–EΔ4 and EΔ16–EΔ17 also contribute to regulated inclusion of E10.

INTERACTIONS BETWEEN E10 AND I10 SPLICING ELEMENTS

Multiple regulatory elements that interact with each other are usually involved in alternative splicing (Wang and Manley, 1997). The collaboration between *tau* E10 and I10 splicing elements was tested in templates containing mutations in single elements and compared with those containing mutations in two different elements. The deletion data show that E10 splicing is severely reduced when any of the three ESEs are individually compromised. Thus all three ESEs are required to compensate for a weak 5′ splice site and to overcome inhibitory ESS and ISS sequences. In the absence of the PPE (EΔ5) almost no E10 transcript is detected. However, FTDP-17 mutations that strengthen the weaker splicing signals or disrupt inhibitory splicing signals are able to compensate for loss of PPE function, as seen in double mutants containing the E10 5′ splice site ($E\Delta5/92^{G\rightarrow A}$, EΔ5/E10+3), the ACE sequence (EΔ5/$E30^{T\rightarrow C}$ not shown) or the ISS (EΔ5/E10+12, EΔ5/E10+13, EΔ5/E10+14, and EΔ5/E10+16), respectively (see Figure 9.2). Inhibition of E10 splicing by the ISS is relieved by FTDP-17 intronic mutations. One hypothesis for ISS function is through a stem-loop structure that sequesters the E10 5′ splice site, rendering it unavailable to the splicing apparatus. Data supporting a stem-loop are derived from structural and mutational studies where I10 mutations are shown to reduce the thermodynamic stability of a 25-nucleotide stem-loop template by NMR spectroscopy (Varani et al., 1999), and alterations that over-stabilize the stem-loop can compensate for intronic FTDP-17 mutations (Grover et al., 1999). In addition, over-expression of 4R tau in other mammalian species is explained by the absence of such a structure (Grover et al., 1999). An alternate hypothesis is that the ISS functions as a linear sequence and inhibits splicing through its association with potential *trans*-acting factors. Indirect data in support of a linear hypothesis are derived from lack of an effect on E10 splicing by compensatory

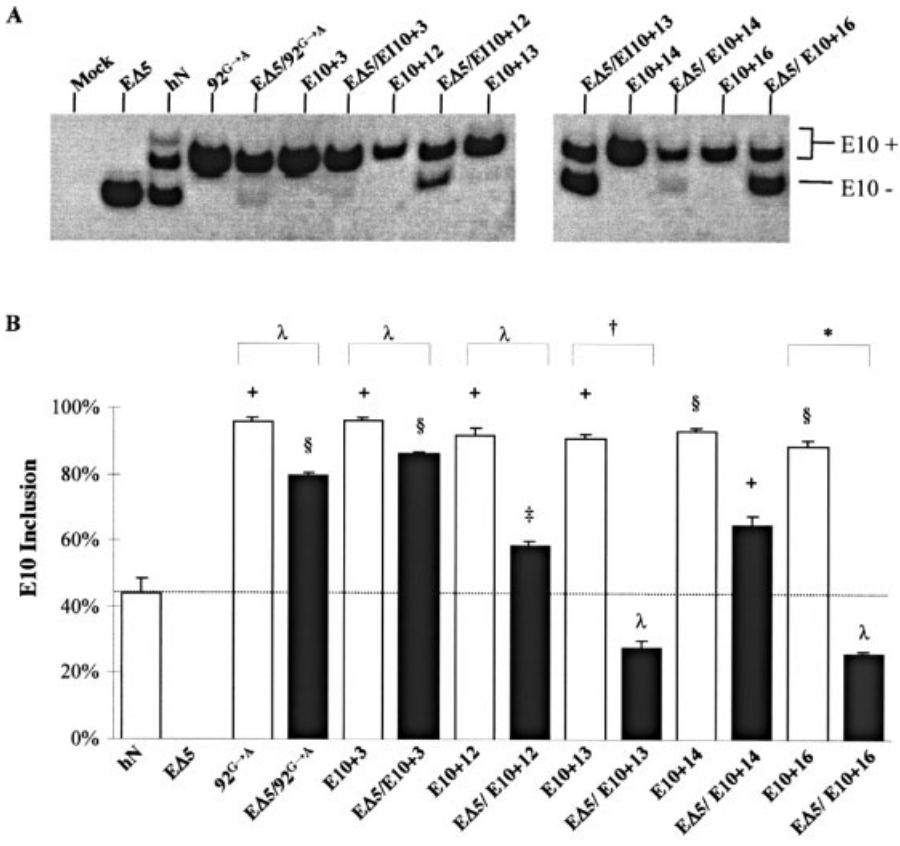
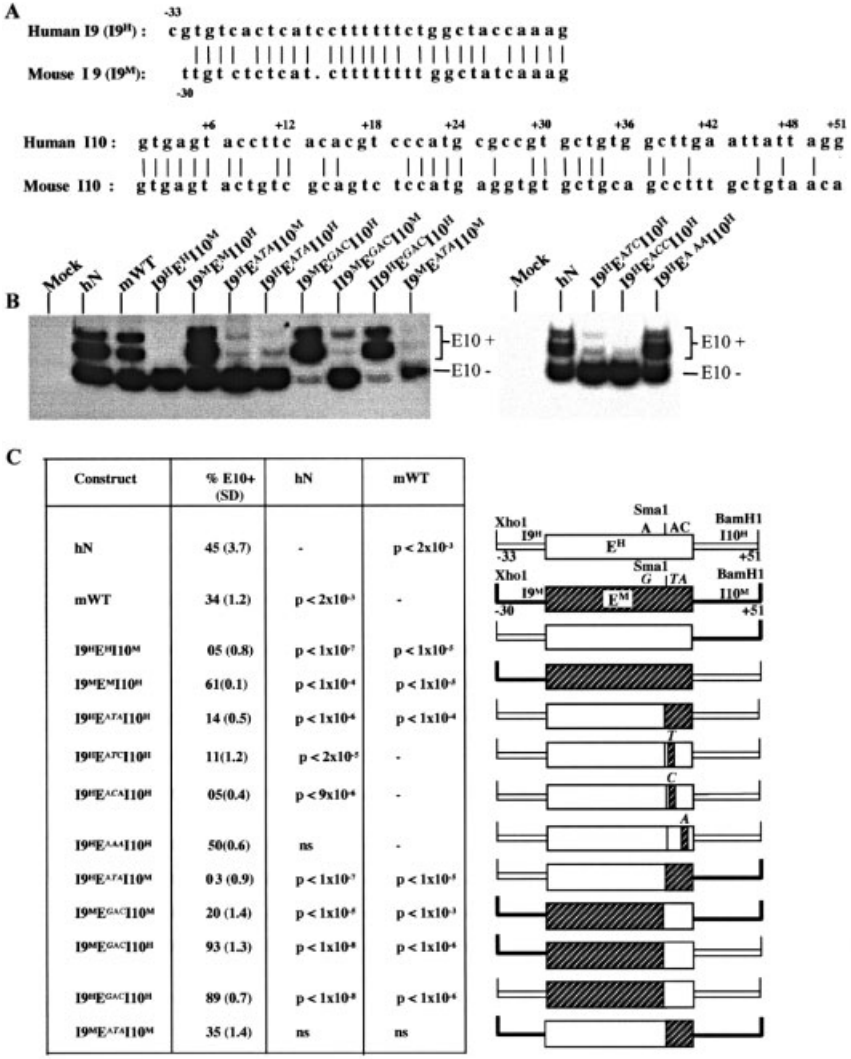


Figure 9.2. Interactions between exon and intron splicing elements. The effects of 5′ splice site ($92^{G\rightarrow A}$ and E10+3) and intronic (E10+12, E10+13, E10+14, and E10+16) FTDP-17 mutations on E10 splicing were analyzed in the absence of a functional ESE sequence. ESE function was disrupted using the EΔ5 deletion. (A) Autoradiograph of E10 splicing by RT-PCR of total RNA isolated from transiently transfected COS-7 cells. (B) Quantitation of E10 inclusion. A corrected significance criteria of $p < 0.002$ was used. Assays were performed as in Figure 9.1. Significance levels for comparison of each mutant to normal human E10 are indicated by symbols above bars as described in Figure 9.1. Comparisons between mutant constructs are shown with lines connecting for constructs compared and significance levels shown above the line (D'Souza and Schellenberg, 2000)

mutations or E10 substitutions predicted to destabilize the stem region (D'Souza et al., 1999). The varied effects on E10 inclusion presented by I10/EΔ5 double mutants provide additional evidence against a stem-loop and suggest that not all nucleotides within the ISS are equivalent. Since the I10/EΔ5 double mutants produce stem-loops with similar free energies, their splicing phenotypes do not correlate with stem-loop stability as a predominant mechanism for ISS function.



Construct	% E10+ (SD)	hN	mWT
hN	45 (3.7)	-	$p < 2x10^{-3}$
mWT	34 (1.2)	$p < 2x10^{-3}$	-
$I9^{H}E^{H}I10^{M}$	05 (0.8)	$p < 1x10^{-7}$	$p < 1x10^{-5}$
$I9^{M}E^{M}I10^{H}$	61(0.1)	$p < 1x10^{-4}$	$p < 1x10^{-5}$
$I9^{H}E^{ATA}I10^{H}$	14 (0.5)	$p < 1x10^{-4}$	$p < 1x10^{-4}$
$I9^{H}E^{ATC}I10^{H}$	11(1.2)	$p < 2x10^{-5}$	-
$I9^{H}E^{ACA}I10^{H}$	05(0.4)	$p < 9x10^{-6}$	-
$I9^{H}E^{AAA}I10^{H}$	50(0.6)	ns	-
$I9^{H}E^{ATA}I10^{M}$	03 (0.9)	$p < 1x10^{-7}$	$p < 1x10^{-5}$
$I9^{M}E^{GAC}I10^{M}$	20 (1.4)	$p < 1x10^{-5}$	$p < 1x10^{-3}$
$I9^{M}E^{GAC}I10^{H}$	93 (1.3)	$p < 1x10^{-8}$	$p < 1x10^{-6}$
$I9^{H}E^{GAC}I10^{H}$	89 (0.7)	$p < 1x10^{-8}$	$p < 1x10^{-6}$
$I9^{M}E^{ATA}I10^{M}$	35 (1.4)	ns	ns

Figure 9.3. Comparison between mouse and human E10 splicing. (A) Human and mouse I9 and I10 sequences shown are those present in normal human (hN) and wild-type mouse (mWT) constructs. (B) Autoradiograph of RT-PCR assays showing E10 inclusion in transiently transfected COS-7 cells. (C) Quantitation of E10 inclusion. Human E10, I9, and I10 sequences (E^{H}, $I9^{H}$, and $I10^{H}$ respectively) are represented by open boxes. Mouse E10 (E^{M}) and intron sequences ($I9^{M}$ and $I10^{M}$) are represented by a hatched box and solid bold lines, respectively. Nucleotides divergent between mouse and human at E10 nucleotides 57, 84, and 87 are indicated by superscript above human E10 as $E10^{AAC}$ and mouse E10 as E^{GTA}. SD is the standard deviation. A corrected significance criteria of $p < 0.003$ was used. Significance levels are given for comparison for each construct to results for both hN and mWT constructs (D'Souza and Schellenberg, 2000)

COMPARISON OF HUMAN AND MOUSE SEQUENCES ON E10 SPLICING

The regulation of E10 splicing differs in adult human and mouse brain. Unlike in adult human brain where equal proportions of 4R and 3R tau are produced, adult mice exclusively produce 4R tau. In fetal brain of both species only 3R tau is expressed. Human and mouse E10 sequences differ by only three nucleotides (positions 57, 84, and 87) and contain identical 5′ and 3′ splice sites. However, the adjacent intron sequences are more divergent. The wild-type mouse E10 splicing template (mWT) is analogous in sequence to the human E10 splicing construct (hN) but splices less efficiently than hN in transiently transfected COS-7 cells. Using human/mouse chimeric constructs, the effects of divergent E10 and flanking intronic sequences were assessed (Figure 9.3) (D'Souza and Schellenberg, 2000). First, mouse I10 sequences are more inhibitory than human I10 sequences (compare hN with $I9^{H}E^{H}I10^{M}$ and mWT with $I9^{M}E^{M}I10^{H}$). Since mouse I10 is predicted to form a less stable stem-loop than human I10, the pronounced inhibitory effect of mouse I10 compared with human I10 on E10 inclusion must be explained by the association of *trans*-acting factors with I10. Second, the human E10 ESS sequence does not seem to function in mouse, as replacing the mouse G at position 57 with human A shows no effect on mouse E10 inclusion although the exchange restores an identical ESS sequence (compare mWT with $I9^{M}E^{ATA}$). Conversely, replacing the human A with the mouse G in human E10 appears to destroy the ESS as E10 oversplices (compare hN and $I10^{M}I9^{H}E^{GAC}I10^{H}$). Of the remaining two E10 divergent positions, replacing only the human A at position 84 with the mouse T or another pyrimidine severely cripples E10 inclusion (compare hN to $I9^{H}E^{ATA}I10^{H}$ and $I9^{H}E^{ACA}I10^{H}$). Nucleotide position 84 in human E10 lies within a purine-rich stretch whose integrity (see also EΔ17, Figure 9.1) appears critical for E10 inclusion. Exchanging the mouse nucleotides at positions 84 and 87 with human reduces mouse E10 inclusion but this inhibitory effect disappears when the downstream intron is human. Collectively, these data suggest that mouse E10 sequences, although more enhancing than human E10 sequences, are kept in check by strongly inhibitory mouse I10 sequences. Both hN and mWT constructs also splice at similar levels in transiently transfected fetal rat neurons (D'Souza and Schellenberg, unpublished work). Thus, both developmental and species-specific regulation of E10 inclusion must involve sequences that extend farther into the flanking introns and perhaps exons as well.

CONCLUSION

FTDP-17 splicing mutations affect four of at least six *cis*-acting splicing regulatory sequences in human E10 and I10. Together, the biochemical and

splicing data suggest that subtle perturbations in the normal 4R:3R tau ratio cause disease as in some cases the elevated 4R tau produced has the normal tau amino acid sequence (as in $^{L}284^{L}$ and I10 mutations). As expected, when E10 usage is increased, the aggregated tau formed has excess 4R tau isoform. Elucidating the regulatory mechanisms involved in E10 splicing is therefore critical for understanding not only FTDP-17, but also other tauopathies like PiD, CBD, and PSP which also show altered 3R:4R tau ratios. However, no overt sequence alterations in the *tau* gene have been detected as yet for these diseases. Another essential feature of identifying potential regulatory sequences for E10 splicing is that the effects of new *tau* mutations may be easily predicted.

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III Diagnosis and Clinical Course

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10 Preclinical Prediction of AD: Relation Between Neuropsychological and Neuroimaging Findings

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INTRODUCTION

The discrimination of those destined to develop Alzheimer's disease (AD) from the larger pool of individuals with mild memory loss is of increasing importance, as strategies for the prevention or delay of dementia are developed. We sought to determine whether neuropsychological tests administered to individuals with evidence of mild but progressive difficulty in daily life could differentiate those individuals destined to develop dementia from normal controls, and from participants who had cognitive problems but did not develop AD within three years of follow-up. We hypothesized, based on previous findings, that tests of memory and executive function would be more discriminating than tests of other cognitive domains. We also hypothesized that brain regions related to tests of memory and executive function would, likewise, differentiate these groups.

To test these hypotheses, we administered a neuropsychological battery, a magnetic resonance imaging (MRI) scan, and a single photon emission computerized tomography (SPECT) scan to 165 subjects, 123 of whom met criteria for 'questionable' AD when the study began. After three years of follow-up, 23 of the 123 questionable individuals had progressed to the point where they met clinical research criteria for probable AD (McKhann et al., 1984). It was therefore possible to determine which neuropsychological measures at baseline could be used to predict the status of the subjects at the end of the following three years, and to evaluate their relationship to MRI and SPECT measures in the same individuals.

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SUBJECTS

RECRUITMENT AND SELECTION PROCEDURES

A total of 165 individuals were participants in the present study. To be included in the study, participants needed to be aged 65 and over, to be free of significant underlying medical, neurologic, or psychiatric illness, to have a collateral source who was willing to participate on an annual basis, to have a Clinical Dementia Rating (CDR) (Hughes et al., 1982) of either normal (CDR = 0) or questionable AD (CDR = 0.5), and to be willing to participate in the study procedures. These subjects were selected on the basis of a multistage screening procedure which has been described elsewhere (Johnson et al., 1998; Killiany et al., 2000; Albert et al., 2000). At baseline, the study procedures included a medical evaluation (consisting of a physical exam and medical history, EKG, and standard laboratory tests), a semistructured interview, neuropsychological testing, an MRI scan, a SPECT scan, and blood for genetic analysis. Only the semistructured interview was repeated annually; the remaining study procedures were repeated in a subset of the participants. All subjects provided informed consent prior to the initiation of the study.

The CDR ratings, central to the categorization of the subjects, were derived from the annual semistructured interview, which was administered to each subject and his or her collateral source. This interview was based on the Initial Subject Protocol which was used for the initial development of the CDR scale (Hughes et al., 1982). It includes a semistructured set of questions regarding functional status administered to both the subject and the collateral, and a neurologic, psychiatric, and mental status evaluation of the subject. In the present study, each interview was administered by a skilled clinician (e.g. psychiatrist, neuropsychologist, physician's assistant) and took approximately 1.5–2 h to complete. The mean inter-rater reliability of the CDR ratings was high ($r^2 = 0.99$, $p < 0.0001$), as was the inter-rater reliability of the six CDR subcategories ($r^2 = 0.90$) that were used to generate the overall CDR rating (Daly et al., 2000).

BASELINE CHARACTERISTICS

At baseline, the subjects were divided into two groups, based on their functional status, as indicated above. One group consisted of 42 subjects with normal cognition (CDR = 0.0) and the other group consisted of 123 individuals with 'questionable AD' (CDR = 0.5). They had a mean age of 71.4 and 72.2 years, respectively. The educational level of the two groups was equivalent (14.4 years and 14.9 years, respectively), as was the mean Mini-Mental State Examination (MMSE) (Folstein et al., 1975) score (29.4 ± 0.7

and 29.1 ± 1.2, respectively). The gender distribution within both groups was also similar, being approximately 60% female and 40% male.

FOLLOW-UP CHARACTERISTICS

After three years of follow-up, nine subjects had died. One of these individuals died before the first follow-up assessment and is not included in this report. For those who remained alive, the annual follow-up rate was 99%.

Based on their three-year trajectory of functional change, the subjects could be categorized into six groups, three of which were analyzed for the present report:

1. Group 1, Normals: Subjects who had normal cognition at baseline (CDR = 0) and continued to be categorized as normal at follow-up (n = 32). This represented 76% of the normal subjects. Ten of 42 subjects with a CDR rating of 0 at baseline were categorized as questionable after three years of follow-up, but none had converted to AD (CDR = 1).
2. Group 2, Questionables: Subjects who met criteria for 'questionable AD' at baseline (CDR = 0.5) and were still categorized as CDR = 0.5 after three years of follow-up (n = 91). This represented 73% of the questionable subjects.
3. Group 3, Converters: Subjects who met CDR criteria for 'questionable' AD at baseline, but progressed to the point where they were coded CDR = 1 within three years of follow-up and met NINCDS/ADRDA criteria for probable AD (McKhann et al., 1984) (n = 23). The annual medical, neurologic, psychiatric, and laboratory evaluation was augmented, as needed, to insure the subjects met these criteria. Three converters have now died and an autopsy was carried out on two of them, which confirmed a diagnosis of definite AD (NIA–Reagan Work Group, 1997). The Converters represented 19% of the questionable subjects; thus, the annual conversion rate among the Questionables was about 6% per year (as compared with 0% per year for the controls).

Groups 4, 5, and 6 consisted of three smaller sets of individuals. Group 4 consisted of subjects who were questionable at baseline and were coded CDR = 1 on follow-up but did not meet clinical research criteria for probable AD (n = 3). These three individuals had strokes. Group 5 consisted of 'questionable' subjects who improved and were categorized as normal at follow-up (n = 6). Group 6 consisted of normal subjects who declined and were categorized as 'questionable' at follow-up (n = 10). An examination of groups 4–6 will be the subject of separate publications.

METHODS

NEUROPSYCHOLOGICAL PROCEDURES

The neuropsychological battery that was administered to the participants at baseline consisted of 20 test scores based on 17 tests: (1) five memory tests (California Verbal Learning Test, Cued Selective Reminding Test, Rey–Ostereith Complex Figure Test, Delayed Word Recall Test, and Visual Reproduction Subtest of the Wechsler Memory Scale) (Delis et al., 1987; Grober and Buschke, 1987; Rey, 1941; Knopman and Ryberg, 1989; Wechsler, 1988), (2) six tests of executive function (Trail Making Test, Part B; Stroop Interference Test; Self-Ordering Test; Porteus Mazes; Alpha Span Test; and Digit Span Backward) (Reitan, 1958; Stroop, 1935; Petrides and Milner, 1982; Porteus, 1959; Craik, 1986; Wechsler, 1988), (3) three language tests (Controlled Word Association Test for letters and for categories; 15 items from the Boston Naming Test) (Benton and Hamsher, 1976; Kaplan et al., 1982), (4) two tests of spatial ability (copying the Rey Complex Figure; copying the figures from the Wechsler Memory Scale) (Rey, 1941; Wechsler, 1988), (5) three tests of sustained attention (Digit Span Forward; Trail Making Test, Part A; Cued Reaction Time) (Wechsler, 1988; Reitan, 1958; Baker et al., 1985), and (6) an assessment of general intelligence (estimated IQ based on a reduced version of the Wechsler Adult Intelligence Scale–Revised) (Satz and Mogel, 1962). These tests were not administered by the same individuals who conducted the interview that was used to generate the CDR ratings, and the test scores were not used in the assignment of the CDR ratings.

MRI PROCEDURES

The MRI data consisted of regions of interest (ROIs) derived from 3D T1-weighted gradient echo scans of the brain (TR = 35 ms, TE = 5 ms, FOV = 220, flip angle = 45 °, slice thickness = 1.5 mm, matrix size = 256 × 256). Each ROI was adjusted by the total intracranial volume on the scan, calculated by a semiautomated computer program (Sandor et al., 1992).

Two types of MRI measures were employed: a set that was manually drawn, and a set that was defined by an automated algorithm. Both types of measures used images that were normalized so that they could be resliced in standard planes.

The first set of ROIs consisted of five manually drawn ROIs that were obtained from the MRI images, with the operator 'blinded' to the group status of the subject. They included: the volume of the entorhinal cortex, the volume of the banks of the superior temporal sulcus (both of which were calculated on three consecutive coronal slices), and the volume of cingulate gyrus, which was subdivided into three sections, as follows: the rostral portion

of the anterior cingulate, the caudal portion of the anterior cingulate, and the posterior cingulate. The methods for identifying these ROIs have been previously described (Killiany et al., 2000). The semiautomated procedures used to calculate the volume of the ROIs are described elsewhere (Sandor et al., 1990).

The second set of ROIs consisted of six automated measures of cerebrospinal fluid spaces that either provided a reflection of the integrity of the medial temporal lobe (e.g. the temporal horn) or assessed generalized atrophy that is evident in the middle and late stage of disease (e.g. the third ventricle). These latter ROIs, which have been previously used in other studies (Sandor et al., 1992), were as follows: the temporal horns, the suprasellar cisterns, the third ventricle, the lateral ventricles, the sylvian fissures, and the interhemispheric fissure.

SPECT PROCEDURES

The SPECT data were obtained from a brain imaging system (CERESPECT, Digital Scintigraphics, Inc., Waltham, Mass.) consisting of a stationary annular NaI crystal and rotating collimator system (Genna and Smith, 1988). Subjects were scanned 20 min after injection of 20.0 mCi($\pm$1.0 mCi) of 99mTc-HMPAO (Ceretec, Amersham, UK) with the subjects supine at rest, with eyes open, in a darkened room, with ambient white noise. Datasets were displayed as a set of 64 slices (1.67 mm slice thickness) using a 128×128 matrix ($167 \times 1.67 \times 2$ mm pixels). Anatomic orientation, surface contouring, and scaling procedures for the SPECT data were performed as detailed previously (Johnson et al., 1993). The measured resolution was 7.3 mm at 9 mm from center for ^{99m}Tc (Holman et al., 1990).

The statistical method of singular value decomposition (SVD) was used to examine the SPECT data. For each subject, 20 weighted scores were calculated, based on 20 vectors derived from a normative sample ($n = 152$). The normative group was used to compute the weighting coefficients for the 20 vectors, which together represented the covariance pattern of brain activity. The 20 vector scores for each subject were then used in the analyses. This application of SVD to SPECT data is described elsewhere (Jones et al., 1998; Johnson et al., 1998).

GENETIC ASSESSMENT

The apolipoprotein (apoE) gene was also examined in the participants because the E_4 allele of this gene is over-represented in AD patients compared to the general population (Saunders et al., 1993), and is now widely recognized as a risk factor for AD. We therefore sought to determine whether apoE status, either alone or in combination with the neuropsychological or

neuroimaging measures, was useful as a predictor of which individuals would 'convert' to AD over time.

DATA ANALYSIS

A series of parallel discriminant function analyses were performed for the neuropsychological and MRI data (Press, 1972). These analyses were as follows:

1. The first discriminant analysis was conducted to determine whether the primary scores in each of the three domains, when taken together, significantly differentiated the three groups from one another (Controls vs. Questionables vs. Converters). This discriminant function also included a measure of age and gender (years of education was also included in the neuropsychological analyses, because of the potential impact of education on test performance). It should be noted that these covariates (age and gender and years of education) were not significant, but were included in all of the discriminant function analyses. This was done to assure that any subtle, though non-significant, effect of these variables was reduced as much as possible.
2. The second discriminant function (which was only performed if the first analysis was statistically significant) was a stepwise discriminant function analysis. The goal of this analysis was to select the variables within each domain that best differentiated the Controls, Questionables, and Converters from one another. The term 'best' refers here to the process by which each variable is selected by the algorithm, and the order by which the variable improves the significance of the overall function. In the stepwise analysis, the covariates were entered at the first step, and then the algorithm of the discriminant function selected the variables that, when combined, 'best' differentiated the groups from one another.
3. Separate post-hoc discriminant function analyses were then performed within each domain in which the groups were compared pairwise (i.e. Controls vs. Converters, Controls vs. Questionables, Questionables vs. Converters). These pairwise comparisons included only the covariates mentioned above, and the variables that had been deemed 'best' by the stepwise analysis.

The analysis of the SPECT data departed slightly from this procedure due to the use of the SVD method. For the SPECT data, the overall discriminant function analysis was performed (adjusted for age and gender) in the same manner described above, using 20 SPECT vector scores for each subject. The vectors that contributed to the discrimination were then 'back projected' in order to identify the brain regions that were involved in differentiating the groups (see Jones et al., 1998, and Johnson et al., 1998, for additional details).

It should be emphasized that all of the data were obtained at baseline, but group membership was based on the subject's status following three annual follow-up visits. It should also be noted that the sample sizes vary depending upon the domain of analysis, as some subjects were missing relevant data or had data that contained artifact and thus could not be used (for specific details, see Johnson et al., 1998; Killiany et al., 2000; Albert et al., in press).

RESULTS

Neuropsychological test results. Four of the 20 neuropsychological measures obtained at baseline were useful in discriminating the groups on the basis of their status three years after the tests were given. The four discriminating tests pertained to assessments of memory and executive function. They were: (1) the total learning score on the California Verbal Learning Test (CVLT), (2) the immediate recall of the figures from the Wechsler Memory Scale (F-WMS), (3) the time to completion on Part B of the Trail Making Test (TMT), and (4) the total score on the Self-Ordering Test (SOT). The accuracy of discrimination was related to the clinical similarity between the groups. When the controls were compared to the individuals with memory impairments who ultimately developed AD (the Converters), the accuracy of discrimination was 89%, based on the neuropsychological measures at baseline (sensitivity = 0.83, specificity = 0.91). The discrimination of the controls from the individuals with mild memory problems who did not progress to the point where they met clinical criteria for probable AD over the three years of follow-up (the 'Questionables') was 74% and the discrimination of the Questionables from the Converters was 80%.

MRI results. Three of the 11 MRI measures obtained at baseline were useful in discriminating the groups on the basis of their status three years after the tests were given. They were: the entorhinal cortex, the banks of the superior temporal sulcus, and the caudal portion of the anterior cingulate. As with the neuropsychological results, the accuracy of discrimination was related to the clinical similarity between groups. When the Controls were compared to the individuals with memory impairments who ultimately developed AD (the Converters), the accuracy of discrimination was 93% (sensitivity = 0.95, specificity = 0.90). The discrimination of the Controls and the individuals with mild memory problems who did not progress to the point where they met clinical criteria for probable AD over the three years of follow-up (the 'Questionables') was 85% and the discrimination of the Questionables and Converters was 75%.

SPECT results. SPECT perfusion abnormalities in four brain regions were found to differentiate the groups. They were as follows: the hippocampal–amygdaloid

complex, the posterior cingulate, the anterior thalamus, and the caudal portion of the anterior cingulate. When the Controls were compared to the individuals with memory impairments who ultimately developed AD (the Converters), the accuracy of discrimination was 83%. The discrimination of the Controls and the individuals with mild memory problems who did not progress to the point where they met clinical criteria for probable AD over the three years of follow-up (the 'Questionables') was 72.6% and the discrimination of the Questionables and Converters was 84%.

Genetic results. When apoE status alone was used to differentiate the three groups, the discriminant function was not statistically significant. ApoE status was then added to each of the discriminant function analyses for each domain. Addition of apoE status did not significantly improve the discrimination of the three groups above that of the neuropsychological or neuroimaging data alone.

DISCUSSION

The findings concerning the discriminating neuropsychological tests are consistent with previous reports of memory (e.g. Tuokko et al., 1991; Bondi et al., 1994; Petersen et al., 1994; Small et al., 1995a; Jacobs et al., 1995; Small et al., 1997; Howieson et al., 1997; Rubin et al., 1998) and executive function deficits (Tierney et al., 1996) among those destined to develop dementia. Atrophy in the entorhinal cortex and the adjacent hippocampus, likewise, has been reported among individuals with preclinical AD by a few research groups (Convit et al., 1993; Fox et al., 1996; Kaye et al., 1997; Bobinski et al., 1999). Decreases in temporoparietal perfusion among prodromal cases has been reported (Kennedy et al., 1995). In addition, alterations in the posterior cingulate have been reported among individuals who are homozygous for the apoE-4 allele, both with and without memory problems (Small et al., 1995b; Reiman et al., 1996). There are no reports of such a finding, to our knowledge, among a group of individuals who were followed to the point where they met clinical criteria for AD.

The overall importance of these findings primarily pertains to the parallels that are evident between the neuropsychological and neuroimaging results. They suggest that selected group brain regions develop neuropathology during preclinical AD which, in turn, influence the cognitive deficits of the individuals.

The memory deficit seen in preclinical AD is consistent with recent findings that the initial neuronal lesions of AD (e.g. the neurofibrillary tangles and neuritic plaques) develop in the entorhinal cortex (Braak and Braak, 1991), with some layers of the entorhinal cortex undergoing 40–60% neuronal loss even in the earliest phase of disease (Gomez-Isla et al., 1996). The entorhinal

cortex is part of a memory-related neural system in the brain (Squire and Zola-Morgan, 1983). Similarly, the superior temporal sulcus is a multimodal association area that appears to be necessary for holding information during a delay and has thus been hypothesized to play a role in memory or the attentional capacities necessary for normal memory (Eskander et al., 1992; Salzman, 1995).

It has been hypothesized that the caudal portion of the anterior cingulate plays a major role in executive function abilities, primarily through reciprocal connections with the prefrontal cortex (Arikuni et al., 1994). It also has reciprocal connections with the memory-related structures, including the entorhinal cortex (Van Hoesen, 1993). This brain region is known to develop severe neuronal loss in AD (Vogt et al., 1991), but the stage at which this occurs is not yet known. The present findings suggest that neuronal loss in the anterior cingulate may begin early in the disease and may, in part, be responsible for the executive function deficits seen in the early stage of disease.

The negative findings regarding apoE status as a predictor of 'conversion' to AD are consistent with several recent studies, including a large multicenter study that examined the use of apoE genotype as a diagnostic test for AD (Mayeux et al., 1998). Although there is consensus that apoE-4 status confers increased lifetime risk for AD, the present findings suggest that this genotype cannot be used to predict conversion to AD within three years' time.

There is still much to be learned about the development of disease during the prodromal phase of AD. Identification of individuals in a 'preclinical' or prodromal phase will be critical in testing existing therapies for their ability to alter the course of the illness and for developing novel strategies to prevent or delay dementia.

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11 Neuropsychological Detection of Preclinical Alzheimer's Disease: Results of a Neuropathological Series of 'Normal' Controls

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INTRODUCTION

Increasing evidence now suggests that the *early* pathogenetic process of Alzheimer's disease (AD) is protracted and may extend over decades (Braak and Braak, 1991; Snowdon et al., 1996). This 'preclinical stage' of AD appears to be separated into two stages: an extended 'latent period,' where there may be no observable symptoms of the disease, followed by a shorter 'prodromal phase.' During this latter stage, symptoms of memory and cognitive impairment are observable but are mild and preclude definitive diagnosis of AD. Recent clinical series of patients with symptoms consistent with early AD, now typically referred to as 'mild cognitive impairment,' suggest that indeed these individuals develop AD dementia at a greater rate as a group than normal older controls (Petersen et al., 1999). In some clinical series as many as 50% of the subjects with isolated memory impairments progress to identifiable AD over the course of five years (Bowen et al., 1997). Defining the borders of preclinical AD and distinguishing it from other conditions of aging is an area of intense scientific scrutiny with practical applications. Reliable definition of the early stages of the illness would afford more accurate early diagnoses and permit timely implementation of prevention strategies at a point in the trajectory of disease when such treatments are likely to be optimally effective.

A number of studies have demonstrated that it may be possible to identify neuropathological changes suggestive of early AD within highly specific and delimited regions of the brain in some non-demented elderly individuals who have come to postmortem examination (e.g. Crystal et al., 1993, 1996;

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Hulette et al., 1998; Davis et al., 1999; Morris et al., 1996; Arriagada et al., 1992). An elegant model for the trajectory of brain neurofibrillary change in preclinical AD has been proposed (Braak and Braak, 1991). Six stages of neuropathological change are described in relationship to tangle-bearing neurons and severity of change (Figure 11.1). During stages I and II, neurofibrillary changes are confined to the transentorhinal area of the medial temporal lobe. At stages III and IV there is more extensive involvement of the limbic cortices including the entorhinal cortex, transentorhinal regions, and CA1 of the hippocampus. Most areas of the neocortex are not involved. By stages V and VI, pervasive neurofibrillary tangles are seen in virtually all subdivisions of cortex, with particularly severe involvement of the neocortical association areas.

From the clinical perspective, a complementary pattern of preclinical change to full-blown AD is also reported. A number of studies from our laboratory and others indicate that there are detectable cognitive, anatomical, and metabolic markers of preclinical AD (Ohm et al., 1995; Small et al., 1995; LaRue et al., 1995; Reiman et al., 1996; Bookheimer et al., 2000). The neuropsychological studies report a predictable pattern of cognitive loss in the very early stages of clinical presentation. Almost without exception, the published studies report memory as being particularly involved. Poorer performance is seen in normal subjects who later convert to AD on tests such as the Buschke Selective Reminding Test (SRT; Masur et al., 1990, 1994; Tuokko et al., 1991; Grober and Kawas, 1997), the Object Memory Evaluation (OME; Fuld et al., 1990), on list learning procedures such as the California Verbal Learning Test (Bondi et al., 1994), the Rey Auditory Verbal Learning Test (Tierney et al., 1996), the CERAD Word List Memory Test (Welsh et al., 1991, 1992) or on measures of narrative recall (Linn et al., 1995). Commonly, there are also deficits noted in highly selective aspects of expressive language, such as naming (Jacobs et al., 1995) or category fluency (Howieson et al., 1997; Dartigues et al., 1997). Poorer performance is also reported on tests of complex executive functions involving verbal abstraction (Elias et al., 2000) or non-verbal conceptualization and spatial integration (LaRue and Jarvik, 1987; Fox et al., 1998). These observations raise the possibility that antemortem markers of the illness might be developed to allow accurate clinical detection of the earliest stages of AD.

The current study was designed to merge together these two parallel lines of investigation which both focus on defining the preclinical stages of AD. The primary aim was to determine whether reliable neuropsychological changes are associated with the very earliest neuropathological manifestation of AD, that is when cellular pathology is confined to the transentorhinal and entorhinal cortical regions (Braak stages II and III). Through study of neuropathologically defined cases of very early stage AD, we are afforded the opportunity to determine more definitively the cognitive profile corresponding to the pathological trajectory of AD. To address whether there is

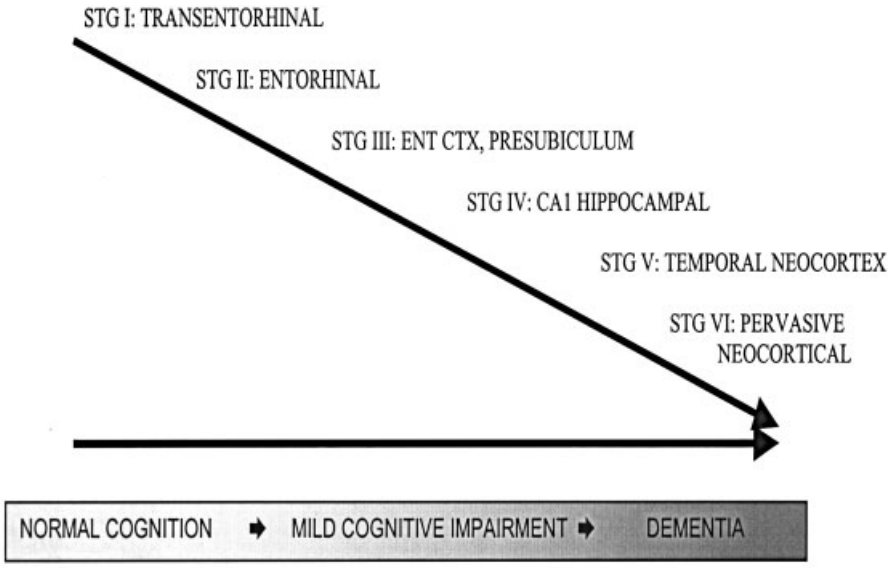

Figure 11.1. Braak staging (Braak and Braak, 1991) of neurofibrillary changes in Alzheimer's disease

a cognitive trajectory paralleling the underlying pathological changes of very early AD, we examined the cognitive performance of a cohort of putatively normal elderly subjects who were enrolled in a normal autopsy control program. These participants who were followed longitudinally until death provide a neuropathologically defined cohort of normal controls and very early AD cases in which the neuropsychological correlates of preclinical AD can be definitively tested.

METHODS

SUBJECTS AND CLINICAL ASSESSMENT METHODS

The 31 cognitively and neurologically normal subjects at entry in the study were all participants in the Bryan ADRC control autopsy program discussed elsewhere (Hulette et al., 1997, 1998). All subjects had at least one complete neuropsychological evaluation and had been clinically assessed in the year prior to death. These individuals were all medically screened at entry and met the following criteria for enrollment into the normal control protocol: (1) they were free of cognitive disorders leading to functional impairments in everyday life, (2) they were living independently without difficulty, operationally defined as a Clinical Dementia Rating of 0 (CDR = 0; see Morris et al., 1989), and (3) none carried a diagnosis of a memory disorder. All subjects

underwent detailed neuropsychological evaluation at entry and at annual re-examination to confirm continued eligibility and to document any changes in cognitive function over time.

The tests administered are part of our standard research battery used routinely in our epidemiological studies of aging and dementia (see Tschanz et al., 2000, for review). The core of the battery consists of the neuropsychological tests developed for the Consortium to Establish a Registry of Alzheimer's Disease (CERAD; see Morris et al., 1989). Briefly, the CERAD battery consists of the Mini-Mental State Examination (MMSE), a test of animal fluency, an abbreviated version of the Boston Naming Test, a test of constructional praxis, and the Word List Memory Test, a ten-item word list recall procedure (see Welsh et al., 1991, for more detail). Additional measures of memory, language, and executive function are added to the CERAD battery in order to increase the instrument's overall sensitivity and specificity to early AD. These add-on measures include Logical Memory I and II from the Wechsler Memory Scale revised, the Benton Visual Retention Test, a delayed recall procedure for the CERAD Praxis items as a test of visual memory, the Controlled Oral Word Association Test from the Multilingual Aphasia Examination, and the Trail Making Tests Parts A and B.

Performance on the neuropsychological examination was considered normal if all test scores fell within normative standards (within 2SD of published control means with age/education correction), or if there were only isolated test abnormalities observed (no more than three tests) with no accompanying cognitive complaint or functional impairments (CDR = 0). Final diagnoses were assigned based on review of all available clinical and medical information by the Bryan ADRC neurologists.

NEUROPATHOLOGICAL METHODS

The neuropathological methods used are well described in our previous work (Hulette et al., 1998). Briefly, the brains were fixed in formalin and examined according to CERAD guidelines (Mirra et al., 1991). Paraffin-embedded sections were obtained from each neocortical region including the primary visual and visual association cortex (Brodmann areas 17 and 18), as well as from the hippocampal formation (at the level of the lateral geniculate nucleus), the amygdala, the basal ganglia (including the insula), the rostral cingulate gyrus, the midbrain, and the cerebellum. Paraffin sections were routinely stained for plaques and tangles using luxol fast blue/hematoxylin and eosin, Congo Red, and Microwave King silver stains. Cortical sections were immunostained with monoclonal antibody to PHF-tau (clone AT8) purchased from Biosource International (Camarillo, Calif.), monoclonal antibody to human β-amyloid (clone 6 F/3D Dako), and a polyclonal rabbit antibody, ubiquitin, purchased from Biomeda (Foster City, Calif.).

Evaluation of plaque and tangle frequencies were estimated by examining silver-stained cortical sections and were graded according to the published criteria (Mirra et al., 1991). Neuritic plaque frequency was graded as sparse, moderate, or frequent in each neocortical section and the scores were then compared to subject age. The age-related plaque scores were then matched with the cognitive status of 'normal.' Controls above age 75 with frequent plaques in one cortical region were thus designated 'Possible Alzheimer's disease.' Controls greater than age 75 with sparse plaques were designated 'Normal 1b.' Controls with absence of plaques were designated as 'Normal 1a' (Mirra et al., 1991).

Neurofibrillary change was staged according to Braak and Braak (1991). The appearance of rare neurofibrillary tangles confined to the entorhinal cortex or hippocampus was considered to represent Braak stage I. More frequent tangles in these areas were representative of Braak stage II. The appearance of tangles in the inferior temporal neocortex constituted Braak stage III. Neocortical tangles outside of the inferior temporal lobe corresponded to Braak stage IV. Stages V and VI were not observed in this study.

ApoE GENOTYPING

In many cases apoE genotype had been determined prior to death from either venipuncture samples or buccal material provided at the time of clinical evaluation. In the cases where apoE genotyping had not occurred prior to death, genomic DNA was isolated from approximately 300 mg frozen cortex or cerebellum. The extracted DNA was amplified using polymerase chain reaction (PCR) methods described elsewhere (Saunders et al., 1993a,b), with the modification of visualizing SYBERGold-stained (Molecular Dynamics) PCR products with a fluoroimager.

RESULTS

Group neuropsychological and demographic data are presented in Table 11.1. There were 19 subjects who were in the neuropathologically normal group as defined by CERAD criteria. The majority had Braak ratings of I ($n = 12$) and II ($n = 4$). There were two individuals in this group who were defined as neuropathologically normal by CERAD criteria but were coded as Braak stage III. Twelve subjects met neuropathological criteria for very early stage, possible AD (CERAD criteria). In this group the majority were Braak stage III ($n = 7$), with a fair number of stage II ($n = 4$). One individual in the possible AD group had Braak staging of IV. Examination of the demographic data by group revealed no differences in age, education, or gender (using ANOVA, p levels <0.05 for significance). With respect to apoE genotypes, a

Table 11.1. Comparison of results for the group rated neuropathologically normal and the group fulfilling criteria for very early stage possible AD[a] (mean ± SD)

	Pathology normal (n = 19)	Pathology possible AD (n = 12)	
Age (years)	81.0 ± 7.6	83.2 ± 8.3	ns
Education (years)	16.6 ± 2.3	15.7 ± 2.8	ns
ApoE ε_4/ non-ε_4	3/16	4/8	ns
CERAD battery			
Mini-Mental Status Exam (MMSE)	28.1 ± 2.0	27.3 ± 3.4	ns
Animal fluency	18.1 ± 4.8	17.1 ± 4.9	ns
Naming test	14.6 ± 0.5	14.0 ± 2.0	ns
Word List Learning, Trial 3	7.6 ± 2.1	7.5 ± 1.8	ns
Word List Memory (delay recall)	6.7 ± 2.1	6.2 ± 2.9	ns
Word List Memory Savings	0.89 ± 0.18	0.77 ± 0.30	ns
Supplemental measures			
Logical Memory I (immediate)	32.1 ± 7.1	26.6 ± 7.4*	$F = 4.27$, $p<0.05$
Logical Memory II (delay)	28.1 ± 8.4	21.4 ± 11.1	$F = 3.65$, $p<0.07$
Benton Visual Retention Test (BVRT)	6.2 ± 1.9	5.1 ± 2.2	ns
Visual Memory (CERAD Praxis)	8.9 ± 2.1	6.9 ± 2.7**	$F = 5.35$, $p<0.03$
Trail Making Part A	52.8 ± 25.1	50.9 ± 19.9	ns
Trail Making Part B	118.8 ± 58.1	144.2 ± 66.0	ns
Change scores			
Logical Memory I (immediate)	1.13 ± 4.8	−2.78 ± 4.8*	$F = 4.18$, $p<0.05$
Logical Memory II (delay)	1.13 ± 3.5	−2.33 ± 4.0**	$F = 5.54$, $p<0.03$
Benton Visual Retention Test (BVRT)	−0.31 ± 1.3	−0.89 ± 2.3	ns
Visual Memory (CERAD Praxis)	−0.46 ± 1.9	−1.67 ± 2.6	ns
Trail Making Part A	2.47 ± 18.6	2.22 ± 8.6	ns
Trail Making Part B	−4.64 ± 46.3	8.22 ± 38.8	ns

[a]CERAD criteria.

somewhat higher proportion of subjects in the very early AD group had an ε_4 allele compared to the neuropathologically normal group (33% vs. 11%). This difference was not statistically significant given the small number of total subjects (Fisher exact test, $p > 0.38$).

Analysis of the cross-sectional cognitive data (test results within one year of death) revealed detectable differences between the two groups. Trends were found in general across nearly all of the memory measures and executive measures. Significant differences were found on the CERAD test of visual memory, which looks at uncued recall of the constructional praxis drawings after a 5-min delay. There was also a significant difference on Logical

Memory I, a test of immediate recall for narrative information. Both these tests assess recent memory function and place demands on effective consolidation of new information for later retrieval. Somewhat surprisingly, differences did not emerge on other similar tests of memory function (e.g. Word List Learning Test) or on all delayed recall tests (e.g. Logical Memory II). However, some of the latter category of tests approached statistical significance. For example, delayed recall of the narratives approached significance ($p < 0.066$). Finally, there were no significant differences on the tests of executive function or on the tests of expressive language ability, as has been suggested in other studies of preclinical AD (see Introduction). The results appear to underscore the importance of memory changes in very early preclinical AD.

Analysis of longitudinal change scores in neuropsychological performance was also possible in the subgroup of subjects which had repeated testing with complete batteries ($n = 17$ in normals; 10 in AD). The results were very consistent with the cross-sectional findings. There was again a fairly uniform trend observed across the cognitive measures on one-year change scores. However, the early-stage AD group showed specific impairments relative to the neuropathologically normal subjects on two specific tests of verbal memory, Logical Memory II, a measure of delayed verbal recall ($p < 0.03$), and Logical Memory I, the immediate recall procedure ($p < 0.05$). This finding is consistent with the cross-sectional data and again underscores the importance of recent memory function in early AD. The specific importance of narrative recall over other types of memory tests may reflect important attributes of the different tasks. Narrative recall may be argued to be a more complex task than other memory tests (such as word recall). With only single exposure to contextually rich information, there is a high demand for efficient extraction of meaning from complex, detailed information. Thus, effective semantic encoding of the story gist and the various details along with consolidation of this information into episodic memory are required for the later effective recall of the learned material.

DISCUSSION

These findings lend support to the notion that there is a preclinical stage in the very early AD neuropathological process that is detectable with highly targeted tests of neuropsychological function. The results indicate that in the very earliest stages of AD pathology when cognition is seemingly normal, recent memory function tested over brief delays may be particularly powerful in detecting the early signs of the disease. Trends in the predicted direction were seen on other cognitive measures of language and executive function. However, none of the differences seen on these other cognitive measures achieved statistical significance. Consequently, we conclude that memory

consolidation is particularly vulnerable to the AD process and may be selectively disturbed by the neuropathological lesions confined to the transentorhinal area, entorhinal cortex, and CA1 area of the hippocampal formation. This interpretation is consistent with a large body of neurobehavioral research, which has articulated a clear role of these medial temporal lobe structures in recent memory processing of AD and other memory disorders (see Troster, 1998; Mesulam, 2000, for review).

The results in this neuropathologically defined group of early AD subjects complement our previous work suggesting that delayed recall is the most sensitive cognitive predictor of early diagnosable AD (Welsh et al., 1991, 1992). The findings also closely parallel the findings obtained in large cohort studies of preclinical AD (e.g. Bondi et al., 1994; Tierney et al., 1996; Petersen et al., 1999). All these studies underscore the importance of recent memory performance in the early clinical presentation of AD. The results from the current study are, however, unique in their neuropathological emphasis, and thereby provide validation for previous models suggesting that memory change presages more fulminant AD. With neuropathologically defined AD as the gold standard, the results clearly support a continuum of cognitive change, which parallels a continuum of neuropathological change.

There are some limitations to the study and the data should not be overstated. The sample size, although reasonable for a neuropathological analysis at a single AD center, is still quite limited in its scope for diagnostic applications. In addition, the study sample was comprised entirely of white individuals who were highly educated and had been carefully screened for any type of potential medical confounding. Consequently, the generalization of the findings to the diversity of situations of early AD may be quite limited. Further study of cognitive predictors over time is needed in much larger samples including diverse patient groups. Within a more expanded study, more refined analytic approaches would also be reasonable. Single-variable, univariate approaches, such as those used here, are practical and useful in small exploratory samples. However, they are somewhat inefficient and pose limitations in terms of general clinical utility (sensitivity and specificity). The application of the more recently emerging and powerful statistical methods such as generalized estimating equations (GEE; Diggle et al., 1994) and generalized linear mixed models (GLMM; Breslow and Clayton, 1993) to longitudinal neuropsychological data appears particularly promising in this regard (Stern et al., 1996). These techniques allow better control over the idiosyncrasies of longitudinal studies, such as missing time points or inconsistent time observation windows.

Future studies examining predictive models which consider not only a combination of neuropsychological outcomes but also incorporate biological measures (such as apoE genotype or brain imaging markers) and longitudinal change over time will likely provide more powerful algorithms for detecting true cases of preclinical AD. This multivariable approach is more closely akin

to standard clinical practice and is likely to eliminate false positive results from impairment on any single measure (e.g. memory, in this instance). Combination of information in this manner will no doubt improve diagnostic detection of early-stage disease and more likely lead to algorithms with acceptable predictive utility. Our current ongoing work is focused in this direction. Armed with useful predictive models, we may be in a position to more accurately identify the early cognitive and biological phenotype of preclinical AD and provide interventions in affected individuals before the disease has gained a foothold within the brain and created irreparable neurological injury.

SUMMARY

Increasing evidence suggests that the underlying pathological process of Alzheimer's disease (AD) may be quite protracted, extending over decades. In this investigation we sought to determine the associated neuropsychological borders of preclinical AD by examining a group of normal older adults who were enrolled in the Bryan ADRC Rapid Autopsy Program and were followed annually with repeat cognitive testing until their death. The results from these studies indicated that clinically normal elderly individuals with early-stage AD pathology (CERAD neuropathological criteria for possible AD) including tangle development in the transentorhinal areas and CA1 of the hippocampus ($n = 12$) perform less well on tests of visual and verbal memory when compared to age- and education-matched subjects with no signs of early-stage AD brain pathology at postmortem examination ($n = 19$). The difference in memory performance was maintained over repeat observations; subjects with early AD decline more rapidly on neuropsychological testing than their normal counterparts. Evaluation of apoE indicated that nearly all genotypes were represented in the groups, but the affected subjects had a slightly higher proportion of ε_4 allele carriers (33%), as predicted, than the unaffected group (15%). We conclude that, in clinical normal elderly individuals, changes in specific aspects of memory function may reliably presage the early stages of AD. In the absence of a biological marker for AD, future multivariate statistical models incorporating targeted measures of memory performance, trajectories of cognitive change over time, and genetic risk variables (e.g. apoE genotype) may prove to be a practical alternative to enhance reliable detection of early AD and guide treatment decisions in early stages of cognitive dysfunction.

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12 Potentially Reversible Conditions in Memory Clinic Patients

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INTRODUCTION

The prevalence of dementia in Europe is 6.4%, with Alzheimer's disease (AD) being the most frequent cause of dementia (Lobo et al., 2000). The approval of cholinesterase inhibitors for the treatment of AD has increased the focus on diagnostic assessment of patients with dementia, particularly in the early stage. To maximize the benefits of acetylcholinesterase inhibitors, treatment should begin as early as possible, ideally in patients with very mild AD. Further, early diagnostic assessment is important for the implementation of a psychosocial support program and caregiver intervention. Finally, diagnostic assessment of patients with cognitive symptoms is crucial for the identification of potentially reversible conditions, non-progressive dementia disorders, and other non-dementia disorders (Waldemar et al., 2000).

The early symptoms of AD comprise increased forgetfulness that interferes with the ability to maintain a job or household, difficulties in finding words, loss of initiative and interest, and impaired judgment. These symptoms are not specific for AD; they may occur in many different kinds of diseases, including potentially reversible conditions. For the patient it is important to know whether the disability is due to a progressive degenerative disorder or to a potentially reversible condition, which may be curable or at least may not progress. In many countries memory clinics have been developed to facilitate the early diagnosis and treatment of patients with possible dementia. With the advent of therapy for AD, many patients referred to memory clinics present with symptoms suggesting either early AD or questionable dementia.

The aim of this study was to investigate the prevalence and classification of potentially reversible conditions in a memory clinic cohort. We defined a potentially reversible condition as a condition known to be (1) potentially reversible or arrestable, and (2) responsible for or contributing to the observed cognitive decline or dementia in the patient.

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METHODS AND PATIENTS

Established in 1995, the memory clinic of the Copenhagen University Hospital is operated as a multidisciplinary outpatient facility in a neurological setting, with specialists in neurology, psychiatry, neurosurgery, and neuropsychology, as well as specialized nurses and social advisors (Hogh et al., 1999). This report describes the diagnostic evaluations with special focus on potentially reversible conditions, in all consecutive patients referred in the clinic during a period of 40 months (September 1995 to December 1998). Referrals were received primarily from general practitioners and from private practice specialists in neurology or psychiatry. Some patients were referred from other hospitals in the Copenhagen district and other Danish counties.

At the first visit, the patient and an informant were interviewed by a neurologist and the following basic study program was performed: a full physical and neurological examination, laboratory screening tests (blood test), electrocardiography, and the Mini Mental State Examination (MMSE) (Folstein et al., 1975). The indications for further diagnostic assessment were determined by the neurologist. Almost every patient underwent brain imaging, either computed tomography (CT) or magnetic resonance imaging (MRI). More than half of the patients also underwent one or more of the following tests: single photon emission computed tomography (SPECT), neuropsychological examination, and psychiatric evaluation. In selected patients, cerebrospinal fluid (CSF) examination, intracranial pressure monitoring and ventricular infusion study, electroencephalography (EEG), blood tests for rare conditions, or other supplemental investigations were performed, if clinically relevant.

After completion of the initial study program, the multidisciplinary staff gathered to complete a consensus report on each patient, including the establishment of a diagnosis and a plan for treatment and follow-up. In the consensus report, patients were classified into the following four groups: (1) dementia, (2) selective amnesia, (3) other cognitive deficits, or (4) no significant cognitive deficits. Some patients could not be classified according to this system, mainly due to language problems or to ongoing abuse of alcohol or drugs. A diagnosis of dementia was determined using the DSM-IV criteria (American Psychiatric Association, 1994).

At the second visit, a final diagnosis was determined to represent the primary underlying etiology. For the clinical diagnosis of Alzheimer's disease the criteria of NINCDS–ADRDA (McKhann et al., 1984) were applied. For a diagnosis of vascular dementia the criteria of NINDS–AIREN (Roman et al., 1993) were applied. All other diagnoses followed the ICD-10 (World Health Organization, 1992).

Patient characteristics and diagnostic classification were registered consecutively in a database.

RESULTS

A total of 785 consecutive patients (376 male and 409 female) were referred during a period of 40 months. Their mean age was 64.6 years (range 17–97 years) and the mean MMSE score was 23.3 (range 0–30). This indicates that the majority of our patients had questionable, mild, or a moderate degree of dementia. Forty-three percent of all patients fulfilled DSM-IV criteria for dementia, 6% had selective amnesia, and 11% had other cognitive deficits. Twenty-eight percent had no cognitive deficits, and 12% could not be classified.

The primary diagnoses in the 785 patients represented a wide spectrum of neurological and psychiatric diseases and internal medicine disorders as the cause of the subjective cognitive symptoms. The four most common specific diagnoses were Alzheimer's disease, depression, and vascular dementia, and no neuropsychiatric disease. These four conditions accounted for almost half of the patients.

Of all 785 patients, 20% had cognitive symptoms due to a potentially reversible primary underlying etiology. Almost half of these patients had depression, which might explain their cognitive symptoms. The second most frequent condition was normal-pressure hydrocephalus, which was found in one-fifth of the patients with a primary potentially reversible cause. Other conditions identified included alcohol dependence syndrome, epilepsy, thyroid disease, cerebral vascular malformation, and other types of hydrocephalus.

Of all patients who fulfilled the DSM-IV criteria for dementia, 6% had a potentially reversible primary underlying etiology. Normal-pressure hydrocephalus represented the majority of these patients, but space-occupying lesions, vascular malformations, epilepsy, traumatic hydrocephalus, and alcohol dependence syndromes were represented in this group of patients.

Of patients not fulfilling the criteria for dementia, 31% had a potentially reversible condition.

DISCUSSION

To our knowledge this is the first prospective study to investigate reversible conditions in a memory clinic cohort of patients. In a cohort of 785 consecutive patients we demonstrated a potentially reversible etiology in 20%. However, among patients fulfilling the criteria for dementia, a potentially reversible etiology was more rare, being observed in 6% of the patients.

A quantitative review of 32 retrospective studies including 2889 patients (Clarfield, 1988) found a prevalence of 13.2% for potentially reversible conditions. Weytingh et al. (Weytingh et al., 1995) reported, in a quantitative

review of 16 studies with 1551 patients, a frequency of potentially reversible dementia that varied widely between studies from 0 to 37.5%, with an average of 15.2%. However, on follow-up, the prevalences of partially and fully reversible conditions were low, 9.3% and 1.5% respectively (Weytingh et al., 1995).

Previous follow-up studies of patients with potentially reversible conditions have shown that in many patients the mental symptoms were not reversible on treatment. However, a significant fraction of patients experienced either full or partial reversibility of symptoms (Clarfield, 1988; Weytingh et al., 1995). We did not examine the actual reversibility of the conditions identified in this study. However, even when a full or partial reversibility cannot be predicted, the search for possible reversibility—or even arrestable conditions—is a crucial element of the management of memory clinic patients (Wahlund et al., 2000).

The higher prevalence of potentially reversible conditions in our study may be explained by the prospective design with systematic registration of predefined classifications and potentially reversible conditions, rather than the retrospective design of previous studies. The higher prevalence in our study may also reflect the fact that many of our patients had only mild cognitive symptoms, and that our memory clinic was based in neurology. Many of the previous studies included in the two reviews (Clarfield, 1988; Weytingh et al., 1995) were carried out in patients with an established diagnosis of dementia. The identification of a reversible or arrestable condition is of obvious benefit to the patient. It is of great medical, ethical, and psychological relevance for the future of the patient and caregiver to know whether the cognitive decline is due to a progressive degenerative disorder like AD or due to a potentially reversible condition, which it may be possible to stabilize or even cure.

Evidence-based guidelines for the diagnostic assessment of patients with dementia focus on recommendations for assessment tools in the early diagnosis of AD and on the identification of other cognitive disorders (Corey-Bloom et al., 1995; Waldemar et al., 2000). Evidence for the occurrence of potentially reversible conditions is highly relevant for the development and revision of guidelines. Most of the reversible conditions identified in our cohort of patients were identified and confirmed by either neurological examination, psychiatric evaluation, structural brain imaging (CT or MRI), routine laboratory tests, or EEG.

With the advent of treatment for AD and the increased awareness of the importance of an early diagnosis, more patients will be referred with mild symptoms and questionable dementia. According to our findings, the prevalence of potentially reversible conditions is significantly higher in this group of patients than in patients fulfilling strict dementia criteria. Thus, an intensive search for reversible conditions and a treatment seems to be particularly relevant in patients with mild cognitive symptoms.

In conclusion, although potentially reversible conditions may not all be fully reversible, the identification and treatment of these conditions represent an essential element of systematic diagnostic evaluation of dementia. Most of the potentially reversible conditions are easily identified by careful clinical examination of the patient, routine laboratory tests, and brain imaging.

SUMMARY

This prospective study describes the prevalence of a potentially reversible primary etiology among 785 consecutive patients referred to a multidisciplinary memory clinic in a neurological setting during a period of 40 months. The mean age of the patients was 64.6 years and the mean MMSE score was 23.3. On clinical and neuropsychological examination, 43% of patients fulfilled DSM-IV criteria for dementia, 6% had selective amnesia, 11% had other selective cognitive deficits, and 28% had no cognitive deficits, while 12% could not be classified. A potentially reversible primary etiology for cognitive symptoms was observed in 20% of all patients. In 6% of patients fulfilling DSM-IV criteria for dementia, and in 31% of patients with cognitive symptoms not fulfilling dementia criteria, a potentially reversible primary etiology was observed. Diagnostic assessment of patients with cognitive symptoms should focus on the identification and treatment of potentially reversible conditions as well as on the early identification of Alzheimer's disease.

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13 The Alzheimer's Disease Centers' Neuropsychological Database Initiative: A Resource for Alzheimer's Disease Prevention Trials

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INTRODUCTION

For the past two decades clinical trials for Alzheimer's disease (AD) have been conducted largely in patients already suffering from the disease. More recently efforts have been directed toward the prevention of AD. Prevention of AD is necessary if we are to forestall the huge economic and societal burden that is predicted to occur due to AD over the next 50 years. Randomized double-blind, placebo-controlled trials to prevent AD in normal elderly individuals are likely to provide the best means for evaluating prevention interventions.

A problem with primary prevention trials, as they are currently designed, is that relatively few normal individuals reach the clinical endpoint of AD over a suitable time frame for a clinical trial. This occurs because of the low conversion rate to AD in the normal elderly population over the relatively short trial period of a few years. This limitation means that primary

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prevention trials must recruit large numbers of participants. The Women's Health Initiative–Memory Study (WHIMS), for example, is recruiting approximately 8000 women who are 65–79 years of age (McBee et al., 1997). These prevention trials suffer from enormous costs due to the large number of participants and the extended duration required for clinical endpoints to be realized. Also, the small number of 'conversions' in such trials limits the conclusions that may be drawn regarding the effectiveness of the intervention for the majority of trial participants who are unlikely to develop AD regardless of which treatment they are given. Finally, the expense and duration of such trials severely limit the number of agents that can be tested.

To overcome these limitations, some approaches have been to enroll only older normal subjects (e.g. aged approximately 80 years and older) or to restrict the study population to those with a positive family history of AD or the presence of the apoE-ε_4 allele. One drawback to these approaches is the limited generalizability of the study results, which can only be extended to individuals with the same characteristics as those who participated in the trial. It is therefore unclear whether an effective intervention in the context of such a prevention trial would be useful for prevention of AD in the general population, particularly those persons who might develop sporadic AD or AD prior to beginning their ninth decade.

Another approach to designing clinical trials for AD prevention is to conduct secondary prevention trials of AD in individuals with 'mild cognitive impairment' (MCI). In this trial design, memory-impaired individuals who otherwise have generally normal cognition and function are randomized to receive active treatment or placebo and are evaluated over a number of years to determine whether the intervention can delay a diagnosis of clinical AD. Through other studies (Flicker et al., 1991; Petersen et al., 1995, 1999b; Tierney et al., 1996) and a previous collaborative data-sharing effort (Grundman et al., 1996), it was determined that the conversion rate from MCI to AD is approximately 12–15% per year for the first few years. The much higher conversion rate to AD in MCI patients than in normals allowed a secondary prevention trial to be designed with fewer subjects and less expense than would be required for a primary prevention trial. These findings led to the development of a National Institute on Aging funded AD prevention trial in MCI subjects involving vitamin E and donepezil (Grundman, 2000).

Similar trials are now being conducted by a number of pharmaceutical companies to determine whether anti-inflammatory agents and cholinesterase inhibitors are effective in delaying the onset of AD in MCI subjects. Although trials enrolling MCI patients offer a more pragmatic approach to AD prevention than primary prevention trials by focusing on early detection and prophylactic treatment, initiating a trial in individuals who already have significant memory or cognitive loss is not optimal. It would be preferable if

AD prevention efforts could be initiated before symptoms of memory loss develop. Also, it is possible that some prophylactic interventions may be less effective if they are started only after AD pathology is present (Mulnard et al., 2000), which appears to be a common feature in many individuals with MCI (Green et al., 2000; Hulette et al., 1998; Jack et al., 1999; Price and Morris, 1999).

Since MCI is known to be a significant risk factor for the development of AD, other trial designs that might be useful for preventing AD are trials to prevent MCI or trials using cognitive or functional decline as the principal endpoints. Previous data suggest that decline on cognitive measures may become apparent before cognitive impairment becomes clinically detectable (Bondi et al., 1994; Howieson et al., 1997; Petersen et al., 1999a; Rubin et al., 1998) and that subtle AD neuropathological changes may be associated with this impairment (Naslund et al., 2000; Schmitt et al., 2000). Primary prevention trials might be designed with neuropsychological endpoints that are highly predictive of AD and therefore suitable surrogates for incipient AD. In addition, age-related memory decline (Small et al., 1999) is a common concern for many older individuals. The Neuropsychological Database Initiative was funded by the National Institute on Aging to enable 16 Alzheimer's Disease Centers to pool data obtained from normal elderly individuals at each center into a common database. This large collaborative database may provide information necessary to define rates of cognitive change that are predictive of incipient AD. It could also describe rates of memory and cognitive change that would be expected in a normal elderly population. Together, these data could be used to estimate the parameters required for conducting clinical trials in the elderly for enhancing memory, slowing cognitive decline, or preventing MCI and AD.

An important objective of this collaboration is to define sensitive neuropsychological and functional measures that could serve as surrogate endpoints for a clinical diagnosis of AD. Some desirable features of a surrogate endpoint are that the surrogate is in the causal pathway of the disease entity (e.g. that the causal pathways leading to early cognitive and functional decline also lead to the diagnosis of AD); that the treatment has an effect on the surrogate similar to that on the disease entity (e.g. delaying or reducing the number of endpoints), and that the surrogate (cognitive deterioration) has clinical significance on its own independent of the disease entity of interest (dementia due to AD). The development of robust neuropsychological trial endpoints, which are more sensitive than a clinical diagnosis of AD, could markedly reduce the cost, number of subjects, and duration of AD primary prevention trials. This project will help determine whether a quantifiable deterioration in memory or other cognitive and functional measures can predict the subsequent development of MCI or AD and whether such a change could serve as a surrogate endpoint for incipient AD in a clinical trial.

The objective of the present article is to outline some of the characteristics of the normal subjects in the database at baseline and to estimate their rate of conversion to MCI and AD.

METHODS

Demographic and diagnostic information as well as neuropsychological and functional data from commonly used instruments were submitted electronically and merged into a central database. The data were previously collected at the 16 participating Alzheimer's Disease Centers on subjects enrolled from the community or referred for evaluation in projects studying normal aging and/or MCI. Demographic information included subjects' age, gender, ethnicity, education and marital status. Family history of AD and apoE-ε_4 allele status were also included. Diagnostic information generally reflected the outcome of a clinical evaluation conducted every one to two years that concluded with each subject being given a diagnosis of normal cognition, MCI, AD, or another type of dementia. Specific test data that were collected spanned multiple cognitive and functional domains and included tests of:

1. General mental status: Mini-Mental State Examination (MMSE; Folstein et al., 1975), Blessed–Roth Information–Memory–Concentration Test (Blessed et al., 1968), Mattis Dementia Rating Scale (Mattis, 1976)
2. Attention: Digit Span (WAIS-R) (Wechsler, 1981), Digit Symbol Substitution Test (Wechsler, 1981)
3. Memory: CERAD Word-List Learning Test (Morris et al., 1989; Welsh et al., 1991), Logical Memory subtest (WMS or WMS-R) (Wechsler, 1987), Visual Reproduction subtest (WMS or WMS-R) (Troester et al., 1993; Wechsler, 1987), Rey Auditory–Verbal Learning Test (Lezak, 1995)
4. Language: Boston Naming Test (Kaplan et al., 1983), Category Fluency Test (Benton and Hamsher, 1976; Morris et al., 1989), Letter Fluency Test (Goodglass and Kaplan, 1983; Newcombe, 1969)
5. Visuoperceptual, visuospatial, and visuoconstructive ability: Clock Drawing Test (Brodaty and Moore, 1997), Block Design subtest (Wechsler, 1987)
6. Problem solving, conceptualization, and executive functions: Trail-Making Test (Parts A and B) (Armitage, 1946; Reitan, 1958)
7. Functional measures: Clinical Dementia Rating (CDR) and the CDR Sum of Boxes (Morris, 1993), Blessed Dementia Scale (Blessed et al., 1968), Pfeffer Functional Activities Questionnaire (Pfeffer et al., 1982).

We are presently collecting additional measures including the Free and Cued Selective Reminding Test (FCSR) (Grober et al., 1997), the Hopkins Verbal Learning Test (Brandt, 1991), the Physical Self-Maintenance Scale (Lawton

and Brody, 1969; Lawton, 1988), the Geriatric Depression Scale (Yesavage et al., 1982), and the Neuropsychiatric Inventory (Cummings et al., 1994).

Survival analysis was used to determine the conversion rates for normal subjects to MCI and AD. This methodology was utilized because it properly accounts for varying lengths of observation. The subjects in this study were enrolled at different times and had unequal lengths of follow-up. For each individual the length of follow-up or time to a clinical diagnosis of MCI or AD was ascertained and Kaplan–Meier survival curves (Kaplan and Meier, 1958) were generated.

RESULTS

To date, longitudinal data on over 4000 normal elderly and 800 individuals with MCI have been cataloged. Table 13.1 lists those instruments currently in the database for which there were at least 500 normal subjects with data at the time of their first visit. Table 13.2 indicates the normal subjects' baseline demographic characteristics as well as their performance on selected measures for which primary data verification was completed. The normal cohort had a mean age of 72.7 (62% women) and a mean baseline MMSE score of 28.5. The group was highly educated, with a mean education duration of 15.0 years. Diagnostic information was available subsequent to baseline for over 2600 normal subjects. Figure 13.1 shows the observed conversion rate from normal to MCI or AD in this cohort. After three years of follow-up approximately 1% of normals developed a diagnosis of AD while 6% developed MCI. After four years of follow-up approximately 2% developed a diagnosis of AD whereas almost 10% developed MCI. Over half of the normal subjects diagnosed with AD received a diagnosis of MCI prior to receiving a diagnosis of AD. This percentage may have been higher except that most centers perform exams annually and some centers perform examinations less frequently. This might allow some subjects to pass through an MCI phase without being detected.

DISCUSSION

These analyses indicate that in a large multicenter cohort of normal individuals followed prospectively, deterioration to MCI occurs more frequently than decline to AD and is a common finding in persons who develop AD. These findings suggest that a diagnosis of MCI would be a more frequent endpoint than AD in prevention trials lasting several years and that a diagnosis of MCI might be a useful surrogate endpoint in such trials. Similar analyses are in process with respect to measures of cognitive and functional decline to determine whether they might also be suitable as surrogate endpoints.

Table 13.1. Neuropsychological and functional measures on normal subjects at baseline

Neuropsychological or functional measure	Number of subjects with data
Mental Status	
Mini-Mental State Examination	3730
Blessed–Roth Information–Memory–Concentration Test	2036
Mattis Dementia Rating Scale	1062
Attention	
Digit Span Test (WAIS-R)	1645
Digit Symbol Substitution Test	1985
Memory	
CERAD Word-List Learning Test	1632
Logical Memory Test (WMS or WMS-R)	1971
Visual Reproduction Test (WMS or WMS-R)	1637
Rey Auditory Verbal Learning Test	543
Language	
Boston Naming Test	1806
Category Fluency Test (Animals)	2449
Letter Fluency Test	937
Visuoperceptual, visuospatial, and visuoconstructive ability	
Clock Drawing Test	890
Block Design	1283
Problem solving, conceptualization, and executive functions	
Trail-Making Test (Parts A and B)	1798
Functional measures	
Clinical Dementia Rating	1787
Blessed Dementia Rating Scale	722
Pfeffer Functional Activities Questionnaire	600

It should be borne in mind that the estimates of 'conversion' provided in this chapter reflect the cohort as a whole, and different conversion estimates would be obtained if a subset of the population with differing baseline characteristics (e.g. age or neuropsychological performance) were analyzed. As noted previously, we are in the process of determining estimates for the rate of development of MCI and AD based on further stratification of this cohort by age, gender, family history, baseline cognitive scores, and apoE status. These analyses have several purposes. First, if we can identify a group of normal individuals who are unlikely to develop significant cognitive decline or MCI in the time course of a clinical trial (e.g. very high performing subjects), it should be possible to design trials to prevent MCI or cognitive decline more efficiently. Individuals who are at very low risk of reaching a trial endpoint are generally not helpful in demonstrating whether an agent is

Table 13.2. Demographics and selected cognitive scores on normal subjects at baseline

Variable	
Age (years)	72.7 ± 10.3[a]
Gender	M 38%: F 62%
Education (years)	15.0 ± 6.0
Ethnicity	93% White; 5% African American; 2% other
Mini-Mental State Examination	28.5 ± 2.2
Logical Memory (delayed recall; WMS-R)	18.8 ± 8.7
CERAD Word-List Delayed Recall	6.8 ± 2.0
Category Fluency (animals)	17.6 ± 6.9
Trails A (s)	49.5 ± 31.9
Trails B (s)	107.1 ± 55.0

[a] Mean ± SD.

effective. This is because the agent may not be able to further reduce the incidence of that endpoint in such subjects (or if it can, it becomes very difficult to measure without a very large sample). It may be recalled that this was the concept behind recruiting MCI subjects for secondary AD prevention trials. MCI subjects provide enough endpoints (i.e. AD diagnoses) to measure a difference between treatment arms during a treatment trial lasting a few years with considerably fewer subjects than a primary prevention trial. By excluding very high functioning normal subjects or other individuals unlikely to show further decline, we may be able to apply a similar strategy in prevention trials where MCI or cognitive decline are the primary endpoints, thereby making these trials more efficient. These analyses will permit more effective planning of primary prevention trials by providing more accurate endpoint estimates based on the demographic, biological, and cognitive characteristics of the study population. They will also be helpful for power calculations where it is necessary to know the expected incidence of endpoints for a given sample size and trial duration.

Ongoing analyses are utilizing Cox proportional hazards models (Cox, 1972) in which baseline cognitive assessments and cognitive change data are entered into the models to determine which cognitive instruments are most useful in predicting a change in the clinical diagnosis. Annual rates of change as well as cumulative rates of change over several years on a variety of sensitive neuropsychological measures are also being computed (Rubin, 1976; Zeger and Liang, 1986). Additional analyses are estimating rates of cognitive change on the overall cohort and subgroups split by baseline cognitive scores, age, gender, education, apoE status, and family history. Cognitive change over the first several years of observation is being subjected to a receiver-operating

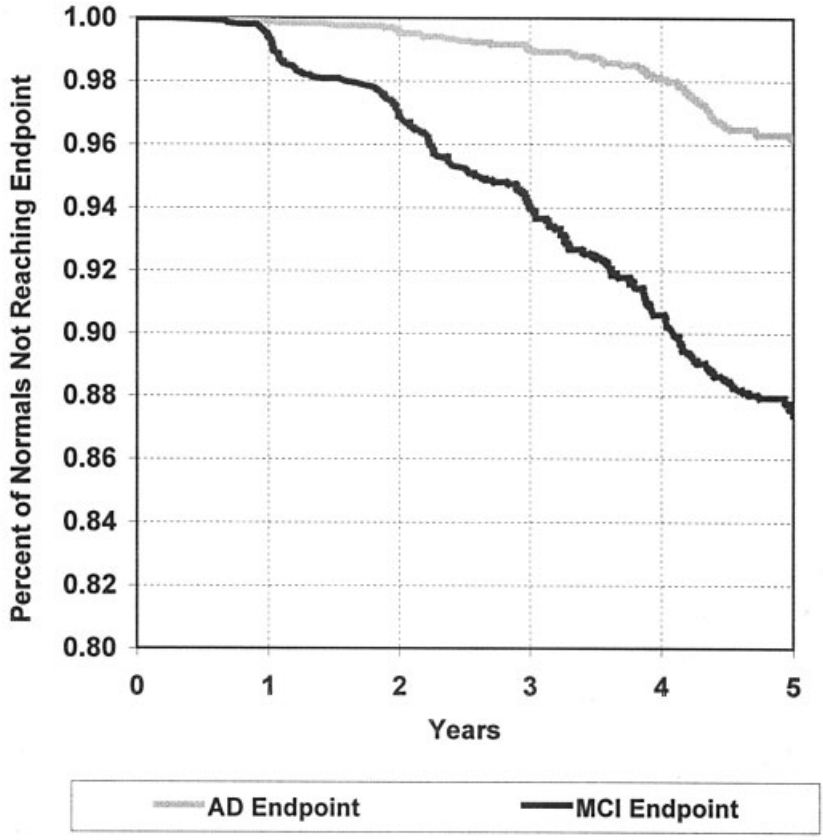

Figure 13.1. Rate of conversion to AD or MCI in normal subjects

characteristic (ROC) analysis to determine whether a certain range of decline can accurately identify future development of MCI or AD.

A new era may be approaching where it will be possible to perform AD prevention trials using more sensitive diagnostic methods (e.g. using a diagnosis of MCI) or by using neuropsychological and functional endpoints that can serve as surrogates for incipient AD. This methodology could have advantages over current AD primary prevention trial designs that require a diagnosis of AD by classical clinical criteria. Surrogates have the potential to make primary prevention trials simpler, shorter, and less expensive and allow more agents to be tested. Other methods to measure early change such as neuroimaging and cerebrospinal fluid biomarkers may also provide early indicators of incipient AD, but it is not clear whether these methods will be advantageous compared with neuropsychological and functional assessments that have more obvious face validity yet are simple, inexpensive, and non-invasive.

SUMMARY

The National Institute on Aging funds a number of Alzheimer's Disease Centers (ADCs) that collect longitudinal data on elderly individuals who are normal or have mild cognitive impairment (MCI). The Alzheimer's Disease Centers' Neuropsychological Database Initiative is an effort by 16 of these federally funded ADCs to merge the diagnostic, neuropsychological, and functional data obtained from these individuals into a single, large, multicenter database. The database is intended to serve as a resource for investigators planning multicenter trials to prevent Alheimer's disease (AD) and cognitive decline associated with aging. The assembled database currently contains clinical and neuropsychological data on commonly collected instruments from over 4000 normal individuals and 800 patients with MCI. A primary objective is to determine the incidence of MCI and AD as well as the annual rate of change on repeated neuropsychological and functional testing in this prospectively evaluated cohort. Also being estimated are rates of change in defined subgroups of normal elderly stratified by age, gender, family history of AD and apoE-ε_4 status. These estimates may be helpful for projecting incidence rates for MCI or AD and cognitive change that might occur in a multicenter primary prevention trial. These data will also allow us to contrast the change on neuropsychological instruments in people who remain normal with that in individuals who eventually develop MCI or AD. This strategy may yield change measures that could identify incipient AD cases and thereby serve as potential surrogate endpoints in primary prevention trials. Surrogate endpoints could provide distinct advantages over current prevention trials that require a diagnosis of AD using standard clinical criteria.

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14 Mild Cognitive Impairment: Transition from Aging to Alzheimer's Disease

RONALD C. PETERSEN

Early identification of persons who might be developing a cognitive impairment which may lead to Alzheimer's disease (AD) has become an increasingly popular topic for investigation. At the previous meetings of what is now the World Alzheimer's Congress, the concept of mild cognitive impairment received scant attention. However, this year there are numerous presentations and scientific studies involving this condition.

A major focus of the basic biology of AD concerns preventive strategies. Work is progressing rapidly on possible immunization therapy and secretase inhibitors which may prevent the deposition of amyloid in the brains of subjects who are predisposed to develop AD (Schenk et al., 1999; Vassar et al., 1999; Wolfe et al., 1999). As this work advances, therapeutic trials are set to begin. It is therefore incumbent upon clinical researchers to have appropriately characterized subject populations available for this intervention research. In particular, most of these clinical research efforts should be aimed at identifying persons who are at the very earliest stages of cognitive impairment even prior to the clinical diagnosis of probable AD. 'Mild cognitive impairment' refers to a state of cognitive function which is abnormal for age and education but does not meet criteria for clinically probable AD (Petersen et al., 1999; Petersen, 2000b). These subjects are seen commonly in clinical practices, and research is clarifying the criteria for their diagnosis and clinical outcome.

CONCEPTUAL FRAMEWORK

As is shown in Figure 14.1, in those persons who are destined to develop AD, there is probably a gradual progression of symptoms. The actual prodromal phase is uncertain but probably spans many years. Neuropathologists are

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quite adept at identifying persons with neuropathologically proven definite AD (Gearing et al., 1995; National Institute on Aging and Reagan Institute Working Group on Diagnostic Criteria for the Neuropathological Assessment of Alzheimer's Disease, 1997). Similarly, clinicians who follow the standard clinical criteria for the diagnosis of probable AD are quite accurate when ultimate clinical–pathological correlations are made (Galasko et al., 1994). However, as Figure 14.1 demonstrates, there is likely to be a period of some impairment in cognition which occurs prior to the actual clinical diagnosis of probable AD. It is this prodromal phase that has been designated as 'mild cognitive impairment.' There is probably an overlap between normal aging and mild cognitive impairment and similarly an overlap between the criteria for mild cognitive impairment and very early AD. These indistinct boundaries accurately reflect clinical practice but represent challenges for the clinician.

Patients with mild cognitive impairment are an important group to study for both theoretical and practical reasons. From a theoretical standpoint, research on mild cognitive impairment will shed light on the very earliest clinical symptoms and signs which are likely to lead to AD. This research will also shed important light on cognition and normal aging (Petersen, 1995). From a practical standpoint, it may be helpful to be able to counsel persons who have various degrees of memory impairment as to the likelihood of this clinical syndrome progressing to a more serious form of impairment and possibly AD. People with a mild cognitive impairment are otherwise normal

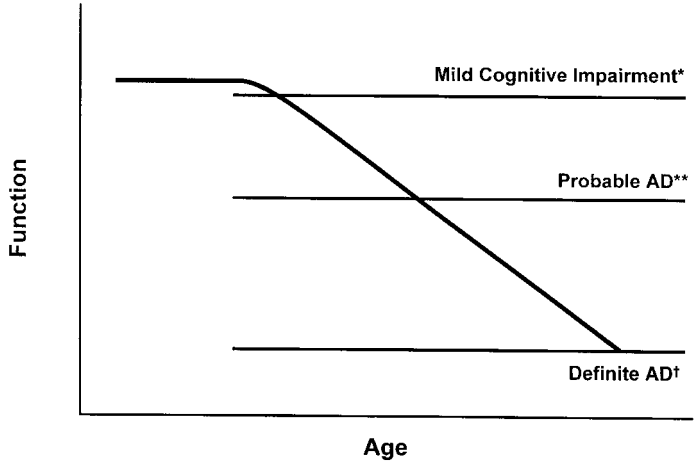

Figure 14.1. Postulated progression of cognitive decline from normal aging to mild cognitive impairment (*), probable Alzheimer's disease (**), and definite Alzheimer's disease (†). [Reproduced from Petersen (2000c) by permission of Saunders Publishing Company]

and functioning quite well and are capable of making important decisions regarding their future.

In all likelihood, most of these subjects manifest the earliest signs of AD. While they do not fulfill the actual clinical criteria for the diagnosis of AD, they probably have features that may progress over the following years. However, it is also incumbent upon the clinician to be accurate with this clinical designation. That is, it would be a disservice for clinicians to label normal elderly individuals with a condition such as mild cognitive impairment when in fact they are just experiencing the changes of normal aging.

The ultimate justification of a designation such as 'mild cognitive impairment' is derived from the longitudinal course. If persons who meet criteria for mild cognitive impairment eventually go on to develop AD, then it becomes self-evident that this condition is important to identify.

Finally, this may be an appropriate group for therapeutic interventions. There are currently five international clinical trials underway on subjects with a mild cognitive impairment. These trials involve over 4000 individuals and are of intense interest to the pharmaceutical industry as well as the scientific community.

HISTORY OF 'MILD COGNITIVE IMPAIRMENT'

Several terms have been used in the literature over the years to refer to various degrees of memory impairment. 'Benign senescent forgetfulness' was a term initially coined by Kral in the early 1960s and largely referred to individuals who were experiencing normal changes in cognition with aging (Kral, 1962). The criteria were so imprecise that the true utility of this concept from a research standpoint was marginal, but nevertheless it focused research interest on this topic.

In 1986, a National Institute of Mental Health Work Group was convened to address the issue of age-associated memory impairment (Crook et al., 1986). 'Age-associated memory impairment' (AAMI) refined the notion of 'benign senescent forgetfulness' and gave criteria for the condition. The key feature of this entity involved the referencing of memory function to that in young adults. Consequently, depending upon which particular memory instrument was used, the majority of the elderly population could be considered to have AAMI (Smith et al., 1991). As such, this limited the utility of the concept, but it still proved to be an important heuristic.

Finally, Levy and colleagues from the International Psychogeriatric Association coined the term 'age-related cognitive decline' to refer to a condition whereby persons had multiple cognitive deficiencies of a mild nature (Levy, 1994). Once again, these deficits are probably not of sufficient

severity to constitute dementia or AD, but this concept did raise the issue of the heterogeneity of cognitive impairments with aging.

CLINICAL CHARACTERIZATION

While there have been many studies involving the concept of mild cognitive impairment, there appears to be a core set of criteria (Petersen et al., 1999). The particular operational definition of the criteria may vary from study to study, but the essential features of the criteria are outlined in Table 14.1. Persons must have a cognitive complaint, and preferably this complaint should be corroborated by an informant who knows the subject well. In general these persons should have normal general cognitive function. Specific neuropsychological testing may reveal that these persons are impaired in other domains besides memory, but generally the degree of impairment is not sufficient to lead the clinician to feel that these deficits are functionally significant. The activities of daily living of these persons are generally normal. They are often living independently in the community and, except for the inconveniences of their memory problem, are functioning well. However, when they are brought into the clinician's office or neuropsychological testing laboratory, they do manifest impaired memory function for age and education (Ivnik et al., 1992; Petersen et al., 1995, 1999). The precise instruments which are used to assess this cognitive function are variable, but generally measures of learning (episodic memory) and delayed recall are most informative. When performance is gauged according to age- and education-appropriate norms, these individuals tend to fall approximately 1.5 standard deviations below their reference group (Petersen et al., 1999). However, these are approximations, and the ultimate diagnosis is clinical. Most importantly, these persons do not meet criteria for clinically probable AD (McKhann et al., 1984).

Figure 14.2 shows typical performance in areas of general cognition such as that on the Mini-Mental State Examination (MMSE) and Full Scale IQ Scores in comparison to measures of verbal and non-verbal delayed recall. As is apparent, the persons with a mild cognitive impairment perform more closely to the normal control subjects on the MMSE and the Full Scale IQ, but they look more like the clinically probable AD subjects on measures of

Table 14.1. Diagnostic criteria for mild cognitive impairment

Memory complaint
Normal general cognition
Intact activities of daily living
Abnormal memory function
Not demented

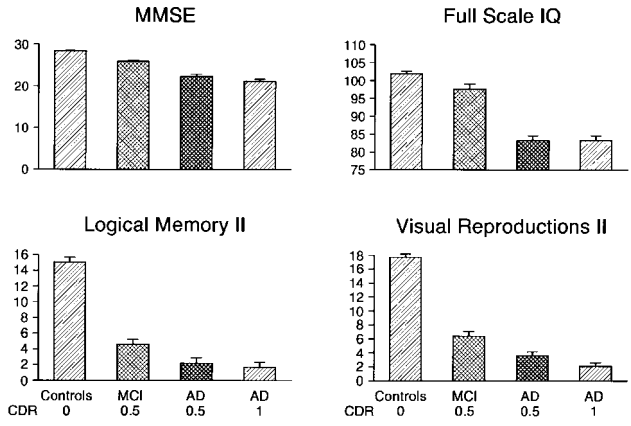

Figure 14.2. Performance of normal control subjects and those with mild cognitive impairment, very mild Alzheimer's disease (CDR = 0.5), or mild Alzheimer's disease (CDR = 1) on measures of general intellectual function (Mini-Mental State Examination, MMSE), Full Scale IQ, and on measures of memory (Logical Memory II, Visual Reproductions II). (Reproduced with permission from Petersen et al., 1999, by permission of the American Medical Association.)

delayed recall. Persons who meet these criteria for mild cognitive impairment tend to progress to clinically probable AD at a rate of 10–15% per year, as is shown in Figure 14.3.

PREDICTORS OF PROGRESSION

Among those individuals who have been diagnosed with a mild cognitive impairment, certain characteristics of their condition allow the clinician to predict which subjects might progress more rapidly. Not a great deal of literature exists on this topic thus far, but the studies that have been conducted longitudinally indicate that there may be qualitative features of the memory performance which may predict progression as well as their apolipoprotein E ε_4 allele carrier status (Petersen et al., 1995, 1999; Tierney et al., 1996a, b). More recently, a great deal of attention has been paid to neuroradiologic measures which may predict progression.

From a cognitive standpoint, it appears that those subjects who are unable to benefit from the use of semantic cues during the recall may be at a greater risk for progressing (Petersen et al., 1995). This finding emanates from the literature concerning the accessibility of semantic networks and the disruption of these retrieval processes in memory impairment leading to

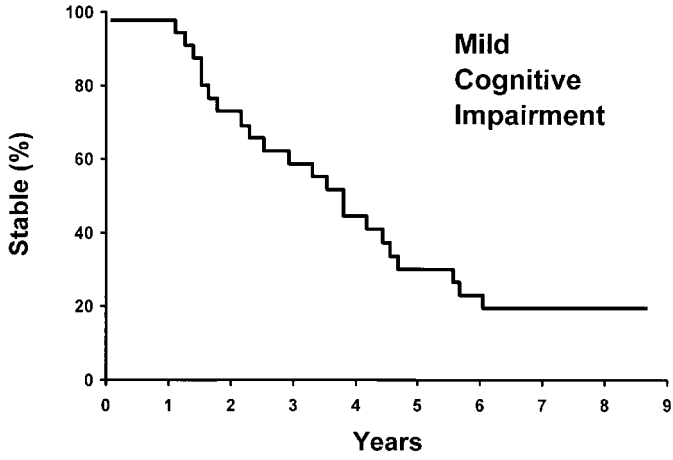

Figure 14.3. Survival curve for subjects with mild cognitive impairment; note that approximately 80% have converted to dementia by six years

AD (Hyman et al., 1990; Welsh et al., 1991). This being so, there can be qualitative features of the memory performance which may predict future outcome.

Studies have been contradictory with regard to the role of apolipoprotein E ε_4 carrier status. Some studies indicate that those individuals with a mild cognitive impairment who are ε_4 allele carriers are likely to progress somewhat more rapidly (Petersen et al., 1995). Others indicate that when this factor is coupled with features of memory performance, the latter seems to predominate (Tierney et al., 1996a, b). The role of the ε_4 allele is not surprising in this situation, but at the present time it cannot be used as a clinical tool.

The use of MRI in classifying and predicting progression in mild cognitive impairment is receiving increasing attention (Jack et al., 1997). Several investigators have indicated that the hippocampal volume at the time of diagnosis may be a useful predictor of subsequent progression (Jack et al., 1999). In addition, rates of change of the volume of the hippocampus have also been useful and, more recently, functional imaging measures have shown some merit (Jack et al., 2000). Kantarci and colleagues have recently reported an MRI spectroscopy study in mild cognitive impairment (Kantarci et al., 2000). These investigators reported that persons with a mild cognitive impairment have a reduced *myo*-inositol/creatine ratio in the posterior cingulate. This, again, differentiated patients with mild AD from those with mild cognitive impairment. In addition, *N*-acetyl aspartate levels were reduced when the subjects developed clinically probable AD.

Probably there will be increasing use of structural MRI as well as functional MRI in elucidating those subjects who are likely to evolve to AD. Functional imaging including positron emission tomography (PET) and single-photon emission computed tomography (SPECT) are also possibly beneficial, and longitudinal studies regarding these modalities are underway (Small et al., 1995; Reiman et al., 1996).

NEUROPATHOLOGY OF MILD COGNITIVE IMPAIRMENT

Relatively few studies have been conducted on the neuropathologic status of persons with a mild cognitive impairment. This is in part due to the fact that this is a relatively mild form of impairment, and consequently subjects do not die of this condition. However, in longitudinal studies, deaths have been reported from other causes while the person's clinical status was mild cognitive impairment (Petersen et al., 2000). Parisi and colleagues report elsewhere the results of a group of individuals who died while their clinical classification was mild cognitive impairment in the Mayo Clinic studies on aging and dementia (Petersen et al., 2000). Approximately half of these subjects had the neuropathologic features of mild AD, including neurofibrillary changes and neuritic plaques in medial temporal lobe structures and largely diffuse amyloid depositions in the neocortex. However, the amyloid was felt to be of sufficient magnitude to constitute the diagnosis of very mild AD. Other subjects had only neurofibrillary tangle involvement of the medial temporal lobe. In these subjects it was uncertain as to whether these persons were in a transitional stage and would have developed neocortical amyloid deposition in sufficient magnitude to warrant the ultimate neuropathologic diagnosis of AD. Finally, a few additional subjects had argyrophilic grain disease of medial temporal lobe structures and similarly, their transition to AD is unknown (Braak and Braak, 1989; Tolnay et al., 1997). In general, these cases were felt to represent transitional states between normal aging and very early AD. All of the subjects had some form of pathologic involvement of the medial temporal lobe accounting for their memory deficit and many of them appeared to have the very earliest stages of neuropathologic AD as well.

Other series reporting mildly demented patients such as those with a clinical dementia rating (CDR) of 0.5 tend to show the features of neuropathologic AD (Morris et al., 1991, 1996). This is probably a more advanced stage of the process than that seen in subjects who are diagnosed with mild cognitive impairment as outlined here. These data taken together imply that mild cognitive impairment may in fact be the earliest stage of pathologic involvement of a process that may eventually lead to AD in many cases.

UNRESOLVED ISSUES

While there is a certain momentum in the field of aging and dementia toward making early diagnoses of clinical impairment, this research approach also raises many questions. For example, while the criteria for mild cognitive impairment as outlined here reflect a constellation of clinical features, these subjects also fall at various points on existing rating scales concerning severity of impairment (Petersen, 2000a). A popularly used rating scale from Washington University is the Clinical Dementia Rating Scale (Morris, 1993). This scale ranges from CDR 0 to CDR 3 although others have extended it to CDR 4 and 5. For the current discussion, the relevant portion of the scale is CDR 0.5, which has been labeled as representing 'questionable dementia.' On this rating scale, subjects with a CDR of 0.5 could meet the current criteria for mild cognitive impairment or they could be very mildly demented. That is, the stage of CDR 0.5 does not preclude the clinical diagnosis of probable AD, and, as is shown in Figure 14.4, many subjects in the Mayo Clinic series did in fact have the clinical diagnosis of probable AD while their CDR was 0.5. Consequently, the CDR is a very useful scale for categorizing subjects along a continuum of impairment, but it does not necessarily coincide with any particular clinical diagnosis such as mild cognitive impairment or AD as outlined by published criteria.

In a similar fashion, the Global Deterioration Scale ranges from 1 to 7 and characterizes subjects along a continuum as well (Reisberg et al., 1982). In this scheme, a GDS of 1 or 2 can be normal, with GDS 2 referring to largely normally functioning subjects who may have a subjective impression of a change in their cognitive status. A GDS of 3 can refer to a subject with a mild cognitive impairment or a very mildly demented person (Flicker et al., 1991). In a fashion similar to that discussed above for the CDR, the GDS 3 may

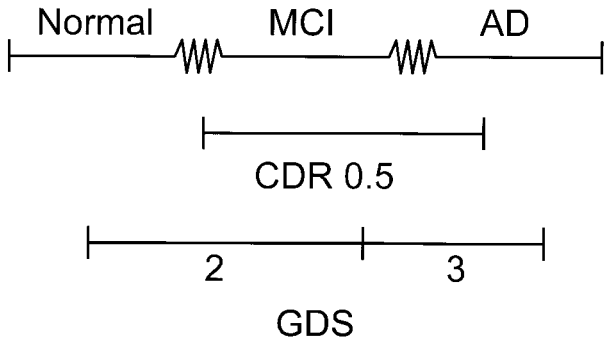

Figure 14.4. Overlap of Clinical Dementia Rating Scale (CDR) 0.5 and Global Deterioration Scale (GDS) 2 and 3 with clinical diagnoses of normal, mild cognitive impairment (MCI), and Alzheimer's disease (AD). (Reproduced with permission from Petersen, 2000a, by permission of the American Medical Association.)

correspond to what is currently called mild cognitive impairment or very early dementia. In summary, these scales are useful for categorizing severity of impairment but do not necessarily coincide with any distinct clinical diagnostic classification (Petersen, 2000a).

Another issue regarding the concept of mild cognitive impairment relates to its heterogeneity. The present discussion has largely centered around the concept of an amnestic mild cognitive impairment. By that we mean that the criteria are developed around a memory impairment. This being so, many of these subjects will likely progress to clinically probable AD. However, one could also characterize subjects who have multiple cognitive domains involved to a slight extent but not of a sufficient magnitude to warrant the diagnosis of clinically probable AD (Mufson et al., 1999). This is a perfectly reasonable definition of mild cognitive impairment but is different from the one discussed here. The outcome for these subjects could in fact include AD or other conditions. In addition, some of these subjects may be manifesting changes of normal aging.

Finally, 'mild cognitive impairment' could refer to a prodromal stage of another condition with a single non-memory cognitive domain being impaired beyond what is felt to be normal. These subjects may go on to develop other neurodegenerative conditions such as a frontotemporal dementia, Lewy body dementia, primary progressive aphasia, or other conditions. This raises the wider spectrum for the concept of mild cognitive impairment as a prelude to a variety of other conditions. Thus, it is important for investigators to be specific with respect to their particular definition of mild cognitive impairment.

CONCLUSION

Mild cognitive impairment is becoming an important topic of investigation in many research settings. It appears that as more longitudinal studies are completed regarding this classification of subjects, a homogeneous set of clinical criteria is emerging. There appears to be a converging body of neuroimaging and neuropathological data which lend credence to the notion that subjects with a mild cognitive impairment have the earliest stages of a degenerative disease which may ultimately evolve to AD. As such, mild cognitive impairment represents a transitional condition between normal aging and very early AD. The literature appears to indicate that this may be an important condition to identify, since as research strategies for early interventions become available, these subjects would be considered prime candidates. Several multicenter clinical trials are currently underway, and their results will be forthcoming. At present, it appears that mild cognitive impairment is a condition that warrants further investigation and attention on the part of clinicians and researchers and will probably become an increasingly popular research topic in coming years.

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15 Brain Functional Imaging in Early and Preclinical Alzheimer's Disease

AGNETA NORDBERG, VESNA JELIC, EVA ARNÁIZ, BENGT LÅNGSTRÖM AND OVE ALMKVIST

INTRODUCTION

With the present conservative approach of the clinical criteria for Alzheimer's disease (AD) the diagnosis is very often given when the clinical symptoms of the disease are quite clear. Tentative AD drug therapies in the future aiming to cure or prevent the disease (e.g. vaccination; Schenk et al., 2000) will prompt a need for diagnosis at preclinical stages of the disease. Symptoms of AD are probably preceded by a period of unknown duration during which neuropathological alterations may accumulate in the brain without detectable changes in cognition (Davis et al., 1999). Mild cognitive impairment is a condition characterized by subtle cognitive deficits before functional impairment is evident and might therefore represent an early stage of AD (Almkvist et al., 1998; Petersen et al., 1999; Arnáiz et al., 2000a).

At present, great interest is being taken in available functional neuroimaging methods, since they provide a potential to reveal neuronal dysfunction before structural changes may appear. Positron emission tomography (PET), a non-invasive neuroimaging technique, allows quantification and three-dimensional measures of distinct physiological variables such as glucose metabolism, cerebral blood flow, and neurotransmitter and receptor function. Regional deficits in cerebral glucose metabolism in parietotemporal regions, assessed by [^{18}F] 2-fluoro-2-deoxy-D-glucose (FDG) as tracer, have consistently been described in patients with AD. The fact that the metabolic impairment correlates to deficits in neuropsychological domains and increases with progression of the disease suggests that PET could provide a sensitive marker of disease progression and severity (Nordberg, 1993; Mielke et al., 1994). The discriminative accuracy of PET between AD and healthy controls was explored in two similar study samples of mildly impaired AD patients; PET

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was found to attain an overall diagnostic accuracy of 87% and 85% in the two studies respectively (Szelies et al., 1992; Jelic et al., 1999) (Figure 15.1). In a recently performed European Union multicenter cohort study, impairment of glucose metabolism in the temporoparietal and frontal association cortex, expressed as metabolic ratio (Herholz, 1995), was observed to be an indicator for severity of dementia but also a prognostic factor of clinical deterioration during follow-up of the disease (Herholz et al., 1999). The finding illustrates the usefulness of PET for diagnosis of AD at an early stage. It may provide insight into the characteristics of subtypes of AD as well as identify responders to certain drug therapies.

PET STUDIES IN SUBJECTS AT RISK OF DEVELOPING AD

When focusing on preclinical and early stages of AD as well as the corresponding functional disturbances in the brain, it is relevant to address the main sources of evidence obtained from PET studies in subjects at high risk of developing AD. In this risk group can be included individuals belonging to families with chromosomal aberrations in chromosome 14 and 21, subjects carrying apoE-ε_4 allele and subjects with mild cognitive impairment. It is important in the context of early diagnostic finding to remember that there appears to be a functional disconnection in early AD between the atrophy observed in the hippocampus, medial temporal lobe (Scheltens et al., 1992; De Leon et al., 1997), and the decrease in glucose metabolism, cerebral blood

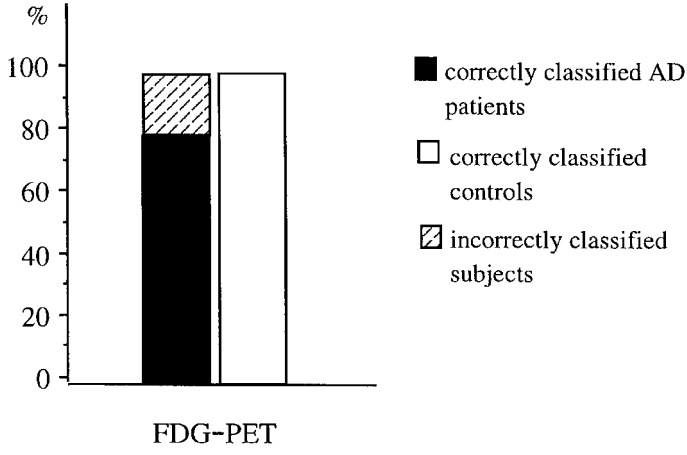

Figure 15.1. Diagnostic accuracy of cerebral glucose metabolism as determined by linear discriminant analysis on a sample of 40 patients with mild AD (mean MMSE = 24) and 14 healthy control subjects. Initial diagnoses were validated through a further two years' follow-up

flow, neurotransmitters, and neuroreceptors in the isocortical association cortex as visualized by PET and single-photon emission computed tomography (SPECT) (Mielke et al., 1994; Johnson et al., 1998) (Figure 15.2).

APP AND PS-1 MUTATIONS

Longitudinal PET studies in families with autosomal dominant inheritance provide a unique opportunity to study individuals who are close to the anticipated age of onset of AD and are in a presymptomatic phase of the disease. PET studies have been performed on at risk individuals from families with known APP 670/671 mutation (Nordberg et al., 1995b; Wahlund et al., 1999) or PS-1 mutation (Kennedy et al., 1995; Nordberg et al., 1995b). Three-year follow-up in the family harboring the Swedish 670/671 APP AD mutation revealed reduced glucose metabolism in the temporal cortex that preceded other studied markers of objective cognitive dysfunction (Wahlund et al., 1999). Impairment of glucose metabolism was detected in APP 670/671 carriers years before clinical symptoms of AD (Wahlund et al., 1999). Kennedy et al. (1995) observed parietotemporal deficits in glucose metabolism in 24 asymptomatic at-risk individuals from families with known PS-1-encoded AD. Similar findings were also observed in a PS-1 AD family of four siblings (two carriers and two non-carriers) longitudinally followed with PET for five years (Nordberg et al., 1995b; Nordberg et al., unpublished data).

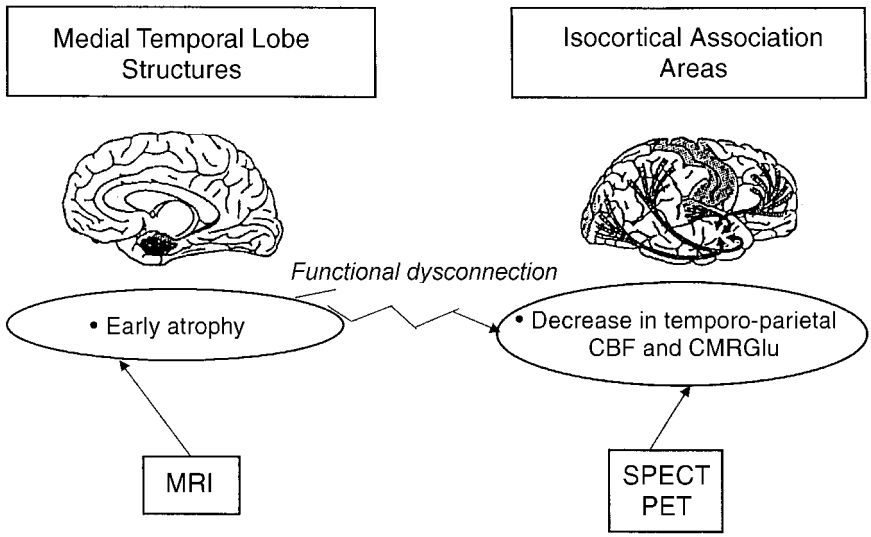

Figure 15.2. Disconnectional hypothesis of early Alzheimer's disease and neuroimaging methods of choice. *CMRGlu*, cerebral glucose metabolism rate; *CBF* cerebral blood flow

APOE-ε_4 CARRIERS

The ε_4 allele of the apoE gene on chromosome 19 is associated with late-onset familial and sporadic AD (Corder et al., 1993). ApoE-ε_4 promotes incipient AD pathology in elderly subjects who have no antemortem signs of clinical dementia (Ohm et al., 1995; Warzok et al., 1998). An impairment of regional cerebral glucose metabolism has been reported in apoE-ε_4 carriers with no cognitive impairments (Small et al., 1995; Reiman et al., 1996). Interestingly enough, apoE-ε_4 carriers with manifest AD dementia do not show any significant differences in regional glucose metabolism compared to non-apoE-ε_4 carriers (Corder et al., 1997; Hirono et al., 1998). Recently, Small et al. (2000) observed that ten non-demented apoE-ε_4 carriers showed a decline in cerebral glucose metabolism in the posterior cingulate cortex and inferior parietal and lateral temporal regions after two years' follow-up, while apoE-ε_4 non-carriers did not show any changes during similar follow-up time. The findings support the earlier observations from the PET studies in families bearing APP and PS-1 mutations that pathophysiological processes in brain begin well before even mild clinical symptoms are recognized. The brain may compensate for a regional dysfunction with normal neuropsychological performance even though pathological changes are ongoing in the brain (Wahlund et al., 1999; Small et al., 2000). Although the apoE genotype solely identifies a subject's risk of developing AD (American College of Medicinal Genetics, 1995), PET studies revealing impairment of brain functional activity may increase our knowledge of clinical outcome as well as defining a population of subjects in whom disease-modifying drugs could be tested.

MILD COGNITIVE IMPAIRMENT

A great deal of interest has been generated concerning the transitional state between normal aging and AD. Mild cognitive impairment (MCI) is an operational term for cognitive deficits present in older subjects without functional impairments who do not satisfy the criteria for dementia or probable AD. The presence of MCI increases the risk of developing AD from 1% to 25% per year (Dawe et al., 1992). In a four-year follow-up study of 76 subjects selected for MCI, the conversion rate to AD was 12%, compared to 1% for normal subjects (Petersen et al., 1999).

Episodic memory is severely disturbed in early stages of AD (Almkvist et al., 1998). When an extended neuropsychological battery of tests was applied to 94 individuals classified as having AD, 90 individuals clinically diagnosed with MCI, and 79 normal subjects, discriminant analysis showed that particularly episodic memory, semantic memory, visuospatial function, and attention contributed to the discriminant functions (Arnáiz et al., 2000a). The test battery correctly classified 95% of the AD patients and 90% of the MCI patients (Arnáiz et al., 2000a). A cross-sectional study comparing more

than one hundred patients with mild AD or MCI and healthy individuals showed a trend to reduced temporoparietal glucose metabolism in the MCI group (Figure 15.3). Similarly, there was an increase in the metabolic asymmetry index of the temporoparietal association cortex, with no directional preference, showing an overlap between mild AD patients and controls (Figure 15.4). However, because patients with MCI form a heterogenous group with respect to clinical outcome, the observed trend in cross-sectional studies did not reach statistical significance. It is plausible to assume that only longitudinal studies could reveal reliable predictive markers of the disease.

When 27 subjects with MCI were followed for two years, 26% converted to AD (Jelic and Nordberg, 2000). At the start of the two-year follow-up period a PET study of brain glucose metabolism had been performed in all patients. The metabolic ratio was found to be significantly lower in the group of MCI patients who progressed to AD ('P MCI') than in those whose MCI remained stable ('S MCI') (Figure 15.5). The posteroanterior ratio of glucose metabolism (parietal association cortex/frontal association cortex), when corrected for the baseline MMSE score, correctly predicted the conversion or non-conversion to AD in 93% of the cases (Figure 15.6). Interestingly enough, when neuropsychological assessment using Block Design gave a 65% correct classification, the combined measure of Block Design and cerebral glucose metabolism gave a maximum correct classification rate of 90% (Arnáiz et al., 2000b).

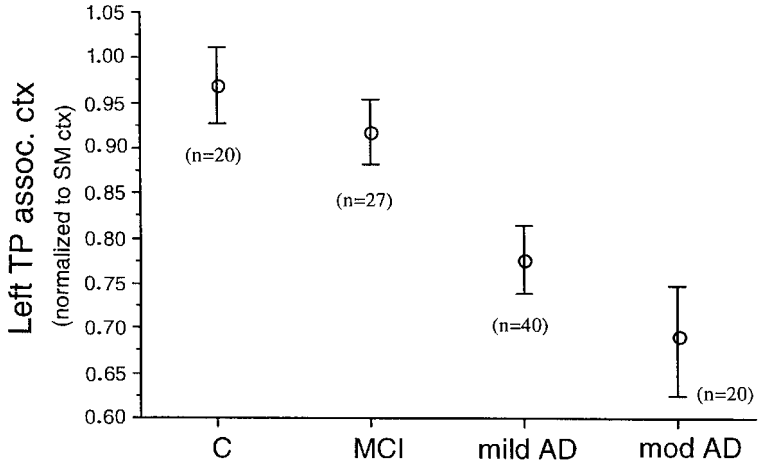

Figure 15.3. Cross-sectional study of cerebral glucose metabolism in AD, MCI and healthy control subjects. *TP ctx*, temporoparietal association cortex; *SM ctx*, sensorimotor cortex, *n* number of subjects

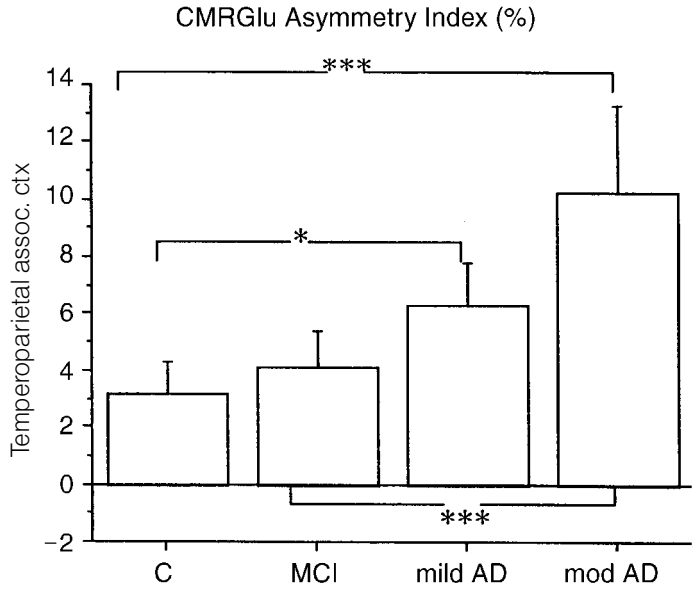

Figure 15.4. Cross-sectional study of asymmetry in cerebral glucose metabolism in AD, MCI and healthy control subjects

PET STUDIES OF NEUROTRANSMITTER ACTIVITY IN MILD AD

Few PET studies have so far been performed regarding transmitter and receptor dysfunction in AD. The cholinergic hypothesis has so far rendered PET ligands for nicotinic receptors, muscarinic receptors (Nordberg et al., 1995a, 1997, 1998), acetylcholinesterase activity (Iyo et al., 1997; Kuhl et al., 1999), and cholinergic terminal density (Kuhl et al., 1996). Detection of acetylcholinesterase activity and cholinergic terminal density by PET has also shown losses in activity in AD patients more pronounced than the impairment seen in glucose metabolism (Kuhl et al., 1996, 1999). It has been well established from autopsy brain studies that the neuronal nicotinic receptors are significantly impaired in AD (Paterson and Nordberg, 2000). It might therefore be useful to image the nicotinic receptors non-invasively in patients with MCI and mild AD. So far (S)(-)nicotine radiolabeled with ^{11}C has been used to target nicotinic receptors *in vivo* in human brain. A significant correlation has been observed between the cognitive impairment and reduction in [^{11}C]nicotine binding sites in the temporal cortex of the AD subjects (Nordberg et al., 1997). The [^{11}C]nicotine binding data suggest that the impairment of nicotinic receptors may represent early phenomena in the course of the disease, supporting the hypothesis of an early cholinergic

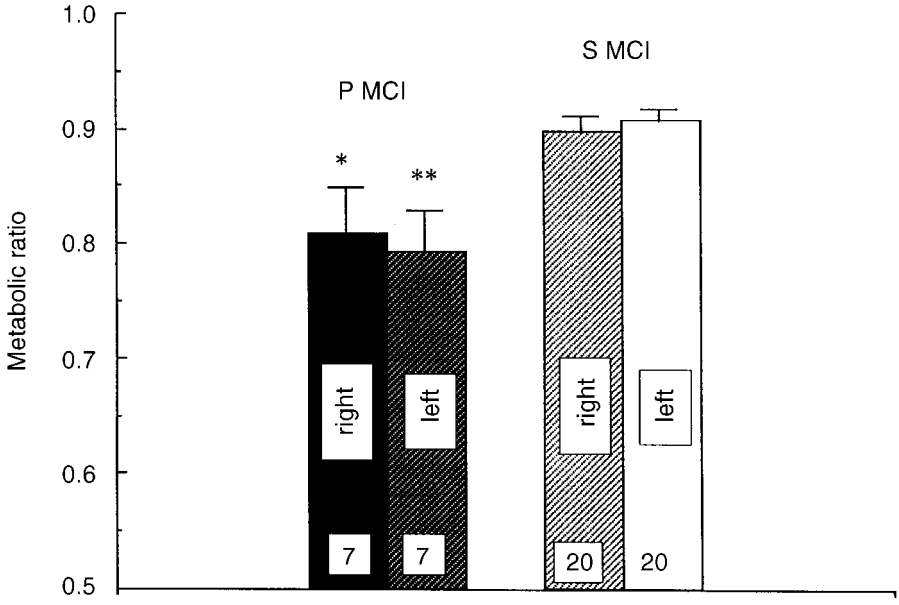

Figure 15.5. Two-year longitudinal study of cerebral glucose metabolism in patients with MCI. *P MCI*, progressive MCI; *S MCI*, stable MCI. Metabolic ratio was calculated according to Herholz (1995). *$p < 0.05$, **$p < 0.01$

vulnerability in the AD brain. The nicotinic receptors exist in several subtypes; the most abundant nicotinic subtype in the human brain is probably of the α_4 nicotinic receptor subtype (Paterson and Nordberg, 2000). Several classes of compounds have been evaluated as potential new PET ligands for visualizing the α_4 nicotinic receptor subtype (Sihver et al., 1999a,b, 2000) (Figure 15.7). When tested *in vivo* in monkeys, analogs of A-85380 so far appear to be the most promising candidates due to a probable high selectivity of the α_4 nicotinic receptor subtype and low toxicity (Sihver et al., 1999a,b, 2000). Analogs of A-85380 will soon be tested in humans. Recent SPECT studies with A-85380 in monkeys look promising for clinical application in the future (Sihver et al., 2000).

PET STUDIES FOR DRUG EVALUATION

In early treatment of AD at the non-symptomatic stage, imaging studies with PET or functional MRI must be considered valuable tools for monitoring drug effects, mechanisms of action, and defining characteristics of responders to the drug therapy. So far relatively few PET studies have been reported in

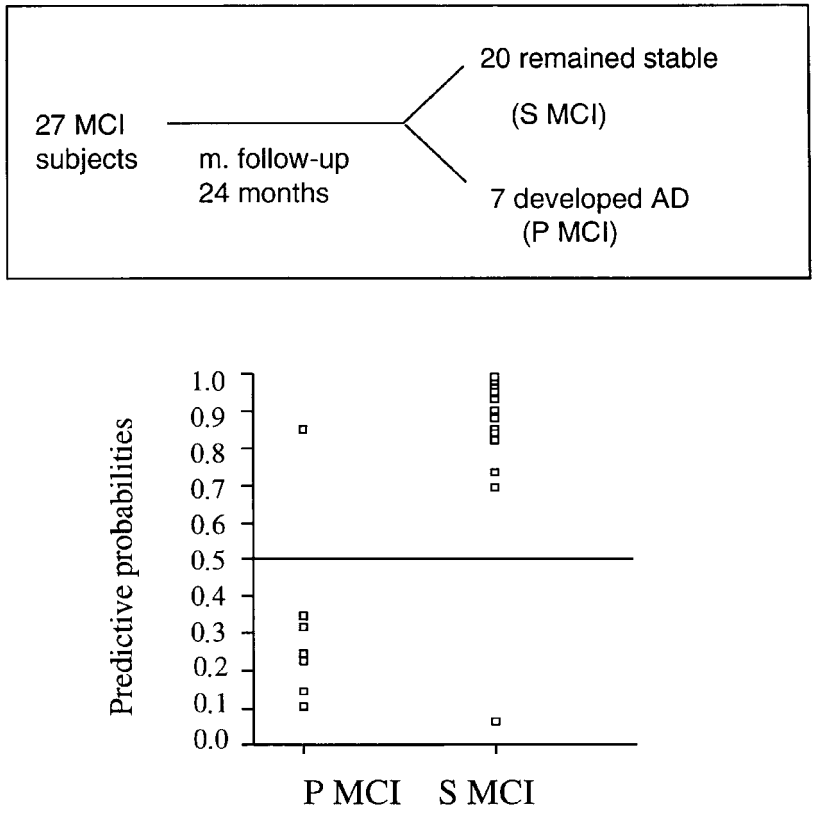

Figure 15.6. Longitudinal study of cerebral glucose metabolism in patients with MCI. Plot indicates predictive probabilities of a logistic regression model using ratio of temporoparietal and association cortex as an independent variable. Only two subjects were misclassified. Predictive power of the model is 93%

AD patients undergoing drug therapy (Nordberg, 2000). Improvement in glucose metabolism has been reported following treatment with cholinesterase inhibitors such as tacrine (Nordberg et al., 1992, 1998) and donepezil (Tune et al., 1999) in AD patients. Restoration of cortical nicotinic receptors has been measured following treatment with cholinesterase inhibitors as well as following treatment with nerve growth factors (Nordberg et al., 1992, 1998; Eriksdotter-Jönhagen et al., 1998). In a recent study Kuhl et al. (2000) reported cortical inhibition of acetylcholinesterase by PET which was less than 30% and thus below the enzyme inhibition measured in red blood cells. The observation stresses the need for functional studies as a complement to clinical trials. Investigation of drug action during functional activation studies in AD and MCI patients is presently ongoing and will provide a new

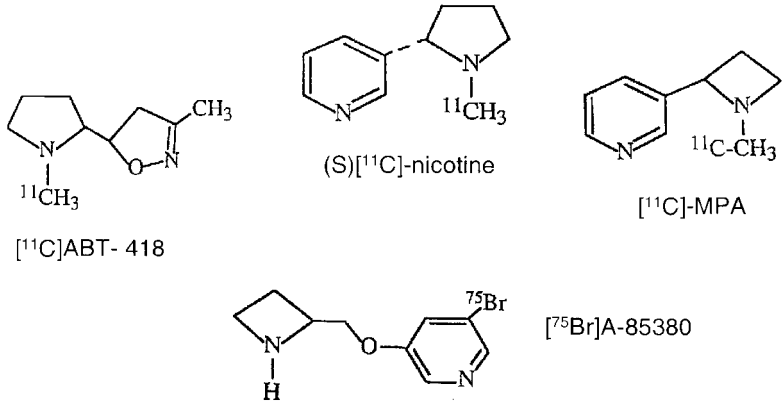

Figure 15.7. Chemical structures of potential PET nicotinic receptor ligands

dimension to our understanding of drug effects in both symptomatic and disease-modifying treatments.

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16 Amyloid, PHF-tau, Ubiquitin and Synaptic Markers in the Progression of Alzheimer's Disease: Immunochemical Analyses of Frontal Cortex from Prospectively Studied Elderly Humans

D. S. WANG, E. COCHRAN, D. BENNETT, E. MUFSON, C. ECKMAN AND D. W. DICKSON

INTRODUCTION

Alzheimer's disease (AD) is characterized by progressive dementia in adulthood accompanied by structural and biochemical changes in the brain, including neuronal and synaptic loss, intracellular aggregates of tau protein in neurofibrillary tangles, and extracellular deposits of β-amyloid protein (Aβ) in senile plaques. Senile plaques also contain reactive microglia and astrocytes, as well as dystrophic neurites that contain tau filaments and lysosomal dense bodies that are immunoreactive for ubiquitin (Dickson, 1997). While quantitative analyses of Aβ, tau, ubiquitin, and synaptic proteins show clear differences between subjects with endstage AD and normal controls, there are few studies of these molecules in brains of subjects with a range of cognitive impairment, and there is uncertainty about the sequence of changes in the course of AD. Furthermore, most studies have focused on one or two proteins, with only a few studies examining multiple measures in the same brains (Lue et al., 1999; Mukaetova-Ladinska et al., 2000).

Defining the earliest clinical stage of AD is a challenge, and separating early AD, which is a progressive neurodegenerative process, from static age-related cognitive changes is an important clinical objective. Clinical studies of elderly people reveal a spectrum of cognitive abilities ranging from no cognitive impairments (NCI) to mild cognitive impairment (MCI) to multiple cognitive

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deficits consistent with dementia of the Alzheimer type (Storandt et al., 1996; Almkvist et al., 1998; Petersen et al., 1999). A number of studies have suggested that MCI may represent the earliest stage of AD, while other longitudinal studies suggest that only some subjects with MCI progress (Gottfries et al., 1998; Petersen et al., 1999; Richie and Touchon, 2000; Celsis, 2000; Milwain, 2000). Characterization of the brains of subjects who die while still in MCI and determining the relationship between the findings in MCI compared to AD remain important research objectives.

In the present study we examined the relationships between NCI, MCI, and AD with respect to quantitative measures of several proteins involved in AD. The patients with AD had early or mild dementia (MMSE 20.0+1.97) rather than endstage disease. The individuals with MCI and NCI did not differ in terms of Mini-Mental State score (MCI: 26.7+0.78, NCI: 27.2+0.57). The subjects in all groups were highly educated individuals (AD: 17.6+0.48; MCI 16.2+1.28; NCI: 19.4+0.97 years) who were volunteers in a prospective longitudinal study. The proteins measured in postmortem brain tissue included $A\beta_{40}$, $A\beta_{42}$, tau, ubiquitin, α-synuclein, SNAP25, and synaptophysin. The analyses were performed in frontal lobe tissue.

MATERIAL AND METHODS

Monoclonal antibodies that were specific to $A\beta_{40}$ and $A\beta_{42}$ (BA27 and BC05, respectively) as well as a monoclonal capture antibody to the carboxy terminus of Aβ (BAN50) (Suzuki et al., 1994) were provided by Dr Steven Younkin, Mayo Clinic, Jacksonville, Fla. Anti-ubiquitin monoclonal antibody, UBI-1 (Shaw and Chau, 1988), was provided by Dr Gerry Shaw, University of Florida, Gainesville, Fla. Anti-synaptophysin (EP10) and anti-SNAP25 (SP12) antibodies (Honer et al., 1993) were provided by Dr William Honer, University of British Columbia, Vancouver, BC. Anti-PHF-tau monoclonal antibody, TG3 (Vincent et al., 1997), was provided by Dr Peter Davies, Albert Einstein Medical College, Bronx, NY. Polyclonal anti-synuclein antibody, NACP98 (Gwinn-Hardy et al., 2000), was from Dr John Hardy, Mayo Clinic, Jacksonville, Fla.

Frozen samples of frontal cortex were obtained from 10 AD, 9 MCI, and 9 NCI subjects who had been part of the Religious Order Study. The study protocol has been previously reported (Gilmor et al., 1999). There were no statistically significant differences between cases and controls with respect to age (AD: 86.8+6.6, MCI: 83.6+5.8, and NCI: 83.4+6.2 years), sex, or postmortem interval (AD: 8.8+5.4; MCI: 5.6+3.3 and NCI: 6.2+4.4 h). Coded samples were processed blinded to clinical and pathological information. Gray matter and white matter were carefully dissected from partially thawed samples.

Brain samples were homogenized on ice in 300 mg/ml Tris-buffered saline (TBS) containing a protease inhibitor cocktail with a PowerGene homogenizer

for 10–20 s. One milliliter of each sample was centrifuged at 28 000 *g* at 4°C for 1 h, and the supernatant was collected as the 'soluble fraction.' The pellet was resuspended in 1 ml 70% formic acid and further extracted after adjusting to 100 mg/ml 70% formic acid for 30 min at room temperature followed by a centrifugation as above. The supernatant was collected as the 'insoluble fraction.' The 'soluble fraction' was diluted (1:10) with EC buffer containing a protease inhibitor cocktail and stored at −70°C until use. Samples from the 70% formic acid fraction were stored at −70°C and neutralized with 1M Tris-base (1:20) immediately before use.

Quantitative analysis of $A\beta_{40}$ and $A\beta_{42}$ utilized a sandwich-ELISA similar to previously published studies (Suzuki et al., 1994). BAN50 was used as the capture antibody and HRP-conjugates of BA27 (specific for $A\beta_{40}$) and BC05 (specific for $A\beta_{42}$) were used as detection antibodies. Since BAN50 captures species based upon a carboxyl-terminal epitope of Aβ, the peptides analyzed largely represent full-length peptides, i.e. $A\beta_{1-40}$ and $A\beta_{1-42}$.

Crude homogenates of gray matter (20 μg wet brain/well) were dried to the bottom of wells of 96-well microtiter plates for PHF-tau ELISA. Anti-PHF-tau antibody, TG3, was used to quantify tau using a procedure similar to previously published methods (Dickson et al., 1995). TG3 recognizes a phosphorylation-dependent epitope of PHF-tau, but its epitope is also conformation-dependent since it does not recognize normal adult tau (Jicha et al., 1997).

Both soluble and insoluble fractions from gray and white matter were assayed for ubiquitin immunoreactivity using the monoclonal antibody UBI-1 and a dot blot assay. Soluble fractions of brain homogenates in TBS or neutralized insoluble fractions were adjusted to 7.5 mg wet brain/ml with EC buffer containing protease inhibitor cocktail. Purified ubiquitin was used as a standard and diluted to desired concentrations with EC buffer containing protease inhibitors. Each sample was blotted in triplicate and detected with UBI-1 antibody as previously described (Swerdlow et al., 1986).

Synaptophysin, α-synuclein, and SNAP25 were quantified using Western blot methods. Crude homogenates of gray matter were dissolved in SDS-PAGE sample buffer and equal amounts (100 μg wet brain/lane) were loaded on 10% SDS-PAGE gels. Proteins transferred to nitrocellulose membranes were immunoblotted with polyclonal anti-human α-synuclein antibody (NACP98), anti-synaptophysin (EP10), and anti-SNAP25 (SP12). The signal detected by ECL system (Amersham) was quantified with Imagecal software.

RESULTS

AMYLOID

Soluble $A\beta_{40}$ and $A\beta_{42}$ levels determined from brain samples extracted with buffer and insoluble $A\beta_{40}$ and $A\beta_{42}$ levels determined from brain tissue

Table 16.1. Comparisons (p value; Student's t-test) of biochemical indices for subjects with no cognitive impairment (NCI), mild cognitive impairment (MCI), and Alzheimer's disease (AD)

	Insoluble $A\beta_{40}$	Insoluble $A\beta_{42}$	Insoluble ubq.	Soluble $A\beta_{40}$	Soluble $A\beta_{42}$	Soluble ubq.	TG3	NACP	EP10	SP12
AD vs. NCI	0.033	0.001	0.0004	0.404	0.048	0.007	0.024	0.041	0.044	0.596
AD vs. MCI	0.021	0.006	0.0002	0.101	0.052	0.018	0.036	0.002	0.355	0.673
MCI vs. NCI	0.945	0.191	0.722	0.355	0.485	0.957	0.345	0.729	0.202	0.938

Ubq., Ubiquitin; TG3, PHF-tau; NACP, synuclein; EP10, synaptophysin; SP12, SNAP25.

extracted with 70% formic acid were both measured, although the most consistent results were obtained with the insoluble fraction. The ELISA system uses carboxyl-specific antibodies to discriminate $A\beta_{40}$ from $A\beta_{42}$ (Suzuki et al., 1994). The assay is not sensitive to amino-terminally truncated or modified Aβ species, such as p3. In general insoluble peptides were more abundant than soluble peptides, especially for $A\beta_{42}$. AD brains had significantly more insoluble $A\beta_{42}$ than both MCI ($p = 0.006$) and NCI ($p = 0.001$) (Table 16.1). Insoluble $A\beta_{40}$ showed a similar trend, although the differences between AD and MCI ($p = 0.021$) and between AD and NCI ($p = 0.033$) were less marked. Neither $A\beta_{42}$ nor $A\beta_{40}$ was different between NCI and MCI (Figure 16.1A).

As was true for insoluble peptides, the average levels of soluble $A\beta_{40}$ and $A\beta_{42}$ were greater in AD than in MCI and NCI, but only soluble $A\beta_{42}$ showed a marginally significant difference between AD and NCI ($p = 0.048$). Greater variance was noted for soluble Aβ species in all three groups of brains, because many samples had undetectable levels of soluble Aβ (Figure 16.1B).

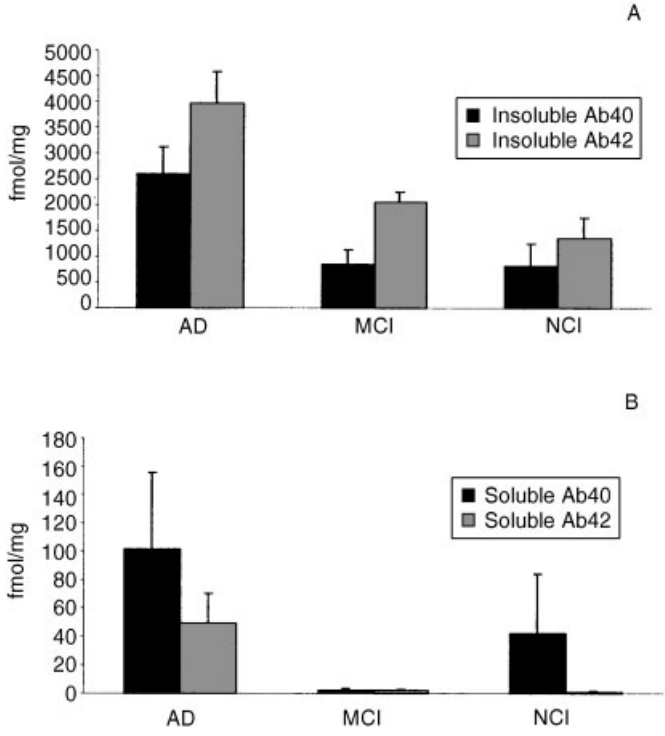

Figure 16.1. **A** Insoluble $A\beta_{40}$ and $A\beta_{42}$ in AD, MCI, and NCI. **B** Soluble $A\beta_{40}$ and $A\beta_{42}$ in AD, MCI, and NCI. Results are expressed as fmol/mg wet brain

As was true for insoluble species, there were no differences between MCI and NCI brains for soluble Aβ species (Table 16.1).

PHF-tau

The assay for PHF-tau utilized a monoclonal antibody (TG3) whose immunoreactivity is dependent upon both phosphorylation and a unique conformation of tau characteristic of paired helical filaments (PHF-tau). The assay does not detect normal tau (Jicha et al., 1997). The average level of TG3 was about four times higher in AD than in MCI ($p = 0.036$) and about eight times higher in AD than in NCI brains ($p = 0.024$) (Figure 16.2A). Although the average TG3 immunoreactivity was higher in NCI than in MCI, there was no statistically significant difference between MCI and NCI in the frontal lobe. Surprisingly, even though half of NCI subjects and two of the MCI subjects had no tau-immunoreactive lesions on immunocytochemistry from adjacent cortices (not shown), TG3 immunoreactivity was detected in low levels in all NCI and MCI cases.

UBIQUITIN

Ubiquitin in both soluble and insoluble fractions of gray matter was measured with a monoclonal antibody to ubiquitin (UBI-1) and a dot blot assay. The dot blot assay was chosen because of the heterogenous nature of the conjugated ubiquitin species. In Western blots the ubiquitin antibody detected an inconsistent diffuse smear in brain samples. The dot blot method consolidated the immunoreactivity into one measurable focus. An inverse relationship was found for the levels of ubiquitin immunoreactivity in soluble and insoluble fractions (Figure 16.2). Ubiquitin in the insoluble fraction was significantly higher in AD than in MCI ($p = 0.0002$) and NCI ($p = 0.0004$), but ubiquitin in the soluble fraction was lower in AD than in NCI ($p = 0.007$) and MCI ($p = 0.018$) (Table 16.1). Ubiquitin immunoreactivity was also measured in subcortical white matter from AD, MCI, and NCI brains. The immunoreactivity was significantly higher than in gray matter; there were no significant differences between AD, MCI, and NCI (data not shown).

SYNAPTIC MARKERS

Of the three synaptic markers studied, only synuclein gave results that differentiated AD from MCI and NCI. The average synuclein immunoreactivity in AD was about 40% lower than in NCI ($p = 0.024$) and MCI ($p = 0.002$) (Table 16.1 and Figure 16.3). The synuclein immunoreactivity in MCI was slightly higher than in NCI, but the difference was not statistically significant ($p = 0.729$) (Table 16.1). Although there was a trend for decreasing

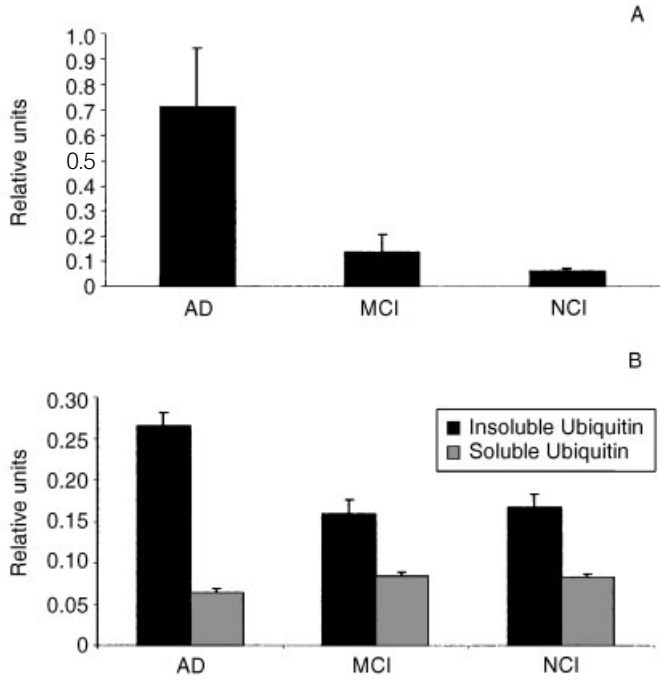

Figure 16.2. **A** Total PHF-tau detected by TG3 antibody. The average level of PHF-tau in AD brains was significantly higher than in MCI ($p < 0.05$) and in NCI brains ($p < 0.05$), but there was no statistically significant difference between MCI and NCI. **B** Insoluble and soluble ubiquitin. Insoluble ubiquitin in AD brains was significantly higher than in MCI ($p = 0.0002$) and NCI brains ($p = 0.0004$). Soluble ubiquitin in AD brains was lower than in MCI brains ($p = 0.018$) and NCI brains ($p = 0.007$)

synaptophysin immunoreactivity from NCI to MCI to AD (Figure 16.3), the only significant difference was between AD and NCI ($p = 0.044$). There was no difference between AD and MCI or between MCI and NCI (Table 16.1). SNAP25 showed a trend similar to synaptophysin (Figure 16.3), but no differences were detected between any of the diagnostic groups (Table 16.1).

CORRELATIONS BETWEEN MARKER PROTEINS AND CLINICAL DIAGNOSTIC CATEGORIES

The strongest correlations between diagnostic categories and marker proteins were for molecules in the insoluble fraction, particularly $A\beta_{42}$ ($r = 0.650$, $p = 0.0002$) (Table 16.2). Ubiquitin and PHF-tau also showed strong correlations ($r = 0.613$, $p = 0.0005$, and $r = 0.515$, $p = 0.006$). All the synaptic

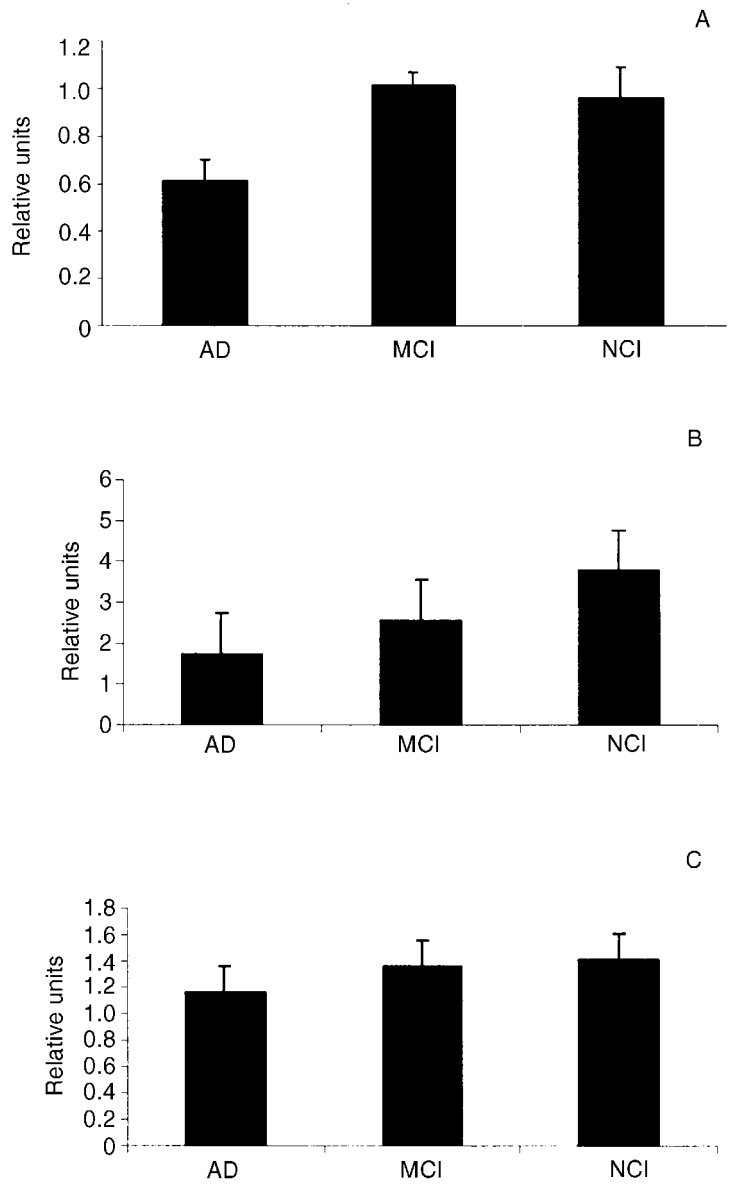

Figure 16.3. The levels of synaptic markers in NCI, MCI, and AD brains. **A** Synuclein quantified by NACP98 polyclonal antibody; **B** synaptophysin detected by monoclonal antibody EP10; **C** levels of SNAP25 detected by SP12 monoclonal antibody. All results shown as relative units compared to a normal control brain

markers showed a negative correlation, but the strength of the correlation was weaker than for amyloid, ubiquitin, and PHF-tau (Table 16.2).

INTERCORRELATIONS BETWEEN MARKER PROTEINS

There were moderate to strong correlations between the various amyloid species (Table 16.3). Brains with high levels of insoluble also had high levels of soluble Aβ species. PHF-tau immunoreactivity was correlated with all amyloid species, but the correlation was strongest for $A\beta_{42}$ (Table 16.4). PHF-tau also showed a similar correlation with insoluble $A\beta_{40}$, although the correlation with soluble $A\beta_{40}$ was weaker (Table 16.4). Given the inverse relationship between ubiquitin immunoreactivity in insoluble and soluble fractions, it was predictable that insoluble ubiquitin levels would correlate directly with $A\beta_{42}$ and PHF-tau levels, while soluble ubiquitin levels would correlate inversely with $A\beta_{42}$ and PHF-tau (Table 16.4).

Synaptic markers were correlated with each other and inversely correlated with Aβ and PHF-tau (Table 16.5). This is in line with the idea that synapse loss is associated with amyloid deposition and neurofibrillary pathology. The strongest correlations were between synuclein and $A\beta_{42}$ and between synuclein and PHF-tau. Interestingly, although synuclein showed an inverse correlation with most of the non-synaptic proteins, synaptophysin and SNAP25 did not show significant correlations (Table 16.5).

DISCUSSION

Given that most of the measured parameters showed values for MCI that are intermediate between those for AD and for NCI, it is reasonable to conclude that MCI may be an intermediate stage in the progression from NCI to AD. In previous autopsy studies MCI has been associated with a range of medial temporal lobe pathology (Parisi et al., 2000), presumably because the earliest structural manifestations of AD are in medial temporal lobe structures, most notably the entorhinal cortex and hippocampus (Price et al., 1991; Braak and Braak, 1995). Despite this prevailing opinion, in this study we focused on, and indeed found, changes in the frontal lobe, which suggests that changes occur in other parts of the brain at early stages of the disease. Most notable was the presence of PHF-tau immunoreactivity in frontal cortex despite the absence of an obvious histopathologic correlate in some of the cases. This suggests that biochemical changes precede morphologic changes. The fact that the brunt of the early pathology may affect medial temporal lobe may explain the fact that there were few or no significant differences between NCI and MCI with respect to most of the quantitative measures. The low effect size when comparing NCI and MCI might be overcome with larger sample sizes. Unfortunately, it is very difficult to collect large numbers of autopsy

Table 16.2. Pearson correlation coefficients (r) between marker proteins and diagnostic categories

	Insoluble $A\beta_{40}$	Insoluble $A\beta_{42}$	Insoluble ubq.	Soluble $A\beta_{40}$	Soluble $A\beta_{42}$	Soluble ubq.	TG3	NACP	EP10	SP12
Corr. coeff.	0.470	0.650	0.613	0.204	0.451	−0.484	0.515	−0.437	−0.382	−0.0984
p value	0.012	0.0002	0.0005	0.30	0.016	0.009	0.006	0.023	0.049	0.63
Sample size	28	28	28	28	28	28	27	27	27	27

Ubq., Ubiquitin; TG3, PHF-tau; NACP, synuclein; EP10, synaptophysin; SP12, SNAP25.

Table 16.3. Intercorrelations of Aβ species

	Insoluble $A\beta_{42}$	Soluble $A\beta_{40}$	Soluble $A\beta_{42}$
Insoluble $A\beta_{40}$	0.773[a] <0.00001[b] 28[c]	0.836 <0.00001 28	0.808 <0.00001 28
Insoluble $A\beta_{42}$		0.376 <0.05 28	0.765 <0.00001 28
Soluble $A\beta_{40}$			0.53 <0.005 28

[a]Pearson correlation coefficient; [b]p value; [c]sample size.

Table 16.4. Correlation of TG3 immunoreactivity with $A\beta_{40}$, $A\beta_{42}$, and ubiquitin

	Insoluble $A\beta_{40}$	Insoluble $A\beta_{42}$	Insoluble ubiquitin	Soluble $A\beta_{40}$	Soluble $A\beta_{42}$	Soluble ubiquitin
Pearson correlation coefficient	0.762	0.822	0.500	0.416	0.942	−0.526
p value	<0.00001	<0.00001	<0.01	<0.05	<0.00001	<0.005
Sample size	27	27	27	27	27	27

Table 16.5. Correlation of synaptic markers with Aβ, TG3, and ubiquitin

	Insoluble $A\beta_{40}$	Insoluble $A\beta_{42}$	Insoluble ubiquitin	Soluble $A\beta_{40}$	Soluble $A\beta_{42}$	Soluble ubiquitin	TG3
NACP	−0.378[a] 0.052[b] 27[c]	−0.525 0.005 27	−0.392 0.043 27	−0.108 0.591 27	−0.580 0.002 27	0.449 0.019 27	−0.599 0.001 26
EP10	−0.055 0.784 27	−0.137 0.496 27	−0.334 0.088 27	0.011 0.957 27	−0.196 0.328 27	0.410 0.034 27	−0.198 0.323 27
SP12	0.152 0.450 27	−0.025 0.900 27	−0.246 0.216 27	0.300 0.129 27	0.103 0.609 27	0.049 0.809 27	0.065 0.747 27

[a]Pearson correlation coefficient; [b]p value; [c]sample size.

samples from normal and minimally impaired subjects. The Religious Order Study is one such opportunity, however, given the highly motivated and educated nature of the study group. The results of the present study support the idea that AD does not necessarily progress in a region-to-region specific manner, but rather that the disease is multicentric or diffuse at its outset and that it is only the degree of pathology that is greater in certain more vulnerable regions such as the medial temporal lobe.

The present quantitative biochemical analyses may also be used to assess the sequence of events in the disease pathogenesis if one extrapolates cross-sectional data from a single neuroanatomic area into a pathogenetic sequence. Until a valid animal model becomes available for AD, this approach will be used, even though it is acknowledged that the concept may be based on uncertain assumptions. If one then uses the strength of the correlations between the various pathologic parameters to reconstruct the sequence of events, one would conclude that amyloid deposition is a relatively early event and that $A\beta_{42}$ may precede $A\beta_{40}$, since the strongest correlation between diagnostic category was for $A\beta_{42}$ ($r = 0.650$), with $A\beta_{40}$ showing a less robust correlation ($r = 0.470$). Ubiquitin ($r = 0.613$) and PHF-tau ($r = 0.515$) changes would be next, and synaptic loss, as assessed by synaptophysin immunoreactivity ($r = -0.382$), a relatively late event.

MCI is of increasing interest to clinicians given the fact that treatments for AD, even symptomatic treatments, are expected to be most effective in the earliest stages of the disease. By the time that a patient has dementia, irreversible brain damage may be so far advanced that all one could hope for would be arresting or slowing the progression of the disease. MCI may be an early stage of AD, but MCI is also heterogenous in that some subjects with MCI progress to dementia, while others remain stable or even improve over time. Nevertheless, MCI is a therapeutically important construct. Despite the great clinical interest in MCI, only a few postmortem studies of MCI have been reported (Price et al., 1991; Storandt et al., 1996; Gomez-Isla et al., 1996; Mufson et al., 1999; Parisi et al., 2000), due in part to the fact that MCI patients progress to dementia before dying.

To explore the relationship between MCI and AD, we used immunochemical methods to quantify marker proteins that are implicated in the pathogenesis of AD. Brains from NCI, MCI, and AD cases were matched in terms of age, sex, apoE genotype, and postmortem intervals, as well as other pathologies. While a few vascular lesions were detected in most of the brains, the severity of vascular pathology was not different between groups. A few of the AD and MCI cases had a small number of Lewy bodies (four of the AD cases and one of the MCI cases). Further studies will be necessary to explore the effect this might have had on the results. When cases with Lewy bodies were excluded from the analysis, the same trends were observed, although the strength of the associations was decreased due to smaller sample size.

For the seven proteins analyzed ($A\beta_{40}$, $A\beta_{42}$, PHF-tau, ubiquitin, and three different synaptic markers), intermediate values were noted for MCI compared to NCI and AD. Differences between AD and MCI or between AD and NCI were statistically significant, but we found no statistically significant difference between MCI and NCI in any of the parameters. The differences were always greater between AD and NCI than between AD and MCI. That values for all the quantitative measures in MCI were intermediate between AD and NCI, but not different from NCI may be due to several factors. First, the frontal lobe may not be an area of the brain that discriminates between MCI and NCI. This would fit with the idea that AD progresses from medial temporal lobe to convexity gray matter. Second, the MCI group may be heterogenous (Parisi et al., 2000). Some subjects with MCI might have early AD and others might have benign senescent cognitive impairment, which is not progressive and not significantly different from normal subjects. Biochemical analyses from tissue from the medial temporal lobe might differentiate between these two possibilities. That the first hypothesis might be true is suggested by the fact that image analysis of Aβ burden in the entorhinal cortex in these cases revealed significant differences between MCI and NCI (Mufson et al., 1999).

The present studies may shed some light on the sequence of changes that occur in proteins during the pathogenesis of AD. Our correlative analyses show that most of the proteins evaluated in this study were highly correlated with each other in a predictable way. AD is characterized by amyloid deposits and neurofibrillary pathology that contains PHF-tau and ubiquitinated proteins. Thus, brains with highest amyloid content also have high PHF-tau and increased insoluble ubiquitin and decreased soluble ubiquitin. We interpret the inverse relationship between ubiquitin in the soluble and insoluble fractions as a manifestation of decreases in free ubiquitin and increases in ubiquitin conjugates. Indeed, the insoluble fractions had more smearing on Western blots and the soluble fractions had more of the 15-kDa band (not shown).

While synaptic pathology is considered by some to be the best structural correlate for cognitive impairment (Terry et al., 1991), other studies have shown that amyloid (Cummings et al., 1996) or PHF-tau are also good correlates (Dickson et al., 1995). Interestingly, the assumption that cholinergic and synaptic losses are early manifestations of AD has come to be questioned as prospectively studied cases are studied at postmortem (Corey-Bloom et al., 2000). Synaptic markers may not be decreased, especially in cortical areas like the frontal lobe, at stages of the disease where there is already considerable cognitive impairment, presumably due to pathology in other brain regions, most notably the medial temporal lobe. There are only a few reports of changes in multiple AD-related proteins in brains from subjects with a range of cognitive impairment (Lue et al., 1999; Mukaetova-Ladinska et al., 2000). The present study is one of the few that

have performed these analyses on prospectively studied individuals. Most previous publications evaluated a limited set of markers in AD subjects compared to normal controls (Wang et al., 1991; Masliah et al., 1994; Wang et al., 1999). In this study synaptic markers correlated with each other and inversely with amyloid and PHF-tau, with the strongest correlations between synuclein and $A\beta_{42}$ and between synuclein and PHF-tau. Unlike amyloid, PHF-tau, and ubiquitin, synaptic markers were not highly correlated with clinical status, suggesting that synaptic loss, at least in the frontal lobe, may not be an early feature of AD type pathology.

On the basis of these data we conclude that amyloid deposits as well as PHF-tau pathology and ubiquitin mark significant differences between NCI and AD, and that MCI has intermediate values. These results are consistent with the hypothesis that MCI may be a precursor to AD, but possible heterogeneity of MCI needs to be considered as well. Given the strong correlations between $A\beta_{42}$ and PHF-tau and clinical diagnoses, it reinforces the idea that these two molecules are important in the pathogenesis of AD. The fact that correlations between clinical states were consistently higher for $A\beta_{42}$ than PHF-tau is consistent with the idea that amyloid deposition is an earlier event in AD pathogenesis than PHF-tau formation.

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17 Imaging the Consequences of Alzheimer's Disease Pathology

YAAKOV STERN AND SCOTT SMALL

INTRODUCTION

The pathology of Alzheimer's disease (AD) is probably present long before it is expressed clinically. Several studies have documented subtle cognitive changes in elders years prior to the clinical diagnosis of AD (Jacobs et al., 1995; Masur et al., 1994). Thus, it is important to understand the relationship between the presence of AD pathology and its clinical expression. In this paper, we discuss two studies that have relevance to this issue. The first (Small et al., 1999) attempts to identify the functional effects of AD pathology at its earliest stages, when it is restricted to the entorhinal cortex. On a practical level, this approach may provide for determining whether a subtle memory change is due to AD pathology or not. In addition, it may be important for defining an alternate condition of non-AD age-related impairment. The second study (Stern et al., 2000) explores the changes that occur in AD in the brain networks underlying memory. While AD pathology is responsible for the impairment that is observed clinically, there is individual variability in the degree to which a specific amount of pathology produces clinical symptoms. It is therefore important to understand how AD pathology impacts on normal brain function, and to determine how the brain might attempt to cope with or compensate for AD pathology. Together, the two studies begin to provide a comprehensive picture of the impact of AD pathology on brain function.

STUDY 1

The first brain structure to be targeted by AD is the hippocampal formation (Braak and Braak, 1996), and, correspondingly, prospective studies have found that memory deficits are the first signs of AD (Belanger et al., 1994). Recent studies have showed that entorhinal cell loss occurs in the predementia stage of the disease (Gomez-Isla et al., 1996). AD cannot

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account for all cases of elderly people with memory decline, however. Recent post-mortem studies have documented age-related cell loss in the hippocampal formation among brains free of AD pathology (Simic et al., 1997; West, 1993). There are age-dependent changes in some physiological processes—such as hormone levels and cerebrovascular supply—that can affect the hippocampal formation and result in memory impairment (Belanger et al., 1994; de la Torre et al., 1990; Lupien et al., 1998). Aside from structural lesions, non-AD processes might also cause functional damage to hippocampal neurons. Extensive animal research has documented age-dependent changes in the physiological properties of hippocampal neurons (Barnes, 1994; Geinisman et al., 1995), and some may account for non-AD memory decline found pervasively in aging animals.

In its transverse axis, the hippocampal formation is made up of different regions that are interconnected to form a complex circuit (Amaral and Witter, 1989). These regions include the entorhinal cortex, which serves as the main gateway into the hippocampal circuit, the 'hippocampus proper,' comprised of the dentate gyrus and CA subfields, and the subiculum. The first hippocampal region to be targeted by AD is the entorhinal cortex, as evidenced by changes in synaptic integrity (Masiliah et al., 1994), reduction in cell density (Gomez-Isla et al., 1996), and the formation of neurofibrillary tangles (Braak and Braak, 1996). In contrast, both post-mortem studies (Simic et al., 1997; West, 1993) and electrophysiological studies in aging animals (Barnes, 1994; Geinisman et al., 1995) suggest that non-AD processes spare the entorhinal cortex, with selective targeting of other hippocampal regions.

Dissociating early AD from non-AD causes of memory decline, therefore, may necessitate a regional analysis of the hippocampal formation. Hippocampal changes could be missed by volumetric MRI analysis (Golomb et al., 1994) because lesions to hippocampal regions can disrupt the physiological function of hippocampal neurons, and not necessarily result in cell loss. PET and SPECT have detected whole brain activation patterns that discriminate individuals who are at risk for developing AD dementia (Johnson et al., 1998; Reiman et al., 1996; Small et al., 1995), and have found a decrease in global hippocampal function associated with memory impairment (Grady et al., 1995), but these methods do not possess the spatial resolution to selectively assess different regions within the hippocampal formation. We therefore attempted to determine whether a functional MRI (fMRI) activation approach with sufficient resolution to capture differences across hippocampal sub-regions can subdivide individuals with memory decline into those who are and those who are not at risk for AD.

METHODS

Three groups of individuals over the age of 65 participated in the study: 4 subjects with normal memory, 12 subjects with isolated memory decline, and

4 subjects with AD. The 16 subjects in the first two groups had been evaluated annually with the Selective Reminding Test (Buschke and Fuld, 1974). They were assigned to the memory decline group if their memory performance worsened with time; and were assigned to the normal memory group if their memory did not decline over time. These subjects were not demented and did not meet criteria for 'questionable dementia' [CDR (Hughes et al., 1982) = 0.5]. Subjects in the AD group met NINCDS–ADRDA (McKhann et al., 1984a) criteria for probable AD, and had mild dementia (CDR = 1).

The fMRI task lasted 4 min during which time an activation phase, made up of 12 black-and-white photographs of faces, alternated with a baseline phase consisting of a fixation point. Activation phases occurred during the first and third minutes, and baseline phases during the second and fourth minutes. Thus, in two activation phases a total of 24 different faces were used. Subjects were instructed to push one button if a face was male and the other button if a face was female. All subjects, including the AD patients, were able to discriminate gender with 100% accuracy. Subjects were instructed to remember the faces for future testing.

Scanning was done on a 1.5-T magnetic resonance scanner retrofitted for echo planner imaging. A gradient echo sequence [echo time (TE) = 60 ms; repetition time (TR) = 2.5 s; flip angle = 30°] and a standard quadrature head coil were used to acquire T2*-weighted images with an inplane resolution of 2.3 × 2.3 mm (64 × 64 matrix; 15 cm field of view). High-resolution images were also acquired using the same spatial coordinates. Six 5-mm slices were selected that were oriented along the long axis of the hippocampal formation. The choice of the most anterior image was based on identification of the alveus and on the image where the temporal horn resides both laterally and superior to the hippocampus proper (Francis et al., 1997). Thus, the most posterior slice was approximately 35 mm caudal to the amygdala, ensuring incorporation of the entorhinal cortex (Loewenstein, 1990).

The structural MR images were used to localize three hippocampal regions for each subject: the entorhinal cortex, the hippocampus proper, comprised of the dentate gyrus and CA subfields, and the subiculum. Pixel-by-pixel t-tests were performed for each subject, comparing the average signal intensity acquired during the activation phases to the average signal intensity acquired during baseline. Within each region, the number of pixels whose hemodynamic response significantly increased in association with facial processing were counted. The number of significant pixels for each hippocampal region was aggregated for each experimental group.

RESULTS AND DISCUSSION

The AD subjects had significantly diminished activation compared to normal elderly persons in all the hippocampal regions (entorhinal cortex: $F = 22.97$,

$p < 0.005$; hippocampus proper: $F = 26.72$, $p < 0.001$; subiculum: $F = 11.48$, $p < 0.01$). There was no overlap in entorhinal activation between the normal and the AD groups. The 12 subjects with isolated memory decline were therefore dichotomized into two subgroups: four with diminished entorhinal activation (i.e. entorhinal activation at least 2 standard deviations below that of the normal elderly; −EC subgroup); and eight with normal entorhinal activation (+EC subgroup).

Representative scans of individuals with normal memory, isolated memory decline with normal entorhinal activation, and isolated memory decline with abnormal entorhinal activation are presented in Figure 17.1 (see Plate II).

The −EC subgroup had diminished activation in the hippocampus proper ($F = 26.72$, $p < 0.005$) and the subiculum ($F = 12.31$, $p < 0.01$) compared to normal elderly subjects. Because the entorhinal cortex is the input subregion to the hippocampal circuit, a lesion there would be expected to result in diminished activity throughout the circuit. Alternatively, it is possible that the presumptive AD pathology in these subjects had already progressed beyond the entorhinal cortex to include other hippocampal regions.

The +EC subgroup had diminished activation restricted to the subiculum ($F = 8.99$, $p < 0.01$). This subgroup of subjects is unlikely to have early AD. Nevertheless, these subjects were found to have selective hippocampal dysfunction. Selective subicular dysfunction may result from physiological or structural lesion to this region, which would fit well with recent post-mortem findings from brains without AD, showing age-dependent subicular cell loss (Simic et al., 1997; West, 1993). Alternatively, diminished activation in the subiculum may arise from lesions upstream in the hippocampal circuit in the CA regions, where animal studies have demonstrated age-related physiological deficits (Barnes, 1994; Geinisman et al., 1995).

We have begun prospective follow-up to validate whether those subjects with memory decline and entorhinal dysfunction are more likely to progress to AD dementia than are those subjects with normal entorhinal function. If so, the fMRI protocol may be developed as a tool for detecting AD at its earliest stages, when it presents with mild memory decline. The rigorous study of non-AD age-related memory decline has been hindered, and remains a controversial entity, in large part because of the inability to select out those subjects with early AD. The fMRI protocol may be used to isolate a group of elderly individuals with non-AD memory decline. This would allow better characterization of its clinical presentation and course, and the testing of candidate processes as underlying etiologies.

STUDY 2

Following brain injury, the same brain networks that normally mediate performance of a cognitive task may continue to operate at reduced efficiency,

or the normal networks may be replaced by alternate networks in an attempt to compensate for the loss of normal function. More extensive recruitment of brain areas during task performance has been noted in AD patients than in age-matched controls, and has been interpreted as patients' attempt to compensate for pathology by using alternate cognitive processes (Backman et al., 1999; Becker et al., 1996; Grady et al., 1993). However, studies of healthy individuals have demonstrated similar alterations in activation as tasks are made more difficult (Grady et al., 1996; Grasby et al., 1994; Gur et al., 1988; Rypma et al., 1999). Since almost any task is more difficult for AD patients than healthy elders, it is important to determine whether observed changes in activation in AD simply represent modulation in the use of normal networks. In this study, we matched task difficulty for each study participant. We hypothesized that some AD patients would continue to use the same networks as healthy elders, while others would use an alternate network.

METHODS

Eleven healthy elderly controls and 14 patients who met research criteria for mild probable AD (McKhann et al., 1984b) participated in this $H_2{}^{15}O$ PET study.

The activation task was a continuous performance verbal recognition test consisting of serial presentation of one or more single words (study block) followed by a series of recognition probes (test block). There was an alternating sequence of study and test blocks. A subject was instructed to make a 'New' or 'Old' response on each test trial by pressing one of two microswitches with the left or right thumb.

Two versions of the recognition task were used. In the low-demand condition, the study list size (SLS) was one (i.e. one study word followed by one recognition probe). The titrated demand condition used a predetermined SLS where the subject's recognition accuracy was 75%. One day prior to the PET scan, the particular SLS value at which a subject achieved 75% accuracy was determined using a staircase method of SLS adjustment. This SLS value was then used in all blocks of the titrated demand condition on the day of the scan.

Employing a Siemens EXACT 47 PET camera, six scans were obtained in the following order: rest, low demand, titrated demand, titrated demand, low demand, rest. After standard processing of the PET images, 21 regions of interest (ROIs) per hemisphere, plus one midline ROI, comprising major cortical and subcortical regions (Figure 17.2, see Plate II) were defined. To identify the brain networks underlying task performance, the ROI activation data were analyzed using the Scaled Subprofile Model (SSM) (Alexander and Moeller, 1994; Moeller et al., 1987, 1998). SSM is a form of principal component analysis that extracts one or more topographies, which consist of regional weights that are independent of a global mean effect and that represent covarying patterns of regional cerebral blood flow (rCBF). Each of

these covariance patterns potentially represents a pattern of functional interactions among specific brain regions, i.e. a brain network. Each brain region has an associated weighting that expresses the degree to which it participates in the specific topography, either by increasing or by decreasing rCBF. SSM was applied to the change in rCBF from the low to the titrated demand condition. The topography is assumed to represent a network of brain areas engaged by the task, and a change in the degree of expression of a topography across the two conditions is assumed to be related to the increased demand presented by the titrated condition. A subject scaling factor (SSF) is also calculated for each subject, which represents the degree to which the subjects expresses the particular topography. The SSF is offset relative to the mean subject expression of network activity. That is, subjects with midrange expression exhibit an SSF value near zero. Subjects with higher scores show a relative increase in flow in some areas and decreases in others, while the opposite pattern of flow increases and decreases is seen in subjects with negative scores.

To evaluate the patterns of activation associated with performing the word recognition task, our strategy was to separately identify the networks underlying task performance in the normal elderly and AD groups. SSM was applied to the titrated-minus-low-demand subtraction data. This approach identifies networks whose expression changed across these two conditions. The SSF scores obtained from the SSM analysis were subsequently correlated with a behavioral index of performance, SLS in the titrated demand condition. A significant correlation provides independent evidence that the identified regions operate as a network that mediates task performance.

RESULTS AND DISCUSSION

During the scans, both groups performed the low-demand task at approximately 95% accuracy, and the titrated demand task at 75% accuracy. The mean SLS in the titrated demand condition was 13.9 ± 1.5 in the controls and 7.4 ± 4.2 in the patients ($t = 4.89$, $p < 0.001$). SLS ranged from 12 to 17 in the healthy elders, and from 2 to 15 in the AD patients. For 11 of the patients, the SLS was below 10, while three had an SLS of 11 or higher.

The SSM analysis identified a network whose differential expression (i.e. individual differences in SSF values) correlated with the SLS attained in the titrated demand condition ($R^2 = 0.94$; $p < 0.0001$). Achieving higher SLS was associated with increased CBF in the left anterior cingulate and anterior insula and decreased CBF in the left basal ganglia (Figure 17.2A, see Plate II).

We calculated the degree to which the AD patients expressed the normal elders' topography. For most patients, the SSF for expression of this topography was close to zero, indicating that these patients did not express this activation pattern. The lack of expression of the healthy elders' topography was not simply due to reduced ability to recruit CBF in the

constituent brain regions; the difference between the two groups was due to differences in how activation covaried among the implicated brain regions. In contrast, the data for the three AD patients who had achieved relatively high SLS (i.e. >10) fit well on the regression line between the healthy elders' expression of topography and SLS, suggesting that they were effectively using the healthy elders' brain network.

Our findings suggest that healthy elders engaged a specific brain network when performing a continuous verbal recognition task. This contention is bolstered by the observation that differential expression of this network was strongly related to the SLS attained by elders in the titrated demand condition. Consideration of the brain regions that participated in this network suggests that it is involved in transforming the recognition task from one which requires attentional resources to a more automated task (Augustine, 1996; Chertkow and Murtha, 1997; Gray, 1999; Houk and Wise, 1995; Kapur et al., 1996; Marsden and Obeso, 1994; Petersen et al., 1990; Petersen and Fiez, 1993; Posner and DiGirolamo, 1998; Posner and Petersen, 1990; Raichle et al., 1994; Saint-Cyr et al., 1995; Vogt et al., 1992).

Three AD patients also expressed this network in the same manner, suggesting that they were performing the task similarly to healthy elders. We hypothesize that AD pathology had not yet disrupted the normal network in these patients. Performance (i.e. SLS) across the healthy elders and the three AD patients was variable, but it was related to modulation in the expression of the same network. Individual differences in performance may represent differences in the ability to effectively recruit this network. It is possible that individuals who can recruit this normal network to a greater degree might be able to continue to do so more effectively in the face of significant brain injury, but this possibility was not assessed in this study.

In a separate analysis of the PET activation data in the 11 AD patients with lower SLS (i.e. SLS < 10), a different topography was identified whose expression by these patients also correlated significantly with SLS in the titrated demand condition ($R^2 = 0.81$, $p < 0.001$). Within this AD subgroup, attaining a larger SLS was associated with increased CBF in left posterior temporal cortex, calcarine cortex, posterior cingulate, and the vermis (Figure 17.2B, see Plate II).

The healthy elders and the three high-SLS AD patients did not express the topography in a manner that predicted SLS.

For the majority of the AD patients, task performance was associated with activation of a different network than that used by controls. In response to the increased load of the titrated demand task, the patients recruited a network of posterior areas that are involved in the initial processing and encoding of the word stimuli (Demonet et al., 1992; Desmond et al., 1997; Petersen et al., 1988; Price et al., 1996; Vandenberghe et al., 1996). However, this posterior, perceptual processing network could be working in tandem with memory-related areas to mediate the increase in the encoding and retrieval demands across the two task conditions.

The alternate network did not mediate task performance in the healthy elders and the three better performing patients, as indicated by the lack of correlation between subjects' SLS and the SSF scores which measure their expression of this network. Still, this network was expressed by controls during task performance, suggesting that this perceptual processing network was engaged, but was not responsible for the elders' ability to work with larger study lists. This observation is consistent with the notion that both brain networks were operating concurrently in the healthy elders.

Our analytic approach took account of the fact that the relative utilization of brain regions in a network may vary across individuals even when pathology is not present. Thus, we could determine whether the network engaged by the AD patients was within this normal range of variability. Also, by matching task difficulty across subjects, we ensured that differences in the utilization of a network were not simply due to differential task demand. More typically, the activation task remains the same and the actual difficulty demands it places on each subject can vary widely.

Whether the patients' use of the alternate network represents compensation is a matter of definition. The alternate network was used by patients in the place of the normal network in an attempt to mediate task demands, suggesting that its use was compensatory. However, this network was also activated by the healthy elders, indicating that it was not unique to the patients. If the term 'compensation' is reserved for the use of a novel network that emerges in response to pathology, then the alternate network does not meet this criterion. On the other hand, the role played by the alternate network differed in patients and controls, in that it appeared to be mediating the ability to achieve larger study list sizes in the patients but not in the elders. This novel use of the network may arise out of the inability to use the standard network, and thus may be considered compensation.

These findings provide a framework for further investigation of compensation in response to brain pathology, and of individual differences in compensation. Also, the use of an alternate network may indicate a point at which pathology has irreversibly altered brain function. The ability to determine whether a patient expresses a normal or alternate brain network may therefore have implications for remediation or therapeutic intervention, with the transition to an alternate network indicating a point where interventions are less likely to be effective.

GENERAL DISCUSSION

The earliest appearance of AD pathology in the entorhinal cortex may produce subtle memory decline in the non-demented elder. We may be able to use fMRI techniques to more definitively establish whether memory decline in an individual subject is an early indication of AD. The brain may

actively attempt to compensate for AD pathology, producing individual variability in the degree of the clinical manifestation of any specific degree of pathology. Our PET studies have begun to provide some understanding of the individual variability in the expression of the networks that normally mediate cognitive functions. In addition they have begun to delineate how the brain may attempt to compensate for AD pathology. Together, these two studies complement each other in advancing our understanding of the relation between AD pathology and the observed clinical features of the disease.

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18 Influence of apoE Genotype and PET Brain Imaging on Preclinical Prediction of Alzheimer's Disease*

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INTRODUCTION

As people age, they nearly always develop mild memory complaints. If they live long enough, these memory changes gradually worsen until they develop a full dementia syndrome usually characteristic of Alzheimer's disease (AD). The neuropathological hallmarks of AD, neurofibrillary tangles (Braak and Braak, 1991) and neuritic plaques (Price and Morris, 1999), are present years prior to clinical diagnosis. Decades before dementia onset (Arai et al., 1999) preclinical amyloid deposits are observed, and diffuse plaques in non-demented elderly persons are associated with accelerated age-related cortical cholinergic deficits, which some investigators consider to be a preclinical AD condition (Beach et al., 1997).

Such accumulating evidence has led to the concept that a long, preclinical phase exists prior to the development of a full dementia syndrome characteristic of AD. If indeed the pathological process leading to AD begins prior to the actual clinical diagnosis, then it follows that treatment interventions that prevent or delay the onset of AD might be feasible. In fact, randomized, placebo-controlled trials of cholinesterase inhibitors, antioxidants, and anti-inflammatory agents are already in progress for such preclinical conditions as mild cognitive impairment (Petersen et al., 1999) or age-associated memory impairment (Blackford and La Rue, 1989). Such

*Based in part on Small et al., 2000; see reference list.

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studies generally use clinical measures to identify subjects at risk for future cognitive decline and to measure primary outcomes of the trials. Many people with mild memory complaints, however, do not progress to AD, and more sensitive and specific tools that predict future cognitive decline would heighten accuracy in detecting preclinical AD and facilitate more effective early intervention. Our group has combined genetic risk and functional brain imaging measures in efforts to develop surrogate markers for early AD detection and monitoring (Small et al., 1995).

Studies of AD genetics have identified an association between the apolipoprotein E-4 (apoE-4) allele on chromosome 19 and the common form of AD that begins after age 60 (Saunders et al., 1993). ApoE has three allelic variants (2, 3, and 4) and five common genotypes (2/3, 3/3, 2/4, 3/4, and 4/4). The apoE-4 allele has a dose-related effect on increasing risk and lowering onset age for late-onset familial and sporadic AD (Corder et al., 1993), while apoE-2 appears to confer protection (Corder et al., 1994). Although the apoE-4 allele may modestly influence cognitive decline prediction in older persons, the apoE genotype alone is not considered a useful predictor in non-demented persons (Relkin et al., 1996).

Surrogate biological brain imaging markers for AD have included both structural and functional approaches. An extensive literature has shown that positron emission tomography (PET) (Phelps et al., 1979), using 2-deoxy-2-[^{18}F]fluoro-D-glucose (FDG) to measure local cerebral glucose metabolic rate, provides a consistent pattern of reduced cerebral glucose utilization beginning in parietal and temporal regions and later spreading to prefrontal cortices in AD. The parietal deficit has been observed in people with questionable dementia (Kuhl et al., 1987) years prior to the development of clinically confirmed AD. Such PET measures also differentiate AD from other dementias, including Lewy body dementia, vascular dementia, and frontotemporal dementia (Silverman et al., 1999). The data support the use of PET for monitoring experimental therapeutic treatments early in the course of AD before substantial neuronal death occurs.

Our initial observation of parietal hypometabolism in non-demented people with a single copy of apoE-4 and a familial risk for AD (Small et al., 1995) has been replicated in apoE homozygotes (4/4 genotype) and extended to other brain regions, including posterior cingulate, dorsolateral prefrontal, and temporal cortices (Reiman et al., 1996). In the present study, we determined the consistency of such findings in a separate cohort at baseline and after two years of follow-up.

METHODS

The subjects and methods have been detailed previously (Small et al., 2000). In brief, subjects included 54 right-handed non-demented persons, aged

50–84 years, who were recruited through newspaper advertisements, media coverage, and other referrals. All subjects were aware of a gradual onset of mild memory complaints (e.g. misplacing familiar objects, difficulty remembering names). Any volunteer with a neurological, medical, or psychiatric condition (e.g. depression) that could affect memory or cognitive processing was excluded. A total of 573 persons volunteered, and 519 were excluded because of medication use, concurrent medical or psychiatric or neurologic illness. Treatable causes of cognitive impairment were excluded and cognitive performances were detailed through neurologic, psychiatric, and neuropsychological evaluations and routine screening laboratory tests (Wechsler, 1945; Benton, 1974; Buschke and Fuld, 1974; Folstein et al., 1975; van Gorp et al., 1990; Small et al., 1997). Previously described standard techniques were used to determine apoE genotypes (Saunders et al., 1993).

Subjects underwent PET imaging as previously described (Small et al., 2000). In brief, the subjects were scanned in the supine position with low ambient noise, and their eyes and ears were not occluded. Intravenous lines were placed 10–15 min before tracer injection, 370 MBq FDG. Scans were performed 40 min after FDG injection using the CTI/Seimens 831-08 (Seimens Corp., Hoffman Estates, Ill.) EXACT HR or EXACT HR+ tomograph (15–63 image planes). Scans were acquired parallel to the canthomeatal line, a transmission measurement was used for attenuation correction, and subjects were scanned 40–80 min after FDG injection.

Baseline MRI brain scans were obtained using either a 1.5-T magnet (General Electric Signa, Milwaukee, Wis.) or a 3-T magnet (General Electric Signa). Thirty-six transaxial planes were collected throughout the brain volume, superior to the cerebellum. A double echo, fast spin echo series using a 24-cm FOV and 256×256 matrix with 3 mm/0 gap [TR = 6000 (3 T) and 2000 (1.5 T); TE = 17/85 (3 T) and 30/90 (1.5 T)] was acquired for MRI registration with PET for anatomical metabolic data localization with reference to individual structural anatomy.

The PET data were co-registered with baseline MRI scans, and statistical parametric mapping (SPM) analyses were performed (Friston et al., 1995) using the SPM96 software package (Wellcome Department of Cognitive Neurology, Functional Imaging Laboratory, London, UK). Briefly, images were co-registered and reoriented into a standardized coordinate system, spatially smoothed, and normalized to mean global activity as previously described (Small et al., 2000). The pooled data were then assessed with the *t*-statistic on a voxel-by-voxel basis to identify the profile of voxels that significantly changed between conditions or co-varied with other parameters. The probability of finding by chance any region containing its voxel of maximal significance was assessed after adjusting for multiple comparisons, and the region as a whole was considered significant only if that adjusted probability was less than 0.05. Written informed consent was obtained in accordance with the procedures set by the UCLA Human Subjects Protection

Committee. Clinical diagnoses were made with investigators blind to genetic data; image data were analyzed with investigators blind to clinical and genetic findings.

RESULTS

At baseline assessment, the non-demented apoE-4 carriers (n = 27) and non-carriers (n = 27) showed similarities in mean ages at examination, dementia onset ages within families, educational achievement level, and frequencies of women or family history of AD (Table 18.1). The apoE-4 carriers were all heterozygotes (3/4 genotype) except for two subjects who were homozygotes (4/4). Of the subjects without apoE-4, 25 had the 3/3 genotype and two had the 2/3 genotype. The subjects had memory performance scores similar to those of cognitively intact persons of the same age and educational level, and there were no significant differences in scores according to genetic risk (Table 18.1).

Baseline PET measures of the non-demented subjects showed that the apoE-4 carriers had significantly lower metabolism than those without apoE-4, particularly in the left inferior parietal, lateral temporal, and posterior cingulate regions (Figure 18.1). Of the 54 subjects assessed at baseline,

Table 18.1. Baseline demographic and clinical characteristics of subject groups

Characteristic	Subject group	
	With apoE-4 (n = 27)	Without apoE-4 (n = 27)
Age (years)	65.9 ± 8.8	66.8 ± 8.9
Women	16 (59%)	13 (48%)
Family history of AD[a]	18 (67%)	13 (48%)
Mean onset age[b]	70.3 ± 6.2	68.8 ± 6.1
Education (years)	15.5 ± 2.6	16.0 ± 2.7
Mini-Mental State Examination	28.4 ± 1.6	29.2 ± 1.2
Delayed Paragraph Recall[c]	18.7 ± 8.5	21.8 ± 6.7
Buschke–Fuld Total Recall[d]	94.7 ± 18.2	101.6 ± 18.8
Benton Visual Errors[e]	4.3 ± 2.4	4.7 ± 2.8
Complex Figure (3 min delay)[f]	17.2 ± 7.1	19.1 ± 6.6

Values are reported as mean ± SD or number (percent).
[a]Calculated only from subgroup with a family history (>1 first-degree relative with clinical or autopsy-confirmed AD).
[b]Onset age of demented family members.
[c]Norms: 16.8 + 8.1 (65–69 years) (Wechsler, 1945).
[d]Norms: men: 81.4 + 17.4, women: 95.9 + 19.1 (Buschke and Fuld, 1974).
[e]Norms: 5 ± 5 (65–74 years) (Benton, 1974).
[f]Norms: 14.1 ± 7.8 (66–70 years) (van Gorp et al., 1990).

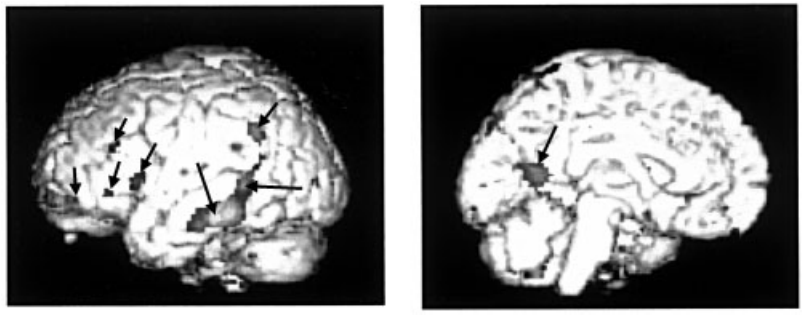

Figure 18.1. Differences in cerebral metabolism in non-demented subjects according to genetic risk (SPM analysis). Lower metabolic levels are seen for the apoE-4 group (arrows) in left lateral temporal ($p < 0.001$), posterior cingulate ($p < 0.001$), and inferior parietal ($p < 0.006$) cortex. The region of peak significance ($z = 3.24$) lies in the temporal cortex in Brodmann's areas 20 and 21

Table 18.2. Baseline and two-year follow-up cognitive performance scores of non-demented subjects in longitudinal study[a]

	Subject group	
	With apoE-4 (n = 10)	Without apoE-4 (n = 10)
Mini-Mental State Examination		
Baseline	28.0 ± 1.3	29.0 ± 1.3
Follow-up	28.0 ± 2.1	28.7 ± 1.3
Delayed Paragraph Recall		
Baseline	14.7 ± 6.6	18.4 ± 4.3
Follow-up	13.6 ± 7.5	20.2 ± 8.3
Buschke–Fuld Total Recall[b]		
Baseline	87.9 ± 14.0	95.2 ± 20.9
Follow-up	70.4 ± 29.4	94.2 ± 21.5
Benton Visual Errors		
Baseline	4.7 ± 2.6	6.1 ± 3.1
Follow-up	5.4 ± 3.7	3.8 ± 2.1

[a]An ANCOVA model using follow-up scores as outcomes and baseline scores as covariates yielded no significant difference between groups in any of the measures. Values are reported as mean ± SD.
[b]Baseline vs. follow-up for apoE-4 carriers: $t = 2.5$, $df = 9$, $p = 0.036$ (uncorrected).

longitudinal memory performance and PET data were available for 20 of them (10 with apoE-4, 10 without apoE-4) two years later (mean ± SD for follow-up was 27.9 ± 1.7 months). Memory performance scores did not differ significantly according to genetic risk either at baseline or follow-up (Table 18.2). PET scans performed after two years showed the greatest magnitude (5%) and extent of metabolic decline in the inferior parietal and lateral

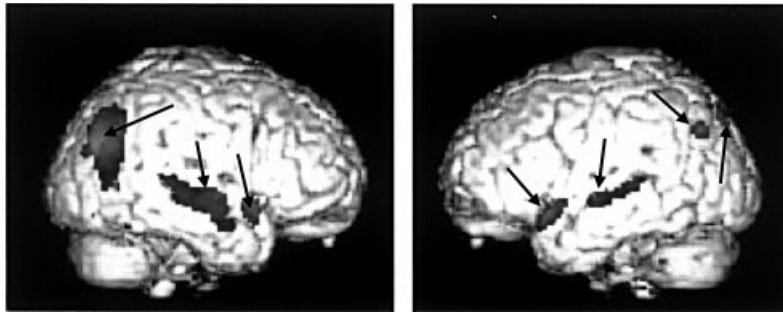

Figure 18.2. Regions showing the greatest metabolic decline after two years of longitudinal follow-up in non-demented subjects with apoE-4 (SPM analysis) included the right lateral temporal and inferior parietal cortex. Voxels undergoing metabolic decline ($p < 0.001$, before correction) are displayed shaded (arrows), with peak significance ($z = 4.35$) occurring in Brodmann's area 21 of the right middle temporal gyrus

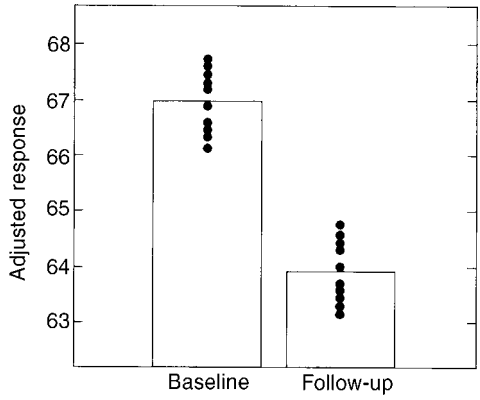

Figure 18.3. Right lateral temporal metabolism for the apoE-4 subjects declined significantly at follow-up two years after baseline scans. Individual values (circles) at the voxel of most significant decline (Talairach coordinates 68 -30 0, normalized to a mean voxel value of 50 for each brain) uniformly decreased, with no overlap for the two points in time ($z = 4.35$, $p = 0.022$, corrected for multiple comparisons). Histograms represent means at each time point. The same analysis for the non-apoE-4 group did not show consistent metabolic decline ($z = 2.33$, $p = 0.998$, corrected), nor did any other voxel in temporal cortex of that group decline significantly

temporal cortices (Figure 18.2). After correction for multiple comparisons, these metabolic changes remained significant for the apoE-4 group ($p = 0.02$), wherein every subject had observable metabolic decline in these regions (Figure 18.3). The group without apoE-4 had a non-significant (after correction) decline in these same regions.

DISCUSSION

These results confirm that middle-aged and older apoE-4 carriers have decreased inferior parietal, temporal, and posterior cingulate cortical metabolism compared with their counterparts without apoE-4 (Small et al., 1995; Reiman et al., 1996). The two-year follow-up component of this study also indicated that these apoE-4 carriers all show continued metabolic decline and that the greatest magnitude and extent of metabolic decline is observed in the parietal and temporal cortices, areas that show extensive and early deposition of neuropathological lesions in patients with AD.

These observations have practical implications for the design of clinical trials for preventing future cognitive decline and eventually delaying the onset of a clinical diagnosis of AD. Such prevention trials may currently include thousands of subjects to determine the primary outcome measure of incipient AD. The present results suggest that using PET scan measures of regional glucose metabolism in apoE-4 carriers will allow testing of an intervention to delay parietal and temporal metabolic decline in smaller numbers of subjects with mild age-related memory complaints.

Comparison of the adjusted right lateral temporal metabolism for the apoE-4 group at baseline and two-year follow-up indicated no overlap between data points in a relatively small sample of ten subjects (Figure 18.3). These data yield an estimated power under the most conservative scenario (i.e. assuming that the points are connected exactly in reverse order) of 0.9 to detect a 1-unit decline from baseline to follow-up in a one-tailed test. Such results are consistent with previous PET studies showing stable and replicable results (Andreasen et al., 1996) and suggest that combining PET and AD genetic risk measures will allow investigators to use relatively small sample sizes when testing antidementia treatments in preclinical AD stages. Based on data from the present study, in a prevention trial over two years with only 34 subjects, we would be able to detect 1 unit difference in lateral temporal metabolism across testings between groups with a power of 0.8 using PET, compared to a detectable difference of 18 units for a more traditional measure of decline such as the Bushke–Fuld test. Such a study would make it unnecessary to follow subjects until they develop AD, and the results would determine whether the treatment would slow regional brain metabolic decline rather than delay the onset of incipient dementia. The longitudinal PET literature indicates that people with parietal and temporal metabolic deficits generally show eventual metabolic decline in these same regions, which often precede the clinical diagnosis of AD if followed subjects are followed long enough (Kuhl et al., 1987; Silverman et al., 1999, 2000a,b).

Findings that metabolic patterns predict cognitive decline in presymptomatic persons are consistent with the notion that the pathophysiologic process begins well before even mild or questionable dementia can be recognized in clinical settings. PET measures of hypometabolism reflect decreased synaptic

activity due either to loss or dysfunction of synapses, and regional metabolic deficits observed on PET may reflect projections from dysfunctional neurons in other brain regions. Lesions of entorhinal cortex in nonhuman primates cause neocortical and hippocampal glucose hypometabolism (Meguro et al., 1999), suggesting that the parietal and temporal cortical deficits observed in our study indicate underlying deficits in brain areas showing the earliest neuropathological changes in AD (Braak and Braak, 1991). Our finding of decreased cerebral metabolism in apoE-4 carriers with normal neuropsychological function is consistent with observations (Grady et al., 1993; Bookheimer et al., 2000) that the brain compensates for regional neuronal dysfunction leading to normal clinical neuropsychological performance.

This work, then, demonstrates the consistency of cerebral metabolic deficits in middle-aged and older people with the apoE-4 genetic risk for AD and shows that baseline PET deficits worsen over two years of follow-up. Our approach of combining apoE genotypic data with PET measures provides a strategy to determine the time course for cerebral metabolic disease progression and to monitor experimental prevention therapies, thereby allowing more efficient testing of promising new treatments in the preclinical phases of Alzheimer's disease.

SUMMARY

The major known genetic risk for Alzheimer's disease (AD), apolipoprotein E-4 (apoE-4), is associated with lowered cerebral glucose metabolism in brain regions showing AD neuropathological damage. To determine metabolic decline patterns according to genetic risk, we investigated cerebral metabolic rates using positron emission tomography (PET) in middle-aged and older persons with normal memory performance. We collected PET, magnetic resonance imaging (MRI), neuropsychological, and apoE data on 54 non-demented subjects (27 with apoE-4, 27 without apoE-4) at baseline, and 20 of them (10 with apoE-4, 10 without apoE-4) two years later (mean, SD for follow-up was 27.9, 1.7 months). The MRI scans were co-registered to PET scans, and statistical parametric mapping was performed. Baseline PET measures indicated significantly lower metabolism in non-demented subjects with a single copy of the apoE-4 allele compared to those without apoE-4 in the inferior parietal, lateral temporal, and posterior cingulate regions. PET scans performed after two years showed the greatest magnitude (5%) and extent of metabolic decline in the inferior parietal and lateral temporal cortices in subjects at genetic risk for AD. After correction for multiple comparisons, these metabolic changes remained significant for the apoE-4 group, wherein every subject had observable metabolic decline in these regions. These results indicate that combining cerebral metabolic rate and genetic risk measures provides a means for preclinical AD detection that will

assist in response monitoring during experimental treatments. Moreover, this strategy could allow testing of relatively small sample sizes when assessing treatments aimed at preventing cognitive decline and delaying dementia onset.

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19 Overview of Vascular Dementia

WILLIAM R. MARKESBERY

The concept that atherosclerosis and impaired cerebral blood were the causes of dementia dates back to the late nineteenth century (Blass et al., 1991). Our modern understanding of dementia was greatly influenced by Tomlinson et al. (1968, 1970) who clarified that Alzheimer's disease (AD) was the most common cause of dementing disorders. They also showed that there was a relationship between the volume of infarcted tissue and dementia, supporting the idea that cerebrovascular dementia was due to brain destruction and not to a gradual reduction of neurons due to diminished blood flow. Subsequently, it has been shown that a broad spectrum of cerebrovascular lesions can result in cognitive function decline. These disorders have been referred to as multi-infarct dementia (Hachinski et al., 1974), vascular dementia, vascular cognitive impairment (Hachinski and Bowler, 1993), and, more recently, dementia associated with stroke (Gorelick, 1997). Although Hachinski and colleagues provide strong reasoning for the use of 'vascular cognitive impairment,' the term 'vascular dementia' (VaD) has become entrenched in the literature and will be used in this brief overview. In the broadest sense, VaD refers to cognitive dysfunction caused by cerebral injury secondary to a wide spectrum of vascular pathology.

CLINICAL DIAGNOSIS OF VaD

The Hachinski Ischemic Score (HIS) (Hachinski et al., 1975), the initial clinical diagnostic criteria for VaD, has remained a reliable, easy-to-use scale that is relatively sensitive in differentiating pure AD from VaD but less sensitive for differentiating mixed AD/VaD (Moroney et al., 1997). The HIS assigns one or two points to the following clinical findings: abrupt onset, stepwise deterioration, fluctuating course, nocturnal confusion, personality preservation, depression, somatic complaints, emotional incontinence, hypertension, history of strokes, associated atherosclerosis, and focal neurologic symptoms and signs. Scores above seven distinguish VaD and scores below four define neurodegenerative disorders. Several modifications

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of the HIS have been suggested (Rosen et al., 1980; Loeb and Gandolfo, 1983; Fischer et al., 1991).

More recently, several diagnostic criteria have been developed in an attempt to widen the concept of VaD. The criteria of the state of California Alzheimer's Disease Diagnostic and Treatment Centers (California ADDTC) (Chui et al., 1992) include: dementia; evidence of two or more ischemic strokes by history, neurologic signs or neuroimaging studies; the occurrence of a single stroke with temporal relationship to the onset of dementia; and evidence of at least one infarct outside the cerebellum by neuroimaging.

The criteria of the National Institute of Neurological Disorders and Stroke and Association Internationale pour la Recherche et l'Enseignement en Neurosciences (NINDS–AIREN) (Román et al., 1993) used the presence of dementia, presence of cerebrovascular disease defined by the presence of focal neurologic signs consistent with stroke and verified by brain imaging, and a causal relationship between these two criteria.

The criteria suggested by the *Diagnostic and Statistical Manual of Mental Disorders* of the American Psychiatric Association (1994) (DSM-IV) include: development of multiple cognitive deficits, an impairment of social or occupational function representing a decline from previous level of functioning, focal neurologic signs and symptoms, laboratory evidence of cerebrovascular disease etiologically related to the clinical picture, and a lack of delirium.

Gold et al. (1997), using autopsied subjects, compared the sensitivity and specificity of the California ADDTC, NINDS–AIREN, and HIS criteria. Sensitivity was 0.63 and specificity was 0.64 for the ADDTC, 0.58 and 0.80 for NINDS–AIREN, and 0.43 and 0.88 for the HIS. The population of mixed dementia cases clinically misclassified as VaD was 54% by the ADDTC, 29% by the NINDS–AIREN, and 18% by the HIS.

Chui et al. (2000) assessed the concordance in classification and interrater reliability for the above three criteria and the DSM-IV criteria for VaD in seven ADDTCs. The frequency of the diagnosis of VaD was highest using the modified HIS and DSM-IV criteria. The interrater reliability was highest for the HIS. They concluded that the clinical criteria for VaD were not interchangeable, and the prevalence of VaD will vary considerably depending on the criteria used.

It should be underscored that the clinical diagnosis of VaD is not necessarily determined by a specific set of criteria. In the end, it requires a thorough clinical history and examination, neuropsychologic testing, brain imaging studies, and interpretation of these data by a clinician experienced with dementing disorders and stroke.

FREQUENCY OF VASCULAR DEMENTIA

The true prevalence of VaD is difficult to determine because of variable diagnostic criteria, geographic differences, age of the populations studied, and

patient selection bias. For example, the prevalence of VaD evaluated in a memory disorders clinic, such as occurs in many AD centers, might be much lower than that from a vascular disease clinic. In addition, it is difficult to compare the prevalence of VaD in clinical studies with recent autopsy studies of dementia patients where a low rate is present.

Evaluation of 11 pooled European population-based clinical studies of persons 65 years and older revealed an age-standardized prevalence of 6.4% for all causes of dementia, 4.4% for AD, and 1.6% for VaD (Lobo et al., 2000). VaD accounted for 15.8% of all dementia cases. The prevalence of VaD ranged from 0.0% to 0.8% at ages 65 to 69 and from 2% to 8.3% in the 90 and older age group in the different countries.

In a Canadian clinical study of 603 dementia patients, 12.1% had VaD and 12.6% had mixed AD/VaD (Rockwood et al., 2000).

In Asian countries, VaD may be more common than AD. Studies from Japan revealed that the prevalence of VaD was more than double that of AD (Hasegawa et al., 1986; Ueda et al., 1992; Yoshitake et al., 1995). White et al. (1996) studied demented Japanese-American men, ages 71–93, living in Hawaii and found that 34% had AD and 30% had VaD. The prevalence of VaD was similar to that found in Japan, but the rate of AD was higher than in Japan. In China, VaD was reported to be 1.5 times that of AD (Li et al., 1991); however, a more recent study found 31% of dementia patients had VaD and 61% had AD in China (Chiu et al., 1998).

A review of autopsy studies of patients with dementia from 1962 to 1995 from many different countries revealed an overall mean rate of 17.3% for VaD (Markesbery, 1998). However, more recent reports show a prevalence rate of 2–5%. Recent studies may reflect a referral bias heavily weighted with AD subjects from clinics or hospitals where AD predominates. However, this was not the case in the Nun Study in which we found only three pure VaD cases out of 118 demented subjects at autopsy (Snowdon and Markesbery, 1999).

RISK FACTORS

The risk factors for VaD are shown in Table 19.1. Several recent reviews of the subject are available (Gorelick, 1997; Skoog, 1998). As expected, many of the risk factors for VaD are the same as for stroke. Age is an extremely important risk factor for VaD. The prevalence of VaD doubled every five to ten years after age 65 (Hofman et al., 1991). Other non-modifiable risk factors include genetic predisposition and Asian background. However, modifiable risk factors for VaD include hypertension, diabetes mellitus, atrial fibrillation, elevated cholesterol, alcohol abuse, and cigarette smoking.

Table 19.1. Risk factors for vascular dementia

Advancing age	Cerebral atrophy
Hypertension	Alcohol abuse
Diabetes mellitus	Cigarette smoking
Myocardial infarction	Low educational attainment
Cardiac disease (especially atrial fibrillation)	Asian background
Elevated LDL cholesterol	CADASIL
Prior strokes	Familial cerebral amyloidosis

GENETIC RISK FACTORS

A few examples of VaD are transmitted on an inherited basis. Small numbers of patients with familial amyloid angiopathies develop dementia after multiple cerebral hemorrhages or infarcts. Patients with cerebral hemorrhage with amyloidosis have been described in Iceland (HCHWA–I) and the Netherlands (Dutch form) (HCHWA–D). Both are dominantly inherited disorders and have amyloid deposited in cerebral arteries and arterioles. HCHWA–I causes intracerebral hemorrhages in younger individuals (20–30 years old). Cystatin C, a cysteine protease inhibitor, is present in blood vessel walls in HCHWA–I. A single mutation on the cystatin C gene on chromosome 20 has been demonstrated in this disorder (Abrahamson et al., 1992). HCHWA–D affects normotensive patients in the age range of 40–60 years. The amyloid fibrils are similar to those found in AD. A mutation in the amyloid precursor protein gene at codon 618 has been described in this disorder (Levy et al., 1990).

In 1993, Tournier-Lasserve et al. described an autosomal dominant disorder linked to chromosome 19, termed 'cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy' (CADASIL), which is increasingly recognized as a cause of stroke and cognitive alterations in mid-adult life. The major clinical features are migraine headaches, subcortical strokes, psychiatric symptoms, and cognitive decline. Pathologically, there is a widespread arteriopathy in medium and small arteries that contain a granular osmiophilic material (GOM). Identification of GOM in skin, muscle, or nerve by electron microscopy allows a specific diagnosis of CADASIL. CADASIL is due to a mutation in the Notch-3 gene, which encodes a transmembrane receptor protein (Joutel et al., 1996). Notch-3 is expressed in vascular smooth muscle cells. A 210-kDa Notch-3 cleavage product is present at the cytoplasmic membrane of vascular smooth muscle cells (Joutel, 2000).

Numerous studies have shown that the ε_4 allele of apolipoprotein E (apoE) is a major risk factor for AD; however, studies of apoE in VaD have yielded variable results. Although several recent studies have described an increased frequency of apoE-ε_4 in patients with dementia with stroke (Slooter et al., 1997; Hofman et al., 1997; Hebert et al., 2000), other studies from different

countries have not confirmed this (Traykov et al., 1999; Alafuzoff et al., 2000; Barba et al., 2000). Again, varying definitions and criteria and the potential overlap with AD in clinical studies make interpretation of these data difficult.

PATHOLOGY

Multiple, highly variable vascular lesions can cause VaD, including large vessel atherosclerotic occlusion, thromboembolism, small vessel disease, intracerebral hemorrhage associated with hypertension and other causes, subarachnoid hemorrhage, hypoperfusion with ischemic injury, vasculitides, and others. In many patients with cognitive decline associated with cerebrovascular disease who have focal destructive lesions, there is also diffuse, widespread vascular disease throughout much of the brain, which may play a role in altering brain reserve.

Neuropathologic criteria have not been established for VaD. The California ADDTC (Chui et al., 1992) and the NINDS–AIREN criteria (Román et al., 1993) did not suggest specific details for the neuropathologic diagnosis of VaD, but indicated that histopathologic confirmation of the brain was necessary to confirm the presence of multiple infarcts. Complicating the diagnosis of VaD are other pathologic entities coexisting with vascular lesions that could lead to cognitive decline. Many of these can be found by postmortem examination; thus, autopsy examination of the brain is critical in the definitive diagnosis of VaD. However, it should be emphasized that the presence of vascular lesions found at autopsy does not prove they cause cognitive decline, underscoring that thorough clinical–pathologic correlation is essential to establish a definitive diagnosis of VaD.

Large pale or hemorrhagic infarctions resulting from atherosclerotic occlusion or thromboembolism in large vessels, such as the internal carotid, middle, anterior, and posterior cerebral arteries can be associated with VaD. These infarcts are often bilateral and multiple and involve strategic regions related to cognitive abilities (see below). A report of the Stroke Data Bank cohort of ischemic stroke patients revealed that the prevalence of dementia was higher in subjects with large artery infarctions than in those with small artery infarcts (Tatemichi et al., 1990).

Infarcts from small vessel occlusion or lacunar infarcts are most often found in cerebral white matter, basal ganglia, thalamus, pons, and cerebellar white matter. Lacunar infarcts are small cavitary lesions (<1.5 cm in diameter) most often caused by occlusion in small arteries or arterioles and are associated with hypertension or diabetes mellitus. Some may be associated with hemorrhage. Román (1981) found lacunar infarcts in 36% of demented patients and Yoshitake et al. (1995) found multiple lacunar infarcts in 42% of Japanese patients with dementia. The prevalence of cognitive decline in those with multiple lacunar infarcts is variable and probably dependent on multiple

factors including widespread diffuse cerebrovascular disease, cognitive reserve, and the presence of large infarcts.

Tiny cerebral cortical microinfarcts often related to small emboli can, when multiple, be associated with dementia (Kaplan et al., 1985).

More recent studies have placed emphasis on deep white matter lesions in VaD. This has been catalyzed by CT and MRI studies showing alterations in deep white matter. The term 'leukoaraiosis' has been used to describe the white matter rarefactions found on CT and MRI scan (Hachinski et al., 1987). The true meaning of these lesions is controversial, and rarefactions in the white matter can be found in VaD, AD, and in aged normal subjects. Merino and Hachinski (2000) summarized data about leukoaraiosis and concluded that it occurred most frequently in association with age, hypertension, lacunar and subcortical strokes, and carotid artery atherosclerosis.

Several neuropathologic alterations are associated with leukoaraiosis. One explanation is incomplete non-cavitary ischemic zones in deep white matter that are characterized microscopically by myelin, axon, and oligodendroglia loss, mild reactive astrocytosis, sparse macrophage reaction, and hyaline fibrosis of arterioles. Brun (1994) suggested that these result from hypotension and narrowing of arterioles. Our clinical–imaging–neuropathologic study of periventricular white matter hyperintensities in a series of autopsied elderly female subjects demonstrated that they were not related to white matter volume, stroke, or dementia (Smith et al., 2000).

Other white matter alterations include frontal and parietal lacunar infarcts or large or small cavitary infarcts. Focal white matter changes and arteriolosclerosis were more frequent in a series of 22 VaD patients compared with 20 AD patients (Erkinjuntti et al., 1996). Erkinjuntti (2000) suggested a subclassification of subcortical VaD in which lacunar infarcts and ischemic white matter lesions related to small vessel disease are the major pathologic features. Clinical findings include white matter lesions on imaging studies, a history of small strokes or multiple transient ischemic attacks, and mild or occasionally absent focal neurologic signs.

Positron emission tomography studies demonstrate that subcortical vascular lesions can cause a reduction in cerebral cortical metabolic activity. Sultzer et al. (1995) showed that periventricular and deep white matter hyperintensities and subcortical lacunar infarcts in patients with VaD caused a decline in mean global cortical metabolism. The reduction in metabolic rate was lower in patients with lacunar infarcts in basal ganglia or thalamus. Lower cognitive function tests correlated with the extent of the white matter lesions. Kwan et al. (1999) found that cortical global metabolic rates were lower in patients with subcortical strokes and cognitive impairment or dementia compared with patients with subcortical stroke and no cognitive impairment. The right frontal cortex showed a lower metabolic rate in all stroke groups.

The entity entitled 'subcortical arteriosclerotic encephalopathy of Binswanger' is controversial and poorly understood and beyond the scope

of this overview, but excellent reviews are available (Caplan, 1995; Pantoni and Garcia, 1995).

Spontaneous non-traumatic intracerebral hemorrhages can be associated with cognitive alterations, but in our experience are uncommon causes of dementia. They are most often related to hypertension or cerebral amyloid angiopathy.

Bilateral hippocampal sclerosis can be the pathologic substrate for dementia (Zola-Morgan et al., 1986), usually involves the CA-1 field of the hippocampus, and results from ischemia. Dickson et al. (1994) described it as a common postmortem finding in elderly (>80 years old) demented subjects, a conclusion with which our autopsy experience agrees. It can be found in isolation or in association with other vascular lesions and/or neurodegenerative changes of AD. When found in conjunction with other vascular lesions or in AD, its precise role in the dementing process is unknown, although it would appear to be a significant additive factor.

OTHER FACTORS INVOLVED IN VaD

Most likely, multiple factors cause VaD, especially the level of brain reserve in those with vascular lesions as described below. Earlier VaD literature emphasized volume of brain destroyed, location of vascular lesions, and number of vascular lesions.

VOLUME OF BRAIN DESTRUCTION

Tomlinson et al. (1968, 1970) showed that infarcts (>100 ml) of brain caused dementia and volumes between 50 and 100 ml were less likely to produce dementia. More recent autopsy studies demonstrated that smaller infarct volume can lead to dementia (Erkinjuntti, 1987; Del Ser et al., 1990; Erkinjuntti et al., 1996). Imaging studies also show that smaller infarct sizes are associated with dementia (Gorelick et al., 1992; Liu et al., 1992; Pohjasvaara et al., 2000).

LOCATION OF VASCULAR LESIONS

The location of vascular lesions causing VaD appears to be more important than the size of the lesion. Multiple brain regions have been implicated in VaD, including dominant angular gyrus, anterior or posterior cerebral artery territory, superior middle cerebral artery territory, left anterior corona radiata, bilateral medial thalamus, dominant caudate nucleus, anterior internal capsule, hippocampus, amygdala, and basal forebrain (reviewed in Markesbery, 1998). Imaging studies demonstrate contradictory results regarding stroke location and VaD (Bowler and Hachinski, 1995; Erkinjuntti

et al., 1999). More recently, Pohjasvaara et al. (2000) suggested that poststroke dementia is not caused by a single factor but by a combination of factors including volume of infarct, right or left superior middle cerebral artery infarcts, left thalamic–cortical connection infarcts, frequency of left hemispheric infarcts, extent of white matter lesions, medial temporal lobe atrophy, and level of education of the subject.

NUMBER OF VASCULAR LESIONS

A common concept about VaD is that multiple small infarcts or lacunar infarcts, especially those involving deep gray nuclei or deep white matter, can lead to dementia. Few studies have addressed the question of the number of infarcts involved in causing cognitive decline. Two studies suggest that VaD patients have between 5.8 and 6.7 infarcts (Erkinjuntti et al., 1988; Del Ser et al., 1990), but Kwan et al. (1999) found no correlation between number of subcortical strokes and score on the Mini Mental State Examination.

MIXED VASCULAR DEMENTIA AND ALZHEIMER'S DISEASE

'Mixed dementia' can refer to the presence of two or more causes of dementia; however, it generally refers to the coexistence of the clinical and pathologic alterations of both AD and VaD. Tomlinson et al. (1968, 1970) first called attention to this disorder when they suggested that the combined effects of AD histopathologic lesions and cerebral infarctions might lead to intellectual decline. Criteria for the diagnosis of mixed AD/VaD have not been developed; thus, the true frequency is not known. A recent review of autopsy studies found the prevalence of mixed VaD/AD to range from 3.7% to 36% in different autopsy series (Markesbery, 1998). The mean of these studies was 17.7%.

The Canadian Consortium for the Investigation of Vascular Impairment of Cognition found that out of 603 dementia patients, 149 had VaD and, of these, 76 had mixed AD/VaD (Rockwood et al., 2000).

Cohen et al. (1997) reviewed different approaches to the clinical diagnosis of mixed dementia. Characteristics of AD by history, examination, and imaging studies revealing vascular lesions are most commonly used for this diagnosis. A definite diagnosis of mixed AD/VaD is best made by autopsy; however, the presence of the pathologic findings of AD plus vascular lesions occurs quite frequently, and without precise clinical information the clinical role of the vascular lesions is not known.

Approximately one-third of pathologically confirmed AD brains contain vascular lesions (Gearing et al., 1995). Infarcts superimposed on AD could potentially worsen cognitive function, although only a few studies document

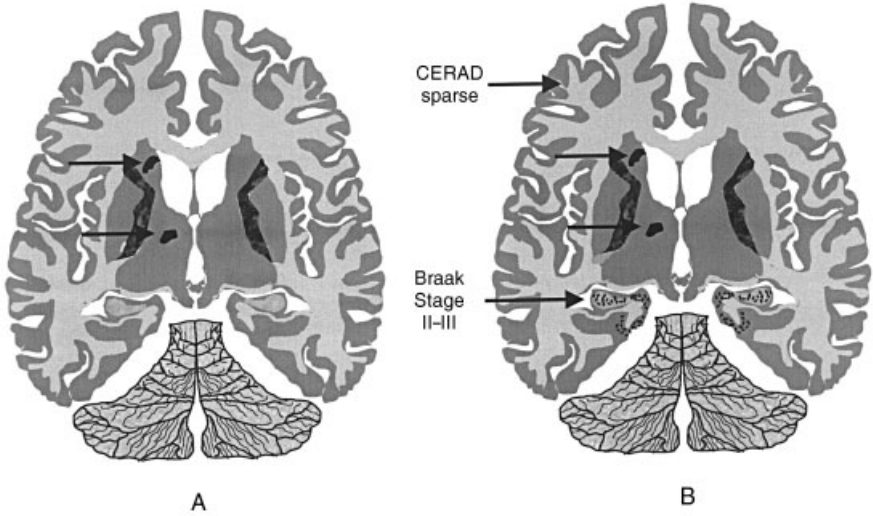

Figure 19.1. **A** Lacunar infarcts in the caudate nucleus and thalamus of a theoretical patient with normal cognitive reserve who shows no evidence of cognitive decline because of the infarcts. **B** Similar lacunar infarcts in a theoretical patient with neurofibrillary tangles sufficient to be Braak stage II or III and CERAD sparse neuritic plaques, which together could lead to cognitive decline

this. In a prospective study of autopsied aged nuns (mean age 87 years), 39% met the neuropathologic criteria for AD and had one or more cerebral infarcts (Snowdon et al., 1997). Subjects who met AD neuropathologic criteria and had cerebral infarction were significantly more cognitively impaired than were AD subjects without infarcts. Fewer neurofibrillary tangles and senile plaques appeared to cause dementia in those with brain infarctions than those without infarcts. Nagy et al. (1997) demonstrated that in autopsied mixed AD/VaD patients and in AD patients with other neurodegenerative pathology (Parkinson's disease changes, motor neuron disease, or normal-pressure hydrocephalus), the density of total senile plaques or neuritic plaques was significantly lower in those with AD and other pathologic alterations of the central nervous system compared with AD and no other pathologic changes for any given level of cognitive deficit. This indicates that the combination of two pathologic processes influences the severity of the cognitive deficit.

Brun (2000) suggested that a summation dementia of old age resulted from a combination of AD changes, synapse loss, trauma, and cerebrovascular disease. He placed emphasis on selective incomplete white matter infarcts in the summation dementia of old age.

One interpretation of the above studies is that superimposing vascular lesions in critical brain regions in individuals with declining functional brain

reserve can lead to decline in cognitive function. That is, individuals with abundant entorhinal and hippocampal neurofibrillary tangles, such as might be found in Braak stage II or III (Braak and Braak, 1991) and infrequent neocortical senile plaques as described in the CERAD sparse classification (Mirra et al., 1991), who experienced critically located infarcts, could start to show more marked intellectual decline than those without these changes (Figure 19.1). For example, individuals showing mild cognitive impairment might show a greater decline in function following cerebral infarction, which could lead to a diagnosis of dementia. Similarly, subjects with early or mid stage AD, who develop cerebral infarcts, might experience a more rapid decline in cognitive function through further depletion of brain reserve.

CONCLUSIONS

Although progress is being made in understanding VaD—risk factors have been identified, attempts have been made to develop and evaluate diagnostic criteria, imaging studies have been improved, and there is a greater awareness of this disorder—as Bowler and Hachinski (2000) pointed out, there is still a great need for replacement of dogma with more hard data. Large multi-institutional cooperative, longitudinal clinical–neuroimaging–pathologic studies would enhance development of crisp clinical, imaging, and neuropathologic criteria and place VaD on a firmer footing. Although these studies are not centered on molecular biology/genetics, which are attractive to funding agencies in this era, they are critical to establishing the appropriate infrastructure for understanding this disorder, as has been done for AD.

Although we lack a depth of understanding of VaD and specific treatment is not available, it is clear that VaD is a preventable disorder. Many risk factors for VaD are similar to those for stroke, and prevention of stroke could lead to a reduction of VaD. Those at risk or in the earliest phase of impairment need to be identified and treatment of hypertension, atrial fibrillation, diabetes mellitus, and elevated cholesterol should be initiated, and cigarette smoking and excessive use of ethanol be eliminated. In addition, in the mixed AD/VaD form, treatment of the cause of the vascular disease component might slow the decline. This may become especially important as newer treatments of AD become available. Prevention becomes critically important in view of the aging of our population with increasing numbers of individuals living into the over-80 age range. Without prevention, the prevalance of VaD and mixed AD/VaD will increase dramatically.

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20 Clinical and Imaging Characteristics of Vascular Dementia in a Memory Clinic

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INTRODUCTION

In Western countries, vascular dementia (VaD) is considered the second most common cause of dementia after Alzheimer's disease (AD). It accounts for about 15% of all dementia cases in autopsy series, while another 10% are explained by a combination of VaD and AD (Zuber, 1994). VaD is a potentially preventable dementia, although it may be due to variable cerebrovascular pathologies, often combined. Multiple infarcts are not the only type of vascular event to cause dementia. Single infarcts in strategic areas, white matter changes (WMC), and hemorrhage are all causes of dementia (Roman et al., 1993).

Diagnostic criteria for VaD are various and questionable. Two types of criteria currently exist. The first are criteria derived from general diagnostic tools, the *Diagnostic and Statistical Manual of Mental Disorders*, fourth edition (DSM-IV; American Psychiatric Association, 1994), and the *International Classification of Diseases*, tenth revision (ICD-10; World Health Organization, 1993). The second type of criteria are specifically constructed tools such as those of the State of California Alzheimer's Disease Diagnostic and Treatment Centers (SCADDTC; Chui et al., 1992) and of the National Institute of Neurological Disorders and Stroke in association with the Association Internationale pour la Recherche et l'Enseignement en Neurosciences (NINDS–AIREN; Roman et al., 1993). The aim of the present study was to describe clinical and imaging characteristics of outpatients attending a memory clinic with the clinical diagnosis of VaD and to compare several sets of clinical criteria. This is a prerequisite for the constitution of homogeneous groups of patients for clinical and pharmaceutical research.

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METHODS

The patients were selected from the prospectively constituted standardized database of the Lille memory clinic. For each patient, a standardized file was completed by a multidisciplinary staff including a neurologist, a psychiatrist, a neuropsychologist, a speech therapist, a nurse, and a social worker. The clinical diagnosis was made by consensus between all the participants based on clinical and cerebral imaging data.

We selected patients who came for their first visit between January 1, 1992 and December 31, 1997, to secure the clinical diagnosis with at least one year of follow-up, and who were given a clinical diagnosis of VaD (possible or probable), alone or associated with another diagnosis, usually AD. We excluded patients whose cerebral imaging was not available for the study and who were not followed up for at least one year.

We collected demographic data (sex, age, way of life), personal and family history (especially vascular risk factors, previous history of stroke and of dementia), onset, duration and course of the cognitive decline, physical examination, scores on Mini-Mental State Examination (MMSE; Folstein et al., 1975), Dementia Rating Scale (DRS; Mattis, 1976), and Activities of Daily Living (ADL; Weintraub, 1986), previous cerebral imaging, treatments, and follow-up (course of the disease, death and other events, imaging).

Cerebral atrophy, WMC, and focal lesions were analyzed on computed tomography (CT) and magnetic resonance imaging (MRI) scans by two independent raters (a neurologist and a neuroradiologist) using reference scales. Final evaluation was made by consensus between the two raters. Medial temporal lobe atrophy (MTLA) was evaluated when CT was oriented to the temporal lobe (Pasquier et al., 1997) and on coronal T1-weighted MRI (Scheltens et al., 1995). Global cerebral atrophy on CT was rated subjectively on a scale of 0–3 (Leys et al., 1989). Atrophy on MRI was rated subjectively in 13 regions on a scale of 0–3 and the sum of the subscores was called the cerebral atrophy score (range: 0–39) (Pasquier et al., 1996). WMC were evaluated, using the 0–3 scale of Blennow on CT (Blennow et al., 1991) and the two scores of the 0–3 scale of Fazekas on MRI (Fazekas et al., 1987; Scheltens et al., 1998). The type of focal lesions (lacunar infarct, territorial infarct, or other lesion) as well as their location (using the classification of Tatemichi et al., 1990) and their side (except for subtentorial lesions) was noted.

We compared three sets of clinical criteria: DSM-IV, NINDS–AIREN, and ICD-10 research criteria.

Statistics were mainly descriptive. We made comparisons in accordance with specific a priori hypotheses. Categorical data were compared using χ^2 test and continuous data were compared using analysis of variance (with Bonferroni correction when needed) and correlation coefficient.

RESULTS

Of 2655 consecutive patients first examined at the memory clinic between January 1, 1992 and December 31, 1997, 1719 were demented. VaD was diagnosed in 306 patients (17.8% of all dementias). Imaging was available in the database for 208 patients, who constituted the study population. Ninety-eight patients were excluded because their imaging was not available. We compared the demographic characteristics (sex and age) and the history of this group with those of the study population and found no significant difference.

The male : female ratio was 1.24. At first visit, mean (standard deviation) age was 72.2 (7.5) years, 72.0 (6.9) for men and 72.5 (8.2) for women. Vascular risk factors were reported in 175 (84.1%) patients and previous history of stroke was reported in 81 (38.9%). Mean age at onset was 68.7 (8.9) years and duration of the disease was 3.7 (3.5) years. A memory impairment was the first symptom in 154 (74.0%) patients, frontal lobe dysfunction in 39 (18.8%), and language impairment in 7 (3.4%). Clinical onset was reported to be progressive in 129 (62.0%), acute in 60 (28.8%), and subacute in 19 (9.1%). Course was progressive in 98 (47.1%) patients, fluctuating in 46 (22.1%), stable in 33 (15.9%), stepwise in 25 (12.0%), and showed improvement in 6 (2.9%) patients. Focal signs were found on neurological examination in 75 (36.1%) patients. Table 20.1 summarizes the main cognitive disorders that were observed. At first visit, mean MMSE score was 22.1 (6.1), mean DRS score was 114 (22.7) and mean ADL score was 42.4 (22.9)%. Cerebral imaging was performed before the first visit in 96 (46.2%) patients: CT in 81 cases, MRI in 10 cases, and both in 5 cases. Patients with a history of stroke were more likely to have a cerebral imaging performed before their first visit to the memory clinic [χ^2 (18) = 31.2; p = 0.027].

The mean follow-up was 3 (1.8) years. The course of the disease during the follow-up period was stable in 100 (48.1%) patients, progressive in 35 (16.8%), fluctuating in 30 (14.4%), stepwise in 20 (9.6%), and showed improvement in 23 (11.1%). Death occurred in 21.1% of patients, 2.2 (1.3)

Table 20.1. Main cognitive disorders

Cognitive disorders	n	%
Memory	192	92.3
Language	186	89.4
Temporal orientation	178	85.6
Praxis	176	84.6
Spatial orientation	168	80.8
Calculation	167	80.3
Gnosis	145	69.7

years after their first examination and 5.2 (3.6) years after onset. For those carers where a cause of death was reported (12 cases), the most common etiology was stroke (4 cases). Hospitalization occurred in 14.7% of patients, stroke in 6.8%, seizure in 6.2%, and delirium in 4%. MRI was performed in 68.5% and CT in 49.8% of the patients.

For the 208 patients, we possessed 187 MRI and 164 CT. MTLA was assessable on 50 (30.5%) CT and 126 (67.4%) MRI. For those cases where it was assessable, MTLA was found in 14 (28.0%) CT and 81 (64.3%) MRI. Mean global cerebral atrophy score on CT was 1.3 (0.8). It was absent in 26 cases (15.9%), mild in 74 (45.1%), moderate in 54 (32.9%) and severe in 10 (6.1%). Cerebral atrophy score on MRI was 18.2 (8.8). There was no significant difference in atrophy between regions (Table 20.2). On CT, global atrophy was significantly higher in patients with MTLA [$F(2, 161) = 7.46$; $p < 0.001$] than in patients without MTLA. On MRI, cerebral atrophy score was significantly higher in patients with MTLA than in patients without MTLA or who were non-assessable [$F(2, 184) = 21.421$; $p < 0.001$]. The mean Blennow score (Table 20.3) was 1.5 (1.1). Mean Fazekas periventricular score (Table 20.4) was 2.2 (0.85), and mean white matter score was 1.9 (0.96). Global atrophy on CT correlated positively to the score of Blennow [$r = 0.313$; $t(162) = 4.19$; $p < 0.001$] and the cerebral atrophy score on MRI correlated positively to the periventricular score [$r = 0.430$; $t(185) = 6.48$; $p < 0.001$] and the white matter score [$r = 0.376$; $t(185) = 5.52$; $p < 0.001$].

Table 20.2. Cerebral atrophy on MRI ($n = 187$)

Regional atrophy on MRI	Mean	Standard deviation	Range
Sulcal dilatation			
Frontal (right)	1.316	0.735	0–3
Frontal (left)	1.316	0.735	0–3
Parieto-occipital (right)	1.289	0.689	0–3
Parieto-occipital (left)	1.299	0.693	0–3
Temporal (right)	1.283	0.733	0–3
Temporal (left)	1.305	0.732	0–3
Ventricular dilatation			
Frontal (right)	1.481	0.757	0–3
Frontal (left)	1.476	0.764	0–3
Parieto-occipital (right)	1.476	0.764	0–3
Parieto-occipital (left)	1.497	0.750	0–3
Temporal (right)	1.503	0.758	0–3
Temporal (left)	1.481	0.785	0–3
Third ventricle	1.460	0.771	0–3
Cerebral atrophy score	18.182	8.836	0–39

Table 20.3. White matter changes on CT (scale of Blennow et al., 1991)

	n	%
Extent of white matter changes		
0 (No hypodensity in white matter)	44	26.8
1 (Hypodensity at the margins of the ventricular horns)	31	18.9
2 (Extension towards the centrum semiovale)	32	19.5
3 (Extension around the whole lateral ventricles)	57	34.8
Severity of white matter changes		
0 (No hypodensity)	44	26.8
1 (Mild hypodensity)	50	30.5
2 (Moderate)	33	20.1
3 (Severe)	37	22.6

Table 20.4. White matter changes on MRI (scale of Fazekas et al., 1987)

	n	%
Periventricular score		
0 (No hyperintensity on T2-weighted images)	2	1.1
1 (Caps or pencil-thin lining)	45	24.1
2 (Smooth halo)	47	25.1
3 (Hyperintensities extending into the deep white matter)	93	49.7
White matter score		
0 (Absence)	11	5.9
1 (Punctate foci)	61	32.6
2 (Beginning confluence of foci)	46	24.6
3 (Large confluent areas)	69	36.9

Focal lesions were lacunar infarcts alone in 45.9% of patients, lacunar and territorial infarcts in 14.8%, territorial infarcts alone in 10.8%, lacunar infarcts and hemorrhages in 3.1%. There were WMC only—no focal lesion—in 25.4% of patients. Most of the focal lesions were deep and involved the basal ganglia–capsule, the thalamus, and the cerebellum–brain stem (Table 20.5). Patients with lacunar and territorial infarcts had a significantly higher score of atrophy on MRI compared to patients without focal lesion [$F(4, 182) = 3.13$; $p = 0.016$]. Other comparisons were not significant. Patients with lacunar infarcts and hemorrhages had a significantly higher Blennow score than other groups [$F(4, 159) = 4.68$; $p = 0.001$]. Other comparisons were not significant.

Of these 208 VaD cases, 181 (87%) met the criteria for VaD according to the DSM-IV, 52 (25%) those according to the ICD-10, and 56 (27%) those according to the NINDS–AIREN. Twenty-seven (13%) had a vascular cognitive impairment not severe enough to meet DSM-IV criteria for

Table 20.5. Locations of cerebral lesions on CT and MRI

Site	n	%[a]	Right side	Bilateral	Left side
Frontal	17	4.8	7	5	5
Frontoparietal	2	0.6	2		
Insula–operculum	11	3.1	7	1	3
Frontotemporal	4	1.1	2		
Parietal	16	4.6	9	3	4
Occipital	29	8.3	10	3	16
Temporal	14	4.0	6		8
Parieto-occipital	15	4.3	6		9
Temporoparietal	15	5.1	12	1	5
Temporo-occipital	9	2.6	3	4	2
Thalamus	91	25.9	18	29	44
Basal ganglia–capsule	160	45.6	37	100	23
Cerebellum–brainstem	94	26.8			
Total			119	146	121

[a]Percentage of 351 scans (187 MRI, 164 CT).

dementia. They had memory impairment alone or other cognitive impairment alone or no significant impairment in occupational or social functioning.

During the period of the study, 39 patients followed up at the memory clinic came to autopsy. All the main clinical diagnoses were confirmed, but the contribution of vascular lesions was often clinically underestimated in cases of mixed dementia. In one patient, the course was progressive and the clinical diagnosis was VaD combined with AD. The histological examination concluded the presence of VaD without enough Alzheimer pathology to confirm the diagnosis of AD.

DISCUSSION

We investigated clinical and imaging characteristics in patients with VaD attending a memory clinic and measured them against the main international diagnostic criteria. VaD accounted for less than 18% of all dementias, whereas the Canadian Study of Health and Aging, a population-based study, found that VaD was responsible for 29% of all cases of dementia (Canadian Study of Health and Aging Working Group, 1994). Memory impairment was the most frequent symptom in our patients but there is a selection bias: the patients came to an identified 'memory clinic,' and memory loss is the prominent early symptom in the main AD and VaD diagnostic criteria (Almkvist, 1994). There were more men than women in this population of VaD, unlike what is observed in the overall population attending the memory clinic. This is in agreement with most studies (Meyer et al., 1988; Kase 1991;

Rocca et al., 1991; Gorelick et al., 1993). A progressive onset or course was often reported in our patients. A progressive course is not rare in VaD with small vessel disease (Pasquier, 1999). An association with AD is possible but needs confirmation with neuropathological analysis.

Imaging techniques are of major value in detecting a vascular origin of dementia: lack of focal lesions or WMC excludes a vascular origin (Roman et al., 1993; Leys and Bogousslavsky, 1994). MRI is more sensitive and is necessary in certain conditions such as cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) (Bowler and Hachinsky, 1994; Bousser and Lasserve, 1994; Tournier-Lasserve et al., 1993). The severity of dementia may prevent the cooperation of the patients needed to perform MRI, and some of them underwent only CT. MRI was more sensitive than CT in evaluating MTLA. In our series MTLA was associated with more severe global atrophy. Cerebral atrophy was observed in more than 80% of patients. It is known to be associated with a higher prevalence of poststroke dementia (Tatemichi et al., 1990; Liu et al., 1992). In this study cerebral atrophy correlated positively with WMC. This association is reported in poststroke dementia (Henon et al., 1996). We showed WMC in three-quarters of CT and in more than 90% of MRI scans. WMC on CT are associated with a higher prevalence of poststroke dementia (Liu et al., 1992). WMC, more frequent in VaD than in AD (Steingart et al., 1987), were even more frequent and severe in this study than previously reported (Leys et al., 1999). Lacunar infarcts in subcortical areas were the main focal lesions. A previous history of stroke (38.9%) was much less frequent than the focal lesions observed on imaging (74.8%), suggesting an important contribution of silent infarcts in the cognitive decline. Tatemichi et al. (1990) have already emphasized the role of silent infarcts in VaD.

Only one-quarter of the patients of this observational study fulfilled the NINDS–AIREN and the ICD-10 diagnostic criteria for VaD, while 85% of them fulfilled the DSM-IV criteria for VaD. The DSM-IV criteria are known to be the most sensitive (Wetterling et al., 1996) and are probably the most clinically relevant. We noticed that patients failed to fulfill the ICD-10 research criteria mainly because of the requirement for at least one focal sign out of a list of four. They failed to fulfill the NINDS–AIREN criteria because of the requirement for focal signs and severe cognitive decline including memory and two other domains. Thirteen percent of patients were considered as having possible VaD by the clinicians but their cognitive impairment was not severe enough to meet the DSM-IV criteria. Current VaD criteria exclude many patients with vascular cognitive impairment attending a memory clinic. Our data support several recent reports suggesting that the concept of VaD should be redefined (Amar et al., 1996; Verhey et al., 1996; Wetterling et al., 1996; Erkinjuntti, 1997; Gold et al., 1997; Rockwood et al., 1999).

Better characterization of patients with VaD attending a memory clinic is desirable to achieve homogeneity of the study population in pharmaceutical

trials. We think that the concept of VaD should be extended to include patients with vascular cognitive impairment.

SUMMARY

Vascular dementia (VaD) is a preventable dementia due to various cerebrovascular pathologies. From a prospectively constituted standardized database, we studied demographic, clinical, and imaging data in patients with possible or probable VaD first attending the Lille memory clinic between 1992 and 1997. We compared three sets of diagnostic criteria: DSM-IV, NINDS–AIREN, and ICD-10 research criteria. Cerebral atrophy, white matter changes (WMC), and focal lesions were rated on CT and MRI scans using reference scales. Of 306 patients with a clinical diagnosis of VaD, imaging was available for 208. Of these 208 VaD cases, 87% fulfilled the DSM-IV criteria, 25% the ICD-10 criteria, and 27% the NINDS–AIREN criteria; 13% had vascular cognitive impairment not severe enough to meet DSM-IV criteria of dementia. The male : female ratio was 1.24. At first visit, mean (standard deviation) age was 72 (7.5) years, duration of the disease was 3.7 (3.5) years, and Mini-Mental State Examination score was 22 (6). Clinical onset was progressive in 62% of patients, acute in 29%, and subacute in 9%. Course was progressive in 47%, fluctuating in 22%, stable in 16%, stepwise in 12%, and showed improvement in 3%. Previous history of stroke was reported in only 39%. Death occurred in 21% of patients during the follow-up period (mean three years). WMC were observed in 94% and focal lesions in 75%. WMC without focal lesions were observed in 25%. Lacunar infarcts were present in 64%, territorial infarcts in 26%, and hemorrhages in 3%. Better characterization of patients with VaD attending a memory clinic is desirable for assessing treatment efficacy. Traditional VaD criteria exclude patients with vascular cognitive impairment at the beginning. The concept of VaD should be extended to include patients with vascular cognitive impairment without dementia.

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21 MRI of Entorhinal Cortex and Hippocampus in Alzheimer's Disease, Subcortical Ischemic Vascular Dementia and Mixed Dementia

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INTRODUCTION

After Alzheimer's disease (AD), cerebrovascular pathology is the next most frequent cause of dementia, accounting for perhaps one-fifth as many cases as AD (Esiri and Morris, 1997). Furthermore, both types of pathology often occur together to varying extents, making it difficult to determine which type played the major part in causing dementia in a particular case. Therefore, there is much interest in improving the accuracy of a differential diagnosis between AD and vascular dementia.

Early *in vivo* magnetic resonance imaging (MRI) studies of AD reported hippocampal atrophy that could to a high degree distinguish AD patients from cognitively normal elderly subjects (Saab et al., 1988; Kesslak et al., 1991). However, additional research has shown that these measurements may not be as accurate at least for patients in mild stages of the disease (Jack et al., 1992; Lehericy et al., 1994). Furthermore, hippocampal atrophy is not specific to AD, limiting its usefulness for a differential diagnosis between AD and other types of dementia. MRI studies reported hippocampal atrophy in vascular dementia without concomitant AD pathology, as confirmed by autopsy (Chui et al., 1999) as well as in frontotemporal dementia (Frisoni et al., 1999) and Parkinson's disease (Laakso et al., 1996). In accordance with the theory that early AD pathology may start in the entorhinal cortex (ERC) before spreading to the hippocampus (Braak and Braak, 1995), several MRI studies also measured atrophy of ERC in AD (Bobinski et al., 1999;

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Juottonen et al., 1999) and in non-demented subjects at risk of AD ((Xu et al., 2000). Quantitative MRI studies of ERC changes in vascular dementia have not been reported. Therefore, the first aim of this study was to compare the volumes of ERC in patients with subcortical ischemic vascular dementia (SIVD) and mixed SIVD/AD with the volumes of ERC in AD patients and cognitively normal elderly subjects.

The diagnostic value of MRI-based volume measurements of ERC for AD is under debate. Bobinski et al. (1999) found that measurements of ERC atrophy in addition to hippocampus improved discrimination of AD from normal aging. In contrast, other studies found no advantage of ERC measurements (Juottonen et al., 1999; Xu et al., 2000). Comparing AD with frontotemporal dementia (FTD), another MRI study (Frisoni et al., 1999) found ERC atrophy in both AD and FTD, while hippocampal atrophy did not occur in FTD, implying that the relationship between ERC and hippocampal changes might aid the diagnostic process. In general, one would expect some information gain if ERC and hippocampal changes were dissociated and little improvement if the changes were strongly correlated. Therefore, the second aim of this study was to explore the relationship of ERC and hippocampal atrophy in AD, SIVD, and mixed SIVD/AD and, furthermore, to assess the value of using ERC and hippocampus findings together for differentiation between the groups.

METHOD

This study included 12 SIVD patients (7 men, 5 women, 76 ± 4 years of age), 17 patients with mixed SIVD/AD dementia (9 men, 8 women, 79 ± 7 years), 25 AD patients (12 men, 13 women, 77 ± 5 years), and 40 cognitively normal (CN) elderly controls (20 men, 20 women, age 75 ± 4 years). MRI results from this population have been reported previously in short publications (Chui et al., 1999). Diagnosis of SIVD and mixed SIVD/AD was established according to the criteria of Chui et al. (1992) and diagnosis of AD was established according to the NINCDS/ADRDA criteria (McKhann et al., 1984). The level of cognitive impairment, as measured by the Mini-Mental State Examination (MMSE) score (Folstein et al., 1975), was similar among the patient groups, with a mean MMSE score (standard deviation) of 20 ± 4 for SIVD, 21 ± 6 for SIVD/AD, and 20 ± 4 for AD. The Committee of Human Research at the University of California, San Francisco approved this study, and all subjects or their legal guardians gave written consent before participation in the study.

The MRI studies were performed on a 1.5-T MR scanner (Vision, Siemens Inc., Iselin, NJ) and consisted of sagittal T1-weighted images (3D MP-RAGE, TR/TI/TE = 10/250/4 ms, 15° flip angle) with $1.0 \times 1.0 \times 1.4\,mm^3$ resolution and axial proton density and T2-weighted MR images (TR/TE1/

TE2 = 3000/20/80 ms) with $1.0 \times 1.25 \times 3.0\,mm^3$ resolution. The volumes of ERC and hippocampus were determined manually by outlining the boundary of the structures on oblique coronal T1-weighted MR images. One rater (A.T.D.), who had no knowledge of the diagnosis and other clinical information, performed the volume measurements. Volumes of the ERC were measured following the editing protocol developed by Insausti et al. (1998), which starts one section caudal to the level of the limen insulae and ends one slice behind the posterior limit of the gyrus intralimbicus. Volumes of the hippocampus were measured following the guidelines by Watson et al. (1992), which include hippocampus proper, dentate gyrus, subiculum, fimbria, and alveus. Rater reliability was 2.9% for ERC and 1.8% for hippocampus. Finally, the volumes of ERC and hippocampus of each subject were normalized to the total intracranial volume.

Effects by group on ERC and hippocampal volumes were tested using analysis of variance (ANOVA) with adjustment for age and sex. Associations between ERC and hippocampal volume changes were tested using Pearson correlation coefficients. The power of ERC and hippocampal volumes to discriminate between the groups was tested using stepwise logistic regression.

RESULTS

Figure 21.1 shows representative oblique coronal images through the temporal lobe of a 73-year-old man with SIVD (left) and a 68-year-old man with AD (right), subvolumed to include ERC and hippocampus. The tracings indicate the boundaries of the entorhinal cortex (left) and hippocampus (right). Despite comparable levels of cognitive impairment (AD: MMSE = 21/30; SIVD: MMSE = 19/30), the patient with AD had a markedly smaller entorhinal cortex and a smaller hippocampus than the patient with SIVD. Mean volumes and standard deviations for ERC and hippocampus of each group are listed in Table 21.1. Differences between the groups are summarized in Table 21.2. This shows that AD patients had 25% ($p < 0.001$) smaller hippocampal volumes and 39% ($p < 0.001$) smaller ERC volumes than CN. The SIVD group had 18% ($p < 0.001$) smaller hippocampal volumes and 19% ($p < 0.05$) smaller ERC volumes than CN. Compared with AD, however, SIVD patients had 25% ($p < 0.001$) larger ERC volumes and no significantly different hippocampal volumes. Finally, patients with mixed SIVD/AD had 27% ($p < 0.001$) smaller hippocampal volumes and 34% ($p < 0.001$) smaller ERC volumes than CN, similar to AD. Compared to SIVD, mixed SIVD/AD had 11% ($p < 0.05$) smaller hippocampal volumes and no significantly different ERC volumes.

There was no significant correlation between the volumes of ERC and hippocampus in CN. In contrast, the volumes of ERC and hippocampus correlated weakly in SIVD ($r = +0.33$, $p < 0.05$), and strongly in both AD

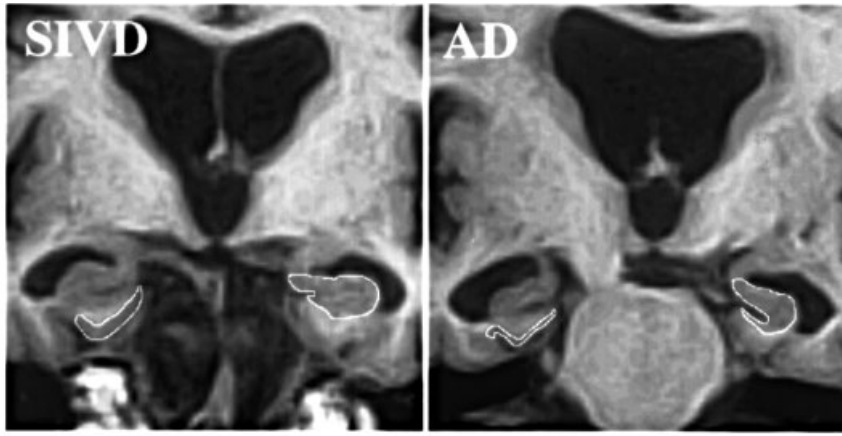

Figure 21.1. Oblique coronal T1-weighted MRI sections through the entorhinal cortex and hippocampus of a 73-year-old man with subcortical ischemic vascular dementia (left) and a 68-year-old man with Alzheimer's disease (right). The tracings indicate the boundaries of the entorhinal cortex (left) and hippocampus (right). Despite comparable levels of cognitive impairment, the AD patient has a markedly smaller entorhinal cortex and a smaller hippocampus than the SIVD patient

Table 21.1. Volumes (mm^3) of entorhinal cortex (ERC) and hippocampus (HP)

Group	ERC	HP
Normal	2720 ± 606	6314 ± 797
AD	1661 ± 456	4734 ± 972
SIVD	2211 ± 611	5188 ± 505
SIVD/AD	1796 ± 548	4602 ± 704

($r = +0.73$, $p < 0.001$) and mixed SIVD/AD ($r = +0.91$, $p < 0.01$). The correlation by group interaction was significant [$F(3,94) = 20$, $p < 0.04$].

Finally, Table 21.3 lists sensitivity, specificity, and overall classification of ERC and hippocampal volumes for discrimination between the groups, tested using stepwise logistic regression. This shows that using hippocampus alone helped to discriminate between AD and NC with an overall classification of 82% ($p < 0.01$, 68% sensitivity, 90% specificity), between SIVD and CN with an overall classification of 87% ($p < 0.01$, 67% sensitivity, 93% specificity), and also between mixed SIVD/AD and CN with overall classification of 72% ($p < 0.05$, 67% sensitivity, 76% specificity). In contrast, SIVD and AD could not be classified by hippocampus better than by chance. However, adding ERC helped to discriminate between SIVD and AD with an overall classification of 81% ($p < 0.05$, 96% sensitivity, 50% specificity). Finally, adding ERC improved further the discrimination between AD and CN with an overall classification of 85% ($p < 0.05$, 76% sensitivity, 90% specificity).

Table 21.2. Percentage differences in entorhinal cortex (ERC) and hippocampus (HP) volumes between the groups

		AD	SIVD	SIVD/AD
Normal	ERC	−39*	−19†	−34*
	HP	−25*	−18*	−27*
AD	ERC		+25*	+8
	HP		+9	−3
SIVD	ERC			−18
	HP			−11†

*$p < 0.001$; †$p < 0.05$.

Table 21.3. Discriminations between the groups using volumes of hippocampus (HP) and entorhinal cortex (ERC)

	HP alone			HP+ERC		
	Sensitivity	Specificity	Overall	Sensitivity	Specificity	Overall
Normal vs. AD	68	90	82*	76	90	85*
Normal vs. SIVD	67	93	87*	58	93	85
Normal vs. SIVD/AD	76	93	88*	76	95	89
SIVD vs. AD	88	8	62	96	50	81†
SIVD vs. SIVD/AD	67	76	72†	67	82	72

Values in percentage of number of subjects in the groups.
*$p < 0.01$; †$p < 0.05$.

DISCUSSION

A major finding of this study was that the volume of ERC was reduced in SIVD compared to CN. This implies that ERC atrophy is not a specific marker for AD. However, autopsy data is necessary to determine that these SIVD subjects had no AD pathology. Recently, ERC atrophy was also reported in frontotemporal dementia (Frisoni et al., 1999), where—unlike in AD—amyloid deposition does not occur.

Another finding was that ERC and hippocampal volume losses were of similar magnitude in SIVD, while the volume losses of ERC markedly exceeded those of hippocampus in both AD and mixed SIVD/AD. In addition, ERC and hippocampal volumes were moderately correlated in SIVD and much stronger correlated in AD and mixed SIVD, while healthy subjects showed no correlation. Greater atrophy of ERC than hippocampus in AD and mixed SIVD/AD is consistent with the distribution of AD pathology, which is thought to arise in the ERC before progressing to the hippocampus (Braak and Braak, 1995). Furthermore, as AD progression impacts ERC and hippocampus equally, one would expect a strong correlation between changes

in these two structures. In contrast, cerebrovascular pathology can impact ERC and hippocampus randomly, inducing changes that are not expected to correlate strongly. These differential patterns of ERC and hippocampal changes might potentially be helpful to differentiate between AD and vascular dementias.

Finally, this study showed that discriminations between SIVD and AD, as well as between AD and NC, were significantly improved after hippocampal volume was combined with ERC volume. This implies that ERC and hippocampus provide partially independent information about dementia that assists classification. However, 100% discrimination between the groups could not be achieved.

Our results imply that ERC atrophy is not a specific marker for AD, but volume measurements of ERC assist classification, particularly the discrimination between AD and vascular dementia.

SUMMARY

MRI shows hippocampal atrophy in both Alzheimer's disease (AD) and subcortical ischemic vascular dementia (SIVD), limiting the ability to differentiate between AD and SIVD of hippocampal measurements. MRI shows also atrophy of the entorhinal cortex (ERC) in AD, but little is known about ERC volume changes in SIVD and mixed SIVD/AD dementia. Therefore, the aims of this study were: (1) to measure ERC volumes in SIVD and mixed SIVD/AD, and (2) to test whether ERC measurements help to discriminate SIVD and mixed SIVD/AD from AD and cognitive normal elderly. Quantitative MRI volume studies were performed on 12 patients with SIVD, 17 patients with mixed SIVD/AD, 25 patients with AD, and 40 cognitively normal elderly subjects. Dementia severity was similar among the patients and all groups were comparable with respect to age and sex. Results showed that both SIVD and SIVD/AD patients had significantly smaller ERC volumes ($p < 0.001$) and significantly smaller hippocampal volumes ($p < 0.05$) than cognitive normal controls. Compared to AD, however, SIVD patients had markedly larger ERC volumes ($p < 0.001$) and slightly larger hippocampal volumes, while mixed SIVD/AD patients had ERC and hippocampal volumes of a similar size to those with AD. A combination of ERC and hippocampal measurements improved discrimination between SIVD and AD and between AD and controls. MRI measurements of ERC atrophy could be useful for a differential diagnosis between AD and vascular dementia.

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22 Olfactory Function and Event-Related Potentials in Alzheimer's Disease

CLAIRE MURPHY AND CHARLIE D. MORGAN

ALZHEIMER'S DISEASE

Alzheimer's disease (AD) is a progressive, debilitating, neurodegenerative disease from which more than 5 million elderly Americans suffer. The disease is characterized by deterioration of cognitive function, memory, personality, language, and the essence of the person. There is no cure, and efforts to blunt disease progression succeed in only a portion of patients and provide temporary slowing of some cognitive symptoms. The neural degeneration is without remission (Braak and Braak, 1997).

The diagnosis of AD is made only at autopsy or biopsy when the characteristic neuritic plaques and neurofibrillary tangles are identified in the brain. A diagnosis of 'probable AD' in patients who meet the NINCDS–ADRDA criteria is made during life, since the diagnosis is not definitive. The difficulty in identifying individuals who are developing the disease has stimulated appreciable research on the functional expression of the underlying neurodegeneration, with rapid progress made on characterizing genetic risk factors and understanding the mechanisms of degeneration. Development of pharmaceutical agents to prevent or retard the degenerative process is underway, and when successful will mandate a means of assessing functional impairment in those with incipient dementia. Because early detection will help ensure medical and social intervention for the patient and family, identification of preclinical markers and an early diagnosis of AD are critically important.

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Edited by K. Iqbal, S. S. Sisodia & B. Winblad.

EARLY NEUROPATHOLOGY IN ALZHEIMER'S DISEASE

The initial event in AD is the appearance of neurofibrillary tangles in the transentorhinal and entorhinal areas of the brain (Braak and Braak, 1994, 1997). Degeneration then proceeds to other temporal lobe structures, which include the hippocampus. Also affected relatively early in the disease is the frontal cortex, including the orbital frontal area. All of these areas are involved in processing of olfactory information. Price et al. (1991) also described tangles in these areas which mediate olfactory function, particularly the anterior olfactory nucleus, entorhinal cortex, and amygdala, even in AD patients with mild dementia. The most severe lesions are in the entorhinal cortex and perirhinal cortex, hippocampus, amygdala, and the association cortices. The prevalence of lesions in the entorhinal cortex, which forms the primary projection to the hippocampus, suggests the mechanism for the impairment in hippocampal function that gives rise to memory dysfunction. Braak and Braak (1992) have argued that the lesions in the entorhinal area and transentorhinal area disconnect the hippocampus from the isocortex and thus prevent the transfer of information critical to optimum memory function. This hypothesis is readily extended to olfactory areas. That is, early degeneration in the entorhinal and transentorhinal areas that causes dissociation of these areas from the hippocampus and orbital frontal cortex strongly suggests that olfactory tasks should reflect the disease process and be particularly sensitive indicators in the earliest stages of the disease. Hence, olfactory tasks with greater cognitive components should challenge the system most effectively and produce greater sensitivity for detection of AD. This model of brain pathology underlies the present strategy to use olfactory function to assess dementia in AD patients.

OLFACTORY FUNCTION IN ALZHEIMER'S DISEASE

Neuropathological changes in olfactory areas of the brain in patients with AD suggest the theoretical importance and potential diagnostic utility of investigating functional changes in olfaction in these patients. A series of investigations utilizing psychophysical, neuropsychological, and electrophysiological techniques have been conducted to assess olfaction function in both patients with AD and those at risk for the disease. Both Alzheimer's patients and those at risk for AD show significant impairment in olfactory threshold sensitivity, odor identification, and odor memory, with some measures showing more impairment than others in the early stages of the disease process (Murphy, 1999). Functional testing in AD revealed deficits in olfactory threshold (Murphy et al., 1990; Nordin and Murphy, 1996: Bacon

et al., 1998), odor identification (Morgan et al., 1995; Murphy et al., 1998), odor memory (Murphy et al., 1999; Nordin and Murphy, 1996; Niccoli-Waller et al., 1999), odor fluency (Bacon et al., 1999) and olfactory event-related potentials (Morgan and Murphy, in preparation).

ODOR THRESHOLD

Although early reports of deficits in odor identification had appeared in the literature, it was not clear that these deficits derived from degeneration in the olfactory system in AD patients since the anomia found in AD patients could well impact their performance in an odor-naming task. Therefore, odor threshold was assessed in patients who had received the diagnosis of probable AD from two different neurologists at the UCSD Alzheimer's Disease Research Center using the NINCDS–ADRDA criteria (McKhann et al., 1984) and in normal, age-matched controls (Murphy et al., 1990). In a second study we assessed the rhinological status of AD patients and controls to investigate the possibility of peripheral changes in the nasal epithelium. In a third study we examined the patients' awareness of smell loss (Nordin et al., 1995), and in a fourth study we assessed persons with the diagnosis of 'questionable AD' (Nordin and Murphy, 1996). The latter diagnosis was given to those who did not meet the criteria for AD, but who showed impairment in two or more areas of cognition as defined by the DSM-III-R (American Psychiatric Association, 1987) criteria for dementia that could not be explained by other medical or neurological factors. These areas included memory impairment, language disturbance (aphasia), inability to recognize or identify objects (agnosia), disturbance in executive functioning, and impaired ability to carry out motor activities (paraxia).

THRESHOLD PROCEDURES

Detection thresholds were obtained monorhinically by a standard, two-alternative (odorant and blank), forced-choice, ascending method of limits (see Murphy et al., 1994a). The subject was presented with two bottles, one containing the odorant and the other a blank consisting of deionized water. The spout of the bottle was inserted into the nostril of interest. The subject was asked to squeeze the bottle in order to generate a puff of air. The subject did this sequentially with both bottles. The task was to identify the bottle containing the strongest odor. Subjects began at the lowest dilutional step in order to avoid adaptation. Incorrect choices led to presentation of a higher concentration. Correct choices led to continued presentation of the same concentration to a criterion of five successive correct responses. The presentation of the odorant and blank were randomized for each trial. The nostril to be tested first was also randomly determined. There were

approximately 45 s between trials in order to allow enough time for recovery of the olfactory system and to allow enough time for odorant concentration to equilibrate in the head space of the bottle.

IMPAIRMENT IN ODOR THRESHOLD

Odor threshold was significantly impaired in patients with probable AD and the degree of dementia was associated with the degree of threshold impairment (Murphy et al., 1990). The latter finding may partially explain discrepancies among some of the early studies which assessed patients with different levels of dementia. The persons with questionable AD also showed a significant loss in threshold sensitivity (Nordin and Murphy, 1996), but the thresholds of these patients were not as impaired as those with moderate to severe AD. Of some clinical and practical significance, Alzheimer's patients and, indeed, normal elderly persons show very little awareness of their olfactory impairment (Nordin et al., 1995). Thus these patients are at some risk for safety issues related to loss of the ability to smell (e.g., smoke, leaking gas, spoiled food, etc.) as well as for loss of quality of life (e.g., enjoyment of food, awareness of personal odor, etc.). Of note, rhinological examination of patients with AD indicates that impairment in odor threshold sensitivity is most likely due to neurological rather than rhinological status. Finally, performance of AD patients on a control taste (assessment of taste threshold for sucrose) pointed to a sensory loss rather than a lack of comprehension of the detection task per se (Murphy et al., 1990; Nordin et al., 1995; Nordin and Murphy, 1996).

ODOR IDENTIFICATION

The initial reports of olfactory dysfunction in AD suggested impairment in odor identification, or, at least, of confrontational naming (anomia) of odors, typically aided by a written list of possible alternatives (Waldton, 1974; Serby et al., 1985; Doty et al., 1987; Koss, 1986; Knupfer and Spiegel, 1986; Morgan et al., 1995; Murphy et al., 1998). A number of these investigations employed the University of Pennsylvania Smell Identification Test (UPSIT; Doty et al., 1984), a 40-item, multiple choice, scratch-and-sniff odor identification test that relies on reading response alternatives. Thus, it was initially unclear whether performance relied heavily on the lexical demands of the task. In Morgan et al. (1995) we addressed this question in persons with 'questionable AD' or 'at risk' for AD, defined by deficits in two or more areas of cognition but without any impairment in activities of daily living or on clinical assessment, on two different odor identification tests, the UPSIT and the San Diego Odor Identification Test, the latter of which makes no lexical demands. In a later study (Murphy et al., 1998) we

examined a group of persons who had been assessed for dementia and given the diagnosis of normal control, some of whom were at risk for AD because of the apoE e_4 allele, on the shorter test, the San Diego Odor Identification Test.

UNIVERSITY OF PENNSYLVANIA SMELL IDENTIFICATION TEST (UPSIT)

The University of Pennsylvania Smell Identification Test (UPSIT; Doty et al., 1984) is a normed test that employs 40 microencapsulated odors with four written response alternatives for each odor (e.g., gasoline, pizza, peanuts, and lilac). The participant scratched the stimulus, smelled it, and then chose one of the four response alternatives corresponding to that stimulus. The number of correctly identified odors was used for analysis.

SAN DIEGO ODOR IDENTIFICATION TEST

The San Diego Odor Identification Test (Murphy et al., 1994) was used to assess odor identification. For this test, prior to beginning the task, participants are presented with a picture-board consisting of 20 line drawings. Eight drawings are of the target odor stimuli and 12 are distractor items. The participant is first tested on picture identification. Subsequently, each picture is reviewed with the participants until all pictures are correctly identified. Eight odors (mustard, cinnamon, coffee, bubble gum, Playdoh, baby powder, chocolate, and peanut butter) in opaque jars are presented sequentially for 5 s each and participants are instructed to point to the line drawing on the picture board representing that odorant. Odors are presented birhinically in random order. Presentations of odorants are spaced approximately 45 s apart to avoid adaptation. Increased power to detect a significant difference between AD and controls has been observed with this test when two odors (bubblegum, Playdoh) are removed from final analysis (Morgan et al., 1995). Thus identification performance for six odors was used in the present statistical analyses.

IMPAIRMENT IN ODOR IDENTIFICATION

Impairment was observed in the ability to identify odors on both the UPSIT and the non-lexical San Diego Odor Identification Test in both the patients with probable AD and in those at risk for the disease because of mild cognitive impairment (Morgan et al., 1995), suggesting that odor identification and not merely the lexical demands of the task reflected the dementia. Interestingly, odor identification deficits were already present in the persons at risk for AD because of genetic status for the apoE e_4 allele, in spite of their designation as normal controls by standard dementia testing (Murphy et al., 1998). This

early impairment is of clinical significance because of its potential to reflect incipient dementia in those at risk for the disease who may be candidates for pharmaceutical intervention.

EVENT-RELATED POTENTIALS

Early components of the evoked brain response are elicited by exogenous stimuli and are thus considered diagnostic of sensory function. The later (P3) component is considered a cognitive component elicited by endogenous events associated with the attentional allocation and stimulus evaluation engendered by either the expected occurrence or the omission of a stimulus event. It is most often elicited by the 'oddball' paradigm in which a stimulus sequence is presented with random occurrence of both a frequently or a rarely presented stimulus to which a subject either responds, attends or does not attend (Donchin and Coles, 1988). The omission of a stimulus in such a sequence has also been observed to elicit the P3 (Sutton et al., 1965; Polich et al., 1994). The cognitive nature of the P3 suggests its potential usefulness in research on aging and in AD.

The event-related potential (ERP) has been used in clinical diagnosis of the visual, auditory, and somatosensory modalities for some time (Chiappa, 1983). In the auditory system, the latency of the P3 has been reported to reflect the slowing of the brain with age (Goodin et al., 1978), and it has been suggested to have potential in the clinical diagnosis of dementia (Polich, 1991), although the diagnostic sensitivity has been variable, precipitating considerable debate regarding its clinical utility (Ball et al., 1989; Blackwood et al., 1987; Goodin and Aminoff, 1987a,b; Lai et al., 1983; Polich et al., 1990; Pfefferbaum et al., 1990; Polich, 1998). In a number of investigations considering both sensory and cognitive components of the olfactory ERP (OERP), we have observed both longer latencies and lower amplitudes in the elderly (Murphy et al., 1994b; Morgan et al., 1997, 1999; Murphy et al., 2000). The declines in amplitude of the OERP were larger for the later components than for the earlier components. Amplitude declines over the life-span were greater for males than for females, and this interaction between age and gender showed its influence in accelerated decline in amplitude in middle age in the males, whereas females exhibited a consistent, albeit lesser, decline in amplitude over the life-span. Latency increases were consistent over decades, suggesting the utility of these measures to characterize functional changes in the normally aging individual.

In Morgan and Murphy (in preparation) we address the following goals: (1) to examine OERPs in AD patients compared to age-matched normal controls, (2) to compare OERPs to auditory ERPs in AD patients relative to controls, (3) to compare OERPs to traditional tests of olfactory function using an odor threshold test and an odor identification test, and (4) to

compare OERP results to performance on neuropsychological measures of dementia. It is hypothesized that AD patients will show increased OERP latencies. In addition, OERP measures are expected to correlate significantly with traditional measures of olfactory function, memory, and measures of dementia severity. Participants were 12 persons diagnosed with probable AD using the NINCDS–ADRDA criteria (McKhann et al., 1984) and the DSM-III-R criteria (American Psychiatric Association, 1987), applied by two senior staff neurologists from the ADRC at UCSD and 12 age/gender-matched controls. Average Dementia Rating Score (DRS) for the AD patients was 119.

PSYCHOPHYSICAL MEASURES

Detection thresholds were obtained monorhinically by a standard, two-alternative (odorant and blank), forced-choice, ascending method of limits (see above and Murphy et al., 1994a). Odor identification ability was assessed with both the San Diego Odor Identification Test (Murphy et al., 1994) and the University of Pennsylvania Smell Identification Test (UPSIT, Doty et al., 1984). See above for details of these testing procedures.

OERP RECORDING PROCEDURE

Methodology similar to that used by Kobal (see review, Kobal and Hummel, 1991, and Murphy et al. 1994) has been adopted to record OERPs. Stimulation is accomplished with an olfactometer similar to that described in Murphy et al. (1994). Timing and concentration of the odor stimulus are fully controlled by the experimenter and a computer interface triggers the ERP-recording computer, providing a signal that the stimulus has been presented. Our previous work as well as that of others has demonstrated most robust OERPs from amyl acetate, hence that was the stimulus employed in this study (Kobal and Hummel, 1991; Evans and Starr, 1993; Murphy et al., 1994; Morgan et al., 1997; Murphy et al., 2000). Breathing air supplies the olfactometer with a constant flow, adjustable via flowmeters. The air is humidified to 80% relative humidity, by leading the airstream through a gas-washing bottle, filled with deionized water maintained at a constant temperature. The airduct which leads to the subject's nostril is surrounded by resistance tape which ensures that the air is heated to body temperature (36.5°C) when it reaches the output piece of Teflon tubing, which is placed just inside a single nostril during stimulation. For each stimulus, a solenoid valve (acoustically isolated) opened for 200 ms, during which a small portion of the air flow is replaced by an equally large odor flow, thus keeping the flow rate through the nostril constant at 7.0 l/min both during and between periods of stimulation. This procedure ensures that participants respond to odor cues and not to pneumatic or auditory cues. Opening the air-flow valve initiates

OERP recordings with a signal. Rise time of the stimulus does not exceed 20 ms. Velopharyngeal closure was employed to restrict breathing to the mouth and thereby maintain the odorant flow rate constant (Kobal and Hummel, 1988; Thesen and Murphy, in press). Stimuli were applied randomly during breathing.

EEG activity was recorded with active polarizing gold-plated electrodes from three midline positions of the international 10/20 system, Fz, Cz, and Pz, referenced monopolarly to linked electrodes at the A1 and A2 sites, grounded to the forehead. Impedances were typically below 5 kΩ, never exceeding 10 kΩ. EEG activity was recorded for 2000 ms (500 ms prestimulus and 1500 ms poststimulus), amplified 20 000 times (Astro-Med Grass Instrument Company, Model 12 Neuro-Data Acquisition System) and filtered (bandpass filter: 0.1–30 Hz, 6 dB/octave). EEGs were digitized at 1000 Hz and recorded. Electro-ocular (EOG) activity was monitored at the outer canthus of the left eye and supraocularly. Trials were screened for artifactual responses by rejecting any trial on which the activity exceeded 50 μV. Artifacts prompted repeat measurements. Averages were baseline-corrected such that the 500-ms prestimulus baseline average equalled zero and amplitudes were computed from this baseline.

Auditory ERPs were elicited for comparison with OERPs using binaural 500 Hz tones (10 ms rise/fall and 60 ms plateau) presented via headphones at 65 dB SPL. Both olfactory and auditory procedures employed a single-stimulus paradigm wherein only one stimulus was presented multiple times as described and implemented in previous OERP studies (Morgan et al., 1997, 1999; Murphy et al., 2000). A total of 20 artifact-free trials were collected in each modality during separate recording sessions. Participants responded to the olfactory and auditory stimuli via a button press.

IMPAIRMENT IN ODOR IDENTIFICATION

The Alzheimer's patients showed significantly poorer performance than normal controls on both odor identification tests, the UPSIT and the San Diego Odor Identification Test. In addition, those who performed poorly on the odor identification tests also showed longer latencies for the later components of the OERPs, suggesting a relationship between poor odor identification performance and slowing of olfactory processing. Alzheimer's patients showed generally poorer performance in the olfactory threshold task than normal controls, but the difference in the sample size was not statistically significant (Morgan and Murphy, in preparation).

IMPAIRMENT REFLECTED IN OERPs

All waveforms were baseline-corrected and analysis was performed at the Fz, Cz, and Pz electrode sites. N1, P2, and P3 amplitudes were measured in

microvolts as baseline to peak, and latencies were measured in milliseconds as the time from which the stimulus was delivered until maximum peak for each component. OERP latency windows for the N1, P2, N2, and P3 components were 300–500, 450–700, 600–900, and 750–1100 ms (Morgan et al., 1997, 1999; Murphy et al., 1994b, 2000). Auditory latency windows for the N1, P2, N2, and P3 components were 75–150, 150–260, 190–360, and 300–600 ms, as used in previous studies (Polich et al., 1990).

Figure 22.1 illustrates the major findings from Morgan and Murphy (in preparation). AD patients evinced much longer P3 peak latencies than normal controls in response to olfactory stimulation ($F = 34.1$, $p50.001$; Figure 22.1, left). The magnitude of this effect is quite remarkable and approximately five to eight times the magnitude of the differences observed in auditory or visual ERP studies of Alzheimer's patients (e.g., Polich et al., 1990). The effect size (η^2) for the latency difference between the AD patients and the controls for the P3 component at Cz in response to olfactory stimulation was 0.63 (200 ms); whereas in response to auditory stimulation (control task) it was 0.27 (50 ms). The sensitivity of a test indicates the power to classify a patient as a patient and the specificity indicates the ability to classify a normal control as a normal control. The correct classification rate (CCR) reflects both properties of the test. Sensitivity and specificity of the P2 and P3 latency measures in response to odor stimulation were substantially higher than for the auditory measures. Higher CCRs (92% and 88%) were observed for olfactory P2 and P3 than for

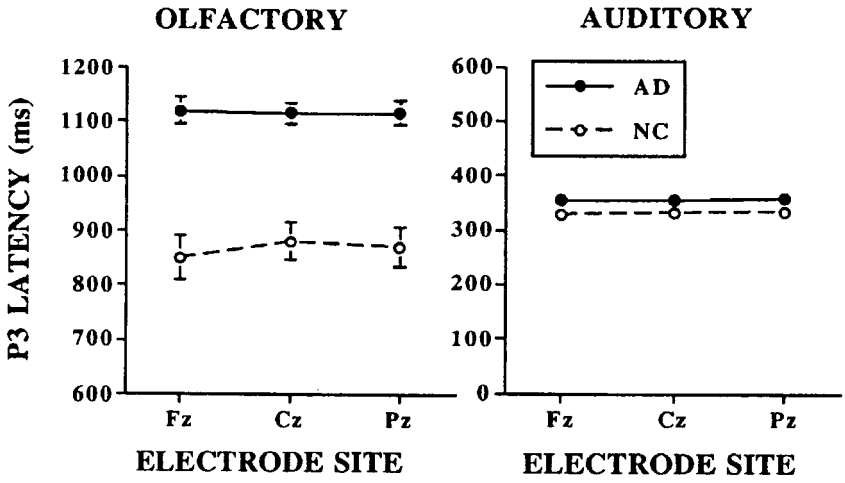

Figure 22.1. Mean latency for the P3 component of the event-related potentials at Fz, Cz, and Pz for Alzheimer's patients and normal controls. The response to olfactory stimulation is illustrated on the left; the response to auditory stimulation on the right. Data from Alzheimer's patients are plotted with a black symbol, data from normal controls with a white symbol

Table 22.1. Correlations (correct classification rates, CCR) between the P3 component of the OERP at Cz and the percent correct odor identification scores for Alzheimer's patients and normal controls

	CCR (%)
Olfactory P3 latency with UPSIT and SD Odor ID	100
UPSIT	92
San Diego Odor ID	88
Olfactory P2 latency	92
Olfactory P3 latency	88
Auditory P2 latency	75
Auditory P3 latency	71

auditory stimulation (CCR of 75% and 71%; Table 22.1). Although AD patients have shown increased latency of P3 to auditory stimuli, the clinical utility has been a subject of debate (Pfefferbaum et al., 1990; Goodin, 1990). The magnitude of the latency increase in AD for the P3 in response to odorants is substantially larger than for auditory stimuli and suggests the potential for OERPs to aid in diagnosis of AD.

Stepwise logistic regression with backward elimination revealed that when all significant olfactory measures (UPSIT, San Diego Odor Identification, auditory P2 and P3 latency, olfactory P2 and P3 latency) were entered into the regression, a correct participant group classification rate of 100% was obtained. In addition, a logistic regression was performed on each measure independently to better understand the unique contribution of each test in predicting group membership. Results indicated overall CCRs of 92% for both olfactory P2 latency and the UPSIT, CCRs of 88% for both the San Diego Odor Identification Test and olfactory P3 latency, and CCRs of 75% and 71%, respectively, for the auditory P2 and P3 latencies. Thus, the olfactory measures, including odor identification tests and OERPs, better differentiate AD patients and normal controls than do auditory ERPs, and they do so with relatively high sensitivity and specificity.

The high correlations between odor identification performance and the latency of the cognitive components of the OERP suggest that the two measures tap the same underlying neuropathology. Simple, easily administered odor identification tests, particularly the rapidly administered San Diego Odor Identification Test, are strong candidates for the Alzheimer's diagnostic battery.

SUMMARY

(1) Alzheimer's patients had significantly longer latencies for olfactory P2 and P3 relative to normal controls; (2) OERP was significantly correlated with

dementia status (DRS), indicating that the participants who had longer latencies also performed more poorly on the DRS, indicating greater dementia; (3) the latency differences between AD patients and normal controls were remarkably larger for OERPs (approximately 200 ms) than for auditory ERPs (approximately 50 ms); (4) OERP measures alone correctly classified up to 92% of patients and controls; (5) odor identification measures (UPSIT and San Diego Odor Identification Test) also classified patients and controls at a high rate, confirming previous work. A correct classification rate of 100% was obtained when odor identification scores were combined with olfactory P3 latency measures. The results strongly support the use of olfactory measures in the assessment of AD.

CONCLUSION

A series of studies investigating olfactory dysfunction in AD indicates significant impairment in olfactory tasks: odor threshold, odor identification, odor memory, odor fluency, and odor-evoked brain responses. Among these, one of the tasks affected earliest in the disease is the odor identification task. A simple, rapidly administered odor identification task such as the San Diego Odor Identification Test reflects impairment even in the early stages of the disease. Electrophysiological assessment with event-related potentials correlates well with the results of this test and in combination with it provides very good sensitivity and specificity for AD. The concordance between psychophysical assessment and electrophysiological assessment implies that the two measure the same underlying substrate. The early neuropathology in areas of the brain affected in AD in concert with these results suggests that assessment of olfactory function may aid in the diagnosis of Alzheimer's disease.

SUMMARY

The initial event in Alzheimer's disease (AD) is the appearance of plaques and tangles in entorhinal and transentorhinal cortex, which suggests the potential for olfactory functional impairment to serve as a preclinical marker that can be useful in assessment of early AD. A series of studies investigating olfactory dysfunction in AD indicates significant impairment in olfactory tasks: odor threshold, odor identification, odor memory, odor fluency, and olfactory event-related potentials. Among these, one of the tasks affected earliest in the disease is the odor identification task. A simple, rapidly administered odor identification task such as the San Diego Odor Identification Test reflects impairment even in the early stages of the disease. Electrophysiological assessment with event-related potentials correlates well with the results of this

test and in combination with it provides very good sensitivity and specificity for AD. The concordance of impairment in olfaction from both neuropsychological and objective, psychophysiological measures implies that both reflect the same underlying substrate and supports the hypothesis that the olfactory system is particularly vulnerable to the disease and can thus serve as a useful modality for diagnostic assessment.

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23 Phenotypic Differences in Cholinergic Markers Within the Nucleus Basalis in Individuals with Mild Cognitive Impairment

ELLIOTT J. MUFSON, MICHELE GILMOR, SHUANG Y. MA, ALAN I. LEVEY AND JEFFREY H. KORDOWER

INTRODUCTION

A major tenet in Alzheimer's disease (AD) research has been that cholinergic neurons within the nucleus basalis degenerate, resulting in a cortical cholinergic deficit (Davis and Maloney, 1976; Whitehouse et al., 1981; Wilcock et al., 1982; Arendt et al., 1985; Mufson et al., 1989). Cholinergic basal forebrain (CBF) neurons provide the primary source of cholinergic innervation to the entire cortical mantle (Mesulam et al., 1983), and the loss of choline acetyltransferase (ChAT) activity is consistently correlated with cognitive decline (Bierer et al., 1995). CBF neurons contain several phenotypic markers other than ChAT, such as the vesicular acetylcholine transporter (VAChT), which is responsible for the accumulation of acetylcholine in synaptic vesicles in cholinergic terminals (see Gilmor et al., 1999). CBF neurons also express the low-affinity p75 neurotrophin receptor as well as the high-affinity tyrosine kinase receptor (trkA) which is specific for nerve growth factor signal transduction (see Mufson and Kordower, 1999). Studies of these markers in late-stage AD indicate that cortical VAChT remains stable (Kish et al., 1990; Kuhl et al., 1996), whereas ChAT- and trkA-containing neurons decline (Whitehouse et al., 1981; Boissiere et al., 1997; Mufson et al., 1996, 1997). Whether similar changes are seen in individuals with mild cognitive impairment and early AD is unclear. In an effort to clarify the degree of neuronal degeneration and phenotypic changes within the CBF in the early stages of cognitive decline, we compared the total number of neurons containing ChAT, VAChT, and trkA within the nucleus basalis of individuals clinically classified as displaying no cognitive

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impairment, mild cognitive impairment without dementia, or early AD. These cases are part of a longitudinal clinical pathological study of aging and AD of retired Catholic clergy at Rush Presbyterian Medical Center in Chicago (see Gilmor et al., 1999; Mufson et al., 1999, 2000, for further discussion of this cohort). Each participant agreed to an annual detailed clinical evaluation and brain donation at the time of death and neuropathological evaluation. The study was approved by the Human Investigation Committee of Rush Presbyterian St Luke's Medical Center. All subjects were randomly chosen from each diagnostic category by the Rush Clinical Core and not accessioned sequentially.

CHOLINERGIC (Ch4) CELL PRESERVATION IN MILD COGNITIVE IMPAIRMENT AND EARLY AD

The groups for this analysis consisted of 33 subjects meeting the criteria for no cognitive impairment (NCI; $n = 11$), mild cognitive impairment (MCI; $n = 11$), or mild AD ($n = 11$). No individuals had a co-existing condition judged to be contributing to cognitive performance. In the first experiment, unbiased stereological counting methods were used to estimate the number of ChAT- and VAChT-immunoreactive (-ir) neurons within the nucleus basalis of individuals from these three groups. Of the 33 cases, complete series of sections were available from 22 cases (6 NCI, 7 MCI, and 9 AD), making them suitable for stereological evaluation. Intensely stained ChAT-ir and VAChT-ir neurons were seen within the nucleus basalis neurons in all three groups. Individuals without cognitive decline (NCI) displayed on average $210\,540 \pm 15\,240$ ChAT-ir and $174\,000 \pm 12\,773$ VAChT-ir nucleus basalis neurons. Individuals with MCI displayed $167\,879 \pm 17\,903$ ChAT-ir and $192\,637 \pm 34\,737$ VAChT-ir nucleus basalis neurons, a change that was not statistically different from MCI cases. Individuals with mild AD also displayed numbers of ChAT-ir ($155\,585 \pm 17\,949$) and VAChT-ir ($149\,423 \pm 17\,615$) nucleus basalis neurons that were similar to those seen in NCI and MCI individuals (see Table 23.1; Figure 23.1). Thus the viability of CBF neurons displaying a ChAT or VAChT phenotype is preserved in people with mild cognitive decline and the early manifestations of dementia.

These data were subsequently reanalyzed using pathological criteria to distinguish AD and non-AD cases. As there has been some controversy regarding the best pathological definition of AD, especially as it relates to segregating AD from normal age-related changes (Mirra et al., 1993), two standard pathological criteria were applied to the 22 cases used for stereological analysis. There was no significant difference in the number of ChAT-ir or VAChT-ir neurons (both $p > 0.05$) when grouped by CERAD (normal, possible or probable AD, definite AD) or Khachaturian (AD, non-AD) criteria (all comparisons, $p > 0.05$).

Table 23.1. ChAT- and VAChT-immunopositive neurons within the nucleus basalis

Cases	ChAT (mean ± standard deviation)	VAChT (mean ± standard deviation)
No cognitive impairment	210 540 ± 15 240 (n = 6)	174 000 ± 12 773 (n = 6)
Mild cognitive impairment	167 879 ± 17 903 (n = 7)	192 637 ± 34 737 (n = 5)
Alzheimer's disease	155 585 ± 17 949 (n = 9)	149 423 ± 17 615 (n = 9)

ChAT, choline acetyltransferase-immunopositive perikarya; VAChT, vesicular acetylcholine transporter-immunopositive perikarya.

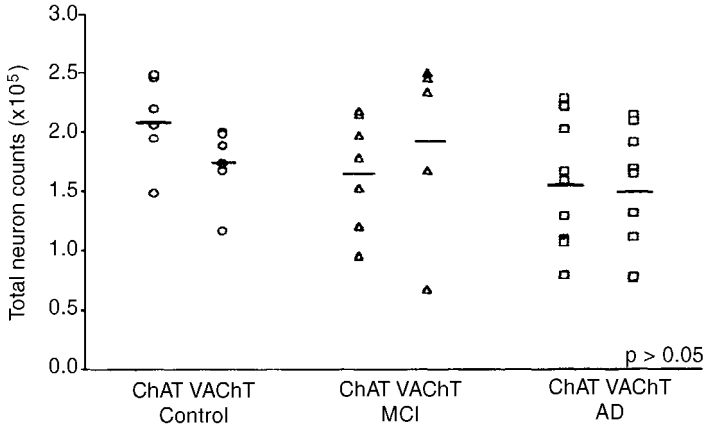

Figure 23.1. Summary of ChAT- and VAChT-immunoreactive (-ir) total neuron counts. ○, NCI cases; △, MCI cases; □, AD cases. Total neuron counts were estimated in the same NCI, MCI, and AD cases for ChAT and VAChT. There was no significant difference between the number of ChAT-ir and VAChT-ir neurons for any of the three clinically diagnosed groups. Bars represent the mean. (Reproduced from Gilmor et al., 1999, by permission of Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc.)

CORRELATION BETWEEN ChAT- AND VAChT-IMMUNOREACTIVE PERIKARYA WITH OTHER VARIABLES

The total number of ChAT-ir and VAChT-ir perikarya was compared to several demographic variables (Gilmor et al., 1999). Each variable was correlated with neuron counts by combining data from all cases as well as by analysis within each clinically designated group. Combining all clinically diagnosed groups, a significant correlation between the score on the Mini-Mental State Examination (MMSE), a brief cognitive test rating dementia

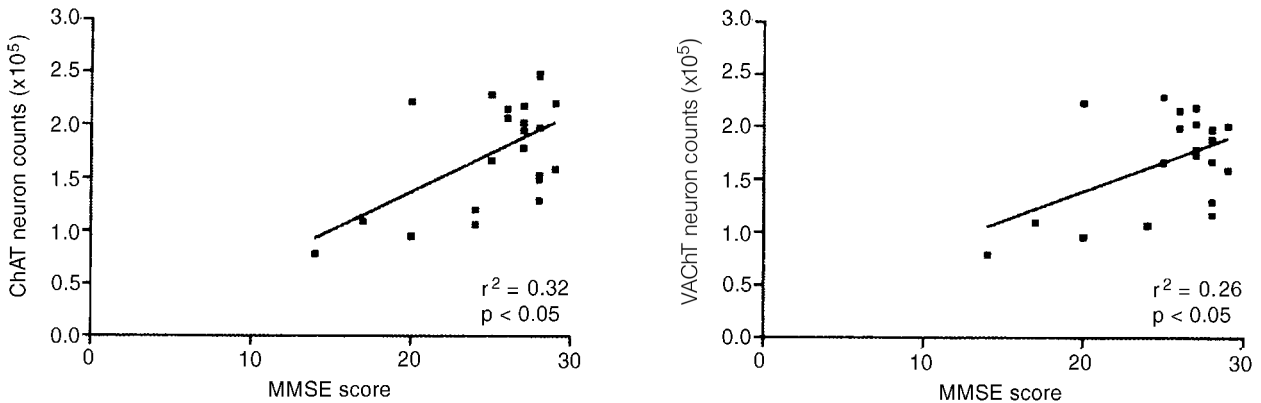

Figure 23.2. Correlation of ChAT-ir and VAChT-ir total neuron counts with each other and MMSE scores. (Reproduced from Gilmor et al., 1999, by permission of Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc.)

severity (Folstein et al., 1975), and the number of ChAT-ir ($r = 0.56$, $p < 0.01$) and VAChT-ir ($r = 0.6$, $p < 0.01$) neurons was observed (Figure 23.2). When examining each diagnostic category, we found that there was a significant correlation between the MMSE score and the number of ChAT-ir ($r = 0.76$, $p < 0.05$) and VAChT-ir ($r = 0.90$, $p < 0.05$) neurons for the MCI group, but not the NCI or AD cases (Figure 23.2). In the present study, there was not a significant association between possession of an apoE 4 allele and the number of ChAT-ir or VAChT-ir neurons, whether the three clinically diagnosed groups were combined or evaluated separately.

CONCLUSIONS

These data, taken together with recent reports indicating that there are no alterations in cortical ChAT activity in early cognitive decline, support the emerging concept that CBF neurons are relatively preserved in individuals with MCI and mild AD. Previous studies citing cholinergic deficits and CBF degeneration (e.g. Davies and Maloney, 1976; Whitehouse et al., 1981; Mufson et al., 1989) were probably examining end-stage cases. The preservation of CBF neurons early in the disease process has important therapeutic implications as the presence of these neurons provides an opportunity for interventions aimed at enhancing cholinergic function.

REDUCTION OF NUCLEUS BASALIS PERIKARYA CONTAINING trkA IMMUNOREACTIVITY IN INDIVIDUALS WITH MILD COGNITIVE IMPAIRMENT

CBF neurons synthesize the mRNA and protein for the trkA receptor (Mufson et al., 1996, 1997). This receptor transduces the signal for the trophic effects of NGF (see Mufson and Kordower, 1999, for review). Unbiased stereological counting procedures determined whether the loss of trkA neurons seen in end-stage AD also extends to individuals with MCI without dementia from our cohort of people enrolled in the Religious Orders Study (Mufson et al., 2000). Thirty people (average age of 84.7 years) came to autopsy. All individuals had been cognitively tested within 12 months of death (average MMSE score 24.2). Clinically, 9 had NCI, 12 subjects were categorized with MCI, and 9 had probable AD. The average number of trkA-ir neurons in persons with NCI was $196\,632 \pm 12\,093$. This was significantly more than in individuals with MCI ($106\,110 \pm 14\,565$) and mild AD ($86\,978 \pm 12\,141$) (see Figures 23.3 and 23.4A) [$F\ (2,27) = 17.38$; $p < 0.001$]. Post-hoc comparisons showed that individuals with MCI or AD had significant reductions in the number of trkA-ir cells as compared to those with NCI (46% reduction for MCI, 56% for AD; see Figure 23.5). In contrast, the number of trkA-ir neurons did not differ between the MCI and

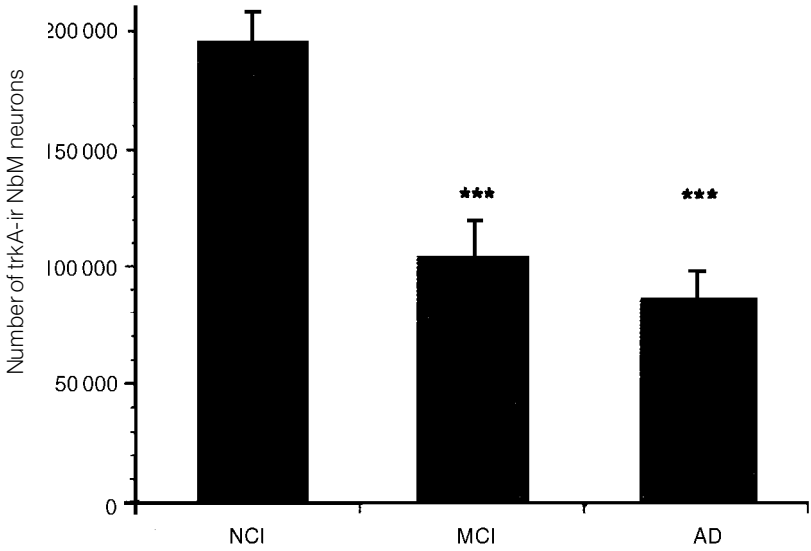

Figure 23.3. Significant reduction in trkA-ir nucleus basalis neurons between the NCI group and the MCI and AD groups, but not between MCI and AD subjects. The standard errors are indicated above each diagnostic group. ***$p < 0.001$. (Reproduced from Mufson et al., 2000, by permission of Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc.)

AD groups ($p > 0.05$). The density of the trkA-ir neurons also differed significantly across the three diagnostic groups in a pattern similar to that seen with the trkA-ir counts [$F\ (2,27) = 15.9$, $p < 0.001$] (Figure 23.4A). The mean density for those clinically classified as having NCI was 1425.1 ($SD = 415.9$), significantly higher than for individuals with either MCI (mean = 723.1, $SD = 328.7$) or AD (mean = 627.3, $SD = 228.3$; Figure 23.4B). No significant differences were found among the mean volumes by ANOVA (Figure 23.4C).

RELATION OF NUMBER OF trkA-ir NEURONS TO COGNITIVE FUNCTION

There are many pathological changes that occur in the MCI and AD brain. Thus, it is critical to determine whether a particular change is spurious or is associated with the cognitive decline. We analyzed the relation between the number of trkA-ir neurons and a global measure of cognitive function based on all tests administered (see Mufson et al., 2000). A global cognitive test score has the advantage of reducing random variability and minimizing floor and ceiling effects. This global score significantly correlated with the number of trkA-ir neurons (Spearman rank correlation $\rho = 0.38$, $p = 0.048$; Figure 23.6A). Since the nucleus basalis has projections to the frontal and temporal

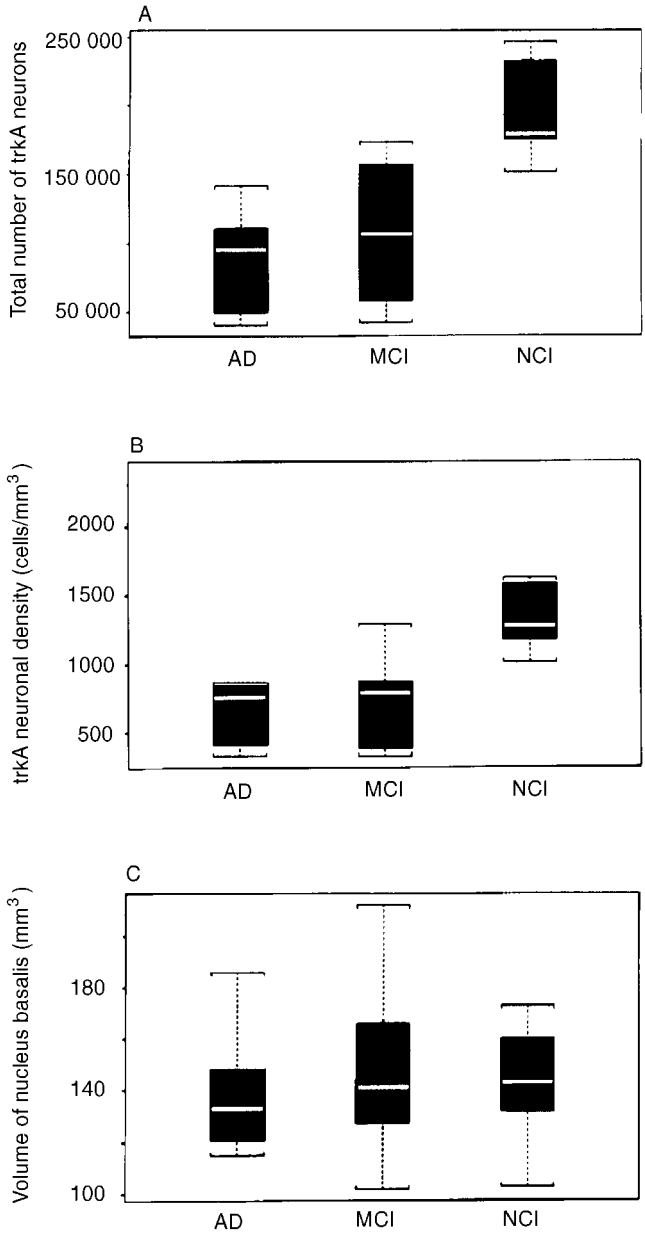

Figure 23.4. Differences in trkA-ir total number of neurons (**A**) and neuronal density (**B**) and volume (**C**) between the clinical diagnostic groups. The white line within the black boxes indicates the mean for each group

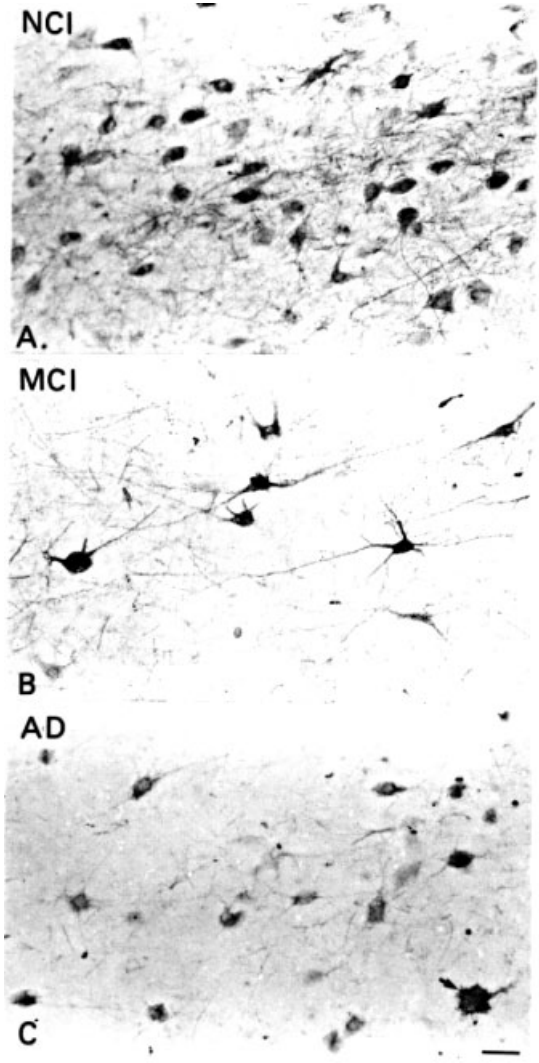

Figure 23.5. Photomicrographs showing the differences in trkA-ir in individuals with **A** NCI, **B** MCI, and **C** AD. Note the striking decrease in trkA-ir in **B** and **C** compared to **A**, but not between **B** and **C**. The arrow in **C** indicates an atrophic neuron. **A**–**C** Scale bar = 50 μm. (Reproduced from Mufson et al., 2000, by permission of Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc.)

cortices, we then examined the relationship of the trkA-ir neuron number with two tests of language and two tests of attention. Higher scores on the Boston naming test, a measure of language function, were associated with higher numbers of trkA-ir neurons ($\rho = 0.42$, $p < 0.05$; Figure 23.6B). In contrast, correlations between trkA-ir neuronal number and

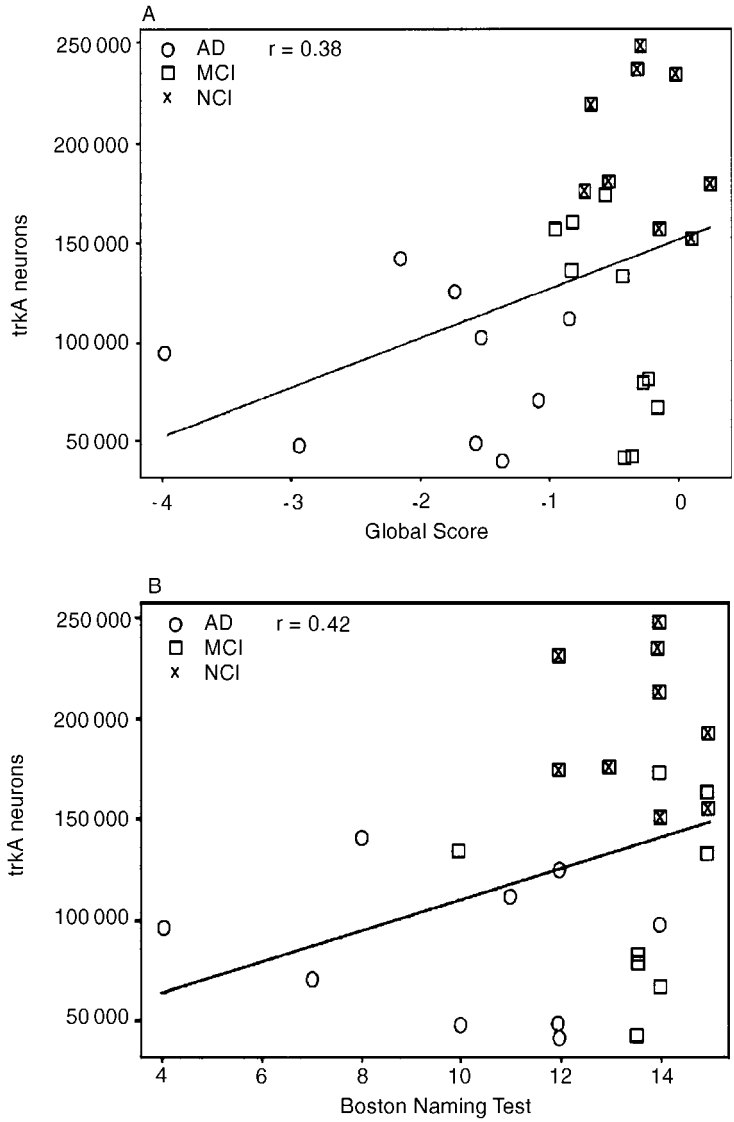

Figure 23.6. Relation of trkA-ir neurons to global score (**A**) and Boston naming test (**B**) for all subjects examined. (Reproduced from Mufson et al., 2000, by permission of Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc.)

Category fluency ($\rho = 0.27$, $p = 0.16$) the Symbol digit test ($\rho = 0.11$, $p = 0.60$), the Digits backward test (-0.03, 0.85), or the MMSE ($\rho = 0.029$, $p = 0.12$) all failed to correlate with the number of trkA-ir neurons.

CONCLUSIONS

The present investigation reveals a significant decrease in neurons containing immunoreactivity for the high-affinity trkA receptor within the nucleus basalis in subjects with MCI. The magnitude of this change is not greater in individuals with mild AD. These changes are associated with deficits on specific tasks of language and memory. Taken together, these data indicate that alterations in the number of trkA-ir neurons occur early in the disease process, even prior to the development of clinically defined dementia. It is interesting that the loss of trkA-ir neurons seen in individuals with MCI is no worse in individuals with mild AD. This indicates that pathological events that underlie the progression of cognitive decline from a non-demented state to dementia may not involve progressive changes in trkA expression.

SUMMARY

Using stereological neuronal counting procedures we have demonstrated a preservation of ChAT- and VAChT-immunoreactive neurons versus a loss of trkA immunoreactive neurons within the nucleus basalis in individuals with MCI or mild AD. The absence of a significant loss of perikarya containing ChAT in these mildly impaired individuals is supported by the fact that ChAT levels within the cerebral cortex, the target regions of nucleus basalis perikarya, are within normal limits in these cases (DeKosky et al., 1998; see also Davis et al., 1999). Since ChAT and trkA extensively co-localize within nucleus basalis neurons in rodents and primates (Sobreviela et al., 1994; Kordower et al., 1994), the preservation of ChAT, but reduction of trkA expression, indicates that there is not a significant decrease of cells per se in MCI and mild AD. Instead, these findings suggest a phenotypic down-regulation of trkA immunostaining in existing CBF cells. The present data suggest that the downregulation of trkA expression occurs early in the disease process when symptoms of MCI without dementia first appear. The relevance of changes in trkA immunoreactivity to the ultimate degeneration of CBF neurons in end-stage disease remains to be established. Additional studies are needed to define the exact mechanisms responsible for the alterations in the expression of trkA receptors within neurons of the nucleus basalis and whether all areas of the cortex also display a decrease in this protein in individuals with MCI and early AD.

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24 Evaluating CNS Biomarkers for Alzheimer's Disease

JOHN H. GROWDON

There are more than 50 medical, neurological, and psychiatric diseases considered in the differential diagnosis of dementia (Mayeux et al., 1993). Alzheimer's disease (AD) is by far the most common cause of dementia; DSM-IV and ICD-10 criteria follow closely on the NINCDS–ADRDA guidelines first published in 1984 (McKhann et al., 1984). Because of these common standards, diagnostic procedures for AD are remarkably uniform throughout the world. In a survey I conducted of 26 centers specialized for AD care in the US, Europe, and Japan, 6 items out of a menu of 16 procedures were specified as essential steps in the diagnosis of AD. These procedures were: history of illness, generally obtained from a spouse, caregiver, or other knowledgeable informant; physical examination, including a neurological examination and psychiatric assessment as indicated; laboratory blood tests to exclude underlying medical or metabolic conditions that can masquerade as dementia; a mental status test; psychometric testing; and a CT or MR anatomic brain scan. There was high (greater than 50%) frequency of use and importance ascribed to results obtained from these diagnostic measures regardless of the geographic region. Thus, there is worldwide consensus on the core diagnostic evaluation of AD-type dementia.

In centers that specialize in the diagnosis and care of patients with dementia and AD, these diagnostic procedures lead to a clinical diagnosis of AD that is confirmed pathologically in about 90% of cases. Although no data exist, it is suspected that diagnostic accuracy is much less in medical practices outside specialized academic centers. Even in the best circumstances, however, the conventional diagnostic procedures are labor intensive, long, and expensive. Thus, there is a need for a biomarker, or set of biomarkers, that would quickly and accurately diagnose AD and circumvent the current practice of expensive and time-consuming testing. The search for biomarkers builds upon advances in understanding the biology of AD, and ranges from uncovering genetic risk factors to documenting the pathology of AD and describing the resultant molecular and biochemical changes that result from the AD process. These

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advances have sparked the hope of detecting some of these characteristic changes, especially those noted in postmortem brain tissue, during life. In 1998, the Reagan Institute/National Institute on Aging Workshop on AD Biomarkers outlined steps to establish a biomarker and reviewed the then-current status of proposed markers (Reagan Institute, 1998). Although there are scores of biological correlates for AD, no biological marker has yet gained full acceptance in practice as the sole basis on which to diagnose AD. Among the 16 items in the informal survey on diagnostic procedures, many of the 10 procedures employed less than 50% of the time are those proposed as biomarkers. Comparing the results of this survey with an identical questionnaire completed in 1994 revealed that the results are remarkably the same: the same 6 procedures remain the core of the diagnostic practice, while the 10 supporting or biomarker tests are still used infrequently (Figures 24.1 and 24.2).

This finding was the most surprising. In 1994, biomarkers were just being considered, but during the past five years there has been a torrent of reports extolling the diagnostic power of many molecular and neuroimaging markers (Black, 1999; Growdon, 1999). On the one hand, it is gratifying to see such

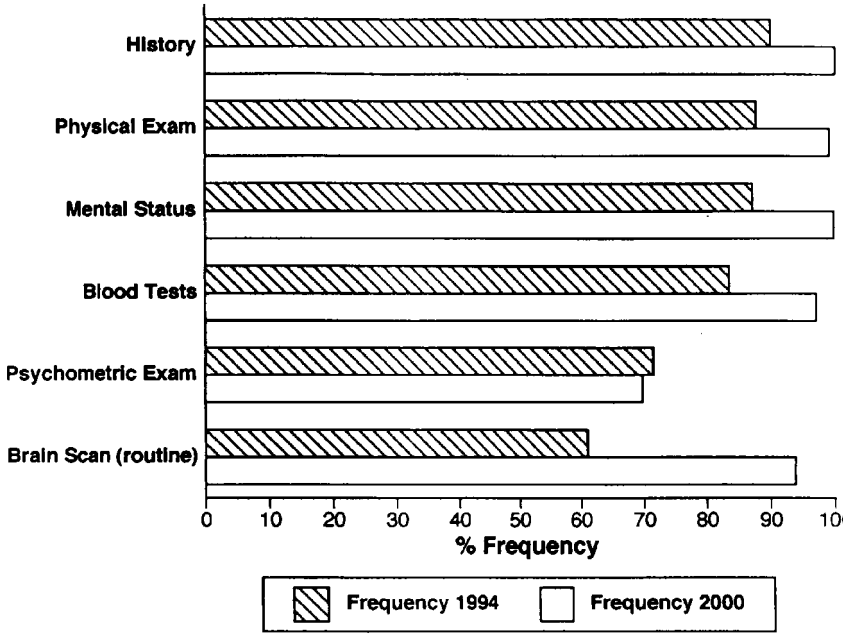

Figure 24.1. AD diagnostic practices: the mean frequency of use for procedures rated greater than 50% by AD centers in 1994 (37 sites; hatched bars) vs. frequency ratings from 2000 (26 sites; open bars). There is close correspondence between surveys conducted in 1994 and 2000 in the consensus regarding essential steps in an AD diagnostic assessment

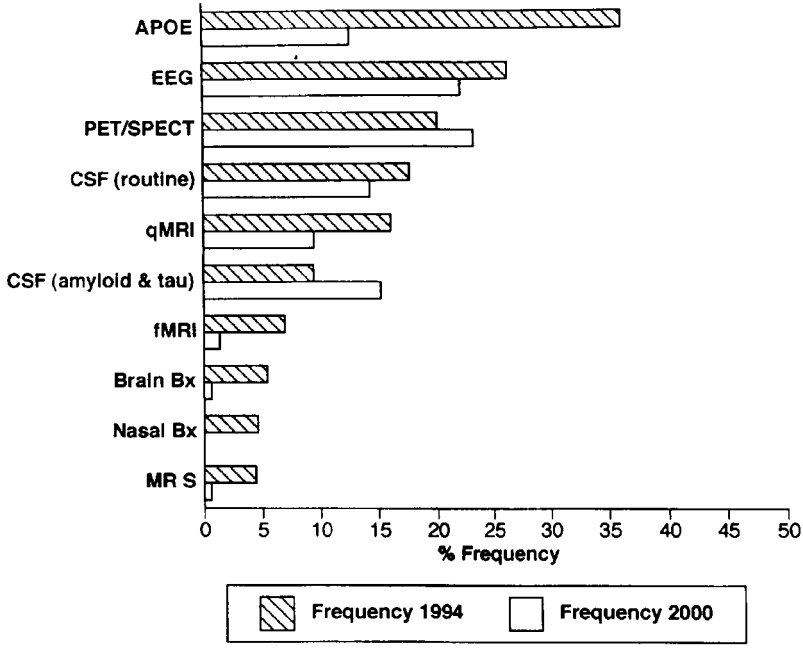

Figure 24.2. AD diagnostic practices: the mean frequency of procedures used less than 50% of the time by AD centers in 1994 (37 sites; hatched bars) and in 2000 (26 sites; open bars). Many of the tests proposed as AD biomarkers, including quantitative analyses of MR brain scans (qMRI), CSF measures of amyloid fragments and tau, and various neuroimaging procedures such as SPECT and PET scans, functional MRI (fMRI), and magnetic resonance spectroscopy (MRS) are used as infrequently in 2000 as they were in 1994

worldwide consensus regarding the necessary steps in an AD evaluation. On the other hand, it is astonishing how little impact biomarker development has made on daily clinical practice. In exploring this discrepancy, this chapter develops a three-tiered concept of biomarkers: *biological correlates*, *diagnostic correlates*, and *surrogate markers.* In the first tier, there are scores of *biological correlates* of AD; in this category, all that is necessary to establish a relation between a marker and AD is statistical significance. Examples of biological correlates of AD range from low levels of somatostatin in cerebrospinal fluid (CSF) (Beal et al., 1986) to pupillary dilation induced by tropicamide (Scinto et al., 1994; Growdon et al., 1997). Although linked to AD, most biological correlates such as these lack specificity and overlap with normal control subjects or with neurologic disease subjects. For these reasons, measures in the biological correlate class are not useful as diagnostic aids, and won't be further discussed in this chapter. A much smaller number of markers fall in the second category and show promise as *diagnostic correlates* of AD. Here, the biomarker must have 80% or greater sensitivity and specificity for AD. A

surrogate marker of AD is the most stringent form of biomarker. Tests in this category will be those that accurately reflect disease activity, course of illness, or treatment outcome. This chapter will focus on the top two tiers: *diagnostic correlates* of AD and *surrogate markers* of AD.

DIAGNOSTIC CORRELATES OF AD

To be useful for diagnosis, a biomarker should detect a fundamental aspect of AD, such as the genetic, morphological, or biochemical pathologies of AD (Table 24.1). An additional requirement for a diagnostic correlate is that methods exist to detect these abnormalities in life. Several of these biomarkers, such as genetic tests, measures of Aβ and tau, quantitative neuroimaging, and physiological neuroimaging, meet these requirements and can be used as confirmatory aids in the conventional diagnostic assessment of a patient with dementia. Detecting a pathologic mutation in the *APP*, *PS1*, or *PS2* gene carries 100% specificity for the diagnosis of AD, but such screening cannot be recommended as a routine measure in clinical practice because of extremely low sensitivity (there are very few individuals with any of these genetic mutations in the total AD population). Thus, searching for a mutation should generally be limited to instances of familial AD in which the age of onset is 50 years or less. Detecting the ε_4 allele of apoE can add a small percent of confidence to the AD diagnosis when used in conjunction with conventional diagnostic workup, but by itself has low diagnostic sensitivity and specificity (Mayeux et al., 1997). There are more than 20 proposed genetic risk factors in addition to apoE that are under investigation, including α_2-macroglobulinemia (Blacker et al., 1998), interleukin-1 (Grimaldi et al.,

Table 24.1. Detecting biological correlates of AD

Abnormality	Method of detection
Genetics	
Early-onset AD	*APP*, *PS1*, *PS2*
Late-onset AD	ApoE, α-2-M, LRP, IL-1 risk factors
Brain morphology	
Senile plaques and neurofibrillary tangles	
Inflammatory response	
Synaptic and neuronal loss	Proton MR spectroscopy
Brain atrophy	Quantitative MRI
Brain biochemistry and metabolism	
Amyloid deposits	Aβ fragments in blood and CSF
Neurofibrillary tangles	Tau in CSF
Altered transmitters	Neurotransmitters in CSF
Decreased metabolism	PET FDG brain scan
Decreased bloodflow	PET/SPECT/fMRI brain scans

2000), interleukin-6 (Papassotiropoulos et al., 1999), lipoprotein receptor (Hyman et al., 2000), and cystatin C (Crawford et al., 2000; Finckh et al., 2000). There is much less consensus regarding these risk factors than the better known and accepted apoE effects, and assays for polymorphisms in these genes have not yet entered routine clinical practice.

Because of the central position of amyloid deposits in brain as the potential toxic exciting event initiating a cascade of neurodegeneration leading to dementia, many molecular biomarkers center on amyloid fragments in blood and CSF. Characteristic findings are increased levels of $A\beta_{42}$ in blood of patients with familial AD due to mutations in the *APP*, *PS1*, and *PS2* genes (Scheuner et al., 1996); plasma $A\beta_{42}$ levels are also increased in some non-familial cases (Mayeux et al., 1999); and the APP ratio in platelets of AD patients is decreased compared to control subjects (DiLuca et al., 1998; Baskin et al., 2000). CSF levels of $A\beta_{42}$ are reduced in AD, whereas CSF levels of the hyperphosphorylated tau protein that forms neurofibrillary tangles in brain are increased in AD (Motter et al., 1995; Kanai et al., 1998; Hulstaert et al., 1999). Although promising as diagnostic correlates, these blood and CSF tests have not yet achieved widespread diagnostic use, either because in some cases they lack sufficient diagnostic sensitivity and specificity, or because in other instances they are available only in an experimental setting.

Neuroimaging procedures, especially quantitative measures of regional structures and whole brain volume, are the most promising biological correlates in the diagnosis of AD. In general, brain scans are obtained in clinical practice to exclude such disorders as stroke, tumor, and hydrocephalus that can cause dementia. Reports from most radiology departments are generally silent, however, as to a positive AD diagnosis. A number of studies now indicate that quantitative analyses of routine brain scans can extract data leading to an enhanced diagnosis of AD. Quantitative magnetic resonance imaging (MRI) to detect regional brain atrophy, especially in medial temporal lobe structures, has proved useful in diagnosing AD and tracking AD progression (Jack et al., 1997). The recent study by Killiany et al. (2000) demonstrates the power of quantitative MRI to detect AD changes. They applied quantitative regional MRI measurements to determine whether people in the prodromal phases of AD could be accurately identified before the onset of clinical manifestations. They examined 11 brain regions in 119 individuals: 16 had mild AD, 24 were cognitively normal, and 79 had mild memory impairment short of dementia, generally corresponding to CDR 0.5. Decreased volumes of entorhinal cortex, superior temporal sulcus, and cingulate gyrus best discriminated these subject groups. Atrophy of the entorhinal cortex and superior temporal sulcus alone accurately discriminated all 16 AD patients from the 24 normal subjects. After three years, 19 of the 79 mild cognitive impairment subjects had progressed to AD; decreased volume measures obtained at baseline accurately identified 93% of these 19.

Functional measures are also sensitive to AD pathology, even when gross atrophy is not apparent. PET scans and SPECT scans reveal a characteristic pattern of bilateral posterior temporal and parietal perfusion and hypometabolism in 80% or more of AD cases (Kennedy, 1998). Functional measures can also detect reduced perfusion at baseline in subjects who progress to AD. In the same 19 subjects who progressed to AD in the study by Killiany et al. (2000), SPECT scans revealed substantial perfusion deficits in entorhinal cortex, superior temporal sulcus, and cingulate gyrus as well as hippocampus and amygdala (Keith Johnson, personal communication). As methods for quantitative analyses of neuroimaging modalities become standardized and disseminated, it is likely that these biological correlates of AD will gain use in diagnostic practice.

SURROGATE MARKERS OF AD

A surrogate marker should accurately reflect disease activity, track course of illness, or index treatment outcome. To fulfill the requirement of a surrogate marker or endpoint, the marker should be directly in the causative pathway to disease outcome (Fleming and DeMets, 1996). Such biomarkers could be important in diagnostic practice, but most will have their major value in tracking the course of disease or monitoring effects of therapeutic interventions. Examples of surrogate markers include CD4 cell counts and measures of viral load in HIV research, and the number of hyperintense plaques on MR brain scan in multiple sclerosis. In AD, there are as yet no accepted biomarkers as surrogate outcomes, although measures of brain atrophy show promise. The basis of dementia in AD is neuronal loss and decreased synaptic contacts, which are the proximate causes of dementia (Gomez-Isla et al., 1996, 1997). If there were a treatment that prevented or even slowed neuronal death and atrophy, how could this effect best be detected? Quantitative analysis of MR brain scans suggest an answer. Although there is regional specificity in the distribution of neuronal loss, the entire brain shrinks over time; at autopsy, brain weight is usually 10% or less than in control brains. In serial measures of whole brain volume using MRI, Fox et al. (1999a) documented a 2–3% per year decrease in brain volume in AD patients compared to less than a 0.5% per year decrease in non-demented control subjects. In a subsequent study, they found that the rate of brain atrophy was significantly correlated to decline on a measure of cognition (Fox et al., 1999b). This observation reinforces the clinical relevance of this surrogate marker in detecting brain atrophy for tracking the course of illness and possibly for monitoring effects of treatment.

Surrogate markers can also be used to verify mode of drug action. The next generation of drugs developed for AD will probably target amyloid and seek to alter APP processing (Felsenstein, 2000) or block amyloid deposition in brain

(Schenk et al., 1999). The rationale for this approach rests upon the fact that β-amyloid (Aβ) deposits in the neuropil are one of the defining histopathological signatures of AD. Because an excessive amount of Aβ may be toxic to neurons (Yankner et al., 1990), attempts to lower the amyloid burden and prevent Aβ deposits is a major goal in drug development for AD. In testing these therapies, clinical trials should incorporate efforts to detect the desired effect of treatment by measuring amyloid derivatives in either blood or CSF. A recent study by Nitsch et al. (2000) illustrates that this strategy is feasible. They administered the selective m_1-agonist AF102B to 19 AD patients based upon preclinical studies showing that activation of the m_1 receptor subtype inhibited Aβ secretion from cultured cells (Hung et al., 1993). In the human studies, they measured total Aβ levels in CSF obtained before treatment and again 4 weeks later during treatment and found that overall CSF total Aβ levels decreased significantly by 20% during treatment with AF102B. CSF Aβ levels did not change significantly in nine AD patients treated according to identical protocols with the acetylcholinesterase inhibitor physostigmine nor in another ten patients treated with the anti-inflammatory drug hydroxychloroquine. These findings confirmed the hypothesized specificity of m_1 receptor activation in affecting APP processing and illustrate the value of CSF Aβ measures as a way to detect the effect of a drug in vivo.

SUMMARY

Biomarkers proposed for AD can be conceptually divided into three tiers; each tier has distinctive relevance for understanding AD biology and clinical use. *Biological correlates* of AD constitute the first tier. These associations and measures are statistically related to AD but will not enter clinical practice because they lack sufficient sensitivity and specificity for AD. They are nonetheless important and have their greatest scientific impact in unraveling the pathophysiology of AD. *Diagnostic correlates* of AD are the second level. They can aid in the clinical investigation of dementia but by themselves are generally insufficient to diagnose AD accurately. Promising markers in this category include causative genetic mutations and risk factors, molecular markers of Aβ and tau in blood and CSF, and neuroimaging techniques, especially quantitative regional MR analyses and functional PET and SPECT scans. In the third tier with the most stringent qualifications for biomarkers are *surrogate outcomes* in AD, which accurately mirror the disease. Although none is accepted now, developing surrogate biomarkers will be vital to tracking the course of illness, verifying mode of drug action, and demonstrating treatment effects.

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25 CSF Markers for Early Alzheimer's Disease

KAJ BLENNOW, PIA DAVIDSSON AND EUGEEN VANMECHELEN

INTRODUCTION

Several acetylcholine esterase inhibitors are available today for symptomatic treatment of Alzheimer's disease (AD). Drugs that might have beneficial effects on the disease process, e.g. γ-secretase inhibitors and β-amyloid (Aβ) vaccination, are under development. These possibilities for therapeutic intervention have highlighted the importance of early and accurate diagnosis of AD.

However, current criteria for the clinical diagnosis of AD are largely based on the exclusion of other dementing illnesses (McKhann et al., 1984). Although many papers report a relatively high accuracy rate for the clinical diagnosis of AD (80–90 %), these studies emanate from expert research academic centers and are most often based on patients in the later stages of the disease who were followed for several years before the confirming autopsy. The diagnostic accuracy rate is probably considerably lower in general hospitals, especially in the earlier stages of the disease when the symptoms often are vague and indistinct. This is unfortunate, as pharmaceutical therapy is probably most effective early in the course of disease, before neurodegeneration is too severe and widespread. Thus, there is a great need for biochemical diagnostic markers (biomarkers) that could aid in the diagnosis of AD early in the course of the disease.

Since the cerebrospinal fluid (CSF) is in direct contact with the brain extracellular space, biochemical changes in the brain are reflected in the CSF. Since AD pathology is restricted to the brain, CSF is the obvious source of biomarkers for AD. Biochemical markers for AD should reflect the central pathogenic processes of the disorder, i.e. the neuronal degeneration, the disturbance in the metabolism of Aβ and its subsequent deposition in senile plaques, and the hyperphosphorylation of tau with subsequent formation of neurofibrillary tangles. Suggested biomarkers for these pathogenic processes are, respectively, normal tau protein, $A\beta_{42}$, and phospho-tau (Figure 25.1, see Plate III).

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TOTAL tau

Tau is a microtubule-associated protein located in the neuronal axons. There are six different isoforms and numerous phosphorylation sites of tau in the human brain (Goedert, 1993). Using monoclonal antibodies that detect all isoforms of tau independent of phosphorylation, ELISAs have been developed that measure the 'total' CSF-tau level (Vandermeeren et al., 1993; Blennow et al., 1995; Vigo-Pelfrey et al., 1995).

An increase in CSF-tau in AD has consistently been found in numerous studies covering more than 2000 AD patients and 1000 controls. Most studies have found an increase in CSF-tau in AD of approximately 300%. The sensitivity, i.e. the ability of CSF-tau to identify AD, varies between reports, but in several large studies has been around 80–90% (Arai et al., 1998; Andreasen et al., 1999a; Hulstaert et al., 1999). Its specificity in differentiating AD from normal aging has also been in the range of 85–90%. However, high CSF-tau levels are also found in a proportion of cases with other dementia disorders, such as vascular dementia (Blennow et al., 1995; Andreasen et al., 1998) and frontotemporal dementia (Arai et al., 1997a; Green et al., 1999; Molina et al., 1999). In contrast, elevated CSF-tau levels are found only occasionally in patients with other types of dementias such as alcoholic dementia (Morikawa et al., 1999), chronic neurological disorders such as Parkinson's disease (Molina et al., 1997), progressive supranuclear palsy (Urakami et al., 1999) and amyotrophic lateral sclerosis (Kapaki et al., 2000), and in psychiatric disorders such as depression (Blennow et al., 1995).

The level of CSF-tau probably reflects the degree of neuronal degeneration and damage (Blennow et al., 1995). This suggestion is supported by the findings that a marked transient increase in CSF-tau is found after acute stroke, with a positive correlation between CSF-tau and infarct size as measured by CT (Hesse et al., 2000a). Further, the degree of increase in CSF total tau is higher in disorders with more extensive and/or rapid neuronal degeneration, such as Creutzfeldt–Jakob disease (Otto et al., 1997), while normal levels are found in patients with Parkinson's disease with limited degeneration (Molina et al., 1997).

β-AMYLOID (Aβ42)

Several different assays have been developed that are specific to $A\beta_{42}$, with minimal cross-reactivity against peptides ending at residues 43 or shorter peptides. Similar results are found using assays specific for $A\beta_{1-42}$ and $A\beta_{x-42}$ (Tamaoka et al., 1997). Using five different assays, a marked decrease in $A\beta_{42}$ in AD to approximately 50% of control level has been consistently found in 15 studies covering more than 900 AD patients and 500 controls. However, one study found an increase in $A\beta_{42}$ in AD (Jensen et al., 1999), which may

be due to methodological differences (e.g. assay specificity for mono- versus oligomers) or differences in patient and control groups.

The sensitivity with which CSF-Aβ_{42} identifies AD varies between reports, but in several large studies has been approximately 80–90%, while its specificity in differentiating AD from normal aging has been in the range of 85–90% (Hulstaert et al., 1999; Galasko et al., 1998; Andreasen et al., 2000a; Sjögren et al., 2000).

The specificity of CSF-Aβ_{42} in distinguishing AD from other dementias and neurological disorders has to be further studied. Low levels are also found in Lewy body dementia (Andreasen et al., 2000a; Kanemaru et al., 2000), a disorder also characterized by the presence of senile plaques. However, low CSF-Aβ_{42} is also found in a relatively large percentage of patients with frontotemporal dementia and vascular dementia (Hulstaert et al., 1999; Sjögren et al., 2000).

The reduction in CSF-Aβ_{42} in AD is often regarded as reflecting the deposition of the Aβ peptide in senile plaques, with lower levels secreted to the CSF. However, a marked reduction in CSF-Aβ_{42} is also found in Creutzfeldt–Jakob disease, even in cases without Aβ-positive plaques (Otto et al., 2000), which puts into question the putative relation between low CSF-Aβ_{42} and senile plaques.

PHOSPHORYLATED tau

Using monoclonal antibodies specific for phosphorylated epitopes of tau (phospho-tau), several ELISAs have been developed for specific measurement of phospho-tau in CSF (Blennow et al., 1995; Ishiguro et al., 2000; Kohnken et al., 2000; Vanmechelen et al., 2000). An increase in CSF-phospho-tau in AD has been found using assays specific for Thr181/Thr231 (Blennow et al., 1995), Thr231/Ser235 and Ser199 (Ishiguro et al., 2000), Thr231 (Kohnken et al., 2000), and Thr181 (Vanmechelen et al., 2000). The sensitivity and specificity figures for AD varies between the reports. Further studies are needed to elucidate which of the phosphorylated epitopes shows the highest increase in CSF and the best sensitivity and specificity figures.

After acute stroke, there is a marked increase in CSF-total tau, while CSF-phospho-tau does not change (Hesse et al., 2000b). This finding suggests that CSF-phospho-tau is not simply a marker for neuronal damage, like CSF total tau, but that it specifically reflects phosphorylated tau, and thus possibly the formation of neurofibrillary tangles.

CSF BIOMARKERS IN EARLY AD

Several studies have found high CSF-tau and/or low CSF-Aβ_{42} in early AD, i.e. in AD patients with high (above 23–25) Mini-Mental State Examination

(MMSE) scores (Table 25.1). The sensitivity figures for identifying early AD cases are similar to those for patients with more severe dementia.

Interestingly, high CSF-tau was found in memory-impaired patients who later progressed to AD, but not in those who did not progress (Arai et al., 1997b). A high sensitivity of the combination of high CSF-tau and low CSF-Aβ_{42} for predicting the progression from mild cognitive impairment (MCI) to AD with clinical dementia has also been found (Andreasen et al., 1999c, 2000a). A first study of CSF-phospho-tau in MCI also found an increase, although the sensitivity was relatively low (Andreasen et al., 2000b). These findings show that these CSF markers are present very early in the disease process, even before the clinical dementia.

CSF BIOMARKERS IN CLINICAL PRACTICE

Numerous scientific papers have evaluated the diagnostic potential of CSF-tau and CSF-Aβ_{42}, revealing high sensitivity and specificity figures. However, most of these studies have been performed in research centers, with selected patient samples and CSF analyses run on one occasion, i.e. under conditions providing figures on the optimal sensitivity and specificity of these analyses. However, two studies have been performed on prospective consecutive patient samples from one clinic, with ELISAs run each week in the clinical neurochemical routine, which may give figures closer to the true performance of CSF-tau and CSF-Aβ_{42}. The analytical variation and stability (analyzed over the course of one year) for these CSF analyses were adequate. In these studies, too, the ability of CSF-tau (Andreasen et al., 1999a) and the combination of CSF-tau and CSF-Aβ_{42} (Andreasen et al., 2000a) to differentiate AD from normal aging, depression, and Parkinson's disease was high, while its specificity against other dementias was lower.

One problematic issue is that CSF studies are most often performed on clinically diagnosed patients. Although the positive predictive value for the clinical diagnosis of AD (i.e. the probability that AD is present when the criteria are met) has been relatively high, about 85%, the negative predictive value (i.e. the probability that AD is not present when the diagnostic criteria are not met) has been considerably lower (Tierney et al., 1988; Jellinger, 1996; Galasko et al., 1994). This is especially true for some of the non-AD dementias (vascular dementia and frontotemporal dementia), where high proportions of the clinically diagnosed patients have notable concomitant AD pathology (Jellinger, 1996; Kosunen et al. 1996). Further, even if they are asymptomatic, age-matched control subjects harbor different degrees of presymptomatic AD lesions in their brains (Tomlinson and Henderson, 1976; Davies et al., 1988; Price and Morris, 1999). Thus, in clinically diagnosed patient and control groups, it is difficult to get high specificity figures for CSF biomarkers.

Table 25.1. CSF biomarkers in early Alzheimer's disease

Clinical criteria	Number of cases	Mean sensitivity (%)	Mean specificity (%)	Reference	Comments
Tau					
AD with MMSE > 25	11	91		Riemenschneider et al., 1996	Approximate sensitivity
AD with MMSE > 20	36	81		Galasko et al., 1997	
AD with MMSE > 25	12	75		Galasko et al., 1997	
AD with MMSE > 25	19	89		Kurz et al., 1998	
AD with MMSE > 23	205	94		Andreasen, 1999a	
MCI, mean MMSE = 25	10	90		Arai, 1997b	MCI cases with progression
	5		100	Arai, 1997b	MCI cases without progression
$A\beta_{42}$					
AD with MMSE > 25	24	88		Andreasen et al., 1999b	Approximate sensitivity
AD with MMSE > 25	25	n.g.		Riemenschneider et al., 2000	Sensitivity not given. Decrease to 63% of controls
Tau + $A\beta_{42}$					
AD with MMSE > 23	24	62		Galasko et al., 1998	
AD with MMSE > 23	23	70		Hulstaert et al., 1999	
MCI with MMSE > 28	16	88		Andreasen et al., 1999c	MCI cases with progression
MCI with MMSE > 28	20	75		Andreasen et al., 2000a	
Phospho-tau					
MCI with MMSE > 28	15	40		Andeasen et al., 2000b	

Sensitivity and specificity figures were given in papers, or were determined from scatterplots. AD, Alzheimer's disease; MCI, mild cognitive impairment; MMSE, Mini-Mental State Examination; n.g., not given.

Much effort has focused on finding a single neurochemical marker for AD. First, this may be elusive unless the marker is related to a pathogenic step that is unique to AD. For example, neuronal degeneration is not only found in AD but also in most chronic degenerative disorders of the brain, and consequently, increased CSF-tau is not specific for AD. Similarly, deposition of Aβ is not specific to AD, but is also found in normal aging and Lewy body dementia, and consequently, reduced CSF-$A\beta_{42}$ is not specific to AD. We suggest that the combination of several CSF biomarkers (e.g. tau, $A\beta_{42}$, phospho-tau, and possibly others such as α- and β-secretase-cleaved APP)—each reflecting a specific pathogenic process—may increase the specificity.

Even today, however, the CSF markers tau and $A\beta_{42}$ have clinical value as adjuncts in the clinical diagnosis of AD, to help to differentiate early AD from some problematic differential diagnoses, especially age-associated memory impairment, depressive pseudodementia, Parkinson's disease, and alcoholic dementia.

SUMMARY

Cerebrospinal fluid (CSF) biomarkers for Alzheimer's disease (AD) would be of great value as diagnostic aids, especially early in the course of the disease, when the clinical symptoms are vague and diagnosis therefore difficult, but when therapeutic compounds have the greatest potential of being effective. Reliable CSF biomarkers will be even more necessary when new therapeutic compounds (e.g. γ-secretase inhibitors and Aβ vaccination) reach the clinical phase. Biomarkers for AD should reflect the central pathogenic processes of the disorder, that is, the neuronal degeneration (e.g. normal tau protein), the disturbance in the metabolism of Aβ and its subsequent deposition in senile plaques (e.g. $A\beta_{42}$), and the hyperphosphorylation of tau with subsequent formation of neurofibrillary tangles (phospho-tau). Today, two CSF biomarkers, tau and $A\beta_{42}$, have been extensively studied and may have a role in the clinical work-up of patients' dementia if used together with the cumulative information from clinical examination and brain imaging techniques. These markers are especially useful for distinguishing early or incipient AD from age-associated memory impairment, depression, and some secondary dementias.

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26 CSF-Phospho-tau (181P) as a Promising Marker for Discriminating Alzheimer's Disease from Dementia with Lewy Bodies

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INTRODUCTION

Increased levels of the microtubule-associated protein tau in cerebrospinal fluid (CSF) is a consistent finding in more than 80% of patients with Alzheimer's disease (AD). As tau protein accumulates in the AD brain in a hyperphosphorylated form, the determination of phosphorylated tau in CSF as opposed to total tau might increase the assay specificity. Several recent studies suggest, but do not yet clearly demonstrate, that assays based on phospho-tau are more specific for AD than those based on total tau (Blennow et al., 1995; Ishiguro et al., 1999; Vanmechelen et al., 2000; Kohnken et al., 2000). For this reason, the phospho-tau (181P)-specific assay (Vanmechelen et al., 2000) was evaluated in AD and closely related dementias. Since the relative absence of phosphorylated tau biochemically distinguishes senile dementia with Lewy bodies (DLB) from AD (Harrington et al., 1994), our primary aim was to determine whether there is a significant difference in phospho-tau levels between these two forms of dementia. Abnormally phosphorylated tau is also increasingly found in a number of non-AD dementias, including several forms of frontotemporal dementia (FTD), Pick's disease, FTD with Parkinsonism linked to chromosome 17, progressive supranuclear palsy (PSP) (Flament et al., 1991), and corticobasal degeneration

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(CBD) (Ksiezak-Reding et al., 1994). Therefore, we also determined CSF-phospho-tau levels in such conditions, including Parkinson's disease without dementia (PD) and multiple system atrophy (MSA) using established criteria for clinical diagnosis.

MATERIALS AND METHODS

SUBJECTS AND METHODS

Eight centers involved in neurological research on CSF participated in this study. A total of 283 CSF samples representing different diagnostic groups (80 AD, 40 controls, 69 FTD, 43 DLB, 15 PD, 16 MSA, 15 PSP, and 5 CBD; Table 26.1) were assayed. The clinical criteria used for diagnosis were based upon well-accepted standards: AD (McKhann et al., 1984); DLB (McKeith et al., 1996); FTD (Anonymous, 1994); PSP (Golbe and Davis, 1993); CBD (Rinne et al., 1994); and MSA (Colosimo et al., 1995). Controls were carefully selected on the basis of age, unimpaired cognition, and the absence of neurological disease. The study was performed on stored CSF samples available for research purposes; when required, the protocol was reviewed and approved by the local Independent Ethics Committee/ Institutional Review Board (IEC/IRB) prior to the start of the study.

Samples were uniformly collected in polypropylene tubes after lumbar puncture. Whenever available, apoE genotype and Mini-Mental State Examination (MMSE) data were obtained from patients. CSF samples that contained more than 500 red blood cells per microliter were not included.

All determinations were performed at Innogenetics (Gent, Belgium) using standard commercial kits for CSF-tau (Innotest hTau antigen, Innogenetics), and a research version of the Innotest Phospho-Tau (181P). A precision profile was used to determine the working assay range between 2 and 40 pM. The reliability and performance of the phospho-tau assay was monitored using five pooled CSF samples covering the performance range of the assay. Values for all quality control samples fell within the established criteria (results not shown).

STATISTICAL METHODS

Normal distributions of CSF-tau and CSF-phospho-tau were tested using the Shapiro–Wilk test; if normality was rejected, non-parametric tests were employed. For comparisons with the AD or control group, p values were adjusted using Dunn's multiple comparison test. Receiver operating characteristic (ROC) curve analysis was used to examine the discriminatory power of tau and phospho-tau between the different diagnostic groups (Hanley and McNeil, 1983). ROC curve analysis was also used to obtain

Table 26.1. Demographic data and CSF results in different diagnostic groups

	Subjects		Age (years)		MMSE			CSF-tau (pM)		CSF-phospho-tau (pM)	
Group	n	(M/F)	Median	(Range)	n	Median	(p25–p75)	Median	(p25–p75)	Median	(p25–p75)
AD	80	(35/45)	72	(53–86)	78	22	(14–24)	13.2	(9.4–17.0)*	14.7	(11.6–19.1)*
Controls	40	(20/20)	70	(56–84)	20	30	(29–30)	3.0	(2.1– 4.0)***	7.8	(6.4–8.9)***
FTD	69	(42/27)	67	(40–94)	61	22	(16–25)	7.5	(5.2–10.8)**,***	9.4	(8.2–12.3)**, ***
DLB	43	(35/8)	72	(61–87)	37	19	(14–24)	5.7	(1.6– 9.0)***	8.1	(6.1–10.0)***
PD	15	(8/7)	70	(51–79)	1	23	(23–23)	4.2	(2.2– 7.6)***	7.4	(6.9–8.8)***
MSA	16	(11/5)	64	(42–77)	3	20	(20–22)	5.3	(3.8– 8.3)***	7.6	(6.2–10.9)***
PSP	15	(11/4)	67	(64–76)	4	26	(21–27)	2.8	(2.0– 4.5)***	6.9	(6.1–7.5)***
CBD	5	(0/5)	70	(57–75)	4	13	(12–15)	12.9	(9.8–15.4)	12.7	(9.1-13.1)

AD, Alzheimer's disease; FTD, frontotemporal dementia; DLB, dementia with Lewy bodies; PD, Parkinson's disease; MSA, multiple system atrophy; PSP, progressive supranuclear palsy; CBD, corticobasal degeneration; MMSE, Mini-Mental State Examination; p25, lower quartile; p75, upper quartile.
*Significantly different from controls ($p < 0.001$; Kruskal–Wallis test with Dunn's multiple comparison test).
**Significantly different from controls ($p < 0.05$).
***Significantly different from AD ($p < 0.001$).

cut-off values comparing AD versus non-AD cases. Possible correlations were determined with the Spearman rank correlation coefficient.

RESULTS AND DISCUSSION

Since the study was designed to investigate a relatively small, but clinically well-characterized number of patients, not all confounding factors potentially affecting CSF-tau and CSF-phospho-tau levels could be accounted for. Significant center effects were observed for CSF-tau, while none were present for CSF-phospho-tau. CSF-tau was found to be correlated with the MMSE in the total AD group [Spearman, $r = -0.28$ $(-0.49, -0.06)$, $p = 0.01$], while neither apoE genotype nor subject age correlated with either CSF-tau or CSF-phospho-tau.

A strong correlation between CSF-tau and CSF-phospho-tau for all patients was observed, independent of the diagnostic group ($r = 0.904$). As normality was rejected for both biomarkers in all groups, analyses were performed using non-parametric tests. Median CSF-phospho-tau levels were significantly different between AD and all the other conditions except CBD, but these differences were also observed for CSF-tau (Table 26.1). Therefore, specificities were compared using a cut-off value for both markers which give a well-defined sensitivity for AD. Parkinson-related conditions (PSP, MSA, and PD) except for CBD were grouped together for this analysis because in these conditions median CSF-tau and CSF-phospho-tau were not significantly different from controls. Specificities for discriminating AD from DLB and AD from FTD tended to be increased for CSF-phospho-tau (Table 26.2). As seen in Table 26.3, a small statistically significant improvement for CSF-phospho-tau for discriminating AD from DLB ($p = 0.039$) and AD from FTD ($p = 0.049$) was confirmed by comparing the ROC curves. Since

Table 26.2. Specificities for CSF-tau and CSF-phospho-tau at a cut-off level of the marker with a defined sensitivity (84%) for AD

	CSF-tau (cut-off = 7.5 pM)	CSF-phospho-tau (cut-off = 10 pM)
AD (n = 80)*	84% (95% CI: 74–90%)	84% (95% CI: 74–90%)
FTD (n = 69)	51% (95% CI: 39-63%)	57% (95% CI: 50-78%)
DLB (n = 43)	65% (95% CI: 45–69%)	74% (95% CI: 60–85%)
Controls (n = 40)	93% (95% CI: 76–97%)	88% (95% CI: 73–96%)
Parkinson-related conditions (n = 46)	80% (95% CI: 67–90%)	84% (95% CI: 71–93%)

AD, Alzheimer's disease; FTD, frontotemporal lobe dementia; DLB, dementia with Lewy bodies; 'Parkinson-related conditions' includes Parkinson's disease without dementia (n = 15), multiple system atrophy (n = 16), and progressive supranuclear palsy (n = 15); CI, confidence interval.
*Sensitivity.

Table 26.3. Comparison of discriminatory power of different markers and combination of markers using ROC analysis

Groups	CSF-tau (AUC ± SE)	CSF-phospho-tau (AUC ± SE)	p-Value
AD vs. controls (n = 80)	0.862 ± 0.038	0.897 ± 0.032	0.191
AD vs. FTD (n = 69)	0.711 ± 0.045	0.754 ± 0.044	0.049
AD vs. DLB (n = 43)	0.782 ± 0.048	0.839 ± 0.042	0.039
AD vs. Parkinson-related conditions (n = 46)	0.873 ± 0.035	0.864 ± 0.037	0.319

Areas under the curve (AUC) and standard errors (SE) of receiver operating curves (ROC) were compared according to Hanley and McNeil.
AD, Alzheimer's disease; FTD, frontotemporal lobe dementia; DLB, dementia with Lewy bodies; 'Parkinson-related conditions' includes Parkinson's disease without dementia (n = 15), multiple system atrophy (n = 16), and progressive supranuclear palsy (n = 15).

the differential diagnosis between AD and DLB is difficult in a clinical setting but important for patient management (McKeith et al., 1999), CSF-phospho-tau might be of potential clinical value here. Also, elevated levels of CSF-tau and CSF-phospho-tau in some of the FTD and LBD patients might be related to coexisting AD pathology, which needs further investigation in autopsy-confirmed cases.

Although the increased CSF-phospho-tau in AD suggests that abnormal phosphorylated tau in CSF is a marker for tau pathology in the brain, CSF-phospho-tau levels in FTD or CBD were only moderately increased. Furthermore CSF-phospho-tau levels are normal in PSP, a condition pathologically characterized by abnormal phosphorylated tau deposition (Flament et al., 1991). This indicates that either phosphorylation at threonine 181 is less specific for tau pathology in FTD or CBD or that other phosphorylation sites on tau might be more important (Ishiguro et al., 1999). Alternatively, an increase of the tau/phospho-tau ratio might be related to other pathological mechanisms: it has been suggested that an increase in CSF levels of cytoskeletal proteins is related to differential involvement of these proteins in the pathophysiology of AD and FTD (Sjögren et al., 2000). Very high levels of CSF-tau in stroke (Hesse et al., 2000) and Creutzfeldt–Jakob disease (Otto et al., 1997) are not accompanied by an increase in phospho-tau levels (C. Hesse, unpublished), or the increase is only moderate (B. Van Everbroeck, unpublished). This suggests that the increased phospho-tau *and* tau levels in AD are related to the damage to specific cellular compartments/populations which are rich in hyperphosphorylated tau, while in other neurodegenerative conditions, such as some of the DLB and FTD cases, the increase in total tau but not in phospho-tau is related to a loss of cells, with tau phosphorylated to a lesser extent. Thus, combining different CSF markers

and relating them to clinical outcome might be especially relevant to our understanding of disease-induced changes in CSF proteins.

In conclusion, CSF-phospho-tau was confirmed as differentiating between AD and DLB, with about 80% of the patients correctly classified. Based on the analysis of other clinical conditions such as FTD, CBD, and PSP, in which tau pathology is present in the brain, CSF-phospho-tau does not seem to change in parallel with tau pathology in the brain. The altered levels of tau/phospho-tau may be more closely related to the loss of specific cellular compartments/populations in the cerebral cortex.

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27 Increased Levels of a Minor Glycoform of Acetylcholinesterase in Alzheimer's Disease Brain and Cerebrospinal Fluid

JAVIER SÁEZ-VALERO, SU SAN MOK, LISA FODERO, ALBERTO MARCOS, MARIA-SAGRARIO BARQUERO, CATRIONA McLEAN AND DAVID H. SMALL

INTRODUCTION

With the advent of effective therapeutic agents for the treatment of Alzheimer's disease (AD), accurate diagnosis will become increasingly important. To date, clinical diagnosis based on neuropsychological assessment is time consuming and often inaccurate. Therefore, there is a need to develop an accurate and objective biochemical test that can be used to increase confidence in diagnosis (Growdon, 1999).

A number of studies have shown that certain cerebrospinal fluid (CSF) proteins are altered in AD. Probably the best studied of these proteins are $A\beta_{42}$ and tau (Kanai et al., 1998). The level of $A\beta_{42}$ is decreased, and the level of tau increased in AD CSF. However, neither $A\beta_{42}$ nor tau provides sufficient sensitivity and specificity of detection to warrant routine use for clinical diagnosis. Therefore, there is a need to identify new biomarkers which can be used alone or in combination with other biomarkers such as $A\beta_{42}$ and tau to increase confidence in clinical diagnosis.

EXPRESSION OF ACETYLCHOLINESTERASE IN THE BRAIN

Acetylcholinesterase (EC 3.1.1.7) (AChE) is chiefly responsible for the hydrolysis of acetylcholine (Small et al., 1996). Although a single gene on chromosome 7 encodes AChE, the enzyme exists in multiple molecular species, which can be distinguished by their different hydrophobicities and

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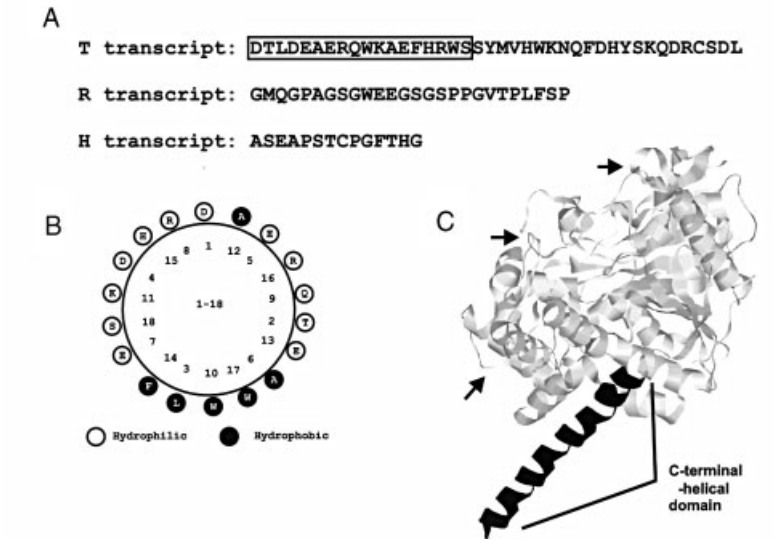

Figure 27.1. Different C-terminal polypeptides obtained by alternative pre-mRNA splicing dictate different membrane anchoring and hydrophobicity characteristics of human AChE. **A** Most brain isoforms are encoded by the T transcript that directs expression of a 40-amino-acid residue C-terminal polypeptide. The first 18 residues of this C-terminal peptide (shown enclosed in a box) are predicted to be α-helical. Construction of an α-helical wheel **B** shows that this region would be amphiphilic in character. Forms encoded by the R transcript lack a C-terminal cysteine residue necessary for subunit association and are therefore hydrophilic monomers. H transcripts encode forms with a hydrophobic C-terminal peptide that directs attachment of a GPI anchor. In **C**, a ribbon representation of the structure of monomeric AChE is shown. The region containing the first 18 amino-acid residues encoded by the T exon is shown in a model of the three-dimensional structure as an α-helix in black. Arrows show glycosylation sites

molecular weights (Massoulié and Bon, 1982). Some of this molecular heterogeneity derives from alternative RNA splicing, which generates three major transcripts encoding polypeptides with different C-terminal sequences (Figure 27.1A) (Taylor and Radic, 1994; Grisaru et al., 1999). A major transcript found in brain (T transcript) encodes forms containing a 40-amino-acid residue C-terminal polypeptide. Although the precise three-dimensional structure of this C-terminal polypeptide is unknown, a model has been proposed (Giles, 1997). Using a number of different secondary structure prediction algorithms, it can be estimated that the first 18 residues of the C-terminal region form an α-helix (Figure 27.1B,C). Construction of a helical wheel shows that this region is probably amphipathic. The hydrophobic regions of the C-terminus allow monomers to self-associate into tetramers. Monomeric and dimeric isoforms are therefore amphiphilic in character.

A second transcript (R transcript) can be generated, particularly under conditions of cell stress, through a 'read-through' mechanism, in which the intronic sequence after the last catalytic exon is retained (Grisaru et al., 1999). This transcript lacks a C-terminal cysteine residue for dimerization and results in hydrophilic monomeric isoforms. Splicing of a hydrophobic sequence encoded by the H exon generates the H transcript encoding isoforms that can be anchored to membranes via a glycosylphosphatidyl inositol (GPI) linkage (Grisaru et al., 1999). These isoforms are either monomeric or dimeric and are amphiphilic in character due to their interaction with lipids via the GPI anchor.

EXPRESSION OF AChE in AD

The level of AChE is decreased in the AD brain, partly because of the overall decline in cholinergic activity (Small et al., 1996). However, as noted first by Friede (1965) and studied extensively by Mesulam, Geula, and co-workers (Mesulam et al., 1987), the level of AChE is increased around amyloid deposits. The increased level of AChE in amyloid plaques may be due to a direct action of the amyloid protein on AChE levels. For example, we have found that Aβ peptides can increase AChE levels in neuronally differentiated P19 cells (Sberna et al., 1997). This effect on AChE is only seen with peptides that aggregate to form amyloid fibrils, and it can be blocked by L-type voltage-dependent calcium channel (L-VDCC) antagonists (Sberna et al., 1997). Thus, the effects of Aβ on AChE expression may be due to an agonist action on L-type channels. Consistent with this view, it has previously been shown that the entry of calcium through L-VDCCs can increase AChE expression in muscle cells by stabilizing AChE mRNA (Luo et al., 1994).

We have also found that AChE expression is increased in APP C100 mice expressing a transgene containing the C-terminal 100-amino-acid residues of the amyloid protein precursor (APP) (Sberna et al., 1998). Although APP C100 mice do not produce amyloid plaques because the level of Aβ production is not sufficiently high, there is a significant increase in AChE levels in the brain. This increase in total AChE is caused solely by an increase in a minor monomeric amphiphilic isoform (G_1^a AChE). The major brain form of AChE is tetrameric (G_4^a AChE) and is not altered in APP C100 mice.

The level of G_1^a AChE is also increased in AD brain (Sáez-Valero et al., 1999). Our studies indicate that the increase in the G_1^a isoform is due to a large increase in a minor subspecies of G_1^a AChE. This subspecies of G_1^a AChE can be distinguished from other G_1^a isoforms by its lack of binding to a monoclonal antibody (HR2) and its unusual glycosylation pattern (Sáez-Valero et al., 2000a). Human AChE possesses three potential N-linked glycosylation sites, which occur in loop regions on the surface of the protein (Figure 27.1C). The structure of the carbohydrate moieties of AChE varies

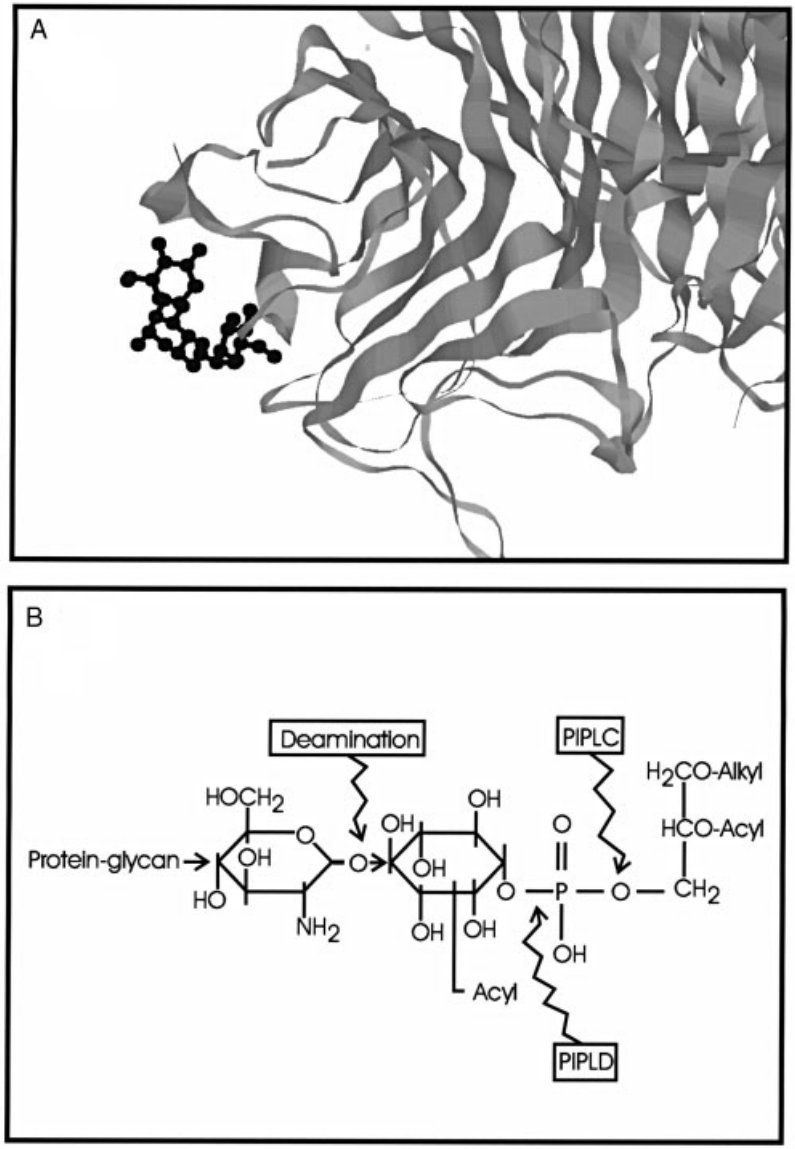

Figure 27.2. Protein–carbohydrate interactions. **A** Binding of trimannoside to the plant lectin concanavalin A (ConA). The structure of trimannoside is represented by a ball and stick model, while the polypeptide backbone in the binding site region of ConA is represented as a ribbon. The carbohydrate-binding site is formed by a number of loops and regions of β-structure reminiscent of the antigen-binding domains of immunoglobulins. **B** The structure of the glycosylphosphatidyl inositol anchor of human AChE. Because lipids are also esterified to the inositol moiety in the anchor, AChE is not released from the lipid by phospholipase C (PIPLC) or phospholipase D (PIPLD) alone. Deamination is necessary to release the anchor from the protein

depending upon the tissue of origin and the specific isoform (Liao et al., 1992; Vidal, 1996). Most species of brain AChE can bind to the plant lectin concanavalin A (ConA), which recognizes carbohydrates containing α-mannose (Figure 27.2A). However, the minor G_1^a isoform of AChE increased in the AD brain does not bind to ConA (Sáez-Valero et al., 1999, 2000a).

Since the minor isoform that is elevated in the AD brain is amphiphilic, this rules out the possibility that it is a product of the R transcripts, which encode hydrophilic monomers. Therefore, we have examined the possibility that the enzyme may be produced by an H transcript, i.e. that its partial hydrophobic character derives from the presence of a GPI anchor. GPI anchored proteins can normally be converted to hydrophilic forms by incubation with phospholipase C or D (Figure 27.2B). However, human AChE is resistant to this treatment, owing to the presence of lipid esterified to the inositol ring (Roberts et al., 1988). Therefore, deamination with alkaline hydroxylamine is required for complete solubilization. When AChE from AD human brain was treated with alkaline hydroxylamine and phospholipase C, there was no shift in its sedimentation coefficient in the presence of Triton X100 (Figure 27.3). This indicated that very little, if any, of the brain AChE is GPI-anchored. In contrast, AChE from human erythrocytes, which is known to be GPI-anchored, was converted to a hydrophilic form upon the same treatment. Therefore, almost all of the G_1^a AChE in the brain, including the isoform that is increased in AD, is probably encoded by T transcripts.

GLYCOSYLATION OF AChE IN AD CSF

To determine whether the glycosylation of AChE can be used as a diagnostic marker of AD, we have examined AChE glycosylation in CSF (Sáez-Valero et al., 1997, 1999). The results obtained for AChE glycosylation in CSF were very similar to those obtained for the glycosylation of AChE in brain. Studies on CSF collected postmortem from cases with confirmed pathology showed that the glycosylation of AChE is altered in AD CSF but not in other neurological diseases, including other dementias (vascular dementia, Lewy body dementia, frontal lobe dementia) (Sáez-Valero et al., 1997, 1999). As observed in our previous studies, the shift in glycosylation was due to an increase in a minor G_1^a AChE glycoform which does not bind to ConA (Sáez-Valero et al., 2000a).

To examine whether the glycosylation of AChE is altered in lumbar CSF collected antemortem, CSF was collected from patients at the Hospital Universitario San Carlos of Madrid after patients were diagnosed with probable AD disease according to NINCDS–ADRDA criteria (McKhann et al., 1984). CSF was incubated with ConA–Sepharose and the amount of enzyme remaining unbound after incubation was assessed. The percentage of AChE unbound to ConA was significantly higher ($p < 0.05$, Student's t test)

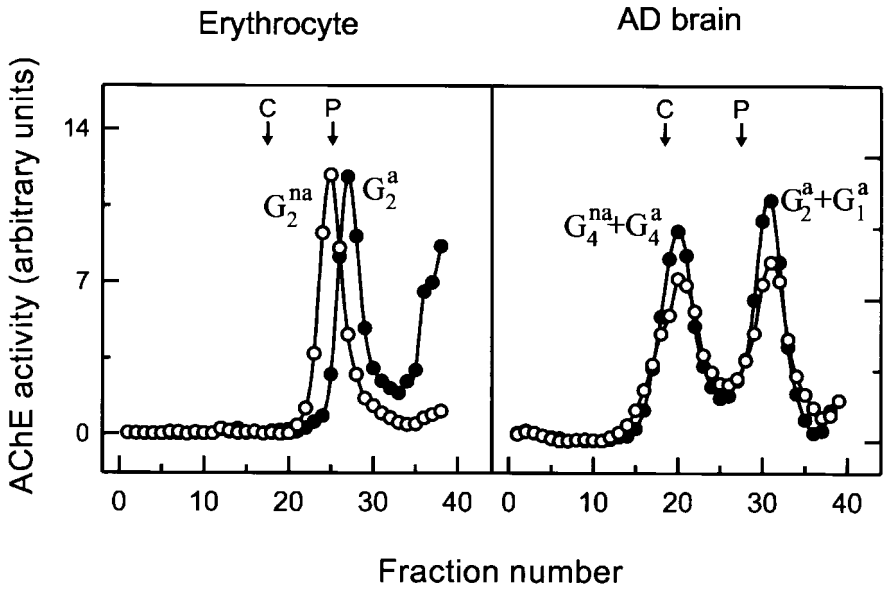

Figure 27.3. Treatment of AChE from human erythrocytes and AD frontal cortex with alkaline hydroxylamine and PIPLC. AChE extracts from erythrocyte or AD brain were analyzed for AChE isoforms by sucrose density gradient ultracentrifugation (●) or subjected to deamination by treatment with alkaline hydroxylamine followed by PIPLC cleavage before sucrose density gradient ultracentrifugation (○). AChE was assayed by the method of Ellman et al. (1961) and sucrose density gradient analysis performed as described by Sáez-Valero et al. (1999). Treatment of extracts with alkaline hydroxylamine and PIPLC was essentially as described by Roberts et al. (1988). Note that only G_2^a AChE from human erythrocyte was converted into the hydrophilic isoform (G_2^{na}). Under identical conditions, none of the G_2^a or G_1^a from AD frontal cortex displayed a shift in the sedimentation values as would be expected for the conversion of GPI-anchored species into their hydrophilic derivates. The internal standards were catalase (C, 11.4S) and alkaline phosphatase (P, 6.1S)

in the AD group than in the control group (no neurological disease) or in patients with other neurological diseases including cases of cerebrovascular accident, normal-pressure hydrocephalus, benign intracranial hypertension, epilepsy, and optic neuritis or multiple sclerosis (Figure 27.4) (Sáez-Valero et al., 2000b). No significant elevation in the percentage of unbound AChE was observed in any of the non-AD groups, indicating that the glycosylation of AChE is not altered in many non-AD diseases.

Analysis of the lumbar CSF data indicates that AChE glycosylation detects AD with at least 60% sensitivity and specificity (Figure 27.4, inset), values which fall slightly below the 80% specificity and sensitivity suggested as necessary for an excellent diagnostic marker (Davies et al., 1998). However,

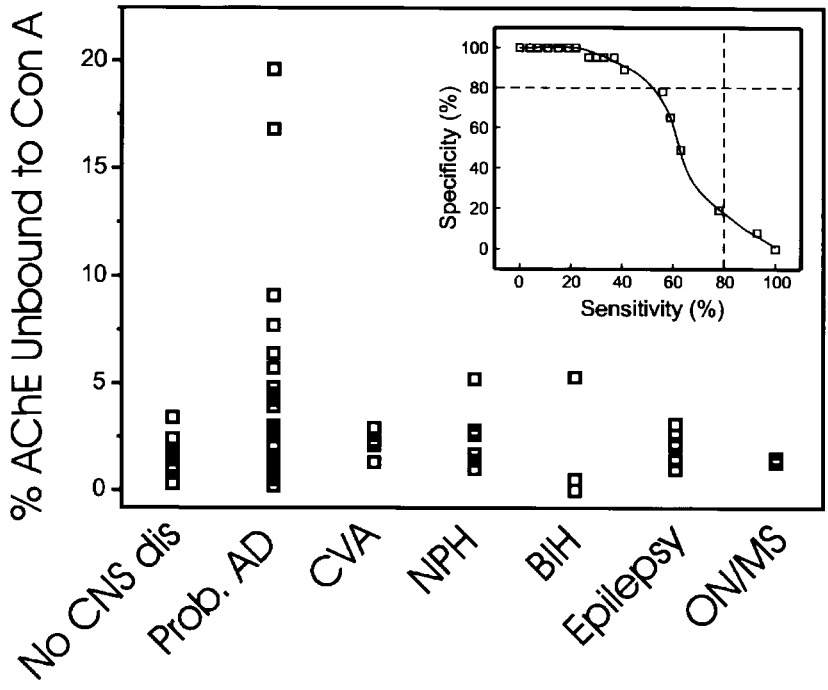

Figure 27.4. Analysis of the glycosylation of AChE in lumbar CSF taken from patients with various diseases. No CNS dis, no CNS disease; Prob. AD, probable AD; CVA, cerebrovascular accident; NPH, normal pressure hydrocephalus; BIH, benign intracranial hypertension; ON/MS, optic neuritis or multiple sclerosis. Inset shows an analysis of the data in which specificity is plotted against sensitivity. Dashed lines show the limits of 80% sensitivity and specificity deemed necessary for a diagnostic marker of AD (Davies et al., 1998)

the value of 60% must be viewed as a minimum, because of the inherent uncertainty of clinical diagnosis. Our postmortem data, in which a firm diagnosis was obtained by histopathological examination, suggest that AChE glycosylation can detect AD cases with 80% sensitivity (Sáez-Valero et al., 1997).

Although no single CSF biomarker has achieved the required 80% sensitivity and specificity of detection to be viewed as useful for diagnosis on its own, it seems likely that when several biomarkers are used in combination, the level of sensitivity and specificity may be improved substantially. For example, the measurement of $A\beta_{42}$, tau, and AChE glycosylation together could substantially improve diagnostic accuracy, although this has not yet been demonstrated. Further studies will be needed to examine this possibility.

SUMMARY AND CONCLUSIONS

Our studies demonstrate that Aβ peptides induce the expression of a minor glycoform of AChE which can be distinguished from other isoforms by its lack of binding to ConA. The minor glycoform is an amphiphilic monomer, but unlike amphiphilic species expressed in cells of hemopoietic origin, it is not GPI-anchored. The minor glycoform is increased in the brain and CSF of patients with AD, but not many other neurological diseases, suggesting that it may be useful, on its own or in combination with other CSF biomarkers, as part of a diagnostic test for AD.

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28 Plasma β-Amyloid as a Surrogate Genetic Marker in Late-Onset Alzheimer's Disease

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INTRODUCTION

Plasma amyloid β_{42} peptide ($A\beta_{42}$) levels are significantly elevated in all genetic forms of early-onset Alzheimer's disease caused by familial Alzheimer's disease mutations or Down's syndrome (Scheuner et al., 1996). Moreover, recent studies have determined that both plasma $A\beta_{42}$ and $A\beta_{40}$ levels are significantly elevated in a subset of late-onset Alzheimer's disease (LOAD) patients, their cognitively normal first-degree relatives, and members of typical LOAD families when compared to appropriate controls (Kuo et al., 1999; Matsubara et al., 1999; Younkin, 2000). To determine the magnitude of the genetic component affecting plasma Aβ levels we estimated the heritability of plasma $A\beta_{42}$ and $A\beta_{40}$ in 16 extended, multigenerational LOAD pedigrees, using a variance components method implemented in the computer package SOLAR (Almasy and Blangero, 1998). Heritability estimates as high as 62% and 49% were found for plasma $A\beta_{42}$ and $A\beta_{40}$ levels, respectively. Inclusion of the apolipoprotein E (apoE) ε_4 allele dosage as a covariate was not found to have a significant effect on the heritability of these traits. These results suggest that genetic determinants other than apoE account for a very substantial percentage of the phenotypic variance in plasma Aβ levels.

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SUBJECTS AND METHODS

SUBJECTS

We collected 16 typical LOAD families composed of 438 individuals (Table 28.1), 435 of whom had complete phenotypic information. Typical LOAD families were characterized by a lack of autosomal dominant family history of AD and an age at onset greater than or equal to 61 years. Among all of the AD probands only one had age at onset below 61 years. Family 13, which was composed of only 7 individuals, had a proband with onset of AD at 51 years. This person also had an $\varepsilon_4/\varepsilon_4$ genotype. We repeated all of the analyses excluding this family and found no significant difference with the results where they were included (data not shown).

Three different ascertainment schemes were utilized for these collections (Table 28.1). Six families were ascertained via a proband who is 'a first-degree relative of an AD patient.' The first-degree LOAD relative had to be a cognitively normal individual with high plasma Aβ levels. The second group of families was selected via a typical LOAD proband without previous plasma measurements of Aβ levels. These were multigenerational extended pedigrees with multiple cases of AD in all older generations. Finally, the last group was ascertained based on an 'LOAD proband with extreme high plasma Aβ levels,' where the proband had plasma $A\beta_{40}$ and/or $A\beta_{42}$ levels in the top tenth percentile of the distribution of all of the LOAD patients measured in our series. The ascertainment schemes were corrected for by excluding all of the probands from the analyses.

The diagnosis of AD in the probands or in the first-degree AD relatives of cognitively normal probands was made as follows: Families 1, 2, 4, and 21 via medical records, family 3 via medical records including an autopsy, families 6, 7, and 12 via family history. The rest of the AD patients were diagnosed after an exam by the neurologist in our group (N. Graff-Radford) according to the guidelines of NINCD–S–ADRDA (McKhann et al., 1984).

The plasma levels of $A\beta_{40}$ and $A\beta_{42}$ were measured using a previously published ELISA method (Suzuki et al., 1994). This study was approved by the Institutional Review Board at Mayo Clinic.

STATISTICAL ANALYSES

We estimated the additive genetic heritability of plasma $A\beta_{42}$ and $A\beta_{40}$ in the family members from the total collection of 16 typical LOAD families (Table 28.1). We corrected for ascertainment by excluding the probands from the analyses. To determine the contribution of genetic factors to plasma $A\beta_{42}$ and $A\beta_{40}$ levels, we analyzed the typical LOAD families with the SOLAR software

package (Blangero and Almasy, 1996). The 'Polygenic Analysis' program was used to detect the proportion of variance of these quantitative traits due to additive genetic factors, environmental factors, and covariates. SOLAR was chosen for its capability of utilizing all of the information that is provided by pedigrees of large size and complexity, and its power in discriminating the genetic and environmental effects in such pedigrees more so than nuclear family or sibship-based heritability analyses. We included 'sex, age, sex*age, age^2, sex*age^2' as covariates in all of the analyses and evaluated the significance of each. We repeated all of the analyses including apoE ε_4 allele dosage as an additional covariate for the subset of individuals who were genotyped for the apoE gene using conventional restriction fragment analysis methods (Crook et al., 1994). During our analyses we calculated the significance of each covariate and kept them in the analysis regardless of their significance level. Logarithmically transformed plasma $A\beta_{42}$ and $A\beta_{40}$ values were used as the quantitative traits in all of the analyses because the untransformed values severely violated the assumption of multivariate normality underlying variance components analysis method. In addition, the highest extreme outliers were excluded from the estimations. Analysis was restricted to the 20- to 65-year-old group to avoid the confounding changes in plasma $A\beta_{42}$ that occur in subjects over

Table 28.1. Family profiles

Family codes	n[a]	Proband
1	13	First-degree relative
2	34	First-degree relative
3	17	First-degree relative
4	19	First-degree relative
6	16	First-degree relative
21	42	First-degree relative
7	24	AD patient
9	13	AD patient
11	75	AD patient
12	48	AD patient
13	7	AD patient
15	20	AD patient
16	19	AD patient with high Aβ
17	48	AD patient with high Aβ
18	7	AD patient with high Aβ
20	36	AD patient with high Aβ[b]
	438	

[a]Total number of family members. [b]This AD proband had high but non-extreme plasma Aβ levels but extreme high Aβ production of fibroblasts.

age 65 in association with aging and cerebral Aβ deposition (Jensen et al., 1999; Kuo et al., 1999; Matsubara et al., 1999; Younkin et al., 2000).

RESULTS

The heritability of plasma $A\beta_{42}$ and $A\beta_{40}$ estimated in the family members from 16 typical LOAD families is depicted in Table 28.2. Plasma $A\beta_{42}$ was found to have a highly significant heritability estimate of 62.3% in the LOAD families analyzed. The heritability of plasma $A\beta_{40}$ was estimated to be 49.1%. None of the covariates analyzed (sex, age, age^2, age*sex, age^{2*}sex) was found to be a significant component for the variance of plasma $A\beta_{42}$ or $A\beta_{40}$.

To determine the effect of 'apoE ε_4 allele' dosage on the levels of $A\beta_{42}$ and $A\beta_{40}$, we included it as a covariate in the analysis. A total of 271 family members were genotyped for apoE, using published methods (Crook et al., 1994). The analyses were conducted for log$A\beta_{42}$ and log$A\beta_{40}$ (Table 28.3) in these individuals. The analyses that exclude and include 'apoE ε_4 allele dosage' as a covariate were compared. The 'apoE ε_4 allele dosage' covariate effect was not significant in any of these analyses (p levels $= \sim 0.5$ for $A\beta_{40}$ and ~ 0.2 for $A\beta_{42}$). None of the other covariates were found to be significant in these estimations.

DISCUSSION

We determined the heritability of plasma $A\beta_{42}$ and $A\beta_{40}$ levels in a total of 306 individuals from 16 families. The heritability estimates indicated that a substantial percentage of the variance in plasma Aβ levels is due to genetic

Table 28.2. Heritability estimates of plasma $A\beta_{42}$ and $A\beta_{40}$ in 20- to 65-year-old family members from 16 typical LOAD families. These are the results of the 'polygenic–screen–all' analyses in SOLAR. The p value depicts the significance of difference between the models where the heritability of the quantitative trait ($A\beta_{42}$ or $A\beta_{40}$) is estimated, and the models where heritability is fixed at zero. The covariates estimated in this analysis were age, sex, age*sex, age^2, age^{2*}sex

Trait	Heritability $\pm$ SE[a]	p Value	Covariate effect[b]	Significant covariates (p value)
Log$A\beta_{42}$ (n = 306)	62.3% $\pm$ 11.7%	10^{-8}	0.7%	None
Log$A\beta_{40}$ (n = 302)	49.1% $\pm$ 12.3%	0.000003	2.3%	None

[a]Proportion of variance due to additive genetic effects.
[b]Proportion of variance due to covariates.

Table 28.3. Heritability estimates of plasma $A\beta_{42}$ and $A\beta_{40}$ in family members genotyped for apoE, from 16 typical LOAD families. Significance level set for the covariates = 0.1. None of the covariates were found to be significant in these estimations

	ApoE ε_4 allele dosage not included as a covariate			ApoE ε_4 allele dosage included as a covariate		
	Heritability $\pm$ SE[a]	p Value	Covariate effect[b]	Heritability $\pm$ SE[a]	p Value	Covariate effect[b]
$LogA\beta_{42}$ (n = 267)	64.4% $\pm$ 12.6%	10^{-8}	0.4%	63.7% $\pm$ 12.6%	10^{-8}	1.2%
$LogA\beta_{40}$ (n = 261)	52.6% $\pm$ 14.3%	0.00002	1.5%	52.5% $\pm$ 14.3%	0.00002	1.7%

[a]Proportion of variance due to additive genetic effects.
[b]Proportion of variance due to covariates.

factors in the typical LOAD families analyzed. Since apoE is the only genetic factor proven to be a risk factor for LOAD, we next determined the influence of apoE genotype on the heritability of plasma Aβ levels. If a substantial percentage of the heritability of plasma Aβ levels is due to the apoE ε_4 allele dosage, then we should expect to see a significant decline in the proportion of variance due to additive genetic factors, and a significant covariate effect for the 'apoE ε_4 allele dosage.' As depicted in Table 28.3, the 'apoE ε_4 allele dosage' covariate effect was not significant in any of these analyses (p levels = ~0.2–0.5). There was essentially no difference in the heritability estimates of any of the quantitative traits between the group where 'apoE ε_4 allele dosage' was included as a covariate and where it was excluded. There was also no significant increase in the covariate effect when 'apoE ε_4 allele dosage' was included as a covariate. These results suggest that apoE ε_4 allele dosage is not a significant determinant of plasma Aβ levels. However, this analysis does not account for the possible interaction between different loci (epistatic effects) or for allelic interaction at a single locus (dominance effects). ApoE may possibly interact with other genetic loci in determining the levels of plasma Aβ levels, and due to the inability of the SOLAR program to detect this effect it would be included within the phenotypic variance due to undetermined environmental effects [environmental effects = 1 − (heritability + covariate effects)].

It is important to note that the heritability estimates for these two traits may in fact be somewhat lower than their true values, because gene–gene and gene–environment interactions are not accounted for in the analytical models. These interactions may therefore be increasing the apparent environmental and thus decreasing the apparent genetic effects in our heritability estimates. In future studies, it will be important to incorporate other covariates that have been suggested to correlate with plasma Aβ levels (Kuo et al., 1998), such as low-density lipoprotein, high-density lipoprotein, total cholesterol, and apoE levels, into the analysis to delineate the importance of these factors in the phenotypic variance of plasma Aβ levels. Once the effects of such covariates are determined and accounted for, the genetic proportion of variance may increase.

Based on the data presented in this chapter and our previous data (Younkin, 2000), we propose to use plasma Aβ levels as a quantitative trait that may underlie the complex genetic susceptibility to Alzheimer's disease. This method has the merit of utilizing data from more family members than do methods simply looking at disease state, thereby increasing the power of the study substantially. Other groups have successfully conducted linkage analysis on quantitative risk factors to identify genetic determinants that contribute to common complex diseases including reading disability and obesity (Cardon et al., 1994; Clement et al., 1996; Comuzzie et al., 1997; Almasy et al., 1999). Several groups have detected successful linkage to major loci for complex disorders using quantitative traits with heritability levels of

22–63% (Comuzzie et al., 1997; Rainwater et al., 1999). Our studies reveal that elevated plasma Aβ levels have a high heritability in typical LOAD families. Therefore, they may well represent quantitative traits that can be used as surrogate markers in linkage analysis of extended LOAD pedigrees. Indeed, we have already mapped one LOAD risk locus on chromosome 10 using $A\beta_{42}$ as a surrogate trait (Ertekin-Taner et al., 2000; Myers et al., 2000) in five extended LOAD pedigrees identified through a LOAD patient with extremely high plasma Aβ. It is likely that additional LOAD loci will be detected by this method as we evaluate the rest of the genome in our collection of pedigrees.

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29 Levels of Total and Deposited Aβ are Correlated with Dementia

S. PARVATHY, J. NÄSLUND, V. HAROUTUNIAN AND J. D. BUXBAUM

INTRODUCTION

Immunocytochemical studies using antibodies against amyloid β-protein (Aβ) have established that Aβ is deposited in neuritic plaques, which are spherical clusters of altered neurites that typically surround well-defined amyloid cores. Aβ is also deposited in large numbers of diffuse plaques, which are poorly circumscribed, immunoreactive lesions showing minimal neuritic change (Masliah et al., 1990).

The carboxy-terminal of Aβ is heterogeneous and may end at residues 40, 42, or 43 (Fukumoto et al., 1996; Mori et al., 1992). By using synthetic peptides it has been shown that the length of the carboxy-terminal of Aβ peptide is a critical determinant for the rate of amyloid fibril formation (Jarrett et al., 1993). Virtually all antibodies that have been developed so far as specific for Aβ ending at residue 42 do not, in fact, distinguish between Aβ ending at residue 42 and Aβ ending at residue 43 (Gravina et al., 1995; Iwatsubo et al., 1994; Mann et al., 1996; Wong et al., 1999). Furthermore, most studies in Alzheimer's disease examine only controls and advanced Alzheimer patients, where there has been extensive, chronic neurodegeneration. For these reasons, the role of the various Aβ species in dementia, and particularly early dementia, are not fully understood.

We have examined the deposition of $A\beta_{x–40}$, $A\beta_{x–42}$, and $A\beta_{x–43}$ in relation to dementia. In addition, the levels of total $A\beta_{x–40}$ and $A\beta_{x–42}$ in relation to dementia severity were studied. The results showed that Aβ accumulation and deposition starts early in the dementia process and that the deposition correlates with cognitive decline.

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METHODS

SUBJECTS

Seventy-nine subjects who had been residents of the Jewish Home and Hospital in Manhattan and the Bronx, New York, were included in the postmortem study. Only subjects with evidence of no significant neuropathology, or neuropathology consistent only with AD, were included in this study. Cognitive and functional status during the last six months of life was rated using the Clinical Dementia Rating (CDR) scale. Since one aim of this study was to identify whether there is a relationship between amyloid plaque deposition and clinical dementia, five groups of subjects were formed, consisting of cases falling into CDR score categories of 0.0 (non-demented), 0.5 (questionable dementia), 1.0 (mild dementia), 2.0 (moderate dementia), and 4.0 or 5.0 (severe dementia). The methods used for subject selection and cognitive and neuropathologic assessment have been described in detail previously (Davis et al., 1999; Haroutunian et al., 1998, 1999). The demographic data for the final selection of the subjects is shown in Table 29.1.

PREPARATION OF ANTIBODIES

Carboxy-terminal specific antibodies were developed by injecting mice with the peptides corresponding to the last six amino acids of $A\beta_{x-40}$, $A\beta_{x-42}$, and $A\beta_{x-43}$, which had been coupled to keyhole limpet hemocyanin. Specificity of the antibodies used was confirmed by western (immuno-) blots, dot blots, and ELISA.

Table 29.1. Demographic characteristics of subjects

	CDR scores				
	0.0 (n = 16)	0.5 (n = 11)	1.0 (n = 22)	2.0 (n = 15)	4&5 (n = 15)
Postmortem interval, mean (SD) (h)	8.6 (6.0)	5.5 (4.6)	4.8 (4.0)	6.1 (6.0)	5.8 (7.4)
Age, mean (SD) (years)	83.1 (10.0)	85.8 (8.3)	82.9 (8.2)	89.1 (5.7)	85.4 (10.3)
Age range (years)	64–99	69–94	74–103	74–97	62–103
Men, no. (mean age, years)	2 (82.3)	2 (77.5)	6 (88.0)	3 (83.7)	3 (69.0)
Women, no. (mean age, years) (89.0)	14 (84.1)	9 (87.7)	16 (89.7)	12 (90.4)	12

IMMUNOHISTOCHEMISTRY

Immunostaining was performed in sections of frontal cortex (5 μm) that were cut from paraffin-embedded tissue blocks, deparaffinized with xylene, and rehydrated. Endogenous horseradish peroxidase activity was quenched by incubating in hydrogen peroxide, and formic acid was used to enhance immunoreactivity. Following blocking the sections were incubated in the relevant antibodies, and the bound antibodies were detected using HRP-conjugated secondary system with Dab (diaminobenzidine) used as substrate. To quantify amyloid load the staining pattern was used to categorize labeling of each section into one of four levels (0, 1, 2, or 3) for both diffused and cored plaques. The accuracy of this analysis was confirmed by computer aided quantitative analysis for cored plaques in 25 out of the 79 cases. The quantitative values correlated very well with semiquantitative counts, with r values of 0.81, 0.89, and 0.75 for antibodies against $A\beta_{x-40}$, $A\beta_{x-42}$, and $A\beta_{x-43}$ respectively.

ENZYME-LINKED IMMUNOSORBENT ASSAY

ELISA from neutralized, formic-acid-extracted brain homogenates was carried out as described (Näslund et al., 2000).

DATA ANALYSIS

The five CDR score categories were used as independent variables against the dependent variables amyloid plaque load or soluble Aβ for subsequent data analysis. Spearman rank-order correlation was used to calculate the r and p values, and $p < 0.05$ was considered significant.

RESULTS AND DISCUSSION

CORRELATION OF LEVELS OF Aβ WITH DEMENTIA

Levels of both $A\beta_{x-40}$ and $A\beta_{x-42}$ increased with progression of dementia (Figure 29.1; Näslund et al., 2000). The levels of these peptides correlated very well with dementia in all brain areas, with r values ranging from 0.27 to 0.72 and p values ranging from <0.001 to 0.01 (Näslund et al., 2000).

CORRELATION OF AMYLOID PLAQUE DEPOSITION WITH DEMENTIA

Levels of diffuse and cored plaques in frontal cortex sections of these cases were categorized with arbitrary values ranging from 0 to 3 depending on the load of amyloid plaques. Amyloid deposition for each antibody, both as

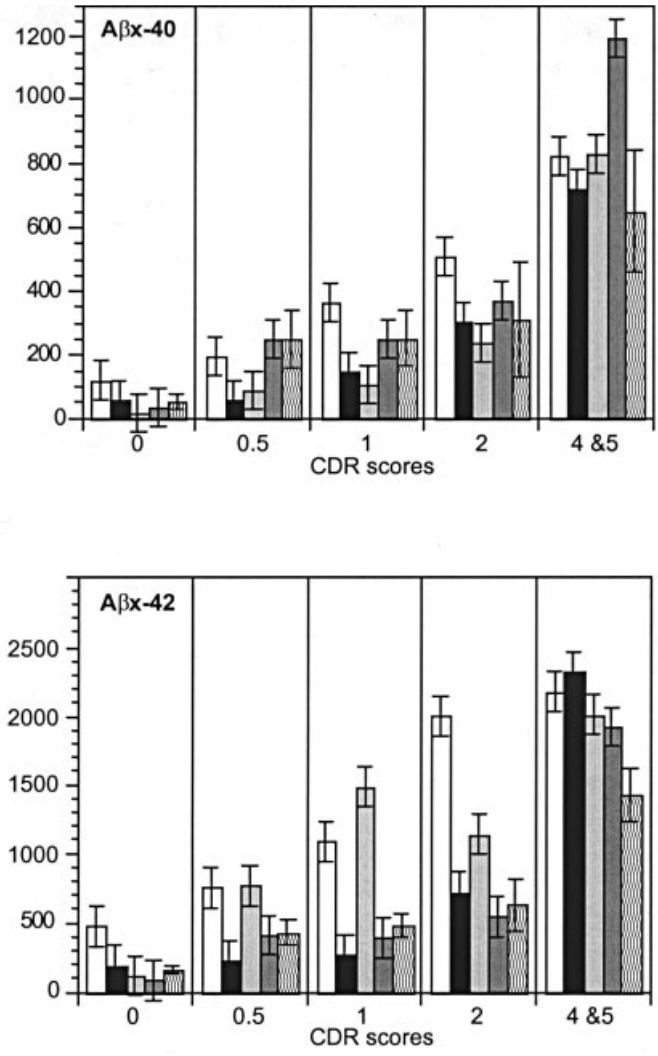

Figure 29.1. Levels of $A\beta_{x-40}$ and $A\beta_{x-42}$ in different cortical regions as a function of dementia severity. Levels of $A\beta_{x-40}$ and $A\beta_{x-42}$ were measured in the middle frontal gyrus, superior temporal gyrus, entorhinal cortex, inferior parietal lobule, and primary visual cortex using ELISA. Data are presented as mean $\pm$ SEM

diffuse and cored plaques, in relation to CDR scores is shown in Table 29.2. Generally amyloid plaque load increased with the CDR score. With all three antibodies there was a strong correlation between CDR scores and cored plaques, with p values <0.0001 and r values >0.47 (Table 29.2). In the case of diffuse plaques, levels of $A\beta_{x-42}$ and $A\beta_{x-43}$ plaques correlated ($p<0.0001$ and

Table 29.2. Relationship between levels of diffuse or cored plaques and CDR score. Sections were incubated with each of the carboxy-terminal specific antibodies and the bound antibodies were detected using HRP-conjugates and Dab-substrate. Sections were given arbitrary scores for both diffuse and core plaques ranging from 0 to 3, and average ± SEM scores are shown. Diffuse and cored amyloid plaque load was correlated with CDR scores using Spearman rank-order analysis. Correlation values (r) and p values are shown

	$A\beta_{x-40}$ staining		$A\beta_{x-42}$ staining		$A\beta_{x-43}$ staining	
	Diffuse	Cored	Diffuse	Cored	Diffuse	Cored
CDR 0	0.20 ± 0.11	0.00 ± 0.00	0.31 ± 0.15	0.25 ± 0.11	0.13 ± 0.09	0.38 ± 0.13
CDR 0.5	0.00 ± 0.00	0.00 ± 0.00	0.45 ± 0.21	0.27 ± 0.14	0.36 ± 0.15	0.82 ± 0.23
CDR 1.0	0.25 ± 0.09	0.25 ± 0.14	0.95 ± 0.25	0.48 ± 0.15	0.66 ± 0.17	0.90 ± 0.18
CDR 2.0	0.23 ± 0.11	0.69 ± 0.24	1.20 ± 0.31	0.93 ± 0.28	1.20 ± 0.26	1.60 ± 0.27
CDR 4&5	0.40 ± 0.13	1.13 ± 0.32	2.47 ± 0.24	1.80 ± 0.33	1.66 ± 0.30	2.33 ± 0.27
r	0.2372	0.5312	0.5508	0.4723	0.5253	0.5986
p	0.0418	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

$r > 0.52$), while levels of $A\beta_{x-40}$ did not correlate significantly ($p = 0.04$ and $r = 0.24$) (Table 29.2). Interestingly, levels of $A\beta_{x-40}$ diffuse plaques remain relatively unchanged across CDR scores. Amyloid plaque load for each species increased significantly early in the dementia process.

CONCLUSION

There is a correlation between clinical dementia and both total Aβ and amyloid plaque deposition in the cortex. In addition, total Aβ and deposited Aβ apparently increase in the earliest stages of dementia. Our studies show that Aβ load plays an important role in early dementia and in cognitive decline.

SUMMARY

Accumulation of senile plaques containing abundant amyloid β-protein (Aβ) fibrils is a pathologic hallmark of Alzheimer's disease. It remains controversial whether these pathological changes lead to dementia or cognitive decline. The major component of the senile plaques is Aβ, a 40- to 43-residue protein. We developed carboxy-terminal specific antibodies that specifically recognize $A\beta_{x-40}$, $A\beta_{x-42}$, and $A\beta_{x-43}$ species, and do not cross-react with any other Aβ

forms. We examined the staining pattern with these antibodies in the frontal cortex of elderly patients with Clinical Dementia Rating scores ranging from 0.0 to 5.0 (representing cases with no dementia to severe/terminal dementia, respectively). In addition, in the same set of subjects we measured the levels of total (deposited and soluble) $A\beta_{x-40}$ and $A\beta_{x-42}$ in five neocortical brain regions. We observed that levels of total $A\beta_{x-40}$ and $A\beta_{x-42}$, and levels of $A\beta_{x-40}$, $A\beta_{x-42}$, and $A\beta_{x-43}$ deposited in cored plaques, correlated strongly with dementia severity. Levels of diffuse $A\beta_{x-42}$-reactive and $A\beta_{x-43}$-reactive plaques correlated with dementia as well. These studies support a role for Aβ in early dementia.

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30 What Should We Tell Patients Attending a Memory Disorders Clinic About Their Diagnosis?

CONOR P. MAGUIRE AND REBECCA M. SLINN

INTRODUCTION

The accuracy of the diagnosis of cognitive impairment, Alzheimer's disease (AD), and other forms of dementia has improved over the past ten years and, using standardized diagnostic techniques, most centers achieve a high rate of correct diagnosis. Progress has been made in delineating the genetic, neurophysiological, and neuropathological basis of Alzheimer's disease, bringing closer the possibility of presymptomatic testing, diagnosis, and treatment. Despite these advances, dementia and particularly Alzheimer's disease is still publicly regarded as progressive, incurable, and resulting in the erosion of an individual's intellect and personality. Possibly as a result of this public perception, there is a lack of consensus among health professionals as to whether and in what manner patients with dementing illnesses should be told their diagnosis. Less than 40% of Memory Clinics in Britain and Ireland have guidelines for sharing the diagnosis and in only 56% are patients told their specific diagnosis (Gilliard and Gwilliam, 1996).

As clinicians we are obliged to be truthful to our patients, but we are also obliged to protect them from harm. Two main approaches to medical ethics have been used to address the issue of whether doctors should inform a patient of a grim diagnosis: one takes a strong rights-oriented position; the other takes a best outcome position (Salsibury, 1985). Most doctors would agree that any patient should have the opportunity to know what is wrong with them, but the evidence on what course of action produces the best outcome for the AD patient is sparse. The patient with AD may have impaired insight, altered decision-making capabilities and be more susceptible to behavioral problems. Telling such a patient their diagnosis may either have little behavioral effect or may plunge them into a spiral of agitation and

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depression: this lack of evidence and uncertainty results in a reluctance among family members and clinicians to inform patients of their diagnosis.

ATTITUDES OF CARERS AND POTENTIAL PATIENTS: RESEARCH EVIDENCE

This quandary is reflected by recent surveys that demonstrate a paternalistic approach by doctors and family members in withholding the diagnosis from patients. In a 1996 study, 100 family members of patients with AD consecutively attending a memory disorders clinic were asked whether the patient should be told their diagnosis (Maguire et al., 1996). Of these, 17 felt that their relative should be told their diagnosis and 83 said that they should not. The family members were asked to give the single main reason why the patient should or should not be informed. The main reason given for not revealing the diagnosis (51 family members) was that it would upset or depress the patient. Only five family members stated that it was the 'right' of the patient to be told their diagnosis.

When subsequently asked whether they themselves would want to be told the diagnosis should they ever develop AD, 71 family members said that they would want to be informed and 29 would not. The majority of those who expressed a wish to be told (36 family members) said that it would be their right to be told their diagnosis. This study illustrates the paradox between what relatives of AD sufferers wish for the patient and what they would wish for themselves.

In determining the attitudes of older adults on being told the diagnosis of dementia, 156 community-dwelling healthy older persons were presented with vignettes of two patients, one with AD and one with terminal cancer, and then questioned about their attitudes towards these illnesses (Holroyd et al., 1996). Most participants (79.5%) responded that they would prefer to know if they had AD, but the number was significantly fewer than those who would want to know if they had terminal cancer (91.7%). Among married subjects, 80.2% would want their spouse to know if they had cancer but only 65.7% would want their spouse to know if the spouse had AD. Paternalism is common in situations where one individual feels overprotective towards a more vulnerable second individual. In a condition such as dementia where there may be an erosion in insight and reduction in control of emotion, paternalism is frequent in carers and this is an understandable and predictable psychological response.

In a study by Erde et al., 224 healthy adult patients aged over 21 years were asked if they would like to be told that they have a diagnosis of AD (Erde et al., 1988). Ninety percent expressed a wish to be told of the diagnosis, citing such benefits as being able to plan for the future and being able to obtain a second opinion. However, few people within this group knew

someone with dementia-like symptoms (30.5%) and the majority was young (43.7% aged <50 years) so the scenario was largely hypothetical. Interestingly, the survey raised the question of confidentiality: almost one-third (31.9%) reported that they would want no one told if they were not told themselves, 12.1% indicated that they would not want their spouse told, and 18.2% indicated that they would not want adult children told.

ATTITUDES OF PROFESSIONALS: RESEARCH EVIDENCE

Paternalism is not confined to carers. Clinicians have been shown to resist informing patients with dementia about their diagnosis (Drickamer and Lachs, 1992). A survey among primary care physicians demonstrated that 90% of a sample of 498 reported that they would be very likely to inform an adult daughter of a parent's diagnosis of a dementing illness, whereas only one-half of the physicians reported that they would be very likely to disclose the diagnosis to the patient directly (Fortinsky et al., 1995). In a second survey, only 39% of general practitioners would often or always tell patients a diagnosis of dementia, as opposed to 95% who would often or always tell a patient a diagnosis of terminal cancer (Vassilas and Donaldson, 1998). Similarly, psychiatrists nearly always tell carers the diagnosis of dementia, yet patients with moderate and severe dementia are rarely told (Rice and Warner, 1994). Throughout all these surveys two main reasons were given for withholding the diagnosis: the fear that informing the patient will induce depression, anxiety or even suicide, and the presumption that the patient with dementia will not understand the diagnosis and therefore 'there is no point' in telling them. The evidence supporting these views is limited, however, and will be explored further throughout this article.

There are similarities in the way that doctors manage the disclosure of the diagnosis of dementia today with that of cancer 40 years ago. In 1961, the vast majority of doctors (90%) expressed a preference for not telling a patient with cancer his/her diagnosis (Oken, 1961). By 1979, a complete reversal of opinion had occurred with 97% of doctors expressing a preference for informing the patient that he or she had cancer (Novack et al., 1979). The reasons for not telling cancer patients their diagnosis in 1961 were similar to those quoted for not telling patients with AD their diagnosis now: the stigma associated with the condition, the fear that disclosing such a diagnosis might have a detrimental effect on the individual's emotional state, and because treatment options were few.

In the 1961 study, Oken suggested that doctors were reluctant to tell cancer patients a diagnosis with a bleak prognosis, as it would deprive the patient of hope (Oken, 1961). The reasons for the change in policy and

attitude since then are multifactorial, including an improvement in therapy for cancer, an increase in public awareness of the disease, the involvement of cancer patients in research protocols and drug trials necessitating informed consent, and the change in physician–patient relationship with an increased emphasis on patient autonomy. These same factors have been cited to support diagnosis disclosure to demented patients; however, Novack et al. found that the four most common factors considered in the decision to tell the cancer patient his diagnosis were age, intelligence, relative's wish about telling the patient, and emotional stability. Only 10% were inclined to tell the diagnosis to a cancer patient who was old or who had 'poor comprehension' (Novack, 1979). There are valid reasons for comparing the plight of the dementia patient now with that of the cancer patient in the 1960s, but the vast majority of cancer patients retain insight into their deficits and have intact decision-making abilities, factors that are frequently impaired in dementia.

ATTITUDES OF PATIENTS: THE PAUCITY OF RESEARCH EVIDENCE

The psychodynamic effects of telling the diagnosis to seven patients with mild to moderate states of probable AD has been described by Bahro et al. in a clinical experience report (Bahro et al., 1995). Six patients exhibited partial or complete denial, including avoidance in naming their illness, vagueness and somatization. Only one patient who was more aware of her illness both cognitively and emotionally developed depression that appeared to reflect appropriate mourning. A significant correlation has been reported between denial and severity of cognitive impairment. This suggests that denial of probable AD reflects the patient's inability to understand the consequences of the diagnosis. This may be caused by lack of insight due to disruption of their cognitive abilities (Sevush and Leve, 1993). Although the evidence is limited, the degree of insight into or awareness of their dementia in patients with AD appears to decrease progressively as the severity of dementia increases (McDaniel et al., 1995; Mangone et al., 1991; Zanetti et al., 1999). In the study by Zanetti et al., insight was uniformly high for patients with Mini-Mental State Examination (MMSE) scores greater than or equal to 24, showed a linear decrease between MMSE scores of 23 and 13, and was uniformly low for MMSE scores below 13. Most clinicians will acknowledge that patients with a diagnosis of a dementing illness are often oblivious of their deficits. Patients with cognitive impairment insufficient for a diagnosis of dementia are more likely to self-refer for assessment and therefore more likely to have retained insight into their deficits.

THE FEARS OF DISCLOSURE

One of the reasons why relatives are wary of the patient being told their diagnosis is the fear that the dementia sufferer will develop increased symptoms of anxiety and depression. Fifty-one percent of family members in the study by Maguire et al. felt that informing the patient of their diagnosis would depress or agitate the patient; only 4% said that the patient would cope better if told their diagnosis (Maguire et al., 1996). It is known that depression is increased by up to 23% in cognitively impaired geriatric outpatients, most commonly in the early stages (Reifler et al., 1982). Sevush reported a negative correlation between denial and depression, suggesting that depression in AD may be reactive in nature (Sevush and Leve, 1993).

There is no established evidence that informing patients of their diagnosis results in a significant increase in behavioral disturbance. Behavioral disturbance including agitation, depression, and anxiety are common in dementia, and it could as easily be argued that such symptoms might be reduced by informing the patient that they are suffering from an organic disease process and by involving them in the diagnosis and management of such symptoms. Ethical guidelines including those issued by the British General Medical Council state that one should not withhold information necessary for decision making unless one judges that disclosure of some relevant information would cause the patient serious harm. In this context, however, serious harm 'does not mean the patient would become upset, or decide to refuse treatment' (Seeking patients' consent: the ethical considerations, 1999).

Another reason quoted for withholding the diagnosis from cognitively impaired or demented patients is the fear that the patient might consider suicide if told about their illness. Four percent of patients attending a memory disorders clinic report suicide ideation or the 'wish to die' (Draper et al., 1998). Although the incidence of suicide in AD is rare, probably due to impaired decision making capability, cases of suicide in patients told their diagnosis have been described (Ferris et al., 1999; Rohde et al., 1995). One of four patients described by Rohde et al. committed suicide with physician assistance. All four patients were highly educated professionals with mild AD and had good insight as to the nature of their disease and their future severe disability. All were participating in trials of pharmacological treatment for AD and were aware of not responding to the treatment. The traumatic effects of caring for a patient with dementia are illustrated by the finding that 6% of family members in the study by Maguire et al. said that they would want to be told their diagnosis so that they could commit suicide. A further 2% stated that they would not want to be told their diagnosis in case it would lead them to commit suicide (Maguire et al., 1996). This sentiment was also expressed by some older adults in Holroyd's study (Holroyd et al., 1996).

The fact that suicide is raised as a consideration should individuals develop AD indicates that the disease is still perceived as devastating by many, particularly by relatives of sufferers. It does not mean, however, that information should be widely withheld from patients for fear that they might commit suicide. Individuals who are perceived as high suicide risk, irrespective of their underlying diagnosis, should be referred for specialist management and intervention as part of the process of imparting information.

THE ETHICAL DIMENSION: RIGHTS AND WRONGS

Many feel that it is the right of any individual to know their diagnosis no matter what their illness and prognosis. In the study by Maguire et al., only 5% of family members said that their relative suffering from AD had the right to be told their diagnosis (Maguire et al., 1996). Despite this, 36% of family members quoted their 'right to know' as the main reason why they should be told their own diagnosis. This inconsistency may represent a paternalistic desire by family members to protect patients from the harsh reality of their disease, but telling anyone of the patient's diagnosis without first informing the patient may be a breach in patient confidentiality guidelines.

The report of the British Medical Association and The Law Society on the Assessment of Mental Capacity states that 'doctors are bound by a professional duty to maintain the confidentiality of personal health information unless the patient gives valid consent to disclosure or, if the patient is incapable of giving consent, the doctor believes disclosure to be in that person's best interests' (Assessment of Mental Capacity, 1995). The consequence of this guideline is that clinicians must first ask the patient attending the memory disorders clinic whether diagnostic information may be shared with their family members or carers. If the clinician has concerns about the patient's ability to give such consent, these concerns and the reasoning behind these concerns should be documented before diagnostic information is shared. If patients lack capacity they do not forfeit their right to control disclosure of diagnostic information: such patients can authorize or prohibit the sharing of information about themselves if they 'broadly understand the implication of so doing.' It is only in rare circumstances when one feels that the patient would be seriously harmed by the information, that one may disclose information to others without consent.

In 1995 the Fairhill Guidelines on Ethics of the Care of People with AD were published in the United States (Post and Whitehouse, 1995). These guidelines were drawn up by a focus group of professionals working in the field of dementia after a series of meetings with family caregivers and individuals with mild dementia of the Alzheimer's type. The main recommendation of the group was that physicians should inform affected individuals and their families about the diagnosis of probable AD. If a family

objects to the patient being present when the diagnosis is disclosed, the group stressed the importance of clarifying that the patient has a moral and legal right to be present and to receive a specific diagnosis unless he or she waives it.

The ethical dilemma of whether patients with AD should be informed of their diagnosis extends into any research that involves such patients. If a patient is to be involved in a research protocol, whether therapeutic or non-therapeutic, they must give informed consent to participate and, in order to give such consent, they must be aware of what condition they have. When a patient lacks capacity to consent to his or her participation in therapeutic research, no other person has the authority to give such consent on the patient's behalf, but it is generally accepted that it is lawful to carry out therapeutic research involving an adult who lacks capacity to consent provided that such research is in the patient's 'best interests' (Assessment of Mental Capacity, 1995). The guidance for doctors on the involvement of persons lacking capacity in non-therapeutic research states that such research may be unlawful (Law Commission Report 231, 1995).

In most memory clinics around the world, trials are ongoing of cognition-enhancing drugs that may halt the progression of AD. Doctors are obliged to obtain informed consent in order to recruit subjects into any trials or research projects. A survey of researchers at the 15 federally funded US Alzheimer's Disease Research Centers, however, found a significant lack of uniformity on the assessment of subjects to consent for research, proxy informed consent usually supplied informally by family members, and minimal use of durable powers of attorney (High, 1993).

As patients in the early stages of the disease who are still competent are more likely to be recruited into therapeutic trials and research projects, it is ethically correct that they should be fully informed of the reason for and consequences of consenting to participate. More severely affected subjects with cognitive impairment are frequently involved in medical research and decisions by surrogates on their behalf have been shown often not to accurately reflect the patient's wishes (Warren et al., 1986).

A patient's capacity to consent is judged through medical assessment in a clinical setting: 'competence' is a legal term and is decided upon by the court. The capacity to make decisions includes 'capacities to understand and communicate, to reason and deliberate with future consequences in mind, and to possess some stability in values and goals' (Post and Whitehouse, 1995). The manner and degree of capacity assessment should depend on the likely harms or benefits that will follow from the decision, therefore high-risk consequences require a more stringent assessment than low-risk consequences. This 'sliding-scale' model for decision making proposes that the patient's capacity to decide is judged with increasing stringency as the decision becomes more potentially harmful (Drane, 1985; Fellows, 1998).

A person with decisional capacity to consent to investigation or treatment needs to be able to understand the medical situation and prognosis, the nature

of the recommended care, the risks and benefits of each alternative, and the likely consequences of any care. This means that such persons should be fully aware of their diagnosis. Where patients have difficulty retaining information, or are only intermittently competent to make a decision, the British General Medical Council recommends that the clinician should provide any assistance that the patient might need to reach an informed decision. The clinician should record any decision made while the patient was competent and this decision should be regularly reviewed in order to establish that the patient's views are consistent (Seeking patients' consent: the ethical considerations, 1999). The importance in reviewing the patient's view is to ensure that their decision remains stable over time—the fact that the decision is forgotten from one day to the next may not be relevant as long as the patient makes the same choice again when presented with the same options and information.

CONCLUSION

Advances have been made in the fields of the cause, accurate diagnosis, and treatment of cognitive impairment and dementia over the past ten years. In many cases, however, the people who are most affected by these illnesses—the patients—are not told their diagnosis. The opinions of family members, spouses, and older adults on whether AD patients should be told their diagnosis have been obtained in various studies. Thus far, the opinions of those diagnosed with cognitive impairment and dementia on being told the diagnosis have not been sought.

The increase in therapeutic options and the greater involvement of cognitively impaired and demented patients in clinical research programs obliges clinicians to examine their own prejudices in regard to diagnostic disclosure and to be willing and able to defend their decision on any given patient. It is vital, therefore, that capacity to consent is assessed for all patients attending memory disorder clinics—not only for participation in research, but also for commencing therapy and planning for future care.

It could be argued that patients with mild dementia and cognitive impairment should be monitored particularly for depressive symptoms after attendance at a memory disorders clinic as they are more likely to retain insight. Determining patients' level of insight into their illness should be an integral part of a memory disorder clinic assessment. It may not be justifiable to completely withhold information from patients with limited insight into their illness, but the experienced clinician should be able, by careful questioning, to ascertain in what detail that information should be conveyed. For some patients, it may be sufficient to disclose that they have a memory problem that may deteriorate with time; for others, it is necessary to discuss the diagnosis in detail, including the prognosis and therapeutic options.

The dementing illnesses and specifically AD present in a myriad different ways and affect no two individuals alike. Clinicians who care for patients with cognitive impairment and dementia need to weigh up the rights of the patient to be told their diagnosis against the rights of those who live with and care for the patient once they are told that diagnosis. There is a spectrum of information between telling patients nothing about their illness and telling them everything. The question of whether AD subjects should be told their diagnosis has to be looked at on an individual basis with clinicians remaining sensitive to the fears and rights of the patient and the family, and a skilled clinician should evaluate what information best fits the individual patient.

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31 Innate Immunity, Autotoxicity and Degenerative Neurologies

PATRICK L. McGEER, KOJI YASOJIMA AND EDITH G. McGEER

The lesions of established Alzheimer's disease (AD) are characterized by the presence of a broad spectrum of inflammatory molecules (for review, see Neuroinflammation Working Group, 2000). They include complement proteins and their regulators, inflammatory cytokines, acute-phase reactants, and numerous proteases and protease inhibitors. Neurons, astrocytes and microglia participate in their production. Several of these inflammatory products are known to be toxic to neurons, providing a rational basis for the hypothesis that neuroinflammation is a major contributing factor to the pathogenesis of AD (McGeer and Rogers, 1992).

This hypothesis has been greatly strengthened by reports that polymorphisms in promoter and other untranslated regions of the inflammatory cytokine genes IL-1α, IL-1β, IL-6 and TNFα significantly enhance the odds ratios for contracting AD (Bagli et al., 2000a,b; Bhojak et al., 2000; Collins et al., in press; Du et al., 2000; Grimaldi et al., 2000; Nicoll et al., 2000; Papassotiropoulos et al., 1999; Rebeck, in press; see other chapters, this volume). These data indicate that the level of expression of inflammatory cytokines influences the onset of AD. It has been additionally strengthened by further epidemiological studies supporting earlier findings (McGeer et al., 1996) that chronic use of non-steroidal antiinflammatory drugs (NSAIDs) reduces the risk of contracting AD. Stewart et al. (1997), in the Baltimore longitudinal study, found that patients who were using NSAIDs for more than 2 years had the risk of AD reduced by 60% (Figure 31.1). Veld et al. (2000), in the Rotterdam aging study, after verifying NSAID use by prescription records, found that the reduction in AD following more than 2 years' use of NSAIDs was 80%, even higher than that reported from the Baltimore study. The sparing was close to that originally found in our study of rheumatoid arthritics, who had presumably been taking NSAIDs and other inflammatory drugs for many years (McGeer et al., 1990) (Figure 31.1).

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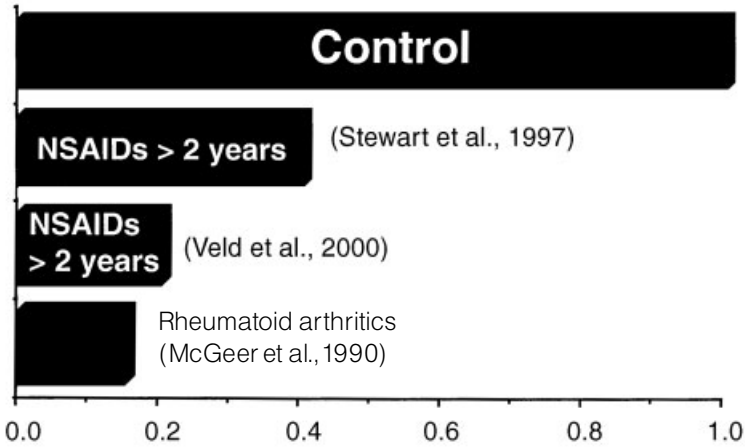


Figure 31.1. Odds ratios for the development of AD noted in three studies of populations taking NSAIDs as compared to that in age-matched controls (Stewart et al., 1997; Veld et al., 2000; McGeer et al., 1990). See McGeer et al. (1996) for data from other epidemiological studies

Thus, there is a strong correlation in AD between post mortem pathology showing neuroinflammatory damage, genetic polymorphisms influencing inflammatory cytokine upregulation, and epidemiological studies revealing sparing of disease amongst those using antiinflammatory drugs.

The absence of antibodies and minimal presence of lymphocytes indicate that the elements of a classical autoimmune disease are absent in AD tissue. The neuroinflammation must then be viewed as evidence of an innate immune response which has self-damaging effects. We define this phenomenon as 'autotoxicity' in order to distinguish it from 'autoimmunity'.

We describe here some of the characteristics of autotoxicity as found in AD tissue. Among the most important are the complement system and activated microglia. The tangles and plaques of AD are marked by the opsonizing complement components, C4d and C3d, and dystrophic neurites in AD brain are immunostained for the membrane attack complex (C5b-9) (Neuroinflammation Working Group, 2000). This lytic molecule is designed to destroy foreign bacteria and viruses. There are multiple protective molecules which defend host cells against self-attack. However, if the concentration of the membrane attack complex exceeds the levels of defensive proteins in host tissue, then self-attack can occur in a process called bystander lysis. It is the smoking gun of autodestruction in AD, since dystrophic neurites are observed immunostained for this complex.

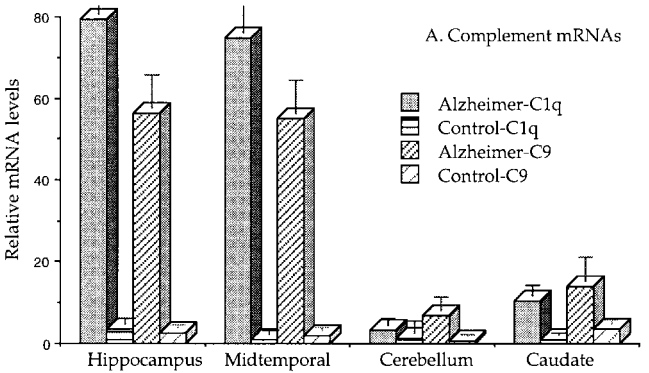
It is of great interest to know which cells are responsible for generating the complement proteins and how they become activated. Neurons (Terai et al., 1997), astrocytes and microglia (Neuroinflammation Working Group, 2000)

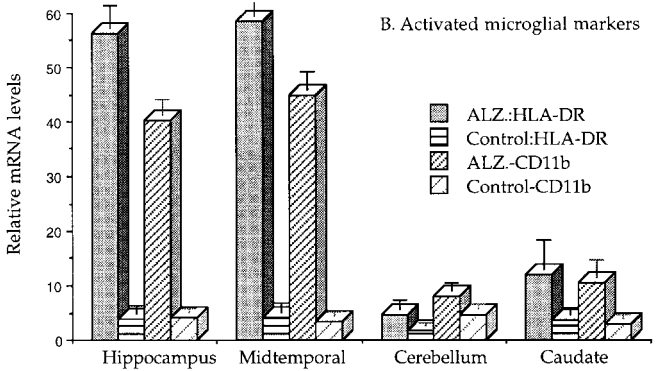
and even endothelial cells (Klegeris et al., 2000) are producers of complement components, so there are many potential sources for the activated complement fragments in brain. Neurons appear to be the most abundant generators. As far as activation is concerned, β-amyloid protein and the pentraxins, amyloid P and C-reactive protein, have all been shown to activate the classical pathway *in vitro* (Neuroinflammation Working Group, 2000). All are associated with AD senile plaques. We have recently shown that neurons are also the major producers of amyloid P and C-reactive protein (Yasojima et al., in press), and it has long been assumed that β-amyloid protein comes from neurons. Thus, neurons are the source of both the complement proteins and the activators that ultimately result in self-attack by the membrane attack complex.

Microglia constitute a few percent of the glia of brain and are normally in a quiescent state. When activated by an insult or injury to the brain, they change their shape and upregulate a large number of proteins, such as the complement receptor CD11b and the major histocompatibility class II glycoprotein HLA-DR. Activated microglia also produce massive amounts of oxygen radicals and other materials which may, in themselves, damage host cells. *In vitro*, cultured microglia secrete materials that are directly toxic to neurons (Klegeris and McGeer, 2000; Giulian et al., 1996). Thus, the complement system and activated microglia are both potential sources of neuronal destruction.

In order to estimate the intensity of inflammatory reactions, we have measured the levels of the mRNAs for complement proteins (Yasojima et al., 1999), two microglial markers (HLA-DR and CD11b) and the pentraxins, C-reactive protein and serum amyloid P, in normal and AD brain, as well as in osteoarthritic joints, atherosclerotic plaques, infarcted hearts and adjacent normal tissue. We have also measured levels in the liver and other peripheral organs. To obtain such data, total RNA is extracted from post mortem tissue, and the relative levels of mRNAs determined by RT–PCR. Messenger RNAs have been found to be surprisingly stable so that useful data can be obtained from post mortem tissue (Yasojima et al., 1999; Johnson et al., 1986; Morrison and Griffin, 1981). The results indicate an innate inflammatory reaction in pathological material from each of these diseases.

In AD brain, the regional upregulation is highly related to the degree of pathological involvement. In areas with a heavy burden of plaques and tangles, such as the entorhinal cortex, hippocampus and temporal cortex, there are very large increases in the mRNAs, while only small increases are observed in areas such as the caudate or cerebellum, with little pathological involvement (Yasojima et al., 1999). Figure 31.2A illustrates this for complement C1q and C9, Figure 31.2B for HLA-DR and CD11b, and Figure 31.2C for C-reactive protein and amyloid P. It was long thought that these last two were only synthesized in the liver but we have now shown that





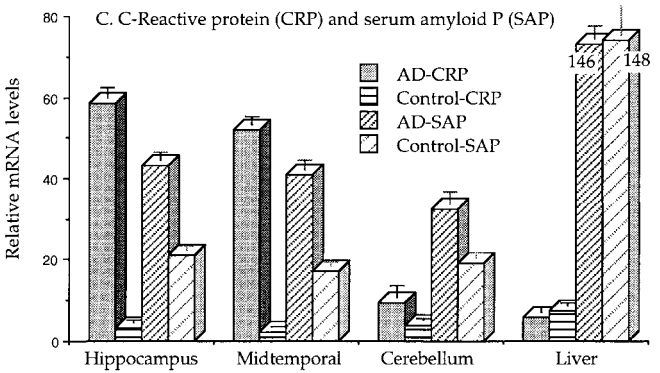


Figure 31.2. (A) Relative levels (mean+SEM) of the mRNAs for the complement proteins C1q and C9 in AD and control hippocampus, midtemporal gyrus, caudate and cerebellum. Data on all the complement mRNAs in 11 regions of AD and control brains have been published (Yasojima et al., 1999). (B) Relative levels of the mRNAs for the markers of activated microglia CD11b and HLA-DR in AD and control hippocampus, midtemporal gyrus, caudate and cerebellum. (C). Relative levels (mean+SEM) of the mRNAs for C-reactive protein (CRP) and serum amyloid P (SAP) in AD and control hippocampus, midtemporal gyrus, cerebellum and liver

the mRNAs exist in brain and are upregulated in AD in pathologically affected regions (Yasojima et al., in press). Figure 31.2C also illustrates that the levels of the mRNAs for C-reactive protein and serum amyloid P were unchanged in AD liver as compared to controls. There was also no difference in the mRNA levels for either pentraxin in AD heart, spleen or kidney, as compared to the corresponding control tissues (Yasojima et al., in press).

Upregulation of the mRNAs for these inflammatory markers is also found in osteoarthritic joints, atherosclerotic plaques and infarcted hearts (Figure 31.3). The initial causes of pathology are quite different—plaques and tangles in AD, anoxia in heart disease, too much fat in atherosclerosis, and mechanical grinding in osteoarthritis. But the secondary process is common to all. The innate immune system may be playing a role in all of these conditions, and the level of its involvement can be measured by the general technique described. However, judging from the mRNA levels of these markers (Figure 31.4), the degree of inflammation is as large or larger in AD hippocampus than in any of these peripheral conditions (Figure 31.4). The mRNA levels for these markers in liver, spleen and kidney were unaffected in any of these diseases, emphasizing the local nature of the innate immune response (Yasojima et al., 1998, 1999).

The pentraxins and complement are ancient host defense mechanisms that can trace their lineage back at least as far as the horseshoe crab. They are key components of the innate immune system. The widespread ability of mammalian tissue to generate these proteins is consistent with this role. By contrast, the adaptive immune system, which is an invention of vertebrates, relies on the cloning of lymphocytes. Generation of cells is restricted to peripheral lymphatic organs. Thus, in any inflammatory process, the role of the innate immune system must be carefully evaluated to determine the extent to which it is involved.

Agents which reduce the inflammatory burden should be beneficial not only in AD but in cardiovascular disease, arthritis and other diseases in which chronic inflammation is prominently associated with the pathology. Cyclo-oxygenase (COX) inhibitors (NSAIDs) are a known class that reduces inflammation by lowering prostaglandin production. The epidemiological data reported to date (McGeer et al., 1990, 1996; Stewart et al., 1997; Veld et al., 2000; Figure 31.1) are based on the use of traditional NSAIDs, which are

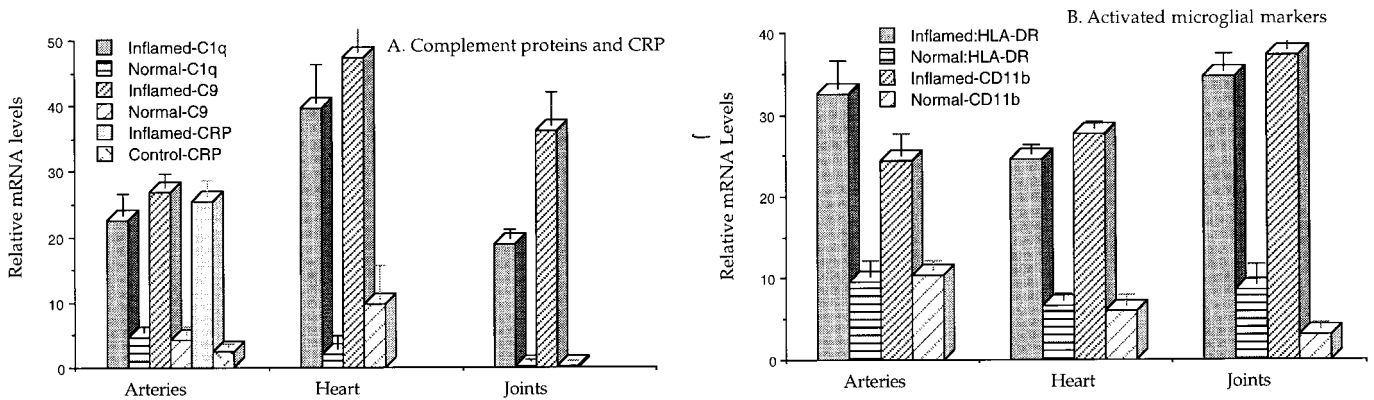


Figure 31.3. (A) Relative levels of the mRNAs for C1q and C9 in osteoarthritic joints, atherosclerotic plaques and infarcted heart tissue as compared with those in normal joints, normal artery and normal heart tissue. (B) Relative levels of the mRNAs for HLA-DR and CD11b in osteoarthritic joints, atherosclerotic plaques and infarcted heart tissue as compared with those in normal joints, normal artery and normal heart tissue. Data for CRP mRNA levels are also given for normal artery and atherosclerotic plaques. All tissues were post mortem except for the joint tissue, which was surgically removed because of intractable pain

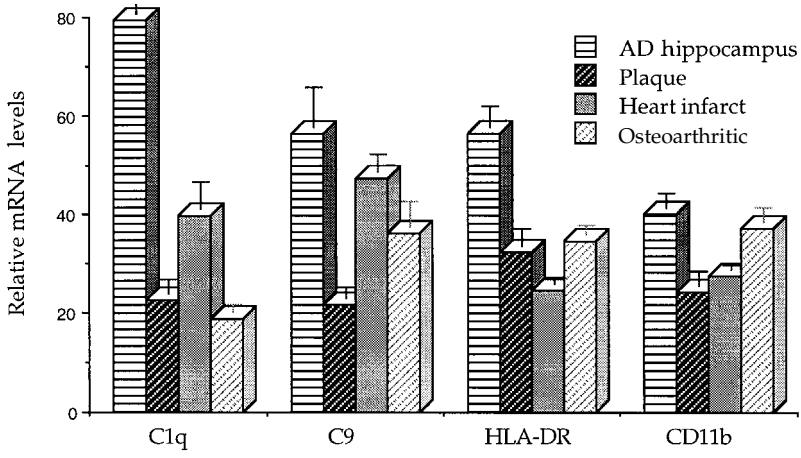


Figure 31.4. Comparison of the levels of the mRNAs for C1q, C9, HLA-DR and CD11b in AD hippocampus, osteoarthritic joints, atherosclerotic plaques and infarcted heart tissue

either inhibitors of COX-1 or mixed inhibitors of COX-1 and COX-2. Considerable interest has been shown in the possible use of selective COX-2 inhibitors because of their lesser gastrointestinal effects. However, COX-2 inhibitors may not be useful because this enzyme shows a high constitutive expression in neurons (McGeer, 2000). Animal experiments suggest that COX-2 may be performing adaptive functions associated with normal neurons and protective functions associated with stressed neurons. COX-1 is found in activated microglia. MacKenzie and Munoz (1998), in assessing the level of microglial activation in post mortem human brain, found that patients taking traditional NSAIDs had significantly fewer activated microglia. This occurred in those with and without tangles and plaques, indicating that such NSAIDs generally reduce the degree of microglial activation. Thus, COX-1 inhibitors may be superior to COX-2 inhibitors for the treatment of AD.

NSAIDs inhibit cyclooxygenase and thereby interfere with the production of prostaglandins. Prostaglandins are known to be inflammatory mediators, although far from the most powerful ones. NSAIDs therefore strike at fringe players in this whole autotoxic and inflammatory scheme. Analysis of lesion-associated inflammatory molecules in AD and other inflammatory conditions suggests more promising therapeutic targets. These include inhibitors of complement production, release and activation, blockers of inflammatory cytokines and anaphylotoxins, and diminishers of microglial activation.

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32 Neuroinflammatory Responses in the Alzheimer's Disease Brain Promote the Oxidative Post-translational Modification of Amyloid Deposits

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INTRODUCTION

Indisputable evidence indicates that an inflammatory response is associated with neuron and neurite damage and the deposition of Aβ and neurofibrillary tangles (NFTs) in Alzheimer's disease (AD; see Neuroinflammation Working Group, 2000, for a comprehensive review). Just as in the periphery, where degenerating tissue and insoluble materials (resulting from trauma, embolism and rupture) promote inflammation, so too these classical stimulants also promote inflammation in the AD brain. From a spatio-temporal perspective, the stimuli promoting neuroinflammation are microlocalized and are present from early preclinical to the terminal stages of AD. Likewise, the upregulation of acute phase proteins, complement, cytokines and other inflammatory mediators also is microlocalized, and chronic. An integral component of the inflammatory response is the localization of microglia to the inflammatory stimuli (protein deposits) and their activation, resulting in the release of the oxidizing system of O_2, myeloperoxidase (MPO) and nitric oxide synthase. While the respiratory burst of microglia during inflammation in peripheral tissues is a response designed to kill invading pathogens or tumor cells, such a (chronic) response within the brain containing a resident population of non-renewing cells could have a devastating impact upon the function and the survival of the organism. Nowhere is this more apparent than in AD, where the loss of large hippocampal neurons and synaptic connections leads to a progressive loss in memory (see

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Atwood 2000a) and is associated with numerous inflammatory and oxidative changes (Smith et al., 1996; Sayre et al., 1997). Similar inflammatory responses to protein accumulation are observed in other neurodegenerative diseases, such as Parkinson's disease, amyotrophic lateral sclerosis (McGeer and McGeer, 1998) following head trauma, and in Down's syndrome (Eikelenboom et al., 1998), and also likely exacerbate neuronal cell loss in these conditions. In this chapter we will review the acute phase response to inflammation, and the resultant oxidative environments that lead to the post-translational modification of amyloid deposits and neurotoxicity in the AD brain.

ACUTE PHASE RESPONSE TO NEURONAL INFLAMMATION

Like other inflammatory diseases, AD is associated with the upregulation of a diverse set of acute phase proteins that arise early in inflammation. Acute phase mechanisms involved in the initiation, clearance and subsequent tissue rebuilding process after injury are coordinated by the pleiotrophic actions of numerous molecules. Acute phase molecules that signal the pro-inflammatory mechanisms of wound healing include interleukin-1 (IL-1), IL-6, TNFα, cell adhesion molecules (ICAM-1), colony stimulating factors (M-CSF) and acute phase proteins such as C-reactive protein, serum amyloid A and transthyretin (Neuroinflammation Working Group, 2000). Simultaneous signaling from other molecules control and assuage inflammation towards the end of the wound healing process. For example, transforming growth factor β1 (TGFβ1) has been implicated in the alleviation of inflammation and the tissue rebuilding process, while α2-macroglobulin, together with its protease inhibitory and removal activity, when bound to low-density lipoprotein receptor-related protein, acts as a clearance system for inflammatory proteins (Borth, 1992), such as apolipoprotein E (apoE), amyloid β protein precursor (AβPP), Aβ (Narita et al., 1997), lactoferrin, tPA, uPA, PAI-1, lipoprotein lipase, receptor-associated protein (Williams et al., 1994; Kounnas et al., 1995), IL-1β, TGFβ, platelet-derived growth factor and fibroblast growth factor (Borth, 1992; Du et al., 1997; Hughes et al., 1998). The oxidative environment induced by inflammatory processes also may act as a signaling mechanism to neurons. Human neurons oxidatively challenged with H_2O_2 upregulate the expression of proteins involved in neurite outgrowth and synapse formation (proliferating cell nuclear antigen, GAP-43, nitric oxide synthase 3 and neuronal thread protein; De la Monte et al., 2001).

ENERGY AND OXIDATIVE STRESSES PROMOTE AβPP SYNTHESIS AND Aβ GENERATION

AβPP is another acute phase reactant upregulated in neurons, astrocytes and microglial cells in response to inflammation and a multitude of associated

cellular stresses. These include axonal injury (Gentleman et al., 1993; Blumbergs et al., 1995), entorhinal cortical lesion (Banati et al., 1994), loss of innervation (Wallace et al., 1993) and trophic factors (Araki and Wurtmann, 1998), excitotoxic stress (Topper et al., 1995; Panegyres, 1998), heat shock (Abe et al., 1991a; Ciallella et al., 1994), seizures (Panicker et al., 1998), oxidative stress (Yan et al., 1994; Frederikse et al., 1996), aging (Higgins et al., 1990; Adler et al., 1991; Nordstedt et al., 1991; van Gool et al., 1994) and inflammatory processes (Brugg et al., 1995). Other pro-inflammatory stimuli that mediate the synthesis and release of AßPP include IL-1β (Goldgaber et al., 1989; Buxbaum et al., 1992) and TNFα-converting enzyme (Buxbaum et al., 1998). In addition to the above-mentioned stresses (and perhaps as a result of them), a shortage of energy supply and Ca(II) overload also induces an upregulation of AβPP expression. Ischemia, hypoglycemia and traumatic brain injury, a condition that has been shown to put neurons under metabolic stress (Xiong et al., 1997), all upregulate AβPP and its mRNA in animal models and culture systems (Abe et al., 1991b; Hall et al., 1995; Jendroska et al., 1995; Yokota et al., 1996; Murakami et al., 1998; Shi et al., 1997, 1998, 2000). Not only does energy shortage and Ca(II) dysregulation promote AβPP expression, but they also direct the metabolism of AβPP from the non-amyloidogenic to the amyloidogenic pathway. Inhibition of mitochondrial energy metabolism alters the processing of AβPP to generate amyloidogenic derivatives (Gabuzda et al., 1994; Mattson et al., 1998), while oxidative stress has been shown to increase the generation of Aβ (Frederikse et al., 1996; Misonou et al., 1999; Paola et al., 2000). Consistent with this response, Aβ has been detected in the human brain a couple of days after traumatic brain injury (Gentleman et al., 1993).

Depending upon its concentration, AβPP may be either toxic or trophic. At subnanomolar concentrations, soluble AβPP (sAβPP) stimulates NF-κB activity, IL-1 and inducible nitric oxide synthase (iNOS) expression, and neurotoxicity (Banati et al., 1993). Conversely, sAβPP has neurotrophic properties, including protection from transient ischemia (Smith-Swintosky et al., 1994) and acute and chronic excitotoxic injury (Mattson et al., 1993; Masliah et al., 1997). In addition, sAβPP has been shown to increase neuronal survival and growth by mediating nerve growth factor-induced neurite extension (Milward et al., 1992; Akar and Wallace, 1998), increasing synaptic density (Roch et al., 1994), having synaptotrophic properties (Mucke et al., 1994), regulating cell growth (Saitoh et al., 1989) and showing general trophic responses (Araki et al., 1991; Yamamoto et al., 1994). Both NGF and neuronal differentiation regulate AβPP expression (Yoshikawa et al., 1990; Fukuchi et al., 1992; Cosgaya et al., 1996). Because of these pleiotrophic properties, it is likely that AβPP is involved in the initial clearance and subsequent rebuilding of tissue after injury (Araki et al., 1991). The prominent growth response induced by sAβPP may reflect attempted regeneration of viable, healthy neurons following synaptic disconnection due

to death of other neurons, a situation that might be expected during the course of AD and following head injury.

Aβ also has neurotrophic properties. Indeed, the increased generation of amyloid β (Aβ) under conditions of energetic stress may be a response to the oxidative challenge observed in the brain in AD and following injury. We have recently found that Aβ has significant antioxidant (superoxide dismutase) activity (Bush et al., 1999), and that nanomolar concentrations of Aβ can block neuronal apoptosis following trophic factor withdrawal (Chan et al., 1999). These findings are consistent with the trophic and neuroprotective action of Aβ at physiological concentrations in deprived conditions and neonatal cells (Whitson et al., 1989; 1990; Yankner et al., 1990; Behl et al., 1994; Stephenson et al., 1992; Koo et al., 1993; Singh et al., 1994; Takenouchi and Munekata, 1995; Luo et al., 1996; Kaltschmidt et al., 1999; Postuma et al., 2000). In support of this role of Aβ as an antioxidant, Aβ amyloid burden of the AD-affected brain has been shown to be significantly negatively correlated with oxidative stress markers (Nunomura et al., 1999; Cuajungco et al., 2000) and *in situ* soluble Aβ levels are inversely correlated with synaptic loss (Lue et al., 1999). Aβ also strongly inhibits autooxidation of lipoproteins in cerebrospinal fluid and plasma by binding metal ions (Kontush et al., 1998, 2001). Moreover, Andorn and Kalaria (2000) have recently shown that low concentrations of Aβ possess significant antioxidant activity in an ascorbate-stimulated lipid-peroxidation assay of post mortem human brain membrane preparations. Together, these data provide a plausible physiological explanation for the increased generation of Aβ in AD and following head trauma, one that is aimed at reducing oxidative damage [thereby preventing reactive oxygen species (ROS)-mediated neuronal apoptosis] and promoting neurite outgrowth.

Since Aβ can bind to the extracellular matrix, a structure that regulates adhesive events such as neurite outgrowth and synaptogenesis, Aβ may be rapidly assembled in the extracellular space by Cu and Zn, which are known to be mobilized to sites of inflammation (reviewed in Atwood et al., 1998, 1999). We have previously shown that Aβ, unlike most proteins, binds Cu under acidotic conditions, in keeping with a role of Aβ as an antioxidant or molecule involved in maintaining structural integrity under stress conditions. It should be noted that the brain maintains high concentrations of both Cu (~70 μM) and Zn (~350 μM; Lovell et al., 1998). In this way, Aβ may dampen oxidative insults by binding excess or loosely bound redox-active metal ions (Smith et al., 1997), which at the same time act to switch on its neurotrophic (antioxidant/sealing) properties. The aggregated Cu/Zn-Aβ would serve as a O_2^- scavenging solid-phase matrix (which disassembles when Zn and Cu levels lower, as the tissue damage resolves). This may explain the rapid cortical deposition of Aβ in stroke and following head injury (Roberts et al., 1994). These activities of Aβ would ensure that minimal numbers of neurons and synaptic connections were lost after head trauma, an important physiological response that would

limit the loss of terminally differentiated neurons. Thus, the acute phase generation of Aβ may have evolved as a secondary antioxidant defense and/or sealant system required during times of excessive ROS generation and/or trauma (Atwood et al., 1998, 1999), while the upregulation of sAβPP/Aβ may act to promote neuronal growth and neurite extension.

Aβ PROMOTES MICROGLIAL ACTIVATION

Studies aimed at understanding the mechanism by which Aβ may drive AD pathogenesis have revealed multiple aspects of the properties of Aβ, one of which is its ability to stimulate inflammation (McGeer and McGeer, 1995). The upregulated expression of cell adhesion molecules, increased cytokine production and activation of the complement system and microglial cells in the brain parenchyma of AD patients are closely associated with Aβ deposits (McGeer and McGeer, 1995; Eikelenboom and Veerhuis, 1996).

Unlike non-demented elderly individuals, in whom resting microglia are typically found in the white matter, the AD brain contains clusters of activated microglia in white and gray matter (Styren et al., 1990; Eikelenboom et al., 1993). Microglia have been shown to cluster at sites of Aβ deposition in AD brain (Rogers et al., 1988; McGeer et al., 1989; Styren et al., 1990) and also at sites of Aβ deposition in AβPP transgenic mice (Frautschy et al., 1998; Stalder et al., 1999). Qualitative histopathological analyses of AD brains indicates that $>80\%$ of core plaques are associated with clusters of reactive microglia, while $<50\%$ of diffuse plaques show such an association (Giulian et al., 1995). Interestingly, diffuse Aβ deposits in elderly individuals contain only quiescent microglia, suggesting a role for microglia in plaque evolution (Eikelenboom et al., 1993; Mann, 1993; Sheng et al., 1995, 1996; Wisniewski et al., 1996). Indeed, it is suggested by many that the activation of microglia seems to increase together with senile plaque maturation, and is first detectable as diffuse deposits making the putative transition to primitive plaques and progresses to clusters of activated microglia in classic plaques (Griffin et al., 1995; Mackenzie et al., 1995; Cotman et al., 1996; Sasaki et al., 1997). Thus, like peripheral macrophages in systemic amyloidosis (Shirahama et al., 1990), microglia appear to be intimately involved in the alteration in plaque morphology. This concept has gained strong impetus from the results of *in vitro* studies, which show that Aβ is capable of priming and/or triggering the respiratory burst of cultured rat microglia and human phagocytes (Meda et al., 1995; Van Muiswinkel et al., 1996; Akama et al., 1998). Activated microglia, the resident brain macrophages, are a major source of ROS (Colton et al., 2000). These cells exist in a quiescent state in normal brain tissue and can become activated in response to neuronal damage or aggregated Aβ (Meda et al., 1995; Giulian et al., 1996; Eikelenboom and Veerhuis, 1996; Paresce et al., 1997; Giulian et

al., 1998). The HHQK domain (residues 13–16) of Aβ has been shown to bind microglia, promote microglial activation and neuron cell death via toxins (see below) released into the culture media (Giulian et al., 1996, 1998). This sequence itself is not neurotoxic and reduces inflammation in the rat brain elicited by injection of Aβ peptides (Giulian et al., 1998). This same cluster of basic amino acids also is known to bind with high affinity to heparan sulfate (Narindrasorasak et al., 1991; Brunden et al., 1993; Snow et al., 1994). Thus, the overproduction of Aβ by the stresses described above, or the lack of sufficient heparan sulfate molecules, would be predicted to lead to Aβ (plaque) accessibility to microglia and microglial activation. Indeed, it has been demonstrated that infusions of Aβ peptides or implantation of native peptide fragments into the rat neocortex induces reactive microgliosis beyond that of a simple needle trauma (Giulian et al., 1989; Frautschy et al., 1992; Giulian et al., 1998). Aβ has been shown to directly activate the NADPH-oxidase complex of inflammatory cells, with increased production of O_2^- and increased generation of H_2O_2 (Klegeris et al., 1994; McDonald et al., 1997; Klegeris and McGeer, 1997; Della-Bianca et al., 1999; Van Muiswinkel et al., 1999a,b). Thus, the release of ROS from activated microglia likely contributes in a significant way to the oxidative challenge observed in the AD brain.

The respiratory burst of activated phagocytes is also accompanied by the release of granule-containing proteins, such as the myeloid-specific enzyme myeloperoxidase (MPO), a heme protein of 150 kDa (Pembe and Kinkade, 1983; Albrecht and Jungi, 1993). Together with H_2O_2, MPO acts as an antimicrobial agent and is present in very high concentrations in monocytes, neutrophils and in some reactive macrophages, where it comprises up to 2–5% of the cell mass. The release of MPO and ROS during the respiratory burst of human phagocytes, designed to generate potent cytotoxins that kill invading pathogens and tumor cells, promotes an environment that oxidizes and crosslinks nitrates and chlorinates amino acids, cholesterol, lipids, lipo(proteins), nucleotides and DNA. MPO–H_2O_2 systems promote the synthesis of tyrosine-crosslinked species, such as dityrosine (Jacob et al., 1996), and catalyze the formation of nitrotyrosine-modified proteins (Podrez et al., 1999), as well as advanced end-product modifications (Anderson et al., 1999). In the presence of a halide ion (usually Cl), MPO catalyzes the reaction between H_2O_2 and Cl to generate hypochlorous acid (HOCl), a potent oxidant that chlorinates and oxidizes lipoproteins (Hazen et al., 1996), apoE (Jolivalt et al., 1996, 2000) and cholesterol (Byun et al., 1999), contributes to the formation of reactive nitrogen species (Eiserich et al., 1998) and crosslinks proteins (Jacob et al., 1996).

MPO has been co-localized to both amyloid plaques (Figure 32.1A; Reynolds et al., 1999) and NFTs (Figure 32.1B) in AD-affected brains, co-localizing with $A\beta_{1-42}$, but is not found in the large neurons of the hippocampus or in age-matched control sections (Reynolds et al., 1999).

The highest levels of MPO are detected in the brains of individuals carrying the apoE4 gene (Reynolds et al., 1999). Interestingly, $A\beta_{1-42}$ has been shown to enhance MPO mRNA expression in rat microglia (Reynolds et al., 1999). Furthermore, inheritance of a polymorphism on MPO has been associated with increased incidence of AD in females, but of decreased incidence in males (Reynolds et al., 1999). Enhanced peroxidase immunoreactivity has been observed following the activation of astrocytes and microglia in rat brain (Lindenau et al., 1998).

OXIDATIVE POST-TRANSLATIONAL MODIFICATIONS OF Aβ

Although Aβ is a normally soluble and constitutive protein found in tissue and biological fluids (Atwood et al., 1998, and references therein), Aβ aggregates together with other proteins such as apoE, MPO and amyloid P-component to form diffuse amorphous deposits and dense, focal, extracellular deposits in AD (Glenner et al., 1984; Maury, 1995; Reynolds et al., 1999). Aβ extracted from biological systems normally migrates as an apparent ~4 kDa monomer on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE; Shoji et al., 1992); however, Aβ extracted from AD-affected post-mortem brain specimens migrates on SDS–PAGE as SDS-, urea- and formic acid-resistant oligomers (Masters et al., 1985; Kuo et al., 1996; Roher et al., 1996; Cherny et al., 1999).

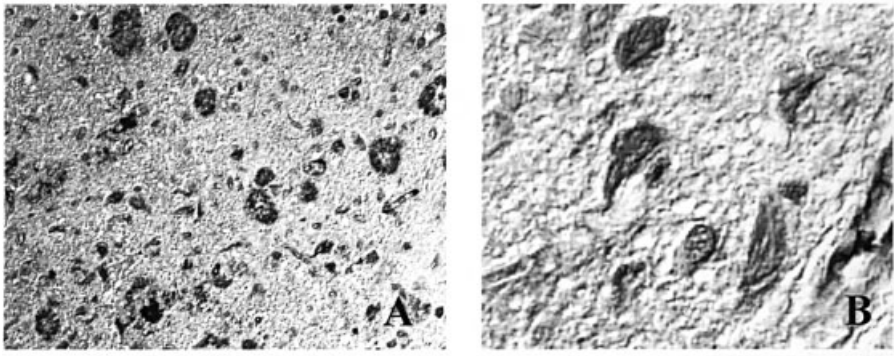

Figure 32.1. Myeloperoxidase co-localizes to amyloid deposits and neurofibrillary tangles. Tissue sections from AD brain were fixed in methacarn, sectioned (8 μm) and stained with an antibody to MPO (Chemicon International, Inc., CA). Showing the characteristic pattern of an amyloid plaque (A) and a neurofibrillary tangle (B) (determined using Congo red) that are immunopositive for MPO. Non-immune staining and control sections were negative (not shown)

Aβ CROSSLINKING

Oligomerized Aβ is a characteristic post-translational modification of Aβ extracted from amyloid plaques of AD brains that may account for up to 10–20% of total Aβ species (Masters et al., 1985; Kuo et al., 1996; Roher et al., 1996; Cherny et al., 1999). Oligomerized Aβ is not normally detected, or is found in very low concentrations in soluble Aβ extracted from cell lines (Podlisny et al., 1995, 1998). Matrix-assisted laser desorption ionization–mass spectrometry of these SDS-resistant oligomers extracted from neuritic plaque and vascular amyloid indicate the presence of covalently crosslinked dimeric and trimeric Aβ species (Roher et al., 1996). We recently reported that these SDS-resistant oligomers of Aβ extracted from amyloid core plaques are tyrosine-crosslinked at position 10, forming dimeric and trimeric Aβ oligomers (Atwood et al., 2000b, and submitted). Tyrosine crosslinked Aβ may contribute to the elevation of total DT (five- to eight-fold) in the AD brain, compared to normal control tissue (Hensley et al., 1998). In support of this, Hensley and colleagues only found DT in those regions affected by senile plaque pathology (hippocampus, inferior parietal lobule, superior/middle temporal gyri) and others have determined that both peroxidase- and metal-catalyzed oxidation systems promote the crosslinking of human Aβ (Galeazzi et al., 1999; Atwood et al., 2000b,c), but not rat Aβ *in vitro*. That tyrosine-crosslinked human Aβ forms *in vivo* indicates that tyrosine residues of Aβ are proximate within the amyloid deposit. Benzinger et al. (2000) have recently provided evidence that the tyrosines of fibrillized Aβ may be in close proximity.

Tyrosine-crosslinking of proteins occurs in mammalian systems, most notably in connective tissues and structural proteins (Amado et al., 1984). The DT crosslink is resistant to cleavage (resistant to 6 N HCl at 110 °C for 24 h and to protease digestion, trypsin, chymotrypsin and pronase; Smail et al., 1995). Therefore, it is not surprising that tyrosine-crosslinkage of proteins is a mechanism normally utilized by various organisms to increase the structural strength of cell membranes and walls for protection against proteolysis and physical trauma. For example, a DT content of only five to eight residues per 10 000 amino acid residues is sufficient to make the fertilization membrane of the sea urchin embryo resistant to proteolysis and physical trauma. Likewise, DT in the cell wall of ascospores of *Saccharomyces cerevisiae* makes these organisms 30- to 100-fold more resistant to heating to 55.5 °C, 100-fold more resistant to ether and extremely resistant to physical trauma and proteolytic degradation by numerous proteases (Deits et al., 1984; Nomura et al., 1990, Briza et al., 1990a,b; Smail et al., 1995). While this protective mechanism is advantageous in single-celled organisms and peripheral tissues, increased tyrosine crosslinkage may not be a usual feature of proteins within the cellular structures of the brain, except perhaps during head trauma, when it may serve a structural function. The DT load of Aβ amyloid suggests that amyloid plaques should be very resistant to resolubilization (e.g. Masters et al., 1985) and may greatly stabilize Aβ deposits.

OTHER POST-TRANSLATIONAL MODIFICATIONS

Other modifications identified in Aβ extracted from amyloid core plaques include the identification of pyroglutamated and racemized amino acids (Shapira et al., 1988; Mori et al., 1992; Roher et al., 1993), carbonyl groups (Atwood et al., submitted) and alterations in the amino acid composition of human amyloid Aβ (Atwood et al., 2000d). Alterations in the composition of certain amino acids (decreases in histidine and tyrosine residues) are consistent with metal- and MPO-catalyzed oxidation of Aβ. We have previously reported that Cu is coordinated to the histidine residues of Aβ (Atwood et al., 1998) and this complex is redox-active (Huang et al., 1999a,b). Therefore it is likely that $OH^{\bullet}$ generation in this coordination site promotes histidine oxidation.

MICROGLIAL ACTIVATION AND Aβ POST-TRANSLATIONAL MODIFICATION

All the components (peroxidases, H_2O_2, ROCl and Cu) necessary for the oxidative modification of Aβ and associated proteins in amyloid deposits are present in the neurochemical environment promoted by the chronic inflammatory response to amyloidosis and trauma. Our results indicate a central role for this neurochemical environment in the post-translational modification of amyloid plaques. Thus, the activation of microglia in response to Aβ accumulation may promote tyrosine crosslinkage of depositing Aβ, thereby inhibiting its clearance and leading to a vicious cycle of enhanced microglial activation. In this context, HOCl-oxidized LDL has been shown to induce and amplify inflammatory reactions by the induction of chemokine synthesis, and induces chemoattraction of neutrophils (Woenckhaus et al., 1998). HOCl–LDL also stimulates enhanced production of ROS, adhesion to endothelial cells (Kopprasch et al., 1998) and alters the aggregation and release reactions of activated platelets (Zabe et al., 1999).

It is not clear why these resident macrophages, which have been shown to digest amyloid plaques *in vitro* (DeWitt et al., 1998), do not clear amyloid deposits *in vivo*. It is possible that microglia *in vivo* receive dual (or incomplete) signals from their interaction with amyloid plaques, one that activates and one that prevents phagocytosis (and the removal of normal structures). Alternatively, their rate of amyloid removal may be slower than the rate of amyloid deposition. It is known that amyloid plaque clearance is markedly suppressed by the presence of astrocytes and is in part due to a diffusable factor (DeWitt et al., 1998). In addition, alterations in the recognition sequence of Aβ as a result of its oxidative modification may prevent the normal uptake and clearance of Aβ by microglia. Both amyloid-derived monomeric and oligomeric Aβ extracted from AD-affected brains

promote neuron cell death at concentrations of around 100 nM, but only in the presence of microglia, indicating that the cytotoxic actions of Aβ may be mediated by activating microglia. Therefore, these inflammatory mechanisms may be central not only to the evolution of hard-core amyloid plaques, but to the progressive synaptic and neuronal cell loss of the AD brain that results from the oxidative attack. Whether the oxidative crosslinking of Aβ is a means of concentrating and insulating toxic soluble Aβ from neuronal damage is unclear.

Similar inflammatory environments exist in the atherosclerotic lesions of the heart, where MPO has been colocalized to HOCL-modified epitopes of human atheroma (type IV), fibroatheroma (type V) and complicated (type VI) lesions (Malle et al., 2000) and where there is selective enrichment of protein DT crosslinks and 3-chlorotyrosine (Leeuwenburgh et al., 1997). *In vitro,* HDL exposed to peroxidase-generated tyrosyl radical undergoes tyrosylation and crosslinking of its apolipoproteins (Francis et al., 1993). Therefore, the MPO–H_2O_2–Cl system has been suggested to play a critical role in converting LDL into an atherogenic form (Hazen and Heinecke, 1997) and the formation of vascular atherosclerotic lesions.

The chronic activation of oxidant-producing inflammatory enzyme systems could act as an important link between the development of amyloid plaques and atherosclerotic plaques in artery walls, and explain the deposition of proteins in other degenerative conditions. For example, the catabolic resistance of DT modifications of proteins could explain the contribution of tyrosine-crosslink polymers to the crosslinking of α-crystallin in fluorescent cataract formation (Kikugawa et al., 1991), the oligomerization of Mn-superoxide dismutase (SOD) detected in renal graft rejection (MacMillan-Crow et al., 1998), the crosslinking of proteins in sputum from cystic fibrosis patients (Van der Vliet et al., 2000) and to lipofuchsin formation (Kato et al., 1998). MPO also has been implicated as playing a role in multiple sclerosis (MS), where increased MPO expression in brain macrophages accelerates damage to the myelin sheath (Nagra et al., 1997). SOD also is known to deposit in the nervous system in MS (Bruijn et al., 1998), although it is not known if this is a result of DT crosslinkage. Recently, DT crosslinking of recombinant human α-synuclein induced by MPO oxidative conditions was proposed as a mechanism for the formation of Lewy body intraneuronal inclusions seen in Parkinson's disease (Souza et al., 2000).

SUMMARY

The induction of inflammation by protein deposits may be a mechanism that explains the crosslinking of proteins in AD and other inflammatory diseases. It is becoming clear that the activation of oxidant-producing inflammatory enzyme systems in the AD brain plays a central role in the post-translational

modification of amyloid plaques. Blocking inflammatory-induced Aβ modifications (and Aβ generation) may prevent plaque deposition and activation of microglia and could therefore provide an important therapeutic target.

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33 Plasma Antioxidants and Oxidative DNA Damage in Lymphocytes from Normal Aged People and Alzheimer's Disease Patients

PATRIZIA MECOCCI, MARIA CRISTINA POLIDORI, TIZIANA INGEGNI, PAOLA MATTIOLI, ANTONIO CHERUBINI, MARCO CATANI, ROBERTA CECCHETTI AND UMBERTO SENIN

INTRODUCTION

A large body of research suggests that an important role in the pathogenesis of Alzheimer's disease (AD) is played by oxidative stress (Christen, 2000), a condition representing the imbalance between oxidants and antioxidants in favor of the former (Sies, 1999). Neurons are indeed highly susceptible to the attack of free radicals due to their low content of glutathione (a potent intracellular antioxidant), to the considerable content of polyunsaturated fatty acid side-chains of their membrane lipids, and to the elevated oxygen consumption rate of the brain (for review, see Christen, 2000). Furthermore, aging—a major risk factor for AD—is itself known to be associated with free radical hyperproduction, and markers of oxidative DNA damage have been found to be increased in post-mortem brains of healthy aged subjects (Mecocci et al., 1993) as well as of AD patients (Mecocci et al., 1994). In addition, the initial idea of a link between free radical hyperproduction and AD is now corroborated by studies showing the potential pathogenetic importance of a vicious circle between free radical-induced β-amyloid formation and β-amyloid-related oxidative stress (Bush et al., 1999; Stadtman and Levine, 1999; Yatin et al., 1999). In support of this evidence, and to contribute to the definition of a rationale for therapeutic intervention in AD patients (Chadman et al., 1997; Gale et al., 1996; Grundman, 2000; Prasad et al., 1999a,b), we describe our new observations regarding a relationship

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between oxidative DNA damage in lymphocytes from AD patients and some plasma antioxidants.

OXIDATIVE DNA DAMAGE IN AD

When oxygen free radicals—some of which are highly reactive molecules able to damage all cellular key components, including proteins, lipids and nucleic acids—react with and damage DNA, oxidized bases are formed, of which at least 20 are known to date. One of these bases, 8-hydroxy-2′deoxyguanosine (8-oxo-dG), is a reliable marker of oxidative stress to DNA, and can be measured by HPLC with electrochemical detection, a sensitive and specific method of assay (Floyd et al., 1986). As cited above, this technique has allowed the observation of an age-dependent increase in the levels of 8-oxo-dG in DNA extracted from cerebral tissue of human healthy subjects aged 42–97 years, both in nuclear DNA (nDNA) and in mitochondrial DNA (mtDNA). The latter showed a higher susceptibility to oxidative damage compared to nDNA (Mecocci et al., 1993), a difference that might be explained by a number of factors, including the proximity of mtDNA to the inner mitochondrial membrane (where enzymes of the phosphorylative chain are located) and the lack, in mtDNA, of protective histones and of particularly efficient repairing mechanisms (Beal, 1995).

The relevance of DNA oxidative damage in AD, however, has been hypothesized when relatively large amounts of 8-oxo-dG were found in mtDNA and nDNA from cerebral tissue of AD patients as compared to controls (Mecocci et al., 1994). Post-mortem studies are not easy to perform, and the possibility of detecting 8-oxo-dG in peripheral tissues of AD patients was recently tested. In this study, nDNA was extracted from lymphocytes and enzymatically hydrolyzed to nucleosides (Mecocci et al., 1998). After this process, 8-oxo-dG was measured by HPLC with UV and electrochemical detectors serially linked in order to measure both deoxyguanosine and 8-oxo-dG, respectively.

Lymphocyte levels of 8-oxo-dG expressed as the ratio between 8-oxo-dG and deoxyguanosine, multiplied by 10^5, were significantly ($p<0.001$) higher in AD patients than in controls (Figure 33.1), and directly correlated with age in controls ($r = 0.58$, $p<0.05$) but not in AD subjects, confirming previous results (Mecocci et al., 1993).

This study, together with other reports, shows that biomarkers of oxidative stress are present in peripheral tissues of AD patients, even though the large majority of the damage is in the brain (Markesbery, 1997, 1999).

Moreover, the measurement of peripheral antioxidants is considered to be an appropriate way of looking at oxidative stress in various processes and disease states in humans (Polidori et al., in press). It is conceivable, therefore, that the conjunct measurement of a sophisticated marker of oxidative DNA

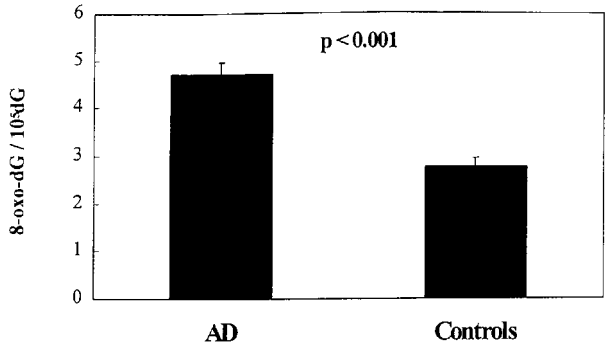

Figure 33.1. 8-oxo-dG levels in lymphocyte DNA from AD patients and healthy controls

damage, such as 8-oxo-dG, and of peripheral antioxidant molecules might provide a reliable estimate of the free radical-induced damage occurring in patients with AD.

The preliminary results of a recent study performed in this sense are presented below.

PERIPHERAL OXIDATIVE DNA DAMAGE AND ANTIOXIDANTS IN AD

The relationship between oxidants and antioxidants in various processes and disease states has been largely evaluated in recent years, and has been found to be strong, at least as far as certain molecules and peculiar conditions are concerned. A significant negative correlation between concentrations of serum carotenoids—which are known to act as antioxidants—and oxidized pyrimidines, however, was found in lymphocytes of healthy humans (Collins et al., 1998), suggesting that some nutritional factors may protect from oxidative DNA damage independently of the health status of the organism. In another interesting study, Lenton et al. (1999) showed that naturally occurring levels of intracellular antioxidants—glutathione and vitamin C—were negatively correlated with oxidative damage in human lymphocytes, as assessed by levels of 5-hydroxy-2′deoxycytidine (5-OH-dCyd) and of 8-oxo-dG. In particular, the strongest inverse relationship was found between glutathione and 8-oxo-dG, and on the basis of these results the authors suggested that intracellular glutathione and ascorbate protect human lymphocytes against oxidative DNA damage. In 88 healthy inhabitants of Mexico City aged 60–94 years, however, DNA damage as assessed by alkaline unicellular electrophoresis (comet assay) was found in more than half of the lymphocyte samples, regardless of the total antioxidant serum levels

quantified colorimetrically (Mendoza-Nuñez et al., 1999). Despite this, the authors found a trend toward greater DNA damage in subjects with low serum antioxidant levels, but concluded that the latter could not be considered predictors of DNA damage in their subject population.

In our study, we measured the content of 8-oxo-dG by HPLC with UV and electrochemical detection, as previously described (Mecocci et al., 1998), in lymphocyte DNA obtained from 40 AD patients (20 females, 20 males, aged 75.9+5.4 years) and 39 healthy aged subjects (20 females, 19 males, aged 74.8+6.3 years). In patients and controls, plasma vitamin C and vitamin E levels were also quantified by HPLC with electrochemical (vitamin C) or UV detection (vitamin E). We found significantly elevated levels of 8-oxo-dG in lymphocyte DNA (Figure 33.2A) and significantly lower plasma vitamin C (Figure 33.2B, black columns) and vitamin E (Figure 33.2B, white columns) concentrations in AD patients as compared to controls. No correlation was found between plasma vitamins and lymphocyte DNA oxidative damage in either patients or controls.

DISCUSSION AND CONCLUSIONS

The renowned importance of oxidative stress in AD has been shown in various studies and widely reviewed (Beal, 1995; Markesbery, 1997, 1999; Christen, 2000). Along with our previous results on the presence of oxidative damage in AD, accumulating evidence in this field has opened new and intriguing perspectives regarding the potential therapeutic application of dietary antioxidants in this devastating dementing disorder. Several studies have indeed shown that dietary supplements, including vitamin C, vitamin E and β-carotene, protect lymphocyte DNA against oxidative damage (Martin and Rademaker, 1987; Anderson, 1996; Stahl and Sies, 1997). In line with other studies and with our previous findings, 8-oxo-dG was found to be significantly higher in lymphocyte DNA from AD patients than in controls. In the same sample of subjects, we found significantly lower levels of plasma

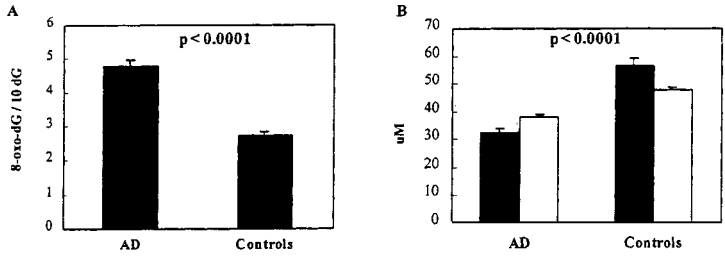

Figure 33.2. Lymphocyte DNA 8-oxo-dG and plasma vitamin C and vitamin E levels in AD patients and healthy controls. (In (B) black columns, vitamin C; white columns, vitamin E)

vitamin C in AD patients than in controls (Figure 33.2B, black columns). This finding is in agreement with the hypothesis that vitamin C may protect against DNA damage in human sperm (Fraga et al., 1991) and in white blood cells (Noroozi et al., 1998; Rehman et al., 1998) as well as in some types of cancer (Byers and Perry, 1992).

Apart from vitamin C, flavonoids (Noroozi et al., 1998), co-enzyme Q10 (Tomasetti et al., 1999) and lycopene contained in tomatoes (Riso et al., 1999) have also been shown to exert protective effects against oxidative DNA damage, either decreasing DNA strand breaks (Noroozi et al., 1998) or increasing DNA resistance towards H_2O_2-induced oxidation (Riso et al., 1999; Tomasetti et al., 1999). Regarding antioxidant supplementation and its potential use in AD, it has been shown that a 20 week supplementation with a daily antioxidant mixture containing 100 mg vitamin C, 280 mg vitamin E and 25 mg β-carotene resulted in a significant decrease in endogenous lymphocyte DNA oxidative damage (measured with the comet assay) in 50–59 year-old smokers and non-smokers (Duthie et al., 1996), suggesting that the positive effect of diets rich in fruit and vegetables on certain cancers may be exerted by dietary antioxidants (Miller, 1990; Negri et al., 1991; Steinmetz and Potter, 1991; Byers and Perry, 1992). The idea that a similar positive effect of antioxidant supplementation might also be seen in AD is supported by epidemiological studies showing that diets rich in fruit and vegetables are protective against AD (Gale et al., 1996), and by trials showing beneficial, although not impressive, effects of antioxidant molecules such as vitamin E (Sano et al., 1997) and *Gingko biloba* (Kanowski et al., 1996; Le Bars et al., 1997; Oken et al., 1998) in patients with AD.

In conclusion, our results support the important role played by oxidative stress in the pathogenesis of AD, highlighting the valence of the measurement of oxidized DNA bases in peripheral tissues and of the relationships existing between free radical-induced damage to DNA and the antioxidant defense system of the organism. Further studies are needed to clarify the usefulness of antioxidant supplementation and diet monitoring in AD.

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34 Oxidative Damage and Antioxidant Responses in Alzheimer's Disease

GEORGE PERRY, AKIHIKO NUNOMURA, JESUS AVILA, MAR PEREZ, CATHERINE A. ROTTKAMP, CRAIG S. ATWOOD, XIONGWEI ZHU, GJUMRAKCH ALIEV, ADAM D. CASH AND MARK A. SMITH

INTRODUCTION

Individuals afflicted by Alzheimer's disease (AD) show increased oxidative damage to every class of biological macromolecule. In this article, we review some of the observations related to oxidative damage in AD. In particular, we focus on the distinction in findings made from systemic vs. subcellular localization studies. We also compare the differences noted when damage occurs to rapidly turned-over biological macromolecules vs. damage to stable structures, some of which arise by oxidative crosslinking.

Analysis of the site of oxidative damage can provide clues to the mechanistic source of such damage. Subcellular localization shows that oxidative damage in AD primarily involves reactive oxygen species (ROS) that are generated in the neuronal cytoplasmic compartment. That this same compartment shows extensive mitochondrial abnormalities as well as redox metal accumulation suggests that metabolic alterations may underlie the extensive oxidative damage.

Finally, that pleotropic oxidative and metabolic changes occur in a chronic state argues that they represent a new homeostatic balance. Induction of heme oxygenase-1, the pentose phosphate pathway, increased free sulfhydryls, as well as induction of the MAP-kinase cascade, argue for a dynamic state that protects neurons from death in AD. With this idea in mind, we also argue that much of the 'pathology' of the disease, specifically amyloid-β and tau (τ) deposits, is a reflection of the compensations necessary to protect neurons from death.

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METABOLISM

Some of the earliest observations of oxidative abnormalities in AD were made by the analysis of the metabolic state of tissues and cells derived from AD patients (Markesbery, 1997). These studies, pioneered by John Blass and Gary Gibson, revealed a substantial blockage of two mitochondrial enzymes in AD, pyruvate dehydrogenase and α-ketoglutarate dehydrogenase (Gibson et al., 1998). Deficiencies in the activity of other mitochondrial enzymes were shown in the same neurons vulnerable in AD by enzyme histochemistry (Wong-Riley et al., 1997). Metabolic deficiencies, as well as neuronal loss, also seem to underlie the reduced glucose utilization demonstrated by metabolic imaging studies (Small et al., 1996) and reduction in the activity of the endothelial glucose transporter (Glut-1) (Kalaria et al., 1988).

OXIDATIVE DAMAGE

One of the correlates of oxidative stress, defined as a breaching of the oxidant defense system, is increased damage to biological macromolecules. While many of the studies focused on malonyldialdehyde and lipofuscin formation (Dowson et al., 1998), these features have, in the former case, high susceptibility to artifacts from sample preparations or, in the latter case, a complex and as yet unestablished relationship to oxidative stress. It was not until the study showing a two- to four-fold increase in reactive carbonyls by Stadtman and Floyd (Smith et al., 1992) that oxidative damage was established as a feature of the disease.

In rapid succession, sugar-derived carbonyls were identified in the specific lesions of AD (Smith et al., 1994a) as well as in their component proteins, amyloid-β (Vitek et al., 1994) and τ (Ledesma et al., 1994; Yan et al., 1994). While these studies suggested that oxidative damage primarily involved the lesions, possibly due to low protein turnover (Mattson et al., 1995), it is also the case that the glycoxidative chemistry underlying the observed carbonyl condensation requires active metal-centered ROS for formation (Smith et al., 1995). Therefore, it was not a surprise when the lipid peroxidation adduction productions of hydroxynonenal (Montine et al., 1996; Sayre et al., 1997) and acrolein (Calingasan et al., 1999) were found in the lesions of AD. However, it was more surprising that the dominant site of damage was not the lesions, but rather the neuronal cytoplasm of neurons vulnerable to death in AD. Since the products of lipid peroxidation and glycation can yield crosslinked molecules, oxidative modification by these pathways can make molecules more resistant to breakdown. In model studies, crosslink formation not only makes proteins resistant to removal by the proteosome but also inhibits proteosome activity (Friguet et al., 1994). Oxidative inhibition of proteolytic

activity underlies the accumulation of ubiquitin conjugates in AD (Mori et al., 1987; Perry et al., 1987), which may in part underlie the cytopathology noted below.

To address the site of ROS formation, we focused our efforts on finding a marker resulting from primary attack, rather than the result of complex reactions involving adduction of secondary metabolites. This necessitated examining damage to a cell constituent with a short half-life. When we analyzed protein-based reactive carbonyl and nitrotyrosine formation, we found that they were essentially confined to the cytoplasm of vulnerable neurons, with less evidence of their formation in amyloid-β or τ deposits. These findings point to the cytoplasm, not the lesions, as the source of ROS. Nevertheless, protein-based modifications are far from ideal, since they are often associated with crosslinking, which slows their turnover. Therefore, crosslink modifications of proteins, while useful to assess history, may reveal less of the current state of oxidative stress. Our observation that RNA, a rapidly turned-over cell constituent, is a target for oxidative stress provided a possible marker. 8-Hydroxyguanosine (8OHG), a nucleic acid modification predominantly derived from •OH attack of guanidine, is greatly increased in cytoplasmic RNA in vulnerable neuronal populations (Nunomura et al., 1999a). Ultrastructural analysis shows that most 8OHG immunodecoration is in the endoplasmic reticulum, with the majority of mitochondria showing little 8OHG.

In quantitative analysis of the extent of 8OHG oxidation, we found that cases of AD with the most extensive amyloid-β deposits show the lowest 8OHG levels, and neurons containing NFT have about half the levels of 8OHG (i.e. current oxidative stress status), despite an obvious history of oxidative damage (i.e. advanced glycation end-products or lipid peroxidation), suggesting that both amyloid-β (Aβ) and neurofibrillary tangles are associated with reduced oxidative stress.

To further examine the relationship of Aβ to oxidative damage, we investigated cases of Down's syndrome, where Aβ deposits begin in the late teens and where oxidative stress has been implicated (Odetti et al., 1998). In Down's syndrome, Aβ deposition follows, rather than precedes, increased 8OHG and, again as with AD, following Aβ deposition, 8OHG levels decline to control levels ($r = 0.98$) (Nunomura et al., 2000).

8OHG is likely to form at the site of •OH production, a process dependent on redox-active metal-catalyzed reduction of H_2O_2, together with cellular reductants such as ascorbate or O_2^-. Both iron and copper in their redox-competent states are bound to neurofibrillary tangles and Aβ deposits (Smith et al., 1997; Lovell et al., 1998; Sayre et al., 2000). That 8OHG levels are inversely related to the extent of Aβ deposits but are distant from the deposits suggests a complex interplay between Aβ and redox metal activity that may be critical to metal dynamics within the neuronal cytoplasm. A possible key element to these dynamics is mitochondria in the cell body. While

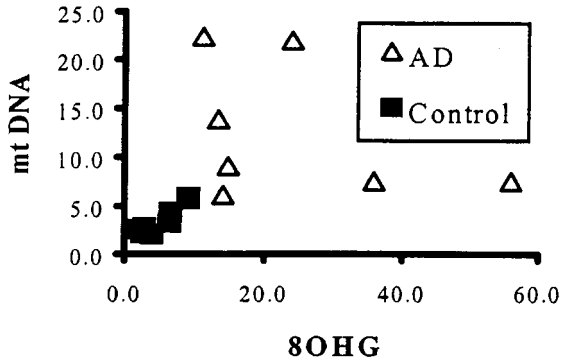

Figure 34.1. The extent of oxidative damage (8OHG) is highly dependent on the degree of mitochondrial abnormalities for normal aging ($r^2 = 0.87$) but displays a non-linear relationship among cases of AD

morphometry of the number of mitochondria in AD shows a decrease, both mtDNA and the protein, cytochrome oxidase 1, are increased several-fold in AD. The extent of mtDNA increase for individual control cases, but not for AD, correlates highly with the extent of 8OHG levels (Figure 34.1). Ultrastructural analysis of the subcellular localization of mtDNA showed that the increase is restricted to the vacuolar portion of lipofuscin, an organelle that increased in AD. These findings suggest a fundamental alteration in mitochondrial dynamics, possibly involving difference in proliferation, transport or turnover related to deficiencies in microtubules (Cash et al., unpublished) or proteolytic insufficiencies. One possibility for the increased oxidative damage observed in the cytoplasm in AD and controls is release of redox-active metal ions as mitochondria are catabolized (Figure 34.2).

COMPENSATORY RESPONSES

Intracellular oxidative balance is tightly regulated and therefore, in the face of stress, an upregulation of compensatory mechanisms would be expected in AD. One of the first reports of oxidative stress in AD came from the finding of pentose phosphate pathway induction in AD (Martins et al., 1986). Reducing equivalents in the form of NADPH are essential to maintain oxidant defenses. Later findings of induction of the NADPH-requiring enzyme heme oxygenase-1 (HO-1; Smith et al., 1994b; Premkumar et al., 1995), the rate-limiting enzyme required for transforming prooxidant heme to anti-oxidant bilirubin, substantiated the view of active antioxidant defense in AD. Since HO-1 induction is synchronous with τ accumulation in neurons (Takeda et al., 2000a,b), reduced oxidative damage in neurons with τ

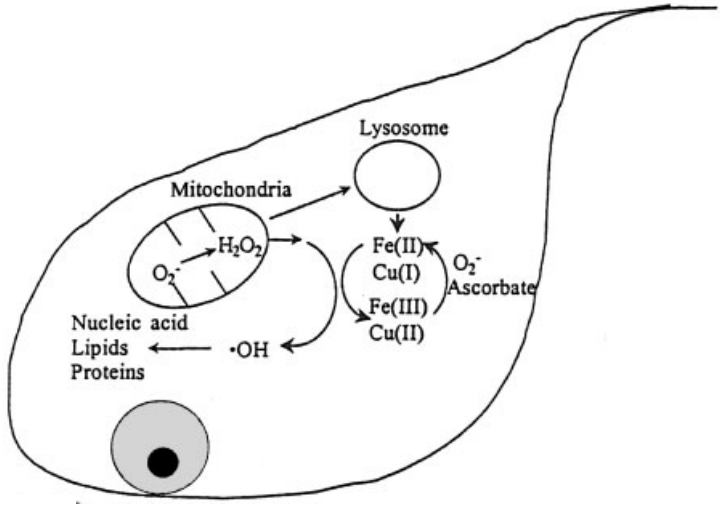

Figure 34.2. The hypothesis of how mitochondrial abnormalities promote oxidative damage is through release of redox-active metal, most likely iron and copper, into the cytosol to catalyze •OH production through the Fenton or Haber–Weiss reactions

accumulation may be a part of this antioxidant response. Another consequence of increased NADPH generation is increased glutathione and free sulfhydryls. The latter are specifically increased in vulnerable neurons (Russell et al., 1999), since sulfhydryls are a major component of the cellular antioxidant defenses to ROS and secondary metabolites.

Additionally, vulnerable neurons show activation of the MAP kinase pathway, including ERK, SAPK and p38 (Perry et al., 1999; Zhu et al., 2000), denoting a survival response consistent with the protracted neuronal death of neurons containing phosphorylated τ (Cras et al., 1995). In fact, the observed decrease in oxidative damage with Aβ and τ accumulation suggests that these may represent important survival responses (Nunomura et al., 1999b). Examination of τ molecular configuration (Takeda et al., 2000b), expression (Takeda et al., 2000a) and localization with HO-1 (Takeda et al., 2000b) supports an important link between cytoskeletal changes and oxidative response. In studies with the epitope of τ defined by proximity of the N- and C-termini and recognized by the monoclonal antibody Alz50, we found an additional important link between oxidative damage and the cytoskeleton. The bifunctional lipid peroxidation product hydroxynonenal stabilized the Alz50 epitope in τ, but only if τ was in the phosphorylated state. These findings suggest that the link between phosphorylation and neurofibrillary tangle formation is oxidative damage, and adoption of the structure defining Alz50 recognition is an early event in the neurodegeneration of AD. Further investigation of the mechanisms of oxidative damage in neuro-

fibrillary tangle formation is required, as well as an understanding of the consequences of these changes to neuronal metabolism.

CONCLUSION

While the prominent increase in neuronal oxidative damage and mitochondrial abnormalities of AD would seem to mark an unstable state leading to cell death, instead, extensive compensatory mechanisms protect neurons from death. We suggest that the pleotrophic nature of changes in AD, broadly considered pathologically, are instead the display of neuronal oxidant defenses.

SUMMARY

Oxidative damage, involving all categories of biological macromolecules, is greatly increased in Alzheimer's disease (AD). However, while there are signs of systemic alterations in oxidative balance, the major site of damage is the cell bodies of neurons at risk of death in AD. This localization is consistent with an abnormality in the neuronal cytoplasm. Ultrastructural analysis of the site of nucleic acid oxidation shows that the damage is restricted to the endoplasmic reticulum and free ribosomes. Analysis to determine whether mitochondria might be involved showed mitochondrial markers, such as mitochondrial DNA or cytochrome oxidase, are increased several-fold. In contrast, morphometric analysis of biopsy specimens showed that mitochondrial number is decreased in AD. Ultrastructural analysis of the mitochondrial DNA and enzymes showed the increased mitochondrial components in AD were primarily confined to lysosomes associated with lipofuscin. These observations suggest that the increased oxidative damage may be a result of altered turnover of damaged mitochondria, leading to increased redox-active metals. The extent of the abnormalities, as well as their chronic nature, suggest that a fundamental metabolic compromise is crucial to disease development.

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35 Cyclooxygenase (COX)-2 and Clinical Progression of Alzheimer's Disease Dementia: Implications in the Role of Neuronal COX-2 in Cell Cycle

GIULIO MARIA PASINETTI

INFLAMMATION AND ALZHEIMER'S DISEASE

A large number of epidemiological studies have addressed the possible protective effect of antiinflammatory drug use with regard to Alzheimer's disease (AD) (McGeer et al., 1996). The most convincing of these studies—the Baltimore Longitudinal Study of Aging—utilized data collected prospectively, thereby minimizing recall bias issues. Corroborated by related studies, their results indicated a protective effect from the use of non-steroidal antiinflammatory drugs (NSAIDs) (Stewart et al., 1997). Available intervention studies support the beneficial efficacy of NSAIDs in AD. One small controlled trial of the NSAID indomethacin suggested that the drug slowed cognitive deterioration (Rogers et al., 1993). Recently, a small controlled trial of diclofenac showed similar results (although not statistically significant because of a high drop-out rate) (Scharf et al., 1999).

Evidence indicates that inflammatory mechanisms are activated in AD. For example, it has been found that amyloid plaques are surrounded by reactive microglia (as well as astrocytes), which have the characteristics of antigen-presenting tissue macrophages, including HLA-DR surface markers. In addition, there is clear evidence of an acute phase response (Vandenabeele et al., 1991), with upregulation of inflammatory cytokines, such as IL-1 and IL-6, and tumor necrosis factor (TNF)α, accompanied by an increase in acute phase proteins, such as α1-antichymotrypsin (ACT) and α2 macroglobulin (α2M). Further, there is an active complement system in the AD brain (Pasinetti, 1996) with generation of the lytic membrane attack complex (Webster et al., 1997) and, presumably, with release of anaphylatoxins. Our

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previous finding (Oda et al., 1995), showing that complement components potentiate amyloid neurotoxicity *in vitro*, is consistent with the evidence that overexpression of the inflammatory cytokine IL-6 in the brain leads to neurodegeneration (Campbell et al., 1993). Finally, upregulation of cyclooxygenase (COX)-2, but not COX-1, in AD neurons (Pasinetti et al., 1998) suggests that inflammatory lipids may also be involved in the pathogenesis of the disease (Pasinetti et al., 1998). However, it remains uncertain whether the inflammatory mechanisms actually cause damage in AD or are merely present to remove the debris of the neurodegenerative events (Aisen, 1997; Pasinetti, 1998).

In this chapter, *new* research directions, which address possible contributions of COX to AD neurodegeneration, are outlined. In particular, recent evidence is discussed, suggesting that neuronal COX-2 in the brain might influence neurodegeneration by promoting abortive attempts to re-enter the cell cycle. In addition, potential interventions are discussed, designed to control mechanisms in neurodegeneration in which COX is implicated. These considerations are critical to the understanding of the role of inflammation in the clinical progression of AD.

INFLAMMATION AND THE CLINICAL PROGRESSION OF ALZHEIMER'S DISEASE DEMENTIA

Prior studies have shown that COX-2, an enzyme involved in inflammatory mechanisms and neuronal activities (reviewed in Pasinetti, 1998), is upregulated in the AD brain and may represent a therapeutic target for antiinflammatory treatments (Ho et al., 1999). COX exists in two isoforms, coded by distinct genes on different chromosomes (Kujubu et al., 1991; Cao et al., 1995; O'Banion et al., 1992). The two isoforms show about 50% homology and have similar catalytic activity, but are physiologically distinct. COX-2 is inducible in inflammatory cells in response to inflammatory signals, such as cytokines and lipopolysaccharides, and is downregulated by glucocorticoids. In contrast, COX-1 expression is generally constitutive. It thus appears that COX-2 mediates inflammatory activity, while COX-1 has housekeeping functions, including gastric cytoprotection and platelet aggregation (Table 35.1).

Recent evidence suggests that different indices of classical inflammatory cascades may have distinct associations with different phases of the clinical progression of AD, as reflected in the clinical dementia rating (CDR) (Luterman et al., 2000). For example, COX-2 (but not COX-1) expression in the neurons of the hippocampal formation, a brain region at risk for AD neurodegeneration, may be a predictor of progression of early AD before neurodegeneration occurs (Ho et al., 2001, in press). Surprisingly, and in contrast to COX-2, other classical markers of inflammatory neurodegeneration,

Table 35.1

COX-1
Brain
. Expression in brain but not regulated in response to experimental neurodegeneration
Expression
. Chromosome 9 (q32 q33.3)
. Constitutive expression
. Stimulated by growth factor in selected cells
Function
. Prostaglandin formation for housekeeping functions
. Gastric cytoprotection
. Vascular homeostasis
. Normal renal maintenance
COX-2
Brain
. Expressed primarily in neurons
. Regulated in response to experimental neurodegeneration
. Localized in dendrites
Expression
. Chromosome 1 (q25.2 q25.3)
. Induced expression by inflammatory mediators, mitogens; elevated expression in tumors
. Transcription inhibited by TGFβ1
Function
. Inflammation
. Cellular differentiation
. Mitogenesis

such as interleukin-6 (IL-6) and transforming growth factor β_1 (TGFβ_1) (Luterman et al., 2000), HLA-DR immunopositive reactive microglia, and complement components gene expression (Xiang and Pasinetti, 2000) showed increased expression, but only at the latest stages of AD dementia (Figure 35.1).

The evidence showing elevation of COX-2 protein content in neurons of the hippocampal formation early in AD dementia suggests that independent segments of inflammatory cascades may play important and possibly independent roles in separate phases of the disease, ultimately influencing the progression of AD dementia. Given these findings, antiinflammatory drugs selected for studies of AD patients at a particular clinical stage should be selected based on their activity against the inflammatory processes most pronounced at that stage; for example, using COX-2 inhibitors in the early phase of AD. Thus, the identification of specific early markers involved in destructive brain inflammation would provide critically important selection criteria and/or possible co-variance for clinical trials of antiinflammatory drugs.

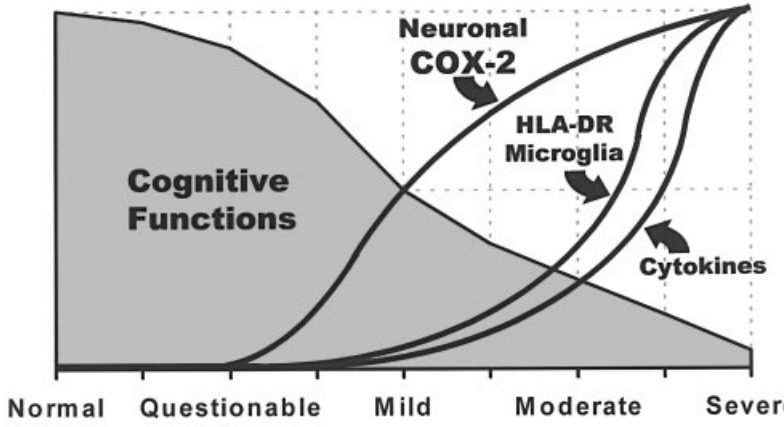

Figure 35.1. Inflammatory markers and clinical progression of Alzheimer's disease dementia

Major efforts are now under way to determine whether COX inhibitors can help control the destructive progression of AD. Large National Institutes of Health (NIH)-supported trials are evaluating whether selective COX-2 inhibitors, or low-dose non-selective NSAIDs, can delay the diagnosis of AD. Industry-sponsored trials of selective COX-2 inhibitors currently target individuals with mild cognitive impairment. Additional trials are in the planning stages as new COX-2 inhibitors continue to be developed. Further elucidation of the role of COX-2 (and COX-1) in various clinical stages of AD, defined by the clinical dementia rating, will clearly aid the clinical design of such trials. As discussed above, the elevation of neuronal COX-2 expression during the early phase (mild dementia) of the clinical progression of AD dementia might set favorable conditions for later inflammatory neurodegenerative conditions. This would be consistent with the apparent early upregulation of COX-2 in neurons of the AD brain, prior to an elevation of cytokine (e.g. IL-6, $TGF\beta_1$) expression (Luterman et al., 2000) and microglial activity (Xiang and Pasinetti, submitted). Moreover, recent evidence suggests that AD patients with a history of NSAID use perform better on neuropsychological test scores than non-users, independently of AD neuropathology (Halliday et al., 2000).

NEURONAL CYCLOOXYGENASE-2: A POTENTIAL TARGET FOR NSAIDs IN THE BRAIN

The apparent protective effect of NSAIDs suggests that COX might be involved in neurodegenerative mechanisms. In view of the great interest in clinical trials of NSAIDs in AD, we have investigated the role of COX in

neurodegeneration (reviewed in Pasinetti, 1998). Traditional NSAIDs are non-selective COX inhibitors; their beneficial effects derive from inhibiting COX-2 activity. COX-1 inhibition, however, is more likely to mediate gastrointestinal, renal and platelet toxicity. Thus, the use of newly developed highly selective COX-2 inhibitors holds promise for maintained efficacy with vastly reduced toxicity (Vane et al., 1995; Warner et al., 1999).

Studies of post-mortem AD, as well as large number of epidemiological and interventional studies point to an important role of COX-2 in the pathophysiology of AD. In particular, the surprising discovery that, in the human brain, COX-2 is expressed primarily in neurons (see below) may have important implications for the treatment of neurodegenerative disorders (Ho et al., 1999, 2001 in press). Moreover, the tremendous resources being devoted by industry and academia to the testing of antiinflammatory drugs for the treatment of AD attests to the growing consensus in favor of the inflammatory hypothesis of the disease (Aisen, 1997).

The potential neuroprotective role of NSAIDs in AD has generally been attributed to suppression of deleterious inflammatory activity. However, evidence from our laboratory indicates a novel role for COX-2 in neuronal death mechanisms other than inflammatory responses. For example, we found that COX-2 might influence cell cycle activities in a model of AD. We found that APPswe/PS1-A246E transgenic mice that develop AD-like neuropathology (Borchelt et al., 1997), when backcrossed with transgenics with neuronal overexpression of human (h)COX-2 (Kelly et al., 1999), show a sharp elevation of cortical neurons immunopositive for phosphorylated serine 795 (S795) retinoblastoma (pRb) tumor suppressor protein and active caspase-3 (Xiang et al., submitted), which are indices of $A\beta_{1\text{-}42}$-mediated apoptotic damage (Giovanni et al., 2000). The elevation of neuronal pRb in the neurons of APPswe/PS1-A246E/hCOX-2 triple transgenics was of particular interest, since pRb regulates cell proliferation by controlling progression through the restriction point within the G_1 phase of the cell cycle (Sherr and Roberts, 1999). While this study provided support to the hypothesis that a principal pathway by which neuronal COX-2 in brain may influence neurodegeneration is by promoting neurons to re-enter the cell cycle (Raina et al., 2000), the data also suggested a novel rational basis for targeting neuronal COX-2 in AD.

COX-2 AND CELL CYCLE ACTIVITIES: IMPLICATIONS IN INFLAMMATORY NEURODEGENERATION IN AD

Four phases comprise the cell division cycle: G_1 (growth), S (DNA synthesis and replication), G_2 (growth for cell division), and M (mitosis) (see scheme below). The main regulators of the progression of the cell cycle are the

cyclin/cyclin-dependent kinase (CDK) complexes. The sequential expression/activation of these proteins not only orchestrate the transition from one phase to another but can also serve as markers of different stages of the cell cycle (Grana and Reddy, 1995). The transition of resting cells from the G_0 to G_1 phase is controlled by the cyclin D/CDK4,6 complexes. Activation of these complexes is triggered by mitogenic growth factors and inhibited by the CDK4,6 inhibitor protein (p)18 (see below).

Little is known about the role of CDK4,6 inhibitors in brain, including p18. Interestingly, using high-throughput microarray DNA techniques, we found that overexpression of hCOX-2 in neurons coincided with decreased mRNA expression of the cell cycle-dependent kinase (CDK)4,6 inhibitor p18 in brain of hCOX-2 transgenics; conversely, the hCOX-2-mediated inhibition of p18 expression was reversed by treatment of mice with the preferential COX-2 inhibitor nimesulide (Pasinetti, unpublished observation). Similarly, we found that the hippocampal expression of CDK4 and COX-2 in hippocampal neurons is co-regulated during the clinical progression of AD dementia, consistent with the evidence that COX-2 may facilitate the re-entry of neurons into the cell cycle (Pasinetti, unpublished observation) (see scheme below). Generally, it is thought that CDK4,6 and cyclin D1 complex regulate the G_0–G_1 transition. As discussed above, one important target substrate for the cyclinD1/CDK4,6 complex is the tumor suppressor retinoblastoma protein (pRb), which is phosphorylated by activated cyclinD1/CDK4,6. Recent evidence shows that pRB can also be phosphorylated during the response to Aβ-mediated neurotoxicity (Giovanni et al., 2000). Once hyperphosphorylated, pRb is released from the transcription factor complex E2F, which then activates genes required for S phase transition. As shown in the diagram below (Figure 35.2), we hypothesize that COX-2, which is induced by glutamate (Kelly et al., 1999) and aggregated Aβ peptides (Pasinetti and Aisen, 1998), may promote cell cycle activities by decreasing the expression of the CDK4,6 inhibitor p18, possibly leading to an unsuccessful attempt to re-enter the cell cycle, influencing caspase activity and neuronal death. We suggest that COX-2-responsive cell cycle activities, possibly facilitating the transition of resting neurons from the G_0 into the G_1 phase, represent an important target for neuroprotection by NSAIDs.

CELL CYCLE, COX-2 AND AD NEUROPATHOLOGY

The search for factors responsible for the formation of neurofibrillary and neuritic plaque pathology has yielded several clues to the hypothesis that the cell cycle may play an important role in AD neurodegeneration. For example, the accumulation of potentially mitogenic growth factors (e.g. epidermal growth factor) in AD amyloid plaques could represent the trigger that initiates

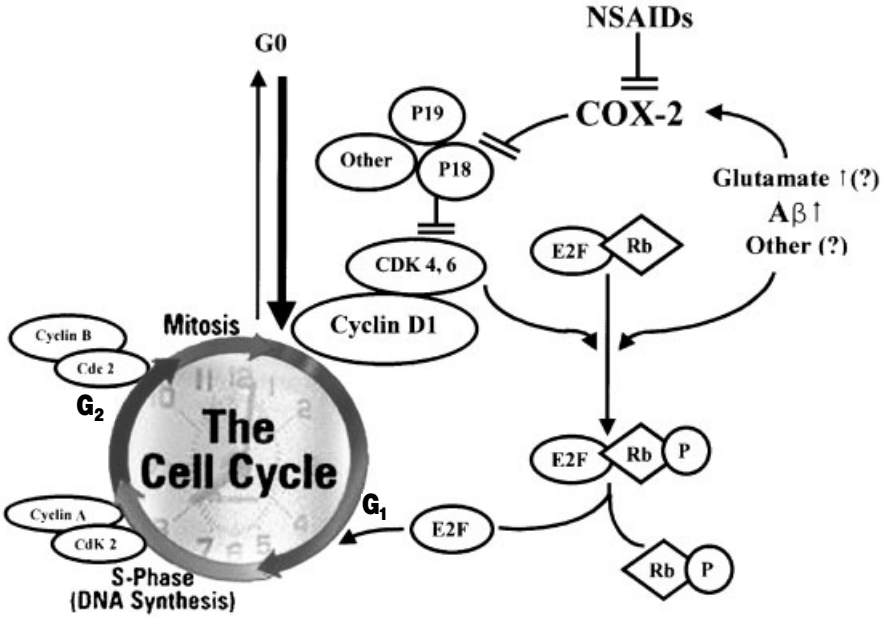

Figure 35.2. The potential role of COX-2 in cell cycle activity in the brain

the re-entry of neurons into the cell cycle. Interestingly, COX-2, whose expression is also regulated by growth factors and tumor promoters (Fletcher et al., 1991), may represent an important link between amyloid pathology and the cell cycle. Another factor associated with amyloid pathology, also known to influence cell cycle pathways, is oxidative stress (Raina et al., 2000). Interestingly, our preliminary studies have established that hCOX-2 transgenics show elevated prostaglandin (PG)$F_{2\alpha}$ levels in brain, along with increased levels of malondialdehyde (MDA), a breakdown product of PGs and a mediator of oxidative stress (Spielman et al., submitted). Thus, it would not be unexpected that neuronal COX-2 overexpression, leading to increased oxidative stress in brain (reviewed in Pasinetti, 1998), may also influence susceptibility to neurotoxic injury through control of cell cycle activities. This hypothesis is consistent with evidence showing that COX inhibitors may also arrest the progression of cell division (Shiff et al., 1996). Another important step toward the understanding of AD-related neuritic pathology was the identification of protein kinases involved in tau (τ) phosphorylation. For example, there is evidence showing that, *in vitro*, CDK 5 can phosphorylate τ into a neurofibrillary tangle (NFT) AD-type state (Baumann et al., 1993). The importance of these findings is that they may provide an explanation for the increased phosphorylation of cytoskeleton

proteins, such as τ neurofilaments, that represent intracellular changes in AD. Interestingly, while destabilization of microtubules and coincidental activation of different kinases that are able to phosphorylate τ are features of the cell division cycle, we note that in the AD brain COX-2 expression is preferentially elevated in NFT positive neurons of the hippocampal formation (Oka et al, 1997). Thus, inappropriate re-entry into the cell cycle and interrupted mitotic processes may be significant factors, not only in neuronal degeneration but also in the cytoskeleton pathology that characterizes the pathology of AD. We hypothesize that COX-2, by promoting cell cycle activities, might also participate in abnormal τ phosphorylation, further influencing AD neuropathology and possibly the clinical progression of AD dementia.

THE POTENTIAL ROLE OF COX-1 IN GLIAL MEDIATED INFLAMMATORY CASCADES IN AD

Inhibition of COX-1 expression might also be beneficial to AD neurodegeneration. While the regulation of COX-1 in the AD brain is still controversial (Pasinetti et al., 1998; Yasojima, 1999), COX-1 appears to be expressed in both glia and neurons of the AD brain (Pasinetti et al., 1998; Yermakova et al., 1999). Unfortunately, little is known about the role of constitutive COX-1 expression in the brain, and its expression in glia might be linked to more traditional inflammatory mechanisms. For example, there is evidence that the number of activated microglia is reduced in non-demented elderly subjects with a history of NSAID use when compared to age-matched control cases with no history of NSAID exposure (Pasinetti, 1998). While inhibiting COX-2 activity in neurons may influence the clinical progression of AD, non-selective NSAIDs might also influence AD progression by simultaneous inhibition of COX-1 (at the cost of side effects associated with non-selective COX inhibitors). However, further research would be necessary to fully clarify the role (if any) of constitutive COX-1 expression in the AD brain.

NEURONAL COX-2 AND ITS EXPRESSION IN THE AD BRAIN

Immunocytochemical evidence indicates that COX-2 expression is elevated in the AD brain. Pasinetti and Aisen (1998) and others (Mackenzie et al., 1998; Oka et al., 1997; Lukiw et al., 1997) found that COX-2 protein content is increased in the post-mortem AD brain and that the elevation of COX-2 signal is localized primarily to neuronal cells. Evidence showing the regulation of COX-2 mRNA in the AD brain is equivocal (Pasinetti, 1998; Ho et al., 1999;

Lukiw et al., 1997), which may be, in part, due to the fact that COX-2 mRNA is an unstable, short-lived RNA species with a half-life of approximately 3–5 h (Lukiw et al., 1997). Thus, COX-2 mRNA may not be the appropriate index for assessing COX-2 regulation in human post-mortem tissues.

Immunocytochemical evidence showed that the increased levels of COX-2 content in subsets of pyramidal layer neurons of the hippocampal formation correlates with neuronal atrophy (Ho et al., 1999), consistent with evidence showing that, in the AD brain (and in Down's syndrome), COX-2 protein content is preferentially elevated in neurons with NFT and damaged axons (Oka et al., 1997). Again, no evidence was found of COX-2 expression in cells other than neurons in the AD brain. This evidence is of great interest, especially in view of the evidence that glial and endothelial cells may also express and regulate COX-2 mRNA/protein in other neurodegenerative or inflammatory conditions *in vivo* (Collaço-Moraes et al., 1996; Sairanen et al., 1998; Hirst et al., 1999; Elmquist et al., 1997; Cao et al., 1997; Thore et al., 1996) or *in vitro* (Bauer et al., 1997; Minghetti et al., 1996; Kelly et al., 1999). Thus, this selective neuronal compartmentalization of COX-2 in the AD brain suggests a potential role of COX-2 in neurodegenerative mechanisms independent of classical inflammatory cascades. It is possible that COX-2 inhibitors may not necessarily exert their actions by suppressing inflammation in the AD brain (i.e. cytokine-driven acute phase response in glia), but by influencing the role of COX-2 in neurons and possibly in cell cycle activities.

If the hypothesis is that neuronal COX-2 is the target of NSAIDs in AD, a selective COX-2 inhibitor with central nervous system penetration would be a good candidate for therapeutic trials in AD. Recently approved selective COX-2 inhibitors have been largely successful because these drugs share COX-2 inhibitory efficacy with older non-selective COX inhibitors (such as indomethacin), with remarkably fewer side effects. These drugs are now being developed for AD treatment.

COX-2 AND EXCITOTOXICITY

There is evidence that several of the prostanoid products of arachidonic acid metabolism by COX potentiate glutamate excitotoxicity, representing another mechanism by which COX-2 overexpression in neurons of the AD brain might accelerate neurodegeneration (Pasinetti, 1998). Evidence also indicates that excitotoxicity may potentiate Aβ neurodegeneration in rats (Morimoto et al., 1998), and that NMDA-mediated neuronal death is prevented in a dose-dependent manner by COX-2 inhibitors in primary neuronal cultures (Hewett et al., 2000). We used a transgenic mouse model overexpressing neuronal hCOX-2 in neurons to explore the role of COX-2 in excitotoxic neurodegeneration. We found that overexpression of neuronal hCOX-2 in neurons of transgenic mice potentiates the intensity and lethality

of kainic acid (KA) neurotoxicity. This finding coincidentally occurred with the potentiation of expression of the immediate early genes *c-fos* and *zif-268* (Kelly et al., 1999). The evidence suggests that COX-2 expression in neurons may play a causal role in excitotoxic neuron death, possibly through control of expression of immediate early genes. This formulation is also consistent with the recent hypothesis that, in AD, chronic excitotoxic activity may be responsible for the widespread pattern of neurodegeneration (Olney et al., 1997).

ANTIINFLAMMATORY DRUG TRIALS IN AD: FUTURE DIRECTIONS

Toxicity remains a major roadblock to a study of antiinflammatory dosage of a traditional NSAID. One way to reduce NSAID toxicity is to add a cytoprotective agent to the treatment regimen. Misoprostol is a synthetic prostaglandin known to reduce the incidence of NSAID gastropathy. However, a recent report of a trial of diclofenac plus misoprostol in AD patients indicated that this combination was not tolerated any better than indomethacin; the drop-out rate in this 25 week trial was 50% in the active drug group, rendering the results of the study inconclusive (Scharf et al., 1999). It is unlikely that a study of a treatment regimen that is tolerated (over the course of a year) by a minority of subjects will yield definitive results. The ADCS has opted to study two NSAID-type regimens that are expected to have substantially less toxicity than the indomethacin and diclofenac regimens reported. The first is the new selective COX-2 inhibitor rofecoxib. As discussed above, COX-2 may be the target of action of NSAIDs in the AD brain and, since COX-2 inhibitors appear to carry much reduced risk of serious gastrointestinal toxicity, rofecoxib may be effective.

In conclusion, the characterization of inflammatory processes in the AD brain has led to efforts to develop antiinflammatory treatment strategies to slow the rate of disease progression. These strategies target one or more of the processes discussed above: COX-2, cytokines and complement cascade. The recent elucidation of the respective physiologic roles of COX-1 and COX-2 has resulted in great interest by the pharmaceutical industry in the development of selective inhibitors of COX-2 (Warner et al., 1999). These agents may be equally effective in suppressing inflammation in diseases such as rheumatoid arthritis, but with reduced gastrointestinal toxicity. Selective COX-2 inhibitors may be excellent candidates for therapeutic trials in AD because of their improved safety and because they target the COX isoform, which may be involved in AD neurodegeneration. Perhaps low doses of the non-selective COX inhibitor, ibuprofen, without an antiinflammatory effect, can influence neuronal COX-2 function in AD brain and limit oxidative stress (e.g. by aggregated Aβ or by the COX-2 activity itself) to

sub-threshold levels (Pasinetti, unpublished observation). Thus, it would not be unexpected that non-selective COX inhibitors, such as ibuprofen, might also affect COX-2 neuronal metabolic activities independent of glial inflammatory. We also note that ibuprofen may suppress plaque pathology and inflammation in a mouse model of AD neuropathology (Lim et al., 2000). In addition, we will address the possible contribution of COX-2 on cytokine expression and activation of the complement cascade during the clinical progression of AD dementia. Two pharmaceutical companies are now conducting large-scale trials of selective COX-2 inhibitors to slow the progression of AD; one of these trials, using rofecoxib (Vioxx), targets the earliest stage of the disease (i.e. slowing the progression from questionable to mild dementia).

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36 Parallels between the Redox Properties and Toxicity of Aβ in Alzheimer's Disease and Mutant Cu/Zn-SOD in Familial Amyotrophic Lateral Sclerosis

ASHLEY I. BUSH

INTRODUCTION

Over the last decade, the pendulum of scientific opinion has swung towards the implication of Aβ as the protein whose mismetabolism is linked to Alzheimer's disease (AD). This is because:

1. Mutations of the β-amyloid protein precursor are linked to familial AD (FAD) (Murrell et al., 1991).
2. Aβ is the principal insoluble component of plaques, the most dramatic feature of AD neuropathology (Glenner and Wong, 1984).
3. Aβ is neurotoxic in cell culture (Yankner et al., 1990).
4. Genetic lesions linked to AD (e.g. apolipoprotein E genotype or presenilin mutations), alter the metabolism of Aβ (Suzuki et al., 1994; Scheuner et al., 1996).

The factors that cause Aβ accumulation, and the mechanism by which the peptide causes neuronal demise, are important for targeting therapeutics. The length of the Aβ species is considered to be one important factor in AD pathogenesis, since $A\beta_{1-42}$, a minor free soluble species in biological fluids (Vigo-Pelfrey et al., 1993), is enriched in amyloid deposits (Masters et al., 1985; Kang et al., 1987; Prelli et al., 1988; Roher et al., 1993) and its concentration is elevated as a result of familial AD-linked mutations. Synthetic $A\beta_{1-42}$ appears to be more self-aggregating than $A\beta_{1-40}$ in solution (Hilbich et al., 1991; Jarrett et al., 1993). These findings have led to two dominant hypotheses posited to explain the association of Aβ with AD:

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1. That amyloid deposition is caused by the generation of excess $A\beta_{1-42}$ alone.
2. That Aβ fibril formation is responsible for the molecular insult that causes neuronal dysfunction, and the attendant dementia.

As a consequence of these hypotheses, much effort has gone into elaborating the catabolic pathway that generates Aβ from APP, with the aim of inhibiting Aβ production as a therapeutic measure. This approach assumes that Aβ has no physiological function. As research in AD moves forward, it is becoming increasingly more clear that these two hypotheses are incorrect. First, we have found that the self-aggregation of $A\beta_{1-42}$ in neutral buffers does not occur if the peptide is strictly quarantined away from metal ions (Cu^{2+}, Zn^{2+}, Fe^{3+}). Since trace concentrations of metals contaminate all buffers, such decontamination can only be achieved by introducing high-affinity chelators to the peptide preparations (Atwood et al., 2000; Huang et al., in preparation). Second, not only is there evidence that Aβ is toxic in its soluble form (Lambert et al., 1998) but also there is evidence that the soluble (and not the insoluble) forms of Aβ correlate with both mortality and dementia-associated neuropathological features, such as tangles and neuritic changes (McLean et al., 1999).

There are further reasons to suggest that other neurochemical reactions apart from Aβ production must contribute to amyloid formation in AD. Were elevated cortical Aβ concentrations to be solely responsible for the initiation of amyloid, then it would be difficult to explain why the amyloid deposits are focal (related to synapses and the cerebrovascular lamina media) and not uniform in their distribution. Microanatomical neurochemical factors are also likely to play a role in initiating the amyloid of hereditary cerebral hemorrhage with amyloidosis–Dutch disease, in which a mutation at residue 22 of Aβ (Glu→Gln) (Levy et al., 1990) causes amyloid to form only in the lamina media of the cerebrovasculature and not in the brain parenchyma. Another observation to argue against elevated concentrations of Aβ as being solely sufficient to induce cortical Aβ precipitation is that, although a handful of transgenic mouse models with amyloid pathology related to overexpression of APP and Aβ species have been reported, similar degrees of overexpression have often failed to induce amyloid deposition in other reported attempts to induce cortical amyloid pathology in transgenic mice (Hsiao et al., 1995). To attribute amyloid initiation to the presence of $A\beta_{1-42}$ alone is problematic, since the peptide is a normal component of healthy CSF. Finally, amyloid deposition is an age-dependent phenomenon, even where accelerated in FAD cases. Since amyloid deposition does not occur in childhood, and since there is no clear evidence that Aβ levels rise with age, it is likely that other age-related stochastic neurochemical changes play an essential role in amyloidogenesis.

These observations argue for neurochemical factors other than Aβ length or concentration to be considered essential in the pathogenic cascade of AD.

Much data now supports the possibility that abnormal combination of Aβ with Cu^{2+}, Zn^{2+} or Fe^{3+}, and the consequent reactive oxygen species (ROS) that are generated, could contribute to AD pathophysiology. In this sense, we see intriguing parallels between Aβ and copper/zinc superoxide dismutase 1 (Cu/Zn-SOD, or SOD1), which is mutated in familial amyotrophic lateral sclerosis (FALS)(Rosen et al., 1993).

Aβ DEPOSITION AND CEREBRAL OXIDATION ARE BOTH CLOSELY ASSOCIATED WITH AD PATHOGENESIS

Increasing evidence has implicated oxidative stress in the pathogenesis of AD. Metabolic signs of oxidative stress in the neocortex of AD patients, oxygen radical-mediated damage of brain proteins, lipids and DNA, systemic signs of oxidative stress and the response of antioxidant systems have all been observed in AD (Mecocci et al., 1994; Hensley et al., 1995; Ceballos-Picot et al., 1996). A number of oxidative stress markers have been observed in association with amyloid plaques, neuritic plaques and NFTs, including 4-hydroxynonenal (Sayre et al., 1997), pyrraline and pentosidine (Smith et al., 1994b), 3-nitrotryosine (Smith et al., 1997b), AGE-modified tau (Smith et al., 1994b), redox active Fe (Smith et al., 1997a), neurofilament-related protein carbonyls (Smith et al., 1991), as well as elevations in Cu/Zn-SOD (Pappolla et al., 1992), Mn-SOD (Furuta et al., 1995), catalase (Pappolla et al., 1992; Furuta et al., 1995), ferritin (Grundke-Iqbal et al., 1990) and elevated HO-1 (Smith et al., 1994a). Further evidence indicating oxidative stress in the AD brain can be implied from the observation that there is a strong spatial correlation between antioxidant enzyme activity and markers of lipid peroxidation and the areas of the brain particularly affected by AD lesions. These results suggest that antioxidant activity is increased in a compensatory manner for the increased free radical generation (Lovell et al., 1998). Recently, treatment of AD patients with the antioxidants vitamin E and selegiline (Sano et al., 1997) has been reported to delay the progression of clinical AD.

The biochemical relationship between Aβ amyloid deposition in AD and oxidative stress is particularly complex and intriguing. A recent study by Smith et al. (1998) showed that amyloid-bearing transgenic animals develop similar oxidative damage in the neocortex, and have elevated redox-active Fe within amyloid plaques similar to that seen in the AD-affected brain. This finding suggests that Aβ deposition may contribute to the oxidative burden within the brain. However, the mechanisms underlying the association between oxidation and amyloid deposition are not well understood. In contrast to the findings of Smith et al. (1998), the same workers have reported that there is no clear spatial association between histological amyloid plaque

deposits and histological markers of oxidation (Nunomura et al., 1999). Nevertheless, amyloid deposition and oxidative markers in neuronal pathways that are most damaged in AD always coincide. We have found that there is no correlation between total Aβ load in neocortex and histological plaque count (McLean et al., 1999). Therefore, it is not surprising that plaque load does not correlate well with other features of neocortical damage in general. Most recently, in collaboration with the Perry laboratory, we have found that there is an inverse correlation between amyloid plaque load with 8-OH guanosine (8-OHG) adducts in AD-affected neocortical tissue (Cuajungco et al., 2000). We believe that amyloid may represent Aβ that has been precipitated and rendered redox-silent by zinc (explained further below). There is other compelling evidence to consider Aβ accumulation as a major source of oxidative damage in AD.

Synthetic Aβ peptides induce lipid peroxidation of synaptosomes (Butterfield et al., 1994), and exert toxicity through mechanisms that involve the generation of cellular H_2O_2 (Behl et al., 1994) and is abolished by SOD (Thomas et al., 1996) and O_2^-/H_2O_2 scavengers (Bruce et al., 1996). $A\beta_{1–40}$ has also been reported to generate the hydroxyl radical by mechanisms that are unclear (Tomiyama et al., 1996). Vitamin E (Harris et al., 1995) and the spin-trap compound PBN (Goodman and Mattson, 1994), have been shown to protect against Aβ-mediated neurotoxicity *in vitro*. Taken together, the chemical nature of the findings of oxidation stress in AD brain indicate that H_2O_2 levels may be elevated in the brain. We have recently reported that Aβ is redox-active when binding Cu and Fe, and directly generates H_2O_2 using O_2 as the substrate in a reaction that involves the transient reduction of the metals (Huang et al., 1999a,b). When $A\beta_{1–42}$ binds Cu^{2+}, it shifts the redox potential of Cu^{2+} creating reduction potential of +500 mV (vs. the $Ag/AgCl_2$ electrode) (Huang et al., 1999b), which is stronger than that of Cu/Zn-SOD. The cell-free generation of H_2O_2 mediates the neurotoxicity of Aβ, and the toxicity of Aβ variants correlates with the peptide's redox activity ($A\beta_{1–42} > A\beta_{1–40} \geqslant$ rat Aβ) (Huang et al., 1999b). H_2O_2 is freely permeable across membrane boundaries and is a necessary substrate for hydroxyl radical formation, which causes the 8-OHG damage that characterizes AD-affected neocortex. The generation of all of these ROS, O_2^-, H_2O_2 and OH•, require catalytic electron transfer from Cu^+ or Fe^{2+} to molecular O_2 which can be shut down by metal-complexing agents (Bush et al., 1999a). For these reasons, we believe that abnormal interaction of Aβ with Cu and Fe could be an excellent target for potential pharmacotherapeutics for AD.

BRAIN BIOMETALS AND AD

My laboratory's interest in the role of brain biometals in AD stemmed from a series of *in vitro* studies that found that synthetic Aβ and purified APP

Table 36.1. Zn^{2+} and Cu^{2+} binding affinities to $A\beta_{1-40}$ and $A\beta_{1-42}$

K_d (M)	Highest affinity	Lowest affinity
$A\beta_{1-40}/Zn$	1.0×10^{-7}	1.3×10^{-6}
$A\beta_{1-42}/Zn$	1.0×10^{-7}	1.3×10^{-6}
$A\beta_{1-40}/Cu$	4.6×10^{-11}	1.3×10^{-8}
$A\beta_{1-42}/Cu$	7.0×10^{-18}	5.0×10^{-9}

These values are a summary of those published (Bush et al., 1994a,c; Atwood et al., 2000) and unpublished ($A\beta_{1-42}/Zn^{2+}$).

exhibited several interesting interactions with Zn^{2+}, Cu^{2+} and, to a lesser extent, Fe^{3+}, at low micromolar and submicromolar concentrations of these metal ions (Bush et al., 1993, 1994a,b,c, 1995; Huang et al., 1997; Atwood et al., 1998). Since the brain is a specialized organ that concentrates these metals in the neocortex (Lovell et al., 1998), we considered it possible that dyshomeostasis of these metals could contribute to abnormal Aβ behaviour in AD. This hypothesis has been supported by the observations by other laboratories of extreme enrichments of these metals in amyloid plaques in both AD and APP2576 transgenic mice (Lovell et al., 1998; Lee et al., 1999; Suh et al., 2000).

Unlike Zn^{2+}, Cu^{2+} and Fe^{3+} induce greater Aβ aggregation under mildly acidic conditions (e.g. pH 6.8–7.0; Bush et al., 1995; Atwood et al., 1998), such as those believed to occur in AD brain (Yates et al., 1990). Significantly, the solubility of rat/mouse $A\beta_{1-40}$ (with substitutions of Arg→Gly, Tyr→Phe and His→Arg at positions 5, 10 and 13, respectively) is unaffected by Zn(II) or Cu^{2+} at low micromolar concentrations (Bush et al., 1994c; Atwood et al., 1998), perhaps explaining why these animals do not form cerebral Aβ amyloid (Vaughan and Peters, 1981).

Aβ possesses selective high affinity binding sites for both Cu^{2+} and Zn^{2+}, and possesses low affinity sites that may either be selective or represent Cu^{2+} binding at the Zn^{2+} site or vice versa. Evidence so far suggests that the high affinity sites mediate the redox activities of the peptide (although the low affinity Cu^{2+} binding may also be redox competent), and that the low affinity sites mediate precipitation. Although the affinity of $A\beta_{1-42}$ is 11 orders of magnitude stronger for Cu^{2+} than for Zn^{2+} (Table 36.1), when co-incubated with equal concentrations of Zn^{2+} and Cu^{2+}, $A\beta_{1-40}$ binds equal amounts of both metal ions (Atwood et al., 2000), suggesting that the binding sites are highly specific. This selectivity is lost when the incubation is performed under slightly acidic conditions (pH 6.8), whereupon the peptide binds only Cu^{2+} (Atwood et al., 2000). Metal ion binding is mediated by histidine residues (Bush et al., 1994a; Atwood et al., 1998). His 13 is essential for Zn^{2+} coordination (Liu et al., 1999), and Cu^{2+} binding involves coordination with the sole tyrosine residue at position 10 (Miura et al., 2000).

There is a large body of evidence from studies that indicate that the homeostases of Zn, Cu and Fe, and their respective binding proteins, are significantly altered in the AD brain (reviewed in Atwood et al., 1999). For example, a recent well-controlled study using microparticle-induced X-ray emission (PIXE) analysis of the cortical and accessory basal nuclei of the amygdala indicated that these metals accumulate in the neuropil of the AD brain where their concentrations are 3–5-fold increased compared to age-matched controls. Interestingly the concentration of these metal ions, in particular redox active Cu and Fe that are implicated in free radical reactions (Halliwell and Gutteridge, 1984), are normally most concentrated in those regions of the brain most affected by AD pathology. Evidence for abnormal Cu homeostasis in AD includes a 2.2-fold increase in the concentration of CSF Cu (Basun et al., 1991) and an accompanying increase in ceruloplasmin (Loeffler et al., 1996) in the brain and CSF of AD patients. There is an extensive literature describing abnormal levels of Fe and Fe-binding proteins in AD (Robinson et al., 1995). Importantly, the Fe and Cu that is found within the amyloid deposits of human brain and in amyloid-bearing APP transgenic mice has been shown to be redox-active (Smith et al., 1997a, 1998; Sayre et al., 2000).

We have recently reported that Zn/Cu-selective chelators markedly enhance the resolubilization of Aβ deposits from post-mortem AD brain samples (Cherny et al., 1999), supporting the possibility that Cu and Zn ions play a significant role in assembling these deposits. Aβ-associated Zn/Cu-metalloproteins, apolipoprotein E (Moir et al., 1999) and α-2-macroglobulin (Du et al., 1997), also participate in metal-mediated interactions with Aβ.

ZINC MODULATES SOD NEUROTOXICITY: A MOLECULAR INSULT LINKING AD TO FAMILIAL ALS

About 10–15% of all amyotrophic lateral sclerosis (ALS) cases are familial and ca. 20% of the cases of familial ALS (FALS) are associated with a point mutation in Cu/Zn-SOD (SOD1) (Rosen et al., 1993). Substantial evidence suggests that the SOD1 mutation causes disease by a gain of function mechanism, due to an adverse property of the mutant SOD1 protein that induces increased oxidative damage (Gurney et al., 1994). The mechanism of oxidative damage seen in transgenic mice expressing mutant SOD1 is not yet clear, but the mutant protein (including G93A) has been observed to catalyze hydrogen peroxide-mediated oxidation more than the wild-type protein (Wiedau-Pazos et al., 1996). The clinical severity of the FALS phenotype correlates with the degree that the mutation of SOD1 lowers the K_m for hydrogen peroxide-dependent radical formation (Yim et al., 1997). Mutant SOD1 binds zinc with lower affinity (Crow et al., 1997) and tends to lose zinc from its binding site, exposing the active site copper, which then abnormally

catalyzes oxygen-dependent superoxide (and then peroxynitrite) formation, which can be blocked by sustaining zinc levels on SOD1 (Estevez et al., 1999).

Free Cu^{2+} is highly toxic and probably does not exist *in vivo* (Rae et al., 1999). The reason for the high toxicity of Cu^{2+} is that it catalyzes the generation of reactive oxygen species (ROS) such as superoxide and hydrogen peroxide, and its peroxidative reactivity also generates the hydroxyl radical. Therefore, there are several biochemical buffers that are believed to quarantine Cu^{2+} from such abnormal reactivity, while the Cu^{2+} cycles within the cell to participate in essential activities such as respiratory chain electron transfer. SOD1 represents a large fraction of the Cu^{2+} within the cell, and the metallothioneins are responsible for complexing Cu^{2+} under conditions of Cu^{2+} excess. Glutathione (GSH) is needed to transfer Cu^{2+} taken up by the cell to metallothionein, and the fraction of total cytoplasmic Cu^{2+} bound to GSH can reach 60% in some cell lines (Freedman et al., 1989). If GSH is depleted, the amount of Cu^{2+} bound to metallothioneins is correspondingly decreased, which means that more cytosolic Cu^{2+} could be available for ROS generation (Freedman et al., 1989). This may explain why GSH protects the cell from Cu^{2+} toxicity (Freedman et al., 1989), as demonstrated in our reports that β-amyloid depletes neuronal cells of GSH and makes them more vulnerable to Cu^{2+} toxicity (White et al., 1999), while depleting the cell of GSH makes it more vulnerable to Aβ-mediated toxicity (Cuajungco et al., 2000). Since the redox-silencing of Cu^{2+} is achieved by the action of GSH moving Cu^{2+} onto MT, our findings of the beneficial effects of N-acetyl cystine (NAC) on FALS transgenic mice bearing the SOD1 G93A mutation (Andreassen et al., 2000) may also be explained by the effects of increasing the GSH-mediated shift of cytosolic Cu^{2+} onto MT.

Cu^{2+} can also generate ROS when incorrectly or inappropriately bound to protein (as is the case with binding to Aβ) and, even when bound to SOD, Cu^{2+} is a versatile catalyst. Alone, it has a SOD activity with a rate constant the same as SOD1 itself (Bielski et al., 1985). The purpose of the SOD1 protein structure could be to harness this activity of Cu^{2+} without permitting any other reaction, such as the generation of OH• formed by the reaction of reduced Cu^{2+} (transiently made during the disproportionation of O_2^-) with the product, H_2O_2. Hence, the Cu^{2+} at the active site of SOD1 has the potential to be abnormally redox-reactive, generating unwanted toxic species.

The Beckman laboratory has recently proposed a mechanism for the pathogenicity of mutant SOD1 (Estevez et al., 1999). Starting from the observations from Joan Valentine's laboratory that pathogenic SOD1 mutations do not cause a loss (or gain) of function when the protein is fully loaded with Cu^{2+} and Zn^{2+}, but do cause a marked loss of affinity for Zn^{2+} (Lyons et al., 1996), the Beckman group (Estevez et al., 1999) observed that altered Cu^{2+} coordination made Zn-deficient SOD (wild-type or mutant) a more efficient oxidant, able to oxidize ascorbate 3000-fold faster than

Cu/Zn-SOD. The altered reactivity of Zn-less SOD allows it to be reduced by cellular reductants (like GSH) which then donate an electron to O_2 to generate O_2^-, which then reacts with NO to form the strong prooxidant, peroxynitrite. Thus, if SOD1 loses Zn^{2+}, its dismutase catalytic activity is diminished while it simultaneously abnormally develops tyrosine nitration activity mediated by O_2^- formed at the Cu^{2+} catalytic site (Crow et al., 1997). The Beckman group proposed that the O_2^- formed by Zn-deficient SOD1 might not be released as a free intermediate, which could explain why excess SOD1 fails to slow disease progression in FALS/SOD1 transgenic mice (Bruijn et al., 1998). Like Aβ (Cuajungco et al., 2000), Cu-loaded SOD1 is neurotoxic at micromolar concentrations in cell culture when not bound to Zn^{2+} (Estevez et al., 1999). Hence, SOD1, while being traditionally thought of as an antioxidant, has the capacity to become strongly pro-oxidant as a consequence of diminished zinc binding.

Part of the appeal of this model to our laboratory is that it resonates with our own thinking about the possible pathogenic mechanism of Aβ-mediated toxicity in Alzheimer's disease, which, as we have reported, is mediated by cell-free H_2O_2 production from O_2 also dependent upon Cu^{2+} interaction (Huang et al., 1999b) and quenched by Zn^{2+} (Cuajungco et al., 2000). To test how far this mechanistic analogy can go, we have recently assayed SOD1 in its native and its 'zinc-less' Cu^{2+}-loaded state, to determine whether, like Aβ, it can produce H_2O_2 from O_2. We have found that Cu-SOD but not Cu/Zn-SOD or apo-SOD, does indeed produce H_2O_2 from O_2 using ascorbate (or dopamine) as the electron donor. Interestingly, we also found that Aβ loaded with Cu^{2+} also uses ascorbate (or dopamine) as a substrate for H_2O_2 production (Opazo et al., manuscript in preparation). Therefore, the reason why native SOD1 may not be able to rescue the phenotype induced by mutant SOD1 is because the lesion is not just caused by inappropriate O_2^- generation, but also by inappropriate H_2O_2 generation—a ROS that is not scavenged by SOD but rather by catalase and (mainly) GSH/GPx. This insight links Alzheimer's disease and ALS together at the level of the basic molecular oxidative insult, and reinforces our confidence in the need for therapies that both inhibit inappropriate protein-mediated ROS generation and also deal with the downstream consequences of radical generation.

Correlating with our findings that apo-SOD1 does not produce H_2O_2, the Beckman group (Estevez et al., 1999) found that apo-SOD1 was not neurotoxic in cell culture, whereas Zn-deficient, Cu-loaded SOD1 was neurotoxic, an effect that could be rescued by treatment with Cu^{2+} chelators, in agreement with reports of the treatment efficacy of Cu^{2+} chelators TETA (Nagano et al., 1999) and D-penicillamine (Hottinger et al., 1997) upon G93A mutant transgenic mice. We are currently appraising the effects of similar chelators in the treatment of amyloid-bearing transgenic mice.

As described above, Aβ and SOD1 share several physicochemical similarities (Table 36.2). Particularly striking about Aβ is the extremely

Table 36.2. A summary of the similar features of Aβ and SOD1

Aβ vs. Mutant SOD (AD vs. FALS) both:
• Aggregate in neural tissue
• Associated with age-dependent neurodegenerative disease
• Have strong reducing potentials
• Vulnerable Zn binding (Aβ-acidosis; SOD mutation)
• Reduce O_2 in the absence of Zn to generate H_2O_2
• Use vitamin C and dopamine to generate H_2O_2
• Are neurotoxic at μM concentrations in the absence of Zn
• Catalyze O_2^- dismutation when Zn-loaded
• May be physiological antioxidants

strong reducing potential of Aβ–Cu complexes (Huang et al., 1999b), resembling that of SOD1, and the attomolar affinity of Cu^{2+} binding for $A\beta_{1-42}$ (Atwood et al., 2000), again resembling that of SOD1. This has led us to determine that Aβ can form soluble complexes when bound to a specific stoichiometry of Zn^{2+} and Cu^{2+}, and upon doing so develops significant SOD-like activity (Bush et al., 1999b). This activity correlates with the affinities of the peptide variants for Cu^{2+} (unpublished observations). Therefore, when Cu^{2+} binds to Aβ, it is not sequestered, but rather is presented for catalytic redox activities (like 'zincless'-SOD). As with SOD1, co-binding of Zn^{2+} to Aβ abolishes inappropriate electron donation to O_2 and promotes electron transfer to O_2^- (dismutation). The similarities between Aβ and SOD1 have led us to consider a hypothesis that Aβ and the APP biosynthetic and degradative pathway may represent elements of a novel antioxidant system. Supporting this notion are reports that Aβ is released by normal cells under oxidative stress (Frederikse et al., 1996) and that Aβ is neurotrophic at nanomolar concentrations (Yankner et al., 1990; Koo et al., 1993). SOD1 forms a neurotoxic 'rogue' protein as a consequence of loss of Zn^{2+} from its active site, caused by mutation. We hypothesize that Aβ may form a similar neurotoxic 'rogue' protein as a consequence of loss of Zn^{2+}, and recruitment of Cu^{2+} caused by epochs of acidosis (a stochastic consequence of aging), which weaken Zn^{2+} binding (Bush et al., 1994a) and promote Cu^{2+} binding (Atwood et al., 1998, 2000; Miura et al., 2000). This generates H_2O_2, which sets up a vicious cycle by triggering the release of more Aβ into an acidotic environment. This model is currently undergoing experimental scrutiny.

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37 β-Amyloid Toxicity: Diverse Biological Activities Drive Multiple Cellular Mechanisms

C. W. COTMAN, L. TONG, A. ANDERSON, D. CRIBBS AND J. SU

INTRODUCTION

Beta-amyloid (Aβ) is a 40–42 amino acid peptide derived from the processing of the amyloid precursor protein (APP). Aβ assumes many different conformations, which are capable of different biological activities. It has the ability to interfere with signal transduction processes and disturb normal neuronal function. It can be toxic and it can drive molecular and cellular cascades. Yet, the *in vivo* role of Aβ in neuronal degeneration is still controversial, possibly because it can assume so many biological roles or personalities. In this chapter, we propose a multiple variable model for the role of Aβ and suggest that a major part of the controversy surrounding the role of Aβ in neurodegeneration and Alzheimer's disease (AD) is that it does not fit the single factor model that is so pervasive in current research. Although the approach of studying one insult against a constant background is necessary, it is clear that the role of Aβ in neurodegeneration is complex. Thus, we propose that the role of Aβ depends on the coexistence of several factors at once. In addition to its own inherent bioactivity, Aβ drives diverse molecular cascades and amplifies the adverse consequences of other insults, and hence becomes a critical driving factor in a variety of pathological mechanisms in AD.

We suggest that there are different phases in brain aging and the development of AD (Figure 37.1). This is consistent with the overall profile of human aging. Some individuals retain high cognitive function throughout life and live into their 90s (successful aging phase). Others start to show functional loss (initiation phase) in their 60s–70s, possibly associated with age-related memory impairment or mild cognitive impairment (MCI). Finally, some aging individuals make the transition into AD (propagation

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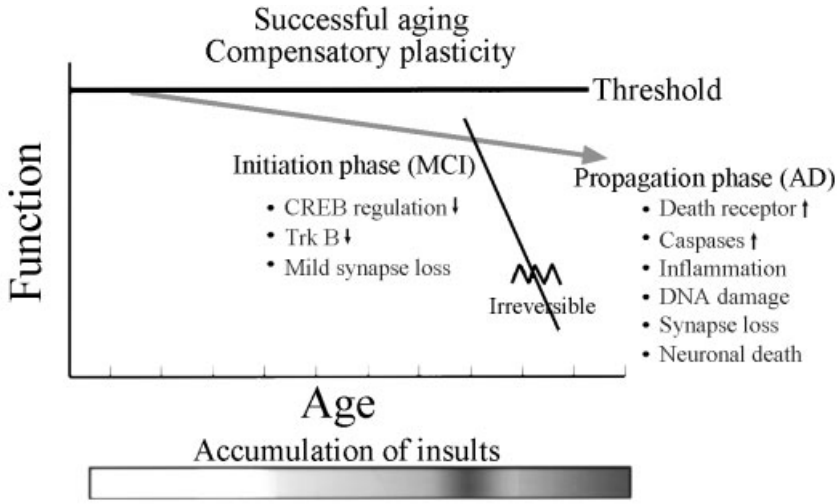

Figure 37.1 A model illustrating phases in brain aging and neurodegeneration: successful aging, initiation and propagation phases. The successful aging phase maintains normal functions which are often protected by cellular plasticity mechanisms. The initiation phase, possibly associated with mild cognitive impairment, is launched once insults reach a critical threshold and cause decreased or altered signaling and mild synapse loss. The propagation phase, characterized by AD, is associated with extensive tangles, plaques and the activation of degenerative cascades. The accumulation of multiple insults underlies the progression. Aβ plays a role in both the initiation and propagation phases

phase). Inherent in this model is that the role of Aβ may vary as brain pathology evolves. In the initiation phase, we propose that Aβ suppresses activity-dependent plasticity mechanisms by interfering with key signal transduction mechanisms. In the propagation phase, we suggest that Aβ and other insults exert an increasing role, including synergism with other risk factors, such that preexisting pathology proliferates in an ever-increasing and irreversible manner. In this brief summary, we will discuss the potential role of Aβ in brain aging and disease propagation through the various phases. Unfortunately, due to space limitations it is not possible to cite all of the many contributions by all investigators that have contributed to this concept.

INITIATION PHASE: Aβ AT SUBLETHAL LEVELS INTERFERES WITH THE REGULATION OF CREB TRANSCRIPTIONAL CONTROL AND GENE EXPRESSION

In the initiation phase, there is an increase in the accumulation of toxic molecules and conditions. These accumulate at sublethal levels over long

periods of time prior to reaching the threshold for having the capacity to cause neurodegeneration. It is generally assumed that these molecules have little effect on neurons at sublethal levels. However, we suggest that insults can compromise neuronal function prior to causing overt degeneration. The initiation phase in this model is hypothesized as being primarily non-degenerative and potentially reversible. It is further suggested that this phase may be initiated locally in limited areas of the neurophil and/or regions of the brain.

Overexpression of mutant amyloid precursor protein (APP) in cells and in transgenic animals mimics some of the features of AD, including increased accumulation of $A\beta_{1-42}$. Most significantly, transgenic mice overexpressing mutant APP also show impairments in neuronal function (Hsia et al., 1999) and develop deficits in learning and memory (Chapman et al., 1999), although neuron loss is not a consistent feature of the phenotype (Calhoun et al., 1998). While it is known that Aβ can be neurotoxic in *in vivo* and *in vitro* animal models (Yankner, 1996), there is growing evidence indicating that Aβ deposition may precede both cognitive impairment in the brain and the majority of neuronal loss in the AD brain (Morris et al., 1996; Lue et al., 1999; Naslund et al., 2000). Although Aβ does not compromise neuron survival at sublethal concentrations for extended periods, it may affect critical signal transduction processes that mediate plastic neuronal changes, including those involved in learning and memory.

A key mechanism in learning and memory is the encoding of signals via signal transduction processes, commonly referred to as 'activity-dependent plasticity'. The transcription factor cyclical AMP response element binding protein (CREB) regulates expression of cAMP response element (CRE)-containing genes and plays an essential role in learning and memory processes in a variety of species, ranging from *Drosophila* to mammals. (Bourtchuladze et al., 1994; Tully, 1997; Abel and Kandel, 1998). Disruption of CREB function specifically interferes with activity-dependent synaptic plasticity, ranging from long-term potentiation (LTP, a synaptic analogue of learning and memory) to long-term memory. Accordingly, we hypothesized that sublethal levels of Aβ may interfere with signal transduction mechanisms involved in activity-dependent plasticity. Specifically, mechanisms that interfere with CREB activation would disrupt downstream gene expression and compromise CREB activity-dependent neuronal function.

Neuronal activity-dependent phosphorylation of Ser-133 of CREB has been well documented and NMDA receptor activation and membrane depolarization in cultured neurons can lead to the activation of CREB. Accordingly, we investigated CREB activation by K^+-induced depolarization and NMDA receptor activation of neurons in the presence and absence of Aβ. The toxic effect of $A\beta_{1-42}$ on neurons shows a sharp threshold. Concentrations over 25 μM caused cell death within 24 hours, whereas those of 10 μM or lower did not result in cell death with our preparations of Aβ peptides. In

the absence of Aβ, high potassium stimulation caused a robust and reproducible increase in phosphorylated CREB (P-CREB). Pre-treatment with 5 or 10 μM $A\beta_{1-42}$ for periods as brief as 1 h decreased P-CREB, induced by K^+-evoked membrane depolarization (Figure 37.2). The effect of $A\beta_{1-42}$ occurred within 1 h of exposure and P-CREB levels were only slightly more suppressed after prolonging the $A\beta_{1-42}$ treatment for 24 h. In order to determine whether the effect was due to an action on a subpopulation of neurons, we further examined P-CREB activation using immunocytochemistry. High K^+ resulted in a pronounced increase in almost all cells in P-CREB immunoreactivity and this was markedly attenuated by exposure to $A\beta_{1-42}$ in nearly all cells. Aβ also suppressed the activation of CREB in response to NMDA receptor activation (Tong et al., submitted).

We next examined Aβ in the form of small diffusible oligomers (ADDLs, Aβ-derived diffusible ligands). ADDLs are postulated to be precursors of fibrillar Aβ and cause degeneration of neurons at nanomolar concentrations (Lambert et al., 1998). At concentrations of 100 nM, ADDLs also suppressed CREB activation but did not produce neuronal death. To determine whether the effect was specific to $A\beta_{1-42}$, the influence of $A\beta_{25-35}$, a toxic fragment of Aβ, was examined. This fragment also elicited neuron loss at 425 μM, but importantly did not interfere with CREB phosphorylation at the sublethal concentration of 10 μM. These data suggest that the mechanism by which Aβ suppresses CREB transcriptional activity is distinct from that mediating toxicity.

Thus, Aβ may be able to initiate early events that contribute to the onset of overt pathogenesis by suppressing normal signaling from the cell membrane to internal gene regulatory mechanisms. This may have a profound effect on the ability of neurons to encode change in the early stage of disorders. Thus, non-degenerative mechanisms may compromise neuronal function in the initiation phase of brain dysfunction with aging. We suggest disruption of CREB transcription as one candidate mechanism that may contribute to the apparent early loss in learning and memory.

PROPAGATION PHASE: ACTIVATION OF MOLECULAR AND CELLULAR PATHWAYS THAT RESULT IN RUNAWAY NEURODEGENERATIVE CASCADES

Following the initiation phase, we propose that there is further irreversible advancement of dysfunction as chronic neuronal injury progresses to neuronal loss. Over a lifetime, amyloid and numerous other insults accumulate in the aging brain, including oxidative damage, reduced metabolism, neuroinflammatory mechanisms, increases in proteases and induction of mechanisms shared with pathways common to apoptosis, such as death receptors and

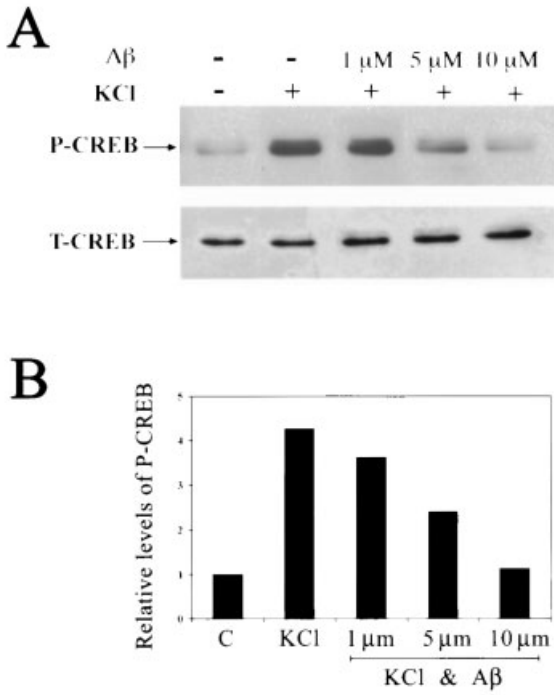

Figure 37.2. Treatment with $A\beta_{1-42}$ decreased CREB phosphorylation in cultured cortical neurons in response to depolarization with potassium but did not change total CREB (adapted from Tong et al., submitted). Pre-treatment with 5 or 10 μM $A\beta_{1-42}$ for 1 h decreased P-CREB induced by K^+-evoked membrane depolarization. (A) Western blot. (B) Quantification

caspases (Figure 37.1). We suggest that the presence of multiple insults acting together overrides compensatory processes, exceeds the threshold for degenerative mechanisms and causes chronic neurodegeneration. Critically, in the propagation phase, a single insult may be insufficient; the cumulative effect of multiple insults, however, may result in progressive loss of synapses, dendrites and eventually neurons. We propose that Aβ is a critical insult in both the initiation and propagation phases, and that it plays a principal role in these phases because of its multiple roles and irreversible accumulation.

It is now well established that there is dramatic neuronal loss during the course of AD (Lippa et al., 1992; West et al., 1994; Gomez-Isla et al., 1996, 1997). However, the mechanisms of neuronal loss remain unclear. We and others have shown that neuronal degeneration caused by Aβ in cultured neurons is apoptotic-like and have proposed that this pathway contributes to neuronal degeneration in AD (Loo et al., 1993; Su et al., 1994, 2000).

Recent work has identified at least two distinct mechanisms for the initiation of apoptosis, a mitochondrial pathway and a death receptor

pathway. Two converging lines of evidence form the basis for the hypothesis that Aβ is a key factor in the propagation of neurodegeneration. First, it is now well accepted that the AD brain exhibits an inflammatory response. One product of inflammation is the activation of apoptosis pathways, either directly via cytokines (i.e. Fas and TNFα) and their death receptors (i.e. Fas and TNFRI), or through bystander injury produced by the release of toxic products produced by activated cells (Giulian et al., 1995). In this regard, we have suggested that Aβ has the capacity to serve as an organizing factor in propagating the inflammatory response. Second, our research suggests that Aβ may be acting as a promiscuous ligand, with cytokine-link activity that binds and oligomerizes membrane death receptors, thereby activating these receptors and initiating intracellular signaling cascades that result in the initiation of apoptosis machinery (Cotman et al., 1996, 1999; Rohn et al., 2000).

One mechanism by which Aβ causes apoptosis involves caspase-8 (Ivins et al., 1999), an initiator caspase that is activated by oligomerization of receptors from the TNF/Fas receptor family that contain death domains. Aβ is toxic when it changes its conformation and assembles into fibrils. The model is that these fibrils span across multiple death receptors and cause their oligomerization. A death receptor role in Aβ toxicity is supported by our recent data, showing that Aβ-induced cell death is blocked by CrmA, a caspase 8 inhibitor, and by dominant negative FADD, an adapter protein that binds to the cytoplasmic tail of TNF/Fas receptors and prevents caspase 8 activation. These death receptor pathways may be particularly critical in the AD brain because of the possibility for soluble diffusible death ligands to affect multiple cells, as well as axons and dendrites in the local microenvironment.

The local microenvironment may be a critical factor in cognitive decline. While it is commonly assumed that the entire cell must undergo cell death, this is not necessarily true. Neurons have extensive processes that extend often over many millimeters even centimeters and are subject to adverse local environments. Recently it has been suggested that apoptotic mechanisms can also act within the processes of neurons to cause their selective degeneration (Ivins et al., 1998; Mattson et al., 1998; Yang et al., 1998). For example, the selective application of Aβ to axons separated from the soma by a diffusion barrier (modified Campenot chamber) will cause local caspase activation and exposure of phosphatidyserine on the external surface of the plasma membrane. In fact, neurites appear more vulnerable to the activation of apoptotic pathways than the soma. Critically, caspase inhibition prevents degeneration in this model. These data suggest the intriguing concept that the loss of neuronal processes and synapses may involve the activation of local apoptotic mechanisms in the absence of overt cell loss. In fact, such local control over neurites may even serve to protect the cell.

In addition to an ability to promote degeneration from outside of the cell, Aβ can also act intracellularly to cause degeneration. One mechanism may be

a lysosomal mediated event (Bahr et al., 1998; Cataldo et al., 2000) and/or one caused by protein–protein interactions and the activation of caspase 8 mechanisms. The activation of caspase 8 by the Huntington protein is not mediated extracellularly by death receptor activation, but intracellularly by polyglutamine repeat-mediated recruitment of FADD and subsequent caspase 8 activation (Sánchez et al., 1999), suggesting another mechanism for activating apoptotic machinery. Caspase activation could be further amplified via intracellular generation of toxic fragments from APP and possibly other proteins, including tau (Gervais et al., 1999; LeBlanc et al., 1999; Fasulo et al., 2000; Lu et al., 2000). In fact, recent studies have shown that caspases 2, 3, 8, 9 and 12 are all capable of promoting Aβ toxicity; hence, caspase activation is likely to reflect converging risk factors, as well as classical apoptosis initiation.

Thus, *in vitro* studies have demonstrated many pathways by which Aβ can drive degenerative mechanisms. While the situation is less clear in animal models, this may be a reflection of deficiencies of the models currently available, rather than the overall Aβ hypothesis, and perhaps the need for multiple insults to exceed the threshold for initiating degeneration.

Aβ IN ANIMAL MODELS CAN BE TOXIC TO NEURONS

While the toxicity of Aβ *in vitro* is no longer debated, the action of this peptide *in vivo* has remained controversial (Harkany et al., 2000). The biological action of Aβ appears to be context-dependent and maybe species-dependent as well. In particular, Aβ injected into the young primate brain produces minimal cell loss and only a small lesion; however, administration into the brain of an aged primate results in both neuronal loss and increased lesion size. A similar age-dependency has been reported in young vs. old rodents; however, the size of the Aβ-induced lesion in the aged rodent brain was comparatively smaller than in aged primate brain, indicating additional vulnerability to Aβ toxicity in primates (Geula et al., 1998). A further example of context on Aβ toxicity is seen in the rodent brain, where co-administration of Aβ and the protease inhibitor leupeptin exacerbates degeneration (Frautschy et al., 1998). Critically, single variable experiments in young animals do not simulate the aging process, since the hallmark of brain aging is the accumulation and coexistence of multiple insults. It is likely that healthy neurons in the homeostatic environment of the young or otherwise healthy brain are able to resist the presence of Aβ. Finally, in mutant APP transgenic animals, synapse loss has been reported and is probably an early sign of degeneration, while neuronal loss is generally absent or minimal (Mucke et al., 2000), supporting the importance of the local microenvironment and neuritic degeneration in the overall role of Aβ in AD pathology.

It is important to learn more about the mechanisms by which Aβ places neurons at risk and can cause their degeneration. Equally essential is the discovery of the endogenous factors that protect the brain from mutant genes in transgenic animal models. In fact, it is remarkable that the rodent brain can tolerate, to the degree it does, overexpression of mutant genes that can cause neuronal loss and dementia in humans.

APOPTOTIC (PROGRAMMED CELL DEATH) MECHANISMS ARE ACTIVATED IN THE AD BRAIN

While cell culture and animal models can help to define critical insults and their associated neurodegenerative mechanisms, the key issue is to define the sequence of events in the AD brain. The operation of neuronal apoptosis in the AD brain continues to be controversial. Yet a common endpoint for many of the insults that exist in the AD brain is the activation of apoptosis or programmed cell death machinery, e.g. Aβ, oxidative damage, low energy, reduced neurotrophic factor support. On the basis of cell culture data and then studies on post-mortem brain tissues, we have suggested that apoptotic-related mechanisms may be a factor in neuronal loss in the AD brain (Loo et al., 1993; Su et al., 1994, 2000). Apoptosis encompasses a highly regulated set of programmed pathways that result in the systematic degeneration of the cell. Four principal lines of evidence support the contention that apoptotic mechanisms may play an important role in neurodegeneration in the AD brain:

1 Pro-apoptotic alterations in the expression of multiple apoptosis-related proteins are observed in the AD brain, including Bcl-2 family members (Su et al., 1996b; MacGibbon et al., 1997), c-Jun (Anderson et al., 1996; Marcus et al., 1998), and Par-4 (Guo et al., 1998).
2. Several studies have shown that downstream effector caspases, such as caspase-3, are increased in AD brain in association with AD pathology (Masliah et al., 1998), and our data suggest that caspase-3 is present in an activated form (Su et al., 2000).
3. Apoptotic bodies and plasma membrane blebs containing condensed chromatin are present in AD and Down's syndrome (DS) brain (Su et al., 1994; Anderson et al., 1996, 2000b). Additionally, numerous examples of cells with apoptotic morphologies are also observed in rapid autopsy samples and in the aged canine model of AD neuropathology, in which potential agonal and post-mortem artifacts can be excluded (Anderson et al., 2000a).
4. Pulsed-field gel electrophoresis of short post-mortem human tissue, a more sensitive method for the biochemical detection of apoptosis than conventional gel electrophoresis for DNA laddering, reveals 50 kb DNA

fragmentation consistent with apoptotic cell death in both AD and DS brain (Anderson et al., 2000b).

While these data do not suggest that apoptosis is an exclusive mechanism of degenerative cell loss in AD brain or necessarily the terminal event in neuronal death, they emphasize the importance of understanding the mechanisms and pathways of this process in this neurodegenerative disorder. Towards this end, the comparison of cell culture models of Aβ-induced neurodegeneration and AD brain is a valuable approach.

As discussed above, we proposed that Aβ may be capable of activating the TNF/Fas superfamily of death receptors. Accordingly, we have examined the possibility that these receptors and ligands are upregulated in the AD brain. We examined FasL and Fas receptors using single- and double-label immunocytochemistry. In AD brain sections, immunocytochemical staining for FasL reveals a remarkable upregulation of this cell death activator. The most striking feature is the appearance of many unusual small spherical structures situated in the vicinity of plaques, often in a line, as if they had originated from a fiber that was in the process of degenerating. Using an antibody to phosphorylated neurofilament, we were able to show that these degenerating neurites could be traced into plaque areas and that the neurites often showed an increased expression of FasL in the region of the plaque (unpublished observations). These spheres resemble those previously identified as an early event in the formation of dystrophic neurites (Su et al., 1996a) and may represent local apoptosis-like processes, as discussed above. These and other changes make the AD brain poised for the activation of apoptotic machinery, but they do not mean the machinery is indeed active, and caspases are cleaving proteins.

In order to evaluate the possible activation of caspases in AD brain, we

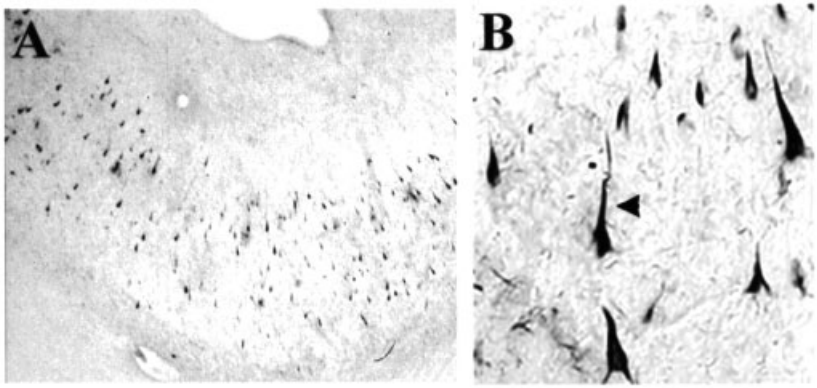

Figure 37.3. A caspase cleavage product (CCP) of fodrin accumulates in neurons in AD brain, suggesting the presence of chronically active caspases (adapted from Rohn et al., 2000). (A) Low magnification image of fodrin CCP immunostaining in AD brain. (B) Higher magnification of the same field shown in (A)

developed an antibody to a caspase cleavage product of fodrin, a prominent cytoskeletal protein and established substrate for caspase cleavage. The presence of a cleavage product is a powerful approach for determining whether or not the machinery is active. Surprisingly, many neurons show the presence of this caspase cleavage product (Figure 37.3). In some cases, the labeling is present in neurons developing tangles, whereas non-tangle-bearing neurons are also found. In fact, the presence of neurons expressing the fodrin cleavage product paralleled the presence of tangle-bearing neurons ($r = 0.84$) (Rohn et al., 2001). This is important because, if one accepts the fact that tangles are related to progressive degeneration and that the accumulation of the caspase cleavage product of fodrin parallels PHF formation, then both may contribute to neuronal dysfunction and disease progression. This, along with correlations between tangles, Aβ accumulation and cognitive dysfunction, argue for multiple factors driving AD, such as chronic caspase activation.

CONCLUSION

We propose that brain aging and neurodegeneration can be resolved into distinct phases; the successful aging phase, the initiation phase and the propagation phase (Figure 37.1). In the initiation phase, mechanisms may operate that are non-degenerative but that compromise neuronal function. We suggest that Aβ at sublethal concentrations can interfere with activity-dependent plasticity through suppressing CREB regulation, a key transcription factor in learning and memory. In the propagation phase, degenerative mechanisms become prominent and progressively irreversible. Aβ plays a key role in initiating propagation and driving it, because Aβ can augment so many different degenerative mechanisms, including the ability to activate caspase death receptors.

Accumulating data on the AD brain supports a role of apoptosis machinery in neuronal degeneration. Insults that accumulate in AD brain, including Aβ, share in common the ability to activate apoptosis. But in the AD brain, classical ('terminal') apoptosis is rare as defined by morphological criteria, e.g. the presence of nuclear apoptotic bodies, chromatin margination, etc. Thus, it can be argued that apoptosis is not active or marginally active in AD brain. On the other hand, the machinery to mediate apoptosis is present, upregulated and active in many neurons. Caspase cleavage products of fodrin, actin and APP, for example, exist in AD neurons and thus caspases must be active. Of course, the terminal phase of apoptosis may be infrequently detected because it is rapid relative to the initiation phase of the mechanism (Cotman et al., 1999). The pathways or programs in AD neurons may be active for years, an unconventional notion. We would suggest that neurons as non-dividing cells activate an 'apoptosis checkpoint cascade' (delayed apoptosis), whereby neurons adopt molecular countermeasures to delay

classical apoptosis. In this context, it may be more appropriate to adopt the term 'caspase-mediated degeneration' to indicate the operation of a particular program of degeneration. In fact, current literature in the field argues that multiple specific programs share in common the apoptosis phenotype (Wolf et al., 1999). Thus, in the propagation phase neurons continue to survive against an increasing number of insults, including Aβ, and induce survival measures that delay final cell loss.

In summary, we suggest that Aβ can shift the balance of pro- and anti-cell death factors, and initiate and amplify cascades, thereby changing its role through the phases of brain aging. In this model of neurodegeneration, arresting progressive brain dysfunction will require preventing the accumulation of Aβ and/or enhancing its clearance.

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38 Mechanisms of Aβ Production and Aβ Degradation: Routes to the Treatment of Alzheimer's Disease

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INTRODUCTION

The more than 1300 scientific papers presented at the World Alzheimer Congress 2000 exemplify the remarkable diversity of basic and clinical approaches to Alzheimer's disease (AD). Even before this Congress, it had become abundantly clear that AD is a syndrome with multiple molecular etiologies, rather than a single disease. One of the greatest challenges of research on AD is to synthesize the disparate, sometimes conflicting bits of knowledge about the Alzheimer clinicopathological syndrome into a coherent and encompassing mechanism of disease. Since before the time of the first International Conference on Alzheimer's Disease (WAC 2000 is the seventh), our laboratory has pursued research on several aspects of the amyloid cascade hypothesis, which we believe provides a unifying mechanism that can explain the full etiology and pathogenesis of this complex disorder.

In this chapter, we summarize recent work from our laboratories on three interrelated aspects of the role of amyloid β-protein (Aβ) as the initiator of AD: its production, its degradation and its aggregation. These three processes represent the life cycle of Aβ. Initial cellular production of these normal, soluble peptides can be followed by degradation inside and/or outside cells, and those peptides which escape degradation can accumulate to form oligomers and polymers (aggregates) that have apparent cytotoxicity. By analyzing each of these three steps in the economy of Aβ independently and then attempting to bring together the findings into a unified cycle, we hope to shed light, not only on multiple mechanisms by which Aβ can accumulate to induce neuronal dysfunction but also on discrete points of therapeutic intervention.

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MATERIALS AND METHODS

All of the methods utilized in the studies summarized in this chapter have been published by us in recent reports (Citron et al., 1997; Esler et al., 2000; Podlisny et al., 1995, 1998; Qui et al., 1997, 1998; Vekrellis et al., 2000; Walsh et al., 2000; Wolfe et al., 1999b; Xia et al., 1997, 1998).

RESULTS

Aβ PRODUCTION

Elevated cerebral levels of Aβ peptides, particularly those ending at residue 42 ($A\beta_{42}$), are an early and invariant feature of all forms of AD. As a result, understanding the detailed mechanism by which two proteolytic activities designated β-secretase and γ-secretase cleave the β-amyloid precursor protein (APP) to liberate Aβ peptides has been a central goal of our work since the original discovery of the normal cellular production of Aβ (Haass et al., 1992; Seubert et al., 1992; Shoji et al., 1992). Because several of the APP mutations, as well as all of the known mutations in presenilin (PS) 1 and 2, selectively alter the γ-secretase cleavage event to heighten $A\beta_{42}$ production, we have focused in particular on the identity and nature of γ-secretase. A key observation from our perspective was the finding that small amounts of holoAPP could be co-immunoprecipitated with presenilin in lysates and isolated microsomal vesicles from cells expressing transfected or endogenous presenilin (Weidemann et al., 1997; Xia et al., 1997). Although this finding initially generated controversy (Thinakaran et al., 1998), it served as a major impetus for our hypothesis that presenilin participates intimately as part of the catalytic complex by which γ-secretase mediates the putative intramembranous proteolysis of APP (Selkoe, 1998). An alternative hypothesis for the role of presenilin in the γ-secretase mechanism is that it does not form complexes with APP, but rather acts as a mediator of membrane trafficking that brings the components of the γ-secretase reaction together (Naruse et al., 1998; Thinakaran et al., 1998). However, when we examined the maturation of holoAPP through the secretory pathway, i.e. the precise timing of the acquisition of N- and O-linked sugars, we were unable to detect any difference in this secretory processing between cells that express presenilin 1 and those that entirely lack it (Xia et al., 2000). Likewise, subcellular fractionation on discontinuous iodixanol gradients showed no definable difference in the distribution of the APP C-terminal fragments that are the immediate substrates for γ-secretase (i.e. C99 and C83) between cells that express or lack PS1 (Xia et al., 1998). We extended the original observation of De Strooper et al. (1998), that the absence of PS1 sharply elevates the amount of these fragments in fractionated microsomes, but we observed no

change in their subcellular localization. Taken together, these results suggested to us that direct participation of presenilin in the γ-secretase catalytic complex was a more tenable mechanism than an indirect role in the trafficking of the components of the reaction.

Another biochemical finding in our laboratory that strongly favored the former hypothesis was the observation that FAD-causing missense mutations in either APP or PS1 led to decreased potency (i.e. increased IC_{50}s) of peptidomimetic inhibitors of γ-secretase that were transition state analogs (Xia et al., 2000). Although the increases in IC_{50} were modest, they were highly reproducible and statistically significant. When we attempted to devise a model to explain this observation, we found it difficult to understand how a function of presenilin in protein trafficking (e.g. of γ-secretase and/or APP) could account for the negative effects of single missense mutations in presenilin on inhibitor potency. It seemed far more probable that these shifts in inhibitor potency when presenilin was mutant denoted a physical (conformational) alteration of a site within the γ-secretase in which presenilin directly participated.

A further finding that turned out to be critical in supporting the first over the second hypothesis was the nature of the designed peptidomimetic compounds that effectively inhibited γ-secretase (Wolfe et al., 1998, 1999a). Wolfe et al. had designed ~4–6 residue peptidomimetics based on the $Aβ_{42–43}$ cleavage site within the APP transmembrane domain. Difluoro-alcohol or difluoro-ketone moieties were installed at the P1 position as non-cleavable transition state mimicking functionalities, and the effects on Aβ generation in intact whole cells were examined. Dose-dependent inhibitions of $Aβ_{40}$ and $Aβ_{42}$ were seen, and a rough structure–activity relationship could be derived by substituting certain of the residues in these peptidomimetics. The inhibitory activity of the difluoro-alcohol moiety signified that these transition state mimics were acting upon an aspartyl protease, rather than on one of the other major classes of known proteases. The results of these designed inhibitor studies, along with molecular modeling, led to a hypothesis that γ-secretase cleavage involves a helical substrate (the APP transmembrane domain) and an unusual aspartyl protease that cleaves the substrate within the phospholipid bilayer (Wolfe et al., 1999a).

Taken together, all of the observations summarized above supported the concept that presenilin was intimately involved in the γ-secretase cleavage of APP and led to close inspection of the presenilin sequence for a possible aspartyl protease motif. The observation of two (and only two) intramembranous aspartyl residues in all presenilins that were predicted to be approximately in the center of TM6 and TM7, flanking the site in the proximal TM6–TM7 loop that undergoes endoproteolysis, led to the hypothesis that presenilin was itself γ-secretase and might also effect the endoproteolytic cleavage (an autoproteolysis) (Wolfe et al., 1999b). Mutagenesis of either of the TM aspartates in PS1 to alanine or glutamate resulted in a marked

decrease in endogenous PS1 endoproteolytic fragments, as a result of replacement (Thinakaran et al., 1996) by the apparently non-cleavable asp-mutant exogenous PS1. Likewise, C83 and C99 levels were markedly elevated and Aβ and p3 levels were reduced sharply. These results suggested that mutation of a single TM aspartate residue produced essentially the same biochemical phenotype in a cell as deleting the entire PS1 gene (De Strooper et al., 1998). Further, the incubation of isolated microsomes from either wild-type or asp mutant-expressing cells with a C99 cDNA in an *in vitro* transcription/translation reaction showed that new Aβ peptide could be generated by wild-type microsomes at pH 6.4, but much less so at neutral pH (7.4); but no Aβ generation was detected from the microsomes expressing asp mutant PS1 (Wolfe et al., 1999b).

These results suggested that PS1 was either an unusual diaspartyl co-factor for γ-secretase or was itself γ-secretase, an unprecedented intramembranous aspartyl protease activated by autoproteolysis (Table 38.1). Because reconstitution of γ-secretase activity, i.e. Aβ generation from purified components (presenilin 1 and the C99 substrate) in phospholipid vesicles,

Table 38.1. Comparison of the predicted properties of γ-secretase and the observed properties of the presenilins

Characteristics of γ-secretase	Corresponding features of PS
Aspartyl protease (requires 2 aspartates)	There are two completely conserved aspartates in presenilins—required for γ-secretase function
Intramembranous proteolysis	The two aspartates are within the membrane
APP cleavage occurs near the middle of the membrane	The two aspartates are near the middle of TM6 and TM7
Aspartyl proteases have an acidic pH optimum	*De novo* Aβ generation in PS-containing microsomes occurs optimally at mildly acidic pH (~6.4)
γ-Secretase binds to its substrates	PS forms complexes with APP and, in particular, with C99 and C83
An intramembranous protease needs a structure for membrane entry of water	PS has an 8 TM structure that could form a pore and admit water
Deletion of γ-secretase must obviate proteolysis	Deletion of PS1 and PS2 obviates all intramembranous proteolysis of Notch and C99
Transition state mimic inhibitors bind directly to the active site of the protease	APP transition state mimics bind directly and specifically to PS heterodimers
Such inhibitors should bind intimately to the target protease	Photoactivatable groups located 5–12 Å from the active site binding moiety at the N- and C-terminal of an inhibitor bind to the NTF and CTF of PS, respectively (Li et al., 2000)

cannot be achieved without knowledge of the protein co-factors required for the γ-secretase reaction, our laboratories took an alternative approach to confirming presenilin as the γ-secretase. Using the aforementioned peptidomimetic transition state analogs, we were able to show that these inhibitors could bind directly to presenilin heterodimers in cell lysates, isolated microsomes and even intact cells (Esler et al., 2000). This work is described in detail in the chapter by Esler et al. in this volume.

When all of the results summarized above are considered together (Table 38.1), there is now very strong evidence that presenilin represents the active site of γ-secretase. Although there is no precedent before this work for an intramembranous aspartyl protease, the cleavage of sterol regulatory element binding protein (SREBP) is effected by an unusual multi-transmembrane domain metalloprotease called site 2 protease, which appears to have its active site within one of its TM domains (Sakai et al., 1998). To ultimately achieve Aβ generation from purified components, we are currently purifying further the presenilin/γ-secretase complex and identifying protein partners that may be necessary for proteolytic activity.

Aβ DEGRADATION

Several years ago, our laboratory conducted an unbiased screen of the conditioned media of several neural and non-neural cell lines for proteolytic activities that could degrade naturally secreted Aβ peptides (Qui et al., 1997). The results of the screen revealed that several cell types released a thiol metalloendopeptidase into their media that actively degraded secreted $A\beta_{40}$ and $A\beta_{42}$ monomers to similar extents. Partial purification and analysis of the inhibitory profile indicated that this activity was indistinguishable from that of insulin-degrading enzyme (IDE) (Qiu et al., 1998). We have conducted a series of experiments examining IDE in various neuronal and microglial cell lines, in primary cortical neurons, in human CSF and in human brain samples (both AD and control) (Vekrellis et al., 2000). In all of these systems, IDE is present. In both neuronal cultures and total human brain homogenates, the major Aβ degrading activity is substantially inhibitable by insulin and 1,10-phenanthroline, consistent with IDE. The use of thiorphan or phosphoramidon, potent inhibitors of neutral endopeptidases, such as neprilysin (NEP) (Iwata et al., 2000), produces very little inhibition of Aβ degradation in total brain homogenates, from both AD and control subjects. When we prepared membrane fractions from these brain homogenates, we observed that ~30% of the Aβ-degrading activity was inhibitable by thiorphan whereas ~70% was inhibitable by insulin. Immunodepletion of IDE from human brain homogenates reduces Aβ-degrading activity by approximately 60–80%, and there is a concomitant reduction in the amount of IDE protein, as determined by Western blotting of the homogenates.

These results strongly suggest that IDE is the principal Aβ-degrading activity in both total human brain homogenates and their membrane fractions, consistent with earlier results that both microglia (Qiu et al., 1998) and primary neurons (Vekrellis et al., 2000) actively degrade Aβ by an insulin-inhibitable protease. In view of the evidence that neprilysin plays a role in Aβ degradation *in vivo* (Iwata et al., 2000), we examined Aβ degradation in total brain homogenates prepared from mice lacking the neprilysin gene (−/−) vs. their wild-type littermates (+/+). Aβ ELISAs using X-40 and X-42 assays showed closely similar levels of peptides in the NEP and −/− and +/+ mice. We also performed Western blotting of these brain homogenates with an antibody specific for the rodent form of Aβ (antibody Y27, kindly provided by F. Kametani and D. Allsop). A 4 kDa band was visualized that was absent after preabsorption of Y27 with rodent Aβ peptide. There was no difference in the amount of the 4 kDa Aβ-specific band in NEP +/+ vs. −/− homogenates. Further, we performed immunocytochemistry with several sensitive Aβ antibodies that are able to detect the rodent form of Aβ and found no Aβ-immunoreactive deposits in 2 and 4 month old NEP −/− mice. We plan to examine older neprilysin-deficient mice when they become available to determine whether the absence of NEP leads to gradual Aβ build-up.

At this juncture, we believe that both IDE and neprilysin play a role in Aβ degradation in the brain. Our quantitative analyses suggest that IDE contributes the major portion of Aβ-degrading activity. The demonstration of neprilysin as an Aβ-degrading protease in rats *in vivo* required the use of SDS extraction to recover the injected Aβ peptide from brain and measure it (Iwata et al., 2000). The authors suggested that the Aβ became rapidly insolubilized upon injection, perhaps via membrane association, and that it was this insoluble form that was apparently cleaved by neprilysin (Iwata et al., 2000). These results are consistent with our finding that neprilysin contributes to some extent to Aβ degradation associated with the membrane (as does IDE). It is of related interest that IDE can actively degrade naturally secreted Aβ monomers (both $A\beta_{40}$ and $A\beta_{42}$) but is much less active against low n oligomers of Aβ found in conditioned media (Qiu et al., 1997). Therefore, we hypothesize that IDE has a principal role in the natural degradation of Aβ monomers following their secretion from cells, whereas once the monomers become insoluble and/or aggregated, neprilysin plays a role in degrading them.

At the time of our initial identification of IDE in our screen for Aβ-degrading proteases in cultured cells, we proposed that genetic alterations in IDE might explain some forms of familial AD not currently linked to known genes (Qui et al., 1998). This suggestion has led to an active collaboration with Rudolph Tanzi and his colleagues, in which they are performing linkage analysis and allelic association studies using markers on chromosome 10 in the vicinity of the IDE gene. Dr Tanzi reported at this meeting that there is an apparent late-onset FAD locus on chromosome 10q (see his chapter in this volume). Two kinds of analyses are under way: (a) further genetic linkage and

association studies in late-onset kindreds, using polymorphisms in the IDE gene as well as various flanking DNA markers; and (b) direct analysis of Aβ-degrading activity in lymphoblasts and fibroblasts cultured from AD families linked to chromosome 10.

Aβ AGGREGATION

Many studies of the aggregation of synthetic Aβ peptides have been conducted, and these have supported the hypothesis that aggregated (oligomeric/polymeric) Aβ, but not monomeric Aβ, can reproducibly induce neuronal injury. Studies of synthetic Aβ peptides have a number of disadvantages, including the requirement for supraphysiological concentrations to achieve aggregation, the use of just a single defined Aβ peptide species, and the difficulty in studying the oligomerization process under physiological buffer conditions. A few years ago, our laboratory discovered apparently stable oligomers (dimers, trimers and tetramers) of naturally secreted Aβ in the conditioned media of APP-transfected Chinese hamster ovary (CHO) cells (Podlisny et al., 1995). We subsequently characterized these naturally occurring low n oligomers in some detail (Podlisny et al., 1998). Recently, we have attempted to detect such oligomers of secreted Aβ in the conditioned media of neuronal cell lines and primary human cortical neurons, and have also examined the hypothesis that the oligomers first arise intracellularly. The latter possibility was suggested by pulse-chase analyses of the APP-transfected CHO cells that indicated that apparent Aβ dimers (~8 kDa) were visible in the media as early as 40 min after pulse-labeling with ^{35}S-methionine, raising the possibility that small amounts of Aβ oligomers were secreted as such from the cells, rather than arising this rapidly from secreted monomers. First, we detected SDS-stable, ~8 kDa putative dimeric Aβ in the lysates of APP-transfected CHO cells, using a highly sensitive immunoprecipitation/Western blotting technique (Walsh et al., 2000). Then, we used this method to examine neuronal-type cells, including primary human corticol neurons, and found small amounts of dimers in total lysates of these cells (Walsh et al., 2000). Of particular interest was the observation that both neuronal cell lines and primary cortical cultures had essentially no detectable extracellular Aβ oligomers. This result indicates that the intracellular dimers we observed could not have arisen from the binding back of extracellular dimers to the plasma membrane. Consistent with this assumption, trypsinization of cells prior to their examination for intracellular dimers does not alter the amount detected.

Taken together, the above results suggest that the critical process of Aβ dimerization is initiated intracellularly, presumably in specific vesicular compartments in which Aβ is generated. We are now conducting analyses of fractionated microsomes to determine to what extent Aβ dimers and higher oligomers can be detected therein. Based on this clear detection of dimeric Aβ

in neuronal lysates, we hypothesize that Aβ dimerization begins intracellularly and that a portion of the dimers can be secreted, along with the soluble monomer. Presumably the released dimers (and perhaps higher oligomers not currently detectable) can serve as seeds for the subsequent addition of monomers and the ultimate formation of high molecular weight oligomers [such as Aβ protofibrils (Harper et al., 1997; Walsh et al., 1997)] and, eventually, large polymers such as amyloid fibrils.

DISCUSSION

The results emerging from these studies of the three major facets of the Aβ life cycle—production, degradation and aggregation—suggest several possible therapeutic strategies to interrupt progressive Aβ accumulation and deposition in the brain. Among these, γ-secretase inhibitors are the most advanced in terms of preclinical development, and one such inhibitor has entered human trials (see chapter by Molinoff and colleagues, in this volume). Our findings predict that many γ-secretase inhibitors, including all that are directed against the active site, will bind to presenilin heterodimers. Given the clear-cut evidence that presenilin mediates not only the intramembranous proteolysis of APP but also that of Notch to release the cytoplasmic signaling domain of Notch to the nucleus, it would be particularly advantageous to find members of the presenilin/γ-secretase complex that principally modulate either the APP or the Notch cleavage. This would offer the prospect of more specificity towards Aβ inhibition, with less likelihood of side effects arising from downregulating Notch signaling. However, there may well be other, still unknown, single transmembrane proteins that are substrates of presenilin, so not all of the toxicity potentially associated with γ-secretase inhibition may be attributable to interference with Notch signaling. We are currently searching for such additional presenilin substrates. Inhibition of β-secretase, recently identified by several groups (Hussain et al., 1999; Lin et al., 2000; Sinha et al., 1999; Vassar et al., 1999; Yan et al., 1999), is an alternative approach for chronically lowering cerebral Aβ levels. However, there are some theoretical considerations that may favor targeting γ-secretase rather than β-secretase. First, chronic inhibition of β-secretase may well lead to increased amounts of APP being processed by α-secretase and thus increased production of p3. There is evidence that p3 can contribute to Aβ deposition in the brain (Lalowski et al., 1996; Roher et al., 1993), and p3 is particularly hydrophobic and thus potentially prone to aggregation. Second, very few inhibitors of β-secretase have arisen out of whole-cell compound screening, whereas numerous γ-secretase inhibitors have been found this way. This raises the possibilities that compounds that are capable of inhibiting β-secretase are poorly cell penetrant, or else are toxic, and thus do not emerge from such whole-cell screens. However, now that β-secretase is in hand, including a crystal structure of the enzyme complexed

with an inhibitor (Hong et al., 2000), inhibitors can be specifically designed or identified by *in vitro* screening, allowing determination of their relative therapeutic indexes, compared to compounds which act on γ-secretase.

Another Aβ-directed therapeutic approach is inhibition of aggregation. Because of recent evidence that relatively small, diffusable oligomeric forms of Aβ may induce neurotoxicity in culture (Hartley et al., 1999; Lambert et al., 1998), an aggregation inhibitor that stabilized such oligomeric intermediates might actually be harmful. It could be that the conversion from oligomers to mature amyloid fibrils leads to less biological activity, so that fibrils are relatively inert compared to low n oligomers. Given our observation (above) that Aβ aggregation appears to be initiated intracellularly by formation of stable dimers that can then be released from cells, a drug that seeks to target aggregation may need to interfere with this early dimerization. If so, the drug would need to penetrate the plasma membrane, whereas anti-aggregation drugs were previously thought to have the advantage that they could act principally in the extracellular space, without the need for cell penetration. In view of these considerations, we believe that lowering the production of Aβ monomer is more likely to be achievable and therapeutically useful than is the anti-aggregation approach. Since Aβ aggregation is dependent on monomer concentration, a relatively modest reduction in [Aβ] should have a marked impact on oligomerization.

Finally, one could envision upregulating Aβ degradation in the brain. However, this also represents a difficult therapeutic target, because there is little precedent for chronically activating an enzyme in humans (e.g. insulin-degrading enzyme or neprilysin). If natural inhibitors of IDE or other Aβ-degrading proteases can be identified, interfering with the production or processing of these inhibitors might theoretically allow a modest increase in the activity of IDE or a similar protease. Much further work is needed before we will have enough information about the biology of IDE and other proteases that degrade Aβ in the human brain in a way that will allow therapeutic targeting.

In conclusion, of the approaches considered in this chapter, we believe that chronic partial (e.g. 30–40%) inhibition of γ-secretase or β-secretase remains a rational and highly attractive strategy to slow and ultimately prevent Alzheimer's disease. A separate approach that is also highly attractive is the use of Aβ immunization to enhance clearance of Aβ from the brain (Bard et al., 2000; Schenk et al., 1999; Weiner et al., 2000).

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39 A High Fat, High Cholesterol Diet Accelerates β-Amyloid Accumulation in the CNS of a Transgenic Mouse Model of Alzheimer's Disease

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INTRODUCTION

In recent years, there has been increasing evidence that cholesterol, or cholesterol metabolism, plays a role in the pathophysiology of Alzheimer's disease (AD). Clinical and epidemiological studies indicate that patients with elevated cholesterol have increased susceptibility to AD (Jarvik et al., 1994, 1995; Notkola et al., 1998; Roher et al., 1999), and that the incidence of AD is higher in countries with high fat and high calorie diets (Kalmijn et al., 1997). In addition, recent data demonstrate elevated total serum and LDL cholesterol in patients with AD compared to age-matched controls, and a positive correlation between the levels of total serum cholesterol, LDL cholesterol and $A\beta_{42}$ (Kuo et al., 1998).

Supporting the link between cholesterol and AD are *in vitro* studies demonstrating that cholesterol levels modulate the enzymatic processing of amyloid precursor protein (APP) and Aβ production (Bodovitz and Klein, 1996; Frears et al., 1999; Howland et al., 1998; Mizuno et al., 1998; Simons et al., 1998). These studies show that cells that have been depleted of cholesterol, using the cholesterol-lowering drug lovastatin, exhibited reduced secretion of Aβ and reduced production of amyloidogenic APP C-terminal fragments (β-CTFs) by β-secretase cleavage (Simons et al., 1998; Frears et al., 1999). Conversely, supplementing these cells with cholesterol resulted in

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increased β-secretase cleavage products, along with increased secretion of $A\beta_{40}$ and $A\beta_{42}$. Moreover, increasing cellular cholesterol with methyl-β-cyclodextrin-solubilized cholesterol showed dose-dependent increases in both mature and immature APP holoprotein and a reciprocal decrease in sAPPα (Simons et al., 1998). Taken together, these data suggest that intracellular cholesterol levels alter APP processing.

There is also a connection between cholesterol, cardiovascular disease, apolipoprotein E (apoE) and AD (Jarvik et al., 1994, 1995; Liu et al., 1999; Notkola, 1998; Poirier, 1996; Sparks, 1997). Significantly, apoE is a cholesterol transport protein that is associated with amyloid deposits and binds Aβ peptide (Poirier, 1996; Strittmatter et al., 1993; Wisniewski and Frangione, 1992) Individuals with two copies of the apolipoprotein E4 allele (apoE4) have elevated serum cholesterol levels and increased risk of both AD and cardiovascular disease (Sparks, 1997).

Based on these data, we hypothesized that high dietary cholesterol is associated with an increased risk of AD because it results in increased production and/or deposition of Aβ peptide in the CNS by an unknown mechanism. To test this hypothesis, we examined the effects of a high cholesterol diet on Aβ production/deposition in the CNS of the PSAPP mouse model for AD amyloidosis (Holcomb et al., 1998; McGowan et al., 1999). Our data provide evidence that diet-induced hypercholesterolemia accelerates the amyloid pathology in this transgenic model. These findings may have important therapeutic ramifications for AD.

RESULTS

A HIGH CHOLESTEROL DIET INCREASES BOTH PLASMA AND CNS CHOLESTEROL LEVELS

To examine the effects of dietary cholesterol on Aβ accumulation, 5 week-old male, PSAPP mice were placed on either a high fat/high cholesterol diet or on a basal diet containing trace cholesterol for 7 weeks. Examination of plasma total cholesterol indicated that the mice fed the high cholesterol diet had a significant increase (two-fold, $p = 0.001$) compared with the basal diet controls (Table 39.1). The mean cholesterol values for the high cholesterol and basal diets were 201.66 ± 10.66 mg/dl and 99.852 ± 10.36 mg/dl, respectively. These data indicate that the high cholesterol diet induced a condition of hypercholesterolemia. In addition, mice fed the high cholesterol diet had significantly higher ($p = 0.011$) CNS total cholesterol levels (Table 39.1). The mean values for the high cholesterol and basal diets were 16.68 ± 0.490 mg/g and 14.76 ± 0.0387 mg/g, respectively.

Table 39.1. Effects of diet on total Aβ, cholesterol and mean β-amyloid deposit number

	Aβ total (pmol/g)	Cholesterol		Aβ deposit no. (mean)
		plasma (mg/dl)	CNS (mg/g)	
High cholesterol diet	386	230	16.2	nd
	202	175	15.5	8.5
	364	195	16.3	21.5
	545	300	19	25
	471	295	16.4	23.5
	279	172	16.7	13.5
	394	195	nd	nd
	150	122	nd	10.5
	335	131	nd	nd
Mean	347.3	201.66	16.68	17.08
SEM	41.3	10.66	0.49	2.9
SD	124.09	27.43	1.2	7.11
Basal diet	156	108	15.2	nd
	165	118	15.4	10.5
	140	83	15.5	8.5
	174	102	14.4	13.5
	309	140	15.1	15
	130	53	nd	7
	124	95	13	7.5
Mean	171.1	99.85	14.76	10.33
SEM	23.98	10.36	0.038	1.34
SD	63.96	24.43	0.95	3.26
p	0.004	0.001	0.01	0.019

Aβ peptides, deposits and cholesterol levels were measured. p values were calculated using an unpaired, Student's t-test (two-tailed). CNS, central nervous system. SEM, standard error of the means. SD, standard deviation. From Refolo et al. (2000), by permission of Academic Press

CNS TOTAL Aβ INCREASES IN RESPONSE TO HYPERCHOLESTEROLEMIA

Brain Aβ from mice fed either high cholesterol ($n = 9$) or basal diets ($n = 7$) were analyzed by three independent methods. Using a sandwich ELISA that detects total formic acid-extracted Aβ, we observed a significant (two-fold, $p = 0.004$) increase in the hypercholesterolemic mice compared with the basal diet controls (Table 39.1). The mean values of total Aβ for high cholesterol and basal diets were 347.3 ± 41.30 and 171.1 ± 23.98 pmol/g of tissue, respectively. The ranges for the high cholesterol and basal mice were 545–150

and 309–124 pmol/g, respectively (Table 39.1). In addition, we observed a positive correlation between the levels of plasma ($r = 0.918$) and CNS ($r = 0.787$) total cholesterol and total Aβ (Figure 39.1).

Next, we determined the effects of high cholesterol diet on formic acid extracted Aβ species ending in amino acids 40, 42, 38 and 34. This was accomplished by performing immunoprecipitation—mass spectrometry (IP–MS), and the various Aβ peaks were identified and quantified using relative peak intensities (Figure 39.2). Data indicate that mice fed the high cholesterol diet had significantly higher levels of $A\beta_{1-40}$ (38%, $p = 0.003$), $A\beta_{1-42}$ (38%, $p = 0.006$), $A\beta_{1-38}$ (32%, $p = 0.004$) and $A\beta_{1-34}$ (26%, $p = 0.003$), than the basal controls. Taken together with the data for total Aβ these data strongly suggest that hypercholesterolemia increases Aβ levels in the CNS of this transgenic model for AD amyloidosis.

β-AMYLOID LOAD, DEPOSIT NUMBER AND SIZE ARE INCREASED IN THE CNS OF HYPERCHOLESTEROLEMIC MICE

Equivalent sets of three serial, coronal sections obtained from two levels of each brain from hypercholesterolemic and basal diet mice were examined using the anti-Aβ antibody, 4G8. A total of 108 and 66 deposits were counted for the high cholesterol and basal groups, respectively. The mean values for deposit number were determined and found to be 17.08 ± 2.9 and 10.33 ± 1.33 for the hypercholesterolemic and basal mice, respectively (Table 39.1) This represents a significant increase (65%, $p = 0.019$) in deposit number in the hypercholesterolemic mice. For both groups of mice, deposits of varying size were readily observed in the cingulate cortex, motor cortex and hippocampus. Moreover, for both groups of mice, $>90\%$ of the deposits were found in the cingulate cortex and $<5\%$ in the CA1 region of the hippocampus. This distribution of deposits is similar to that previously reported for 12-week-old PSAPP (line 8.9) mice (McGowan et al., 1999).

To determine deposit size, image analysis of fluorescent images from 50 high cholesterol and 47 basal deposits were acquired by a computerized, digital video system. The mean areas were 96.35 ± 8.38 and $75.60 \pm 8.13 \mu m^2$ ($p = 0.041$), respectively.

Next, we determined the average amyloid load (% amyloid) in hypercholesterolemic and basal mice and these were $0.80 \pm 0.174\%$ and $0.436 \pm 0.065\%$ ($p = 0.037$), respectively (Figure 39.3). It is of note that the latter number is very close to the 0.3% previously reported for 12 week-old PSAPP (line 8.9) mice (Takeuchi et al., 2000).

Taken together, these data indicate that hypercholesterolemia increased the amyloid load due to increased deposit size and number. Importantly, these

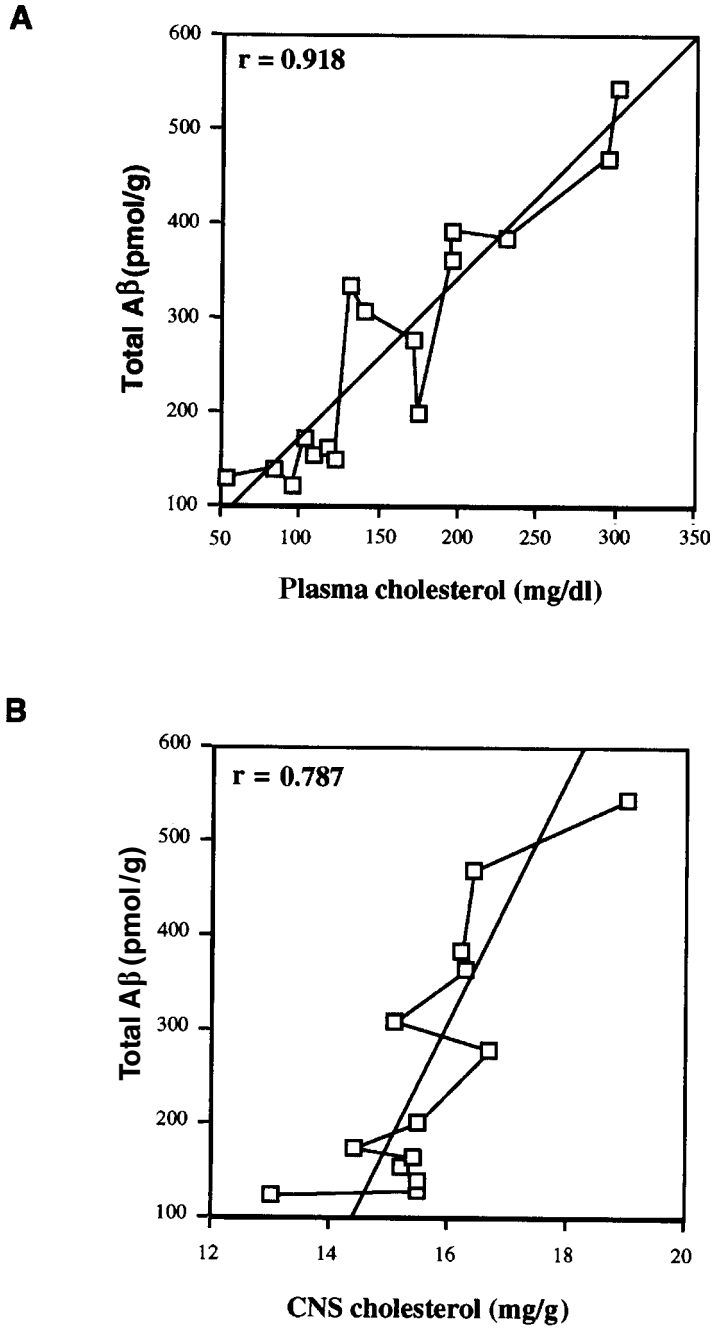

Figure 39.1. Positive correlation between (A) plasma cholesterol and total Aβ levels and (B) CNS cholesterol and total Aβ levels

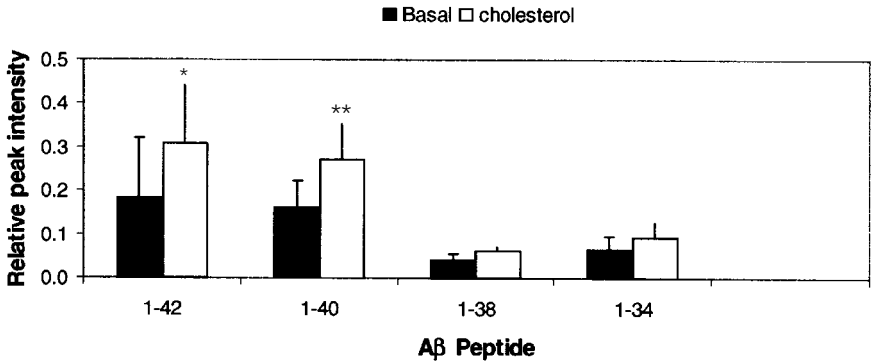

Figure 39.2. Quantitation of individual Aβ species from the CNS of basal diet (control) or hypercholesterolemic mice. IP–MS of formic acid extracts was performed. The *asterisks* denote statistical significance relative to the basal group. $^{*}p = 0.006$ and $^{**}p > 0.005$; p values were calculated using a paired Student's t-test (two-tailed). From Refolo et al. (2000), by permission of Academic Press.

data correlate well with the hypercholesterolemia-induced increase in Aβ peptides as determined by ELISA and IP–MS.

INCREASED AMYLOIDOGENIC PROCESSING OF APP IN THE CNS OF HYPERCHOLESTEROLEMIC MICE

Several recent studies have documented the effects of altered cellular cholesterol levels on the proteolytic processing of APP (Bodovitz and Klein, 1996; Frears et al., 1999; Howland et al., 1998; Mizuno et al., 1998; Simons et al., 1998). Therefore, we determined the effects of high cholesterol diet on APP holoprotein, sAPPα and β-CTF. In addition, since PS1 is a transmembrane protein that is known to affect the proteolytic processing of APP, we examined the effects of hypercholesterolemia on the levels of PS1.

To determine the effects of hypercholesterolemia on APP and PS1 processing, samples were immunoprecipitated with an antibody against APP or PS1, followed by Western blotting with a second antibody to the same protein. Results indicated that hypercholesterolemia had no significant effect on levels of APP holoprotein (Figure 39.4A), but resulted in a significant ($33 \pm 18\%$, $p = 0.005$) increase in β-CTF (Figure 39.4B). In addition, data showed a hypercholesterolemia-related reduction ($56 \pm 16\%$, $p = 0.005$) in the levels of sAPPα (Figure 39.4C). These results suggest that hypercholesterolemia alters APP processing and results in increased amyloidogenic processing.

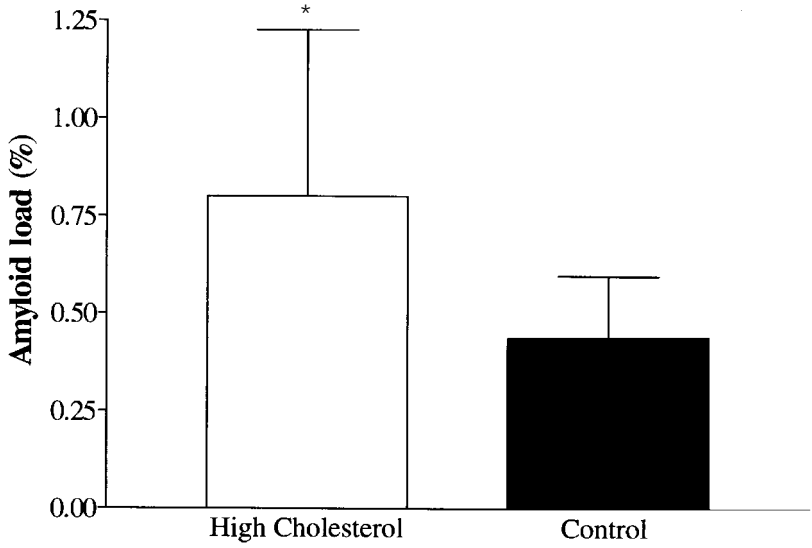

Figure 39.3. Effect of hypercholesterolemia on the amyloid-load. Amyloid load was measured. A total of 66 and 108 amyloid 4G8-positive Aβ deposits were counted for the basal and high cholesterol group, respectively. Amyloid load (%) represents the percentage of total hemispheric section covered by 4G8 immunoreactivity. p values calculated using a two-tailed Student's t-test. From Refolo et al. (2000), by permission of Academic Press

Our analysis of PS1 levels revealed no significant change in the human PS1 N-terminal fragment (NTF) due to hypercholesterolemia (Figure 39.4D). We did not detect measurable levels of PS1 holoprotein in our samples, as expected from our previous data on the parental PS1 mice (Duff et al., 1996). We therefore conclude that PS1-NTF steady-state levels are not affected by diet-induced hypercholesterolemia.

HYPERCHOLESTEROLEMIA INCREASES CNS apoE EXPRESSION

Using rodents, previous studies have shown that diet-induced hypercholesterolemia resulted in increased liver, plasma and brain apoE expression (Howland et al., 1998; Santillo et al., 1999; Srivastava, 1996). To determine the effects of cholesterol on apoE expression in PSAPP mice, we measured, by Western blot, the levels of apoE in the CNS of mice that were placed on either a hypercholesterolemic or a basal diet. Quantitation of the blots showed that the mean level of CNS apoE in the hypercholesterolemic group is $45 \pm 16\%$ greater than in the basal group (Figure 39.5).

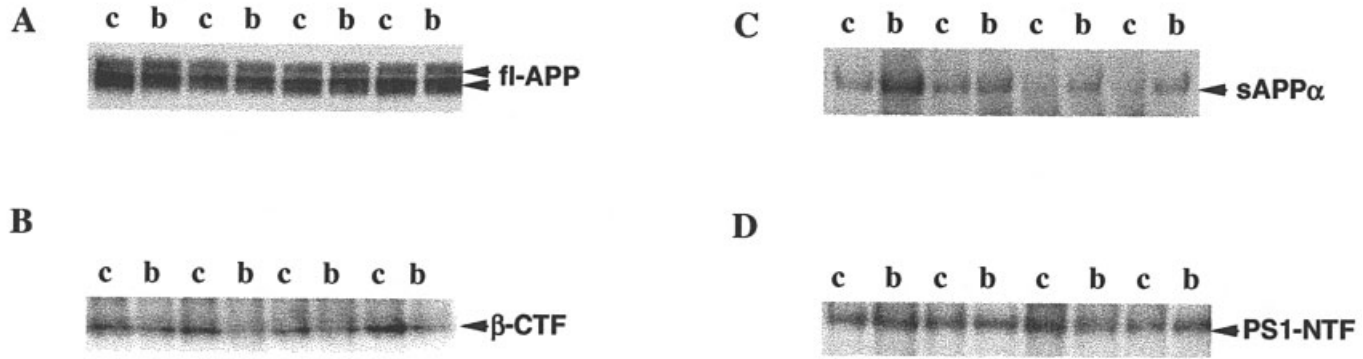

Figure 39.4. Effects of hypercholesterolemia on APP and PS1. IP/immunoblot analysis of brain from mice fed basal (b) or high cholesterol diets (c). Neutralized/lyophilized formic acid extracts were analyzed by IP/ immunoblotting. (A) APP-holoproteins. Arrows indicate the 150 and 130–125 kDa APP holoproteins. (B) β-CTFs. Arrow indicates the 14 kDa CTF. (C) sAPPα. Arrow indicates the 125 kDa sAPPα. (D) PS1 NTF. Arrow indicates the 27 kDa NTF. Statistical significances reported in the text were calculated using an unpaired Student's t-test (two-tailed). From Refolo et al. (2000), by permission of Academic Press

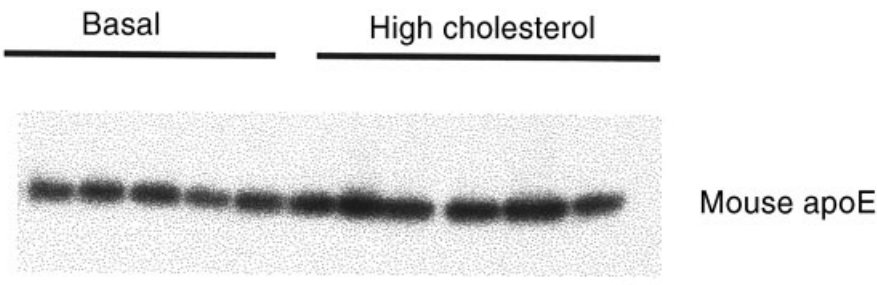

Figure 39.5. Effects of hypercholesterolemia on CNS apoE expression. Immunoblot analysis of neutralized formic acid extracts from mice fed basal or high cholesterol diets. Blots were probed with rabbit, anti-mouse apoE from BioDesign International Inc.(Saco, ME)

DISCUSSION

In the present study, we have examined the effects of diet-induced hypercholesterolemia on amyloid accumulation in a transgenic model for Alzheimer's amyloidosis. Our results demonstrate that hypercholesterolemia results in a significant increase in the accumulation of Aβ as measured by three independent techniques: sandwich ELISA of total formic acid-extracted Aβ, IP–MS performed on formic acid extracts, and IHC/image analysis of serial sections. In addition, we found a positive correlation between the increase in total Aβ and both elevated plasma and CNS cholesterol levels. To our knowledge, these data are the first demonstration that hypercholesterolemia increases amyloid accumulation in a β-amyloid depositing transgenic mouse model.

An earlier study using an APP gene-targeted mouse that did not develop amyloid deposits showed that, in contrast to our results, a similar high cholesterol diet resulted in a significant reduction in Aβ peptides in this model (Howland et al., 1998). The converse experimental outcomes may be attributed to differences between the mouse models used. These include: differences in the APP isoforms expressed, the inclusion of a mutant PS1 transgene in the PSAPP mouse, and the fact that the PSAPP mouse model accumulates high levels of Aβ, which is deposited at an early age. In addition, differences in the genetic backgrounds of these mouse models could make them more or less sensitive to hypercholesterolemia-induced alterations in APP processing and/or Aβ deposition.

Here, we report that hypercholesterolemia resulted in increased amyloidogenic processing of APP, as evidenced by increased levels of β-CTF, decreased levels of sAPPα and increased Aβ. In this regard, our data support earlier, *in vitro* data, which indicated that the pharmacological manipulation

of intracellular cholesterol altered APP processing (Bodovitz and Klein, 1996; Frears et al., 1999; Mizuno et al., 1998; Simons et al., 1998). In these studies, changes in intracellular cholesterol levels, resulted in alterations in Aβ, sAPPα and sAPPβ levels, suggesting that cholesterol levels modulate α and β-secretase activities.

Cholesterol-mediated changes in APP processing are consistent with several experimental observations. Cholesterol is an integral component of all eukaryotic membranes, and dramatically affects the physical properties of membranes including ordering, rigidity and fluidity (Yeagle, 1991). In addition, APP is a transmembrane glycoprotein (Gandy, 1999) and the APP processing enzymes, or secretases, appear to be membrane associated (Amour et al., 1998; Koike et al., 1999; Vassar et al., 1999). The interactions of APP with the secretases are believed to occur within several subcellular compartments including the endoplasmic reticulum (ER), Golgi stacks, trans-Golgi network, post-Golgi vesicles, endosomes and plasma membrane (Gandy, 1999). All of the membranes within these compartments contain cholesterol. However, the cholesterol content of the membranes varies, with the plasma membrane containing the highest and the ER the lowest levels (Bretscher and Munro, 1993; Schroeder et al., 1995).

In considering a mechanism in which cellular cholesterol levels regulate APP processing, it is important to appreciate that the secretase cleavage sites lie within, or near, the intramembranous domain of APP. Therefore, it is possible that a change in membrane cholesterol levels affects how APP resides in the membrane. Interestingly, alterations in membrane lipid and cholesterol content have been reported to affect the activities of membrane-associated enzymes (Klein et al., 1978; Mitchell et al., 1990). Thus, one plausible mechanism for the observed effects of cholesterol on APP processing is that, as membrane cholesterol levels change, so does membrane rigidity and fluidity. This could possibly alter the lateral movement of APP and secretases within the membrane and affect the contact between enzyme(s) and substrate or the intrinsic activities of the secretases.

Another possible mechanism could involve cholesterol-driven alterations in APP sorting. The cholesterol content of a membrane may function in the sorting of transmembrane proteins to specific subcellular compartments (Nickel et al., 1998). Alterations in the cholesterol content of membranes could affect the sorting of APP to the secretase-residing compartments and result in changes in the proteolytic processing of APP.

Recent studies suggest that the caveolae-like microdomains are subcellular sites that subserve APP secretase interactions (Ikezu et al., 1998; Lee et al., 1998; Nishiyama et al., 1999). Caveolae-like microdomains are a subset of non-clathrin-coated membrane domains that have been identified in most mammalian cells, including neurons and neuroglia (Anderson, 1998). These microdomains are involved in vesicular trafficking and signal transduction and are characterized by their unique lipid composition, which includes a high

content of cholesterol, glycosphingolipids and lipid-anchored membrane proteins (Anderson, 1998). Importantly, data suggest that the lipid core, particularly cholesterol, plays a vital role in the signal transduction and vesicular trafficking functions of these microdomains (Anderson, 1998).

Significantly, these microdomains contain several proteins important in the pathophysiology of AD including APP, Aβ, apoE, PKCα, PS1, possibly both α- and β-secretases and GPI-anchored proteins, which modulate β-secretase cleavage of APP (Anderson, 1998; Cole et al., 1999; Ikezu et al., 1998; Lee et al., 1998; Nishiyama et al., 1999; Sambamurti et al., 1999). The caveolae-like domains may subserve the specific interactions between APP, secretases, PKC, Aβ and apoE that are critical for the production and accumulation of Aβ. It is plausible that the cholesterol content of the caveolae-like microdomains may be vital for the biochemical processes involved in Aβ production and accumulation.

Measurements of CNS cholesterol in AD cases vs. aged-matched, non-demented controls have not provided any definitive clues concerning the role of cholesterol in AD. In one study, the cholesterol content of the frontal cortex of AD patients with an apoE4 genotype was found to be significantly increased over non-demented controls with the same apoE genotype (Sparks, 1997). However, another study found that the cholesterol content in the CNS of AD patients was significantly lower than that in non-demented controls (Mason et al., 1992).

Additional studies should be undertaken to establish whether CNS cholesterol levels are affected by AD. To provide the important and necessary data concerning the role of cholesterol in AD, these studies may need to focus on specific subcellular fractions from specific brain regions. It is plausible that specific subcellular fractions, e.g. synaptic membranes or caveolae-like microdomains, exhibit altered levels of cholesterol in AD, and this provides the basis for any cholesterol-mediated amyloid pathology (Wood et al., 1999).

In the PSAPP mouse model, hypercholesterolemia resulted in increased CNS apoE expression. This is not the first data demonstrating a direct connection between cholesterol metabolism and apoE expression. For example, in previously reported studies, diet-induced hypercholesterolemia in rodents resulted in increased liver, plasma and brain apoE expression (Howland et al., 1998; Santillo et al., 1999; Srivastava, 1996).

While the mechanism(s) for the regulation of CNS apoE by cholesterol remain unknown, we believe that cholesterol-mediated alterations in CNS apoE levels may explain the link between cholesterol metabolism, apoE genotype and an increased risk of AD. Supporting this hypothesis are recent data showing that the levels of apoE mRNA and protein are higher in the brains and plasma of patients with AD. Additional studies have demonstrated that certain apoE promoter polymorphisms are associated with increased apoE expression and an increased risk of AD, independent of the risk conveyed by the ε4 allele of apoE (Bullido and Valdivieso, 2000; Laws et al.,

1999). Significantly, it has been proposed that, in the CNS, apoE is involved in Aβ fibril formation and clearance (Bales et al., 1997; Beffert et al., 1999; Holtzman et al., 2000; Kuo et al., 2000).

Taken in the context of these studies, our data showing that cholesterol modulates both Aβ accumulation and CNS apoE suggest a plausible mechanism by which cholesterol metabolism is linked to AD. Our data suggest that cholesterol levels modulate β-amyloid accumulation, by altering APP processing in a manner in which hypercholesterolemia results in increased Aβ production. In addition, high cholesterol levels upregulate CNS apoE expression, which in turn modulates β-amyloid accumulation by promoting fibril formation, impeding Aβ clearance.

In conclusion, the present study supports clinical and epidemiological studies suggesting that a high fat/high cholesterol diet raises the risk of developing AD and, therefore, suggests that diet could be used to reduce the risk of developing the disease. This implies that dietary methods might offer a path to prevention of AD without resorting to drugs that are expensive and may have harmful side effects.

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40 Electron Microscopy and X-ray Diffraction Studies further Confirm the Efficacy of PTI-00703[1] (Cat's Claw Derivative) as a Potential Inhibitor of Alzheimer's β-Amyloid Protein Fibrillogenesis

GERARDO M. CASTILLO, DANIEL A. KIRSCHNER, ANN G. YEE, AND ALAN D. SNOW

INTRODUCTION

Alzheimer's disease (AD) is characterized by the accumulation in the brain parenchyma of insoluble fibrillar deposits 'plaques' that contain the β-amyloid protein (Aβ) (Glenner and Wong, 1984; Masters et al., 1985; Westermark et al., 1999). Aβ also accumulates to form amyloid deposits in the blood vessel walls, which are referred to as cerebrovascular amyloid or congophilic angiopathy (Mandybur, 1986; Pardridge et al., 1987). In addition, AD is characterized by the presence of numerous neurofibrillary 'tangles', consisting of paired helical filaments that accumulate abnormally in the neuronal cytoplasm (Grundke-Iqbal et al., 1986; Kosik et al., 1986; Lee et al., 1991). Recent studies indicate that the accumulation of Aβ is a causative factor for AD and should not be regarded merely as a consequence (Calhoun et al., 1998; Games et al., 1995; Haass et al., 1995; Hardy, 1992; Harrigan et al., 1995; Hsiao et al., 1996; Murrell et al., 1991; Pike et al., 1991, 1995; Sturchler-Pierrat et al., 1997; Van Broeckhoven et al., 1990). These studies suggest that providing agents or compounds that are capable of inhibiting the formation and persistence of Aβ deposits may be therapeutically beneficial to patients with AD.

In our search for possible natural inhibitors of Aβ fibrillogenesis, we have discovered PTI-00703[1], which is a proprietary aqueous extract derived from

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the bark of the rain forest woody vine known as *Uncaria tomentosa* or cat's claw. *U. tomentosa* is a slow-growing woody vine that is native to the Amazon rain forest and other tropical areas of South and Central America (Elkins, 1995). It is referred to as 'cat's claw' because of its distinctive curved claw-like thorns, which project from the base of its leaves. The bark of *U. tomentosa* is known to contain important phytochemicals that have previously been demonstrated to possess antiinflammatory (Sandoval-Chacon et al., 1998) and antioxidant properties (De Matta et al., 1976; Desmarchelier et al., 1997; Ostrakhovich et al., 1997). The former finding could be important, as it pertains to Alzheimer's, since previous studies have indicated that inflammation plays important roles in the pathogenesis of this disease (Hull et al., 1999; McGeer and McGeer, 1999). The latter is also significant, since oxidative free radical damage occurs in a number of neurodegenerative diseases, including AD (Repetto et al., 1999; Yatin et al., 1999).

The purpose of the present study was to further determine the *in vitro* effects of the plant derivative PTI-00703[1] on inhibition of Aβ fibril formation. The major hypotheses tested include: (1) PTI-00703[1] is a potent inhibitor of Aβ fibril formation; (2) inhibition of Aβ fibril formation by PTI-00703[1] is a property not shared by other cognitive enhancing herbal extracts, including *Ginkgo biloba*, Korean ginseng and Gotu kola; and (3) PTI-00703[1] exerts its effects on Aβ fibrillogenesis by inhibition of hydrogen bonding.

MATERIALS AND METHODS

The *in vitro* methods employed in the present study included the use of a Congo red staining assay and negative-stain electron microscopy to assess the effects of PTI-00703[1] on Aβ fibril formation in comparison to other herbal extracts, including *G. biloba*, Gotu kola and Korean ginseng. Studies were also implemented whereby fresh $A\beta_{1-40}$ incubated in the presence or absence of different amounts of PTI-00703[1] were dried and examined by X-ray diffraction to determine a potential mechanism of inhibitory action of PTI-00703[1] on Aβ fibril formation.

For Congo red staining assays, 125 μM $A\beta_{1-40}$ was incubated for 3 days at 37 °C in 100 mM Tris–HCl, 50 mM NaCl, pH 7.0 (TBS), in the presence or absence of PTI-00703[1] at 1:1 weight ratio. Aliquots (5 μl) were analyzed by air-drying on gelatin-coated slides, followed by Congo red staining (Puchtler et al., 1962), as described in our previous studies (Castillo et al., 1998, 1999b). This technique has been effective in providing corroborating evidence of potential Aβ amyloid inhibitors. We qualitatively determined whether the decrease in Aβ fibrillogenesis as a result of treatment with PTI-00703[1] or other extracts observed in thioflavin T fluorometry studies (not shown), correlated with a decrease in Congo red staining (i.e. red/apple-green birefringence, as viewed under polarized light).

For negative-stain transmission electron microscopy (EM), 50 μM $A\beta_{1-40}$ was incubated at 37 °C for 7 days in the absence or presence of PTI-00703[1], *G. biloba*, PTI-00703[1] + *G. biloba*, Gotu kola, or Korean ginseng at a 1:1 weight ratio. Aliquots were taken at 0, 1, 3 and 7 days for EM analysis to observe any time-dependent inhibition of $A\beta_{1-40}$ fibril formation. Negatively-stained Aβ amyloid fibrils were prepared by floating pioloform, carbon-coated grids on peptide solutions. After the grids were blotted and air-dried, the samples were stained with either 2% (w/v) uranyl acetate or 1% (w/v) phosphotungstic acid, visualized with a Phillips CM-10 TEM, using 80 kv accelerating voltage, and photographed.

For X-ray diffraction studies, we determined whether PTI-00703[1] inhibits Aβ fibril formation by inhibition of H-bonding and/or β-pleated sheet packing. To this end, lyophilized $A\beta_{1-40}$ peptide (Bachem Inc.) was dissolved at 1 mg/ml, either in distilled water alone or with PTI-00703[1], at Aβ:PTI-00703[1] weight ratio of 3:1 or 6:1, prior to drying. Approximately, 20 μl aliquots of each solution was slowly drawn into siliconized, thin-walled glass capillary tubes (0.7 mm outer diameter; Charles Supper Co., South Natick, MA) with the aid of negative pressure. The capillary tubes were flame-sealed at the narrow end, and a plug of wax was melted into the wide end and pierced with a hot needle to form a small hole, through which solvent was allowed to evaporate gradually at room temperature. When the solutions were fully dried, the capillary tubes were transferred to the X-ray diffraction sample holder. X-ray diffraction patterns were collected using nickel-filtered, double-mirror-focused Cu/Kα radiation from an Elliot GX-20 rotating anode X-ray generator (GEC Avionics, Hertfordshire, UK), operated at 35 kV and 35 mA. Patterns were recorded on Kodak DEF films with exposure times of 100–105 h. The known Bragg spacing of calcite (3.035 Å) was used to calibrate the specimen-to-film distance (86.0 mm). The Bragg spacings of the reflections were measured directly off the films with a × 6 optical comparator. The films were digitized at 40 μm using a ScanMaker 5 (Microtek Inc.) operated by the program ScanWizard. Scanning non-linearity was corrected using a calibrated Kodak Photographic Step Tablet (No. 2; optical density range 0.04–3.03).

RESULTS

PTI-00703[1] CAUSES A MARKED REDUCTION IN AMYLOID CONGOPHILIA SUGGESTIVE OF AN INHIBITION OF Aβ FIBRIL FORMATION

Our earlier studies (Castillo et al., 1999a), using thioflavin T fluorometry, indicated that increasing concentrations of PTI-00703[1] caused a dose-dependent inhibition of Aβ fibril formation. In the present study, Congo red staining of $A\beta_{1-40}$ peptide dots in the absence or presence of PTI-00703[1] was used to obtain further corroborating evidence for the inhibitory effects of PTI-

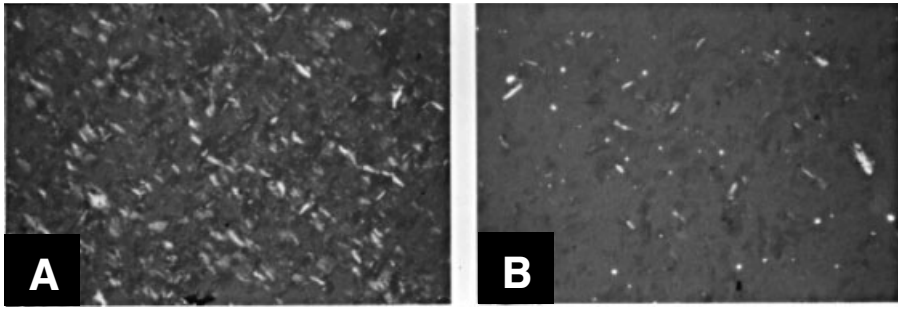

Figure 40.1. Black and white photomicrographs of Congo red-stained $A\beta_{1-40}$ peptide dots in the absence (panel A) or presence (panel B) of PTI-00703[1] at a 1:1 weight ratio. The original red/green birefringence of amyloid fibrils stained with Congo red and viewed under polarized light is shown in (A) (as bright and dark regions in this black and white photomicrograph). As can be seen, there is significant reduction in the red/green birefringence when $A\beta_{1-40}$ peptide was incubated with PTI-00703[1] suggestive of an inhibition of Aβ fibril formation

00703[1] on Aβ fibril formation. 125 μM of $A\beta_{1-40}$ was incubated alone for 3 days at 37 °C in TBS, air-dried and stained with Congo red. Extensive amyloid congophilia was observed when viewed under polarized light, indicative of massive amounts of amyloid fibrils (Figure 40.1A). As shown in Figure 40.1B, when Aβ was co-incubated with PTI-00703[1] at a 1:1 weight ratio, a marked reduction in amyloid congophilia was observed, suggestive of an inhibition of Aβ amyloid fibril formation.

ELECTRON MICROSCOPY REVEALS THAT PTI-00703[1] BUT NOT *G. BILOBA* INHIBITS Aβ FIBRIL FORMATION

In one EM study, 50 μM $A\beta_{1-40}$ was incubated at 37 °C for 7 days in the absence or presence of *G. biloba*, PTI-00703[1], or PTI-00703[1] + *G. biloba*, at a 1:1 weight ratio, with aliquots taken at 0, 1, 3 and 7 days of incubation for EM analysis. Amyloid fibrils were formed by $A\beta_{1-40}$ alone within 3 days of incubation (Figure 40.2A). Similar amyloid fibrils were observed at 3 days following incubation of $A\beta_{1-40}$ with *G. biloba* (Figure 40.2B) suggesting that *G. biloba* alone does not significantly affect amyloid fibril formation. However, as shown in Figure 40.2C,D, $A\beta_{1-40}$ in the presence of PTI-00703[1] only, or PTI-00703[1] + *G. biloba*, demonstrated primarily the formation of amorphous non-fibrillar material. A similar inhibition of Aβ fibril formation by PTI-00703[1] alone, or PTI-00703[1] + *G. biloba*, was observed at all time points analyzed. These results suggest that the inhibitory effect of PTI-00703[1] is neither shared nor abolished by *G. biloba*.

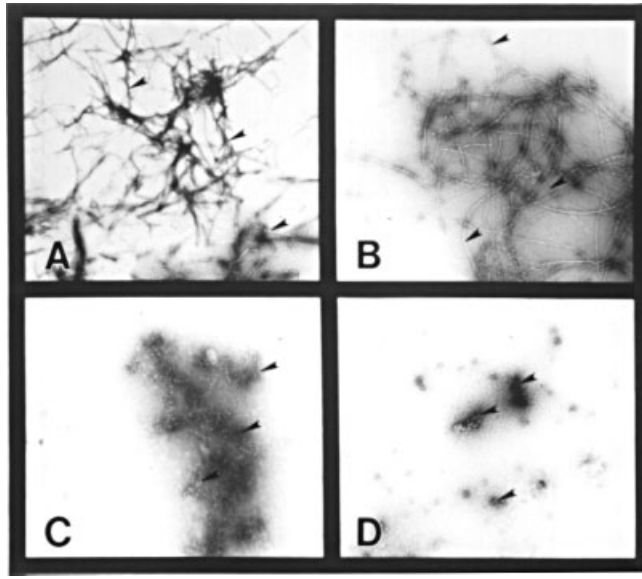

Figure 40.2. EM photomicrographs of $A\beta_{1-40}$ after a 3-day incubation at 37 °C in the absence (A) or presence (B) of *G. biloba*, PTI-00703[1] (C), or PTI-00703[1] + *G. biloba* (D), at a 1:1 weight ratio. Amyloid fibrils were formed by $A\beta_{1-40}$ alone within 3 days of incubation and by $A\beta_{1-40}$ incubated with *G. biloba*. However, $A\beta_{1-40}$ incubated with PTI-00703[1] only, or PTI-00703[1] + *G. biloba* demonstrated few or no fibrils and primarily amorphous non-fibrillar material was observed

PTI-00703[1] BUT NOT GOTU KOLA OR KOREAN GINSENG, INHIBIT Aβ FIBRIL FORMATION

In another EM study, the potent inhibition of Aβ fibril formation by PTI-00703[1] was directly compared to possible amyloid inhibitory effects by other herbs, including the Indian herb Gotu kola, believed to be effective for improving cognition, and Korean ginseng, a herb believed to contain many important phytochemicals. In this latter study, 50 μM $A\beta_{1-40}$ was incubated at 37 °C for 7 days in the absence or presence of PTI-00703[1], Gotu kola or Korean ginseng at a 1:1 weight ratio, with aliquots taken at 0, 1, 3 and 7 days of incubation for EM analysis. As shown in Figure 40.3A, amyloid fibrils were formed by $A\beta_{1-40}$ alone within 3 days of incubation. Consistent with Figure 40.2, PTI-00703[1] was again effective in preventing amyloid fibril formation (Figure 40.3B). However, Gotu kola (Figure 40.3C) or Korean ginseng (Figure 40.3D) did not have any effect on the inhibition of $A\beta_{1-40}$ amyloid fibril formation. This study indicates that extracts from other cognitive-enhancing herbs, such as Gotu kola and Korean ginseng, do not appear to affect Aβ fibril formation.

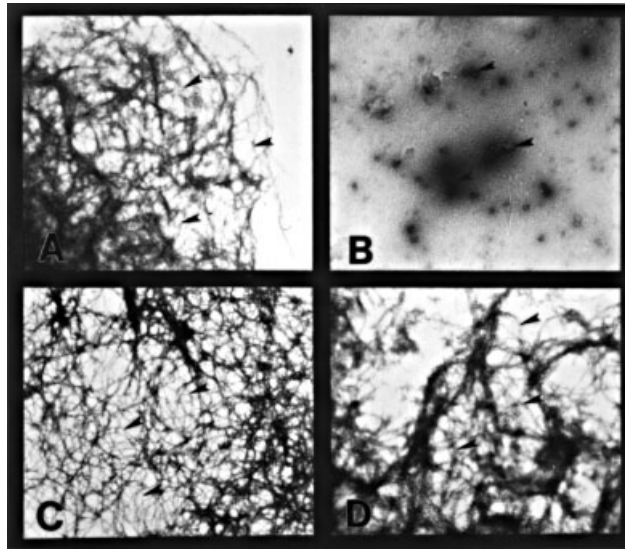

Figure 40.3. EM photomicrographs of $A\beta_{1-40}$ after a 3-day incubation at 37 °C in the absence (A) or presence (B) of PTI-00703[1], Gotu kola (C) or Korean ginseng (D). Amyloid fibrils were formed by $A\beta_{1-40}$ alone within 3 days of incubation but not in the presence of PTI-00703[1]. The presence of Gotu kola or Korean ginseng did not significantly inhibit $A\beta_{1-40}$ amyloid fibril formation

INHIBITION OF H-BONDING DURING Aβ FIBRIL FORMATION IS ONE MECHANISM OF ACTION AS REVEALED BY X-RAY DIFFRACTION

In another study, X-ray diffraction patterns were collected from lyophilized $A\beta_{1-40}$, or $A\beta_{1-40}$ dissolved at 1 mg/ml, either in distilled water or with PTI-00703[1], at Aβ:PTI-00703[1] weight ratio of 3:1 or 6:1 prior to drying. The non-hydrated Aβ peptide gave an X-ray diffraction pattern typical of lyophilized synthetic amyloid peptides, with major reflections at ~4.7 Å (Figure 40.4A, arrow) and ~10 Å (arrowhead in 40.4A). These reflections arise from the array of hydrogen-bonded polypeptide chains responsible for elongated fibrillar structure and the packing of β-pleated sheets, respectively (Fraser et al., 1991a,b; Inouye et al., 1993; Malinchik et al., 1998). When the lyophilized peptide was solubilized in water and dried, the H-bonding reflection became darker and sharper (dashed arrow in Figure 40.4A), indicating a more extensive array of H-bonded chains, while the inter-sheet spacing became weaker and broader. Whereas no obvious effect on the sharpness of H-bonding reflection at ~4.7 Å was observed at an Aβ:PTI-00703[1] weight ratio of 6:1 (dashed arrow in Figure 40.4B), a markedly decreased sharpness of the same reflection was observed when PTI-00703[1]

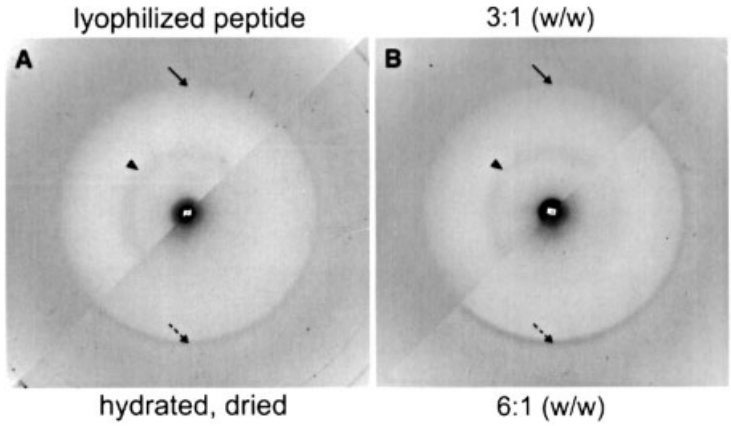

Figure 40.4. X-ray diffraction patterns from lyophilized $A\beta_{1-40}$ peptide (upper panel of A), dissolved in water at 1 mg/ml and dried (lower panel of A), or mixed with PTI-00703[1] and dried at an Aβ: PTI-00703[1] weight ratio of 3:1 (upper panel of B) or 6:1 (lower panel of B). The arrows in each panel indicate the position of the X-ray reflection of ~4.7 Å space coming from the H-bonding between the polypeptide chains in the β-pleated sheet conformation. In the upper panels of (A) and (B), this reflection is broader than it is in their lower panels, indicating that there are fewer chains H-bonded to one another. The arrowheads indicate the position of the reflection of ~10 Å (upper panels) coming from the inter-sheet spacing of the β-pleated sheets. PTI-00703[1] at an Aβ:PTI-00703[1] weight ratio of 3:1, markedly inhibited Aβ fibril formation, as evidenced by a weakened and broadened H-bonding reflection (arrow, upper panel of B). No H-bonding inhibition was observed at an Aβ:PTI-00703[1] weight ratio of 6:1 (dashed arrow, lower panel of B)

was increased, such that the Aβ:PTI-00703[1] weight ratio was 3:1 (arrow in Figure 40.4B). This weakened and broadened H-bonding reflection (arrow in Figure 40.4B) in the presence of PTI-00703[1] is indicative of PTI-00703[1] interfering with the H bonding responsible for growth and extension of amyloid fibrils.

DISCUSSION

Initial screening studies by our group identified PTI-00703[1], which is an extract of *Uncaria tomentosa* (also known as cat's claw or Uña de Gato), to be a potent inhibitor of Aβ fibrillogenesis. Additionally, previous studies have shown that this extract contains compounds capable of inhibiting the formation and growth of Aβ fibrils, as well as causing a disruption/disassembly of pre-formed fibrillar Aβ deposits (both $A\beta_{1-40}$ and $A\beta_{1-42}$; Castillo et al., 1999a). The present study is an extension of these earlier studies to obtain stronger corroborating evidence for the observed inhibition of Aβ fibril

formation by PTI-00703[1] and to try to determine the mode of PTI-00703[1] action by X-ray diffraction experiments.

Although there are many components in the *U. tomentosa* extract, significant knowledge had already been gained from studying *U. tomentosa*, its components and their associated biological activities (Aquino et al., 1991; Harada et al., 1979; Hemingway and Phillipson, 1974; Jones, 1994; Keplinger et al., 1989, 1990; Laus et al., 1997; Laus and Keplinger, 1994; Sheng et al., 1998). Among these, and most important for AD, is *U. tomentosa*'s potent antiinflammatory activity (Sandoval-Chacon et al., 1998), since previous studies have indicated that inflammation plays important roles in the pathogenesis of AD (Hull et al., 1999; McGeer and McGeer, 1999). *U. tomentosa* also contains flavonoids, which act to protect cells from oxidative damage. Two of the flavonoids found in *U. tomentosa*, proanthyanidins and catechin tannins, have been shown to possess potent antioxidant activity (De Matta et al., 1976; Desmarchelier et al., 1997; Ostrakhovich et al., 1997). The antioxidant effects of *U. tomentosa* and its extracts are therefore believed to be beneficial in diseases involving free radical tissue damage. This is also significant, since free radical damage occurs in a number of neurodegenerative diseases, including AD (Repetto et al., 1999; Yatin et al., 1999). However, no component of *U. tomentosa* has so far been tested for the direct inhibition of fibril formation, and the ongoing studies that we are implementing will lead to the structural identification of the Aβ fibril-inhibiting components, which may have desirable pharmacokinetic and toxicity profiles.

Earlier toxicity studies suggest that this plant, and derivatives thereof, are not toxic, even at high dosages. No evidence of *in vitro* toxicity was found when *U. tomentosa* was tested in the neutral red assay, total protein content assay, tetrazolium assay and Microtox assay (Santa Maria et al., 1993). Plant extracts and fractions of *U. tomentosa* exhibited no mutagenic effects, and in fact demonstrated a protective effect against UV light-induced mutagenesis (Rizzi et al., 1993). Lack of organ-specific damage was noted upon histological examination of tissues in rats after prolonged administration of *U. tomentosa* extracts in a number of studies (Keplinger et al., 1999; Sheng et al., 2000). In one recent study, the LD_{50} and maximum tolerable single dose of a *U. tomentosa* aqueous extract in rats was found to be greater than 8 g/kg (Sheng et al., 2000).

The antioxidant and anti-inflammatory activities of *U. tomentosa*, as well as its apparent lack of toxicity, are desirable secondary characteristics, fitted to its use as a potential agent for the treatment of AD. In the present study, Congo red staining, negative-stain electron microscopy and X-ray diffraction studies all confirmed that PTI-00703[1] is a potent inhibitor of Aβ fibril formation. Furthermore, X-ray diffraction studies indicated that one mechanism of Aβ fibril inhibition by PTI-00703[1] involved inhibition of H bonding important for growth and extension of amyloid fibrils. In addition,

we have also recently investigated the ability of PTI-00703[1] to dissolve pre-deposited $A\beta_{1-42}$ fibrils in brain (Cummings et al., 2000). This study involved a 3 day infusion of $A\beta_{1-42}$ (25 μg) into rat hippocampus, followed by a 4 day rest period. After the rest period, PTI-00703[1] (125 μg) was infused continuously for 7 days. At the end of 7 days, amyloid deposits were quantified throughout the infusion site by the scoring of Congo red-stained brain sections (Snow et al., 1994). The results of this study indicated that a significant (p50.01) 64% disruption/dissolution of pre-deposited $A\beta_{1-42}$ fibrils was observed, following the infusion of PTI-00703[1], in comparison to vehicle (i.e. water) controls. All of these studies indicate that PTI-00703[1] and/or its amyloid inhibitory ingredients are anticipated to lead to the identification of a new drug candidate for the treatment of Aβ amyloidosis in AD and related disorders.

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41 Accelerated Aβ Generation in a Cell Model of Alzheimer's Disease-related Endosomal–Lysosomal System Upregulation

PAUL M. MATHEWS, CAROLYN B. GUERRA, YING JIANG, BENJAMIN H. KAO, RAVI DINAKAR, PANKAJ MEHTA, ANNE M. CATALDO AND RALPH A. NIXON

INTRODUCTION

We have previously identified and characterized prominent endosomal and lysosomal changes within neurons, changes that are an invariant and early feature of sporadic Alzheimer's disease (AD) (Cataldo et al., 1995, 1996, 1997, 2000). Lysosomal system activation, which develops in virtually all neurons within at-risk populations, is characterized by increased gene expression and protein expression of lysosomal hydrolases, including such proteases as cathepsin D (Cat D), and a robust increase in the number of lysosomes (Cataldo et al., 1991, 1994, 1995, 1996). We have also shown that neuronal endocytic abnormalities precede substantial β-amyloid deposition in AD brain and begin even decades prior to the development of AD pathology in Down's syndrome (Cataldo et al., 2000). Endocytic pathway abnormalities in AD include increased early endosome volume, increased expression of proteins involved in the regulation of endocytosis and recycling (such as rab5 and rab4), and abnormally increased levels of lysosomal hydrolases within the early endosome (Cataldo et al., 1997).

The partial redistribution of lysosomal hydrolases in AD brain may be, in part, due to changes in the expression of neuronal mannose 6-phosphate receptors (MPR). These receptors bind the majority of lysosomal hydrolases in the trans-Golgi network and direct their transport to the endosomal–lysosomal system (Griffiths et al., 1988). By immunocytochemistry, Cataldo

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et al. (1997) demonstrated an increased neuronal expression of the 46 kDa, cation-dependent MPR (CD-MPR) in sporadic AD cases when compared to control. This increase in CD-MPR immunoreactivity in sporadic AD brain was not accompanied by increased levels of the other MPR, the cation-independent MPR. The CD-MPR delivers newly synthesized lysosomal hydrolases to endocytic compartments, including the early endosome (Chao et al., 1990), which may in part explain the increased localization of lysosomal hydrolases in neuronal early endosomes in AD (Cataldo et al., 1997).

Early endosomes are an important site for APP processing, including the generation of Aβ. When compared to wild-type APP, expression of APP trafficking mutants that fail to undergo endocytosis results in reduced Aβ production (Koo and Squazzo, 1994; Perez et al., 1999). There is also evidence that at least some of the proteases responsible for Aβ generation reside within early endosomes. For example, BACE, an aspartic protease with β-secretase activity, has shown it to be within early endosomes (Capell et al., 2000; Vassar et al., 1999). Cat D, which was recently confirmed to have secretase-like activity (Gruninger-Leitch et al., 2000), is primarily localized to lysosomes. In human AD, however, Cat D can be detected in neuronal early endosomes, particularly in abnormally enlarged early endosomes (Cataldo et al., 1997).

RESULTS

EXPRESSION OF CD-MPR CONSTRUCTS

In order to determine whether increased expression of the CD-MPR in AD might modify intracellular lysosomal hydrolase distribution and therefore the proteolytic processing of APP, we expressed human CD-MPR in murine L cells overexpressing human APP695 (Figure 41.1A). The wild-type CD-MPR is primarily localized to the trans-Golgi network, where it binds newly synthesized lysosomal hydrolases through its lumenal domain, directing them to endosomal compartments. In addition to the wild-type CD-MPR, we constructed and expressed two CD-MPR-trafficking mutants containing modified cytoplasmic domains but an intact hydrolase-binding domain. In the CD-MPRendo construct, the cytoplasmic tail of the CD-MPR was removed and replaced by a 10 amino acid sequence derived from influenza hemagglutinin (HA) containing an artificial tyrosine-endocytosis motif (Lazarovits and Roth, 1988). Immunolabeling of the CD-MPRendo construct showed a punctate pattern distributed throughout the cell body (Figure 41.1B), consistent with an early endosomal distribution. The other CD-MPR trafficking mutant, CD-MPRpm, contains the wild-type HA cytoplasmic tail, which promotes its accumulation at the plasma membrane (Roth et al., 1986). As expected, immunolabeling of CD-MPRpm showed a

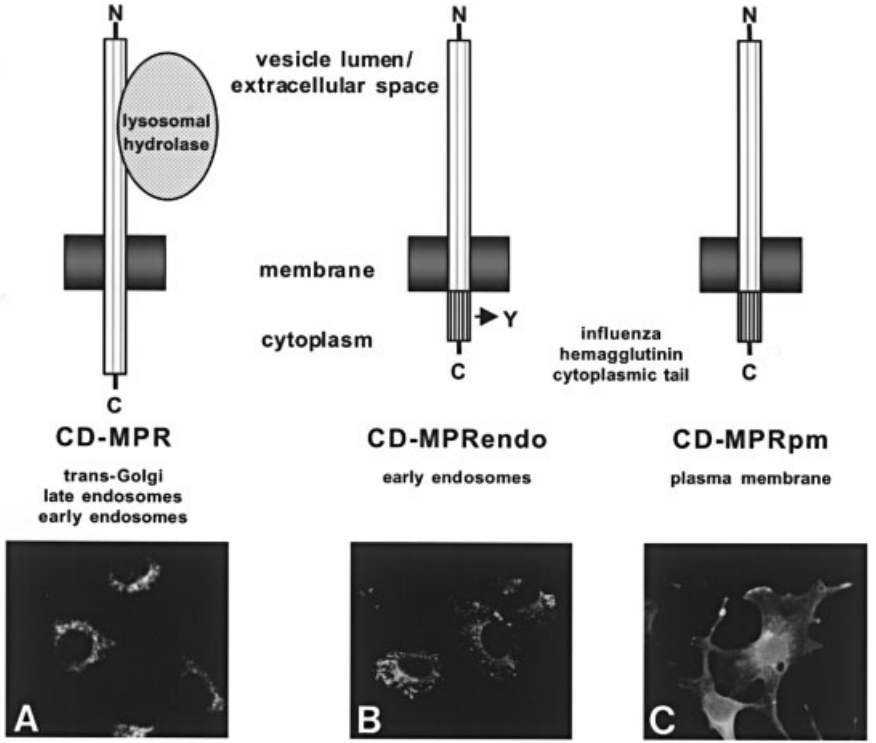

Figure 41.1. Expression of human CD-MPR and CD-MPR constructs in L cells. Human CD-MPR was expressed in a murine L cell line overexpressing human APP (A). In addition, two CD-MPR chimeras were constructed and expressed in the same APP-overexpressing cell line. The CD-MPRendo construct localized primarily to early endosomes (B) while the CD-MPRpm construct was found at the cell surface (C). Immunolabeling was with a monoclonal antibody specific for the hydrolase-binding domain of human CD-MPR

typical cell surface pattern in either permeabilized or intact cells (Figure 41.1C; permeabilized cell shown).

To demonstrate whether overexpression of CD-MPR or expression of CD-MPR-trafficking mutants resulted in a redistribution of lysosomal hydrolases, we characterized the intracellular distribution of Cat D (data not shown). In control cells, Cat D immunolabeling revealed a typical lysosomal pattern. However, in L cells overexpressing CD-MPR, significant Cat D immunolabeling was also seen in small punctate compartments corresponding to early endosomes. A similar pattern of small Cat D immuno-positive compartments was seen in CD-MPRendo-expressing cells. In CD-MPRpm-expressing cells, some cell surface Cat D immunolabeling was detected and, in some cells, depletion of intracellular Cat D signal was evident.

CHARACTERIZATION OF APP METABOLISM IN L CELLS EXPRESSING CD-MPR CONSTRUCTS

Metabolic labeling followed by immunoprecipitation with an antibody directed against the C-terminus of APP showed equal levels of APP biosynthesis in the parental APP overexpressing cell line, as well as in lines co-transfected with each of the CD-MPR constructs. These lines showed similar rates of APP turnover, with the majority of cellular APP degraded within a 2 h chase period (data not shown). Additionally, in each of the lines, similar levels of sAPPα and sAPPβ were seen following chase and immunoprecipitation of the growth media. The results argue that the bulk turnover of APP was not greatly affected by the expression of the CD-MPR constructs.

In each of these cell lines, we determined the amount of human Aβ_{40} and Aβ_{42} secreted into the growth media by sandwich ELISA. Both pulse-labeling followed by immunoprecipitation and Western blot analysis were done to demonstrate comparable levels of APP expression. In the experiment shown in Figure 41.2, cells overexpressing CD-MPR showed a 2.8-fold increase in Aβ_{40} and a 1.7-fold increase in Aβ_{42} secreted into the growth media during an 8 h incubation. A similar increase was seen in CD-MPRendo expressing cells (3.2-fold increase in Aβ_{40} and 2.0-fold increase in Aβ_{42}). Expression of the CD-MPRpm construct, which does not traffic lysosomal hydrolases to the early endosome, had little effect on Aβ secretion from the L cells (1.4-fold increase in Aβ_{40} and 1.1-fold increase in Aβ_{42}). That the two CD-MPR constructs that partially redistribute lysosomal hydrolases to the early endosome result in increased Aβ secretion, while the control construct CD-MPRpm had little effect, argues for a specific role of endosomal proteolysis in this increased Aβ secretion.

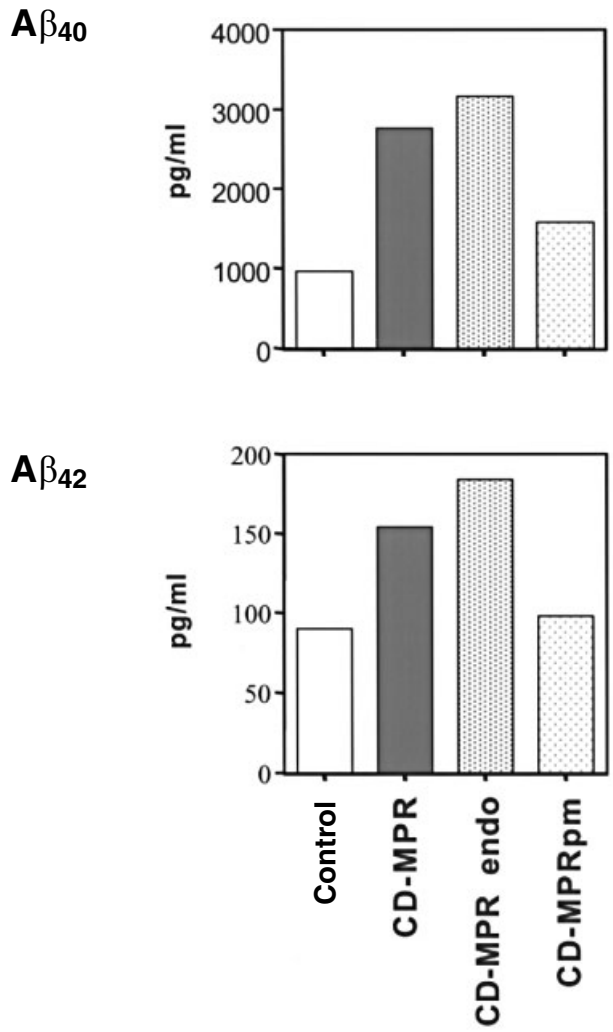

Figure 41.2. Increased Aβ secretion from cells expressing CD-MPR and CD-MPRendo. Secretion into the growth media of $A\beta_{40}$ and $A\beta_{42}$ by each line was measured using a sandwich ELISA (see Potempska et al., 1999, for methods)

CONCLUSIONS

To model the alterations in the hydrolase trafficking we have observed in neurons from sporadic AD brain, we have used cells overexpressing human CD-MPR or a CD-MPR construct that delivers hydrolases to early endosomes. Overexpression of CD-MPR has particular relevance to sporadic

AD because, by immunolabeling, we have detected increased expression of this MPR in pyramidal neurons early in the disease process (Cataldo et al., 1997). Several previous studies using cells in culture have shown that the endocytic pathway is a major site of Aβ generation (Koo and Squazzo, 1994; Perez et al., 1999; Soriano et al., 1999). Our current findings show that the mistrafficking of lysosomal hydrolases to early endosomes, which normally contain low levels of these enzymes, leads to an increased production of $A\beta_{40}$ and $A\beta_{42}$. This suggests that in sporadic AD, where CD-MPR expression is increased and early endosomes contain abnormal levels of lysosomal hydrolases, the generation of Aβ in early endosomes may be pathologically increased. Due to the rapid communication of early endosomes with the extracellular space, relatively subtle changes of APP proteolysis within the early endosome may contribute substantially to Aβ release from the cell without detectably changing APP bulk turnover, as we have seen in this system. The results of this study suggest the dysfunction of the endocytic pathway as seen in sporadic AD can lead to an increase in Aβ secretion and, thus, could shift the balance towards Aβ accumulation within the brain.

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42 The Amyloid Precursor Protein V717I Mutation Increases Susceptibility to Cell Death in a Cholesterol-dependent Manner

LUIGI PUGLIELLI, LAURA A. MACKENZIE INGANO, RUDOLPH E. TANZI AND DORA M. KOVACS

INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by abnormal accumulation of amyloid β-peptide (Aβ) in the form of senile (or amyloid) plaques, neurofibrillary tangles and diffuse loss of neurons and synapses in the neocortex, hippocampus and other subcortical regions of the brain. Genetic linkage studies have identified two genetic polymorphisms that seem to operate as risk factors in late-onset AD (Saunders et al., 1993; Blacker et al., 1998) and three causative genes that are directly involved in the development of early-onset AD (FAD). The latter include the amyloid precursor protein (APP) (Goate et al., 1991), presenilin 1 (PS1) (Sherrington et al., 1995) and presenilin 2 (PS2) (Levy-Lahad et al., 1995). They all seem to accelerate the generation and deposition of Aβ, which in turn is thought to lead to neurodegeneration (for general review, see Selkoe, 1999).

The precise sequence of events underlying AD-associated neurodegeneration still remains unknown. Both apoptosis and necrosis have been shown to occur in AD, but apoptosis is currently thought to be the most prominent event, at least in the early phases of the disease (for review, see Nijhawan et al., 2000). The exact mechanism of programmed cell death in AD still remains elusive. *In vitro*-generated Aβ is able to induce apoptosis and cell death in cultured cells, including hippocampal neurons (Loo et al., 1993; Yankner, 1996). In addition, FAD mutant forms of APP, PS1 and PS2 have been shown to activate the programmed cell death and potentiate the effect of apoptotic stimuli (Kovacs et al., 1999; Thinakaran, 1999; Wolozin et al., 1996).

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Recent data have linked cholesterol to Aβ generation and aggregation. Specifically, studies with cultured cells and transgenic mice expressing FAD-associated APP have shown that cholesterol metabolism is able to modulate APP processing and Aβ generation/aggregation (Bodovitz and Klein, 1996; Simons et al., 1998; Howland et al., 1998). In addition, both APP and Aβ are, at least in part, associated with cholesterol-rich domains in cell membranes (Lee et al., 1998; Parkin et al., 1999; Refolo et al., 1991).

In the present study we have used a genetic approach to assess the mechanism by which cholesterol regulates Aβ generation. Specifically, we have used Chinese hamster ovary (CHO) cells having a defect in the molecular pathway that regulates the expression of the genes involved with the uptake and biosynthesis of cholesterol (for review, see Chang et al., 1997; Brown and Goldstein, 1999). Here we have identified a novel link between intracellular cholesterol compartmentation, FAD-associated forms of APP and cell death.

MATERIALS AND METHODS

CELL LINES AND CONSTRUCTS

Wild-type (WT), 25RA and AC29 CHO cell lines were stably transfected with a V717I (APP-V717I, London mutation) APP expression construct, containing G418 as selection marker. Clones were selected based on similar levels of expression of APP-V717I. 25RA and AC29 cells were a generous gift of Dr T.Y. Chang, Dartmouth Medical School, Hanover, NH.

CHOLESTEROL DETERMINATIONS

For total cholesterol determination, 80–90% confluent cells were washed twice in Dulbecco's phosphate buffered saline (PBS; Sigma Chemicals, St. Louis, MO) and extracted in chloroform:methanol (2:1, vol/vol). The chloroform phase was dried, resuspended again in chloroform and assayed using the enzymatic assay from Sigma Chemicals.

For the determinations of intracellular pools of free and esterified cholesterol, cells were incubated in the presence of [1-^{14}C]acetic acid (Amersham Pharmacia Biotech, Piscataway, NJ) *ad equilibrium* (for 3 days), then washed twice in PBS and extracted as above. The chloroform phase was dried, resuspended again in chloroform and applied, together with standards, to silica gel-G thin layer chromatography (TLC). Plates were developed in hexane:ethyl ether:acetic acid (87:20:1, vol/vol/vol) and visualized in iodine vapor. Spots were scraped and counted in a liquid scintillation counter (Puglielli et al., 1995).

To determine cholesterol distribution in the plasma membrane, cells were pre-incubated in the presence of radiolabeled acetate, as described above, and then subjected to cholesterol oxidase (Sigma Chemicals) immediately before extraction (Puglielli et al., 1995). Cholesterol oxidase oxidizes free cholesterol at the 3β-hydroxyl position to form 4-cholesten-3β-one (cholestenone), which can then be separated from free cholesterol based on the migration on a silica gel-G TLC. Cholesterol oxidase is not able to cross the plasma membrane in living cells and therefore only has access to the pool of cholesterol in the plasma membrane (Lange, 1992).

LIPOPROTEIN-DEFICIENT SERUM

Lipoprotein-deficient serum (LDS) was produced after elimination of total lipoproteins from fetal bovine serum (FBS) (Brown and Goldstein, 1974). Briefly, FBS was brought to a density of 1.25 g/ml with KBr and centrifuged in a Beckman Vti 65 rotor at 45 000 rpm for more than 8 h at 4–10 °C. Total lipoproteins (VLDL, IDL, LDL, and HDL) were discarded at the top of the tubes and LDS was then dialyzed against 15 l NaCl 0.15 M, EDTA 0.3 mM, pH 7.4, for 36 h at 4 °C with five changes. Total cholesterol levels in LDS dropped by more than 97% after lipoprotein elimination.

Aβ DETERMINATIONS

For Aβ determination, cells were grown in six-well tissue culture plates (Fisher Scientific Co, Agawam, MA). The day before the experiments, cells were incubated in 1 ml fresh medium. After 24 h, the medium was collected and $A\beta_{total}$ and $A\beta_{42}$ levels were assessed by a sandwich-ELISA assay (Aβ ELISA Core Facility, Center for Neurological Diseases, Harvard Institutes of Medicine, Boston, MA).

CELL VIABILITY

Cell viability was assessed using ethidium bromide coupled to calcein AM staining (LIVE/DEAD Viability/Cytotoxicity Kit, Molecular Probes, Eugene, OR).

RESULTS AND DISCUSSION

25RA cells have a mutation in the sterol regulatory element binding protein (SREBP) cleavage-activating protein (SCAP), the central regulator of cholesterol metabolism (reviewed in Brown and Goldstein, 1999). Consequently, these cells are resistant to downregulation of cholesterol synthesis by sterols and have an increased content of cell cholesterol (for review, see

Chang et al., 1997; Brown and Goldstein, 1999). We decided to use 25RA cells to assess the role of cholesterol compartmentation in the generation of Aβ. For this purpose, we first characterized cholesterol distribution and metabolism in both wild-type (WT) and 25RA (25RA) Chinese hamster ovary (CHO) cells and then stably transfected them with APP-V717I, a FAD-associated mutant form of APP (London mutation).

When compared to WT, 25RA cells had a ~six-fold increase in cholesteryl-esters (1310 ± 87 vs. 206 ± 32 mg/g protein) with comparable levels of free cholesterol (162 ± 11 vs. 252 ± 32 mg/g protein). Approximately 60% of free cholesterol was found in the plasma membrane (vs. 40% of WT cells). Intracellular membrane cholesterol was mostly associated to fractions containing Golgi markers in both WT and 25RA cells.

We next stably transfected WT and 25RA cells with APP-V717I and several clones were selected, based on similar levels of expression of APP-V717I (data not shown). Transfection with APP-V717I did not alter intracellular cholesterol distribution (data not shown). 25RA-APP-V717I cells showed increased levels of both $A\beta_{total}$ (~1.7-fold) and $A\beta_{42}$ (~2.6-fold) in the medium (Figure 42.1). The $A\beta_{42}/A\beta_{total}$ ratio also increased by ~1.5-fold. Stable transfection of the above cell lines with wild-type APP yielded similar results with regard to Aβ generation (manuscript in preparation). These results confirm data already present in the literature indicating a positive correlation between cellular cholesterol levels and Aβ generation (Racchi et al., 1997; Howland et al., 1998; Frears *et al.*, 1999; Mizuno *et al.*, 1998). In addition, these results also suggest that the pool of cholesteryl-esters, and not free cholesterol, may be the direct mediator of such correlation.

In order to analyze whether or not we could revert the increased production of Aβ, we next depleted 25RA cells of cholesterol. Cholesterol depletion was achieved by growing cells in lipoprotein-deficient serum (LDS)-containing medium. The use of LDS, instead of fast-acting cholesterol binding molecules, like cyclo-dextrins, is preferred since it is not associated with cell damage. After six passages in LDS-containing medium, 25RA cells showed an ~67% decrease in total cholesterol. Such an effect was determined by a marked decrease in cholesteryl-esters (Figure 42.2). Even if we observed a redistribution of free cholesterol from the plasma membrane to intracellular membranes, the overall levels of free cholesterol remained unchanged (Figure 42.2).

Surprisingly, 25RA-APP-V717I cells showed a decreased viability in response to cholesterol deprivation. Figure 42.3A shows the growth rate of 25RA-APP-wt and 25RA-APP-V717I grown in FBS- or LDS-containing medium. The growth rate of 25RA-APP-V717I cells reduced progressively from the first to the third week of cholesterol deprivation. This effect was not observed in 25RA-APP-wt or in untransfected 25RA cells, which remained viable for more than 70 days.

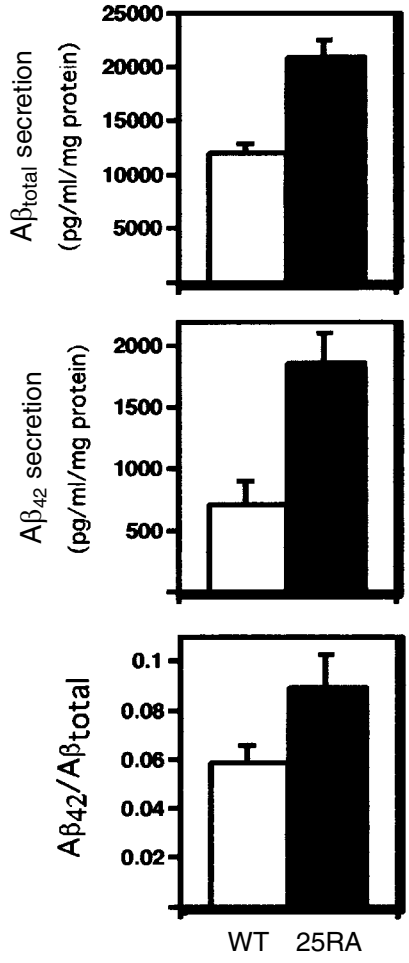

Figure 42.1. Aβ secretion is increased in 25RA as compared to wild-type CHO cells. WT and 25RA cell lines stably transfected with APP-V717I were grown as described in Materials and Methods. Aβ secretion in the medium was assessed by standard sandwich ELISA. Values are the mean ± SD of two separate experiments carried out on at least two different clones

Ethidium bromide (for dead cells) plus calcein-AM (for live cells) stainings showed that the reduction in growth rate observed in 25RA-APP-V717I cells was due to marked cellular death (Figure 42.3B). In this set of experiments, we also used AC29 cells stably transfected with APP-V717I. AC29 cells derive from 25RA cells and have the same defect in the SREBP pathway, which leads to increased cholesterol content. In addition, AC29 cells also have a mutated form of acyl CoA:cholesterol acyltransferase (ACAT), the

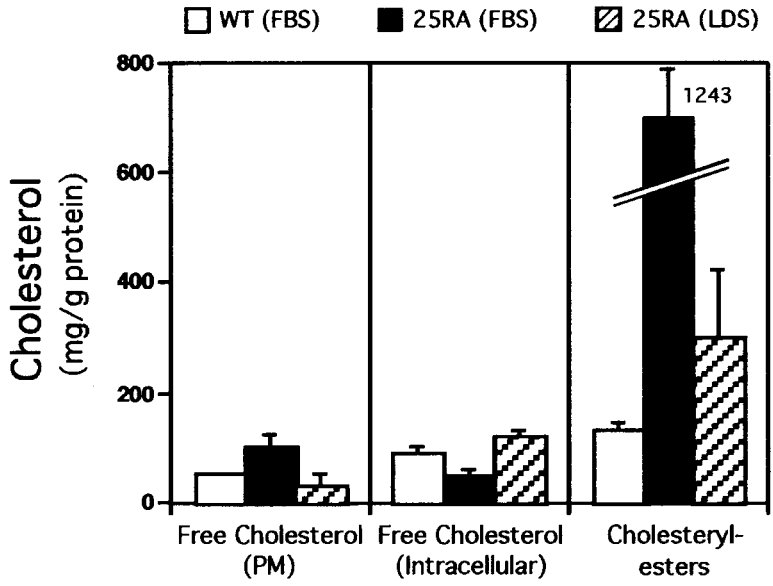

Figure 42.2. Cholesterol depletion reduces cholesteryl-ester levels in 25RA cells. 25RA cells were grown in FBS- or LDS-containing medium for 6 weeks. Cholesterol content and distribution were determined in 80–90% confluent cells. Values are the mean $\pm$ SD of at least three independent determinations. FBS, fetal bovine serum; LDS, lipoprotein-deficient serum; PM, plasma membrane; Intracellular, intracellular membranes

enzyme that controls cholesterol esterification (reviewed in Chang et al., 1997). Consequently, they have a ~six-fold increase in membrane cholesterol with undetectable levels of cholesteryl-esters. Only $<5\%$ of AC29-APP-V717I and untransfected 25RA cells died after 3 weeks in LDS-containing medium (Figure 42.3B). In contrast, $>90\%$ of 25RA-APP-V717I cells were not viable under the same conditions (Figure 42.3B). Taken together, these results suggest that depletion of the pool of cholesteryl-esters and not of membrane cholesterol potentiates the susceptibility to cell death in cells expressing FAD-associated mutant forms of APP.

One possible reason for cell toxicity associated with cholesterol mobilization from intracellular pools of cholesteryl-esters is the generation of cholesterol oxides. Cholesterol oxides have been shown to downregulate the universal caspase inhibitor FLIP, activate CPP-32 (caspase 3) and induce DNA fragmentation and chromatin condensation (Sata and Walsh, 1998; Yin et al., 1999). In macrophages, they originate after hydrolysis of cholesteryl-esters, through the action of not yet identified cholesterol oxidase(s) (Kellner-Weibel et al., 1999). Apoptosis is one of the possible mechanisms of cell death occurring in neurons of AD patients (for review, see Nijhawan et al., 2000). FAD-associated mutant forms of APP, PS1 and PS2 have been shown to

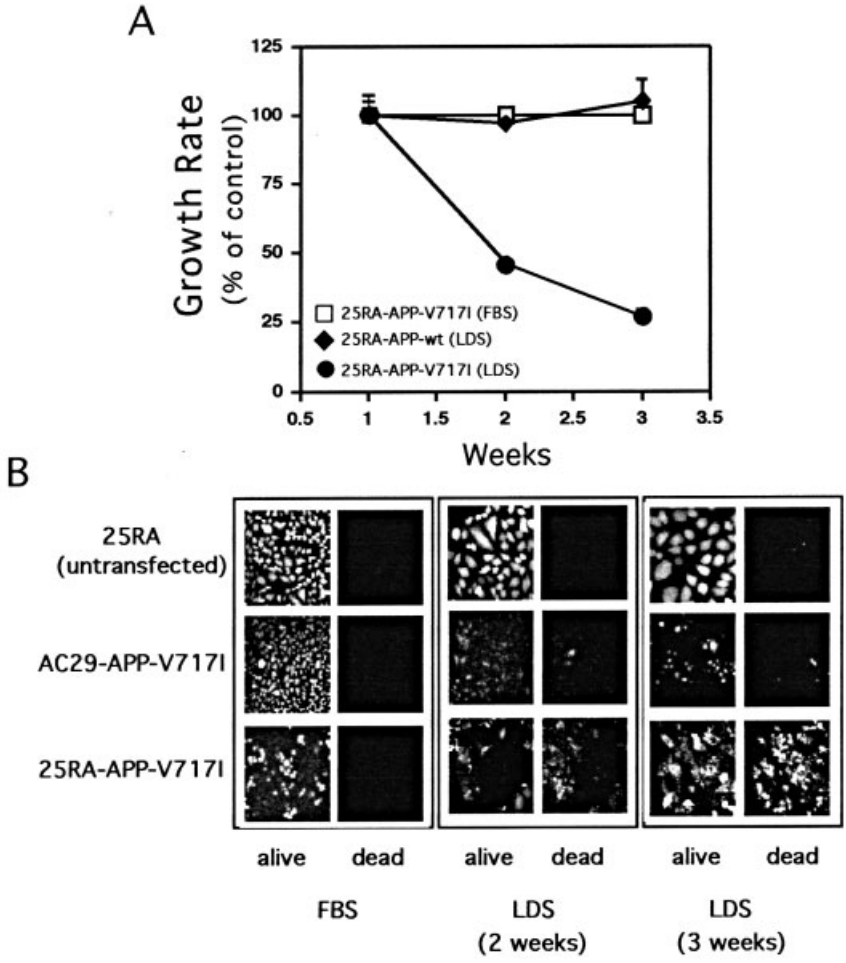

Figure 42.3. Cholesterol deprivation induces cell death in 25RA-APP-V717I cell lines. AC29 and 25RA cell lines stably transfected with APP-V717I were grown in the presence of FBS- or LDS-containing medium for the indicated periods of time. (A) Growth rate was expressed as percentage of 25RA-APP-V717I cells grown in FBS medium. Values are the mean $\pm$ SD of two different determinations from at least three different clones. (B) Cell death was assessed using calcein-AM (alive cells appear green) plus ethidium bromide (dead cells appear red) stainings

activate the programmed cell death pathway and to potentiate apoptotic stimuli (Kovacs et al., 1999; Thinakaran, 1999; Wolozin et al., 1996; reviewed in Nijhawan et al., 2000). However, the exact molecular event that activates apoptosis in AD still remains unknown. The above results suggest a possible link between FAD-associated mutant APP and cholesteryl-esters in the activation of cell death.

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43 Intracellular and Secreted $A\beta_{42/40}$ Ratios Are Differently Influenced by APP Mutations

HEIKE S. GRIMM, STEFAN F. LICHTENTHALER, KONRAD BEYREUTHER AND TOBIAS HARTMANN

INTRODUCTION

Amyloid-β peptide (Aβ), derived from the amyloid precursor protein (APP), plays a crucial role in the pathogenesis of Alzheimer's disease (AD) (Glenner and Wong, 1984; Masters et al., 1985; Kang et al., 1987). Processing of APP by β-secretase generates C99 (Higaki et al., 1995), which is further cleaved by γ-secretase, yielding two major species of Aβ, $A\beta_{40}$ and $A\beta_{42}$, differing in length of the amino acid chain. Aβ is normally secreted ($A\beta_{sec}$) (Haass et al., 1992; Seubert et al., 1992; Shoji et al., 1992; Busciglio et al., 1993); however, experimental evidence shows that significant levels of intracellular Aβ ($A\beta_i$) are produced, remaining inside the cells (Wertkin et al., 1993; Cook et al., 1997; Hartmann et al., 1997; Wild-Bode et al., 1997; Gouras et al., 2000).

In the present study, we investigated the intracellular metabolism of APP, using a truncated γ-secretase-specific Aβ precursor, C99 (Figure 43.1) (Lichtenthaler et al., 1999b). C99 is the natural substrate for γ-secretase (Higaki et al., 1995) and is therefore an excellent substrate for studying γ-secretase specificity and activity independently of β-secretase. Point mutations within the transmembrane domain of C99 close to the γ-cleavage site influence the cleavage specificity of γ-secretase activity, causing an altered product ratio of secreted $A\beta_{42/40}$ (Suzuki et al., 1994; Lichtenthaler et al., 1997, 1999a). Therefore, we have selected two point mutations close to the γ-cleavage site (Figure 43.1) that show a strong but opposite effect on the $A\beta_{42/40}$ ratio of $A\beta_{sec}$, and examined their effects on the formation of $A\beta_i$. Analysis of SH-SY5Y cells stably expressing these constructs led to the unexpected result, that the $A\beta_{42/40}$ ratios of $A\beta_i$ and $A\beta_{sec}$ were differently influenced by the introduced point mutations.

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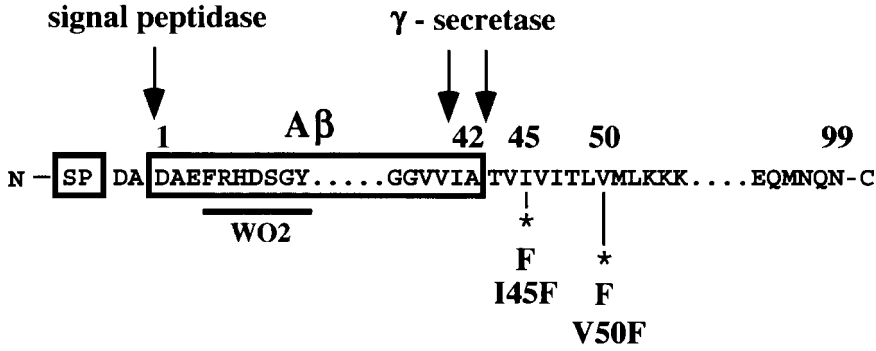

Figure 43.1. Schematic representation of SP-C99 and SP-C99 mutant proteins (I45F and V50F, respectively) used in the study of $A\beta_i$ generation. SP-C99 contains the signal peptide (SP) of APP, two additional amino acids (DA), the Aβ domain (boxed) and the complete C-terminus of APP. SP-C99 only requires γ-secretase activity for Aβ generation, since the N-terminus of Aβ is produced by the activity of signal peptidase instead of β-secretase (Dyrks et al., 1993). Protease cleavage sites are marked by black arrows. Asterisks indicate the position of amino acid exchanges in the SP-C99 mutant proteins. Black horizontal line indicates the epitope of monoclonal antibody WO2. Amino acids are shown in single letter code

EXPERIMENTAL PROCEDURES

CELL CULTURE AND TRANSFECTIONS

The neuroblastoma cell line SH-SY5Y was maintained in Dulbecco's modified Eagle's medium (high glucose; Sigma) containing 10% fetal calf serum (PAA) and 1% MEM non-essential amino acid solution (Sigma); 80% confluent cells were transfected with the expression vector pCEP4 (Invitrogen) alone, or the pCEP4 vector carrying the SP-C99 inserts using Lipofectin (Gibco BRL), as described by the manufacturer. Stable transfectants were selected using 300 μg/ml Hygromycin (Roche). For each construct, at least two independent cell lines were established.

ANTIBODIES

Monoclonal antibodies WO2 (for the precipitation of all Aβ peptides), G2-10 (specific for $A\beta_{40}$) and G2-11 (specific for $A\beta_{42}$) were used (Ida et al., 1996).

PREPARATION OF CELL LYSATES AND COLLECTION OF CONDITIONED MEDIA

Fresh culture medium (5 ml) was added to a confluent monolayer of cells in a 10 cm culture dish. Conditioned media were collected after 14–16 h. The

conditioned media were centrifuged at 4 °C for 1 min at 13 000 rpm and the supernatants were used for immunoprecipitation. In parallel with the conditioned media, cell lysates were prepared. Cells were harvested and lysed in lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 1% Triton X-100, 2 mM EDTA), supplemented with protease inhibitor cocktail (Roche). The 13 000 rpm supernatants were used for immunoprecipitation.

IMMUNOPRECIPITATION

Equal volumes of conditioned media and cell lysates were immunoprecipitated with 20 µl protein-G-Sepharose (Sigma) and the antibody WO2 (5 µg/ml) or the antibodies G2-10 (12.5 µg/ml) and G2-11 (17.3 µg/ml), respectively. The immunoprecipitated proteins were separated on 12% Tris–Tricine gels (Schagger and von Jagow, 1987) or commercial 10–20% Tricine gels (Novex). Western blot analysis was performed with the antibody WO2 (Ida et al., 1996).

To determine whether the same amount of $A\beta_i$ was generated from the WT and mutated C99 proteins, $A\beta_i$ and C99 immunoprecipitated from the cell lysates and detected in the Western blot were densitometrically quantified. The $A\beta_i$:C99 ratios were determined for each stably transfected cell line. The resulting $A\beta_i$:C99 ratios were divided by the corresponding $A\beta_i$-WT:C99–WT ratio of the same experiment. Thus, the value for the C99–WT protein was 1.0 and was set to 100%. To determine whether the same amount of $A\beta_{sec}$ was generated from the WT and mutated C99 proteins, the same calculation was used, except that $A\beta_{sec}$ was densitometrically quantified instead of $A\beta_i$. The $A\beta_{42/40}$ ratios of secreted and intracellular Aβ were determined by densitometrical quantification of $A\beta_{40}$ and $A\beta_{42}$ detected in the Western blot for each stably transfected cell line. The determined $A\beta_{42/40}$ values were divided by the corresponding $A\beta_{42/40}$ ratios obtained for C99–WT in the same experiment. Thus, the values for C99–WT were 1.0.

RESULTS

POINT MUTATIONS CLOSE TO THE γ-CLEAVAGE SITE DO NOT INFLUENCE THE TOTAL AMOUNT OF INTRACELLULAR AND SECRETED Aβ

In order to analyze whether point mutations close to the γ-cleavage site affect the total amount of $A\beta_i$ and $A\beta_{sec}$, SH-SY5Y cell lines expressing the SP-C99–WT or mutant constructs (SP-C99–I45F and SP-C99–V50F) were established. To determine the expression levels of SP-C99 proteins and the amount of $A\beta_i$ and $A\beta_{sec}$, respectively, cell lysates and conditioned media

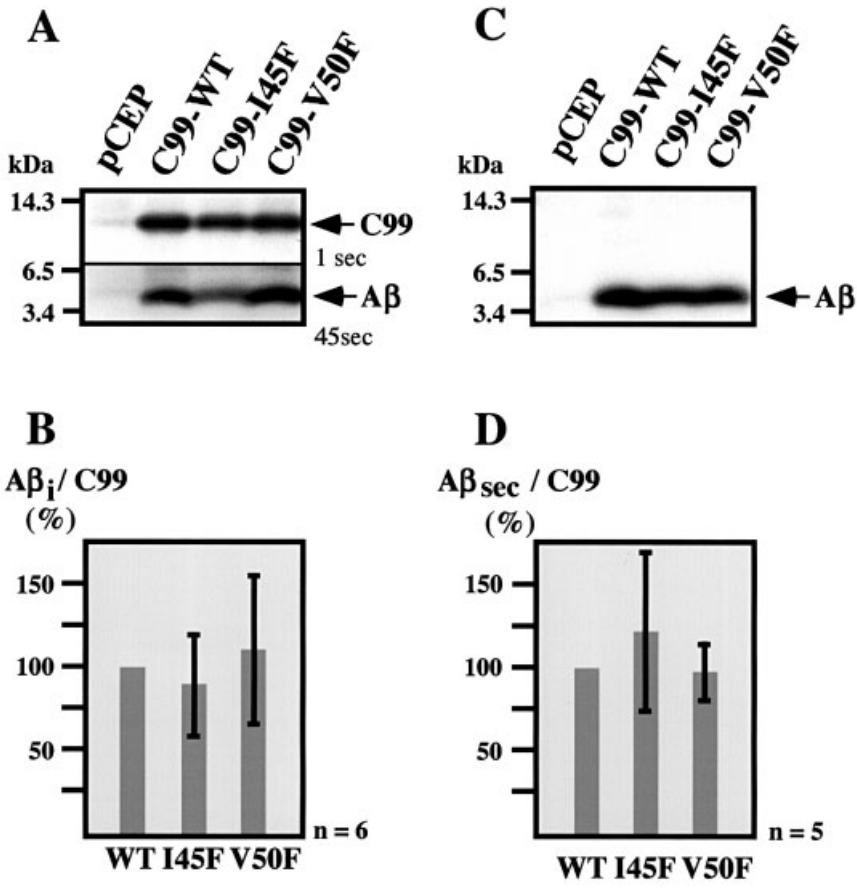

Figure 43.2. Western blot analysis of cell lysates and conditioned media from cells stably expressing the SP-C99 constructs (C99–WT, C99–I45F and C99–V50F). Control cells were transfected stably with the expression vector alone (pCEP). (A) Detection of C99 and $A\beta_i$ in cell lysates. C99 and $A\beta_i$ were immunoprecipitated from cell lysates using antibody WO2, followed by a Western blot analysis with antibody WO2. The figure represents different exposure times of the same Western blot analysis, as indicated. (B) Calculation of $A\beta_i$:C99 ratios normalized to the C99–WT construct. Grey bars represent the mean value $\pm$ SD of six independent experiments. (C) Western blot analysis of $A\beta_{sec}$ in conditioned media. $A\beta_{sec}$ was detected with the same assay described under (A). (D) Calculation of $A\beta_{sec}$:C99 ratios relative to C99–WT construct. For this, the same calculation and graphical presentation were used as in (B). Student's t-test relative to C99–WT showed no statistical significance for the quantifications presented under (B) and (D)

were analyzed by a highly sensitive immunoprecipitation/Western blot assay, using the monoclonal antibody WO2 (Figure 43.1). The expression levels of C99 wild-type and mutant proteins were very similar (Figure 43.2A). The intracellular Aβ levels did not appear to be affected, which was confirmed by

densitometrical quantification of the C99 and $A\beta_i$ bands from six independent experiments, measuring the ratio of $A\beta_i$ to C99 relative to the WT construct (Figure 43.2B). Similar to the generation of $A\beta_i$, neither mutation (I45F and V50F) dramatically influenced the production of $A\beta_{sec}$ compared with the C99–WT protein (Figure 43.2C and D).

INTRACELLULAR AND SECRETED $A\beta_{42/40}$ RATIOS ARE DIFFERENTLY INFLUENCED BY POINT MUTATIONS CLOSE TO THE γ-CLEAVAGE SITE

It has recently been shown that point mutations within the transmembrane domain of C99 influence the cleavage specificity of the γ-secretase activity by leading to an altered product ratio of secreted $A\beta_{42/40}$ ($A\beta_{sec\ 42/40}$) in stably transfected COS7-cells (Lichtenthaler et al., 1997, 1999a). To examine whether point mutations within the transmembrane domain also affect the production of intracellular Aβ species ($A\beta_{i\ 40}$ and $A\beta_{i\ 42}$), equal volumes of cell lysates and conditioned media of SH-SY5Y cells, stably expressing C99–WT and both C99–mutant proteins, respectively, were analyzed in an IP/Western blot assay (experimental details in Figure 43.3).

As expected, both mutations in the C99 proteins (I45F and V50F, respectively) have a clearly opposite effect on the generation of $A\beta_{sec}$ species ($A\beta_{sec\ 40}$ and $A\beta_{sec\ 42}$). The Aβ precursor C99–I45F is mainly processed to $A\beta_{sec\ 42}$, resulting in a dramatic increase of the $A\beta_{sec\ 42/40}$ ratio relative to the $A\beta_{sec\ 42/40}$ ratio of C99-WT-expressing SH-SY5Y cells (Figure 43.3A). In contrast, the V50F mutation results in a strongly reduced $A\beta_{sec\ 42}$ production, causing a decrease of the $A\beta_{sec\ 42/40}$ ratio compared with the $A\beta_{sec\ 42/40}$ ratio obtained for C99–WT protein (Figure 43.3A). These results confirm the data obtained with stably transfected COS7-cells (Lichtenthaler et al., 1999a). Surprisingly, in contrast to the dramatic effect of both point mutations on the $A\beta_{sec}$ species, both mutations have only a marginal influence on the $A\beta_{42/40}$ ratio of intracellularly produced Aβ compared with the C99–WT protein (Figure 43.3B). The C99–WT protein still produced slightly more $A\beta_{i\ 40}$ than $A\beta_{i\ 42}$, whereas nearly equal amounts of $A\beta_{i\ 40}$ and $A\beta_{i\ 42}$ could be detected in cells expressing the C99–I45F and C99–V50F proteins.

DISCUSSION

In the present study we investigated the intracellular metabolism of APP, using SP-C99 (Lichtenthaler et al., 1999b), a direct substrate for γ-secretase. γ-Secretase activity is influenced by different proteins, the presenilins (Borchelt et al., 1996; Scheuner et al., 1996; de Strooper et al., 1998) and the recently identified protein nicastrin (Yu et al., 2000), which form complexes with the APP C-terminal fragments C99 and C83 (α-cleaved

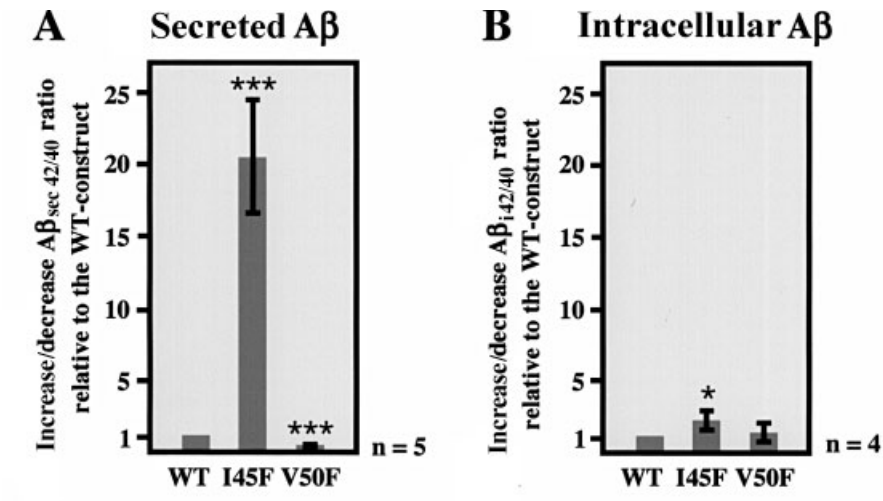

Figure 43.3. Analysis of $A\beta_{40}$ and $A\beta_{42}$ production in SH-SY5Y cells stably transfected with the SP-C99 WT (C99–WT) and SP-C99 mutant constructs (C99–I45F and C99–V50F). $A\beta_{40}$ and $A\beta_{42}$ were immunoprecipitated from conditioned media and cell lysates using antibody G2-10 (specific for $A\beta_{40}$) and G2-11 (specific for $A\beta_{42}$). Both Aβ species were detected by a Western blot using antibody WO2. (A, B) Determination of $A\beta_{sec\ 42/40}$ and $A\beta_{i\ 42/40}$ ratio relative to the $A\beta_{42/40}$ ratios produced by C99–WT protein. Grey columns represent the mean values of five ($A\beta_{sec}$) and four ($A\beta_i$) independent experiments; black vertical bars indicate SD. The asterisks indicate the statistical significance (Student's t-test) relative to C99–WT (*$p < 0.05$; ***$p < 0.001$). (A) Ratios of $A\beta_{sec\ 42/40}$ relative to the C99–WT construct. (B) Ratios of $A\beta_{i\ 42/40}$ relative to the C99–WT construct

C-terminal fragments) (Xia et al., 2000; Yu et al., 2000). Point mutations close to the γ-cleavage site of APP and C99, respectively, have been shown to influence the cleavage specificity of γ-secretase, by altering the ratio of Aβ species ($A\beta_{40}$ and $A\beta_{42}$) secreted in the medium (Suzuki et al., 1994; Lichtenthaler et al., 1997, 1999a). Work from our group and others have demonstrated that Aβ is secreted not only in the environment of the cell (Haass et al., 1992; Shoji et al., 1992; Busciglio et al., 1993), but that there also exists an intracellular pool of Aβ (Wertkin et al., 1993; Cook et al., 1997; Hartmann et al., 1997; Wild-Bode et al., 1997; Skovronsky et al., 1998; Gouras et al., 2000). Therefore, we addressed the question of whether γ-secretase, leading to the formation of $A\beta_i$ exerts the same specificity as γ-secretase leading to the generation of $A\beta_{sec}$. To answer this question, we selected two point mutations (SP-C99–I45F and SP-C99–V50F), known to have a dramatic but opposite effect on the Aβ species secreted in the conditioned medium of stably transfected COS7-cells (Lichtenthaler et al., 1997, 1999a), and analyzed their influence on the Aβ species produced intracellularly ($A\beta_{i\ 40}$ and $A\beta_{i\ 42}$). Stable expression of SP-C99–WT and the

mutant SP-C99 proteins (SP-C99–I45F and SP-C99–V50F, respectively) in SH-SY5Y cells showed that point mutations close to the γ-cleavage site have a dramatic effect on the $A\beta_{42/40}$ ratio of $A\beta_{sec}$, consistent with data obtained for stably transfected COS7-cells (Lichtenthaler et al., 1999a). However, in contrast to the drastic effect of point mutations close to the γ-cleavage site on the Aβ species secreted in the medium ($A\beta_{sec}$), neither mutation dramatically altered the $A\beta_{42/40}$ ratio of $A\beta_i$ compared with the C99–WT protein. From these results, we propose that the γ-secretase activity involved in the formation of $A\beta_i$ differs from the γ-secretase activity responsible for the generation of $A\beta_{sec}$. It remains to be seen whether totally different γ-secretases are involved in the generation of $A\beta_i$ and $A\beta_{sec}$ or whether a single γ-secretase forms complexes with different proteins, resulting in different γ-secretase complexes responsible for the production of $A\beta_i$ and $A\beta_{sec}$.

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44 Familial British Dementia

JORGE GHISO, TAMAS RÉVÉSZ, AGUEDA ROSTAGNO, RUBEN G. VIDAL, GORDON PLANT AND BLAS FRANGIONE

Familial British dementia (FBD) is an autosomal dominant form of congophilic amyloid angiopathy (CAA), clinically characterized by progressive dementia, spastic tetraparesis and cerebellar ataxia, with an age of onset in the fourth to fifth decade and full penetrance by age 60. A single extensive pedigree with the disease occurring in nine generations has been reported (Plant et al., 1990; Mead et al., 2000). The main duration of the disease is 9 years after the appearance of the neurological symptoms. Neuropathologic examination of autopsy cases has revealed a severe amyloid angiopathy of the brain and spinal cord, with perivascular amyloid formation, parenchymal amyloid plaques affecting hippocampus, cerebellum and, occasionally, cerebral cortex, extensive periventricular white matter changes compatible with leukoaraiosis, and neurofibrillary degeneration of hippocampal neurons (Plant et al., 1990; Révész et al., 1999; Holton et al., in press). In spite of the extensive amyloid deposition in the CNS vasculature, large intracerebral hemorrhage appears not to be a common feature of FBD. The disease was originally described by Worster-Drought et al. (1933) as a familial presenile dementia with spastic paralysis; due to the extensive cerebrovascular involvement, the disorder was later designated familial cerebral amyloid angiopathy, British type (Plant et al., 1990), and cerebrovascular amyloidosis, British type (Révész et al., 1999).

The biochemical nature of the amyloid fibrils extracted from leptomeningeal deposits in FBD was recently uncovered (Vidal et al., 1999). The amyloid subunit, named ABri, is a 34 amino acid peptide (pEASNCFAIRHFENKFAVETLICSRTVKKNIIEEN), with no sequence homology to any known amyloid protein, featuring a pyroglutamate N-terminus, a post-translational modification observed in other brain amyloids, i.e. peptide fragments derived from Alzheimer Aβ species (Mori et al., 1992; Saido et al., 1996; Tekirian et al., 1998). ABri is a degradation product of a 277 amino acid precursor molecule whose primary structure resembles a type II single-spanning transmembrane protein, codified by a single gene, *BRI2,* also known

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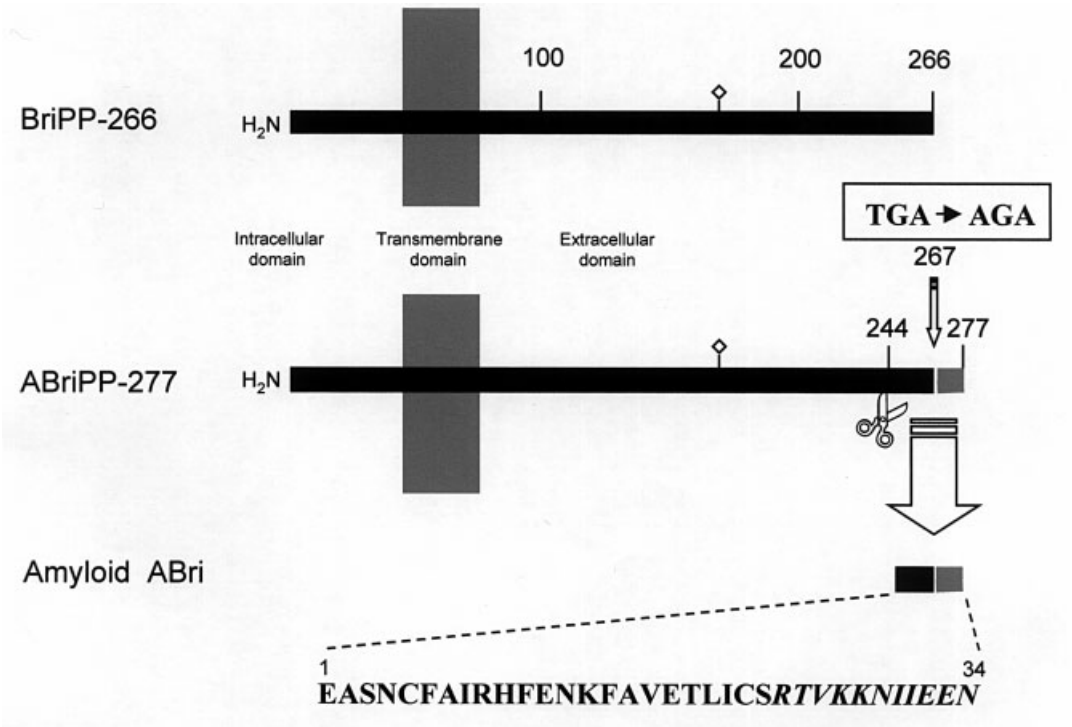

Figure 44.1. Schematic representation of the Bri Precursor Protein (BriPP-266), the genetic variant ABriPP-277 and the generation of the ABri peptide. Residues 52–74 comprise the putative transmembrane domain. A single N-glycosylation site is indicated at position 170. Peptide bond 243–244: degradation of BriPP-266 and ABriPP-277 by furin-like proteolytic processing. A single nucleotide substitution, T to A, is indicated at codon 267, causing the replacement of the normally occurring stop codon for arginine and the generation of a 277 amino acids precursor molecule (ABriPP-277)

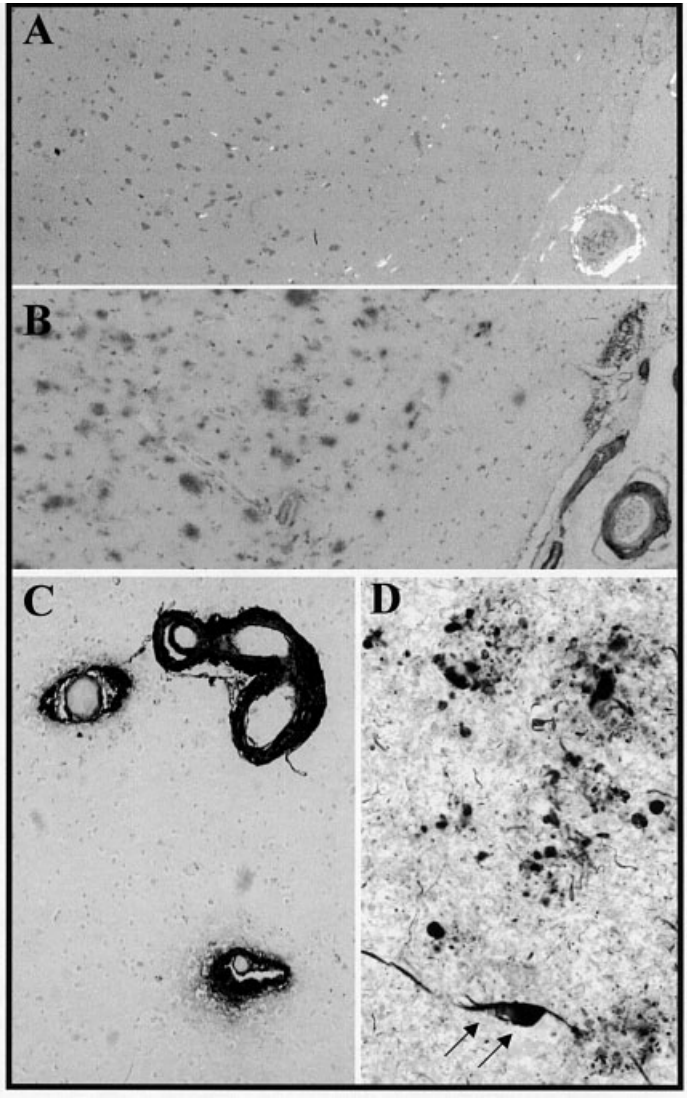

Figure 44.2. ABri lesions and NFT pathology in FBD. (A) Temporal cortex, stained with Congo red and visualized under polarized light. Vascular amyloid, as well as a few parenchymal deposits, are clearly visible. (B) Parallel section, stained with antibody 338 (specific for the C-terminal 10 amino acids of ABri), showing parenchymal immunoreactivity in Congo red-negative regions (panel A), characteristic of pre-amyloid lesions. (C) Perivascular amyloid plaques in the temporal cortex, stained with antibody 338. (D) Dystrophic neurites immunodetected by anti-phosphorylated tau antibody AT8 co-localize with amyloid plaques in the amygdala. A neuron showing immunoreactive intracellular NFTs is indicated by arrows. Magnification: (A, B) 630; (C) 670; (D) 6130

as *ITM2B* (Pittois et al., 1998), located on the long arm of chromosome 13. In patients with FBD, a single nucleotide substitution (**T**GA to **A**GA at codon 267) results in an arginine residue, replacing the normally occurring stop codon in the wild-type precursor molecule, and a longer open-reading frame of 277 amino acids (ABriPP277) instead of 266 (Figure 44.1). The ABri amyloid peptide is generated by furin-like proteolytic processing that releases the 34 C-terminal amino acids of the mutated precursor protein (Kim et al., 1999). Antibodies raised against the purified 4 kDa ABri amyloid, as well as against a synthetic peptide comprising the last 10 residues of the ABri sequence, specifically recognized the vascular and parenchymal amyloid deposits. These anti-ABri antibodies were unable to recognize other cerebral or systemic amyloid deposits, viz. sporadic cerebral amyloid angiopathy (CAA), sporadic Alzheimer's disease (AD), Down's syndrome, hereditary cerebral hemorrhage with amyloidosis (Dutch type), hereditary cerebral hemorrhage with amyloidosis (Icelandic type), Hungarian transthyretin cerebral amyloidosis, and systemic cases of light chain amyloidosis (kidney), light chain deposition disease (kidney) and amyloid A (heart). In FBD brains, the antibodies recognize amyloid and pre-amyloid deposits. As indicated in Figure 44.2, in many instances the immunoreactivities co-localize, with yellow-green birefringent material observed under polarized light after Congo red staining. However, diffuse plaques (Congo red-negative, immunoreactive with anti-ABri) are clearly seen throughout the neocortex and the limbic areas (Figure 44.2A,B). A large number of perivascular plaques (Figure 44.2C) are widely distributed throughout the CNS. Antibodies recognizing distinct hyperphosphorylated tau epitopes are able to detect dystrophic neurites co-localizing with hippocampal plaques. These antibodies also immunolabeled intraneuronal NFTs (Figure 44.2D).

In many aspects FBD resembles AD, the most common form of cerebral amyloidosis and the major cause of dementia in humans. As indicated in Table 44.1, both disorders exhibit vascular and parenchymal amyloid deposition, as well as neurofibrillary pathology. In AD, parenchymal Aβ plaques are mainly present in the hippocampus and the cerebral cortex, while Aβ vascular lesions are seen in cortical and leptomeningeal vessels. In FBD, amyloid plaques affect the hippocampus and occasionally the cerebral cortex; severe amyloid angiopathy with perivascular plaques is found throughout the CNS. Amyloid deposition in the cerebellum is also characteristic in FBD, where the cerebellar involvement is far more severe than in AD patients (Plant et al., 1990; Holton et al., in press). In AD, degenerating neurons containing NFTs, immunoreactive with a spectrum of different anti-phosphorylated tau antibodies, are frequent in limbic areas and in the neocortex and in many instances they co-localize with amyloid deposits (reviewed in Dickson, 1997). In FBD, the neurofibrillar pathology is abundant in the hippocampus, although scarcely found in the cortex. The predominantly limbic distribution of the tau pathology, together with the mild involvement of the temporal

Table 44.1. Neuropathological lesions in familial British dementia and Alzheimer's disease

	Plaques		Congophilic angiopathy	Cerebellar involvement	Neurofibrillary tangles
	Hippocampus	Neocortex			
FBD	Neuritic and non-neuritic, perivascular	Rare (diffuse and perivascular)	Severe (central nervous system and spinal cord)	Severe (vascular amyloid and perivascular plaques)	Limbic areas (rare in neocortex)
AD	Neuritic and non-neuritic	All types frequent	Variable (leptomeningeal and cortical)	Less severe (vascular amyloid and plaques)	Frequent in both limbic areas and neocortex

cortex, suggests that the NFT pathology in FBD corresponds to stage IV in the system recommended by Braak and Braak for AD (Braak et al., 1991; Révész et al., 1999; Holton et al., in press). The electrophoretic pattern of hyperphosphorylated tau isolated from FBD tissue is indistinguishable from that obtained from AD individuals (Hanger et al., 1991; Spillantini et al., 1998; Holton et al., in press).

In summary, the ABri molecule is structurally unrelated to all known amyloids, including those deposited in the brain; however, immunohistochemical and electron microscopical studies on FBD cases demonstrated that the cytoskeletal pathology in these patients is very similar to that seen in patients with AD. Thus, ABri and Aβ cerebral deposition can trigger similar neuropathological changes, leading to the same scenario: neuronal loss and dementia. The ABri data support the concept that amyloid deposition in the vascular wall and brain parenchyma is of primary importance in the initiation of neurodegeneration.

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45 Cellular Metabolism of Familial British Dementia-associated BRI-L

SEONG-HUN KIM AND SANGRAM S. SISODIA

INTRODUCTION

Familial British dementia (FBD), an autosomal dominant neurodegenerative disorder, is characterized by progressive spastic tetraparesis, cerebellar ataxia and dementia (Plant et al., 1990). The principal pathological hallmarks of FBD include the presence of non-neuritic plaques and amyloid angiopathy, hippocampal neurofibrillary tangles and ischemic white matter changes (Worster-Drought et al., 1940; Griffith et al., 1982; Plant et al., 1990). The first insight into the biochemical basis of this unusual disorder recently emerged when Vidal et al. (1999) described the purification of a peptide, termed ABri, that is the major component of highly insoluble amyloid fibrils in leptomeninges and parenchymal deposits from a patient with FBD. Comparison of the sequence obtained from tryptic peptides of ABri with the expressed sequence tag (EST) database revealed the presence of cDNAs encoding a larger, 266 amino acid ABri precursor protein, termed BRI. In fact, *BRI* cDNA is identical to a previously described cDNA encoding integral membrane 2B (ITM2B), a member of a family of proteins identified in a subtraction hybridization screen for markers of chondro-osteogenic differentiation (Deleersnijder et al., 1996; Pittois et al., 1998). Unexpectedly, one of the tryptic peptides generated from ABri contained a C-terminal arginine residue that was not predicted from the EST clones; the *BRI* open reading frame (ORF) typically terminated at this position. Subsequent analysis of *BRI* cDNA from affected individuals from the FBD pedigree revealed a T to A transversion (TGA–AGA) at the termination codon. As a result, an arginine codon was created and translation of the ORF extended for an additional 10 amino acids, terminating at an ochre codon; the extended ORF encodes a mutant BRI (BRI-L), of 277 amino acids. Indeed, the sequence of one ABri tryptic peptide matched the carboxyl-terminal 6 amino acids of the predicted

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BRI-L protein. Matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF-MS) of ABri purified from the leptomeningeal amyloid fraction revealed an M_r of 3935, suggesting that the peptide was generated following endoproteolytic processing of mutant *BRI* between arginine 243 and glutamic acid 244 (Vidal et al., 1999). Secondary structure algorithms, and the presence of a hydrophobic stretch between amino acids 52 and 76, lead to the prediction that BRI is a type II integral membrane protein that has a single transmembrane domain, and N-terminal orientates toward the cytoplasm and C terminus toward the lumen. A potential N-glycosylation site was also identified at asparagine 170.

Interestingly, a different genetic defect of the *BRI* gene was found in another rare neurodegenerative disease, familial Danish dementia (FDD; Vidal et al., 2000). FDD is characterized by progressive development of cataracts and hearing impairments as well as other neurological symptoms and dementia. The same group who had found the BRI mutation in FBD also identified that another 34 amino acid peptide termed 'ADan' was deposited in the FDD brain. Surprisingly, these ADan peptides originated from BRI precursor with a different mutation (BRI-D). In FDD patients, 10 nucleotides (TTTAATTTGT) just before the stop codon were found to be duplicated. This decamer duplication produces the loss of serine266 and a change in the reading frame generating another 11 amino acids longer precursor with different C-terminal sequences.

BRI IS A GLYCOSYLATED TYPE II MEMBRANE PROTEIN

To study the cellular processing of BRI, we generated BRI and FBD-associated mutant BRI-L constructs containing N terminal myc- and C terminal amyloid precursor-like protein 1 (APLP1) tag, which can be recognized by 9E10 and CT11 antibodies, respectively. Transfection of COS cells with these constructs results in the expression of $\sim$40 kDa full-length BRI and $\sim$42 kDa full-length BRI-L. Treatment with tunicamycin, which blocks N-glycosylation, resulted in increased mobility of these bands, indicating that BRI and BRI-L are N-glycosylated proteins as predicted. Next, we performed a proteinase-protection assay to examine the topology of BRI (Kim et al., 1999). Post-nuclear, microsomal membrane fractions were prepared from mouse neuroblastoma (N2a) cells transfected with BRI and digested with increasing amounts of proteinase K (PK). The resulting reactions were subsequently divided, fractionated by SDS–PAGE and subject to Western blot analysis with 9E10 or CT11 antibodies. $\sim$40 kDa full-length BRI was detected with either antibody and this molecule was resistant to digestion with 0.25 μg/ml PK. However, N-terminal, 9E10-immunoreactivity was markedly reduced at 2.5 μg/ml PK and almost completely abolished at

25 μg/ml PK. Coincident with loss of the N-terminal, 9E10-immunoreactivity, we detected a ~34 kDa polypeptide using the C-terminal CT11 antibody. These results indicated that the hydrophobic sequence encompassing residues 52 and 76 serves as a transmembrane domain and, in so doing, affords protection of the carboxyl-terminal portion located in the lumenal side of the membrane. Hence, we concluded that the chimeric BRI molecule adopts the topology of a type II integral membrane protein.

ENDOPROTEOLYTIC PROCESSING OF BRI IS MEDIATED BY FURIN

To examine the metabolism of BRI and BRI-L, we transiently transfected cDNAs encoding these molecules into mammalian cells, and subjected lysates of transfected cells to Western blot analysis using CT11 or 9E10 antibodies. As expected, the CT11 antibody detected ~40 kDa BRI and ~42 kDa BRI-L in lysates of transfected cells. On the other hand, the 9E10 antibody detected a doublet of ~40 kDa and ~37 kDa in lysates of cells expressing BRI and ~42 kDa and ~37 kDa species in lysates of cells expressing BRI-L (Kim et al., 1999). The accumulation of an N-terminal ~37 kDa species derived from either precursor protein strongly suggested that the full-length molecules are being subjected to the same C-terminal truncation event. In parallel, we detected CT11-reactive peptides of ~3 kDa and ~4 kDa in the conditioned medium of cells expressing BRI and BRI-L, respectively. To establish the identity of secreted peptides, we immunoprecipitated the peptides from conditioned medium of N2a cells with CT11 antibody, and subjected recovered material to MALDI-TOF-MS. These analyses revealed that N2a cells expressing BRI or BRI-L secrete discrete peptides of relative molecular mass 3557.7 and 4881.5, respectively, which correspond to the calculated mass of the C-terminal peptides cleaved between arginine 243 and glutamic acid 244.

The sequence immediately N-terminal to the BRI cleavage site, **Arg**-Gly-Ile-Gln-**Lys-Arg**, is highly reminiscent of the consensus recognition site for the subtilisin-like, proprotein convertase (PC), furin (Watanabe et al., 1992, 1993; Molloy et al., 1992, 1999; Takahashi et al., 1994; Nakayama, 1997). To examine the role of furin in BRI endoproteolysis, we transiently transfected cDNA encoding BRI or BRI-L into Chinese hamster ovary (CHO-K1) cells, or a CHO-K1 derivative, RPE.40, that is furin-deficient (Moehring et al., 1983, 1993; Spence et al., 1995). Transfected cells were biosynthetically labeled with [^{35}S]-cysteine for 3 h, and detergent lysates of cells and conditioned medium were subjected to immunoprecipitation with CT11 antibody. As expected, CT11 antibody detected ~40 kDa BRI and ~42 kDa BRI-L in lysates of transfected CHO-K1 cells. Parallel analysis of the conditioned medium of transfected CHO-K1 cells revealed the presence

of ~3 kDa BRI- and ~4 kDa BRI-L C-terminal peptides. However, we failed to detect CT11-precipitable peptide in the medium of RPE.40 cells expressing either BRI or BRI-L, suggesting that endoproteolysis of the precursor proteins required furin. To verify that expression of furin was both necessary and sufficient for processing BRI and BRI-L, we co-transfected *BRI* or *BRI-L* cDNA with cDNA encoding human furin into RPE.40 cells. The ~3 kDa and ~4 kDa CT11-precipitable peptides now appeared in the medium of RPE.40 cells co-expressing furin and BRI or BRI-L, respectively, results that confirm the view that furin mediates endoproteolytic processing of the precursors, resulting in the production of secreted C-terminal peptides. We then examined the kinetics of BRI processing by pulse-chase analysis. CHO or RPE.40 cells expressing BRI were pulse-labeled for 15 min, then chased for various time periods at 37 °C. In CHO cells, ~40 kDa full-length protein was gradually degraded during the chase; a small amount of ~3 kDa C-terminal peptide was detectable in the cell lysates after 15 min chase and secreted peptide appeared after 30 min chase. This result further supports the view that BRI is processed in late compartments of the secretory pathway, a finding consistent with a wealth of information that the preponderant steady-state distribution and activity of furin is within the trans-Golgi network (TGN). In contrast, RPE.40 cells failed to secrete these peptides at any time point examined. In addition, the full-length BRI molecule was much more stable in RPE40 cells than in CHO cells.

ENHANCED PROCESSING OF FBD-ASSOCIATED BRI-L BY FURIN

While it was apparent that BRI endoproteolysis was mediated by furin, it also became clear that the levels of secreted peptides in medium of CHO-K1 or RPE.40 cells that were derived from the BRI-L precursor were markedly elevated compared with peptides generated from the BRI precursor. In order to validate the intriguing observation that the BRI-L precursor was subjected to enhanced endoproteolysis, we quantified the levels of BRI- and BRI-L-derived peptides in the medium of N2a cells. N2a cells were transiently transfected with *BRI* or *BRI-L* cDNA, individually or in combination with *furin* cDNA. Triplicate plates of cells were transfected, biosynthetically labeled with [^{35}S]-cysteine for 3 h, and cellular or secreted BRI derivatives were immunoprecipitated with CT11 antibody. The levels of secreted peptides generated from the BRI-L precursor were consistently and significantly elevated compared to cells expressing BRI (Kim et al., 1999). These results suggested that the C-terminal extension present in the BRI-L molecule had a dominant effect on proteolysis at a scissile bond that, most likely, is mediated by furin.

Since it became clear that the C-terminal amino acid extension present in mutant BRI-L affects the efficiency of furin cleavage, we were worried about whether the C-terminal seven amino acids APLP1 tag in our constructs might confound the results. To resolve this issue, we generated non-tagged BRI and BRI-L constructs and transfected them into HEK293 cells. These non-tagged constructs were expressed as $\sim$37 kDa and $\sim$39 kDa bands, respectively, and, similar to the APLP1 tagged constructs, non-tagged BRI-L-derived ABri peptide accumulated to a higher level in the conditioned medium than the wild-type counterpart, confirming that the bona fide mutant BRI-L molecule is indeed subject to enhanced furin endoproteolysis.

EFFECTS OF α1-PDX ON BRI PROCESSING

We tested the effect of the bioengineered furin inhibitor, α1-antitrypsin Portland (α1-PDX), which contains a furin consensus motif in its reactive site loop (Ala^{355}–Ile–Pro–Met^{358}→Arg^{355}–Ile–Pro–Arg^{358}) (Jean et al., 1998; Benjannet et al., 1997). It is a potent and relatively specific inhibitor of furin ($IC_{50} = 0.6$ nM; Jean et al., 1998). We have generated stable HEK293 cell lines co-expressing BRI-L and pIND-α1-PDX that encode an ecdysone-inducible, FLAG-tagged α1-PDX. In this stable cell line, α1-PDX is readily inducible with increasing dose of muristerone A. With increasing level of expression of this bioengineered furin inhibitor, the level of the secreted ABri peptide is markedly reduced. Notably, full-length BRI-L is accumulated upon induction of α1-PDX. The similar inhibitory effect of α1-PDX was also observed for BRI. These results indicate that α1-PDX is able to inhibit the protease involved in the processing of BRI and BRI-L.

EFFECTS OF OTHER PROPROTEIN CONVERTASES ON BRI PROCESSING

Although α1-PDX is initially designed for a specific furin inhibitor, later reports indicate that it also inhibits the activity of other members of the PC family to a variable level (Benjannet et al., 1997; Jean et al., 1998). Eight members of the PC family are identified so far, including furin, PC2, PC1 or PC3, PC4, PACE4, PC6A, PC6B and LPC (for review, see Seidah and Chretien, 1999). Among these PCs, only furin, PC6B and LPC have a transmembrane domain at their C-terminus. PC1 and PC2 are mainly localized in endocrine tissues and expression of PC4 is restricted to the gonad tissues. Since the rest of the PCs, furin, PACE4, PC6A, PC6B and LPC, exhibit ubiquitous tissue distribution including brain, we decided to test the effect of those PCs on BRI processing. To examine the potential role of other members of the PC family in processing of BRI and BRI-L, we co-transfected

cDNAs encoding PACE4, PC6A, PC6B or LPC (PC7) with *BRI* or *BRI-L* cDNA into RPE.40 cells. Cells were labeled with [^{35}S]-cysteine for 2 h and intracellular and secreted BRI-derivatives were analyzed by immunoprecipitation with the CT11 or BRI-NT antibodies. The latter antibody, generated against the amino-terminal 52 amino acids of human BRI, confirmed the expression of BRI and BRI-L in transfected RPE.40 cells, while no secreted peptides were detected in the conditioned medium. However, the levels of secreted C-terminal peptide were abundant in the medium of cells co-expressing furin, but low levels of peptides were also found in the medium of cells that co-expressed PC6A and LPC. Phosphorimaging quantification revealed that PC6A and LPC process BRI at ~25% of the efficiency of furin. PACE4 fails to show any cleavage of BRI. Interestingly, LPC, like furin, also exhibits enhanced cleavage of BRI-L; more ABri peptide accumulated in the conditioned medium than wild-type peptide. In contrast, PC6A and PC6B cleave wild-type and mutant molecules with similar efficiency. These data support our earlier suggestion that the 11 amino acid extension in BRI-L may induce local structural alterations that differentially effect proteolytic processing by the PCs. Expression of each PC in the co-transfected cells was confirmed by immunoprecipitation analyses using the respective polyclonal antibodies.

CYS247 IS ESSENTIAL IN THE FURIN-MEDIATED ENDOPROTEOLYSIS

Since many bioactive peptides have an intramolecular disulfide bond, we explored the possibility that BRI-derived C-terminal peptide also has a disulfide bond. There are two cysteines at positions 5 and 22 of the peptides. Actually, the mass spectrometric analysis of conditioned medium from BRI-L expressing cells revealed that there are two proton unit differences between the calculated molecular weight of ABri and the detected molecular mass. In addition, treatment with Tris-(2-carboxyethyl)phosphine (TCEP), a strong reducing reagent, increases the two proton units of ABri peptide in MALDI-TOF-MS, suggesting the presence of a disulfide bond in the ABri peptides. To more fully define the role of the disulfide bond in the endoproteolytic processing of BRI, we mutated the first cysteine (Cys247) to serine and expressed the mutant protein. Surprisingly, secretion of the C-terminal peptide is completely abolished in both wild-type *BRI* and FBD-associated mutant *BRI-L* harboring the Cys247Ser mutation. These results suggest that the cysteine located three amino acids downstream of the scissile bond plays a critical role in the proteolytic processing of BRI, probably by forming an intramolecular disulfide bond.

ELECTRON MICROSCOPY OF SYNTHETIC ABri PEPTIDES

Earlier histological studies indicated that the amyloid deposits in FBD patients exhibit yellow-green birefringence under polarized light after staining with Congo red (Vidal et al., 1999). In order to examine the assembly of ABri, we synthesized 34 amino acid ABri peptide and incubated the peptide in PBS at room temperature. Electron microscopy analysis revealed the formation of fibrils with an average diameter of ~ 50 Å , though they were irregular and varied in the range 40–75 Å (Kim et al., 1999). The fibrils were tortuous and showed occasional branch points or crossing; these fibrils differed from the linear, unbranched 90 Å diameter fibrils generated by $A\beta_{1-40}$.

PROTEOLYTIC PROCESSING OF DANISH VARIANT OF BRI

Finally, we examined the proteolytic processing of recently identified Danish variant of BRI (BRI-D; Vidal et al., 2000). We generated APLP1-tagged BRI-D construct and transiently transfected into CHO-K1 or RPE.40 cells. Transfected cells were biosynthetically labeled with [^{35}S]-cysteine for 3 h, and detergent lysates of cells and conditioned medium were subjected to immunoprecipitation with CT11 antibody. As expected, the CT11 antibody detected ~ 42 kDa full-length BRI-D protein in lysates of both cell lines. Parallel analysis of the conditioned medium of transfected CHO-K1 cells revealed the presence of ~ 4 kDa ADan peptides. However, we failed to detect a CT11-precipitable peptide in the medium of RPE.40 cells expressing BRI-D. Again, when we co-transfected *BRI-D* with *furin* cDNA into RPE.40 cells, the ~ 4 kDa CT11-precipitable peptides were now detected in the conditioned medium of RPE.40 cells. These results indicated that furin mediates endoproteolytic processing of all the BRI variants (BRI, BRI-L and BRI-D), resulting in the secretion of C-terminal peptides.

Next we tested whether BRI-D was also subject to enhanced proteolytic processing by furin like BRI-L. HEK293 cells transiently transfected with *BRI*, *BRI-L* or *BRI-D* cDNA were labeled with [^{35}S]-cysteine for 2 h, and cellular or secreted BRI derivatives were immunoprecipitated with CT11 antibody. Surprisingly, in contrast to the BRI-L, the level of secreted C-terminal peptides in the conditioned medium of BRI-D transfected cells was much lower than that of its wild-type counterpart. Parallel analysis of cell lysates revealed that a much higher level of ADan peptides was present intracellularly. These results suggested that BRI-D is also subject to enhanced processing by furin; however, the resulting C-terminal peptides were not secreted efficiently to the conditioned medium but rather accumulated inside

the cells. The exact reason why these two amyloid peptides behave differently is not clear. However, it is possible that ADan peptide tends to aggregate more easily, preventing its secretion, while ABri peptide, which aggregates more slowly, is secreted into the medium. This hypothesis should be tested by structural analysis of synthetic ADan peptides.

CONCLUSION

Recent studies have revealed that two different mutations around the termination codon of the *BRI* gene are the underlying genetic defects in two unrelated, autosomal dominant neurodegenerative disorders, FBD and FDD (Vidal et al., 1999). The point mutation at the stop codon in FBD patients generates an 11 amino acids-longer ORF, BRI-L, the precursor of ABri peptide, while decamer duplication just before the stop codon produces an 11 amino acids-longer BRI-D, the precursor of ADan peptide (Vidal et al., 1999, 2000). ABri and ADan peptides, both of which consist of 34 amino acids, although the C-terminal amino acid sequences are different, accumulate in leptomeningeal and parenchymal deposits in the brains of FBD and FDD, respectively.

We have shown that BRI and BRI-L adopt the topology of type II integral membrane proteins, and that endoproteolytic cleavage of these proteins between Arg243 and Glu244 generates $\sim$3 kDa and $\sim$4 kDa peptides, respectively, which are released into the conditioned medium. In addition, using somatic cells deficient for furin and furin-complementation strategies, we have demonstrated that furin appears to be both necessary and sufficient for endoproteolytic processing of BRI, BRI-L and BRI-D, generating the secreted C-terminal peptides. α1-PDX, a potent furin inhibitor, could inhibit the proteolytic processing of BRI in a dose-dependent manner. Moreover, the levels of secreted peptides derived from the BRI-L precursor were markedly elevated over the peptides generated from the BRI precursor. Different members of PCs effect processing of BRI and BRI-L with different efficiency. Interestingly, BRI-D precursor was also subjected to the enhanced furin-mediated cleavage, but the resulting C-terminal peptides rather accumulated intracellularly. These results suggested that the C-terminal extension present in the mutant molecules introduces subtle conformational transitions within the C-terminal domain, providing a substrate with enhanced susceptibility to furin proteolysis. After cleavage, depending on the amino acid composition, the C-terminal peptides were either efficiently secreted into the medium (ABri) or accumulated in the intracellular compartments (ADan). This could partly explain why two similar peptides originating from the same precursor molecule, although with different types of mutation, lead to similar but distinct clinical and pathological phenotypes.

At present, little information is available pertaining to the cellular distribution or biological function(s) of BRI, or the secreted C-terminal peptide derivative, in brain. In this regard, it is notable that *BRI* cDNA is identical to a previously described cDNA encoding ITM2B, a member of a family of proteins identified in a subtraction hybridization screen for markers of chondro-osteogenic differentiation (Deleersnijder et al., 1996; Pittois et al., 1998). These ITM2 family proteins were upregulated during the differentiation of chondrocytes or T lymphocytes (Pittois et al., 1998, Kirchner et al., 1999), raising the possibility that these proteins are critically involved in the differentiation of specific cell types. Subtilisin-like PCs also play critical roles in regulating the turnover and processing of members of the transforming growth factor β (TGFβ) family of proteins, including TGFβ1, TGFβ2 and TGFβ3, bone morphogenetic proteins (BMP2-7), nodal activin A and DPP (Constam et al., 1999), molecules that are critical for developmental cell fate decisions. It is not inconceivable that BRI may also serve as the precursor of biologically active peptides that play important roles during normal brain development and aging.

Finally, mechanisms by which the role of ABri or ADan peptides initiate the clinical syndromes and pathophysiological cascades in patients are not known. To examine these issues, it will be critical to generate transgenic mice that overexpress human BRI-L or BRI-D in the nervous system. Patients with FBD have very unusual clinical features, including early motor deficits (spastic paraparesis) and cerebellar dysfunction (ataxia), followed by cognitive changes (dementia). Patients with FDD develop cataract first, followed by hearing impairments and cognitive deficits. The histopathological lesions are quite severe. We anticipate that these transgenic mice will recapitulate at least a subset of the histological lesions and behavioral alterations that typify FBD or FDD, information that will serve to clarify the processes that lead to neuronal dysfunction and death in this disorder.

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46 A Decamer Duplication in the *BRI* Gene Originates a *de novo* Amyloid Peptide that Causes Dementia in a Danish Kindred

RUBEN G, VIDAL, TAMAS RÉVÉSZ, AGUEDA ROSTAGNO, TOKE BEK, HANS BRAENDGAARD, GORDON PLANT, JORGE GHISO AND BLAS FRANGIONE

INTRODUCTION

Familial Danish dementia (FDD), formally known as heredopathia ophthalmo-oto-encephalica, was described in the early 1970s (Strömgrem et al., 1970) as a dominantly inherited disorder originating in the Djursland peninsula, north-east of the city of Aarhus, Denmark. The clinical presentation of the disease is cataracts and other ocular symptoms in early adult life, followed by hearing impairment around the age of 30. Neurological symptoms and dementia start after age 40 (Strömgrem et al., 1970; Strömgrem, 1981). Most patients die in their fifth or sixth decade of life.

At post-mortem examination, FDD patients have a uniform, diffuse atrophy of all parts of the brain. Histological examination shows a very severe, diffuse encephalopathy, especially in the cerebellum, cerebral cortex and white matter. The original description of Strömgrem (1970) indicated the presence of an accumulation of large quantities of cholesterol and cholesterol compounds in the tissue and, to a lesser degree, in glial cells, walls and lumena of the vessels. These findings suggested a disorder of cerebral cholesterol metabolism caused by an inborn enzymatic error. Material from three autopsied cases showed the presence of a widespread amyloid angiopathy in blood vessels of the cerebrum, including the choroid plexus, cerebellum, spinal cord and retina (Plant and Esiri, 1997). Neuritic plaques and sparse neurofibrillary tangles are present in the hippocampus.

Since FDD combines vascular amyloid deposition with argyrophilic plaques and neurofibrillary tangle formation, we decided to identify the

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amyloid protein present in the deposits and the genetic defect associated with dementia in this kindred.

MATERIALS AND METHODS

AMYLOID ISOLATION, AMINO ACID SEQUENCING AND MASS SPECTROMETRY ANALYSIS

Amyloid was isolated from leptomeningeal vessels of a patient affected with FDD, as described by Vidal et al. (2000). After extraction with 99% formic acid for 2 h at room temperature, the formic acid-soluble material was dried under a N_2 atmosphere and run on a 16% Tris–Tricine sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Gels were stained with 0.5% Coomassie blue R250 in 40% methanol–1% acetic acid. For amino terminal sequence analysis, proteins were electro-transferred onto polyvinylidene difluoride membranes (Immobilon-P, Millipore) using 10 mM 3-cycloexylamino-1-propanesulfonic acid (CAPS) buffer, pH 11, containing 10% methanol. The bands were subjected to N-terminal sequence analysis on a 477A protein sequencer with an on-line 120A PTH analyzer (Applied Biosystems). Mass spectrometry studies were done on total formic acid extracts at the Harvard Microchemistry Facility, Harvard University.

GENETIC ANALYSIS

Genomic DNA was amplified from samples of living FDD family members and autopsy brain tissue obtained post mortem as described (Vidal et al., 1999, 2000). Restriction enzyme analysis was carried out with XbaI (Gibco, BRL) according to the manufacturer's protocol. DNA sequence analysis was done by automated DNA-cycle sequencing (ABI) in both directions.

IMMUNOHISTOLOGICAL STUDIES

Paraffin sections of formalin-fixed tissues were deparaffinized as described (Vidal et al., 2000). Tissue sections were probed with polyclonal Ab 5282 (residues 22–34 of the amyloid sequence), 1:2000 and 6F/3D (Dako; 1:60). Tau immunohistochemistry was done using polyclonal Ab A024 (Dako; 1:200) or monoclonal Ab AT8 (Innogenetics; 1:50).

RESULTS

AMYLOID PURIFICATION AND AMINO ACID SEQUENCE ANALYSIS

The purified amyloid fraction (Danish amyloid or ADan) consisted mainly of a single peptide of ~4 kDa molecular weight. Direct amino acid sequence analysis retrieved the sequence SNXFAIRHFENKFAVE. This sequence

was identical to the sequence of the recently characterized amyloid, ABri (Vidal et al., 1999), starting at position three, indicating that both amyloids may be the same or closely related proteins. However, mass spectrometry analysis of the purified leptomeningeal ADan amyloid indicated the presence of peptides that did not match the mass of the ABri amyloid (Vidal et al., 2000). Moreover, polyclonal antibodies raised against the ABri peptide failed to recognize the amyloid lesions in FDD patients in immunohistochemical studies of paraffin sections of formalin-fixed brain tissue or in Western blot analysis using purified ADan protein. The MALDI-TOF mass spectrometry analysis of the purified leptomeningeal ADan amyloid indicated the presence of two prominent peaks of 3883.7 and 4046.4 mass units. Other important peaks corresponded to peptides with a mass of 3392.3; 3701.5 and 3864.2.

GENETIC STUDIES

Restriction enzyme analysis, using XbaI as described (Vidal et al., 1999), was negative for the presence of a XbaI restriction site between nucleotides 795 and 801 of the *BRI* gene*. PCR amplification of the *BRI* gene between nucleotides 727 and 868 (141 bp) showed the presence of an additional band of 151 bp (Figure 46.1). DNA sequence analysis of the *BRI* gene revealed the insertion (ins) of 10 nucleotides in the *BRI* sequence (795–796 ins TTTAATTTGT) originated by the duplication of nucleotides 786–795. The 10-nucleotide duplication was present in affected FDD family members (Vidal et al., 2000). All the patients tested so far are heterozygous, carrying one normal allele and one with the duplication. The 10 bp duplication-insertion was not observed in normal individuals, patients with unrelated neurologic disorders or individuals with familial British dementia.

IMMUNOHISTOCHEMICAL STUDIES

We observed Congo red-positive, parenchymal and vascular amyloid lesions as well as Congo red-negative deposits in the brain tissue of three FDD cases, using polyclonal antibody Ab 5282 (Figure 46.2), an antibody raised against residues 22–34 of the ADan amyloid protein sequence (Vidal et al., 2000). Extensive immunoreactive deposits were seen in the hippocampus, subiculum and enthorhinal cortex. In addition to vascular amyloid deposits, there were large loosely formed plaques, mostly Congo red-negative, in the CA4 subregion of the hippocampus, the inner molecular layer of the dentate gyrus and the enthorhinal cortex. Neocortical areas showed immuno-positive

*The sequence of the human *BRI* mRNA has been deposited in the GenBank database (Accession No. AF152462).

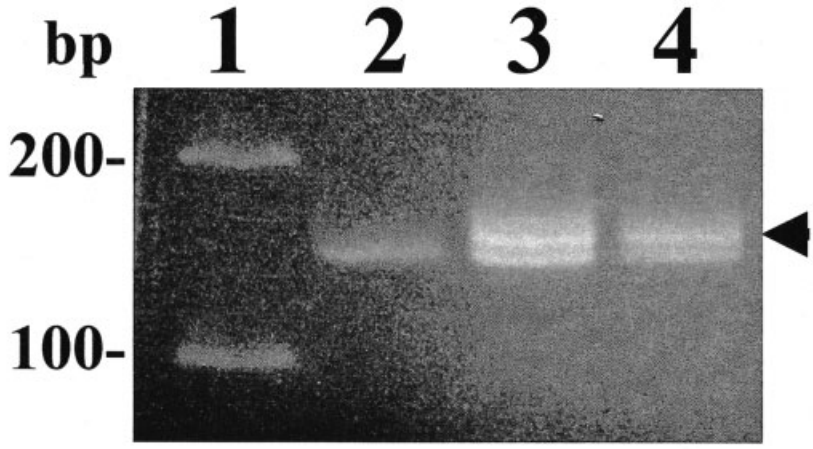

Figure 46.1. PCR amplification of the *BRI* gene. PCR amplified fragments were run on a 4% Metaphor agarose gel. Lane 1, BioLow molecular weight marker. Lane 2, PCR product of amplification of the *BRI* gene in a normal control (141 bp). Lanes 3 and 4, PCR amplification of the *BRI* gene in two patients with FDD. The extra band obtained as a result of the duplication-insertion in the *BRI* gene in the Danish kindred (151 bp) is indicated by an arrowhead

vascular amyloid and perivascular deposits. The cerebellum exhibited severe leptomeningeal and parenchymal amyloid angiopathy, as well as perivascular plaques. Immunohistochemistry using anti-Aβ antibodies showed variable co-localization with ADan-positive vascular amyloid, mainly in the form of perivascular deposits. Tau-immunoreactive structures were numerous in the hippocampus, where abnormal neurites, mostly around affected blood vessels, neurofibrillary tangles and neuropil threads were seen in large numbers. Although neocortical areas were less affected by these changes, neurofibrillary tangles, neuropil threads and an arrangement of abnormal, tau-positive neurites were seen around blood vessels with amyloid deposition.

DISCUSSION

We report the biochemical characterization of the amyloid subunit (ADan) of the leptomeningeal amyloid deposited in the brain tissue of FDD patients. We also identified the genetic defect associated with the development of FDD.

Amino acid sequence and mass spectrometry analysis indicate that the amyloid deposits in FDD are made of a 34 amino acid peptide with some degree of N- and C-terminal heterogeneity. In some instances, we observed by immunohistology the presence of Aβ immunoreactivity that co-localized with ADan-positive vascular amyloid, mainly in the form of perivascular deposits.

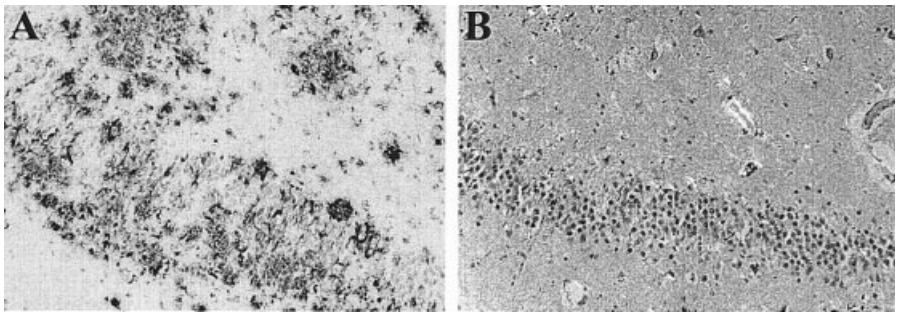

Figure 46.2. Immunohistochemical studies. Staining of the hippocampus with antibody 5282 (A) shows the presence of numerous amyloid plaques and looser deposits, which are mainly Congo red-negative (B)

Genetically, we established the presence of a 10-nucleotide duplication-insertion of the DNA sequence encoded between nucleotides 786–795 of the *BRI* gene sequence (795–796ins TTTAATTTGT). The genetic defect produces the loss of the C-terminal serine at codon 266, changing the reading frame of the BRI molecule, which now is extended at the C-terminus with the addition of 11 amino acids. The wild-type BRI protein has 266 amino acids, while the mutant precursor protein (ADanPP) associated with FDD has 277. The ADan peptide is originated by the proteolytic cleavage of ADanPP between amino acids 243 and 244 (Figure 46.3).

A different genetic defect in the *BRI* gene has been reported previously (Vidal et al., 1999) in patients affected with familial British dementia (FBD). A point mutation at codon 267 (T for A) in gene *BRI* (Vidal et al., 1999) changes the normal stop codon into an arginine residue. As a consequence, the BRI protein extends 11 amino acids into the C-terminus of the molecule, with the amyloid ABri protein being composed by the last 34 amino acids of the mutated precursor. FBD is characterized by progressive dementia, spastic paralysis and cerebellar ataxia, normally occurring in the fifth decade of life. Neuropathologically, these patients have a widespread amyloid angiopathy in the cerebrum, cerebellum and spinal cord, with the presence of mainly non-neuritic and neuritic amyloid plaques and neurofibrillary tangles in the hippocampus (Plant et al., 1990; Révész et al., 1999). The brain lesions in FDD and FBD are neuropathologically similar, although not identical (Plant and Esiri, 1997; Vidal et al., 1999, 2000). Both conditions present with severe amyloid angiopathy in the cerebrum and cerebellum, with extensive hippocampal plaques, some resembling the cotton-wool plaques of familial AD with PS1 Δ9 mutation (Crook et al., 1998), and neurofibrillary tangles.

Although amyloid deposits in FBD and FDD are not formed by Aβ, immunohistochemical and electron microscopical studies indicate that the

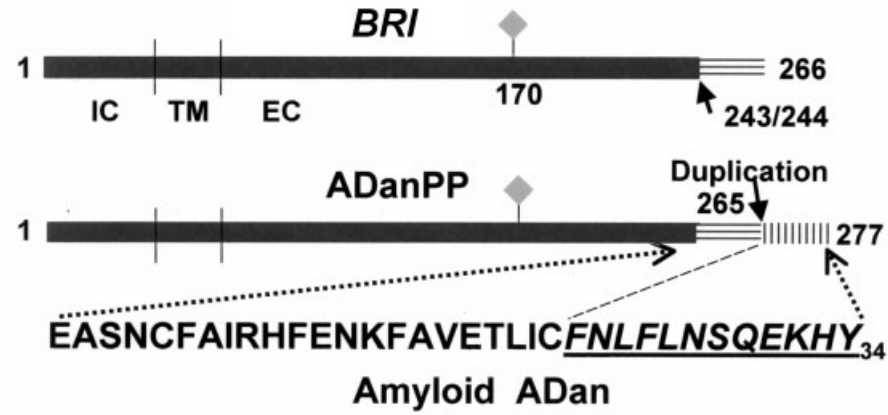

Figure 46.3. Schematic representation of the amyloid precursor protein in patients affected with FDD. The *BRI* gene codifies a putative single transmembrane-type II precursor protein of 266 amino acids (BRI) with a unique N-glycosylation site at asparagine 170. The 10-nucleotide duplication (TTTAATTTGT) in FDD, between codons 265 and 266, originates a mutated precursor protein of 277 amino acids (amyloid ADan precursor protein or ADanPP). Cleavage of the precursor molecules occurs between codons 243 (Arg) and 244 (Glu) in both BRI and ADanPP. As a consequence, the ADan peptide is released from the mutated ADanPP. The additional 12 amino acids are underlined. TM, transmembrane domain; IC, intracellular domain; EC, extracellular domain

cytoskeletal pathology present in FBD and FDD patients is very similar to that seen in patients with Alzheimer's disease, as well as in other neurodegenerative conditions, including prion disorders (Ghetti and Gambetti, 1999). We hypothesize that different amyloid peptides may be of primary importance in the initiation of similar neuropathological changes, leading to cell death and dementia. Further studies are needed to determine whether these diseases share a common neurotoxic mechanism that can be treated using similar therapeutic agents. The development of transgenic animals bearing the *BRI* genetic defects will provide alternative models in which to investigate the mechanisms involved in neurofibrillar degeneration, congophilic angiopathy and amyloid plaque formation in the brain.

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47 Familial Alzheimer's Disease-linked Mutant Presenilins Attenuate Capacitative Calcium Entry

ISAAC CHENG, ANDREW S. YOO, RUDOLPH E. TANZI AND TAE-WAN KIM

INTRODUCTION

Mutations in genes encoding the presenilins (PS1 and PS2) cause up to 50% of the early-onset cases of familial Alzheimer's disease (FAD). Recent studies have shown that both PS1 and PS2 play an essential role in the γ-secretase cleavage of amyloid β (Aβ) protein precursor (APP) and selected other integral membrane proteins, including Notch (reviewed in Vassar and Citron, 2000; Sisodia et al., 1999). Common molecular consequences of presenilin-linked FAD mutations include the increased generation of Aβ-protein x-42 ($A\beta_{42}$). In addition, FAD mutations in the presenilins have been shown to disrupt intracellular Ca^{2+} homeostasis. One of the most consistent effects of presenilin FAD mutations in Ca^{2+} signaling is the potentiation of IP_3-mediated release of Ca^{2+} from internal stores (Ito et al., 1994; Gibson et al., 1996; Guo et al., 1996; Etcheberrigaray et al., 1998; Leissring et al., 1999). However, the contribution of PS1 and PS2 on the intracellular Ca^{2+} signaling pathway has never been fully elucidated. Moreover, a molecular connection between this Ca^{2+}-related phenotype and other presenilin-associated FAD phenotypes, such as increased $A\beta_{42}$ generation, remains to be determined. We therefore studied the effects of FAD mutations on a common Ca^{2+}-regulatory pathway, known as store-operated or capacitative Ca^{2+} entry (CCE), which is triggered by an intracellular Ca^{2+} store depletion (Putney, 1986, 1999; Berridge et al., 2000).

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METHODS

The methods are described in detail in Yoo et al. (2000).

RESULTS

ATTENUATED CAPACITATIVE CALCIUM ENTRY (CCE) IN CELLS EXPRESSING FAD MUTANT PRESENILIN 1

To test whether the autosomal dominant presenilin FAD mutation affects the CCE, we studied the CCE response using SY5Y cells stably transfected with the M146L-PS1 FAD mutant. To induce CCE artificially, cells were incubated in Ca^{2+}-free media containing an ER Ca^{2+}-depleting reagent, cyclopiazonic acid (CPA), and were washed and replenished with Ca^{2+}-containing media. CCE was then monitored by ratiometric imaging using fura-2/AM. When CCE was induced, the amplitude of the CCE response was markedly reduced in the M146L-PS1 cells (~40% reduction) as compared to wild-type PS1- or vector-transfected cells (Figure 47.1). To verify that this

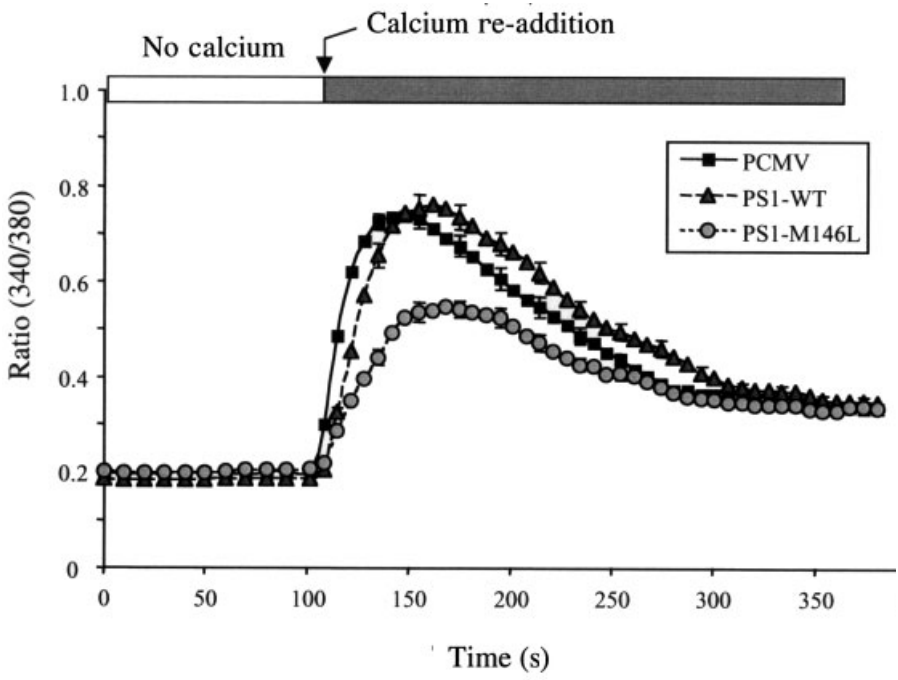

Figure 47.1. Effect of the M146L PS1 FAD mutation on CCE in stable SY5Y cell lines. CCE was measured by ratiometric imaging in fura-2-loaded SY5Y cells stably transfected with vector (PCMV), wild-type PS1 (PS1-WT) or mutant PS1 (PS1-M146L) (n = ~30)

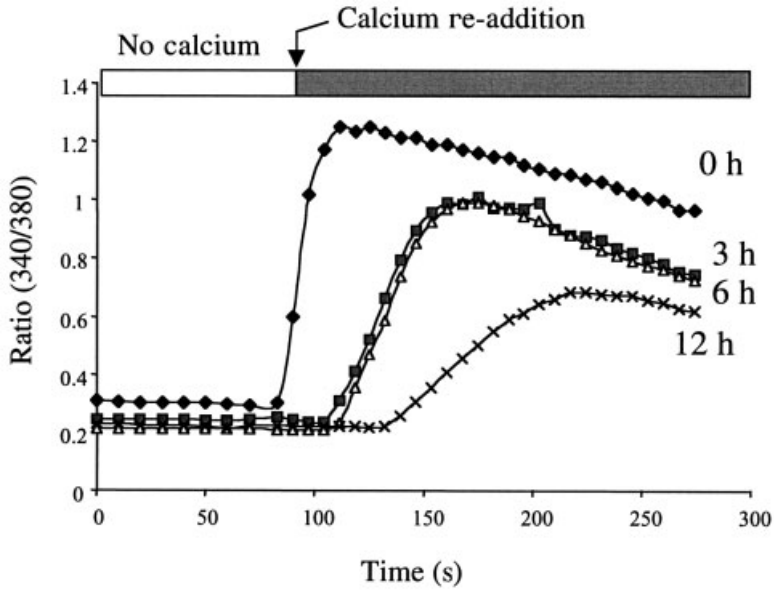

Figure 47.2. Effects of a CCE inhibitor SKF96365 on the CCE. CHO cells stably expressing wild-type PS1 were pretreated with 50 μM SKF96365 for the indicated times (0, 3, 6 and 12 h) and subjected to the Ca^{2+} imaging experiment

reduction of CCE response was not due to the elevated levels of PS1 protein in our cell lines, we also measured CCE in wild-type or mutant SY5Y cells, as well as in CHO cells with higher PS1 expression levels; we found that varying levels of PS1 protein had no detectable effect on the CCE response (data not shown). Our results showed that the M146L PS1 mutation causes a gain of function in overregulating CCE.

CCE INHIBITOR, SKF96365, ABOLISHES THE EFFECTS OF FAD MUTANT PRESENILIN 1 ON CCE

To further characterize Ca^{2+} influx pathway(s) affected by PS1 FAD mutations, we studied the effects of a CCE inhibitor, SKF96365. The Ca^{2+} influxes observed in wild-type PS1 cells (Figure 47.2) as well as M146L-PS1 FAD mutant cells (data not shown), were blocked by pretreatment with 50 μM SKF96365 in a time-dependent manner (Figure 47.2). However, the L-type Ca^{2+} channel antagonist, nifedipine, and N-type Ca^{2+} channel antagonist, w-conotoxin GVIA, had virtually no effect on Ca^{2+} influx (data not shown), suggesting that the mechanism underlying reduced CCE in mutant cells is independent of these types of voltage-operated Ca^{2+} channels.

Our data suggest that the alterations in intracellular Ca^{2+} concentration were likely CCE-specific.

DISCUSSION

Our studies imply that autosomal dominant FAD mutant presenilins exert a gain-of-function effect in downregulating CCE while increasing IP3-mediated release from the ER store, leading to reduced lumenal Ca^{2+} concentration (Waldron et al., 1997; Hofer et al., 1998). It is interesting to note that reduced CCE leads to diminished luminal Ca^{2+} concentrations in Bcl-2-expressing cells or cells grown in a low Ca^{2+} environment (Pinton et al., 2000). CCE involves direct physical interaction between the ER and plasma membrane constituents. According to this conformational coupling mechanism, a conformational change of the IP_3 receptor upon agonist stimulation and subsequent release of Ca^{2+} leads to the formation of a molecular complex containing IP_3 receptor bound to molecular constituents in the plasma membrane harboring CCE channels. This then allows extracellular Ca^{2+} to replenish the ER store. Given a published role for the presenilins in γ-secretase cleavage of APP, the presenilins may regulate or directly mediate the cleavage of protein(s) involved in modulating CCE. Thus, a gain in the biological activity of the presenilins, owing to autosomal dominant FAD mutations, may attenuate CCE while increasing γ-secretase activity. Further experimentation will be necessary to elucidate this connection at the molecular level.

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48 Presenilin-1 is a Regulatory Component of the Cadherin Cell Adhesion Complex: Implications for Alzheimer's Disease*

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INTRODUCTION

Presenilin-1 (PS1) is a polytopic transmembrane protein expressed in many tissues, including embryonic and adult brain, where it is enriched in neurons (Berezovska et al., 1997; Elder et al., 1996; Sherrington et al., 1995). PS1 mutations are responsible for most cases of early-onset autosomal dominant familial Alzheimer's disease (FAD). Structural studies suggest that PS1 crosses the membrane eight times, with the N-terminus, the C-terminus and the large hydrophilic loop all located in the cytoplasm. Most cellular PS1 is cleaved within the large cytoplasmic loop to yield N-terminal fragments (PS1/NTF) of approximately 30 kDa and C-terminal fragments (PS1/CTF) of approximately 20 kDa. Following cleavage of the full-length protein, the PS1 fragments form a stable 1:1 heterodimer (Podlisny et al., 1997; Thinakaran et al., 1996). PS1 facilitates processing of Notch-1 receptor and amyloid precursor protein (APP), stimulates production of Aβ peptide (De Strooper et al., 1998, 1999) and may play a role in protein trafficking (Efthimiopoulos et al., 1998; Naruse et al., 1998), neuroprotection (Giannakopoulos et al., 1997; Shen et al., 1997), and chromosome segregation (Li et al., 1997). Mice lacking PS1 die shortly after birth with skeletal malformations, impaired neurogenesis and brain hemorrhage (Shen et al., 1997; Wong et al., 1997). Theories proposed to explain the mechanism by which PS1 mutations induce FAD include increased

*Dedicated to the memory of Henryk M. Wisniewski.

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production of Aβ, destabilization of β-catenin, inhibition of PS1 proteolysis, and increased apoptosis (Selkoe, 1999).

Recently, we showed that PS1 concentrates at synaptic and epithelial cell–cell contact sites, where it forms complexes with components of the cadherin/catenin adhesion system, including E-cadherin, β-catenin and α-catenin (Georgakopoulos et al., 1999). Classic cadherins, a superfamily of single-pass transmembrane glycoproteins that includes E (epithelial)- and N (neural)-cadherin, mediate Ca^{2+}-dependent cell–cell adhesion and recognition and control critical cellular events in development, neurogenesis and tissue homeostasis (Yagi et al., 2000; Gumbiner et al., 2000). These functions are mediated by the extracellular domain of cadherins, which effects homophilic interactions between cadherins on opposing cell surfaces. The membrane-distal cytoplasmic sequence of cadherins binds either β-catenin or γ-catenin (plakoglobin), which in turn binds α-catenin. β- and γ-Catenin are two highly homologous members of the armadillo family of proteins (Ben-Ze'ev et al., 1998) and bind the same sequence of cytoplasmic cadherin in a mutually exclusive manner (Aberle et al., 1994; Jou et al., 1995). α-Catenin binds polymerized actin either directly or indirectly through other proteins, thus linking the cadherin/catenin adhesion complex to the cortical cytoskeleton (Figure 48.1) (for review, see Yap et al., 1997). Homophilic interactions of the extracellular sequence of specific cadherins with same-class cadherins on the surface of neighboring cells regulate cell–cell adhesion and communication in tissues and organs. In addition, cell surface cadherins mediate the intracellular transduction of extracellular signals (Jou et al., 1995). Cytoskeletal linkage of the cadherin/catenin system is required for the realization of the full adhesive and signal transduction functions of surface cadherins (Yap et al., 1997; Nagafuchi et al., 1989).

RESULTS AND DISCUSSION

We used confocal and immunoelectron microscopy to show that in confluent epithelial cell cultures PS1 is localized at cell–cell adhesion sites in close association with E-cadherin, β-catenin and α-catenin (Georgakopoulos et al., 1999), all of which are components of the cadherin-based cell–cell adhesion system (Yap et al., 1997). In cells not forming cell–cell contacts, PS1 was mostly found in the ER/Golgi network. Immunogold electron microscopy also localized PS1 at intercellular junctions along the lateral plasma membrane. That PS1 concentrates at cell–cell contact sites in close association with E-cadherin and catenins suggests that PS1 may be a part of the cadherin/catenin adhesion system. Indeed, we found PS1 fragments in detergent-stable complexes with E-cadherin, β-catenin and α-catenin. Furthermore, quantitative immunoprecipitations and velocity gradient centrifugation revealed single complexes containing both PS1 fragments, E-cadherin, β-catenin and α-catenin.

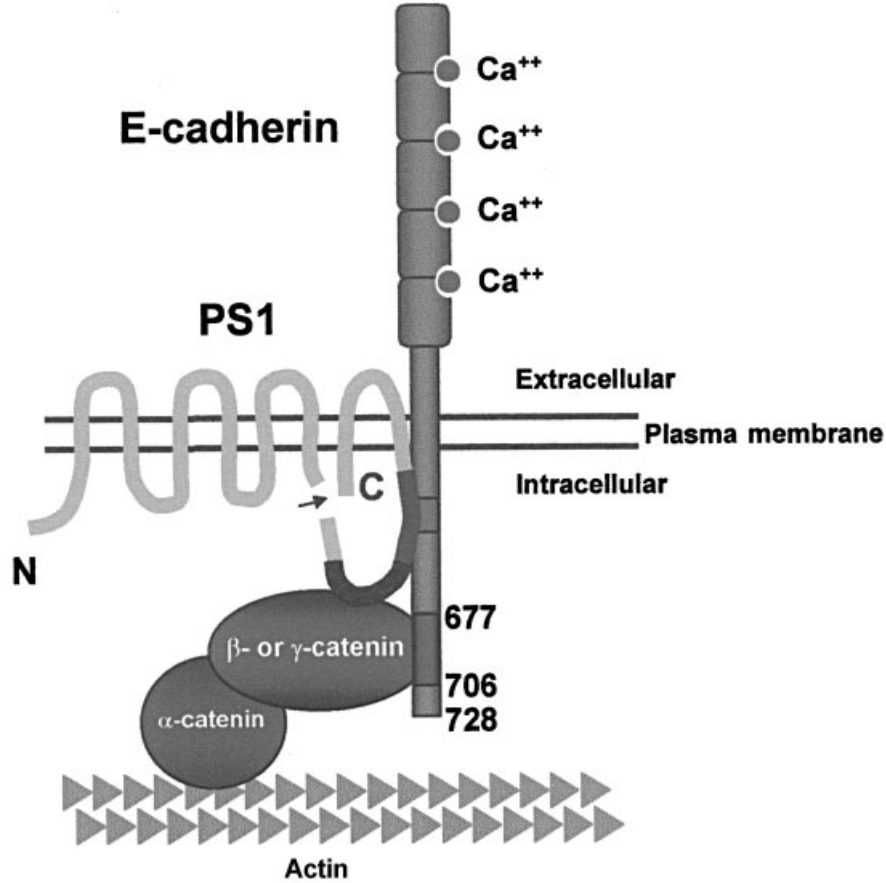

Figure 48.1. Schematic representation of interactions between E-cadherin, β- or γ-catenin and PS1. β- or γ-Catenin binds E-cadherin at amino acids 677–706. PS1 C-terminal fragment binds β-catenin and E-cadherin closer to the membrane. For more details, see text

Thus, detection of complexes containing PS1 and the basic structural components of the cadherin/catenin cell adhesion system indicates that PS1 is a component of this system. In support of this conclusion, we found that PS1 is recruited to Ca^{2+}-induced cell–cell contact sites, where PS1 fragments form complexes with cell surface E-cadherin. Interestingly, removal of extracellular Ca^{2+} resulted in a specific decrease of the cellular PS1/E-cadherin complexes. These data suggest the presence of signal transduction mechanisms that regulate the stability of PS1/cadherin complexes in response to changes in extracellular Ca^{2+}. *In vivo* experiments showed that in epithelial tissue PS1 concentrates at cell–cell contact sites, suggesting a PS1 function in cell–cell adhesion. In support of this suggestion, overexpression of PS1 stimulated

cell–cell aggregation (Georgakopoulos et al., 1999). We obtained evidence that PS1 stimulates cell–cell adhesion/communication by stabilizing the binding of β- and γ-catenins to E-cadherin and by increasing linkage of E-cadherin to the actin cytoskeleton (Baki et al., 2001). Interestingly, FAD mutant ΔE9 failed to stabilize the cadherin/catenin complex and did not stimulate cell–cell aggregation (Baki et al., 2001). These findings indicate that PS1 is an important regulator of the cadherin-related cell adhesion and signaling systems and suggest possible mechanisms by which mutations of PS1 might act to cause FAD.

In brain, we detected PS1 in complexes with E-cadherin, N-cadherin and β-catenin, suggesting that PS1 is an integral component of the brain cadherin/catenin complex. Furthermore, electron microscopy showed PS1 at synaptic junctions, where E-and N-cadherins are also localized (Georgakopoulos et al., 1999) and confocal microscopy showed that in primary neuronal cultures PS1 co-localizes with synaptophysin (Figure 48.2). Together, our results indicate that PS1 is a component of the synaptic cadherin/catenin adhesion complex and suggest that PS1 has a synaptic function. Synapses are specialized contact sites between neurons in the brain. These contacts are vital for the interneuronal communication required for the processing, integration, storage and retrieval of information. Synapses consist of nerve terminals in the pre-synaptic neuron that release chemical messengers that activate receptors in the post-synaptic neuron. Efficient function of the synapse and the synaptic signal transduction mechanisms are critical for proper functioning and information processing in the CNS. That PS1 is a component of the synaptic cadherin/catenin complex makes that complex a potential target for PS1 FAD mutations, as they could affect any of the steps required for the interaction of PS1 with other components of the complex. Such perturbations of protein–protein interactions within multimeric complexes is a mechanism for dominant 'gain of aberrant function or loss of function' effects of disease-associated mutations (Kaushal et al., 1994). Furthermore, the incorporation of a defective (mutant) PS1 within the synaptic structure may influence the transmission of signals from the pre- to post-synaptic terminals, or may otherwise interfere with synaptic plasticity and function. In this context, it is interesting to note that among the neuropathological phenotypes of AD, synapse abnormalities show the best correlation with the degree of dementia (Terry et al., 1994). In addition, synapses also contain APP (Simons et al., 1995) and, although a function for APP has not yet been clearly established, several lines of evidence suggest that APP may actually promote cell adhesion (Williamson et al., 1996; Wu et al., 1997). The synaptic co-localization of PS1 and APP makes this brain structure a candidate locus for the interaction of these two proteins, an interaction that may be of critical importance for the development of AD. This is particularly important in the face of recent evidence that PS1 plays a role in the processing of APP and production of Aβ peptide (De Strooper et al., 1998; Wolfe et al., 1999).

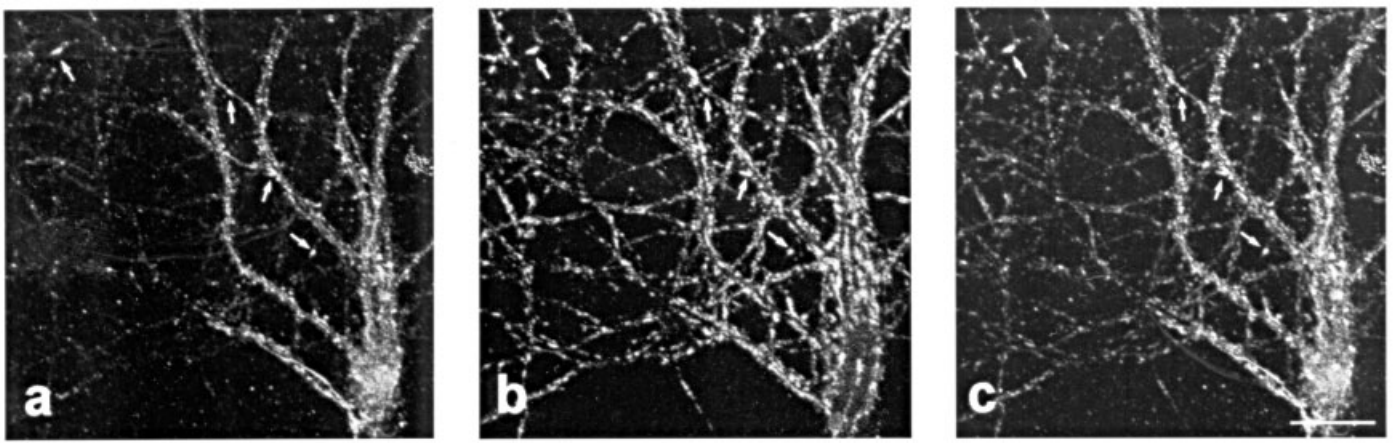

Figure 48.2. Co-localization of PS1 with synaptophysin in primary hippocampal neurons. 4–5 Week-old rat hippocampal neurons were fixed in methanol and stained with affinity-purified anti-PS1/NTF polyclonal antibody (Ab222) (a) and anti-synaptophysin monoclonal antibody (b). (c) Shows superimposed images of (a) and (b), showing the co-localization of PS1 with synaptophysin (arrows). Scale bar = 20 μm

Cadherins are cell adhesion receptors whose complete adhesive and signal transduction functions require their association with the actin cytoskeleton via catenins (Gumbiner, 2000). Since cytoskeletal association of the cadherin/catenin complex is a key event for the full expression of the cadherin functions (Yap et al., 1997; Nagafuchi et al., 1989), it is likely that PS1 binding to E-cadherin has important functional consequences, including the modulation of cadherin-based cell adhesion and signal transduction. This hypothesis is strongly supported by our finding that PS1+/+ fibroblasts display increased Ca^{2+}- and cadherin-dependent cell aggregation compared to PS1−/− fibroblasts, and that reintroduction of PS1 into PS1−/− cells stimulates cell aggregation (Georgakopoulos et al., 1999; Baki et al., 2001). In summary, our data indicate that PS1 functions as a regulatory component of the cadherin-based cell–cell adhesion system.

Cadherins and associated catenins are expressed in highly dynamic and specific patterns throughout embryonic development and the adult life of the vertebrate central nervous system (Redies et al., 1996). At least 15 different classic cadherins have been detected in various developmental stages of the CNS. The cadherin/catenin adhesion system is found in CNS neurons and in synaptic contacts, where it functions in neuronal recognition and in synaptic structure and function (Tanaka et al., 2000; Tang et al., 1998; Suzuki et al., 1997). In early development, various cadherins are regionally expressed in the brain, thus defining specific neuromeric subdivisions. This expression pattern suggests that cadherin-mediated adhesion plays a role in establishing the cyto-architecture of the CNS. Later in development, cadherins are expressed in the elongating neurites and may be involved in target recognition and synapse formation, as the targets of cadherin-expressing neurites usually express the same type of cadherin. Several cadherins, including N- and R (retinal)-cadherin, have been shown to promote neurite outgrowth by functioning as homophilic guidance molecules for the navigation of neuronal processes (for review, see Redies et al., 1996). It is now clear that cadherins are crucial for cell–cell adhesion during embryogenesis (Kintner, 1992), cell motility (Chen et al., 1997) and regulation of paracellular permeability of endothelial cell junctions in blood vessels (Navarro et al., 1998). That PS1 is important for CNS development is verified by PS1 null mice, which exhibit several abnormalities of CNS development and impaired CNS vascular integrity (Shen et al., 1997; Wong et al., 1997). The lethal phenotype of these mice may be due to the malfunction of the cadherin-based cell–cell adhesion system caused by the absence of PS1. Thus, PS1 seems to regulate the function of multiple cadherin adhesion systems, including the neuronal and vascular systems, both of which are affected in AD.

That PS1 binds the cadherin/catenin adhesion complex and regulates its stability suggests an interesting and potentially important connection between this protein and cancer. Free cytosolic β-catenin has been recognized as an important member of the Wnt signal transduction pathway and as an

oncogene (for review, see Ben-Ze'ev et al., 1998). The Wnt pathway involves nuclear translocation of cytosolic β-catenin and activation of gene expression. Increased levels of cytosolic β-catenin and enhanced transcriptional activity of the Wnt pathway have been linked to the development of colon cancers (Morin et al., 1997). Adhesive interactions that sequester β-catenin to intercellular junctions reduce the free levels of this protein, thus antagonizing its signal transduction activities (Ben-Ze'ev et al., 1998). By stabilizing the cadherin/β-catenin complex and favoring adhesive interactions, PS1 could limit the cytosolic pool of free β-catenin and thus modulate its signal transduction and oncogenic activities. Malignant tumors are, in part, characterized by their ability to overcome cell–cell adhesion and to invade the surrounding tissue. E-cadherin is the main adhesion molecule of epithelia, and dysfunction of E-cadherin complexes as well as downregulation of E-cadherin expression has been implicated in tumorigenesis and metastasis of human epithelial cancers (Perl et al., 1998). That PS1 promotes cadherin-based cell–cell adhesion suggests that PS1 may inhibit cancer growth, and loss of PS1 function might decrease cell–cell adhesion and thus promote cancer development. This model provides an explanation for the surprising observation that a PS1 null mutation leads to the formation of epidermal tumors in adult mice (Zeng et al., 2000).

Recent evidence shows that PS1 forms complexes with APP and Notch-1 receptor and regulates production of Aβ (Wolfe et al., 1999). Like E-cadherin, APP and Notch-1 are type I transmembrane proteins. These observations raise the possibility that PS1 forms complexes or regulates the function of many type I transmembrane protein systems. That PS1 forms complexes with APP, Notch-1 and E-cadherin suggests that, in addition to APP processing and Aβ production, PS1 FAD mutations may also target Notch-1, cadherins and possibly other type I transmembrane systems. Which of these potential targets is critical for development of AD remains unclear, as there is good evidence that AD is a heterogeneous brain disorder with multiple neuronal systems affected (Neve et al., 1998; Mesulam, 1999).

SUMMARY

Presenilin-1 (PS1) is an integral membrane glycoprotein involved in the development of familial Alzheimer's disease (FAD). Cadherin-based cell–cell interactions control critical events in cell–cell adhesion, recognition and tissue development. We obtained evidence that PS1 concentrates at cell–cell contacts, where it binds directly to E-cadherin and forms complexes with the cadherin-based cell–cell adhesion system. PS1 fragments are linked to the cortical cytoskeleton and form detergent-stable complexes with E-cadherin, β-catenin, γ-catenin and α-catenin, all components of the cadherin adhesion system. PS1 stabilizes the cadherin/catenin complexes and enhances Ca^{++}-

and cadherin-dependent cell–cell adhesion. Together, our data show that PS1 incorporates into the cadherin adhesion system, stabilizes the cadherin/catenin complexes and modulates cell–cell adhesion. PS1 concentrates at synaptic contacts and forms complexes with brain E- and N-cadherin, which are known synaptic components. These observations reveal a new PS1 function, viz. it regulates cadherin-based adhesion. The PS1 incorporation into the cadherin/catenin complex makes that complex a potential target for PS1 FAD mutations.

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49 Presenilins and Notch Signaling Pathway

WEIHONG SONG AND BRUCE A. YANKNER

INTRODUCTION

Notch-1 is a member of a conserved family of transmembrane receptors that regulate cell fate decisions during development (Artavanis-Tsakonas et al., 1999). Members of the Notch family are activated by ligands of the δ-serrate-LAG2 (DSL) family. Notch-1 undergoes a complex set of proteolytic cleavages during its maturation and upon activation by ligand binding. Ligand binding induces proteolytic cleavage within or proximal to the Notch-1 transmembrane domain, resulting in the release of the Notch-1 intracellular domain (NICD) (Schroeter et al., 1998, Struhl and Adachi, 1998). The NICD can then translocate to the nucleus and activate the transcription of downstream target genes (Jarriault et al., 1995).

Alzheimer's disease (AD) is the most common neurodegenerative disorder leading to dementia. Mutations in presenilin 1 (PS1) and presenilin 2 (PS2) are genetically linked to the autosomal dominant AD and missense mutations in the presenilin genes are the major cause of the early onset familial AD (Sherrington et al., 1995, Levy-Lahad et al., 1995). Mutant forms of both proteins have been shown to increase the production of secreted β-amyloid peptide (Aβ) (Scheuner et al., 1996). Aβ is generated from amyloid precursor protein (APP) by β-secretase (BACE) and an unknown transmembrane cleavage enzyme, γ-secretase. γ-Secretase activity is completely inhibited in PS1/PS2 double deficient cells (Herreman et al., 2000; Zhang et al., 2000) and is catalyzed by a PS1-containing macromolecular complex (Li et al., 2000a,b). The evidence clearly showed that presenilins are absolutely required for γ-secretase activity and mutant PS1 and PS2 play an important role in the generation of pathogenic Aβ in the pathogenesis of AD.

A role for presenilins in the regulation of Notch signaling has been suggested by studies of the *Caenorhabditis elegans* presenilin homolog sel-12 (Levitan and Greenwald, 1995). Furthermore, presenilin-1 (PS1)-deficient mice exhibit decreased expression of Notch and the Notch ligand Dll1 in the

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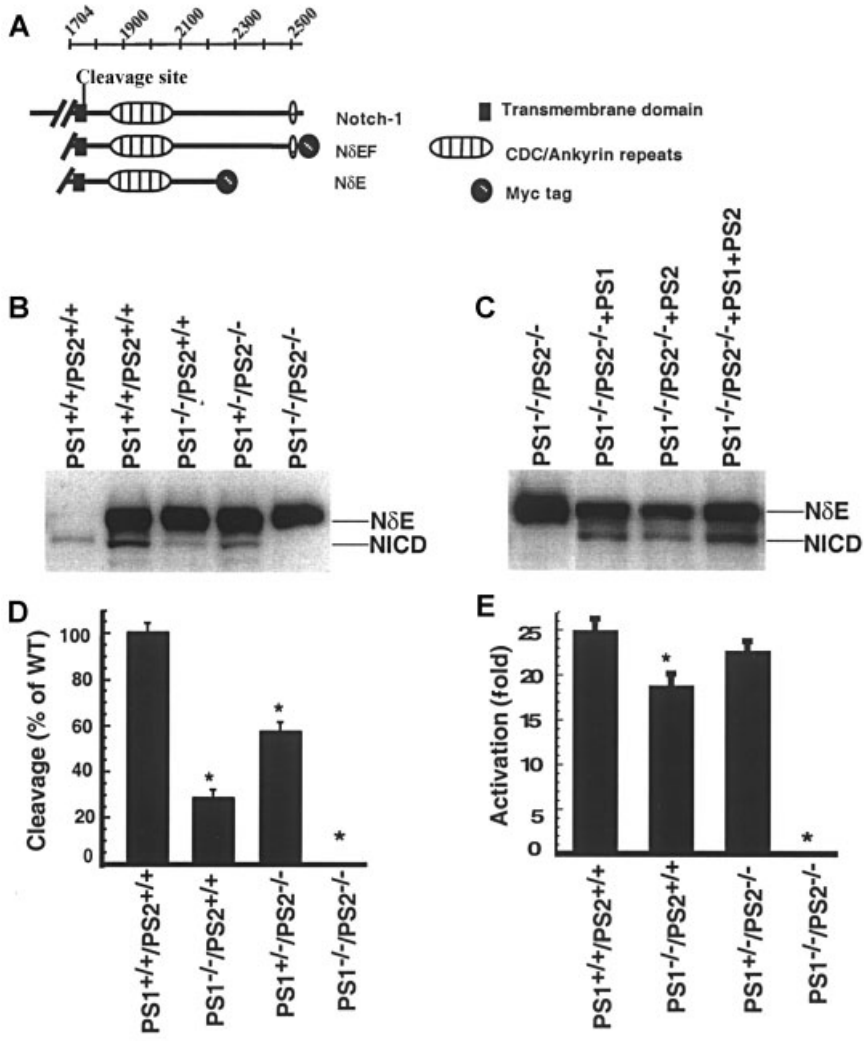

Figure 49.1. Absence of NICD production and activation of Notch signaling in PS1/PS2 double knockout cells. (A) Notch-1 constructs utilized in assays of proteolytic cleavage. (B) Immunoblots of uncleaved Notch-1 NδE and the cleaved NICD fragment in $PS1^{+/+}PS2^{+/+}$, $PS1^{-/-}PS2^{+/+}$, $PS1^{+/-}PS2^{-/-}$ and $PS1^{-/-}PS2^{-/-}$ blastocyst cultures. Lane 1; $PS1^{+/+}PS2^{+/+}$ cells transfected with the NICD expression plasmid. Lanes 2–5, indicated cell types transfected with the NδE expression plasmid. Immunoblotting was performed with anti-Myc to recognize Notch-1-derived proteins with a C-terminal Myc-tag. (C) NICD generation is restored in presenilin double knockout cells by transfection of PS1 or PS2. *cont'd*

pre-somitic mesoderm, as well as defective somite formation and skeletal abnormalities reminiscent of mice with targeted disruption of the Notch-1 gene (Shen et al., 1997; Wong et al., 1997). These findings suggest that PS1 may regulate the Notch signaling pathway during development, although the cellular mechanism is unknown.

Since Notch-1 undergoes a similar proteolytic cleavage in response to ligand binding (Schroeter et al., 1998), we explored the possibility that PS1 and PS2 may also regulate this proteolytic event. We now report that presenilins are absolutely required for the proteolytic release and nuclear translocation of the NICD. PS1 mutations associated with FAD and aspartyl mutants impair this function. Thus, presenilins play a central role in the activation of Notch signaling, which is impaired by pathogenic PS1 mutations.

RESULTS AND DISCUSSION

PRESENILINS ARE ABSOLUTELY REQUIRED FOR THE PROTEOLYTIC CLEAVAGE OF NOTCH-1 AND ACTIVATION OF NOTCH SIGNALING

To explore the role of presenilins in Notch-1 signaling, we analyzed the proteolytic processing of a constitutively active membrane-bound derivative of Notch-1 (NδE) in $PS1^{+/+}PS2^{+/+}$, $PS1^{-/-}PS2^{+/+}$, $PS1^{+/-}PS2^{-/-}$ and $PS1^{-/-}PS2^{-/-}$ blastocyst cultures. NICD production was observed in $PS1^{+/+}PS2^{+/+}$ cells, but was significantly reduced in $PS1^{-/-}PS2^{+/+}$ cells, and was reduced to a lesser extent in $PS1^{+/-}PS2^{-/-}$ cells (Figure 49.1B,D). Similar to γ-secretase activity, NICD release was not detectable in $PS1^{-/-}PS2^{-/-}$ cells, despite robust expression of uncleaved NδE (Figure 49.1B,D). Generation of NICD was restored in $PS1^{-/-}PS2^{-/-}$ cells upon transfection of either PS1 or PS2 (Figure 49.1C).

Figure 49.1 *continued* $PS1^{-/-}PS2^{-/-}$ cells were co-transfected with NδE alone (lane 1) or NδE together with PS1 or PS2 expression plasmids as indicated, and immunoblot analysis was performed on cell lysates with anti-Myc. (D) Quantitative analysis of the generation of NICD. NICD generation was determined as the ratio of NICD to NδE and is expressed as a percentage of the value for $PS1^{+/+}PS2^{+/+}$ (100%). Values represent the mean+SEM, $n = 3$ independent experiments; $^{\star}p < 0.05$ relative to the $PS1^{+/+}PS2^{+/+}$ control. (E) Transcriptional activation of the HES-1 promoter is absent in $PS1^{-/-}PS2^{-/-}$ cells. Cells were co-transfected with NδEF and HES-1-luciferase together with a β-galactosidase expression plasmid. Values represent the ratio of normalized luciferase activity to normalized activity determined with the luciferase plasmid alone, and represent the mean+SEM, $n = 9$. $^{\star}p < 0.01$ relative to PS1-WT. Reprinted by permission from *Nature Cell Biology* 2000; **2**: 463–5. Copyright 2000, Macmillan Magazines Ltd

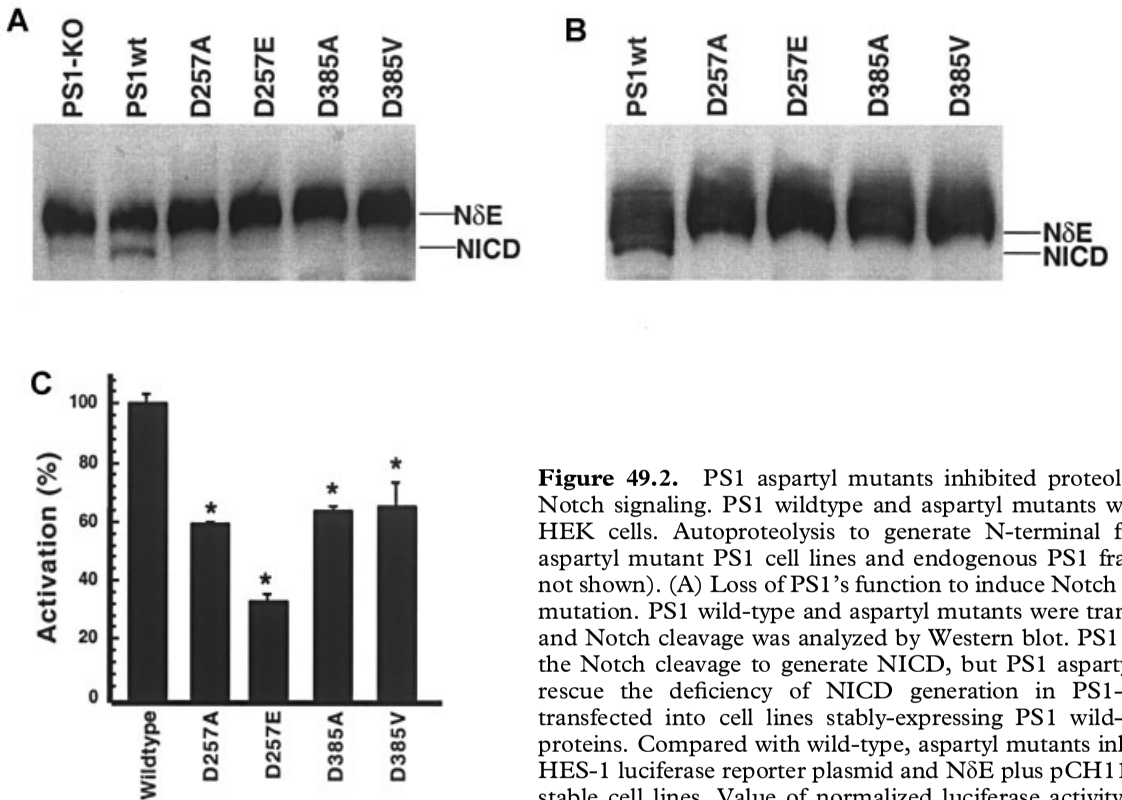

Figure 49.2. PS1 aspartyl mutants inhibited proteolytic release of NICD and Notch signaling. PS1 wildtype and aspartyl mutants were stably-transfected into HEK cells. Autoproteolysis to generate N-terminal fragment was inhibited in aspartyl mutant PS1 cell lines and endogenous PS1 fragment was replaced (data not shown). (A) Loss of PS1's function to induce Notch proteolysis by PS1 aspartyl mutation. PS1 wild-type and aspartyl mutants were transfected into PS1-KO cells and Notch cleavage was analyzed by Western blot. PS1 wild-type can fully restore the Notch cleavage to generate NICD, but PS1 aspartyl mutants were unable to rescue the deficiency of NICD generation in PS1-KO cells. (B) NδE was transfected into cell lines stably-expressing PS1 wild-type and aspartyl mutant proteins. Compared with wild-type, aspartyl mutants inhibited the proteolysis. (C) HES-1 luciferase reporter plasmid and NδE plus pCH110 were co-transfected into stable cell lines. Value of normalized luciferase activity was converted to relative transcriptional activation unit with wild-type as 100%. The HES-1 promoter activity was significantly reduced in aspartyl mutant cells. Activation units are mean $\pm$SEM, n = 3, and *$p < 0.05$ comparing to PS1-WT

To determine whether presenilins affect the biological activity of Notch-1, we assayed for transcription mediated by the HES-1 promoter, a downstream target of the Notch signaling pathway that can be activated by the NICD (Jarriault et al., 1995). Membrane-bound mNotch δE and HES-1-luciferase reporter plasmids were co-transfected into cultured $PS1^{+/+}PS2^{+/+}$, $PS1^{-/-}PS2^{+/+}$, $PS1^{+/-}PS2^{-/-}$ and $PS1^{-/-}PS2^{-/-}$ blastocyst and luciferase activity was measured to reflect the HES-1 promoter activity. HES-1 promoter activity in PS2-KO ($PS1^{+/-}PS2^{-/-}$) cells was similar to that in the wild-type cells ($p > 0.05$). The promoter activity was greatly decreased in PS1-KO ($PS1^{-/-}PS2^{+/+}$) cells and completely inhibited in $PS1^{-/-}/PS2^{-/-}$ cells (Figure 49.1E). It demonstrated that presenilins play an essential role in Notch signaling.

INHIBITION OF NOTCH TRANSMEMBRANE CLEAVAGE AND NOTCH SIGNALING BY ASPARTYL MUTANT PS1

Aspartyl mutants of PS1 reduce Aβ generation by inhibiting γ-secretase activity (Wolfe et al., 1999). To determine whether PS1 aspartyl mutations also affect Notch signaling, we generated aspartyl mutant PS1 expression plasmids and established stably transfected cell lines to express the mutant proteins. To find out whether aspartate 257 and 385 site mutations in PS1 resulted in a loss of PS1 function in the Notch signaling pathway, we assayed the PS1 wild-type and aspartyl mutants' role in NICD proteolytic release in PS1-knockout (KO) cells. Consistent with previous findings, we found that wild-type PS1 can fully rescue Notch transmembrane cleavage deficiency in PS1-KO cells, resulting in NICD proteolytic generation. In contrast, PS1 aspartyl mutants were incapable of restoring the Notch cleavage in PS1-KO cells (Figure 49.2A). It indicated that PS1 aspartyl mutations at sites 257 and 385 cause PS1 to lose its function on Notch transmembrane cleavage. By transfecting NδE into the stably transfected HEK cells, we found that transmembrane proteolytic cleavage of membrane-bound mNotch was inhibited in those PS1 aspartyl mutant cell lines (Figure 49.2B), despite robust expression of the holoprotein (Figure 49.2B). This demonstrated that PS1aspartyl mutants act as a dominant-negative form and inhibit Notch cleavage to generate NICD. These results are consistent with the report of Capell et al. (2000). To further investigate the aspartyl mutants' effect on Notch biological activity, we performed a HES-1 reporter assay to determine their role in Notch transcriptional activation. HES-1 promoter activity was reduced by about half in D257A and D257E cell lines, 59.15+0.56% and 33.65+2.16% ($p < 0.001$), respectively, and one-third in D385A and D385V cell lines, 65.17+1.43% and 66.53+8.08% ($p < 0.05$), respectively (Figure 49.2C). These results suggest that PS1 aspartyl mutations affect Notch signaling.

PROTEOLYTIC RELEASE OF THE NICD IS IMPAIRED BY FAD-ASSOCIATED PS1 MUTATIONS

We then determined whether PS1 mutations associated with FAD affect the proteolytic release of the NICD. As described above, PS1-KO cells exhibit markedly reduced proteolytic release of NICD, which is restored by transfection of PS1. Transfection of PS1 mutants in PS1-KO cells resulted in reduced cleavage of NδE compared with wild-type PS1, despite similar levels of PS1 expression (Figure 49.3A). Quantitative analysis of proteolytic cleavage was performed by determining the ratio of cleaved to uncleaved Notch-1 proteins. This analysis showed that six different FAD mutations significantly reduced Notch-1 cleavage (Figure 49.3B). Mutations in the PS1 C-terminal domain inhibited Notch-1 cleavage by 70–100%, whereas PS1 N-terminal mutations inhibited Notch-1 cleavage by approximately 40–60% (Figure 49.3B). The PS1 mutation C410Y also markedly reduced nuclear translocation of the cleaved NICD fragment (Song et al., 1999). These results suggest that PS1-induced proteolytic release of the NICD is significantly reduced by FAD mutations.

It has been reported that PS1 regulates Notch transmembrane cleavage and γ-secretase activity (De Strooper et al., 1998, 1999; Song et al., 1999). These experiments indicate that presenilins are absolutely required for proteolytic release of the NICD, and that both PS1 and PS2 contribute to these cleavage events. The functional redundancy of PS1 and PS2 is consistent with the observation that Notch-1-related developmental defects are more severe in PS1/PS2 double knockout mice than in PS1 or PS2 single knockout mice (Donoviel et al., 1999; Herreman et al., 1999). Moreover, our findings on the relationship of PS1/PS2 genotype to Notch-1 cleavage suggest that Notch-related developmental defects in the corresponding mice may relate to relative levels of NICD generation. The absolute requirement for presenilins in γ-secretase and Notch-1 cleavage events is consistent with the hypothesis that presenilins are themselves γ-secretases or co-factors for γ-secretase (Wolfe et al., 1999), but does not exclude the possibility that presenilins could affect proteolytic cleavage indirectly through effects on protein trafficking, folding or stability.

Several lines of evidence suggest that Aβ accumulation plays a central role in the pathogenesis of AD (Yankner, 1996). Therapeutic approaches to reducing presenilin function in order to reduce Aβ production are therefore of great interest, but may be limited by the toxic effects of inhibiting Notch signaling. However, the presence of only a single PS1 allele is sufficient to maintain significant NICD generation (Figure 49.1B; $PS1^{+/-}PS2^{-/-}$), and does not result in Notch-related defects *in vivo* (Herreman et al., 1999). But the presence of only a single PS1 allele significantly reduces γ-secretase activity (Zhang et al., 2000). Thus, partial inhibition of presenilin function may constitute a viable therapeutic approach to AD.

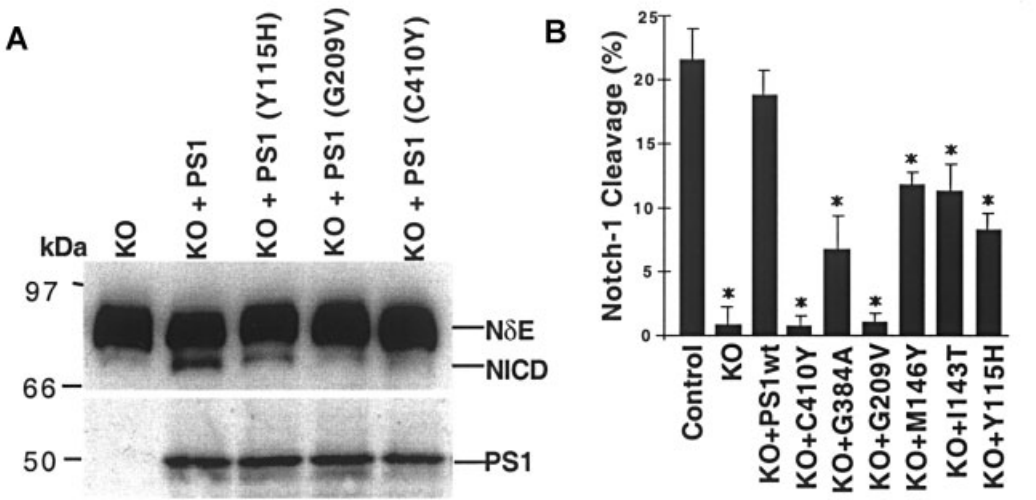

Figure 49.3. PS1 mutations associated with familial Alzheimer's disease impair proteolytic release of the NICD. (A) PS1 mutations associated with familial Alzheimer's disease impair PS1-induced cleavage. PS1-KO cells transfected with the indicated PS1 mutants exhibit reduced Notch-1 cleavage relative to PS1-KO cells transfected with wild-type PS1. Upper panel, NδE immunoblot. Lower panel, PS1 immunoblot showing transfected holo-PS1. (B) Quantitative analysis of the effects of six PS1 mutants on Notch-1 cleavage. The ratio of cleaved to uncleaved forms of NδE was quantitated after transfection of NδE in PS1-WT (Control) and PS1-KO (KO) cells, and in PS1-KO cells co-transfected with wild-type PS1 (PS1wt) or the indicated PS1 mutants. Shown is the mean+SEM, $n = 4$. *$p < 0.05$ relative to KO + PS1wt. Reprinted by permission from Proc Natl Acad Sci 1999; 96: 6959–63. Copyright 1999, National Academy of Sciences, USA

METHODS

CELL CULTURE, PLASMIDS AND TRANSFECTION

$PS1^{+/+}PS2^{+/+}$, $PS1^{-/-}PS2^{+/+}$, $PS1^{+/-}PS2^{-/-}$ and $PS1^{-/-}$ $PS2^{-/-}$ blastocyst cultures and PS1-WT and PS-KO immortalized embryonic fibroblast were obtained and cultured as described (Donoviel et al., 1999; Song et al., 1999). The Notch-1 NδE, NICD (ICv1744) and PS1 expression plasmids have been described (Schroeter et al., 1998; Song et al., 1999). NδE is a membrane-bound constitutively active Notch-1 construct that is Myc-tagged at the C-terminus. PS2 cDNA was cloned into the pcDNA3 expression vector (Invitrogen). Plasmid DNA was transfected using Lipofectamine Plus (Life Technologies, Inc), together with the pCH110 β-galactosidase expression plasmid (Pharmacia) to control for transfection efficiency. Cells and media were harvested 48 hours after transfection for subsequent analysis.

IMMUNOBLOTTING, REPORTER ASSAYS, IMMUNOFLUORESCENCE MICROSCOPY AND SUBCELLULAR FRACTIONATION

Cell lysates were resolved by 10% SDS–PAGE and immunoblotting was performed as described (Busciglio et al., 1997). Myc-tagged Notch-1 derivatives were resolved with monoclonal anti-Myc (9E10 from ATCC). PS1 was resolved with rabbit polyclonal antibody 231 to the PS1 N-terminus (Busciglio et al., 1997). Cleaved and uncleaved Notch-1 proteins were quantitated, using Molecular Dynamics software, by analysis of immunoblots, in which bands were demonstrated to be present in a linear concentration range, as determined by standard curves. The HES-1 promoter reporter assay was performed as described (Jarriault et al., 1995). Immunofluorescence microscopy and subcellular fractionation of PS1-WT and PS1-KO cells were performed as described (Song et al., 1999).

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50 Functional Consequences of the Association of PS1 with β-Catenin

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INTRODUCTION

Mutations within PS1 and PS2 account for the majority of the identified mutations of familial Alzheimer's disease cases. The mechanisms by which the mutations cause the disease phenotype are unknown. The prevailing hypothesis centers on increased and presumably pathologic production of $A\beta_{42}$ species. The recent hypothesis that the presenilins are the actual γ-secretases implies that the mutations alter the normal protein conformation in such a way as to perturb APP cleavage. Related to APP proteolysis is the activity of PS1 in regulating cleavage of the Notch family of receptors in the transmembrane region (discussed elsewhere in this volume). In addition to its activity in APP and Notch processing, PS1 has also been shown to associate with the family of armadillo proteins, including β-catenin. While the interaction of PS1 with β-catenin has been consistently found in a number of laboratories, the consequences of this interaction remain controversial. While we and others have shown that wild-type PS1 negatively regulates β-catenin stability (Murayama et al., 1998), other investigators (Zhou et al., 1997; Weihl et al., 1999) have presented evidence that wild-type PS1 stabilizes β-catenin.

Modulation of β-catenin levels by PS1 is likely to have implications during Wnt signaling. Wnt proteins are involved in embryonic development and cell growth by regulating the levels of cytosolic β-catenin (reviewed in Willert and Nusse, 1998). In the absence of Wnt signaling, cytosolic β-catenin is rapidly turned over by proteosome-mediated degradation (Hart et al., 1998; Ikeda et al., 1998). Wnt stimulation antagonizes the β-catenin degradation machinery, leading to β-catenin stabilization and subsequent translocation to the nucleus,

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where β-catenin binds to the T-cell factor/lymphoid enhancer factor-1 (TCF/LEF abbreviated herein as LEF) family of transcription factors and mediates transcriptional activation of downstream target genes (Behrens et al., 1996), two of which are c-myc and cyclin D1 (He et al., 1998; Shtutman et al., 1999; Tetsu and McCormick, 1999).

In this report, we summarize our recent results showing that lack of PS1 expression is associated with increased stability of cytosolic (and hence signaling) β-catenin, higher cyclin D1 protein levels, and increased cell proliferation. Moreover, the role of PS1 as a modulator of β-catenin levels appears to be independent of its functions in Notch and APP processing. Similarly, PS1 FAD mutations show partial loss of function with regards to β-catenin turnover and, as a result, show increased proliferation as compared to wild-type PS1. Furthermore, in transgenic mice, where PS1 expression is absent from skin, a variety of hyperproliferative changes, including tumor formation, have been noted. Thus, in the absence of PS1, a known downstream gene of the β-catenin signaling pathway responds as predicted based on our model that PS1 accelerates β-catenin turnover.

RESULTS AND DISCUSSION

LOSS OF PS1 FUNCTION LEADS TO UPREGULATION OF β-CATENIN/LEF-DEPENDENT SIGNALING

In our previous study, we showed that the turnover rate of β-catenin was markedly slowed in fibroblasts derived from PS1-deficient mice. The turnover rate can be fully restored with the reintroduction of wild-type PS1 but only partially with a PS1 mutation (ΔX9), suggesting that PS1 mutations may represent partial loss of function with regard to β-catenin stability. A number of downstream target genes have now been identified as being activated by the β-catenin/LEF family of transcription factors, including c-myc and cyclin D1 (He et al., 1998; Shtutman et al., 1999; Tetsu and McCormick, 1999). Accordingly, based on our previous evidence that turnover of β-catenin is in part regulated by PS1, we predicted comparable alterations in cyclin D1 transcription in primary mouse embryonic fibroblasts deficient in PS1. Indeed, Western blotting showed that $PS1^{-/-}$ cells contained higher amounts of β-catenin and cyclin D1 protein (Figure 50.1). In contrast, levels of cdc2 and cyclin A, two other related genes not subject to β-catenin-mediated transcription (Tetsu and McCormick, 1999) were not elevated in $PS1^{-/-}$ cells (not shown).

We have previously shown that, in inducible EcR293 cells, overexpression of wild-type PS1 resulted in ~40% decrease of β-catenin levels, whereas

Figure 50.1. Immunoblot analysis of cyclin D1 and β-catenin from $PS1^{-/-}$ and $PS1^{+/-}$ primary mouse embryonic fibroblasts. PS1-deficient cells expressed higher amounts of cyclin D1 and cytosolic β-catenin

induction of M146L and ΔX9 increased β-catenin levels by ~50% and ~75%, respectively, compared to the non-induced controls (Kang et al., 1999). We therefore analyzed BrdU incorporation in these cells as a measure of the passage from G_1 to S phase of the cell cycle. As shown in Figure 50.2, muristerone induction resulted in significantly higher BrdU incorporation in cells expressing M146L or ΔX9, but not PS1 wild-type ($F = 3.315$; $p = 0.0025$; *post hoc* Tukey, mutants compared with wild-type, $^{\star}p < 0.05$), indicating that overexpression of FAD PS1 mutants results in hyperproliferation in EcR293 cells.

From the above results, it is of particular interest that skin tumors have been described in the majority of PS1 null mice rescued with human PS1 transgene (hPS1) driven by the neuronal-specific human Thy-1 (hThy) promoter (Zheng et al., 2000). Transgenic mice expressing hPS1 under the hThy-1 promoter were able to rescue the embryonic lethality associated with PS1 deficiency. As the hThy-1 promoter is enriched in neurons, PS1 expression cannot be detected in skin. In these animals, epidermal hyperplasia and neoplasia were seen and these changes were associated with increased β-catenin stability and protein levels *in vivo* and in primary keratinocyte cultures. Furthermore, consistent with the results obtained from $PS1^{-/-}$ cells, there was evidence of accelerated proliferation of the epidermal cells *in vivo* (Zheng et al., 2000).

MODULATION OF β-CATENIN SIGNALING BY PS1 REQUIRES THE INTERACTION OF BOTH PROTEINS

The initial report describing the association of PS1 and β-catenin demonstrated that the interaction occurred in the PS1 loop domain (residues 263–407). Subsequently, using a yeast two-hybrid screen, the sequence 299–

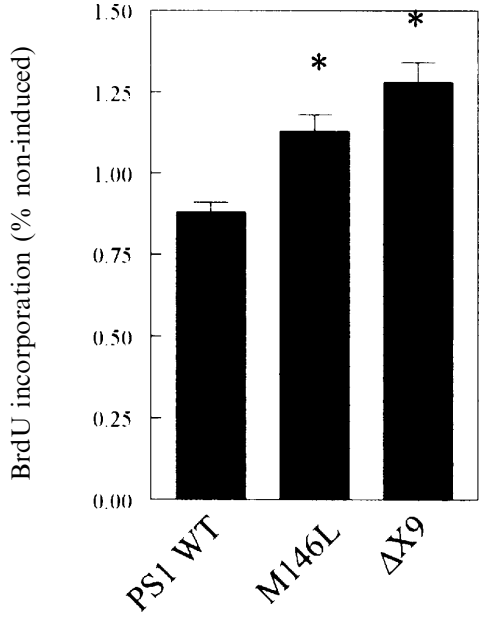

Figure 50.2. Cells treated with or without muristerone for 20 h were incubated for 45 min with bromodeoxyuridine (BrdU) and positive nuclei, scored after immunofluorescence detection as a percentage of total nuclei. The ratios of induced vs. non-induced cultures for each cell line are shown ($F = 3.315$; $p = 0.0025$; *post hoc* Tukey, mutants compared to wild-type, $^{*}p < 0.05$)

362 was shown to interact with p0071, another member of the armadillo family (Stahl et al., 1999). To further narrow down the cytoplasmic loop region with β-catenin, we performed pull-down assays using GST–PS1 loop fusion proteins in cell homogenates. By this assay, the region between amino acids 330–360 within the loop of PS1 is required for its association with β-catenin (Saura et al., 2000). This result is consistent with the report that, following caspase cleavage at position 345, PS1 no longer associated with β-catenin (Tesco et al., 1998). Next, we confirmed that this site is required for β-catenin interaction by expressing a PS1 deletion mutant lacking residues 330–360 (PS1Δcat) in 293 cells. Muristerone induction of wild-type PS1 resulted in the presence of full-length PS1 and an increase in NTF and CTF forms, the latter occasionally appearing as a doublet, which may represent phosphorylation by PKA or PKC (Seeger et al., 1997; Walter et al., 1997). In contrast, muristerone induction of PS1Δcat resulted in the production of a shorter full-length PS1 (Figure 50.3A), together with an increase in NTF and the appearance of a truncated band corresponding to the CTF fragment lacking residues 330–360 (ΔCTF) (Figure 50.3B). Co-immunoprecipitation

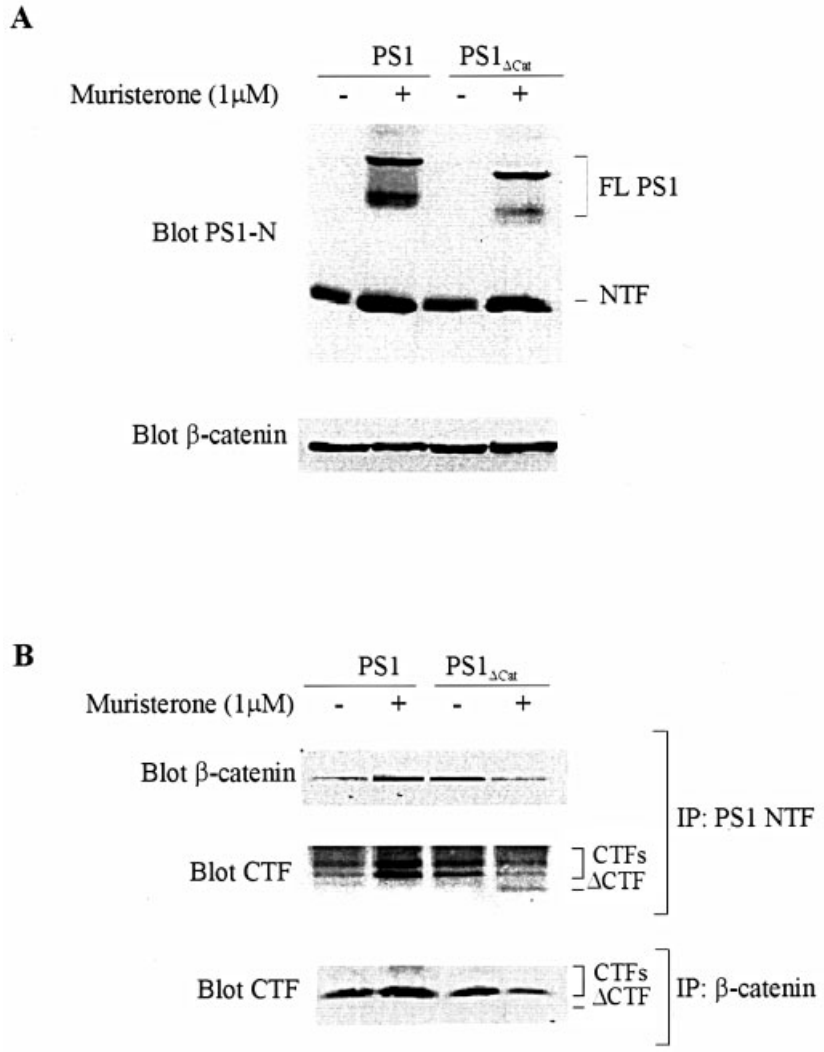

Figure 50.3. Induction profile of PS1 and PS1Δcat in stably transfected EcR293 cells. (A) The cells were incubated overnight with 1 μM muristerone or vehicle and expression of PS1 forms analyzed by immunoblotting (Kang et al., 1999). Induction of the full-length and the NTF fragment was similar in wild-type (WT) PS1 and PS1Δcat. Consistent with a loss of 30 amino acids from the cytoplasmic region, full-length PS1Δcat migrates faster than WT PS1. β-catenin levels were not affected by muristerone induction (bottom panel). (B) Co-immunoprecipitation results show a lack of association between PS1Δcat and β-catenin (top and bottom panels). In contrast, the truncated CTF (ΔCTF), lacking amino acids 330–360, co-immunoprecipitated with the NTF fragment, indicating that PS1Δcat could form stable NTF-ΔCTF fragment complexes

results showed that wild-type PS1, but not PS1Δcat, interacted with β-catenin (Figure 50.3B). Importantly, ΔCTF was efficiently co-immunoprecipitated with PS1-NTF (Figure 50.3B, middle panel), indicating that NTF and ΔCTF were able to form stable complexes. In summary, full-length PS1Δcat was processed into a ~28 kDa NTF indistinguishable from wild-type NTF and a ~14 kDa CTF lacking amino acids 330–360, the latter containing the region required for association with β-catenin.

We next analyzed β-catenin homeostasis in $PS1^{-/-}$ primary fibroblasts retrovirally infected with constructs containing PS1 wild-type and PS1Δcat sequences. In PS1Δcat-infected $PS1^{-/-}$ fibroblasts, the N- and C-terminal fragments, but not full-length PS1, were detected, and lack of binding of PS1Δcat to β-catenin was confirmed. β-catenin turnover was increased in wild-type PS1-infected $PS1^{-/-}$ cells, as expected, but expressing PS1Δcat in $PS1^{-/-}$ cells, did not have a significant effect (data not shown).

BOTH WILD-TYPE PS1 AND PS1ΔCAT RESTORE NORMAL PROTEOLYTIC CLEAVAGE OF NOTCH-1 AND γ-SECRETASE ACTIVITY

PS1 plays a central role in the proteolysis of Notch-1 and the modulation of γ-secretase activity. Therefore, while the studies described above have provided evidence that one of the functions of PS1 is to regulate cytosolic β-catenin turnover, we cannot exclude the possibility that the cellular consequences we have defined may be due to the concomitant impairment of γ-secretase activity or Notch signaling. However, using a larger deletion construct (residues 304–371), Saura et al. (2000) recently showed that Notch proteolysis and Aβ production were not impaired when this PS1 mutant was expressed in N2a cells. Our results are essentially identical, as Notch-1 cleavage and APP processing were normal in 293 or $PS1^{-/-}$ cells expressing PS1 ΔCAT (not shown). Thus, the upregulation of cytosolic β-catenin level and cyclin D1 transcription is unlikely to be related to impaired Notch-1 processing or γ-secretase activity.

In summary, our observations provide evidence that PS1 is part of the β-catenin degradation machinery and that this activity is independent of the known role of PS1 in Notch proteolysis and γ-secretase activity.

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51 A Novel Protease Active Site Motif Conserved in Presenilins and Polytopic Bacterial Aspartyl Proteases?

HARALD STEINER AND CHRISTIAN HAASS

INTRODUCTION

The Alzheimer's disease (AD)-associated presenilins are required to support the cleavage of membrane-bound proteins, either within or close to the cytoplasmic side of the membrane (Brown et al., 2000; Haass and De Strooper, 1999). Several substrates have been identified, which include members of the APP family and Notch, and a PS1 gene ablation results in the inhibition of endoproteolysis of these proteins (De Strooper et al., 1998, 1999; Naruse et al., 1998). A Notch-like phenotype is observed in the PS1 knockout (Shen et al., 1997; Wong et al., 1997), which is strongly augmented if, in addition to PS1, the homologous PS2 is deleted as well (Donoviel et al., 1999; Herreman et al., 1999). Moreover, the double knockout of both presenilins results in a complete inhibition of amyloid β-peptide (Aβ) generation and Notch endoproteolysis (Herreman et al., 2000; Zhang et al., 2000).

Two conserved critical aspartate residues within transmembrane (TM) domains 6 and 7 of PS1 (Wolfe et al., 1999) and PS2 (Kimberly et al., 2000; Steiner et al., 1999) are functionally required for proteolysis of PS targets. Wolfe et al. (1999) demonstrated that mutagenesis of these aspartate residues in PS1 results in the accumulation of βAPP CTFs, which are believed to be the precursors for the ultimate γ-secretase cleavage. Strikingly, a significant reduction of Aβ generation was observed, which appeared to correlate with the lack of endoproteolysis of the aspartate mutant PS derivative. Based on these findings, Wolfe et al. (1999) postulated that PS1 may be an aspartyl protease, which is identical to the γ-secretase. This hypothesis is now supported by the recent finding that γ-secretase inhibitors can be cross-linked to the proteolytic fragments of PS1 and also PS2 (Esler et al., 2000; Li et al., 2000b). However,

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no homology of PSs to any known protease or, in particular, to the active site of aspartyl proteases has been detected so far (Wolfe et al., 1999).

We have identified a critical glycine at residue 384 of PS1. In striking contrast to mutations of aspartate 385, which all block Aβ production and Notch cleavage (Capell et al., 2000; Ray et al., 1999; Steiner et al., 1999), substitution of G384 either results in an inhibition of γ-secretase function or a dramatic increase of its pathological activity (Steiner et al., 2000). Glycine 384 is conserved in all presenilin proteins and is part of a novel putative protease active site motif that is shared with a recently identified family of unusual prokaryotic polytopic aspartyl proteases.

MATERIALS AND METHODS

Materials and methods have been described in detail previously (Capell et al., 2000; Steiner et al., 1999, 2000).

RESULTS AND DISCUSSION

MUTATION OF GLYCINE 384 AFFECTS PS1, βAPP AND NOTCH ENDOPROTEOLYSIS

Immediately adjacent to the critical aspartate at residue 385 of human PS1, a FAD-associated mutation (PS1 G384A) has been identified (Cruts et al., 1995; Tanahashi et al., 1996). Since that mutation occurs so close to the critical aspartate residue 385 and results in an increase of $A\beta_{42}$ generation (De Jonghe et al., 1999; Murayama et al., 1999), we hypothesized that the glycine may have an important function in addition to the critical aspartate at position 385, and may affect the putative active site of presenilins. We therefore mutagenized residue 384 by inserting a variety of different amino acids and analyzed PS endoproteolysis and γ-secretase function. These PS1 derivatives were stably transfected into K293 cells overexpressing Swedish mutant βAPP (Citron et al., 1992). Pooled cell lines were generated and analyzed for PS endoproteolysis. Consistent with previous results (Thinakaran et al., 1996), almost no PS1 holoprotein could be identified in untransfected control cells expressing endogenous PS1 (Figure 51.1A). In contrast, cell lines overexpressing ectopic PS1 accumulated the PS1 holoprotein (Figure 51.1A). Efficient endoproteolysis of PS1 holoproteins was observed in cell lines expressing the G384A, G384I, G384P and G384W mutants (Figure 51.1A). In contrast, expression of G384K and G384D resulted in reduced amounts of the PS1 CTF, indicating diminished PS1 endoproteolysis by the introduction of charged amino acids at this position. As reported previously (Wolfe et al., 1999), the PS1 D385A mutant also accumulated as an uncleaved holoprotein

(Figure 51.1A). Overexpression of all PS1 G384 mutants, as well as PS1 D385A and wild-type PS1, led to an almost complete displacement of endogenous PS2 CTFs (Steiner et al., 2000).

We next investigated the influence of the PS1 G384 mutations on βAPP processing. Since the aspartate mutations lead to an accumulation of βAPP CTFs, probably due to reduced γ-secretase activity (De Strooper et al., 1998; Wolfe et al., 1999), we first analyzed the levels of these amyloidogenic precursors. A significant accumulation of βAPP C-terminal fragments was observed in cell lines expressing the PS1 derivatives G384I, G384P, G384W, G384K and G384D. These fragments accumulated to similar levels as in cells expressing the PS1 D385A mutant (Figure 51.1A). In contrast, cells expressing the G384A mutation generate much lower levels of βAPP CTFs (Figure 51.1A), indicating that all mutations, except the FAD-associated G384A variant, are loss-of-function mutations that directly interfere with γ-secretase activity.

Since G384 mutations can affect βAPP CTF generation, we analyzed the functional consequences of these variants on Aβ generation. For further analysis, three different G384 mutations were selected: first, the naturally occurring FAD-associated G384A mutation (Cruts et al., 1995; Tanahashi et al., 1996), which undergoes PS endoproteolysis and accumulates low levels of βAPP CTFs; second, the artificial G384P mutant, which, like the G384I and G384W mutants, does not affect PS endoproteolysis and causes the accumulation of high levels of βAPP CTFs; and third, the artificial G384K mutant, which, like the G384D mutant, undergoes reduced PS endoproteolysis and accumulates high levels of βAPP CTFs. Cell lines were labeled with ^{35}S-methionine and conditioned media were investigated for Aβ levels. The FAD-associated G384A mutant produced substantial amounts of total Aβ, including an exceptionally high amount of abnormal $A\beta_{42}$ (~ six-fold increase of the $A\beta_{42}/A\beta_{total}$ ratio (Steiner et al., 2000; Figure 51.1B). In contrast, the proline and lysine substitution significantly reduced total Aβ production (Figure 51.1B). In agreement with previous results (Wolfe et al., 1999) the PS1 D385A mutation strongly inhibited total Aβ production (Figure 51.1B). None of the mutations analyzed affected the secretion of APPs or expression of the βAPP holoprotein (Figure 51.1B). Analysis of the Aβ concentrations in conditioned media of unlabeled cells by ELISA confirmed the decreased total Aβ production by the proline and lysine mutation and the extreme $A\beta_{42}$ production by the alanine mutation (Steiner et al., 2000).

Since it has been shown previously that PS1 and PS2 are required to facilitate endoproteolysis of Notch (Capell et al., 2000; De Strooper et al., 1999; Ray et al., 1999; Song et al., 1999; Steiner et al., 1999), we investigated whether expression of PS1 G384A, G384P and G384K affects NICD formation. HEK 293 cells expressing endogenous PS1 or the indicated mutant derivatives were stably co-transfected with the NotchΔE cDNA construct, described previously (Schroeter et al., 1998; Steiner et al., 1999)

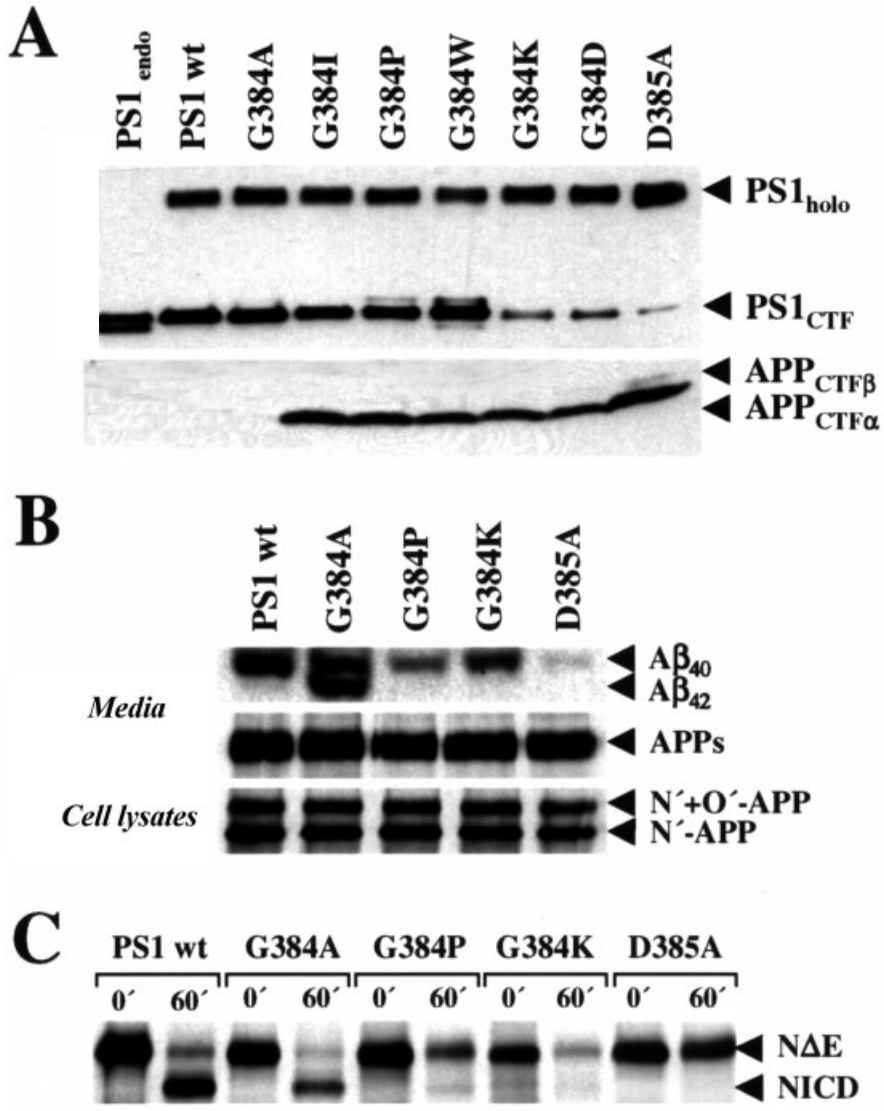

Figure 51.1. Mutation of glycine 384 affects PS1, βAPP and Notch endoproteolysis. (A) Upper panel: expression and endoproteolytic processing of the mutant PS1 derivatives. Cell lysates were immunoprecipitated with antibody 3027 and precipitated PS1 derivatives (CTFs and holoprotein) were visualized by immunoblotting, using antibody BI.3D7. Note that endoproteolysis of PS1 is diminished in cell lines expressing the G384K, G384D and D385A mutants. Lower panel: accumulation of βAPP CTFs in G384 mutant cell lines. Aliquots of the lysates used were immunoblotted with antibody 6687 to the C-terminus of βAPP. *cont'd*

and analyzed for Notch endoproteolysis. In control cells expressing wild-type PS1, proteolytic release of NICD from NotchΔE was observed during the chase period (Figure 51.1C), which is consistent with previous results (Capell et al., 2000; Steiner et al., 1999). Similarly, substantial NICD production was observed in cell lines expressing the FAD-associated G384A mutant, which is also in line with previous results (Kulic et al., 2000). In contrast, overexpression of PS1 G384P or PS1 G384K showed significantly reduced proteolytic generation of NICD (Figure 51.1C). Consistent with previous results (Capell et al., 2000; Steiner et al., 1999), an almost complete block of NICD generation was observed in the cell line expressing PS1 D385A (Figure 51.1C). Therefore, the mutagenesis of codon 384 to proline or lysine not only affects Aβ generation but also efficient NICD generation and consequently Notch signaling.

Taken together, in contrast to the mutagenesis of the critical aspartate residue 385, mutagenesis of the neighboring glycine residue can have two fundamentally different effects. Depending on the amino acid inserted, it can promote extreme levels of pathological $A\beta_{42}$ generation or reduce total Aβ production. In addition, the very same mutations either inhibit or block Notch endoproteolysis. Therefore, it is likely that G384 may directly affect the active center of presenilins (Esler et al., 2000; Li et al., 2000b; Wolfe et al., 1999).

G384 AND D385 ARE PART OF A MOTIF CONSERVED IN BACTERIAL ASPARTYL PROTEASES

The above-described data demonstrated that G384 is functionally important for γ-secretase-mediated cleavage of βAPP and Notch. Based on these data,

Figure 51.1 *cont'd* βAPP CTFs generated by α- and β-secretase (longer exposure reveals significant amounts of β-secretase generated βAPP CTFs) strongly accumulated in cells expressing the PS1 G384 artificial mutants, as well as in cells expressing PS1 D385A, as observed previously (Wolfe et al., 1999). (B) Effects of selected mutant PS1 derivatives on βAPP endoproteolysis. Upper panel (conditioned media): Aβ species were immunoprecipitated from conditioned media of metabolically labeled cells with antibody 3926 and separated on a previously described Tris–Bicine gel system, which allows the specific identification of $A\beta_{40}$ and $A\beta_{42}$ (Wiltfang et al., 1997). APPs were immunoprecipitated with antibody 5313. Lower panel (cell lysates), full-length APP (N′/O′-glycosylated APP and N′-glycosylated APP) was immunoprecipitated from cell lysates with antibody 5313. (C) Effects of the mutant PS1 derivatives on Notch endoproteolysis. Cell lines expressing PS1 derivatives were transfected with the NotchΔE cDNA (Schroeter et al., 1998). NICD formation was analyzed in pulse-chase experiments, as described (Steiner et al., 1999). Note the significant change of the ratio of NΔE:NICD in cells expressing the G384P and G384K mutation, as compared to those lines that express wild-type PS1 or PS1 G384A. Consistent with previous results, NICD formation was blocked in cell lines expressing PS1 D385A (Capell et al., 2000; Steiner et al., 1999). Note that NΔE is not only processed to NICD but also degraded during the cold chase

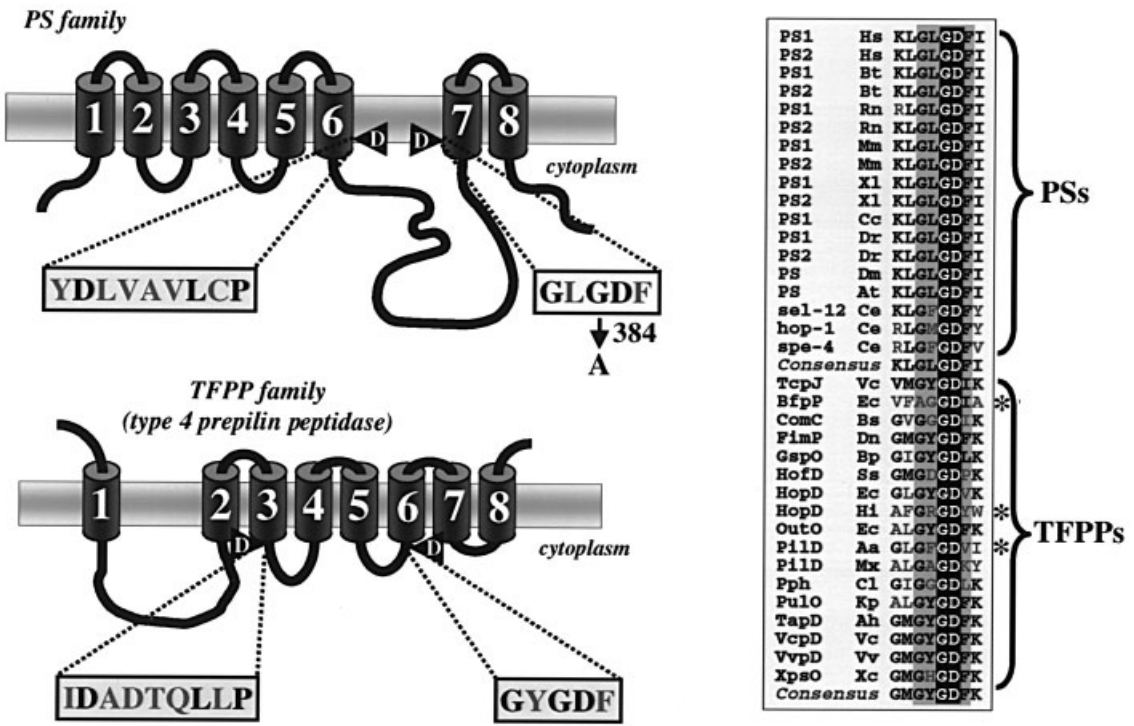

Figure 51.2. Sequence homology around the active site aspartates of PSs and TFPPs. Hs = *Homo sapiens*; Bt = *Bos taurus*; Rn = *Rattus norwegicus*; Mm = *Mus musculus*; Xl = *Xenopus laevis*; Dr = *Danio rerio*; Dm = *Drosophila melanogaster*; At = *Arabidopsis thaliana*; Ce = *Caenorhabditis elegans*; for the abbreviations of the bacterial TFPPs, see LaPointe and Taylor (2000). The conserved glycine and the aspartate residue of the C-terminal G(A)X′GDX'' motif (dark gray box) are highlighted. The FAD-associated G384A mutation is indicated. Arrowheads indicate the critical aspartate residues of PSs and TFPPs. For details, see text

we hypothesized that the glycine residue, together with the critical aspartate residue, may be functionally conserved during evolution. Sequence comparison revealed that, like aspartate 385, glycine 384 was conserved in all members of the presenilin family, including the very distant worm homologue, spe-4 (Figure 51.2). Even in the putative presenilin from *Arabidopsis*, the critical glycine is conserved (Figure 51.2). We therefore searched databases for bilobed aspartase proteases lacking the classical D(T/S) G(T/S) motif, using the sequence RLGFGDF derived from spe-4, the most distant member of the presenilins. Strikingly, this search revealed numerous members of a novel family of bacterial aspartyl proteases (Figure 51.2). These proteases belong to the recently described family of type 4 prepilin peptidases (TFPP) (LaPointe and Taylor, 2000). The TFPPs have eight TM domains and two critical aspartate residues located close to TM3 and TM6 (Figure 51.2). Mutagenesis of the critical aspartates blocks the proteolytic function of the TFPPs (LaPointe and Taylor, 2000). The conserved motif, including the critical glycine residue, is observed around the C-terminal but not the N-terminal aspartate residue (Figure 51.2), which is consistent with the conservation of the same motif in TM7 but not TM6 of all members of the presenilin family. Moreover, TFPPs do not only contain a very similar active site but also mediate an endoproteolytic cleavage, which is reminiscent of the cleavage reactions supported/mediated by PSs. TFPPs are known to remove leader peptides of selected substrates by cleaving between hydrophobic and hydrophilic domains close to the cytoplasmic side of the membrane (LaPointe and Taylor, 2000), a cleavage reaction that is strikingly similar to the γ-secretase cleavage of Notch and βAPP (Haass and De Strooper, 1999). TFPPs and PSs appear to share a G(A)X′**GD**X″ (X′ = variable; X″ = F>I>V>L; bold type indicates amino acids conserved in all members of both families) motif around the C-terminal active site aspartate (Figure 51.2). Some sequence homology may also be found around the N-terminal active site aspartate (Figure 51.2).These data indicate a possible convergent evolution of the protease active sites in TFPPs and presenilins. In higher organisms polytopic 'PS-proteases' are required to cleave βAPP and Notch, either within the membrane or close to the cytoplasmic side of the membrane. This protease activity occurs as a high molecular weight complex (Capell et al., 1998; Li et al., 2000a; Thinakaran et al., 1998; Yu et al., 1998) which contains Nicastrin and probably additional proteins (Yu et al., 2000). Taken together, these data provide evidence that presenilins may indeed be aspartyl proteases, as originally proposed.

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52 The Unfolded Protein Response-mediated Upregulation of BiP and CHOP Is not Affected by Presenilin Expression

NAOYUKI SATO AND GOPAL THINAKARAN

INTRODUCTION

Alzheimer's disease (AD) is associated with several risk factors, including age, genetic factors and environmental factors. In the last decade, much progress has been made in our understanding of the genetic factors responsible for the predisposition of individuals to early- and late-onset AD (Price et al., 1998). However, the pathogenic mechanisms involved in sporadic AD are still largely unclear. It is known that the pathogenesis of several neurodegenerative disorders, including AD, involves protein misfolding and aggregation (Cohen, 1999; Dobson, 1999; Sanders and Nagy, 2000). For example, genetic mutations in *APP*, PS1 or *PS2* lead to the production of highly amyloidogenic $A\beta_{42}$ peptides that are largely secreted, promoting the extracellular deposition of Aβ in senile plaques (Thinakaran, 1999). In addition, it was recently reported that intracellular oligomerization and accumulation of Aβ peptides might precede extracellular Aβ deposition in human AD brains and in cell culture models (Gouras et al., 2000; Walsh et al., 2000). These latter findings raise the possibility that intracellular accumulation of Aβ may be an important factor that might contribute to neuronal cell death, both in familial and sporadic AD. In this study, we examined whether PS1 and familial AD-linked PS1 variants play a role in the cellular response to the accumulation of misfolded proteins.

Newly-synthesized transmembrane proteins and lumenal proteins undergo post-translational modifications, initial folding and oligomerization within the endoplasmic reticulum (ER). A number of genotoxic agents, including tunicamycin, thapsigargin, Ca^{2+} ionophores, and reducing agents such as dithiothreitol affect protein folding process by perturbing the ER homeostasis. The subsequent accumulation of misfolded proteins within the ER initiates

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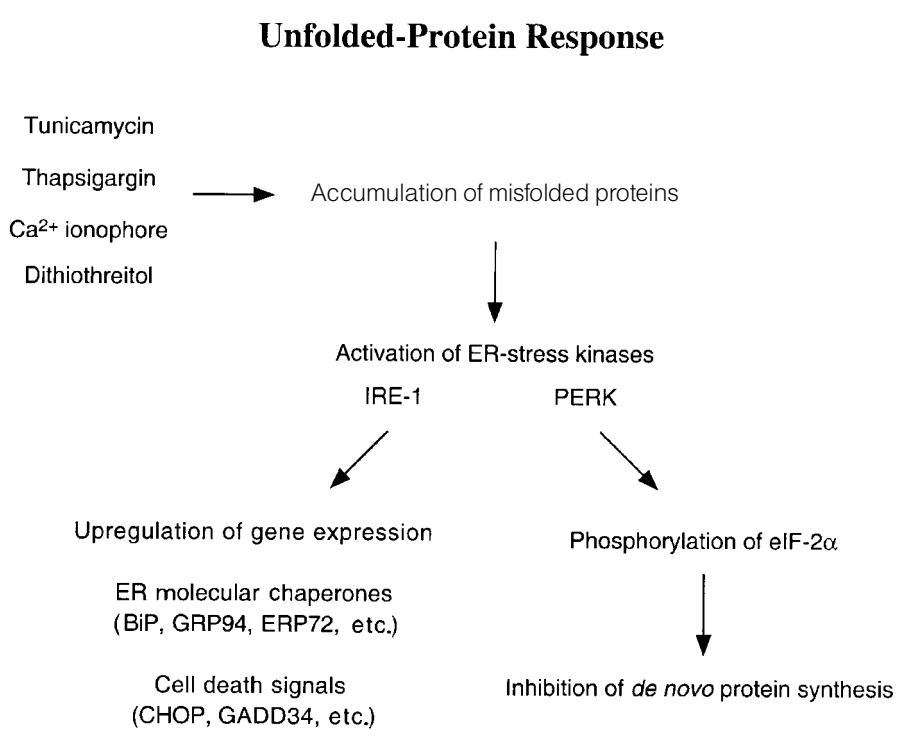

Figure 52.1. Schematic representation of the UPR. The UPR occurs as a result of the accumulation of misfolded proteins within the ER. In eukaryotic cells, the UPR is activated by exposure to agents that affect endoplasmic reticulum (ER) homeostasis, including tunicamycin, thapsigargin, Ca^{2+} ionophores and reducing agents such as dithiothreitol. Accumulation of misfolded proteins triggers the activation of transmembrane kinases, IRE1α and IRE1β, and leads to the transcriptional upregulation of genes that encode protein chaperones, such as BiP, GRP94 and Erp72, and cell death signal proteins, such as CHOP and GADD34. Accumulation of misfolded proteins induces another ER-stress kinase, PERK. Activated PERK phosphorylates the translation initiation factor eIF2α, thereby inhibiting an early step in mRNA translation. Both the upregulation of ER chaperones and inhibition of *de novo* protein synthesis are compensatory mechanisms; the former increases the capacity of ER to fold proteins, and the latter attenuates the stress by decreasing new protein synthesis

the cellular response, termed the unfolded-protein response (UPR), which culminates in the coordinated transcriptional activation of a set of genes that encode ER chaperones and certain cell death signals (Kaufman, 1999; Pahl, 1999) and the repression of *de novo* protein synthesis (Figure 52.1).

The most proximal components of the UPR are type I transmembrane protein kinases termed IRE1α, IRE1β and PERK. Accumulation of misfolded proteins in the ER causes the dissociation of BiP from the

lumenal, stress-sensing domain of IRE1α and IRE1β and leads to their oligomerization and activation by *trans*-autophosphorylation (Sidrauski et al., 1998; Bertolotti et al., 2000). Subsequently, via downstream signaling steps that are not clearly defined, activation of IRE leads to the transcriptional activation of selected genes that contain ER-stress elements within their promoter regions. The well-characterized UPR genes encode protein chaperones such as BiP/GRP78 (IgG binding protein), GRP94, and Erp72, and the transcription factor CHOP (C/EBP-homologous protein) (Price and Calderwood, 1992; Wang et al., 1996). In addition to the IRE-mediated upregulation of certain genes, ER stress activates a parallel pathway that signals the inhibition of *de novo* protein synthesis. This latter pathway is dependent on the activity of PERK, a type I transmembrane protein kinase that phosphorylates the translation initiation factor eIF2α, thereby inhibiting an early step in mRNA translation (Shi et al., 1998; Harding et al., 1999, 2000).

RESULTS

LACK OF REQUIREMENT OF PS1 AND PS2 FOR THE INDUCTION OF UPR

It was recently reported that in a $PS1^{-/-}$ fibroblast line, the induction of *BiP* mRNA was impaired under conditions that induce the UPR (Niwa et al., 1999). To re-examine this issue, we incubated monolayers of primary fibroblasts generated from $PS1^{-/-}$ and $PS1^{+/-}$ mouse embryos with tunicamycin for various periods of time, and performed Northern blots. Tunicamycin, a nucleoside antibiotic that inhibits N-glycosylation of target asparagine residues in the lumenal domains of proteins expressed in the ER, is a potent inducer of the UPR. The levels of *BiP* and *CHOP* mRNA were induced and accumulated in a time-dependent fashion, after treatment with tunicamycin, to comparable levels in both primary $PS1^{-/-}$ and $PS1^{+/-}$ fibroblasts (Figure 52.2). These results were further confirmed by Western blot analysis of the accumulation of CHOP polypeptide in $PS1^{-/-}$ and $PS1^{+/-}$ fibroblasts that were subject to ER stress (Sato et al., 2000). In a similar set of studies, Urano and Ron documented that a time-dependent accumulation of *BiP* and *CHOP* mRNA was comparable in wild-type and $PS1^{-/-}$/ $PS2^{-/-}$ mutant lines (Sato et al., 2000). These data strongly support the view that PS1 and PS2 are not required for UPR-related upregulation of *BiP* and *CHOP* mRNA.

To assess whether the early steps in the activation of UPR may be influenced by PS1, we and others examined the activation of IRE1 and PERK in $PS1^{-/-}$ or $PS1^{-/-}/PS2^{-/-}$ cells. For these studies, cells were exposed to either tunicamycin or thapsigargin, an inhibitor of the ER Ca^{++} ATPase

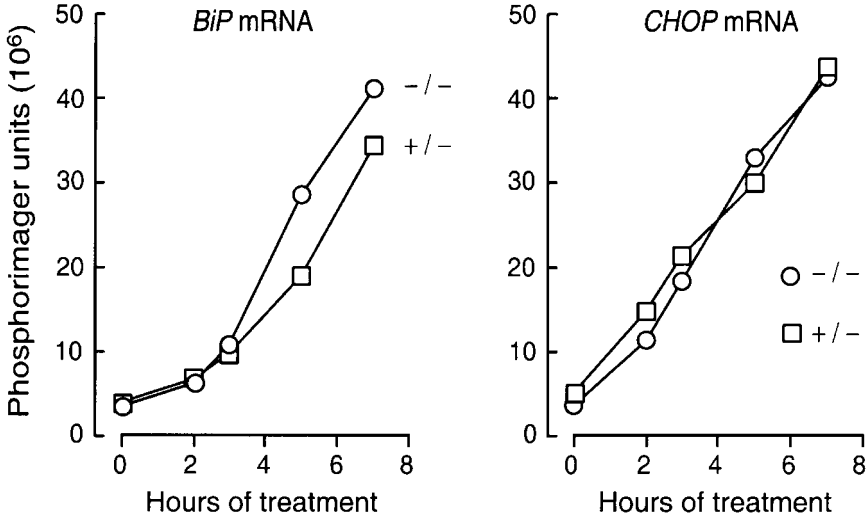

Figure 52.2. UPR-mediated induction of *BiP* and *CHOP* expression in primary $PS1^{-/-}$ and $PS1^{+/-}$ fibroblasts. Primary mouse fibroblasts derived from $PS1^{-/-}$ and $PS1^{+/-}$ embryos were incubated with tunicamycin (2 μg/ml) for various periods (0, 2, 3, 5 or 7 h). Total RNAs isolated from the fibroblasts were examined by Northern blot analysis. Blots were sequentially hybridized with *BiP*, *CHOP* and *GAPDH* probes and the signals were quantified by phosphorimaging. *BiP* and *CHOP* mRNA levels were normalized to *GAPDH* mRNA and plotted

known to induce the UPR. As predicted from Northern blot studies, phosphorylation of IRE1α and PERK was unaffected in $PS1^{-/-}/PS2^{-/-}$ cells (Sato et al., 2000). Collectively these results demonstrate that PS1 and PS2 are not required for either the activation of ER stress kinases IRE1α and PERK, or the induction of *BiP* and *CHOP* mRNA following ER stress.

UPR INDUCTION IN STABLE CELL LINES EXPRESSING FAD-LINKED PS1 MUTANTS

Recent studies revealed that expression of FAD-linked PS1 impairs the UPR (Katayama et al., 1999). We re-examined this issue in stable mouse N2a neuroblastoma cell lines that express FAD-linked PS1 C410Y variant. In previous studies, we and others reported that expression of PS1 C410Y mutant causes ~two-fold increase in the secretion of $A\beta_{42}$ peptides (Thinakaran, 1999). To determine whether activation of the UPR is impaired in stable PS1 C410Y cell lines, we examined ER stress-mediated activation of IRE1α or PERK, and the increased accumulation of *BiP* and *CHOP* mRNA.

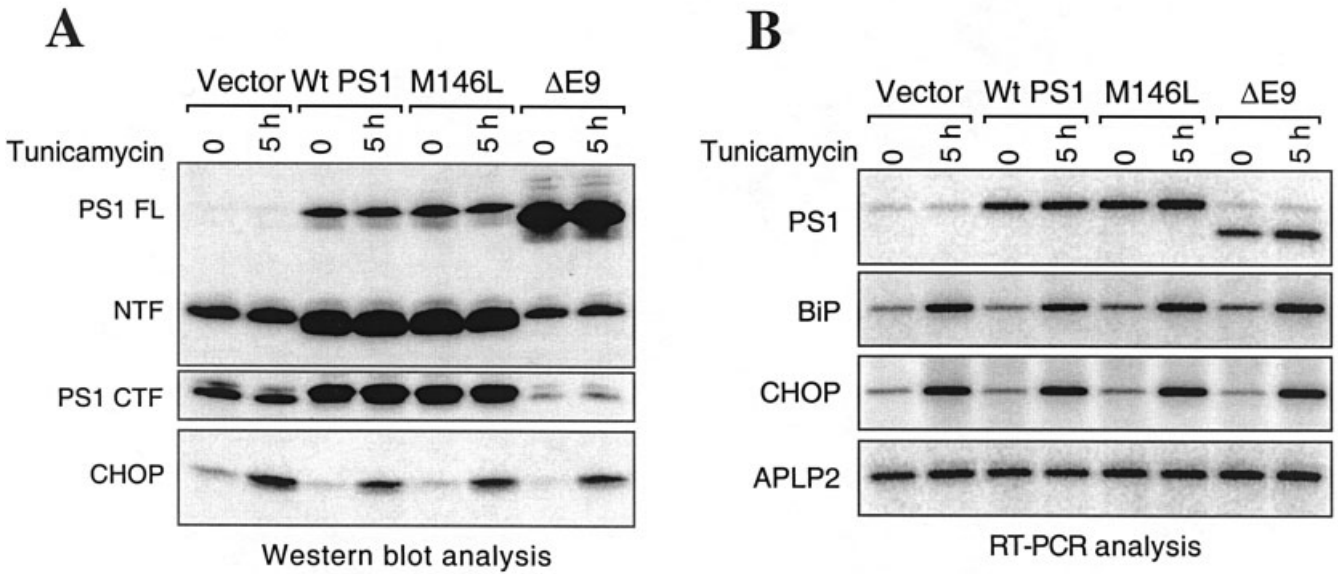

Figure 52.3. UPR induction in stable N2a cells that express wild-type or FAD-linked mutant PS1. (A) Stable pools of N2a cells expressing PS1Wt, M146L or ΔE9 were incubated with tunicamycin (2 μg/ml) for 5 h. Detergent lysates of tunicamycin-treated and control cultures were analyzed by immunoblotting with $PS1_{NT}$, αPS1Loop or anti-CHOP antibodies. Western blot analysis revealed that the levels of full-length PS1 or derivatives were not affected by ER stress. In contrast, tunicamycin markedly increased the comparable levels of CHOP polypeptide in cells expressing wild-type and mutant PS1. (B) Semi-quantitative RT–PCR analysis of *BiP* and *CHOP* mRNA levels in stable pools of N2a cells expressing Wt or mutant PS1. Subconfluent cultures were incubated with tunicamycin (2 μg/ml) for 5 h. Total RNAs isolated from the cells were subjected to RT–PCR (20 cycles of PCR) in reactions supplemented with radiolabeled sense primers. Phosphorimager quantification of signals from RT–PCR revealed that, regardless of the expression of wild-type or mutant PS1, *BiP* and *CHOP* mRNA levels were induced to similar levels by the ER stress agent, tunicamycin

Our studies revealed that, upon exposure to tunicamycin, IRE1α and PERK were activated to comparable levels in wild-type PS1 or mutant PS1 cell lines. Furthermore, quantification of *BiP* and *CHOP* mRNA levels by Northern blots, followed by normalization to *GAPDH* mRNA, revealed that tunicamycin treatment resulted in increased accumulation of *BiP* mRNA to comparable levels in wild-type and PS1 C410Y cell lines. Finally, *BiP* promoter activity was also induced to comparable levels in the wild-type PS1 and C410Y PS1 stable lines, indicating that the UPR-mediated increase in *BiP* transcription is not influenced by the expression of FAD-linked PS1 C410Y variant (Sato et al., 2000).

INDUCTION OF THE UPR IN STABLE POOLS OF MOUSE NEUROBLASTOMA N2a CELLS THAT EXPRESS MUTANT PS1

In order to exclude clonal or cell-type variability and obtain unambiguous confirmation that the UPR is unimpaired by the expression of several PS1 variants, we generated stable pools of mouse N2a cells transfected with bicistronic expression plasmids that encode PS1 and hygromycin selection marker. Western blot analysis using PS1 N-terminal and C-terminal antibodies revealed transgene-derived expression of full-length and cleaved PS1 derivatives in cells transfected with wild-type, M146L PS1 cDNA, and uncleaved mutant PS1 polypeptide (due to the lack of the site of endoproteolysis) in cells transfected with *PS1ΔE9* cDNA (Figure 52.3A). Western blot analysis revealed that the levels of full-length PS1 or processed PS1 NTF were not affected by the treatments. To assess the levels of *BiP* and *CHOP* mRNA, we performed quantitative RT–PCR by adding small amounts of radiolabeled sense primer. For controls, we measured the levels of *APLP2* mRNA; note that expression of *APLP2* is not affected by ER stress. Radiolabeled *BiP* and *CHOP* PCR products were quantified by phosphor-imaging and normalized to *APLP2* products. These studies revealed that, regardless of the expression of wild-type or mutant PS1, *BiP* and *CHOP* mRNA levels were induced to similar levels by the ER stress agents tunicamycin (Figure 52.3B). Western blots also confirmed that ER stress markedly increased the levels of CHOP polypeptides in wild-type and mutant PS1 cells (Figure 52.3A).

Our findings were further confirmed in N2a cells that express PS1 under an inducible promoter, and in stable pools of human embryonic kidney 293 cells that express PS1 wild-type, M146L, H163R and ΔE9 variants (Sato et al., 2000). Collectively, our results demonstrate that expression of FAD-linked mutant PS1 has no influence on UPR-related gene expression in cultured mammalian cell lines.

DISCUSSION

The cellular response to the accumulation of misfolded proteins involves the transcriptional induction of genes encoding ER chaperones and selected transcription factors involved in cell death pathways. Recent studies in PS1-deficient fibroblasts have alluded to a role for PS1 in the induction of the UPR (Niwa et al., 1999), while analysis of cells expressing FAD-linked PS1 variants have indicated that these cells may be rendered more susceptible to ER stress by impairing the UPR signaling pathway (Katayama et al., 1999). In the present study, we have re-examined the involvement of PS1 in the UPR using a variety of experimental models. Contrary to previous reports, our findings show that neither the activation of ER-stress kinases IRE1α and PERK nor the coordinate induction of *BiP* and *CHOP* mRNA and protein are impaired in cells lacking PS1 function, including PS1 single knockout cells and PS1/PS2 double knockout cells, and in stable cell lines expressing a dominant loss-of-function variant (PS1D385A). Furthermore, we also document that UPR is not impaired in stable N2a neuroblastoma and human 293 cells expressing FAD-linked PS1 variants. In addition, our recent studies also failed to document significant decreases in the levels of BiP in the brains of individuals with sporadic AD, or patients with FAD harboring PS1 mutations I143T and G384A, compared to controls. These latter findings were further strengthened by our demonstration that, in brains of transgenic mice expressing wild-type human PS1 or PS1 M146L and A246E variants, the levels of BiP protein are nearly indistinguishable (Sato et al., 2000).

Analysis of FAD-linked PS1 and PS2 variants in a number of cell culture models provide evidence that expression of mutant PS1 perturbs calcium homeostasis and likely causes ER stress (Mattson et al., 1998). It is well known that the UPR triggers both survival responses (inhibition of protein synthesis, induced expression of ER chaperones, etc.), as well as activated expression of certain genes associated with cell death (e.g. *CHOP*). As such, a model in which PS1 plays a role in the cellular response to ER stress, and an impairment of the UPR pathway by mutant PS1, seems highly attractive. However, our results provide strong evidence that induction of BiP (survival response) and CHOP (cell death signaling) expression by ER stressors is not effected by the lack of PS1 function or the expression of FAD-linked PS1 variants. Alternatively, we suggest that if FAD-linked PS variants alter ER stress signaling *in vivo*, these processes may be driven by mechanisms that are distinct from the classical UPR pathway.

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53 Mechanisms of α-Synuclein and NAC Fibrillogenesis

MAKOTO HASHIMOTO, EDWARD ROCKENSTEIN, TAKATO TAKENOUCHI, MARGARET MALLORY, AND ELIEZER MASLIAH

INTRODUCTION

The role of abnormal protein folding and aggregation in the central nervous system (CNS) is being extensively explored as one of the central mechanisms leading to neurodegeneration in disorders such as Alzheimer's disease (AD), Lewy body disease (LBD), Huntington's disease (HD) and Creutzfeldt–Jacob disease (CJD) (Ferrigno and Silver, 2000; Ramassamy et al., 1999). Lewy body disease is a common cause of dementia and motor dysfunction in the elderly, second only to AD (Galasko et al., 1994). This heterogeneous group of disorders presents with parkinsonism, cognitive deficits and formation of Lewy bodies (LBs) (McKeith et al., 1996). In accordance with the CDLB International Workshop (McKeith et al., 1996), this group includes Parkinson's disease (PD), diffuse Lewy body disease (DLBD), Lewy body variant of AD (LBV) and combined PD+AD. Recent studies have shown that, in LBD, α-synuclein accumulates in Lewy bodies (LBs) (Spillantini et al., 1997; Takeda et al., 1998b; Wakabayashi et al., 1997) and that LB neurites are major components of these lesions (Spillantini et al., 1998). Furthermore, mutations in the α-synuclein gene are associated with rare familial forms of PD (Kruger et al., 1998; Polymeropoulos et al., 1997).

α-Synuclein, a 140 amino acid (aa) synaptic molecule, was originally identified in human brains as the precursor protein of the non-β-amyloid (Aβ) component (NAC) of AD plaques (Masliah et al., 1996; Ueda et al., 1993; Iwai, 2000) (Figure 53.1). NAC is a highly hydrophobic 35 aa domain within the α-synuclein molecule that is involved in amyloid formation (Iwai et al., 1995). α-Synuclein is related to other molecules, including human β-synuclein/bovine phosphoneuroprotein 14 (Jakes et al., 1994; Nakajo et al., 1993) which, although highly homologous with α-synuclein,

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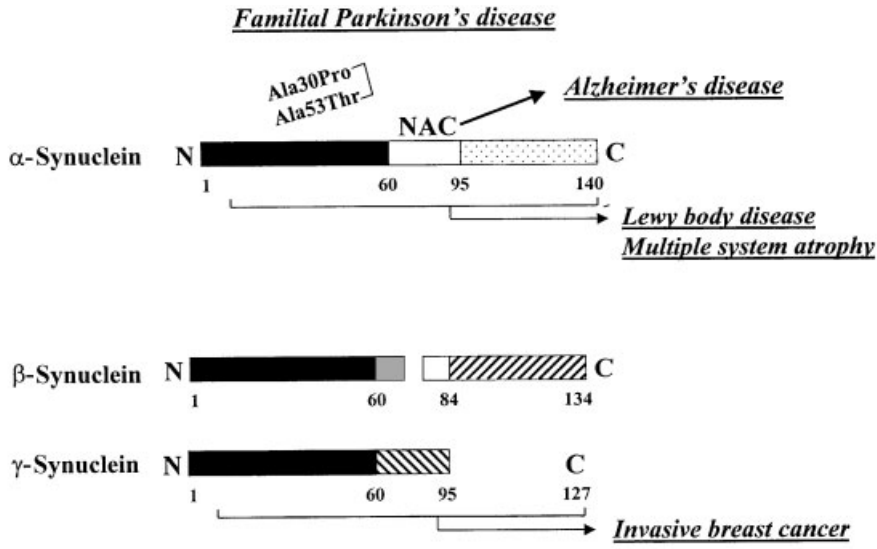

Figure 53.1. Diagrammatic representation of the synuclein family of molecules

do not possess an amyloidogenic domain (Iwai et al., 1994) (Figure 53.1). More recently, γ-synuclein was isolated as a D3 synuclein-like molecule predominantly expressed in peripheral sympathetic neurons (Clayton and George, 1998). γ-Synuclein was later cloned from the EST library as the breast cancer-specific gene (BCSG1) whose expression was observed in the invasive types of breast cancer (Jia et al., 1999). Furthermore, synoretin, the fourth member of the synuclein family was shown to be abundantly expressed in both retinal cells and the brain (Surguchov et al., 1999).

α-Synuclein has been independently identified in a variety of biological systems in Torpedo (Maroteaux et al., 1988), rat (Maroteaux and Scheller, 1991) and human (Jakes et al., 1994) and as 'synelfin' in the zebra finch (George et al., 1995). α-Synuclein is a naturally unfolded (Weinreb et al., 1996) major presynaptic nerve terminal protein (George et al., 1995; Iwai et al., 1994; Jakes et al., 1994) that might interact with other synaptic proteins such as synphilin-1 (Engelender et al., 1999) and with cytoskeletal proteins (Jensen et al., 1999). These findings have led to a hypothesis that α-synuclein may play a critical role in synaptic events, such as neural plasticity during development, learning (George et al., 1995) and degeneration of nerve terminals under pathological conditions in LBD, AD and other disorders (Hashimoto and Masliah, 1999; Masliah et al., 2000). Thus, identifying the role of factors promoting and/or blocking the aggregation of α-synuclein is critical for the understanding of LBD pathogenesis.

MECHANISMS OF α-SYNUCLEIN AGGREGATION

FACTORS PROMOTING α-SYNUCLEIN AGGREGATION

The α-synuclein molecule is capable of self-aggregating to form oligomers, as well as fibrillar polymers with amyloid-like characteristics (Hashimoto et al., 1998). Aggregated full-length α-synuclein oligomers, fragments and fibrils have been identified in LBs (Baba et al., 1998; Hashimoto et al., 1998). While oligomers are usually the soluble and potentially toxic components, fibrils are the insoluble and ubiquitinated components that might be entrapped and isolated representing a less toxic form of the aggregates (Masliah, 2000) (Figure 53.2). As previously described for Aβ (Yang et al., 1999), polymerization of α-synuclein might occur in several stages including formation of protofibrils, nucleation (Wood et al., 1999) and fibril formation (Hashimoto et al., 1998) (Figure 53.2). Conditions promoting this aggregation include: mutations (Conway et al., 1998; Narhi et al., 1999); oxidative stress mediated by iron, cytochrome *c* or copper(II) (Hashimoto et al., 1999a,b; Hsu et al., 2000; Paik et al., 1998); failure to bind to lipid membrane vesicles caused by mutation (Jo et al., 2000; Perrin et al., 2000); and interactions with NAC; Jensen et al., 1997; Paik et al., 1998) and Aβ (Masliah et al., unpublished; Jensen et al., 1997; Paik et al., 1998). In addition, apolipoprotein E has been shown to bind the NAC region of α-synuclein (Olesen et al., 1997). The properties and formation of these fibrils have been recently reviewed (El-Agnaf and Irvine, 2000).

Taken together, this suggests that both mutations and stress conditions act in a similar manner by promoting protein misfolding and aggregation which,

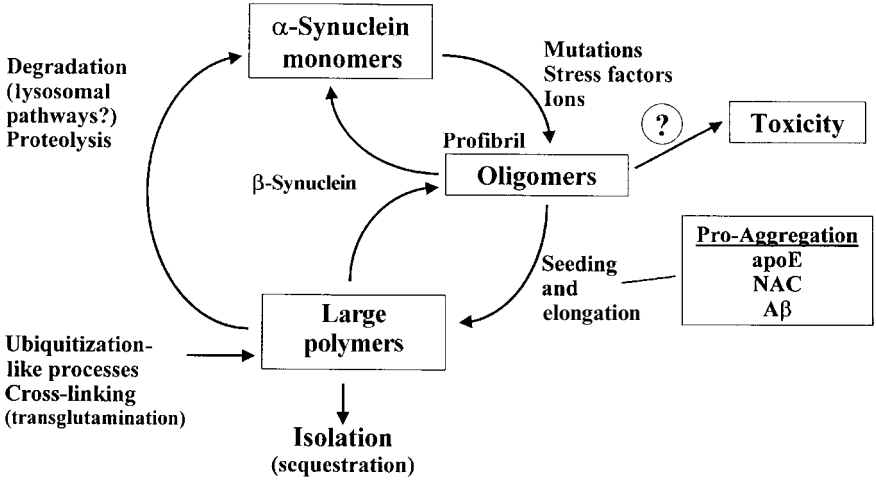

Figure 53.2. Potential mechanisms of α-synuclein aggregation and anti-aggregation

in turn, could result in the formation of either inactive fibrils (polymers) or toxic oligomers (Figure 53.2). This is supported by recent studies in transgenic mice (Masliah et al., 2000) and *Drosophila* (Feany and Bender, 2000), showing that expression of wild-type or mutant α-synuclein protein leads to dopaminergic synapse loss, inclusion body formation (Figure 53.3) and motor deficits. In addition to our group, other groups have also developed transgenic mice expressing wild-type and mutant α-synuclein (Kahle et al., 2000; van der Putten et al., 2000). While we used the platelet-derived growth factor B (PDGFB) promoter to drive wild-type human α-synuclein expression, others have used the Thy-1 and the mouse PrP promoters. These studies have shown that, in addition to the dopaminergic and limbic systems (Masliah et al., 2000), α-synuclein might also affect the neuromuscular junction and that the A53T mutation may be particularly damaging (Kahle et al., 2000; van der Putten et al., 2000).

These observations are further supported by recent studies in α-synuclein-transfected neuronal cell systems, where accumulation of this molecule results in dopaminergic cell death (Zhou et al., 2000), caspase cleavage (Alves da Costa et al., 2000), mitochondrial dysfunction and oxidative stress (Hsu et al., 2000; Osterova-Golts et al., 2000). In addition to α-synuclein, NAC has also been shown to be toxic to neuronal cell lines (El-Agnaf et al., 1998a,b,c).

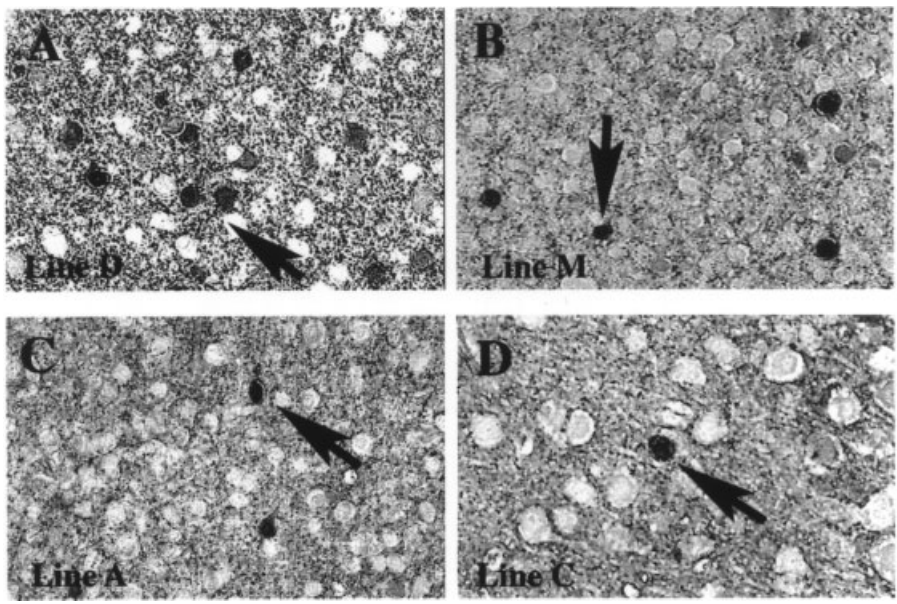

Figure 53.3. Inclusion body formation (arrows) in α-synuclein transgenic mice. Expression of human wild-type α-synuclein under the regulatory control of the PDGFβ promoter results in the formation of Lewy body-like inclusions. Lines A and C = low expressers; line M = intermediate expresser; line D = high expresser

CHARACTERIZATION OF α-SYNUCLEIN IN DETERGENT-SOLUBLE AND -INSOLUBLE FRACTIONS

Although Aβ exists both in detergent-soluble and -insoluble fractions, it is more abundant in the insoluble fraction in cell cultures and in human brain (Lee et al., 1998; Skovronsky et al., 1998; Yanagisawa et al., 1995). This raises the possibility that insoluble Aβ fractions might be involved in intracellular aggregation and amyloidogenesis. In this context, we determined whether α-synuclein exists in the detergent-insoluble fractions, where it might interact with Aβ. Surprisingly, we detected levels of α-synuclein immunoreactivity in virtually all types of cells. Figure 53.4 shows an example in 293 cells with or without transient transfection of α- and/or β-synuclein cDNA. In addition to the 18 kDa fragment of α-synuclein, we found a slightly smaller fragment at 16 kDa in the detergent-insoluble fractions. This fragment was labeled with both C-terminal (aa 131–140) and human-specific (aa 101–124) antibodies, and the positive staining by the C-terminal antibody was abolished by preabsorption with corresponding peptide. In contrast to α-synuclein, expression of β-synuclein in wild-type cells was not detected in the soluble or insoluble fractions and was detected only in the soluble fractions in the β-synuclein-transfected cells. Thus, differential intracellular localization of α- and β-synuclein suggests that these molecules could be differentially involved in the intracellular amyloidogenesis of Aβ in AD.

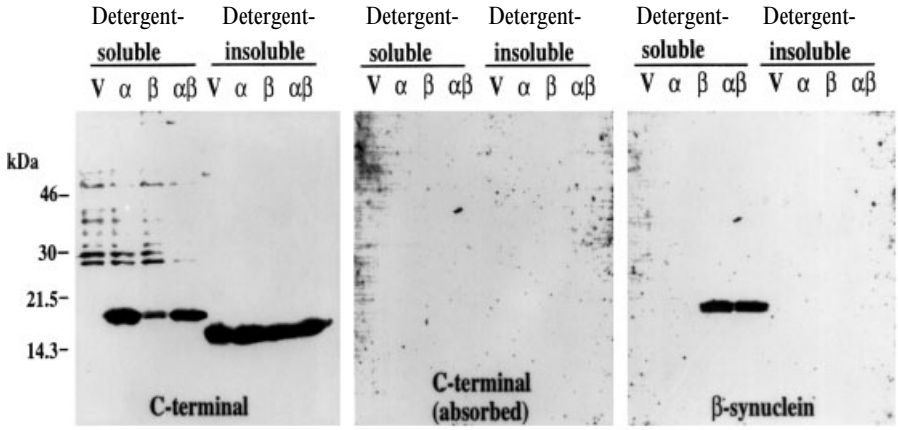

Figure 53.4. Characterization of α-synuclein in detergent-soluble and -insoluble fractions. Immunoblotting analysis of cells transiently transfected with pCEP4 vector, with or without insertion of α- and β-synuclein cDNA, showed that α-synuclein was detected only in the detergent-soluble fractions

ROLE OF NAC IN AMYLOIDOGENESIS

In 1993, Saitoh and colleagues identified NAC as an important component of amyloid-enriched fractions in AD brains (Ueda et al., 1993). NAC is a 35 aa fragment derived from its 140 aa precursor (α-synuclein), which is now recognized to play a major role in LBD pathogenesis (for review, see Hashimoto and Masliah, 1999). Furthermore, studies with the anti-NAC-X1 antibody revealed that NAC immunoreactivity is closely co-localized with Aβ in the AD plaques (Masliah et al., 1996; Ueda et al., 1993) and is present in LBs (Takeda et al., 1998a, 2000). Moreover, biochemical studies showed that NAC is extremely hydrophobic and easily forms amyloid-like fibrils under physiological conditions (Iwai et al., 1994). These results led us to the hypothesis that NAC might play an important role in Aβ aggregation and amyloidogenesis in AD and LBD (Hashimoto and Masliah, 1999).

In contrast to this possibility, a recent study reported that NAC may not be associated with Aβ in the plaques (Culvenor et al., 1999). Using the antibody NAC42580 to label cortical and hippocampal sections of AD cases, the authors found no evidence of NAC immunoreactivity in the plaques. Furthermore, although NAC42580 showed certain immunoreactivity in the urea extracts of the SDS-insoluble fractions in DLBD and PD cases, there was no correlation between NAC and Aβ immunoreactivity in the same AD fractions. Based on this study and a previous report (Bayer et al., 1999) the authors concluded that NAC is not associated with Aβ in AD plaques.

Some possible explanations of the discrepancy between our results and those reported by Culvenor et al. include differences in properties of antibodies used in each study. NAC-X1 and NAC42580 were raised against epitopes derived from different portions of the NAC region: NAC1-9 for NAC-X1 and NAC15-31 for NAC42580. Furthermore, NAC-X1 preferentially immunoreacts with aggregated α-synuclein. In fact, biochemical analysis of the NAC peptide by El-Agnaf et al. (2000) showed that the N-terminal region of NAC (NAC1-18) aggregates to form amyloid fibrils, while the C-terminal region (NAC19-35) remains soluble, suggesting that the N-terminal portion is essential for aggregation of NAC peptide (El-Agnaf and Irvine, 2000).

Furthermore, differences in antibody immunoreactivity might depend on the type of tissue used. In this regard, the NAC-X1 antibody immunolabeled

plaques in paraformaldehyde-fixed vibratome sections, but not in formalin-fixed paraffin-embedded tissue (Takeda et al., 1998a, 2000). In fact, both Bayer and Culvenor used archival formalin-fixed and/or paraffin-embedded (rather than vibratome) tissues for their immunohistochemical studies, raising the possibility that fixation, solvents and paraffin might destroy the NAC-X1 epitope. Further supporting the importance of tissue pretreatment and processing to detect the NAC epitope, we have recently shown that in vibratome sections pretreated with formic acid, the NAC-X1 antibody immunostained not only amyloid plaques and amyloid angiopathy, but also astroglial cells and granular neurons in LBD. Letters to the Editor detailing the positions of the different groups in regard to these issues have been recently published (Culvenor et al., 2000; Hashimoto et al., 2000).

Finally, difficulties in obtaining conclusive evidence may be due to the lack of information as to the mechanisms by which NAC is generated from its precursor, namely the α-synuclein (or NACP) molecule. Better understanding of this process in the future might help to more clearly define the role of α-synuclein and NAC in LBD and AD.

FACTORS BLOCKING α-SYNUCLEIN AGGREGATION

Recent evidence suggests that, in the same way that some conditions promote α-synuclein aggregation, there might be some endogenous factors blocking amyloidogenesis and aggregation (Figure 53.2). Interactions among the synucleins might play a role in this process. For example, β- and γ-synuclein do not seed α-synuclein aggregation (Biere et al., 2000). Thus, a critical balance between pro- and anti-aggregation factors might be at play in disorders such as LBD and AD (Masliah, 2000; Rochet et al., 2000) (Figure 53.2). Here we present evidence showing that β-synuclein blocks the aggregation of Aβ. β-Synuclein is a synaptic protein encoded by a gene in chromosome V (different from α-synuclein, which is encoded by a gene in chromosome IV; Chen et al., 1995). Although these two proteins are homologous, the main difference is that, compared to α-synuclein, β-synuclein lacks part of the NAC region (Figure 53.1). Extensive studies are under way to test the effects of β-synuclein on α-synuclein aggregation and to better understand the mechanisms by which β-synuclein blocks amyloidogenesis.

INHIBITORY EFFECTS OF β-SYNUCLEIN ON Aβ ARE PHYSIOLOGICALLY RELEVANT

Surprisingly, the inhibitory effect of β-synuclein clearly and consistently ameliorated Aβ cytotoxicity. When B103 neuroblastoma cells were incubated for 3 days with Aβ (20 μM) under serum-free conditions, dead cells became prominent due to the cytotoxic effects of Aβ. In addition, co-incubation with β-synuclein (20 μM) blocked cytotoxicity, while α-synuclein (20 μM) was detrimental (Figure 53.5). Further characterization of cell death and the underlying mechanisms is currently in progress. Although synuclein is a cytosolic protein, these results may become a first step in further investigations into the interactions between Aβ and synucleins under pathophysiological conditions *in vivo*.

β-SYNUCLEIN INHIBITS THE AGGREGATION OF NAC

Since NAC forms β-pleated structures (Han et al., 1995; Iwai et al., 1995) and is present in the senile plaques of AD, it was predicted that NAC might be involved in AD amyloidogenesis (Ueda et al., 1993). Therefore, inhibition of NAC aggregation may block the initial step in amyloid formation. In order to determine whether β-synuclein blocks NAC aggregation, NAC (10 μM) was co-incubated with β-synuclein (0, 1, 10 μM) under high-temperature conditions for up to 20 h (Figure 53.6) and the effects were assessed by

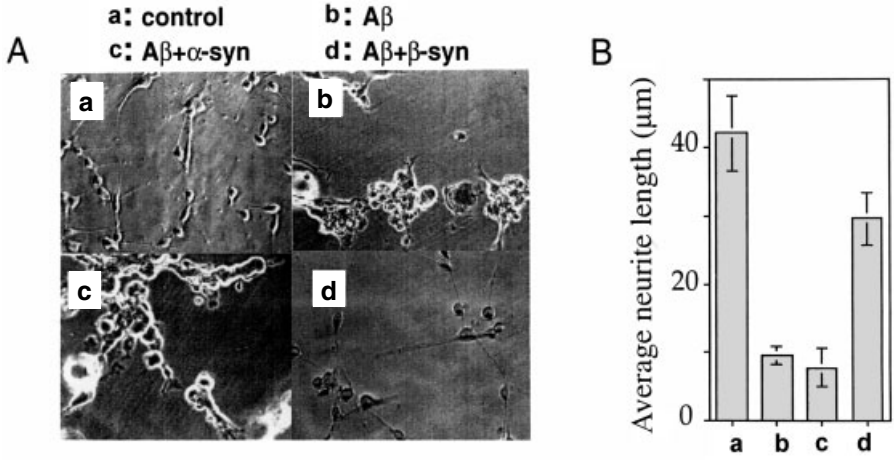

Figure 53.5. Inhibitory effects of β-synuclein on Aβ are physiologically relevant. Cells were incubated for 48 h under serum-free conditions with $A\beta_{1-40}$ (20 μM) in the presence of either human recombinant α- or β-synuclein (20 μM). (A) Phase-contrast microscopy. (B) Neurite assay

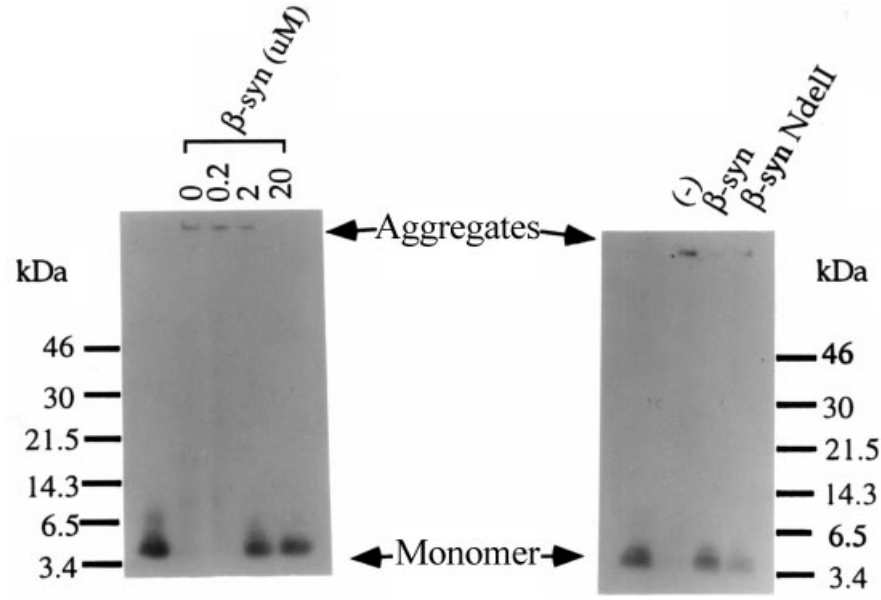

Figure 53.6. β-Synuclein inhibits the aggregation of NAC. Co-incubation of NAC (10 μM) with β-synuclein (0, 1.0, 10 μM) under the high temperature conditions for 20 h showed inhibition of the aggregation of NAC in a concentration-dependent manner. β-SynucleinNdel partly suppressed the aggregation of NAC, further supporting the contention that this β-synuclein region is necessary for modulating its anti-amyloidogenic effects. Immunoblotting analysis was performed using the anti-NAC antibody

immunoblotting analysis, using the anti-NAC antibody. As expected, co-incubation with β-synuclein inhibited the aggregation of NAC in a concentration-dependent manner (Figure 53.6). β-SynucleinNdel partly suppressed the aggregation of NAC, further supporting the contention that this β-synuclein region is necessary for modulating its anti-amyloidogenic effects.

CHARACTERIZATION OF THE BINDING OF SYNUCLEINS TO Aβ

It has previously been described that α-synuclein binds to $A\beta_{1\text{-}38}$ under physiological conditions (PBS, pH 7.4) (Yoshimoto et al., 1995). The formation of an SDS-resistant complex of α-synuclein with Aβ was observed at 72 h. Furthermore, α-synuclein was shown to bind to Aβ through the 81–95 aa portion of the NAC domain (Yoshimoto et al., 1995). Since this region is conserved between α- and β-synucleins (Jakes et al., 1994), we hypothesized that both synucleins might bind to Aβ through this specific domain. In order

to test this hypothesis, α- and β-synucleins and their mutant proteins deleted with this domain (81–95 aa) were created in an *E. coli* expression system (Figure 53.7A). After incubation with $A\beta_{1\text{-}40}$ for 72 h, they were subjected to immunoblot analysis using the 6E10 antibody (anti-$A\beta_{1\text{-}17}$) (Senetek, Maryland Heights, MD). As expected, formation of the SDS-resistant complex of synucleins with Aβ was observed around 20 kDa (Figure 53.7B, lanes 2 and 4). Furthermore, if the 81–95 aa domain is critical for the binding of synucleins to Aβ, mutant proteins deleted with this domain should no

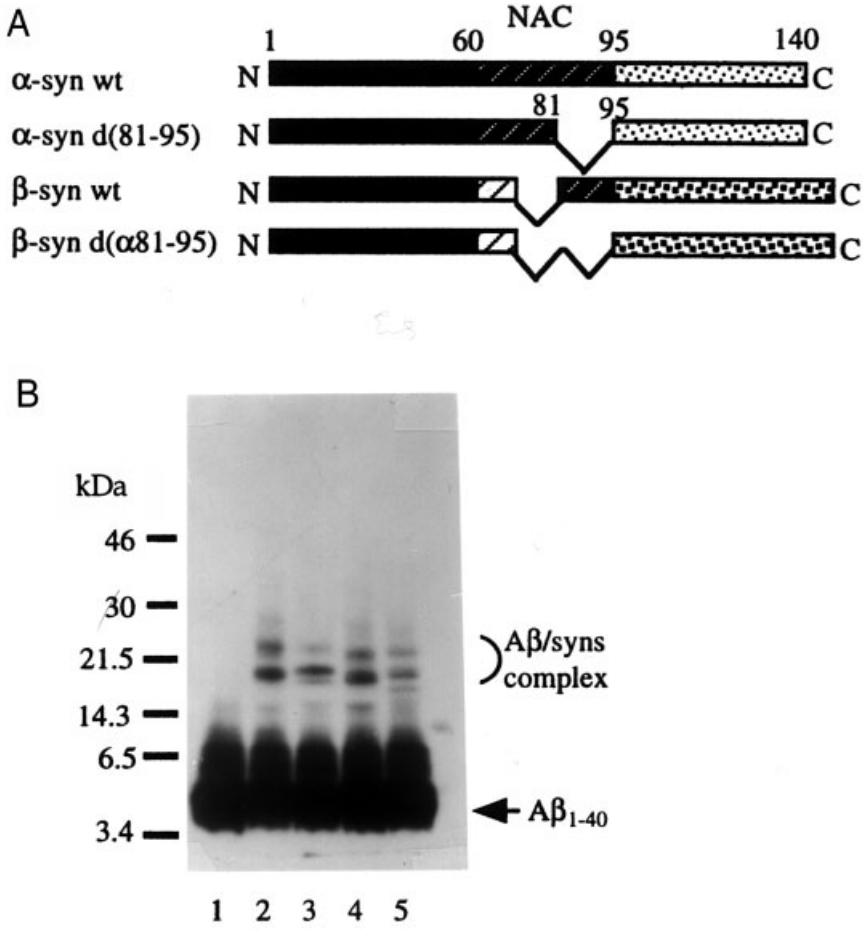

Figure 53.7. Characterization of the binding of synucleins to Aβ. (A) α- and β-Synuclein and their mutant proteins deleted with aa 81–95 were created in an *E. coli* expression system. (B) Immunoblotting analysis with the 6E10 antibody showed that incubation with $A\beta_{1\text{-}40}$ (20 μM) under physiological conditions showed formation of an SDS-resistant complex with synucleins at around 20 kDa (lanes 2–5)

longer bind to Aβ. However, both of the mutant synucleins formed an SDS-resistant complex with Aβ (Figure 53.7B, lanes 3 and 4), indicating that binding sites other than the 81–95 aa region may be critical for the binding of synucleins with Aβ. Thus, these results favor a report by Jensen et al. (1997), showing that more broader domains of α-synuclein, including its N-terminal and NAC regions, could be involved in the binding of α-synuclein to Aβ in the presence of chemical cross-linker BS3. Jensen et al. (1997) further found that sedimentation of ^{125}I-Aβ was significantly increased in the presence of either α- or β-synuclein. Based on this result, they proposed that both synucleins might be able to stimulate Aβ polymerization; however, stimulation of sedimentation does not necessarily lead to stimulation of polymerization.

It is possible, then, that binding and trapping of Aβ may result in either stimulation or inhibition of Aβ aggregation and further amyloid fibril formation. While the former action is observed in several 'pathological chaperone' molecules, including apolipoprotein E, α-1 antichymotrypsin and proteoglycan, the latter action can be compared to that of a 'physiological chaperone', such as heat shock protein. We hypothesize that, while α-synuclein acts as a pathological chaperone of Aβ, β-synuclein could be a physiological chaperone of Aβ. Under long-term incubation conditions, Aβ is known to form amyloid fibrils spontaneously. When Aβ is co-incubated with β- but not α-synuclein, fibril formation is completely abolished (Figure 53.8), supporting the possibility that β-synuclein might be a negative regulator of Aβ aggregation.

A PEPTIDE DERIVED FROM β-SYNUCLEIN INHIBITS INTERACTION BETWEEN α-SYNUCLEIN AND Aβ

It is possible that β-synuclein blocks Aβ protofibril formation via two pathways (Figure 53.2): (1) indirectly by blocking NAC or α-synuclein interactions with Aβ, or (2) directly by blocking Aβ aggregation. In order to test the first possibility, α-synuclein (10 μM) and $A\beta_{1\text{-}40}$ (10 μM) were co-incubated with β-synuclein synthetic peptides (100 μM), corresponding to aa 1–15 up to aa 51–61 of the N-terminal region of β-synuclein. Immunoblotting analysis showed that two peptides corresponding to two distinct regions (aa 1–15 and aa 51–60) significantly suppressed the formation of an SDS-resistant complex of α-synuclein and $A\beta_{1\text{–}40}$ (Figure 53.9A). In order to test the second possibility, β-synuclein (20 μM) was incubated with $A\beta_{1\text{-}40}$ (20 μM) under the physiological conditions (PBS, 37 °C). This study showed that Aβ aggregation was abolished after 5 days incubation (Figure 53.9B). These results were further supported by *in vitro* studies showing that β-synuclein inhibits the formation of Aβ fibrils (Figure 53.8). Studies are under way to elucidate the mechanisms by which β-synuclein blocks amyloidogenesis.

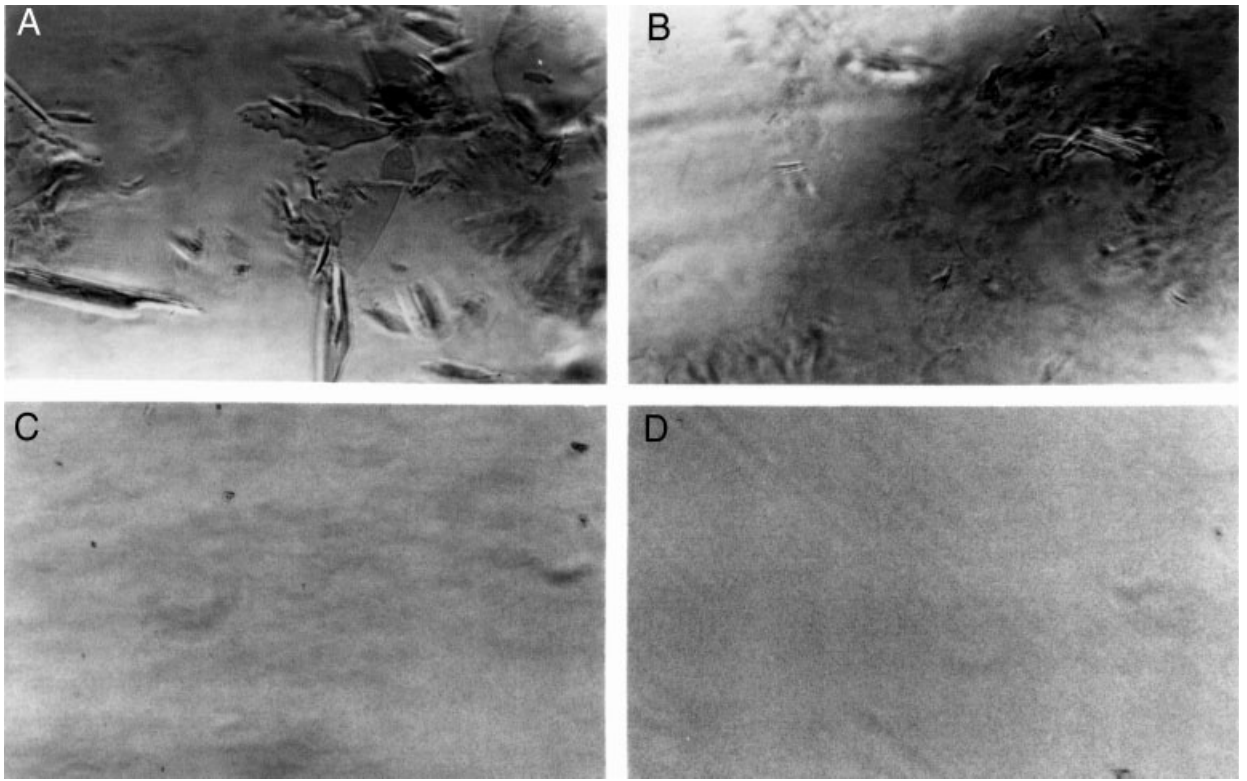

Figure 53.8. Inhibitory effects of β-synuclein on Aβ fibril formation. (A) Aβ (20 μM) alone. (B) Aβ (20 μM) with α-synuclein (20 μM). (C) Aβ (20 μM) with β-synuclein (20 μM). (D) Control

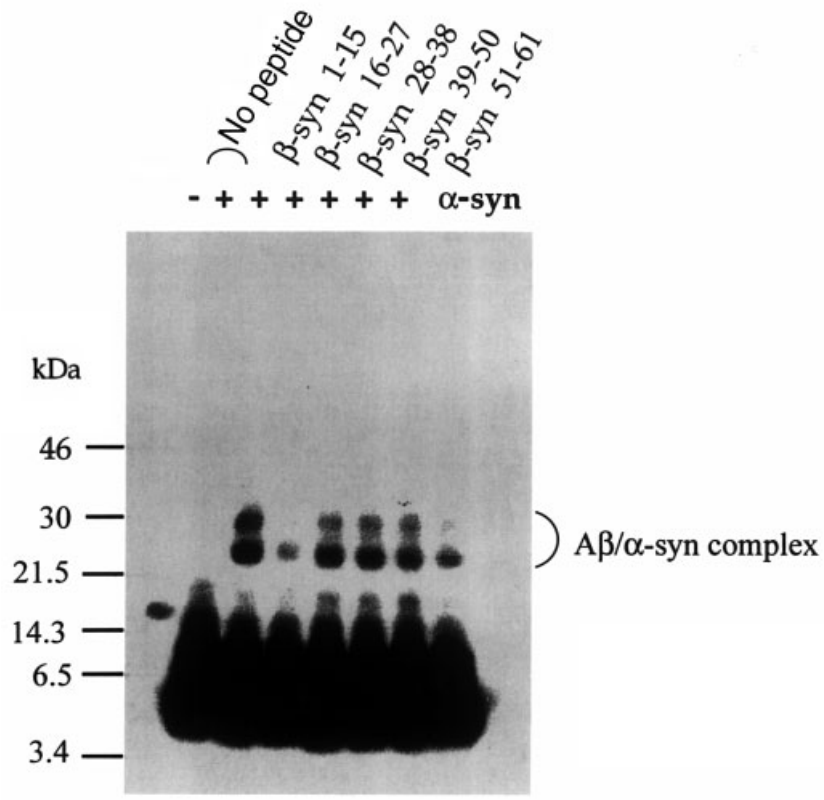

Figure 53.9. A peptide derived from β-synuclein inhibits interaction between α-synuclein and Aβ. Immunoblotting analysis showed that peptides corresponding to two distinct regions of β-synuclein (aa 1–15 and aa 51–60) significantly suppressed the formation of an SDS-resistant complex of α-synuclein and $A\beta_{1-40}$.

CONCLUSIONS

Although it has become evident that, in disorders such as LBD and AD, protein misfolding and aggregation leads to neurodegeneration, neither the underlying mechanisms, nor why this process is selective for specific groups of neurons and requires long periods of time, has been clarified. Understanding the role of factors promoting and inhibiting α-synuclein aggregation is of critical importance for understanding the pathogenesis of this group of disorders. Factors promoting aggregation include mutations, oxidative stress mediated by iron, cytochrome *c* or copper (II), failure of binding to lipid membrane vesicles caused by mutation, and binding to other amyloidogenic

molecules such as NAC. Recent evidence suggests that, in the same way that some conditions promote α-synuclein aggregation, there might exist endogenous factors blocking aggregation and amyloidogenesis. Thus, a critical balance between pro- and anti-aggregation factors might be at play in disorders such as LBD and AD. Studies are under way to identify these anti-aggregation factors, which might hold promise as targets for development of new treatments.

SUMMARY

The role of abnormal protein folding and aggregation in the central nervous system is being extensively explored as one of the pivotal mechanisms leading to neurodegeneration in disorders such as Alzheimer's disease (AD), Lewy body disease (LBD), Huntington's disease (HD) and Creutzfeldt–Jacob disease (CJD). Lewy body disease is a heterogeneous group of disorders presenting with parkinsonism, cognitive deficits, and formation of Lewy bodies (LBs). Recent studies have shown that α-synuclein is centrally involved in the pathogenesis of these disorders because this molecule is a major component of LBs, mutations in this molecule are associated with familial parkinsonism, and expression in transgenic mice and *Drosophila* mimics several aspects of disease. Identifying the role of factors promoting and/or inhibiting α-synuclein aggregation is of critical importance for understanding pathogenesis in this group of disorders. Factors promoting aggregation include mutations, oxidative stress and lipid binding, and binding to other amyloidogenic molecules, such as the non-amyloid β component of AD amyloid (NAC). Recent evidence suggests that, in the same way that some conditions promote α-synuclein aggregation, some endogenous factors might block aggregation and amyloidogenesis. Thus, a critical balance between pro- and anti-aggregation factors might be at play in disorders such as LBD and AD. Studies are under way to identify these anti-aggregation factors, which might hold promise as potential targets for development of new treatments.

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54 Neurofibrillary Degeneration: Patterns of Tau Isoform Expression

ANDRÉ DELACOURTE

INTRODUCTION

Tau proteins are the basic components of the pathological filaments that accumulate in neurons and glial cells affected by neurofibrillary degeneration (Iqbal et al., 1998; Buée et al., 2000). Tau pathology is observed in more than 20 different diseases, including Alzheimer's disease (AD), progressive supranuclear palsy (PSP), corticobasal degeneration (CBD), Pick's disease (PiD), myotonic dystrophy (MyoD), familial frontotemporal dementia with parkinsonism, associated with chromosome 17 (FTDP-17), etc. Tau is an outstanding marker, well correlated with clinical manifestations. Indeed, pathological tau proteins observed in the neocortical association areas are systematically associated with dementia. Also, tau pathology in the brain association areas is a degenerative process specific to humans. Taken together, tau pathology reveals precisely the intensity and the extent of the degenerative process (Delacourte and Buée, 2000). This pathological entity can be defined according to six different molecular parameters, which are presented here.

NEUROFIBRILLARY DEGENERATION (NFD)

A MODERN DEFINITION

NFD is a degenerative process, visualized at the histological level using silver stains, as demonstrated by Alois Alzheimer and colleagues at the beginning of the twentieth century (Alzheimer, 1907). This technique is still used for neuropathological examination, and reveals in detail the abnormal intracellular fibrils that accumulate in cell bodies and in neurites: neurofibrillary tangles, neuropile threads and dystrophic neurites of senile

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plaques. These lesions are composed of bundles of filaments that result from the aggregation of tau proteins. Using antibodies against tau, it has been shown that NFD is a degenerative process found in numerous neurodegenerative disorders. All approaches combined, the modern definition of neurofibrillary degeneration is as follows: a degenerating process characterized by the abnormal filamentous accumulation of tau proteins in neurons and glial cells.

PATHOLOGIES WITH NEUROFIBRILLARY DEGENERATION

NFD is observed in more that 20 other neurodegenerative disorders (Table 54.1). All these diseases are very different in that they are familial or sporadic, with different origins, from mutations on tau gene to traumatisms. NFD is a many-sided pathological process that can preferentially affect either subcortical nuclei or neocortical areas, neurons or, in addition, astrocytes or oligodendrocytes. The pattern of NFD lesions is also different and characteristic, according to the type of cell affected and the subcellular

Table 54.1. Presentation of the different neurodegenerative disorders with a tau pathology, and their different biochemical tau signatures, from Class I to Class IV

Diseases	Classes of tau pathology
Aging (hippocampal region, patients over 75 years)	I
Alzheimer's disease, familial and sporadic	I
Amyotrophic lateral sclerosis/parkinsonism–dementia complex of Guam	I
Argyrophilic grain dementia	
British type amyloid angiopathy	I
Corticobasal degeneration	II
Dementia pugilistica/autism with self-injury behavior	I
Down's syndrome	I
FTDP-17	II, I and III
Gerstmann–Sträussler–Scheinker disease (rarely)	I
Hallervorden–Spatz disease	
Inclusion body myositis	
Multiple system atrophy	
Myotonic dystrophy	IV
Niemann–Pick disease type C	I
Pick's disease	III
Presenile dementia with tangles and calcifications	
Prion protein cerebral amyloid angiopathy	
Progressive supranuclear palsy	II
Post-encephalitic parkinsonism	I
Subacute sclerosing panencephalitis	
Tangle only dementia	

location, such as the Pick bodies of Pick's disease (PiD) or the neuritic plaques of AD. At the electron microscopic level, the filamentous material of NFD is either helical (AD), twisted (PSP, CBD) or mainly straight (PiD) (Delacourte and Buée, 2000).

THE ROLE OF TAU PROTEINS

Tau proteins belong to the microtubule-associated proteins (MAP) family. The human tau gene is unique and located over 100 kb on the long arm of chromosome 17 at band position 17q21, and contains 16 exons. Exons 2, 3 and 10 are alternatively spliced and are adult brain-specific. In the human brain, the tau primary transcript gives rise to six mRNAs, three of them with exon 10. Translation of exon 10 adds a fourth repeated sequence, which is a binding site to tubulin dimers, the basic components of microtubules. The normal role of tau is to stabilize microtubules, which are the tracks of the intraneuronal transport. Stabilization of microtubules is dramatically increased by tau isoforms with four repeated binding sites (4R tau or tau E10+ isoforms). Conversely, phosphorylation of tau destabilizes microtubules, and it is suggested that abnormal phosphorylation, as observed in AD, provokes a collapse of the microtubule network and neurodegeneration (Buée et al., 2000).

THE PARAMETERS OF TAU PATHOLOGY IN THE HUMAN BRAIN

QUANTIFICATION OF TAU PATHOLOGY

Native tau proteins are normally phosphorylated on numerous serine or threonine sites. In a living cell, there are different pools of tau proteins with different states of phosphorylation. The more tau proteins are phosphorylated, the less they bind to the microtubules. Also, the state of phosphorylation of tau proteins is probably different according to the cell compartments. Tau are less phosphorylated in axons, as demonstrated by monoclonal antibody tau 1. Also, phosphorylation of tau proteins is developmentally regulated. Antibodies such as AT8, AD2 and PHF-1, which are well known in the field of AD to label tau pathology, also strongly label native tau proteins (reviewed in Buée et al., 2000).

AD biochemistry is performed on post mortem human brains. From the work of Matsuo et al. (1994), we know that native tau proteins are almost totally dephosphorylated during post mortem delays. Dephosphorylation results from the strong phosphatasic activity which is released after cell death. But in parallel, aggregated tau proteins that constitute brain lesions are not dephosphorylated, because the phosphatase enzymes are unable to access

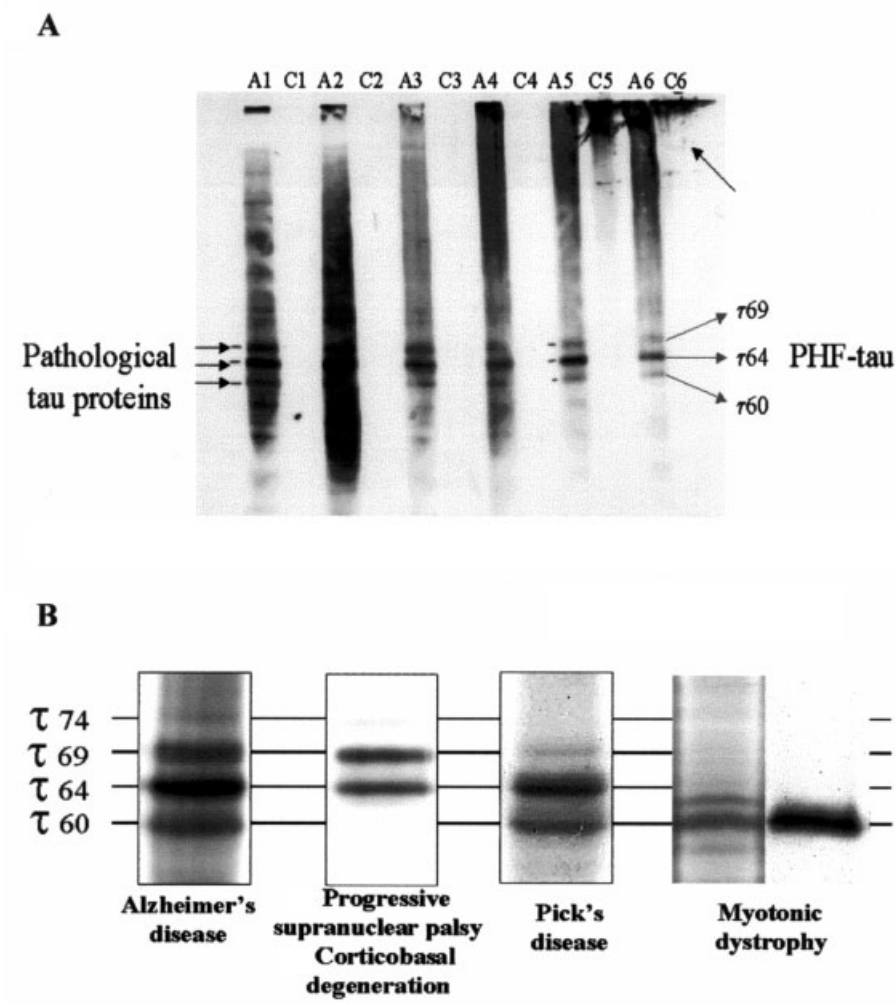

Figure 54.1. (A) Immunoblot detection of pathological tau protein in AD brain extracts, with AD2, a phosphodependent tau antibody, as described in Sergeant et al. (1999). Same amounts of total protein extracts were loaded in each well. The brain area analyzed is the parietal cortex. Controls were non-demented, aged-matched cases. Pathological tau proteins are detected, with a molecular mass of 60, 64 and 69 kDa, exclusively in AD brain extracts. Note the intensity of tau pathology, which is different among the AD patients. A1–A6, Alzheimer's disease parietal cortex samples; C1–C6, control cortex samples. (B) The different tau signatures in several disorders. Note the main triplet of pathological tau proteins in AD, the upper doublet in progressive supranuclear palsy, the lower doublet in Pick's disease and the main Tau 60 in myotonic dystrophy

phosphorylated sites that are buried deep in brain lesions. Therefore, antibodies such as AT8 or AD2 (Buée-Scherrer et al., 1996b) specifically detect tau pathology on post mortem brain tissues (Figure 54.1).

As shown in Figure 54.1A, a characteristic triplet of pathological tau proteins is exclusively detected in AD brain extracts from polymodal association brain areas, while no trace of tau pathology is observed in the same brain areas from non-demented aged-matched controls. Pathological tau proteins from AD, named PHF-tau, are composed of three main electrophoretic variants designated tau 60, 64 and 69, as a function of their molecular mass. A minor fourth band, named tau 74, is also detected at 74 kDa (Mulot et al., 1994; Sergeant et al., 1999). Furthermore the intensity of the detection is proportional to the intensity of NFD. Therefore, a semi-quantification by Western blot is able to easily detect and quantify NFD, in good agreement with immunohistochemical observations.

DIFFERENT BIOCHEMICAL SIGNATURES IN AD, PSP, PiD, MyoD

As shown in the previous section, a characteristic triplet of electrophoretic bands is detected in AD brain homogenates. A different but also characteristic pattern is observed in other diseases with pathological tau and with tau-positive brain lesions (Figure 54.1B). Indeed, we demonstrated that pathological tau proteins from progressive supranuclear palsy (PSP) are composed of a main doublet (tau 64, tau 69; Flament et al., 1991) and a minor tau, 74 (Sergeant et al., 1999), while those from Pick's disease are composed of another doublet (tau 60, 64) and a minor tau, 69 (Delacourte et al., 1998).

Myotonic dystrophy (MyoD), a familial disease with abnormal CTG repeats on chromosome 19, is characterized by a tau pathology with a major band of 60 kDa. Electrophoretic bands at 64 and 69 kDa are also found, but in lower amounts (Vermersch et al., 1996).

TAU ISOFORMS IN BRAIN LESIONS: CLASSES I–IV

The analysis of the tau isoform content, using specific immunoprobes against isoforms with the peptidic regions expressed by exons 2, 3 or 10, combined with 2D gel electrophoreses and Western blots, enabled us to characterize the different patterns of pathological tau. Specific sets of tau isoforms aggregate to constitute four main classes of brain lesions: AD (all six isoforms); PSP/CBD (three $E10^+$ isoforms) (Sergeant et al., 1999); PiD (three $E10^-$ isoforms) (Delacourte et al., 1998) and MyoD (mainly the shortest tau) (Figure 54.2). These different patterns can be reconstructed in cellular models (Mailliot et al. 1998).

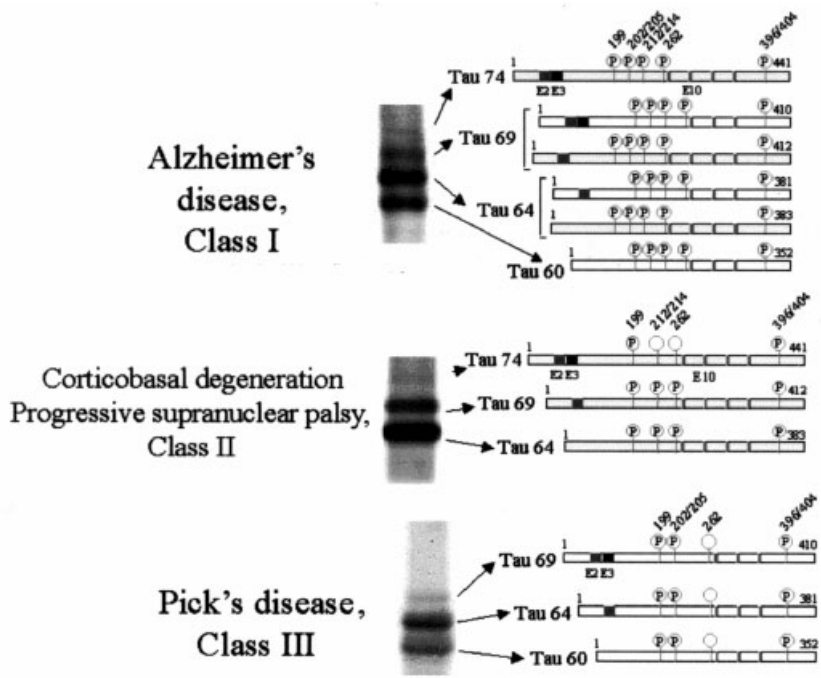

Figure 54.2. The correspondence between the biochemical signature of tau pathology and the tau isoform content. Note that PHF-tau from AD made up the six isoforms, while only 4R-tau isoforms are found in tau filaments of PSP and 3R-tau isoforms in the Pick's bodies

These different classes of tau isoform patterns can be explained at two levels:

1. Neuronal subsets could express specific sets of tau isoforms, e.g. it has been shown that the granule cells of the dendate gyrus do not normally express $E10^+$ tau isoforms. If a given neuronal subset degenerates, the corresponding set of tau isoforms of this neuronal subset will aggregate to constitute a characteristic tau lesion, e.g. neurons that express only $E10^-$ tau isoforms will produce Pick bodies if they are affected by NFD (Delacourte et al., 1998).
2. On the other hand, $E10^+$ tau isoforms are able to dramatically increase the stability of microtubules and therefore to modify the physiological properties of the cell. It is likely that the expression of $E10^+$ tau isoforms can be dysregulated in several diseases, due to an abnormal alternative splicing. This dysregulation is demonstrated in FTDP-17, following a pathogenic mutation in the intron 10 regulating the splicing of exon 10 (reviewed in Delacourte and Buée, 2000). This abnormal processing of

intron 10 could also be boosted by polymorphisms on the tau gene, such as those found in PSP (Conrad et al., 1997).

Together, we observe a number of important neurodegenerative diseases, with different etiologies and classes of tau pathology (Table 54.1). They have all in common a cognitive impairment when tau pathology is present in brain association areas. For some of them, tau is the etiological agent (FTDP-17). For many other diseases, tau pathology is not only a marker but also a motor of the degenerative process (AD, PSP, CBD) (Delacourte and Buée, 2000). For dementia pugilistica, tau pathology is probably a consequence, since the etiology is related to trauma.

HYPER- AND ABNORMAL TAU PHOSPHORYLATION IN DISEASES WITH NEUROFIBRILLARY DEGENERATION

Tau Proteins in AD Brains are Hyperphosphorylated

Tau pathology in the brain at autopsy is visualized with antibodies against phosphorylated sites on tau. However, these phospho-dependent antibodies reveal an aggregation of tau but certainly not an abnormal phosphorylation, as frequently mentioned in the literature. Indeed, a Western blot analysis of a biopsy sample from a human brain reveals a tau triplet that is similar to the triplet of AD. The major difference is that no smears of aggregated tau are observed in the biopsy brain tissue. Following these observations, the question was to determine whether tau proteins in the AD brain are really hyperphosphorylated, as frequently suggested (Buée et al., 2000).

Sergeant et al. (1995) have been able to demonstrate directly the hyperphosphorylation of tau in AD brains, by comparing on 2D gel the isoelectric pattern of tau proteins from a biopsy of a normal human brain tissue with an autopsy sample of an AD brain. The 2D electrophoresis revealed that AD tau proteins are more acidic, and therefore more phosphorylated, than normal native biopsy tau proteins. These results unambiguously demonstrate that pathological tau proteins in AD are hyperphosphorylated (Sergeant et al., 1995).

Tau Proteins are Abnormally Phosphorylated in Alzheimer's Disease

A few sites on tau proteins have been shown to be phosphorylated on PHF-tau, but not present on native tau (from biopsies). These sites are: Ser 212/Ser214, detected by AT100; Ser 231/Ser235, detected by PHF-27 and TG3; and Ser 422, detected by AP422 or Ab988. These monoclonal antibodies demonstrate the aberrant phosphorylation that occurs in numerous neurodegenerative disorders (reviewed in Buée et al., 2000).

Some Phosphorylation Sites are Disease-specific

Many normal phosphorylation sites, such as AT8 or AD2, are observed in all tau lesions of neurodegenerative diseases presented in Table 54.1. In the same way, phosphorylated pathological epitopes such as Ser422 are found in all tau lesions, whatever the pathology (Bussière et al., 1999). However, there are some interesting specificities. Indeed, it has been shown at the immunohistological and biochemical levels that phosphorylated Ser262 is present in tau lesions of numerous neurodegenerative disorders, but not in the Pick bodies of PiD (Probst et al., 1996; Delacourte et al., 1998). Also, oligodendrocyte tau inclusions in multiple system atrophy do not contain pathological sites of phosphorylation (Cairns et al., 1997).

SPATIOTEMPORAL DISTRIBUTION

The mapping of the spatiotemporal distribution of tau pathology in the different brain areas is important to understand how the disease spreads in the brain. Indeed, there is a precise biochemical pathway of tau pathology in aging and in AD. The progression of tau pathology is sequential, invariable, hierarchical and predictable. Ten stages (S1–S10) were defined, corresponding to the 10 brain areas sequentially affected. The degenerative process always starts in the hippocampal region (S1, transentorhinal cortex; S2, entorhinal cortex; S3, CA1 region of the hippocampus), followed by the temporal cortex (S4, temporal pole; S5, inferior temporal cortex; S6, mid-temporal cortex), then the polymodal brain association areas (S7–S8), and finally the primary regions (motor cortex and/or the occipital cortex) as well as many subcortical nuclei (S9–S10) (Delacourte et al., 1999).

These data show that tau pathology is systematically observed in the normal population aged over 75 years. They also demonstrate that tau pathology sometimes occurs independently of amyloid deposits and that the hippocampal area is the most vulnerable area of the human brain. The hippocampal vulnerability is probably a springboard for AD pathology, namely amyloid precursor protein dysfunction, which will exacerbate and extend tau pathology in other brain areas (Figure 54.3). Taken together, the neuropathological and biochemical data indicate that tau pathology is instrumental in AD.

From these data, it is now possible to precisely quantify tau pathology and amyloid deposition at the biochemical level and to determine the criteria to establish a biochemical diagnosis of AD (CEBDAD), that separate aging from preclinical AD, and preclinical AD from clinical AD (Delacourte et al., 1999). In that respect, it should be pointed out that recent criteria for the neuropathological diagnosis have rehabilitated tau pathology, in good agreement with Alois Alzheimer's observations and the natural history of AD (Working Group, 1997).

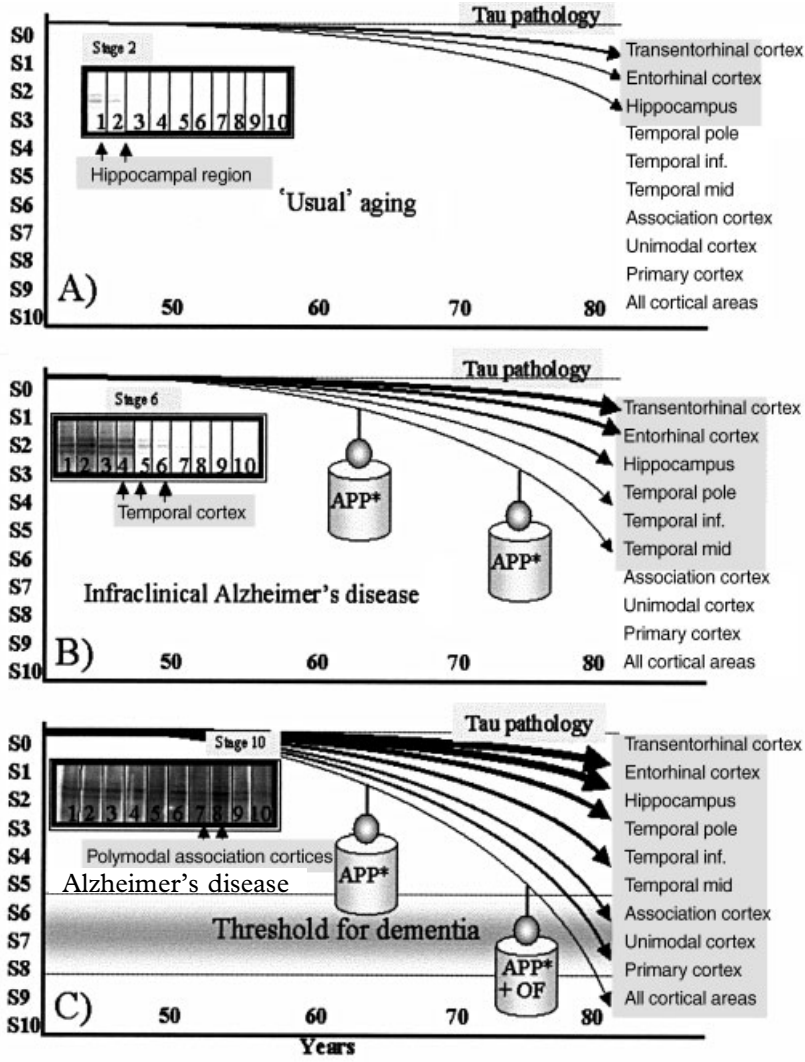

Figure 54.3. The biochemical pathway of tau pathology in aging and Alzheimer's disease (AD). Inserts: Western blot analysis in the 10 brain areas that are successively affected in AD (Delacourte et al., 1999). (A) Tau pathology is systematically observed in the transentorhinal cortex (stage 1), or in addition in the entorhinal cortex (stage 2), and CA1 of hippocampus (stage 3) in non-demented patients aged over 75 years. (B) Tau pathology can be found in other brain areas, along a stereotypical pathway, at the infraclinical stage of AD (stages 4–6). (C) All brain areas are affected by tau pathology at the last stage of AD. The clinical symptoms occur when polymodal association areas are affected (stage 7). Hypothesis: the spreading of tau pathology is fuelled by APP dysfunctions (APP*), and also, progressively, by other factors (OF), such as microglial reaction, inflammation and oxidative stress

The pathway of tau pathology in PSP is different from that in AD, and is roughly opposite, emerging from the subcortical nuclei toward the neocortex, and especially the frontal motor cortex. The specific pattern of tau pathology in PSP, with the upper tau doublet (tau 64, 69), is observed in all brain areas affected, from the subcortical nuclei to the frontal neocortical regions (Vermersch et al., 1994; Buée-Scherrer et al., 1996a).

In all types of neurodegenerative disorders, the spreading of tau pathology follows specific neuronal connections, like a precise neuronal chain reaction. There is also probably a 'dynamic' of spreading, fuelled by different factors, each of them being a lead for neuroprotection (Delacourte, 2000).

THE GENETIC PROFILE OF TAU PATHOLOGY

Tau Pathology as an Etiological Agent

The presence of brain lesions in neurodegenerative disorders usually prompts the same question: is this a cause or a consequence? Some answers are already available for tau pathology. The discovery that tau mutations are directly involved in numerous FTDP-17 cases has been dramatically documented. More than 20 different mutations have been spotted. Most of the pathogenic mutations are responsible for an increase of 4R tau isoforms, giving a class II tau pathology. 4R tau isoforms, with the additional peptide sequence of exon 10, have a much stronger affinity toward microtubules than 3R isoforms, and an excess could modify microtubule properties, which will be stiffer and less dynamic. The other mutations are missense mutations, which also affect microtubule polymerization and stability by decreasing tau–microtubule binding and lead to one of the class I, II or III tau pathologies. The striking feature of these familial tauopathies is the heterogeneity of the phenotype, which results from the different effects on tau (overexpression, loss of function). Surprisingly, for the same mutation in the same family, different onsets and different phenotypes can be observed, showing that numerous additional factors are modulating the clinical and neuropathological phenotypes (reviewed in Delacourte and Buée, 2000).

We note that the neuropathological profile in FTDP-17 is quite different from AD, with a special involvement of astrocytes and cortical white matter, but also with an important heterogeneity for each mutation.

However, not all diseases with a tau pathology have mutations on the tau gene. This has been verified for CBD, PSP, amyotrophic lateral sclerosis/parkinsonism dementia complex of Guam, and AD.

Tau as a Genetic Risk Factor

Conrad et al. (1998) identified a polymorphic dinucleotide repeat sequence in a Caucasian population with PSP. This polymorphism, named A0,

corresponding to an 11 TG dinucleotide repeat in intron 9 of the tau gene, is found in 95% of the PSP cohort (95.5%) and only in 57% of normal controls and 50% of patients with AD. Recently, these data were confirmed by several studies and extended to a haplotype, including a number of polymorphisms in linkage disequilibrium with A0 and named H1. This haplotype corresponds to A0 polymorphism, numerous single nucleotide polymorphisms along the entire tau gene and one intronic 238 bp deletion flanking exon 10. These polymorphisms may influence exon 10 splicing and thus the proportion of 4R:3R tau isoforms, leading to a class II tau pathology. It should be noted that these A0 polymorphisms or H1 haplotypes were recently described in other pathologies including CBD and Parkinson's disease. Some other polymorphisms in the tau gene were also described as being associated with a risk of AD, but these data are still controversial (reviewed in Delacourte and Buée, 2000).

SUMMARY

Tau is an outstanding biochemical marker of neurofibrillary degeneration (NFD), well correlated with clinical manifestations. Tau pathology concerns many familial or sporadic neurodegenerative disorders, such as Alzheimer's disease (AD), progressive supranuclear palsy (PSP), corticobasal degeneration (CBD), Pick's disease (PiD), FTDP-17, myotonic dystrophy (MyoD) and many other diseases. Molecular parameters of tau pathology help to elucidate the involvement of tau in the physiopathology of these diseases and to set up diagnoses and therapeutic strategies. Six main features define tau pathology: (1) the quantitative aspects, using Western blots; (2) the different biochemical signatures observed in AD, PSP, CBD, PiD, MyoD (one to four immunodetected electrophoretic bands); (3) the specific tau isoform content of brain lesions; indeed, 'disease-specific' sets of tau isoforms aggregate to constitute four main categories of tau lesions—class 1, AD (all six isoforms); class 2, PSP/CBD (three exon10^{+} isoforms); class 3, PiD (three exon10^{-} isoforms); and class 4, MyoD, mainly, and sometimes exclusively, the shortest tau isoform; (4) the different states of phosphorylation of tau, with hyper- and abnormal phosphorylation, as well as disease-specific sites of phosphorylation (e.g. ser262); (5) the spatiotemporal progression of tau pathology, which is extremely well correlated with cognitive impairment; (6) the genetic aspects, with pathogenic tau mutations in FTDP-17 or characteristic polymorphisms in PSP. These results emphasize two important physiopathological points. First, neuronal populations are probably distinguished by the expression of different sets of tau isoforms. A dysregulation in the expression could generate vulnerability and neurodegeneration. Second, there is a 'dynamic process' that fuels the precise, sequential and hierarchical spreading of tau pathology in brain areas, along corticocortical projections. This process should be a

target for neuroprotection. Taken together, tau pathology is a many-sided degenerative process that will open diagnostic and therapeutic avenues.

CONCLUSION

Taken together, the correlation between the distribution of tau pathology and clinical manifestation is excellent if we take into account the extent and the function of the brain areas that are affected. In AD, dementia is observed at stage 7 or above, when polymodal brain association areas are affected. In PSP and CBD, dementia is always observed when tau pathology is found in the frontal neocortical areas.

Other parameters of tau pathology might be important, such as the extent of tau phosphorylation and the location of the phosphorylated sites. Other post-translational events, such as glycosylation, that modulate tau functions or sorting, have still to be investigated (Buée et al., 2000). Tau pathologies are also influenced at the genetic level, either by polymorphisms or directly driven by mutations on the tau gene. In conclusion, tau pathology is a many-sided degenerative process that will open diagnostic and therapeutic avenues.

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55 Phosphorylation, Microtubule Binding and Aggregation of Tau Protein in Alzheimer's Disease

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INTRODUCTION

Tau is a protein that was first characterized as a microtubule associated protein (Weingarten et al., 1975). However, it may also be localized at the cell membrane (Brandt and Lee, 1993) and, in pathological conditions, it forms aberrant aggregates like those found in the brains of victims of Alzheimer's disease (AD) and other tauopathies (Goedert and Spillantini, 1990). The binding of tau to microtubules is regulated by phosphorylation (Lindwall and Cole, 1984). Tau with low-level phosphorylation, or unphosphorylated, binds with a low affinity to microtubules, moderately phosphorylated tau binds with a higher affinity to microtubules, whereas hyperphosphorylated tau, like that present in AD, shows a very decreased capacity for microtubule binding (Kosik, 1992; García de Ancos et al., 1993). This hyperphosphorylation takes place at residues located in the flanking regions of the tubulin-binding domain and, to a much lesser extent, inside this domain (Morishima-Kawashima et al., 1995).

Tau associated with the membrane is not phosphorylated at its proline-rich region (Arrasate et al., 2000), suggesting that such an association does not take place in AD, since this region is modified in the disease. It has also been indicated that tau dephosphorylated in this region is present mainly in the axonal compartment of neurons (Papasozomenos and Binder, 1987). Additionally, tau found in the aberrant aggregates present in AD neurons is also phosphorylated, as previously indicated (Grundke-Iqbal et al., 1986).

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It is thus clear that phosphorylation plays a key role in the regulation of different tau functions. In this work we will focus mainly on the study of tau aggregation and association to microtubules in some conditions.

IN VITRO TAU AGGREGATION

About 15 years ago, it was reported that tau protein purified from brain extracts was able to form fibrillar polymers *in vitro* (Montejo de Garcini et al., 1986). This result was subsequently reproduced using recombinant (unmodified) tau (Crowther et al., 1989; Wille et al., 1992). A subsequent study suggested that glycosaminoglycans (GAGs) such as heparin favor this *in vitro* assembly of tau polymers (Goedert et al., 1996; Pérez et al., 1996). Moreover, when heparin was microinjected into SH5Y neuroblastoma cells, aggregates containing tau and heparin were observed (Figure 55.1). When the *in vitro* experiments were repeated with phosphorylated tau, it was found that phospho-tau is also able to assemble under these conditions (Goedert et al., 1996). However, we observed a four-fold reduction in the capacity for assembly for phospho-tau, compared with recombinant unmodified tau, in those conditions where heparin was added. This result suggests that under some conditions phospho-tau could have a higher capacity for assembly, but in the presence of the anionic compounds (e.g. GAG) assembly could be reduced. It has also been found that some mutations in the tau gene observed in frontotemporal dementia associated with chromosome 17 (FTDP-17) could also favor tau self-aggregation (Arrasate et al., 1999).

OVEREXPRESSION OF TAU KINASE-1 IN MOUSE AND ITS CONSEQUENCES FOR TAU PHOSPHORYLATION

Different protein kinases have been involved in the pathological hyperphosphorylation observed in AD (Morishima-Kawashima et al., 1995). Among them, tau kinase 1 (or *GSK3*) (Ishiguro et al., 1993) is probably the one that modifies the largest number of sites in the tau molecule (Morishima-Kawashima et al., 1995), and thus it would be desirable to have an animal model exhibiting overexpression of *GSK3*. GSK-3β is known to play a crucial role in early development. Accordingly, GSK-3β knockout mice die during embryonic life (Hoeflich et al., 2000). Similarly, previous attempts by Miller and collaborators to generate GSK-3β transgenic mice (with either ubiquitous or CNS-specific promoters) failed to yield detectable overexpression of GSK-3β, presumably due to the toxicity of GSK-3β overexpression during embryonic development (Brownlees et al., 1997). This prompted these authors to suggest a conditional transgenesis approach to achieve substantial

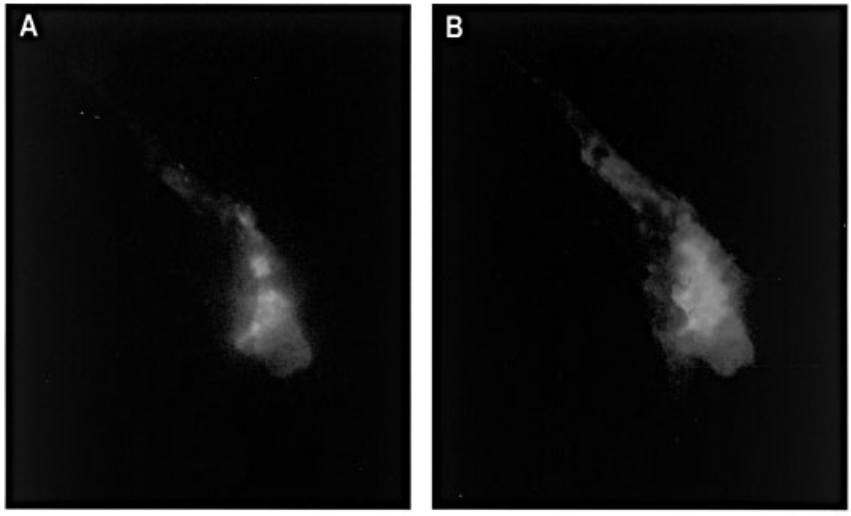

Figure 55.1. Double immunofluorescence of FITC–heparin loaded SH-SY5Y differentiated cells and PHF-1 anti-tau antibody. Human neuroblastoma SH-SY5Y cells seeded on poly-L-lysine coated coverlips were differentiated in neurobasal medium supplemented with B-27 (NB/B-27) and 1 mM dibutyryl cyclic AMP (dbcAMP) for 4 days. The loading of FITC–heparin was adapted from the method described by Okada and Rechsteiner (1982), based on the osmotic lysis of pinocytic vesicles. Briefly, differentiated SH-SY5Y cells were treated with NB/B-27 buffered with 10 mM Hepes (N-[2-hydroxyethyl] piperazine-N′-[2-ethanesulfonic acid]) pH 6.8 for 1 h and then exposed to a range of different concentrations of FITC-heparin (Molecular Probes, Leiden, The Netherlands), prepared in 8% PEG 600 in DEMEM, for 1 h in the CO_2 incubator at 37 °C. An osmotic shock was administered by incubation in a hypotonic medium (DEMEM: sterile distilled water, 6:4), for 10 min. The cells were then recovered in DEMEM supplemented with 2% FCS for 90 min, rinsed with PBS and methanol-fixed and immunostained. (A) Heparin localization inside a differentiated SY5Y cell. (B) The same cell stained by PHF-1 mouse monoclonal antibody (gift of Dr Davies, Albert Einstein University, Bronx, NY) employed directly to stain tau aggregates, using goat antimouse Texas red as secondary antibody (Molecular Probes, Leiden, The Netherlands), diluted 1:400. After washing in PBS, the coverslips were mounted in Fluoromount (Southern Biotech. Assoc. Inc.), observed using a Zeiss Axiovert fluorescence microscope and photographed using 400 ASA Neopan Fujifilm

overexpression of GSK-3β. By using the tet-regulated system, we have now generated conditional transgenic mice that overexpress GSK-3β in the forebrain during adulthood, while avoiding perinatal lethality (a similar approach was recently used by some of us to generate the first conditional model of Huntington's disease; Yamamoto et al., 2000). Transgene expression is restricted to the forebrain by use of the CamKIIa promoter, while a pulse of tetracycline administered to pregnant mothers is used to

silence embryonic transgenic expression and avoid the lethality observed in the absence of tetracycline.

In vivo overexpression of GSK-3β results in increased phosphorylation of tau, as detected with antibodies raised against AD tau. Hyperphosphorylated tau was found in somatodendritic localization, similar to the compartmentalization of tau previously observed in tau transgenic mice. However, the change in subcellular localization of tau that we observed was not dependent on increased levels of tau, as is the case in tau transgenic mice. In our conditional GSK-3β transgenic mice, there is no change in the total level of tau, and the change in subcellular localization is due exclusively to the hyperphosphorylation of tau by GSK-3β. Increased phosphorylation of tau results in decreased affinity of tau for microtubules, as evidenced by the fact that we are able to detect tau in somatodendritic localization using antibody 7.51 in the conditional GSK-3β transgenic mice. This antibody recognizes the tubulin-binding domain of tau and therefore, recognizes tau only when it is not bound to microtubules (Figure 55.2). Our *GSK3* transgenic mice thus reproduce two of the characteristics of AD tau, viz. hyperphosphorylation and decreased interaction with microtubules. However, the aberrant tau aggregation found in AD was not observed in these *GSK3* transgenic mice.

OVEREXPRESSION OF HUMAN TAU BEARING FTDP-17 MUTATIONS

Recent work suggests that FTDP-17 mutations associated with the tau gene facilitate self-aggregation of the protein (Arrasate et al., 2000), and thus we have generated a transgenic mouse bearing three of these mutations (G272V, P301L and R406W) simultaneously, on the basis that their effects would be cumulative.

In our preliminary analysis of transgenic mice bearing mutant human tau, we have observed accumulation of high levels of the mutant protein specifically in the hippocampus and the cortex, closely resembling the spatial pattern of tau lesions in FTDP-17 and AD. The accumulated tau is highly hyperphosphorylated, and is detected by a number of phosphorylation-specific anti-tau antibodies traditionally used as markers in AD diagnosis. Relatively low levels of tau were observed in the spinal cords of these transgenic mice and no motor deficits are apparent (up to 10 months of age), which will facilitate the testing of any cognitive and memory defects caused by the transgene.

AGGREGATION OF MODIFIED TAU

In AD, three main features of tau pathology are apparent: a hyperphosphorylation of the protein, decreased binding to microtubules, and the

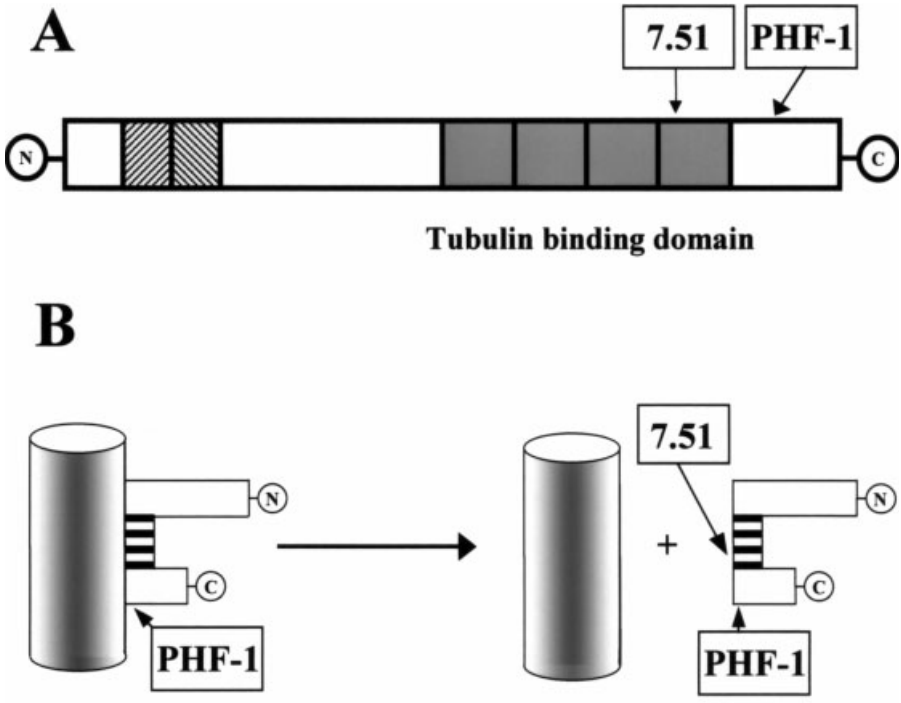

Figure 55.2. Reaction of 7.51 antibody with neurons from *GSK3* transgenic mouse. (A) Schematic representation of tau molecule, with recognition sites for antibodies 7.51 (in the tubulin binding domain) and PHF-1 (phosphorylation-dependent). (B) When tau is bound to microtubules 7.51 antibody epitope is not exposed. PHF-1 antibody epitope, on the contrary is outside the tubulin-binding domain. Thus, PHF-1 antibody can recognize tau, either bound or unbound to microtubules. Immunohistochemistry with 7.51 antibody in brain sections from *GSK3* transgenic mouse revealed an increase in unbound tau

formation of aberrant aggregates. By using as a model transgenic mice in which *GSK3* or tau (bearing FTDP-17 mutations) are overexpressed, the abnormal phosphorylation and decreased binding to microtubules were reproduced in these models, but so far the formation of aberrant aggregates resembling those found in AD patients has not been observed. This result could suggest that in addition to phosphorylation, other factors could be needed to promote the aberrant self-assembly of tau. Our preliminary results suggest that one of these factors is a product resulting from lipid peroxidation. In the presence of this compound, phosphorylated but not unmodified tau is able to self-assemble. Since it has been proposed that amyloid beta protein (Aβ) could promote the formation of oxidative reactions resulting in lipid peroxidation (Huang et al., 1999) and that it could also stimulate *GSK3*

activity (Alvarez et al., 1999), tau aggregation could result from the combination of those two features (Perez et al., 2000).

SUMMARY

In AD there are three main features for tau pathology, a hyperphosphorylation of the protein, a decreased binding to microtubules and the formation of aberrant aggregates. To elucidate the mechanism for tau pathology, we have tried to reproduce these features *in vitro* or by using transgenic mouse models.

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56 Phosphorylation of Protein Tau and Rescue of Protein Tau-induced Axonopathy by GSK-3β in GSK-3β×htau40 Double Transgenic Mice

KURT SPITTAELS, CHRIS VAN DEN HAUTE, JO VAN DORPE, HUGO GEERTS AND FRED VAN LEUVEN

INTRODUCTION

Biochemical and structural analysis of the phosphorylation sites of human protein tau of paired helical filaments (PHF) in brain of Alzheimer's disease (AD) patients revealed that many sites consist of serine or threonine residues followed by a proline residue. This observation focused attention on proline-dependent kinases (Hasegawa et al., 1992). In the brain of AD patients, NFT were demonstrated to be immunoreactive for glycogen synthase kinase-3β (GSK-3β), referring to a potential association with protein tau (Yamaguchi et al., 1996; Shiurba et al., 1996), and identifying GSK-3β as one of the possible kinase candidates. In addition, active GSK-3β is found to accumulate in pre-tangle and tangle-bearing neurons in AD (Pei et al., 1999). Phosphorylation by GSK-3β of bovine (Ishiguro et al., 1992) and human protein tau (Mandelkow et al., 1992) in cell-free systems, resulted in phosphorylation patterns of protein tau that resembled those of the protein isolated from PHF from AD brain. Further evidence for GSK-3β as a potential protein tau kinase has been obtained in transfected cells, in which co-transfection of GSK-3β with protein tau increased its phosphorylation concomitant with loss of prominent bundles of microtubules (Wagner et al., 1996).

The involvement of GSK-3β in the hyperphosphorylation of protein tau, both in cultured neurons and *in vivo* in brain, was indirectly supported by the finding that lithium, as an inhibitor of GSK-3β, caused tau dephosphorylation at the sites recognized by antibodies Tau-1 and PHF-1, which are two of the

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major epitopes typically associated with PHF in AD brain (Muñoz-Montaño et al., 1997; Hong et al., 1997).

Despite the wealth of data *in vitro*, convincing evidence demonstrating the phosphorylation of protein tau by GSK-3β and the functional repercussions it causes *in vivo*, is lacking. Solid support for the hypothesis that GSK-3β is an effective protein tau kinase *in vivo*, is presented here in single human GSK-3β[S9A] transgenic mice and in double transgenic mice, additionally expressing human protein tau.

MATERIALS AND METHODS

GENERATION OF TRANSGENIC MICE

A constitutively active form of the human kinase, i.e. GSK-3β[S9A], with serine at position 9 replaced by alanine, was used to design an expression cassette that was microinjected into 0.5 day-old FVB/N prenuclear mouse embryos. The GSK-3β transgenic mice were crossed with mice that overexpressed the longest isoform of human protein tau (htau40) (Spittaels et al., 1999) to obtain double transgenic animals. In both constructs, the transgene cDNA was placed under the control of an adapted mouse thy1 gene promoter to steer expression to neurons of the central nervous system. Use of the same promoter to overexpress both cDNAs yielded the co-localization of both transgene products.

SENSORIMOTOR TESTS

Homozygous htau40 transgenic mice, denoted as htau40 HH, and double htau40 HH×GSK-3β transgenic mice, were subjected to three sensorimotor tests to assess muscle strength, endurance, coordination and equilibrium (Lamberty and Gower, 1991). Single and double transgenic mice, 2–4 months old, were used in the 'forced swimming' and 'inverted wire-grid hanging' test, as described previously (Spittaels et al., 1999). In the 'uprighting reflex' test, we scored the time that the mice needed to return to the upright position after being forced to lie on their backs.

TISSUE EXTRACTIONS AND WESTERN BLOTTING

To prevent phospho-epitopes from being destroyed by dephosphorylation, all tissues were stored at −70 °C immediately after dissection, homogenized on ice and centrifuged at 4 °C. The supernatant was rapidly stored at −70 °C after snap-freezing in liquid nitrogen.

Brain hemispheres and spinal cords were homogenized as described (Spittaels et al., 1999). After centrifugation, portions of the supernatant

were denatured and reduced before separation on Tris–glycine-buffered polyacrylamide gels (8% SDS–PAGE) and transferred to nitrocellulose filters (Spittaels et al., 1999). To eliminate reaction of the secondary antibody with mouse immunoglobulins in Western blotting, brain homogenates were treated with immobilized protein G at 4 °C for 2.5 h and centrifuged before use.

We used Tau-5 as a phosphorylation-independent monoclonal antibody against protein tau. Monoclonal antibodies directed against phosphorylated protein tau epitopes were AT-8, AT-180, PHF-1 and AD-2.

BINDING OF PROTEIN TAU TO ISOLATED MICROTUBULES (Spittaels et al., 2000)

Taxol-dependent isolation and re-assembly of microtubules and microtubule-associated proteins were performed essentially as described (Vallee, 1982). Briefly, mouse brain hemispheres were homogenized on ice in microtubule assembly buffer (MT-buffer) and the supernatant cleared by centrifugation. Taxol (20 μM final) and GTP (1 mM final) were added to the supernatant. In some experiments, LiCl was added to inhibit GSK-3β activity during tissue processing. The mixtures were incubated at 37 °C for 60 min and the microtubules collected by centrifugation through a cushion of 5% sucrose in MT-buffer containing 20 M taxol. The pellets containing the taxol-stabilized microtubules and associated MAPs were washed in MT-buffer with taxol, re-pelleted and dissolved in MT-buffer.

Equal amounts of proteins were loaded on 8% SDS–PAGE gels after removal of mouse immunoglobulins. Densitometric quantification of Western blots of protein tau was performed as described (Spittaels et al., 1999) and the results were normalized for neuron-specific tubulin and Tau-5.

HISTOCHEMISTRY AND IMMUNOHISTOCHEMISTRY

The mice were anaesthetized with nembutal and transcardially perfused with paraformaldehyde (4%, v/v). The brain and spinal cord were immersion-fixed overnight, cut sagittally into two hemispheres or transversely into four tissue blocks of 9 mm, respectively, dehydrated and embedded in paraffin wax.

ULTRASTRUCTURAL ANALYSIS

For transmission electron microscopy, see Spittaels et al. (1999).

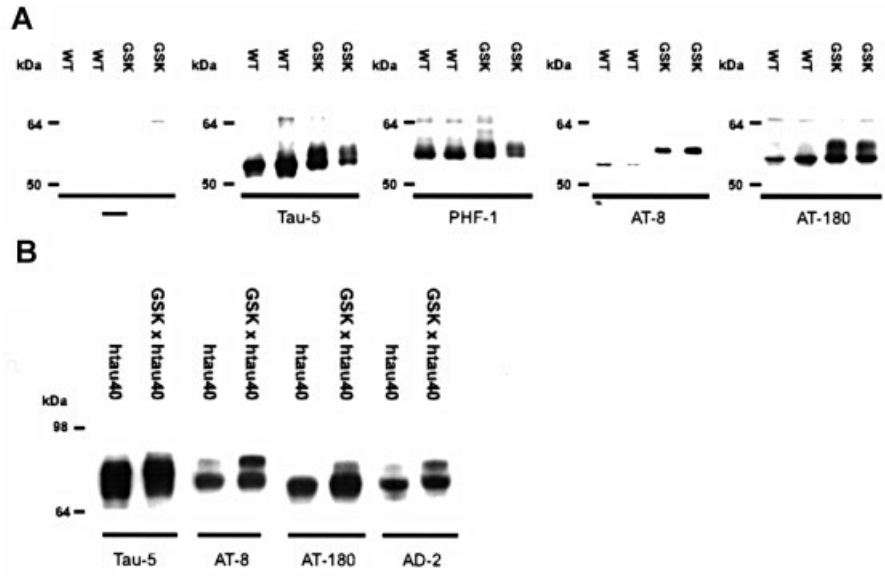


Figure 56.1. Protein tau is hyperphosphorylated in brain of GSK-3β[S9A] transgenic mice. (A) and (B) Western blotting of brain extracts of single GSK-3β[S9A] (A) and double GSK-3β[S9A]×htau40 (B) transgenic mice. In (A) each panel compares brain extracts from two wild-type (WT) and two GSK-3β[S9A] transgenic (GSK) mice. Immunoblots demonstrate retardation in electrophoretic mobility (Tau-5 and PHF-1) and increased immunoreaction with phosphate-dependent antibodies (AT-8 and AT-180) of phosphorylated protein tau. Brain homogenates were purified from mouse IgG prior to electrophoresis, as demonstrated by incubation of the blot with only secondary antibody (−). (B) Brain extracts from htau40 transgenic mice and double htau40×GSK-3β[S9A] transgenic littermates show increased phosphorylation of human protein tau in double transgenic animals when immunoblotted with the specified monoclonal antibodies

RESULTS

GSK-3β PHOSPHORYLATES MURINE AND HUMAN PROTEIN TAU *IN VIVO*

Extensive analysis of mouse brain extracts was performed by Western blotting with a battery of well-characterized antibodies, to reveal the typical slow-migrating isoforms of protein tau. Antibodies known to recognize epitopes on protein tau that are phosphorylated by GSK-3β *in vitro* were evidently included (Sperber et al., 1995). Extracts of brain from GSK-3β transgenic mice (6–7 months old) reacted with both phosphorylation-dependent antibodies AT-8 and AT-180, and revealed the presence of protein tau

isoforms with slower electrophoretical mobility. These isoforms were also detected with antibodies Tau-5 and PHF-1 (Figure 56.1A).

Further studies were mainly devoted to double transgenic mice. These mice were heterozygous for both transgenes and expressed both human protein htau40 and GSK-3β[S9A] in the CNS, in the same neurons. Western blotting demonstrated hyperphosphorylation of human protein tau in brain extracts of double transgenic mice of 5 weeks. Antibodies AT-8, AT-180 and AD-2 reacted with slowly migrating human protein tau isoforms, and these were virtually absent in the brain extracts of the single transgenic littermates (Figure 56.1B).

GSK-3β REDUCED BINDING OF PROTEIN TAU TO RE-ASSEMBLED TAXOL-STABILIZED MICROTUBULI

We examined whether GSK-3β affected the binding of protein tau to microtubules in brain and spinal cord extracts. In the presence of taxol, extracts of tubulin from mouse brain and spinal cord can still assemble into microtubular structures, despite unfavorable *in vitro* conditions. These re-assembled microtubular preparations are suitable for estimating the association of different MAPs (Vallee, 1982).

The binding of protein tau to reassembled microtubules was significantly reduced in preparations derived from brain and spinal cord of htau40×GSK-3β double transgenic mice, compared to their htau40 littermates. The addition of lithium ions in all buffers during the isolation and reassembly process did not affect the reduced binding of protein tau to the microtubules, excluding that reduced binding was due to phosphorylation of protein tau by GSK-3β *in vitro*. We conclude, therefore, that phosphorylation of protein tau had occurred *in vivo* and that these *in vitro* findings reflected the *in vivo* conditions of the brain of double transgenic mice (Figure 56.2). Reduced binding to microtubules was related to phosphorylation of protein tau, which was further supported by the hyperphosphorylation (especially at the AD-2 epitope) of unbound protein tau that remained in the supernatant after microtubule assembly and isolation (Spittaels et al., 2000).

Although more MT-unassociated hyperphosphorylated protein tau is available, neither an increase in insoluble protein tau aggregates nor the presence of paired helical filaments or tangles was observed in the CNS of any of our single and double transgenic mice up to the age of 14 months (Spittaels et al., 2000).

GSK-3β RESCUED THE AXONOPATHY AND MOTORIC IMPAIRMENT OF htau40 TRANSGENIC MICE

The pathological hallmark of the htau40 transgenic mice, i.e. the dilated axonal segments in brain and spinal cord (Spittaels et al., 1999), were

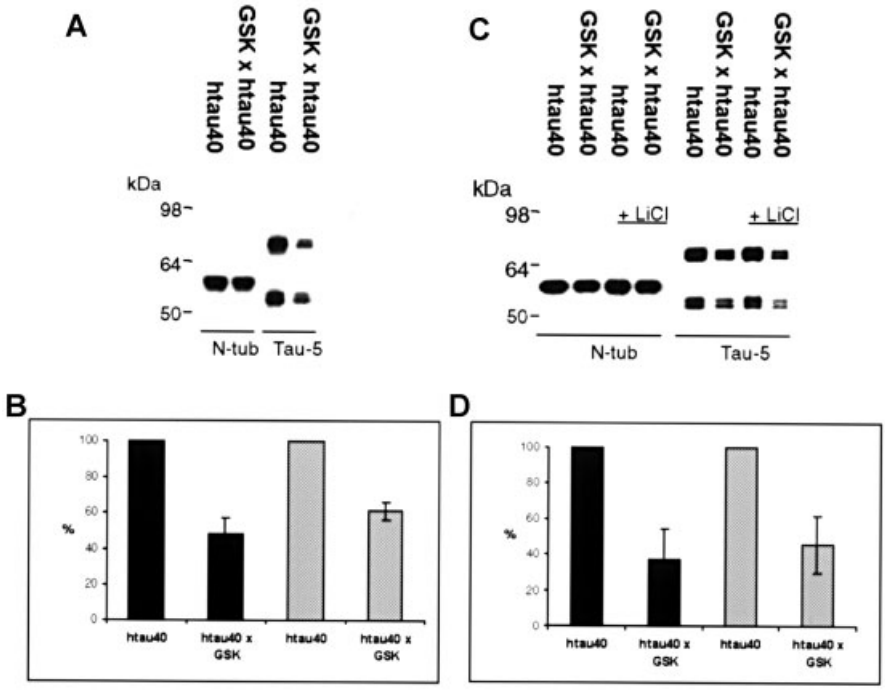


Figure 56.2. Determination of protein tau bound to taxol-stabilized microtubules. (A) Western blots of tubulin and microtubule-associated human and murine protein tau in taxol-stabilized microtubule pellets from brain of single and double transgenic mice. Tubulin and protein tau was determined by immunoblotting with neuron-specific β-III anti-N-tubulin and Tau-5 antibody, respectively. (B) Quantification by densitometric scanning of the amount of associated protein tau/tubulin for each mouse individually, normalized to htau40 transgenic mice ($n = 5$). (C) Western blotting as in (A) with LiCl (10 mM) added during the *in vitro* assembly of microtubules. (D) Quantification of the LiCl-treated samples of panel (C) as in (B) ($n = 3$). The reduction in the amount of protein tau associated with microtubules in brain of double compared to single transgenic mice is significant, both in LiCl treated ($p < 0.001$) and untreated ($p < 0.05$) conditions. Panels (A) and (C) are representative experiments. Black and gray boxes represent human protein tau/tubulin and murine protein tau/tubulin, respectively. Error bars represent SEM. n, Number of htau40−htau40×GSK-3β couples used

demonstrated to also contain synaptophysin. Synaptophysin is normally transported to the synapses by fast axonal transport mediated by kinesin. Moreover, in a number of dilated axons, the cytoskeleton was disrupted and numerous microtubules, randomly orientated, engirdled accumulations of pleomorphic vesicles, dense-cored vesicles and smooth endoplasmatic reticulum (Spittaels et al., 2000). Surprisingly, in the brain and spinal cord of the double transgenic mice, the number of dilated axons was dramatically

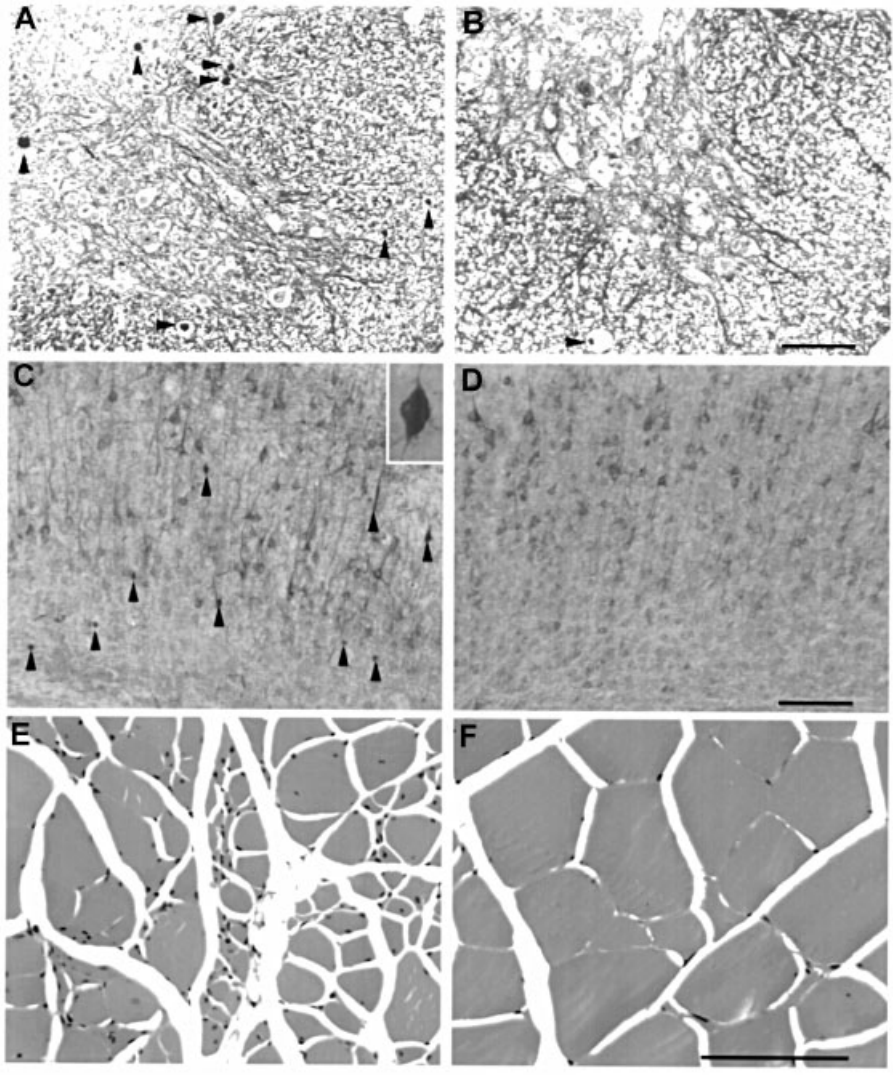
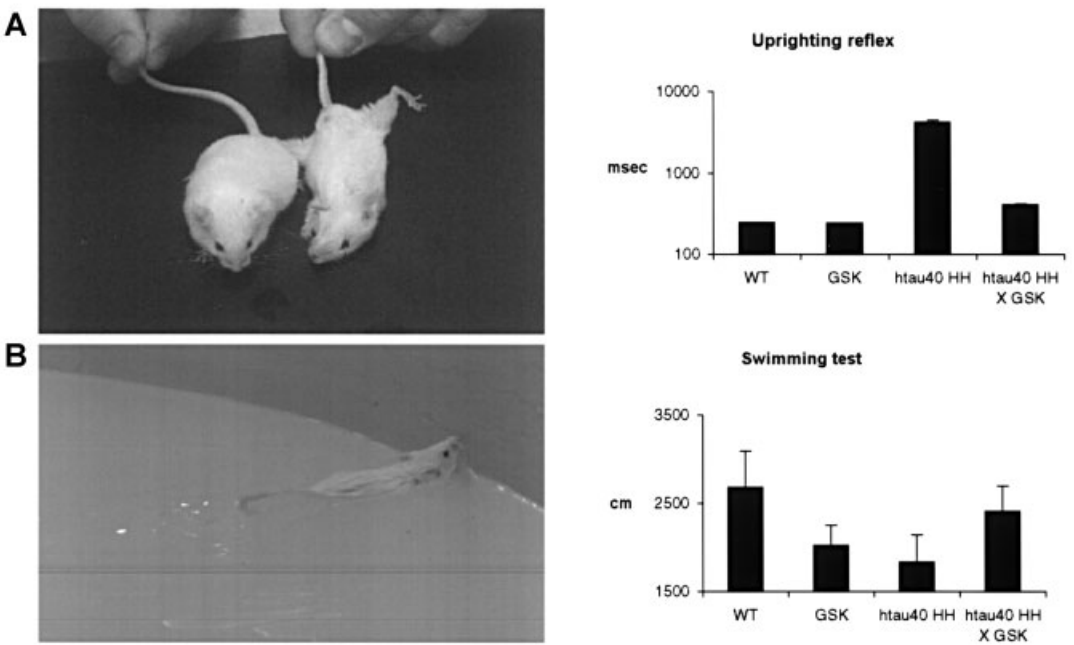


Figure 56.3. Comparison of central nervous system and quadriceps skeletal muscle from homozygous htau40 HH and htau40 HH×GSK-3β double transgenic mice. (A), (C) and (E) are from htau40 transgenic mice, (B), (D) and (F) from htau40×GSK-3β double transgenic mice. (A, B) Reduced number of argyrophilic dilated axons in the gray matter of the ventral horn and surrounding white matter (arrowheads) in double compared to single htau40 transgenic mice. (C, D) Low-power view of the neocortex stained with monoclonal antibody SMI-32, showing dilated axons (arrowheads) only in the cortex of single htau40 transgenic mice. Inset in (C) displays a higher magnification of a dilated axonal segment. (E, F) Hematoxylin/eosin staining of quadriceps muscle. Atrophic fibers are absent in quadriceps of double transgenic mice. Bars, 100 μm. Mice are 2–4 months old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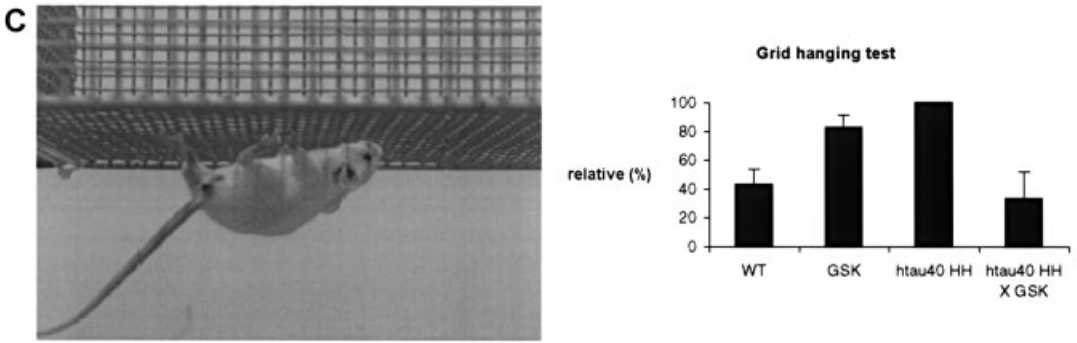

Figure 56.4 Performance of single and double transgenic and wild-type mice in three sensorimotor tasks. (A) Time (ms, logarithmic scale) mice needed to return to four legs after being forced to lie on their backs. htau40 HH mice were significantly slower than WT, GSK and htau40 HH×GSK littermates ($p<0.001$). (B) Swimming speed, defined as distance travelled in 2 min. htau40 HH mice covered shorter distances than WT mice ($p<0.001$). However, htau40 HH×GSK double transgenic and WT mice traversed the same distance ($p>0.05$). Moreover, htau40 HH×GSK mice swam faster than htau40 HH animals ($p<0.05$). (C) Inverted wire grid hanging, expressed as number of mice that did not remain suspended for the entire 1 min test period, relative to the number of mice tested in each group. Significantly more htau40 HH ($p<0.001$) mice lost hold than WT and htau40 HH×GSK mice. The htau40 HH×GSK double transgenic and WT mice performed equally ($p>0.05$) well. WT, wild-type mice; htau40 HH, single homozygous htau40 transgenic mice; htau40 HH×GSK, double homozygous htau40 heterozygous GSK-3β transgenic mice

reduced. Concomitantly, the quadriceps muscle of double transgenic mice was completely normal and devoid of any muscle wasting, which is a pathological hallmark of the htau40 single transgenic animals (Figure 56.3).

We previously demonstrated in three distinct htau40 transgenic founder strains that the severity of the axonopathy closely correlated with the motoric problem (Spittaels et al., 1999). The effect of co-expression of GSK-3β on the motoric phenotype was evaluated by three different tests. Overall, the double transgenic mice behaved in all tests significantly better than the single htau40 parental strain. In the 'uprighting reflex' test, the evident impairment of the single htau40 transgenic mice was nearly completely corrected in the double transgenic mice by co-expression of GSK-3β. In the forced swimming and inverted grid-hanging tests, the double transgenic mice performed equally as well as wild-type mice and significantly better than single htau40 transgenic mice (Figure 56.4).

DISCUSSION

The kinases that phosphorylate protein tau *in vivo* have not been identified. It remains imperative and important, both fundamentally and for the sake of patients suffering from protein tau-mediated dementia, to identify these neuronal kinases that phosphorylate protein tau and thereby control its function(s). The hypothesis that GSK-3β is such a kinase was tested here experimentally by generating transgenic mice that overexpress a constitutively active kinase, i.e. GSK-3β[S9A], in their CNS. Mild overexpression was obtained, resulting in a two-fold increase of GSK-3β kinase activity in brain.

In this study, the ability of GSK-3β to phosphorylate protein tau was revealed in the GSK-3β transgenic mice, and even more so in double transgenic mice, generated by cross-breeding with transgenic mice that overexpress the longest isoform of human protein tau, characterized previously (Spittaels et al, 1999). Evidence for hyperphosphorylation was the appearance of slower migrating isoforms of protein tau, which reacted with specified monoclonal antibodies in Western blotting, i.e. AT-8, AT-180 and AD-2. These are known from *in vitro* studies to define phosphorylated epitopes on protein tau that are generated by GSK-3β. Moreover, the binding of protein tau to MT was reduced by 50% when protein tau was extra-phosphorylated in brain of double transgenic mice. This is the *in vivo* correlate of the reduced binding of protein tau to microtubules in NT2N cells, transfected with GSK-3β (Hong and Lee, 1997).

In addition, the co-expression of GSK-3β had a major effect on the pathology of the single htau40 transgenic mice. Indeed, an important finding of the work presented here is the nearly complete rescue, by the mild overexpression of GSK-3β, of nearly all the pathological defects documented in the htau40 transgenic mice. This 'restoration' comprised: (1) reduction by

an order of magnitude of the number of axonal dilations in brain and spinal cord; (2) reduction in axonal and muscular degeneration; and (3) alleviation of practically all the motoric problems.

The formation of dilated axons in single htau40 transgenic mice supports the hypothesis that excess protein tau inhibits kinesin-mediated anterograde transport (Feiguin et al., 1994) by binding to axonal microtubules (Ebneth et al., 1998; Trinczek et al., 1999). That mild overexpression of GSK-3β prevented the formation of axonal dilatations in the CNS of double transgenic mice suggested that the axonal transport was restored, since hyperphosphorylated protein tau binds less efficiently to MT. Nevertheless, the mechanism of this rescue needs further study. Our current data suggest that drugs that inhibit GSK-3β—and subsequently increase the binding capacity of protein tau to microtubules—could even lead to more axon damage. On the other hand, the fact that GSK-3β is a protein tau kinase *in vivo* in neurons still qualifies it as a target for drug discovery in Alzheimer's disease.

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57 Pathogenic Implication of Altered Tau Properties Caused by FTDP-17 Mutations

P. NACHARAJU, S. YEN, M. DETURE, C. EASSON, M. HUTTON AND S.-H. YEN

INTRODUCTION

Tau is a microtubule (MT)-associated protein encoded by a single gene located in chromosome 17. It is expressed in neuronal tissues as six major isoforms due to alternative gene splicing (Himmler, 1989). Three of these isoforms contain three imperfect sequence repeats (R) in the MT-binding domain and are referred to as 3R tau. The other three isoforms contain an extra repeat encoded by exon 10 and are referred to as 4R tau. Different tau isoforms also differ from each other by the presence of none, one or two inserts in the amino-terminal region, encoded by exons 2 and 3. The expression of various isoforms is developmentally regulated (Kosik et al., 1989). While only 3R, without any amino-terminal inserts, is expressed in fetal brain, adult human brain contains about an equal amount of 3R and 4R tau.

Tau has the ability to promote the polymerization of tubulin into MT and stabilize the MT. Normally, tau proteins are readily extracted from neuronal tissue as soluble proteins. In a number of neurological disorders, however, tau self-interacts and assembles to form polymers. They accumulate in neurons and glia as bundles of filaments and become relatively insoluble. Disorders with tau pathology, referred to as tauopathy, include Alzheimer's disease (AD), Pick's disease (PD), corticobasal degeneration (CBD), progressive supranuclear palsy (PSP), frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17), Down's syndrome, and parkinsonism dementia complex of Guam (for reviews, see Dickson, 1997; Spillantini et al., 1998). Many of these disorders are sporadic in nature. While familial AD has been linked to mutations of genes encoding amyloid precursor protein and presenilin proteins, FTDP-17 has been linked to mutations in the tau gene in recent studies (for review, see Hutton, 1999). The mutations are at

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the 5′ splice of exon 10 and in exons 9–13 of the tau gene. Exons 9–12 encode a region of tau important for the binding of tau to MT (MT-binding domain) and for self-interactions of the tau molecule. The intronic mutations are at positions −2, +3, +12, +13, +14 and +16 relative to the splice site. The missense mutations are K257T, I260V, G272V, N279K, ΔK280, L284L, P301L, P301S, S305N, V337M, G389R and R406W (Figure 57.1).

Intronic mutations and several missense mutations at exon 10 (N279K, L284L, P301L, P301S and S305N) lead to the generation of filaments with straight or twisted ribbon structures, which are similar to tau filaments accumulated in CBD and PSP with respect to morphology (Reed et al., 1998; Spillantini et al., 1997). As revealed by immunochemical analyses of insoluble tau preparations, such filaments contain proportionally far more 4R than 3R tau isoforms (for review, see Yen et al., 1999a). Straight and occasionally twisted ribbon types of tau filaments are also present in Pick's disease as Pick's bodies, but they differ from filaments deposited in CBD and PSP by containing predominantly 3R tau isoforms. Other missense mutations (K257T, I260V, G272V, V337M, and R406W), with the exception of G389R, which forms straight and twisted filaments (Murrell et al., 1999; Pickering-Brown et al., 2000), resulted in the formation of filaments resembling paired helical filaments in AD. These filaments contain a similar ratio of 4R and 3R tau to that found in AD.

Studies of brain tissues and extracts from AD have demonstrated that the accumulation of tau filaments is associated with phosphorylation and conformational changes of the tau molecule. Accumulation of tau filaments in neurons or glia is accompanied by additional biochemical modifications and by aberrant expression/distribution of proteins, suggestive of unsuccessful attempts of the affected cells to degrade the filamentous tau (for review, see Yen et al., 1998). These abnormalities are also observed in non-AD disorders with tauopathy.

How mutations lead to the development of tauopathies has been examined in several recent studies by comparison of the wild-type and corresponding mutant tau for their ability to interact with tubulin, microtubules and other tau molecules, as well as for their susceptibility to proteolysis. The studies described in this paper focus on G389R tau mutation, which was identified recently from screening Pick's disease cases (Pickering-Brown et al., 2000). In addition, we compare the functional consequences of G389R and other tau mutations.

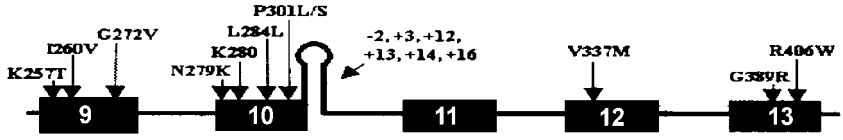

Figure 57.1. Tau mutations associated with FTDP-17. The boxes represent the region of tau encoded by exons 9–13, and the arrows indicate the approximate positions of mutations

RESULTS

BIOCHEMICAL ANALYSES OF INSOLUBLE tau

Tau filament-enriched samples from a case with G389R mutation were prepared by extracting the affected brain tissue with sarkosyl, as described by Ksiezak-Reding et al. (1997). The sarkosyl-insoluble preparations were analyzed by Western blotting. They were demonstrated to contain three tau-immunoreactive bands (Figure 57.2). Two of the bands migrated to positions corresponding to 60 and 64 kDa molecular weight, and the other band has a molecular weight of 68 kDa. The two smaller-sized tau were more intensely labeled than the 68 kDa tau. Tau proteins of similar molecular weights were present in the sarkosyl-insoluble samples derived from AD. In comparison, insoluble tau from sporadic Pick's disease contains mainly the 64 and 60 kDa tau (Figure 57.2). The exact composition of the tau isoform in the G389R mutation was determined by Western blotting of dephosphorylated tau. The results demonstrated that the insoluble tau in G389R mutation contains 3R and 4R tau isoforms, with or without exon 2 (Figure 57.2). Such a composition is different from that of insoluble tau from sporadic Pick's disease, which contains mainly 3R tau. Moreover, even though the G389R and AD tau all contain 3R and 4R tau, the G389R tau have a higher proportion of 4R tau, without the N-terminal insert encoded by exon 2. Whether this is the reason why G389R and AD tau form filaments of different morphology requires further investigation.

Electron microscopic examination showed that the sarkosyl-insoluble tau fraction from G389R brain extracts contains filaments of 40–80 nm diameter. These filaments have a twisted morphology and with a periodicity of about 400 nm (data not shown). The results are consistent with those reported recently (Murrell et al., 1999).

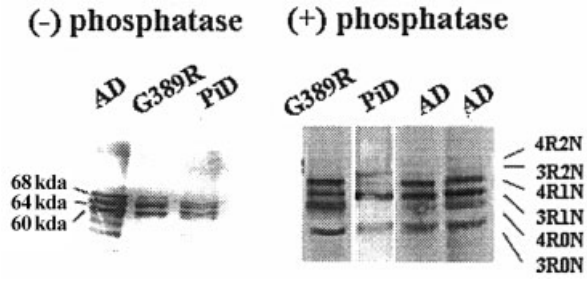

Figure 57.2. Immunoblotting demonstrated differences in tau isoform composition of insoluble tau from AD, G389R and PiD brains. Recombinant tau containing 3R and 4R tau with and without the N terminal inserts were used as references

EFFECT ON MICROTUBULE ASSEMBLY

Several tau mutations have previously been shown to decrease the ability of tau to promote MT assembly. Some of these mutations affected the nucleation phase and others the elongation phase. To elucidate the functional consequences of G389R mutation, we compared the ability of this mutant tau with wild-type tau and other tau mutants in promoting MT assembly. Purified recombinant tau proteins were incubated with bovine brain tubulin under established conditions for microtubule assembly, and the extent of tubulin assembly was monitored by changes in turbidity (DeTure et al., 2000).

The results demonstrated that the G389R tau has a reduced ability to promote MT assembly (Figure 57.3). Of the mutations we studied, P301L , K257T and I260V are more potent than others in affecting the extent of MT polymerization (Table 57.1). V337M and R406W had a moderate effect and G389R the least. Similar results were also obtained in other studies (Hong et al., 1998; Hasegawa et al., 1998), in which the ability of tau to promote tubulin assembly into MT was greatly reduced by the exon 10 mutations of tau, ΔK280, P301L and P301S. The K257T, I260V and P301L mutations are located in the first and second MT-binding repeats, and V337M (encoded by exon 12) is located in the fourth binding repeat region, whereas G389R and R406W (encoded by exon 13) are outside the MT-binding domain. The differences between different mutants in functional properties, therefore, are likely to reflect the importance of different subregions of tau in promoting microtubule assembly. They also appear to be associated with a decrease in the positive charge of the amino acid residue. The deletion of the positively charged residue, lys-280, can reduce the electrostatic interactions of tau with negatively charged tau-binding domains of tubulin. The P301L/P301S mutation is located in a motif (PGGG) which is also present in three other

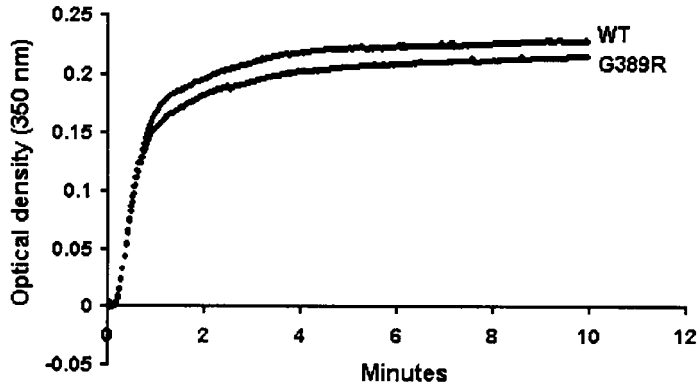

Figure 57.3. Microtubule assembly promoted by tau. G389R mutant is less effective than wild-type tau in promoting microtubule assembly

Table 57.1. Influence of tau missense mutations as compared to wild-type

Mutation	MT assembly inhibition	Polymerization promotion	Calpain I digestion
K257T	+++	ND	++
I260V	+++	ND	+++
ΔK280	ND	ND	++
P301L	+++[a]	+++[b]	−[c]
V337M	++[a]	++[b]	−−[c]
G389R	+	−−−	+
R406W	+[a]	+/+++[b*]	−−[c]

+, Higher/faster; ND, not determined; [a] DeTure et al. (2000); [b] Nacharaju et al. (1999); −, lower/slower; [c] Yen et al. (1999a); *R406W mutation inhibited polymerization more at the later stages of the reaction.

repeats in the MT binding domain, and is crucial for tau–tubulin interactions. The substitution of the proline residue by leucine or serine is likely to alter the conformation of the protein. G272V is present in the PGGG motif of the first repeat and V337M is very close to the PGGG motif of the third repeat. Such mutations do not change the charge of the amino acid residue. Of interest is the N279K mutation, which changes the charge to basic and has very little effect on microtubule assembly. Instead, such mutation leads to a disproportionate production of 4R over 3R tau, due to its effect on alternate gene splicing (Hong et al., 1998).

EFFECT ON PROTEOLYTIC DEGRADATION

Recombinant mutant and wild-type human tau were digested with calpain I at an enzyme:substrate ratio of 1:100, as described previously (Yen et al., 1999b). During the course of incubation, aliquots of the samples were removed and analyzed by SDS–PAGE (Figure 57.4) and Western blotting with anti-tau antibodies. The level of tau that remained undigested was estimated by densitometric scanning of silver-stained gel and immunostained blots. G389R mutant tau was more rapidly degraded than wild-type tau. Besides G389R mutation, K257T, I260V and ΔK280 mutations also lead to a reduced susceptibility of tau to calpain degradation. The results are different from those obtained from degradation of other tau mutants. As reported previously and confirmed in current studies, P301L, V337M and R406W mutants are less susceptible to calpain digestion. Among the mutant tau examined, I260V tau appears to be the fastest in degradation. By probing with antibodies specific to different subregions of the tau molecule, we have demonstrated that wild-type and mutant tau are cleaved at similar sites. Many of the calpain I cleavage sites are mapped in the C-terminal half of the tau molecule (Yen et al., 1999b). The results of the above studies suggest that mutation is likely to alter the accessibility of some cleavage sites for calpain digestion.

EFFECT ON TAU-SELF-INTERACTION

Normal tau proteins are not able to form filaments *in vitro*. In the presence of molecules such as heparin, heparan sulfate, polyunsaturated fatty acids or transfer RNA, normal tau assembles initially to become spherical structures and subsequently to become filaments (Goedert et al., 1996; Kampers et al., 1996; Wilson DM et al., 1997; Nacharaju et al., 1999). Different explanations have been given as to the mode of interactions between these inducer molecules and tau in the initiation of filament polymerization. Mutation of tau was shown in our previous studies to affect tau self-interactions. At low concentrations, P301L mutant polymerized in the presence of heparin without an apparent lag phase (Nacharaju et al., 1999). In comparison, the kinetics of polymerization for wild-type, V337M or R406W tau demonstrate the

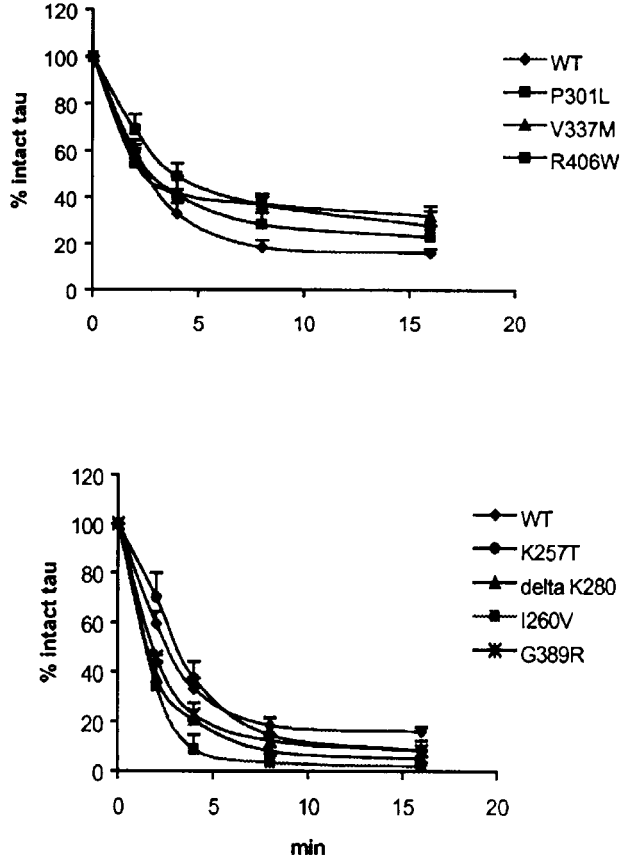

Figure 57.4. Kinetics of four repeat tau degradations by calpain I. The susceptibility of tau to degradation is increased by K257T, ΔK280, I260V and G389R mutations, and decreased by P301L, V337M and R406W mutations. Error bars represent standard errors of the mean

presence of nucleation and elongation phases. P301L mutation was far more potent than either V337M or R406W mutation in increasing tau fibrillogenicity. In other words, filaments were detected in P301L sooner than other tau mutants. V337M tau polymerized faster than wild-type at early stages of reaction and R406W exhibited an enhanced rate of polymerization only at later stages. In the absence of inducers, P301L tau formed spherical structures after prolonged incubation. Such structures were not detected in V337M or R406W mutant tau processed similarly. These differences between tau mutants in their ability for self-interactions have been confirmed (Goedert et al., 1999; Barghorn et al., 2000; Gamblin et al., 2000). P301S mutation was shown to be comparable to P301L mutation in its ability to enhance tau assembly. G272V and V337M mutation also have some enhancement effect, but ΔK280 did not alter the polymerization potential of tau.

Incubation of recombinant G389R mutant tau with heparin unexpectedly resulted in the formation of mostly non-filamentous structures after 2 days and a few short filaments after 4 days of incubation. This is in contrast to wild-type tau, which formed abundant filaments after 2 days of incubation (Figure 57.5). The difference between G389R and the aforementioned other tau mutants in their ability to self-interact may be due to the increase of positive charge caused by replacement of glycine with arginine. Such a change may hinder tau from acquiring a conformation favorable for self-assembly. In this scenario, a decrease of positive charge would increase the potential of tau for self-interactions. Indeed, the replacement of arginine with tryptophan in the R406W mutation, which decreases the positive charge, has rendered R406W mutant tau more susceptible to fibrillization than wild-type controls. Why G389R failed to form polymers *in vitro* will be a subject of future investigations.

SUMMARY

The results of the present and other studies suggest that missense FTDP-17 tau mutations cause diverse neurodegenerative syndromes by multiple mechanisms. A decrease in the ability of tau to interact with tubulin and microtubules would lead to a reduction in the level of microtubules *in vivo* and the stability of the microtubule system, which is important for transport of molecules and organelles vital for neuronal cell survival. An increase in the ability of tau molecules to interact with each other would facilitate the process of tau filament polymerization. Also, a decrease in the susceptibility of tau to proteolysis would result in the expansion of the pool of tau to assemble into filaments. Not all tau mutations, however, have the same effect on the aforementioned three parameters. In some mutations (e.g. P301L, R406W), it is difficult to know which of the changes of tau properties is most significant in the pathogenesis of FTDP-17. In the G389R mutation, its effect on microtubule assembly may be more important than other parameters in the

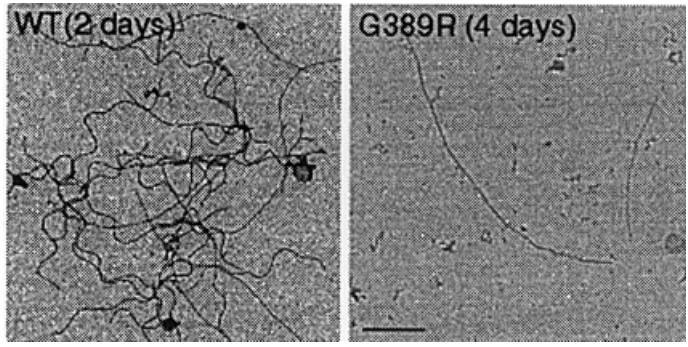

Figure 57.5. Representative EM fields of tau filaments formed in the presence of heparin. Tau (0.1 mg/ml) was incubated with heparin (0.01 mg/ml) in 30 mM MOPS, 2 mM PMSF, pH 7.4. Aliquots of incubation mixtures were placed on EM grids, stained with 2% uranyl acetate and examined with a Philips EM208S electron microscope. Scale bar = 500 nm

development of tau pathology. However, further investigations using transfection and/or transgenic approaches are essential for substantiating the findings made by the *in vitro* studies.

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58 A Hexapeptide Motif (^{306}VQIVYK^{311})-forming β Structure Induces the Aggregation of Tau Protein to Paired Helical Filaments

M. VON BERGEN, J. BIERNAT, E.-M. MANDELKOW AND ECKHARD MANDELKOW

INTRODUCTION

Alzheimer's disease (AD) and other dementias are characterized by abnormal protein deposits in the brain, such as amyloid plaques or neurofibrillary tangles, formed by fibrous assemblies of the Aβ peptide or of tau protein, respectively. These aggregates are thought to be toxic to neurons, either by causing some toxic signaling defect (in the case of Aβ) or by obstructing the cell interior (in the case of tau deposits). It is therefore one of the top priorities in AD research to understand the reasons for the pathological aggregation in order to find methods to prevent it. The aggregated Aβ peptide exhibits an increased content of β sheet structure content (Barrow and Zagorski, 1991); and the molecules in the fibrils are organized in a cross-β structure (Kirschner et al., 1986). This is also found in other disease-related proteins, such as fibrous prion protein (in Creutzfeld–Jakob disease; Pan et al., 1993) or transthyretin fibers (for review, see Sunde et al., 1997; Lansbury, 1999).

By contrast, the principles governing tau aggregation in AD into paired helical filaments (PHFs) have remained elusive (for review, see Friedhoff et al., 2000). The core of these PHFs is built mainly from the repeat domain (Wischik et al., 1988; Ksiezak-Reding and Yen, 1991; Novak et al., 1993) and this domain also promotes PHF assembly *in vitro* (Wille et al., 1992). Despite tau's random coil appearance in solution, tau assembles into well-defined fibers. This process can be enhanced by oxidation (Wille et al., 1992), by polyanions (Perez et al., 1996; Goedert et al., 1996; Friedhoff et al., 1998a;

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Kampers et al., 1996) and can be described by a nucleation–condensation mechanism (Friedhoff et al., 1998b; King et al., 1999).

Because of the long-range periodicity of most PHFs one could speculate that they are built from a reproducible secondary structure element in the microtubule-binding domain of tau, which is responsible for the repetitive and specific interaction that leads to filament aggregation. This element might be too short to be detected reliably by global spectroscopic studies of intact tau. Therefore, we looked for the minimal sequence required for PHF formation. This search led to the identification of the fragment PHF43, containing largely the third repeat R3 plus some adjacent residues. This peptide can self-assemble into filaments (not PHFs in the strict sense) but, significantly, nuclei derived from these filaments efficiently promote the assembly of bona fide PHFs from larger tau constructs or intact tau.

A search for the motif underlying the interactions between PHF43 molecules yielded the hexapeptide ^{306}VQIVYK^{311} (termed PHF6), which also shows a high tendency to aggregate into fibrous structures. Assembly of PHF43 or PHF6 is accompanied by a noticeable shift from random-coil to β-structure, as judged by CD and FTIR spectroscopy. We therefore conclude that tau filaments can assemble from a small stretch of tau containing the PHF6 sequence, capable of interacting with other tau molecules by a β-sheet-like interaction.

MATERIALS AND METHODS

The methods of tau cDNA cloning, protein expression and assays of PHF aggregation have recently been described (Friedhoff et al., 1998a,b; von Bergen et al., 2000).

RESULTS

IDENTIFICATION OF A MINIMAL SEQUENCE REQUIRED FOR AGGREGATION

Previous experiments to reconstitute PHFs *in vitro* had shown that tau constructs containing roughly the repeat domain polymerized much more readily than the full-length protein (Wille et al., 1992), consistent with the observation that the repeat domain forms the core of PHFs from Alzheimer brains (Wischik et al., 1988). To achieve PHF assembly from intact tau, it was necessary to add polyanionic co-factors such as heparin, RNA or poly-Glu (Perez et al., 1996; Goedert et al., 1996; Kampers et al., 1996). In order to find the minimal aggregating sequence, we focused on construct K19, a derivative of the fetal tau isoform (htau23), which contains only three repeats

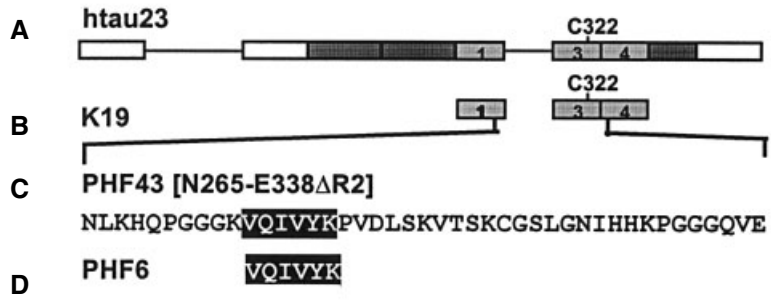

Figure 58.1 Bar diagram of tau constructs and peptides. (A) Human tau23, the smallest and fetal isoform, lacking the N-terminal inserts and repeat R2. (B) Construct K19, comprising R1, R3 and R4. (C) PHF43, containing the end of R1, all of R3 and the beginning of R4. (D) The hexapeptide PHF6, located near the beginning of R3

(R1, R3, R4; the second repeat, R2, is absent due to alternative splicing; Figure 58.1) and analyzed different proteolytic fragments. Construct K19 was digested with chymotrypsin, trypsin and GluC. One of the products was a fragment, generated by GluC digestion and termed PHF43, comprising only 43 residues, essentially the third repeat plus flanking stretches on either side (tau sequence 265–338 without the second repeat, 275–305, Figure 58.1). This fragment assembled much more rapidly than other fragments or intact tau, as judged by electron microscopy and the ThS fluorescence assay (half-time 0.75 min, compared with 12 min for K19 and 180 min for htau23, Figure 58.2). As with other tau constructs, efficient assembly required the presence of polyanions (e.g. heparin, RNA or poly-Glu), as well as initial dimerization by oxidation of Cys322 into disulfide bridges, indicating that dimers form the effective building blocks of assembly (Friedhoff et al., 1998b; Schweers et al., 1995).

THE FRAGMENT PHF43 NUCLEATES PHFS FROM FULL-LENGTH TAU

Judging by electron microscopy, most fibers obtained after self-assembly of the peptide PHF43 appeared as straight thin filaments, often aggregated laterally, and lacking the periodic $\sim$80 nm supertwist that is characteristic of AD PHFs, which is in contrast to fibers assembled from construct K19 or htau23. Since we were interested in studying AD-like PHFs, the absence of a twist could have been taken as a sign of a different assembly form, whose structure might be unrelated to that of PHFs. However, since PHF assembly follows a nucleation–condensation mechanism (Friedhoff et al., 1998b), we prepared fragments of PHF43 fibers by sonication and sought to discover

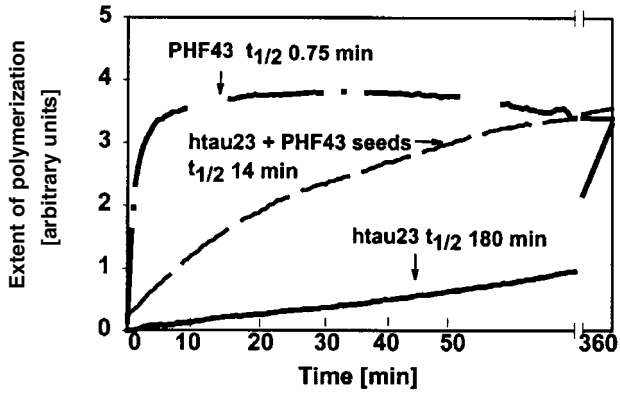

Figure 58.2 Kinetics of PHF assembly. The assembly of tau or tau fragments was measured fluorimetrically, using the fluorescence of ThS. PHF43 assembles rapidly and spontaneously (dash-dotted line, half-time of assembly 0.75 min), but htau23 is very slow by comparison (solid line, half-time 180 min). The aggregation of htau23 can be speeded up more than 10-fold in the presence of seeds from sonicated fibers obtained after PHF43 assembly (dashed line, half-time 14 min)

whether these could function as seeds for PHF assembly from larger tau constructs or full-length htau23. Surprisingly, the seeds made from the fragmented PHF43 filaments were capable of rapidly nucleating bona fide PHFs from K19 or htau23, displaying the typical 80 nm twist (Figure 58.3B). This means that the interaction between PHF43 molecules must be very similar to that of PHFs, both in a kinetic sense (requiring dimerization and polyanions) and in a structural sense (nucleation of twisted fibers). The smaller diameter of PHF43 fibers could be accounted for by the small size of the peptide, compared to the larger tau constructs and isoforms studied previously (Kampers et al., 1996; Wille et al., 1992).

THE POLYMERIZATION OF PHF43 IS ACCOMPANIED BY AN INCREASE OF β SHEET CONTENT

Our earlier studies of tau isoforms had shown that the ordered secondary structure of tau (α-helix or β-sheet) was minimal, below the reliable detection limit of the methods (~10%) (Schweers et al., 1995). This view did not, however, exclude the possibility of a locally ordered structure which should become more visible with shorter tau-derived peptides. Interpreting CD spectra in a quantitative way can be precise in the case of proteins containing a high content of helical structure but gives no exact results in the case of mostly unfolded structure. More significant, however, are the general shapes of the spectra which reveal gross conformational changes.

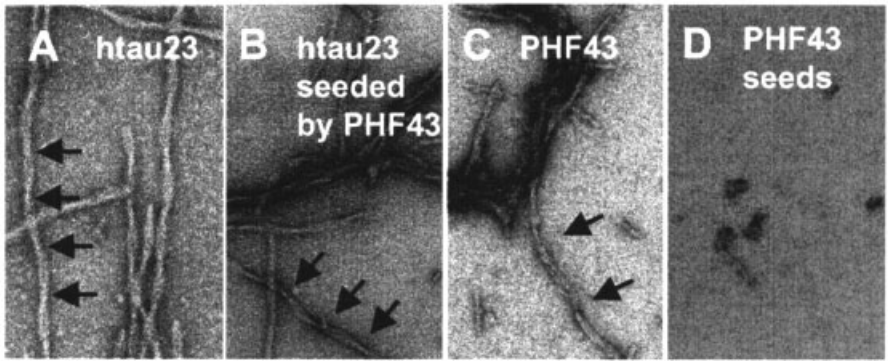

Figure 58.3. Electron micrographs of fibers obtained from the self-assembly of tau or the tau fragment PHF43. Assembly conditions were the same as in Figure 58.2 (20 μM protein, 5 μM heparin). A–C show mostly twisted filaments (width ~10–20 nm) resembling Alzheimer PHFs polymerized from (A) htau23, (B) htau23 plus seeds made from PHF43, (C) PHF43. (D) shows the seeds obtained by sonication of PHF43 fibers

All curves obtained for htau23 have negative molar ellipticities at wavelengths between 190 and 200 nm and show a minimum around 200 nm. This is characteristic of mostly random coil structures and confirms our earlier observations (Schweers et al., 1995). Neither dimerization nor heparin changes the spectra substantially, suggesting that the random coil structure dominates in all cases, even after PHF assembly (solid curve in Figure 58.4A). Even if a local β-structure were formed during PHF assembly, this does not become noticeable with full-length tau. The situation changes as the tau constructs become smaller (Figure 58.4B): K19 also displays a mostly random coil structure in the monomeric or dimeric state, even with heparin, but polymerization induces a noticeable change in the spectra, so that the minimum becomes wider and is shifted towards higher wavelengths, indicating a substantial change from random coil to β-structure. This behavior is reiterated in the case of PHF43 (Figure 58.4C), showing mostly random coil structures, except after filament assembly, when the content of β-structure is increased.

MUTATIONS IN THE HEXAPEPTIDE MOTIF INHIBIT PHF FORMATION

Further confirmation was provided by several additional mutants derived from the three-repeat construct K19, in which residues with high β-propensity in the PHF6 region were replaced or deleted. Thus, residues ^{306}VQI^{308} were deleted (construct K19ΔVQI), exchanged for NAE (construct

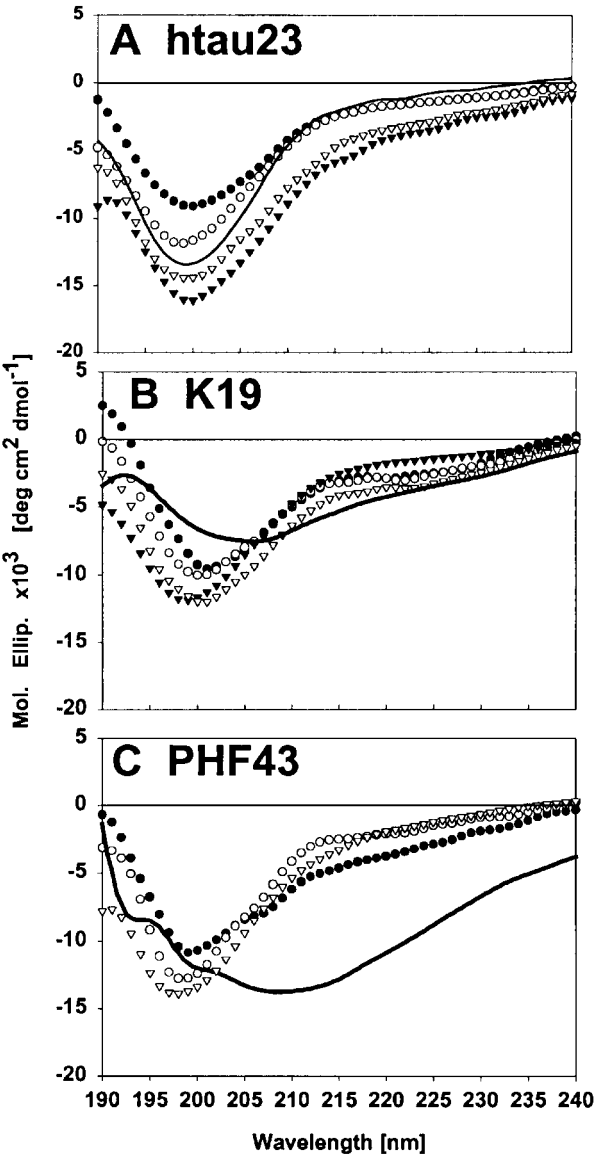

Figure 58.4. CD spectroscopy of tau and tau fragment PHF43. In the diagrams, circles represent CD traces from monomers, triangles the dimeric protein (cross-linked by disulfide bridges at Cys 322) and the solid lines assembled PHFs. Empty symbols show data obtained in the absence of heparin, filled symbols those in the presence of heparin. (A) htau23, (B) K19 monomers and dimers and (C) PHF43 monomers and dimers without heparin, show similar CD curves with or without heparin. Only K19 PHFs and PHF43 dimers in the presence of heparin (solid lines) show a noticeable shift to β-sheet structure, concomitant with aggregation

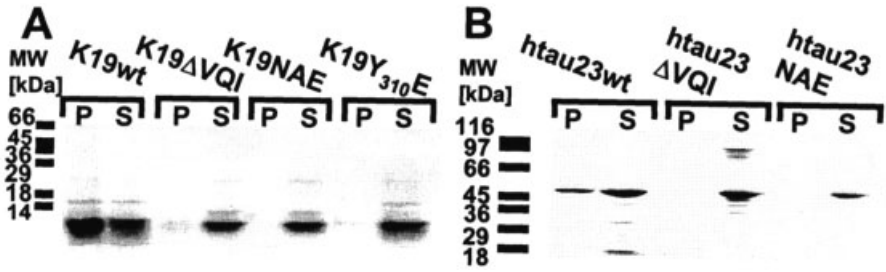

Figure 58.5. Analysis of the ability of tau mutants to form filaments. (A) PHF assembly of K19 wild-type and the mutants K19ΔVQI, K19NAE and K19Y310E was performed for 24 h and the supernatants and pellets, after ultracentrifugation of the reaction mixtures were applied to a 15% SDS–PAGE and stained with Coomassie blue. (B) PHF assembly of htau23 wild-type and the mutants htau23ΔVQI and htau23NAE was performed for 3 days and the supernatants and pellets, after ultracentrifugation of the reaction mixtures, were applied to a 10% SDS–PAGE and stained with Coomassie blue. Note that the mutant proteins remain in the supernatant because they do not aggregate

K19NAE), or Tyr310 was replaced by E (construct K19Y310E). In all three cases, the aggregation of the K19 mutants was strongly suppressed, compared to the K19 control. Thus, even after 24 h, when the aggregation of K19 was complete, the aggregation of the K19 mutants was still at background level (Figure 58.5A) and no fibers were detected by electron microscopy (not shown). Even in the full-length isoform htau23, the deletion of ^{306}VQI^{308} and the substitution of these amino acids to ^{306}NAE^{308} led to a complete loss of PHF formation (Figure 58.5B).

DISCUSSION

In this study we have attempted to define the smallest element of tau that is capable of initiating the assembly of tau into the pathological paired helical filaments. Here we show that: (1) a contiguous sequence motif supporting PHF assembly does indeed exist. (2) a small peptide supporting PHF assembly more efficiently than any other tau construct is PHF43, representing the third repeat plus short flanking sequences. This peptide assembles within seconds into thin fibers under the same conditions as full-length tau (favored by dimerization and anionic co-factors such as heparin; Figure 58.2). Seeds derived by sonication from such fibers are capable of greatly accelerating the assembly of full-length tau into bona fide PHFs, arguing that the PHF43 interactions are equivalent to the interactions in PHFs (Figure 58.3). Thus, the nucleation capacity appears to reside within PHF43, even though the morphology of the assembled fibers may be influenced by sequences which lie outside of PHF43; (3) PHF assembly is accompanied by a substantial

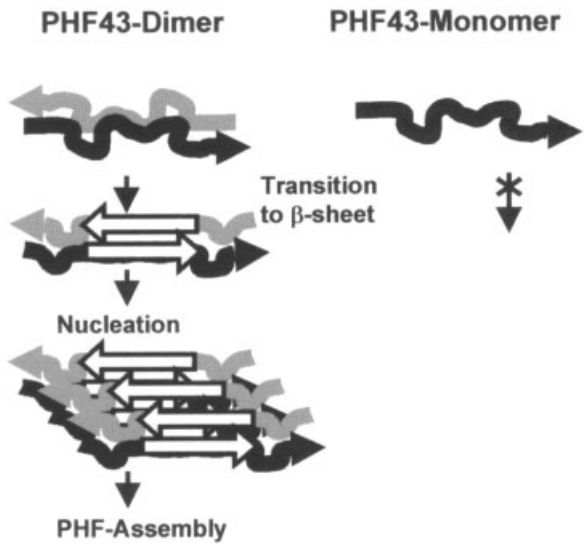

Figure 58.6. Model of the conformational transition from random coil to β-sheet during PHF assembly. Dimers and Monomers (black and dark gray) of PHF43 can bind polyanions (light gray), but only the dimers undergo a conformational transition from random coil (sinuous arrows) to β-sheet (straight arrows) in the sequence of the hexapeptide (^{306}VQIVYK^{311})

increase in β-sheet formation (Figure 58.4). This is not observed upon dimerization or addition of anionic co-factors alone, indicating that β-sheet formation is an essential part of PHF assembly. The pronounced tendency for β-structure is explained by the local clustering of β-sheet-inducing residues, such as V, I, Y and Q, whereas the corresponding hexapeptides in R1 (^{244}QTAPVPM^{250}) and R4 (^{337}VEVKSE^{342}) contain β-sheet breakers such as P and E (Street and Mayo, 1999). When the propensity for β-conformation is lowered by mutations in the PHF6 motif, PHF aggregation is inhibited as well. The corresponding motif in R2 may also be affected in some of the mutations found in FTDP-17, which are located near to it. Indeed, in the case of the P301L mutation, an accelerated PHF formation was found in several studies (Nacharaju et al., 1999; Barghorn et al., 2000; Gamblin et al., 2000).

These observations argue that the residues of tau centered around the hexapeptide motif PHF6 within the PHF43 fragment form the backbone of PHF assembly by a limited β-sheet interaction, while other sequences of tau remain more peripheral and retain a mostly random-coil structure (Figure 58.4). PHF43 itself forms only slender fibers, but larger constructs (e.g. K19) or full-length tau show the typical PHF-like twisted appearance; in each case, the kernel of the interaction is centered around the PHF6 sequence. These insights into the structural base of PHF formation provide

useful information for the design of tools for inhibition of the polymerization of tau protein and its possibly toxic effects on neurons in AD.

SUMMARY

Tau protein is overall a highly soluble and 'natively unfolded' protein which shows little tendency to aggregate into paired helical filaments *in vitro*. However, we show here that the microtubule binding domain contains one or two hexapeptide motifs, which promote the formation of β-sheet-like interactions and hence PHF assembly. This interaction may be responsible for the regular structure of PHFs found in AD brains.

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59 Formation of Neurofibrillary Tangles in Mouse Brain

AKIHIKO TAKASHIMA AND KENTARO TANEMURA

INTRODUCTION

Neurofibrillary tangles (NFTs) are the neuronal inclusions of microtubule-binding protein tau. NFT-bearing neurons show immunoreactivity for ubiquitin and phosphorylation-dependent tau, and a Congo red birefringency and thioflavin S reactivity, because NFTs are composed of a phosphorylated and ubiquitinated tau aggregation forming a β-pleated sheet structure. Tau dysfunction and filamentous tau aggregates are key markers of neurodegenerative pathologies that display NFTs, the most common pathway that leads to degeneration of neurons in several neurodegenerative diseases, including Alzheimer's disease (AD). Discovery of the molecular mechanisms of NFT formation may lead to more insight about events occurring during neurodegeneration in AD. Early efforts to produce animal models for AD involved the production of transgenic (Tg) mice that expressed amyloid precursor protein (APP) mutations associated with familial AD (Games et al., 1995; Hsiao et al., 1996). The brains of these mice displayed diffuse and neuritic beta-amyloid (Aβ) plaques like those found in human AD brains, but tau-positive NFTs were never observed. Aβ plaques alone, therefore, are not sufficient to induce tau pathology and cause neuronal death (Gomez-Isla et al., 1999; Murayama et al., 1999). In frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17), genetic studies revealed that tau is a causative gene, and tau mutation was found in exons and introns of tau gene. The patient who possesses this mutation exhibits NFT and loss of neurons. Thus, tau abnormalities cause NFT, leading to neurodegeneration. Intronic mutation and some missense mutation of tau gene alter the splicing and increase the expression of tau transcript with exon 10. In light of the important link between NFT formation and neurodegenerative diseases, a concerted effort has been made to produce tau Tg mice. Intronic mutation and some missense mutations of the tau gene alter the splicing and increase the expression of the tau transcript within exon 10, which encodes four-repeat

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tau (Goedert et al., 1998; Lee and Trojanowski, 1999). Based on this evidence, three lines of transgenic mice expressing human tau have been produced (Gotz et al., 1995; Brion et al., 1999; Ishihara et al., 1999). Neurons of these lines express a 2–10-fold increase in tau, and display tau inclusions. However, none of these lines exhibit the Congo red birefringent or thioflavin S reactive tau aggregations characteristic of NFTs observed in neurodegenerative diseases. This suggests that the robust overexpression of tau does not necessarily indicate that filamentous tau with β-sheet structures will be formed.

This prompted us to develop an animal model that closely mimics the formation of NFTs using another missense mutation of tau: V337M of FTDP-17. This mutation does not affect *tau* gene expression (Poorkaj et al., 1998). In the present study, although the expression level of mutant tau was less than one tenth of endogenous levels, neurons from our transgenic mice contained phosphorylated and ubiquitinated tau aggregations with a β-sheet structure. This was demonstrated by Congo red and thioflavin S positive staining, a histological criterion used to identify NFTs observed in neurodegenerative disorders.

GENERATION OF TRANSGENIC MICE EXPRESSING V337M HUMAN tau

A cDNA construct of the V337M human tau with myc and FLAG epitope tags at the N- and C-terminal ends was generated by PCR-based site-directed mutagenesis (Figure 59.1A). Three sets of PCR primers were used:

(1) sense:
CTGATCTCCGAGGAGGACCTGATGGCTGAGCCCCGCCAGGAG;
antisense:
AGATTTTACTTCCATCTGGCCACCTCC;
(2) sense:
GGAGGTGGCCAGATGGAAGTAAAATCT;
antisense:
ATCGTCCTTGTAGTCCAAACCCTGCTTGGCCAGGGA;
(3) sense:
CCCCTCGAGCCACCATGGAGCAGAAGCTGATCTCCGAGGAG
GACCTG;
antisense: CCGCGGCCGCTCACTTATCGTCATCGTC

The entire sequence of cloned cDNA (1.4 kb) was confirmed by an ABI PRISM377 DNA sequencer. This cDNA was inserted into the PDGF-β chain expression vector at the XhoI and NotI sites. A 4.3 kb BglII and NaeI fragment containing PDGF-β promoter, V337M human tau cDNA, and 3′

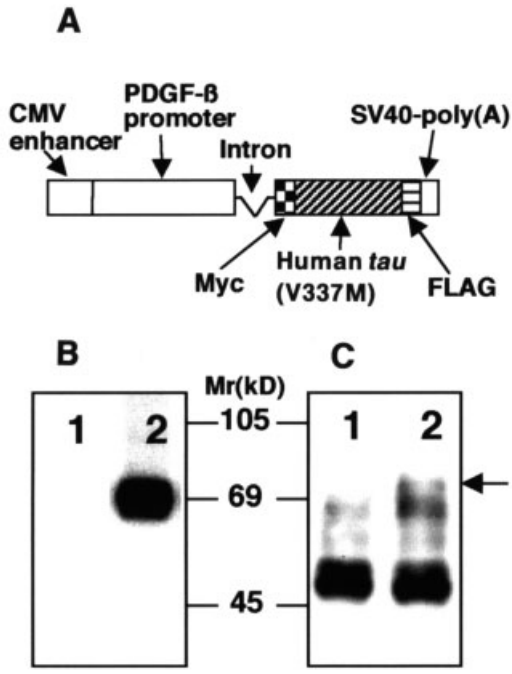

Figure 59.1. Analysis of mutant human tau (V337M) expression in brain of Tg mice. (A) Map of the mutant human tau construct used to generate Tg mice. (B) Western blot analysis of mutant human tau in brain of Tg mice. The same protein (20 μg) of RAB fraction from mice brains was applied to 8% acrylamide gel. Transferred membranes were probed by anti-myc antibody, which specifically recognizes the mutant human tau (V337M) at 70 kDa. Lane 1, *non*-Tg littermate of Tg214; lane 2, Tg214. (C) The same samples were probed by the anti-tau antibody JM, which recognizes both endogenous and mutant human tau. Lane assignments as in (B)

untranslated sequence was used as the transgene to create the mutant tau Tg mice on a B6SJL background. Microinjection of the transgene and generation of Tg mice were performed at DNX Transgenic Sciences (Cranbury, NJ, USA).

EXPRESSION AND LOCALIZATION OF HUMAN tau IN Tg MICE

Southern blot analysis showed that 5–10 copies of the transgene were inserted at a single site in Tg mice. The expression of mutant tau was confirmed by Western blot analysis. Brains taken from Tg mice and littermates (8–10 months old) were homogenized in reassembly buffer (RAB), and then tau was

recovered as the heat stable fraction. These fractions were separated on SDS-PAGE gel (8% acrylamide). Separated proteins were transferred onto a PVDF membrane (Millipore). Anti-myc antibody recognized the 70 kDa band in Tg mice but not in littermates (Figure 59.1B). Treatment with JM, an anti-tau antibody, recognized both 70 kDa human tau from the transgene and 50 kDa endogenous tau (Figure 59.1C). Although the amount of mutant tau varied among mice, even within the same Tg line, the levels of Tg tau expressed were generally less than one tenth of the endogenous tau levels. The regional localization of mutant tau in the brains of Tg mice was demonstrated by immunostaining with anti-myc antibody and anti-tau antibody, JM. Although PDGF-β chain promoter activity is found throughout mouse brain, anti-myc immunoreactivity localized to some neurons in the cerebral cortex (Figure 59.2A) and hippocampal region (Figure 59.2B). Myc-immunoreactive neurons were also double-labeled by anti-tau antibody JM (Figure 59.2C–F), suggesting that myc-positive neurons do express human mutant tau.

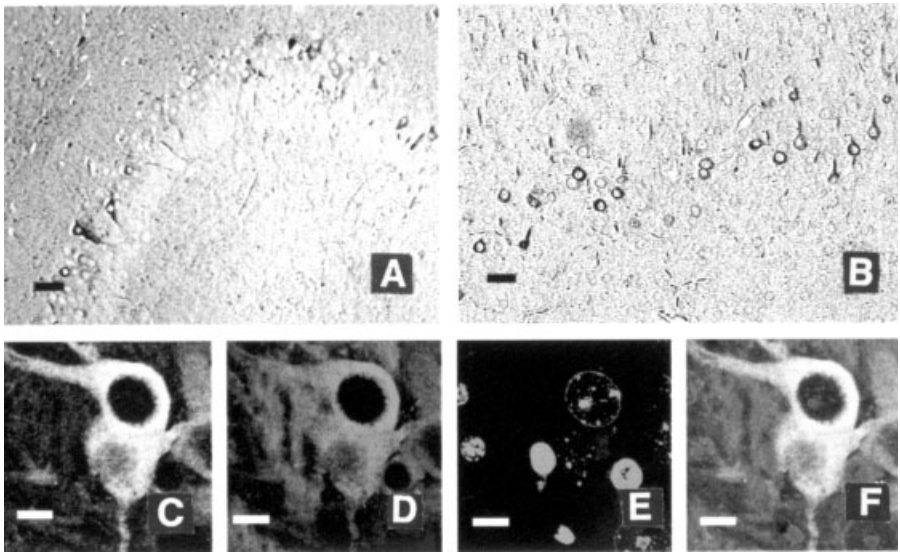

Figure 59.2. Location and distribution of human mutant (V337M)- and wild-type *tau*-containing neurons of 11-month-old Tg mice and non-Tg mice. (A–F) Photomicrographs of anti-myc immunostained sections of the cerebral cortex (A) and hippocampus (B) showing the location and distribution of mutant tau-containing cells in mutant tau Tg mice. The myc immunoreactivity was visualized with HRP (A, B) Scale bars, 50 μm. (C–F) Double-labeled indirect immunofluorescence of a cell in the hippocampal region from a 10-month-old Tg214 mouse. (C) Image of anti-myc immunostaining. (D) Image of anti-tau immunostaining (JM antibody). (E) Image of nuclear counterstaining using To-PRO-3 dye and pretreatment with RNase. (F) Merged image of myc immunoreactivity (A), JM immunoreactivity (B), and nuclear counterstain (C). (C–F) Scale bars, 10 μm

PATHOLOGICAL DETERMINATION OF NFT IN Tg MICE

To determine whether these Tg mice form NFT, mouse brain was histologically investigated by Gallyas silver staining, phosphorylation or conformational change of PHF-tau dependent anti-tau antibodies (Alz50, PS199, and AT8), and Congo red birefringence. Neurons were immunoreactive with tau antibodies recognizing conformational (Alz50) and phosphorylated epitopes (PS199 and AT8) (Figure 59.3A–C) in CA1 and CA2 regions of hippocampus. In the same region, Gallyas silver staining positive neurons were detected (Figure 59.3D), suggesting that Tg mice form NFTs in the hippocampus region. This was confirmed by Congo red birefringency. We stained using Congo red, and made observations under polarizing filter. Apple-green Congo red birefringence was detected in

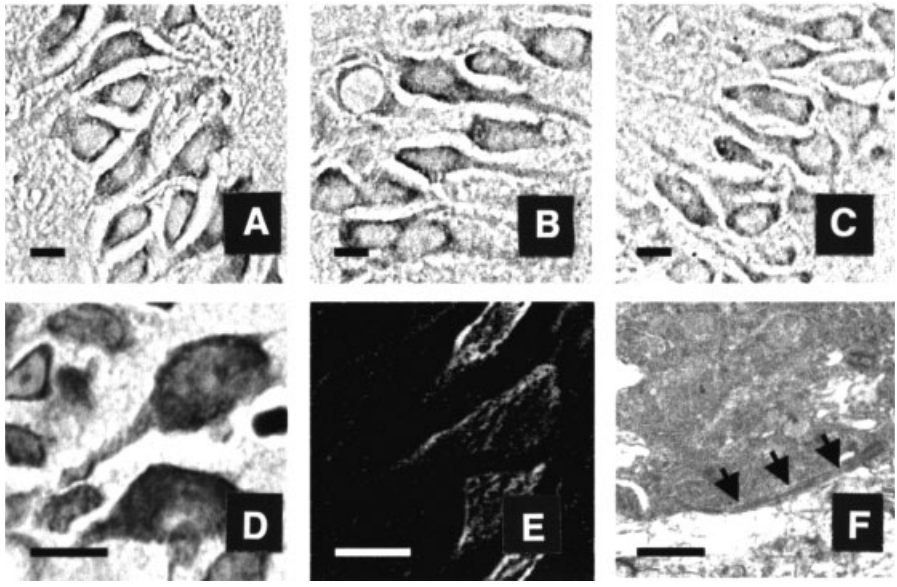

Figure 59.3. Histochemical characterization of neurons in mutant Tg mice. Sections of the hippocampal region from Tg214 (10-month-old) showing (A) immunostaining with mouse monoclonal antibody Alz50, an antibody specific for a conformational epitope found in PHF; (B) immunostaining with phosphorylation-dependent rabbit polyclonal anti-tau antibody PS199; (C) immunostaining with phosphorylation-dependent mouse monoclonal antibody AT8. (D) Photomicrograph showing Gallyas silver stained sections of hippocampi taken from mutant tau (V337M) Tg mouse. (E) Congo red birefringence in hippocampal neuron of Tg214 with polarizing filter. (A–E) Scale bars, 10 μm. (F) Ultrastructural images of hippocampal neurons of a Tg214 mouse. There are bundles of straight tubules (diameter 15 nm) in the cytoplasm of this neuron. Scale bar, 1 μm

neurons of the hippocampus region (Figure 59.3E). Ultrastructually, hippocampal neurons with NFT showed filamentous aggregates in cytoplasm, and most filaments were straight with 15 nm diameter (Figure 59.3F). Thus, the expression of mutant tau V337M in mouse brain induced NFTs in neurons of hippocampus, similar to human NFTs observed in neurodegenerative disorders. Since the expression level of mutant tau is less than 10% of endogenous tau in our Tg mice, the nature of tau, rather than the intracellular concentration of tau, may be the determining factor in the formation of NFTs. The exact biochemical events underlying the formation of NFTs from tau in these Tg mice is yet to be determined.

SUMMARY

Formation of NFTs is the most common feature in several neurodegenerative diseases, including AD. Here, we report the formation of filamentous tau aggregations with β-sheet structure in Tg mice expressing human tau with mutation of FTDP-17, in which valine is substituted with methionin residue 337. The aggregation of tau in these Tg mice satisfies all histological criteria used to identify NFTs common to human neurodegenerative diseases, and the mice showed the emotional changes and cognitive impairment. These mice, therefore, provide a preclinical model for the testing of therapeutic drugs for the treatment of neurodegenerative disorders that exhibit NFTs.

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60 Inducible Transgenic Expression of Wild-Type tau in H4 Neuroglioma Cells

MICHAEL DeTURE, LI-WEN KO, COLIN EASSON, MIKE HUTTON AND SHU-HUI YEN

INTRODUCTION

Tau is the principal component of paired helical filaments that form neurofibrillary tangles (NFTs) in Alzheimer's disease (AD). These NFTs accumulate in the cell bodies of dying neurons and glial cells and accordingly their abundance correlates positively with the severity of cognitive decline in AD patients (Nagy et al., 1995). The role of tau in AD neurodegeneration has been speculative, but the linkage of tau gene mutations to frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17) demonstrates that abnormalities in tau can compromise neuronal functions (Van Slegtenhorst et al., 2000). Still, the relationship of tangle formation to cell death is not understood. With the identification of FTDP-17 mutations, it is tempting to speculate that NFTs are not simply a marker of cell death, but there is no direct evidence for this. The formation of tangles might be a mechanism employed by the cell to sequester toxic, soluble FTDP tau. Alternatively, neurodegeneration might result from the detrimental activation of signal transduction pathways as the cell attempts to correct abnormal functions of FTDP tau (Yen et al., 1999; DeTure et al., 2000).

The cause and effect of tau filament assembly in AD and related disorders are poorly understood. These issues could be resolved by analyses of animal and cell culture model systems that generate paired helical filaments. Animal models for senile plaque formation in AD have been developed through transgenic expression of human familial AD genes, but these mice form only plaques and not NFTs (Goedert and Hasegawa, 1999). Transgenic FTDP-17 P301L mice have been created that exhibit a motor neuron disease phenotype and tau NFTs, but in these mice the mutant tau exerts a dominant effect over the wild-type tau (Lewis et al., 2000). This is representative of what happens

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in FTDP-17, but it is unlike tangle formation in AD, where all the wild-type isoforms of tau accumulate. Transgenic mice overexpressing human wild-type tau have not been found to form NFTs with morphological and biochemical properties characteristic of the tauopathies even though axonopathy and high levels of hyperphosphorylated, somatodendritic tau were observed (Grundke-Iqbal and Iqbal, 1999). This increased somatodendritic tau is observed in AD, but the absence of tau tangles in the transgenic mice suggests that other factors are involved.

Likewise, cell lines with stable wild-type tau expression have been developed, but these cells have not been shown to form tau filaments (Matsumura et al., 1999; Sahara et al., 2000). The perceived problem is that constitutive expression of the microtubule-stabilizing protein tau dictates that tau levels can only accumulate so much before they interfere with cell division. This results in the selection of cells that express low levels of tau during the cloning process. The generation of the H4 neuroglioma cell lines with inducible tau expression presented here circumvents this problem. These cells express high tau levels upon induction. As a result of such transgenic expression, tau binds the microtubules and eventually saturates the microtubule network with the majority of the tau being free in the cytosol. Using quantitative Western blot analysis, the levels of four-repeat (4R) tau are calculated to approach 80 μM concentrations in the cytosol after five days of induction. Additionally, the expression levels of tau can be manipulated by varying the tetracycline concentration in the feeding media. The abundance of microtubule-free, cytosolic tau is presumably the first step in Alzheimer paired helical filament formation (Alonso et al., 1997); and therefore, this system is likely to be useful in identifying the stresses and signaling pathways that lead to tau aggregation *in vivo*.

CLONING OF TRANSFECTED H4 CELL LINES WITH INDUCIBLE tau EXPRESSION

The H4 neuroglioma founder line containing the tetracycline-repressible transactivator pUHD15-1neo plasmid (Clonetech Laboratories Inc.) was generously provided by Dr R. E. Tanzi (Massachusetts General Hospital). These H4 founder cells, referred to as H4-15neo, were propagated in feeding media comprised of Dulbecco's MEM (Life Technologies, Gaithersburg, MD) containing 10% FBS (Life Technologies), 200 μg/ml G418 (Life Technologies) and 2 μg/ml tetracycline (Sigma) in a humidified incubator at 37 °C with 5% CO_2. The cDNAs for three- and four-repeat wild-type (2-, 3-) tau (Figure 60.1) were kindly provided by Dr A. Andreadis (E. K. Shriver Center for Mental Retardation) in pBlueScript. These wild-type cDNAs were removed from pBlue Script with SalI and cloned into pcDNA3 using the XhoI site. The tau cDNAs from pcDNA3 were then cloned into the

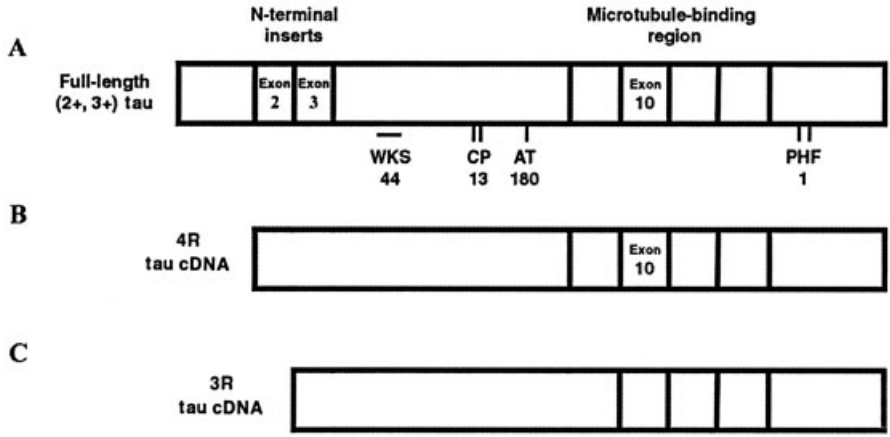

Figure 60.1. Schematic presentation of human brain tau. (A) Full-length 4R tau containing 441 amino acid (aa) residues. The localization of microtubule-binding repeats and the positions of epitopes for the antibodies used were indicated. (B) Tau cDNA used to express 383-aa 4R tau. (C) Tau cDNA used to express 352-aa 3R tau

tetracycline-responsive expression pTRE plasmid (Clonetech) using EcoRI (Gossen and Bujard, 1992). DH5α competent cells (Clonetech) were transformed using the above pTRE constructs and plated to select positive clones. Colonies were picked, grown up, and the pTRE plasmid containing the cDNA of interest was isolated using the Qiagen Maxi-prep Plasmid Purification Kit (Qiagen). The sequence was determined with an ABI377 automated sequencer and the Big Dye Terminator Sequencing Kit (Perkin Elmer) while cDNA orientation was verified by restriction enzyme size analysis. The H4-15neo founder cells were plated overnight in six-well plates before being transfected to the pTRE constructs containing cDNAs for 3R or 4R wild-type tau and pBabe-Puro, a kind gift of Dr T.-W. Kim (Massachusetts General Hospital). This was done using the transfection reagent Tfx-20 (Promega). The following day, cells were transferred to 100 mm dishes (Becton Dickinson Labware) and selected with the feeding media supplemented with 1 μg/ml puromycin (Sigma) for three weeks. Colonies were picked and eventually expanded for screening. Cells were plated on glass coverslips in feeding media without tetracycline for three days to screen for tau expression using a tau/tubulin double-immunofluorescence procedure.

Tau IMMUNOFLUORESCENCE IN TRANSFECTED H4 NEUROGLIOMA CELLS

To determine the time course of tau induction, immunofluorescence was performed. The expression of tau was monitored using the rabbit polyclonal

WKS44. This antibody was generated to the tau peptide 162–178 and recognizes all six isoforms of tau. As tau is a microtubule-associated protein, the microtubule network was monitored simultaneously with the mouse monoclonal TUB2.1 (Sigma). Fluorochrome-conjugated secondary antibodies Alexa 488 goat anti-rabbit IgG and Alexa 594 goat anti-mouse IgG (Molecular Probes) were used in conjunction with these primary antibodies. Dual-labeling of the cells not only allowed the percentage of cells expressing tau to be estimated, but it also provided a means of examining the relative levels of tau expression in different cell lines during screening. Tau was observed in some cells in as little as one day, but it usually required two or three days for most of the cells to express tau. The expressed tau was observed to co-localize with the microtubule network, and it promoted bundling and reorganization of microtubules within the cell, providing evidence for a tau–microtubule interaction (Figure 60.2). During prolonged induction, the tau

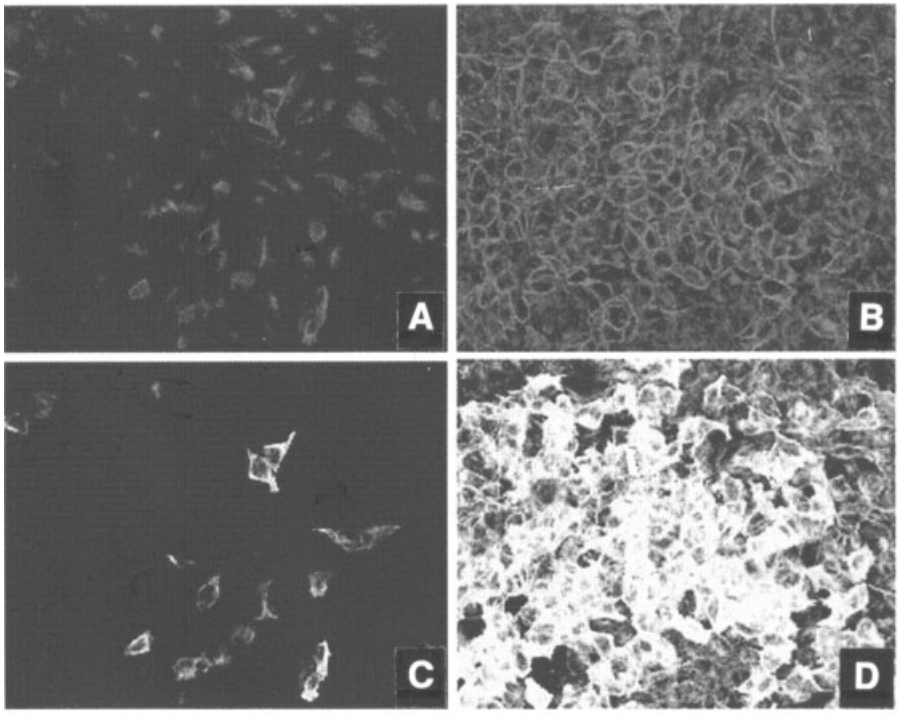

Figure 60.2. Immunofluorescence double-staining of (C,D) tau with WKS44 and (A,B) tubulin with TUB2.1 in H4 cells with induced 4R tau expression. After 3 days of induction most of the cells expressed a low to moderate level of tau (C). Low levels co-distributed with microtubules in a network pattern similar to that found in non-transfected cells while moderate levels induced some bundling (A). After 6 days of induction, most of the cells expressed high levels of tau (D) that bundled microtubules into a ring around the cells (B)

was observed not only with microtubules but also in the cytosol, suggesting saturation of the tau-binding sites on microtubules within a cell.

WESTERN BLOT ANALYSIS OF tau IN TRANSFECTED H4 NEUROGLIOMA CELLS

Post-nuclear cell lysates were prepared from induced H4 cells. Cells were washed in PBS, collected, counted and homogenized at 20 000 cells/μl in lysis buffer (20 mM MES at pH 6.8, 80 mM NaCl, 1 mM $MgCl_2$, 2 mM EGTA, 10 mM NaH_2PO_4, 20 mM NaF, 1 mM PMSF, and 10 μg/ml leupeptin). Western blot analysis of these cell lysates demonstrates that there is a rise in protein expression through five days that depends on the presence of tau cDNA in pTRE (Figure 60.3A,B). Use of recombinant (2-, 3-) tau standards demonstrates that the unphosphorylated band for 3R tau runs at 52 kDa while

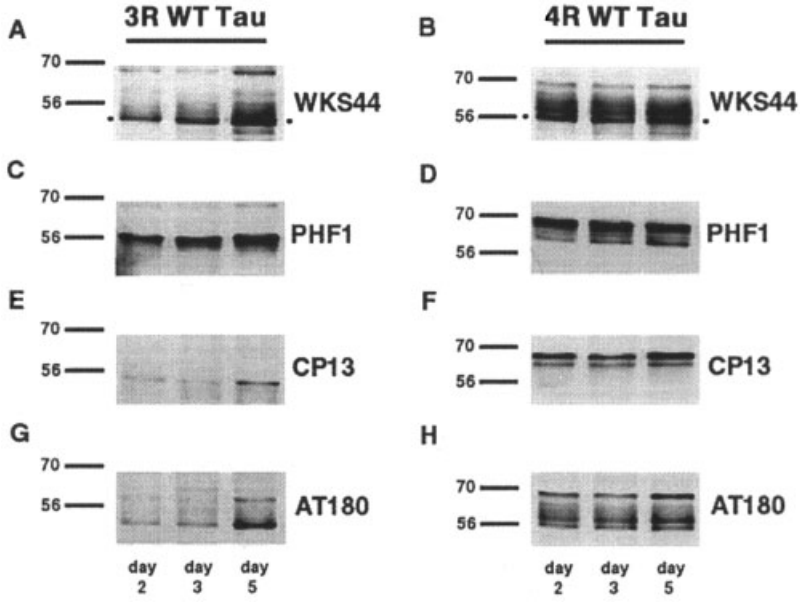

Figure 60.3. Expression of phosphorylation-sensitive tau epitopes. (A,B) Lysates from cells expressing 3R or 4R tau, respectively, were immunoblotted with a tau antibody, WKS44, after 2, 3, or 5 days of induction. The lysates contained tau with different electrophoretic mobilities (A,B). One of the tau bands co-migrated with recombinant standards marked by dots. Duplicate blots were stained with PHF1, which strongly labeled bands running above the position of the recombinant markers (C,D). In 4R tau lysates, bands also displayed strong CP13 and AT180 immunoreactivities (F,H). In 3R tau samples, only weak CP13 and AT180 immunoreactivities were detected (E,G)

the 4R band runs at 56 kDa. Additionally, there are a number of tau bands with retarded mobility. The 3R tau migrates predominantly as a single band, but there is a small smear above this band and a larger species at 68 kDa. The 4R tau also has this 68 kDa band in addition to several bands between 60 and 64 kDa. Quantitative Western blot analysis using recombinant tau standards indicates that the 4R tau protein levels reach 80 μM in the cytosol while the 3R concentrations reached 50 μM. These numbers were calculated based on diameter measurements of trypsin-treated, rounded cells after five days tau induction similar to the method used earlier for calculating the tubulin concentration in similar cell lines (Hiller and Weber, 1978). From these diameter measurements, the volume of the H4 cells was calculated to be 2.7610^{-12} liters, and the cell volume was not found to change appreciably with tau induction. Corrections for the volume of the nucleus were not made, so cytosolic concentrations of tau might be slightly higher than those reported here.

Tau PHOSPHORYLATION IN TRANSFECTED H4 NEUROGLIOMA CELLS

Alkaline phosphatase treatment of transfected H4 lysates confirmed that the higher molecular weight bands described above were phosphorylated tau (data not shown). Subsequently, Western blots of non-treated post-nuclear cell lysates were probed with several mouse monoclonal antibodies that recognize specific phosphorylated sequences in tau (PHF1, CP13, and TG3 were a gift of Dr P. Davies; AT100 and AT180 were purchased from Pierce Endogen). The numbering of phosphorylation sites is traditionally based on the longest (441-amino acid) isoform of human brain tau and is reported here in that manner. The Ser396 and Ser404 sites were robustly phosphorylated in both 3R and 4R tau as determined with the PHF1 antibody, but the Ser202 and Thr205 sites (CP13) and Thr231 (AT180) were significantly more phosphorylated in 4R tau than 3R tau (Figure 60.3C–H). Immunofluorescence of fixed H4 cells with these same antibodies confirmed the generation of phospho-epitopes at these sites, and the co-localization of these tau phospho-epitopes and microtubule bundles suggests that phosphorylation of these sites does not interfere with microtubule binding (data not shown). Neither Western blot nor immunofluorescence staining of 3R or 4R tau was observed with either AT100 or TG3, suggesting that tau expressed in H4 cells is not phosphorylated at the Thr212, Ser214 or Ser235 sites at levels above our detection limits.

MICROTUBULE BINDING OF tau IN TRANSFECTED H4 NEUROGLIOMA CELLS

The immunofluorescence data suggest that phosphorylation of induced tau does not interfere with microtubule binding. This was confirmed by analysis

of cell lysates containing taxol to stabilize the microtubules. The post-nuclear cell lysates were prepared as before with 35 °C lysis buffer containing 1 mM GTP and 50 μM taxol to stabilize the microtubules. These lysates were then centrifuged at 200 000 **g** for 10 min in a Beckman TLA 100.2 rotor to collect a microtubule-bound pellet and a microtubule-free supernatant. By running controls in lysis buffer lacking GTP and taxol, the stabilization of microtubules was observed as a shift in tubulin from the soluble to the insoluble fractions (Figure 60.4A,B). This tubulin shift was accompanied by a small concurrent shift in tau to the microtubule fraction, but the majority of the tau remained soluble in the microtubule-free fraction (Figure 60.4C,D). To determine if the soluble tau was unable to bind microtubules or if the amount of tau expressed had saturated the microtubule-binding sites, the ability of the soluble tau to bind microtubules was determined. Exogenous taxol-stabilized microtubules prepared from purified bovine brain tubulin (Cytoskeleton) were added to the supernatant, incubated for 20 min at 35 °C and collected as before by centrifugation (Figure 60.4E,F). The soluble tau that did not bind to endogenous microtubules was able to bind exogenous

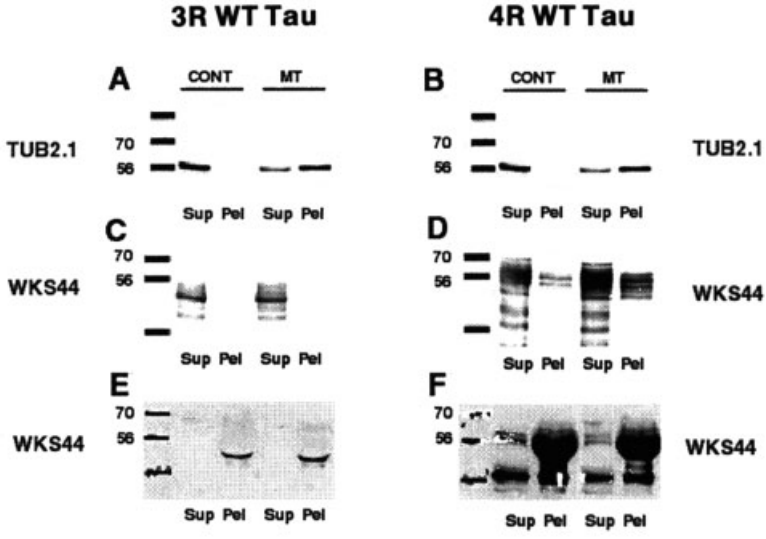

Figure 60.4. Binding of tau to microtubules. Antibodies to tubulin (TUB2.1) and tau (WKS44) were used for immunoblotting of samples prepared in lysis buffer without GTP and taxol (CONT) or with GTP and taxol (MT) after 3 days of induction. Pellets (Pel) containing microtubules (MT) and supernatants (Sup) devoid of MT were obtained by high-speed centrifugation. As expected, most of the tubulin was detected in the pellet fraction prepared in the presence of taxol and GTP (A,B) from 3R and 4R tau. Most tau did not segregate with microtubules although there was an increase of tau in the pellet fraction when compared to controls (C,D). Tau present in the supernatants was able to bind bovine microtubules prepared in the presence of 50 μM taxol (E,F)

microtubules, and this demonstrated that the endogenous microtubules were saturated with tau as suggested by immunofluorescence. This also suggests that the transfected H4 cells expressing tau contained a large pool of soluble tau in the cytosol, but thioflavin S staining did not show any differences between transfected cells, with induction and control cells indicating the absence of tangle formation (data not shown).

DISCUSSION

Tau does not readily assemble into filaments under normal physiologically relevant conditions. In healthy neurons, tau is found to bind to microtubules in the axons via electrostatic interaction. This tau binding to microtubules stabilizes microtubules and spaces them adequately while permitting vesicular transport. In many cells of neuronal and glial lineage the tubulin concentration is estimated to be less than 20 μm (Hiller and Weber, 1978), but this is probably higher in the axon where microtubules are tightly packed. The stoichiometry of tau binding to microtubules under the first-order saturation condition is 0.2–0.4 (Ackmann et al., 2000), and this implies that the tau concentration is likely to be below 10 μM in most cells. *In vitro,* this concentration of tau can self-assemble to form filaments only in the presence of heparin, RNA or arachidonic acid (Goedert et al., 1996; Kampers et al., 1996; Wilson and Binder, 1997). *In vivo,* microtubules most certainly interfere with tau polymerization as the levels of microtubules are greater than those of tau. Therefore, it is reasonable to assume that tau must be released from microtubules or produced in excess amounts to give rise to a pool of free tau for tau filament assembly. This release may result from aberrant kinase or phosphatase activity in AD, but microtubule depolymerization or upregulation of tau could also give similar results.

It is tempting to speculate that somatodendric tau and axonal degeneration in AD brains may result from abundant tau accumulation through its interference with axonal transport (Ebneth et al., 1998). However, H4 cells expressing tau at as high as 80 μM (observed in the present study) failed to form thioflavin S positive structures. Moreover, transgenic mice overexpressing wild-type human tau (Grundke-Iqbal and Iqbal, 1999) did not generate thioflavin S positive tangles. The results indicate that simply having a large pool of unbound tau in neurons or glial cells is not sufficient for formation of tau filaments. Initiation of filament assembly probably requires the participation of non-tau molecules to lower the critical concentration necessary for tau polymerization. It is plausible that the expression and the abundance of such inducer molecules is associated with aging, since prominent tauopathies affect mostly aged populations. The inducer molecules could be produced or become elevated in concentration in cells exposed to age-related stress, such as oxidative stress, or to cytotoxic microenvironments.

These could be addressed by exposing the transfected H4 cells expressing abundant tau to various agents, including those capable of inducing lipid and protein oxidation, perturbing calcium ion homeostasis, or activating different signal transduction pathways.

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61 Lewy-Like Pathology in Mice Transgenic for Mutant (A53T) and Wild-Type Human α-Synuclein

BERND SOMMER, SAMUEL BARBIERI, KATJA HOFELE, KARL-HEINZ WIEDERHOLD, ALPHONSE PROBST, CLAUDIA MISTL, SIMONE DANNER, SABINE KAUFFMANN, WILLIBRORDUS SPOOREN, MARKUS TOLNAY, GRAEME BILBE AND HERMAN VAN DER PUTTEN

INTRODUCTION

Idiopathic Parkinson's disease (PD), dementia with Lewy bodies (DLB), and a Lewy body variant of Alzheimer's disease (LBVAD) are characterized pathologically by proteinaceous inclusions commonly referred to as Lewy pathology in postmortem brain tissue samples (Braak et al., 1999; Forno, 1996; Ince et al., 1998; Irizarry et al., 1998; Spillantini et al., 1998; Takeda et al., 1998). These inclusions occur in the dystrophic (Lewy) neurites (Braak et al., 1999; Spillantini et al., 1998), in neuronal perikarya (Lewy bodies) (Baba et al., 1998; Braak et al., 1999; Forno, 1996; Ince et al., 1998; Irizarry et al., 1998; Mezey et al., 1998; Spillantini et al., 1997, 1998; Takeda et al., 1998; Wakabayashi et al., 1997), and occasionally extracellularly (Den Hartog and Bethlem, 1960). α-Synuclein is the major constituent of Lewy inclusions (Forno, 1996), which contain to lesser and varying degrees neurofilament proteins (Forno, 1996; Ince et al., 1998; Takeda et al., 1998), ubiquitin C-terminal hydrolase (Lowe et al., 1990), proteosomal subunits (Li et al., 1997), and ubiquitin (Gai et al., 1995). α-Synuclein immunoreactivity is the most sensitive and reliable diagnostic for Lewy-type pathology throughout PD, DLB, and LBVAD brains (Baba et al., 1998; Braak et al., 1999; Forno, 1996; Ince et al., 1998; Irizarry et al., 1998; Mezey et al., 1998; Spillantini et al., 1997, 1998; Takeda et al., 1998; Wakabayashi et al., 1997). The distribution of α-synuclein containing inclusions together with the recent

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discovery of two mutations in the α-synuclein gene linked to early-onset familial PD (Krüger et al., 1998; Polymeropoulos et al., 1997), and the ability of the protein to self-aggregate (Conway et al., 1998; Giasson et al., 1999; Narhi et al., 1999; Wood et al., 1999), strongly support a central role for α-synuclein in the pathophysiology of diseases with Lewy pathology.

METHODS

PRODUCTION AND CHARACTERIZATION OF TRANSGENIC MICE

Human α-synuclein cDNA (396 bp) was PCR amplified from 20 ng human brain cDNA (Clontech). The A53T mutation was introduced by PCR oligonucleotide-directed mutagenesis. Expression constructs were generated by cloning wild-type and mutant α-synuclein cDNAs into a murine Thy-1 cassette (Lüthi et al., 1997; Sturchler-Pierrat et al., 1997; Wiessner et al., 1999). Linear NotI DNA fragments comprising transgene without plasmid vector sequences were injected into homozygous C57BL/6 mouse eggs. Genotyping was performed by PCR (van der Putten et al., 2000).

RNA AND PROTEIN ANALYSIS

Northern blot analysis was carried out with total brain RNA (TriZol method). Probes were either 364 bp or 111 bp of the human α-synuclein cDNA or a 600 bp of Thy-1 3′-untranslated sequences (Lüthi et al., 1997; Wiessner et al., 1999). Standard procedures were used. 14 000×**g** supernatant fractions of half-brain homogenates were used for Western blot analysis. Protein samples were separated on a 15% SDS-PAGE. After blotting and blocking non-specific binding, membranes were incubated with rabbit anti-α-synuclein polyclonal antibody (1:1000; AB5038, Chemicon), followed by AP-conjugated anti-rabbit IgG (1:50 000; Sigma AO418), and chemiluminescent detection (Clontech) (van der Putten et al., 2000).

IN SITU HYBRIDIZATION

The spatial distribution pattern of transgene versus endogenous synuclein expression was determined by *in situ* hybridization (Lüthi et al., 1997; Wiessner et al., 1999) using cRNA derived from either a 364 bp complete coding cDNA template or a 111 bp cDNA template, corresponding to codons for the 37 carboxy-terminal amino acids of α-synuclein (van der Putten et al., 2000).

IMMUNOCYTOCHEMISTRY

Mice (aged 3.8–6 months) were perfused transcardially with 0.01 M phosphate buffered saline (PBS) followed by 4% paraformaldehyde in PBS. One brain hemisphere, spinal cord and hind limb muscle were embedded in paraffin and cut as 4 μm thick sections, and 25 μm vibratome sections were cut from the other hemisphere for free-floating immunocytochemical staining using anti-α-synuclein mouse monoclonal antibody (1:500; S63320, Transduction Laboratories), biotinylated anti mouse IgG (1:500; E0464, Dako) and the avidin-biotin peroxidase method (Elite standard kit SK6100, Vector). Deparaffinized sections were used for Campbell Switzer silver staining (Campbell et al., 1987), Holmes Luxol staining (Holmes, 1943), and immunostaining with rabbit anti-ubiquitin Ig fraction (1:200; Z0458, Dako), rabbit anti-glial fibrillary acidic protein (GFAP) Ig fraction (1:500; Z0334, Dako), anti-phosphotyrosine mouse monoclonal antibody (1:1500; P3300, Sigma) and anti-α-synuclein mouse monoclonal antibody (1:2000; 18-0215, Zymed). Antigenity was enhanced by treating paraffin sections with concentrated formic acid for 5 min and microwave heating at 90 °C for 60 min before incubation with anti α-synuclein; microwave heating at 90 °C for 30 min before anti-GFAP and anti-phosphotyrosine, and pronase treatment (37 °C, 30 min) before anti-ubiquitin. Non-specific binding sites were blocked using normal serum. Bound antibody was visualized using the avidin-biotin peroxidase method (Elite standard kit SK6100, Vector) and DAB substrate (1718096 Boehringer) or Vector VIP substrate (SK-4600, Vector).

IMMUNOELECTRON MICROSCOPY

For immunoelectron microscopy transgenic and wild-type C57BL/6 mice were perfused transcardially with a mixture of 1.5% picric acid, 0.1% glutaraldehyde, and 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Vibratom sections were stained free-floating with antibody to α-synuclein (18-0215, 1:2000) dehydrated in ascending series of ethanol and acetone, and flat-embedded between glass slides and coverslips in Embed-812 (Electron Microscopy Sciencies, Fort Washington, PA, USA). Fragments of the spinal cord were then dissected out, ultrathin sections were cut from the tissue surface, and these were mounted on copper grids and analyzed with a Zeiss EM900 microscope (van der Putten et al., 2000).

ROTATING ROD

The mice were trained twice daily and on two successive days to stay on a rotating rod (TSE, Bad Homburg, Germany) for 150 s (speed 12 rpm). Subsequently, the animals were tested on the rotarod once weekly, three times

and at three different speeds (i.e. 12 rpm, 24 rpm, and 36 rpm). The cut-off time used for measuring the endurance performance in all of these experiments was 60 s. The plotted mean endurance performance on a particular test day was the mean of the three performances at the given speed. Statistical evaluation of the data was done using repeated measures ANOVA (van der Putten et al., 2000).

RESULTS AND DISCUSSION

Several transgenic C57BL/6 lines were produced expressing human mutant (A53T) and wild-type α-synuclein under the control of murine Thy-1 regulatory elements to a different level. Two of the (A53T) mutant lines (9813 and 9956) and one wild-type line (S969) expressed abundant transgene mRNA and protein levels in brain. In these lines, transgene expression levels were similar, occurring in neurons throughout the telencephalon, brainstem, and spinal cord. In contrast, endogenous α-synuclein mRNA expression was prominent mainly in the telencephalon.

In all of the mice of lines 9813 and 9956 expressing mutant α-synuclein as well as in mice from line S969, we observed an early onset (>3 weeks of age) of motor deficits. Consequently, a progressive decline of motor performance was measured in the rotating rod experiment comparing a group of transgenic ($n = 7$) versus non-transgenic littermates ($n = 12$) of line 9813, as illustrated (Figure 61.1A; see Plate III). The results depict the impressive reduction in endurance of the transgenic mice to stay on the rotating rod.

This pronounced motor phenotype found in all of the mice of both A53T as well as the matching wild-type transgenic lines, and the documented expression of Thy-1-driven transgenes in motor neurons (Aigner et al., 1995), prompted us to examine the pathological changes more closely. Approximately 80% of the motor neurons expressed the α-synuclein transgene in the anterior horns of the spinal cord. Many of these cells showed diffuse perikaryal α-synuclein staining (Figure 61.1B,E). In addition Lewy-like pathology with pronounced ubiquitin immunoreactivity could be found in a subset of neurons (Figure 61.1C,F). Furthermore, staining with the routinely used Campbell-Switzer pyridine silver technique (Campbell et al., 1987), to detect Lewy-type changes in human brain tissue (Braak and Braak, 1999; Sandmann-Keil et al., 1999), revealed intense signals indicating that (rodent) motor neurons are susceptible to Lewy-like changes. Other changes associated with the development of the motor neuron pathology included astrocytic gliosis and microglial activation. Immunostaining for α-synuclein was also observed in spinal roots and nerve fiber bundles in muscles. In spinal roots axonal degeneration was observed, indicated by nerve fibers showing breakdown and segmentation into ellipsoids of the myelin sheath (Figure 61.1D). Skeletal muscles contained small angular fibers

indicating neurogenic muscular atrophy and consistent with denervation and neuropathology observed to varying degrees at neuromuscular junctions of gastrocnemius muscle taken from different 9813 and S969 mice. In individual mice up to 40% denervation was seen, and thinning of preterminal nerves and/or swellings occurred in at least 50% of innervated synapses. Overall, no significant differences could be observed between the pathological changes in A53T mutant and wild-type α-synuclein transgenic mice. Therefore, our findings provide substantial evidence for a pathological influence of human α-synuclein on motor neurons and offer a likely explanation for the observed reduction in complex motor performance. The presence of the A53T mutation does not seem to be required for the development of motor neuron pathology in transgenic mice. In agreement, others also reported that transgene expression of wild-type human α-synuclein can cause neuropathology. It was observed in dopaminergic neurons of mice (Masliah et al., 2000) and flies (Feany and Bender, 2000).

To investigate whether pathological changes extend to central areas, animals of both mutant lines and the wild-type transgenic mice aged 12 weeks and older ($n = 15$), were examined immunohistochemically. Techniques routinely used to assess Lewy pathology in human brain (Baba et al., 1998; Forno, 1996; Gai et al., 1995; Ince et al. 1998; Mezey et al., 1998; Spillantini et al., 1997; Takeda et al. 1998; Wakabayashi et al., 1997) were applied. In transgenic mouse brains high α-synuclein levels in cell bodies and dendrites were detected in many neurons in telencephalon and brainstem. In sharp contrast, endogenous α-synuclein distributes mainly to axonal and presynaptic locations in the nervous system of non-transgenic mice (Figure 61.2; see Plate IV). Increased perikaryal and neuritic staining of neurons by α-synuclein antibodies is also one characteristic feature in the diseased human brain (Baba et al., 1998; Braak et al., 1999; Irizarry et al., 1998; Mezey et al., 1998; Spillantini et al., 1997, 1998; Wakabayashi et al., 1997). Interestingly, α-synuclein staining in transgenic brain showed heterogeneous changes in neurites, very similar to those observed in human brain (Baba et al., 1998; Braak et al., 1999; Forno, 1996; Ince et al. 1998; Irizarry et al., 1998; Mezey et al., 1998; Spillantini et al., 1997, 1998; Takeda et al. 1998; Wakabayashi et al., 1997) including sausage-like enlargements of proximal and distal neuritic segments, thick or fine thread-like inclusions as well as beaded or spindle-shaped neurites. Such changes were most prominent and frequent in areas including the nucleus centralis oralis pontis, the nucleus vestibularis lateralis, the deep cerebellar nuclei, and the deep aspects of the tectal plate.

Immunostaining for ubiquitin is also frequently used to visualize Lewy pathology in human brain (Forno, 1996; Gai et al., 1995). In the transgenic mice, dystrophic neurites and cell bodies occasionally stained intensely for ubiquitin. The stained neurites displayed morphological features similar to those seen in human brains with Lewy pathology

(Forno, 1996). Ubiquitin-stained neurons with Lewy-like features in the transgenic mouse brain were less frequent when compared to cells with Lewy-like α-synuclein-positive features. Similar observations have been made in human brains with Lewy pathology (Braak et al., 1999; Forno, 1996; Ince et al. 1998; Irizarry et al., 1998; Spillantini et al., 1998; Takeda et al., 1998). These inclusions occur in the dystrophic (Lewy) neurites (Braak et al., 1999; Spillantini et al., 1998), suggesting that ubiquitination is a late event in the development of Lewy pathology in both mouse and human. This effect appears to be independent of mutation, gender, expression levels, and neuronal subpopulations and needs further investigation.

Both mutant and wild-type transgenic mice cover a remarkable spectrum of the Lewy pathological changes that are seen in postmortem brain tissue of DLB, PD and LBVAD patients. Our results identify the A53T mutant as well as wild-type of human α-synuclein as a pathogenic agent driving the formation of Lewy-like pathology in rodent central neurons. In both rodents and in humans, subsets of central neurons show α-synuclein-stained perikarya and Lewy-like neurites. A smaller subset of these cells also stains for ubiquitin. This suggests that ubiquitination is a late modification in cells with α-synuclein pathology. In addition, a selective and topographically-confined vulnerability of neurons to develop Lewy-like pathology is a feature of both the transgenic animals and human diseases with Lewy pathology (Braak et al., 1996; Forno, 1996; Ince et al., 1998; Takeda et al., 1998). It needs to be noted, however, that the pattern in the mouse is defined a priori by the properties of the transgene cassette, which fails to express in dopaminergic neurons of the substantia nigra pars compacta (S. Bischoff and van der Putten, unpublished observations).

Using transmission and α-synuclein immuno-electron microscopy, mainly small granules were detected in cytoplasm, dendrites, and occasionally in axons (not shown). We failed to see α-synuclein stained filaments typically seen in human Lewy inclusions (Baba et al., 1998; Braak et al., 1999; Irizarry et al., 1998; Mezey et al., 1998; Spillantini et al., 1997, 1998; Wakabayashi et al., 1997), which could relate to insufficient aging and/or species differences, since only granular but not fibrillar deposits have been described in transgenic mice expressing human wild-type α-synuclein (Masliah et al., 2000) whereas in transgenic α-synuclein *Drosophila* fibrillar material was seen (Feany and Bender, 2000). Perhaps species differences also account for the prominent, diffuse, ubiquitin staining seen in the cytoplasm of mouse but not human neuronal cell bodies with Lewy-like pathology. In human cell perikarya, the staining is usually confined to Lewy bodies (Braak et al., 1999; Forno, 1996; Gai et al., 1995; Ince et al., 1998; Irizarry et al., 1998; Spillantini et al., 1998; Takeda et al., 1998). But, interestingly, ubiquitin-staining of dystrophic neurites is remarkably similar in these species.

It is not known whether Lewy bodies in human diseases are cytotoxic, harmless side products, or markers of cell damage. However, other Lewy

pathology-like features seen in the transgenic mice correlate with cell damage and loss of function as particularly evident in motor neurons. Expression of mutant and/or wild-type human α-synuclein in different neuronal subpopulations may reveal additional pathological features shared between human and mouse. It could also help to elucidate the role of the wild-type protein in the pathophysiology of sporadic forms of diseases with Lewy pathology. α-Synuclein transgenic mice provide the means to address fundamental aspects of processes underlying the development of disorders with Lewy pathology and a novel animal model for synucleinopathy to test therapeutic strategies.

CONCLUSIONS

We have identified mutant and wild-type human α-synuclein as pathogenic agents driving Lewy-like neuropathology. Our animal models support a central role for α-synuclein in pathogenic events leading to changes resembling Lewy-like pathology. Brains of α-synuclein transgenic mice show a topographically confined vulnerability of selective neurons. The transgenic mice resemble hallmark features of Lewy-like pathology in human disease. The mice provide the means to address fundamental aspects of disease pathophysiology, to explore surrogate markers, and to test therapeutic strategies with behavioral and biochemical read-outs. Thy-1α-synuclein mice are particularly useful to address extranigral aspects of α-synucleinopathy commonly found in Parkinson's disease brains.

SUMMARY

Lewy pathology is a defining hallmark of degenerating neurons in postmortem brain tissue of patients with neurodegenerative disorders including idiopathic Parkinson's disease (PD), dementia with Lewy bodies (DLB), and a Lewy body variant of Alzheimer's disease (LBVAD). Lewy lesions appear to be central and may contribute mechanistically to dysfunction and degeneration of neurons in these diseases. A prime suspect for contributing to the pathophysiology of diseases with Lewy pathology is the presynaptic protein α-synuclein. It is a prominent constituent of Lewy structures in most sporadic idiopathic as well as familial forms of PD, DLB, and LBVAD. Furthermore, α-synuclein has the ability to self-aggregate, forming insoluble proteinaceous Lewy body-like assemblies. Recently, two mutations (A53T and A30P) in the α-synuclein gene were linked to early-onset familial PD with Lewy pathology. Also, they are known to accelerate self-aggregation of the cognate proteins and, in the A53T mutant, to slow down degradation. We now have been able to

recapitulate striking features of Lewy pathology in the mouse brain by neuronal expression of either the A53T mutant or wild-type human α-synuclein. Our animal models support a central role for α-synuclein in diseases with α-synucleinopathy and Lewy-pathology. They are the first rodent models with hallmarks of such diseases, and provide the means to address underlying fundamental aspects and to test therapeutic strategies.

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62 Somal and Neuritic Accumulation of the Parkinson's Disease-Associated Mutant [A30P]α-Synuclein in Transgenic Mice

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INTRODUCTION

Synucleins (SYNs) are 15–20 kDa proteins predominantly expressed in brain. Members of the family include αSYN and βSYN, and the more distantly related γSYN and synoretin (Kahle et al., 2000a). The central domain of αSYN had originally been identified as the non-amyloid β-protein component of Alzheimer's disease (AD) plaques (Uéda et al., 1993). Full-length αSYN is the major component of Lewy bodies and Lewy neurites, the characteristic brain lesions of patients with Parkinson's disease (PD) and related disorders (Hashimoto and Masliah, 1999). Two missense mutations in the αSYN gene have been linked to familial PD (Krüger et al., 1998; Polymeropoulos et al., 1997).

The physiological function of SYNs is unknown. αSYN is anterogradely transported (Jensen et al., 1999) to presynaptic terminals where it is enriched (Maroteaux and Scheller, 1991). Targeted disruption of the αSYN gene in mice caused a subtle perturbation in dopaminergic (DA) neurotransmission (Abeliovich et al., 2000). Like several other regulatory synaptic vesicle associated proteins, αSYN is phosphorylated (Okochi et al., 2000).

We have investigated the cellular expression of SYNs in transgenic mouse brain. The human PD mutant [A30P]αSYN was expressed in transgenic mouse brain under the control of a pan-neuronal promoter (Thy1; Moechars et al., 1996), and a DA neuron-specific promoter, tyrosine hydroxylase (TH; Schimmel et al., 1999). Thy1-driven [A30P]αSYN was

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detected in synaptophysin-positive fractions, and immunohistochemically detectable in neuropil. Specific (striatal) synaptic transport of [A30P]αSYN was demonstrated in TH-driven mice. Interestingly, [A30P]αSYN also accumulated in neuronal cell bodies and abnormally shaped neurites, in sharp contrast to the endogenous mouse αSYN. Thus, transgenic [A30P]αSYN did not fail to be transported to synapses, but its overexpression apparently caused pathological accumulations in neurons.

MATERIALS AND METHODS

Immunological tools and methods for generation and analysis of transgenic mice were described by Kahle et al. (2000b). The constructs allowing expression of h[A30P]αSYN under the control of the Thy1 and TH promoters are shown in Figure 62.1.

RESULTS

Lysates from brains of non-transgenic and (Thy1)-h[A30P]αSYN mice were subjected to a sucrose gradient flotation assay (Jensen et al., 1998). SYNs partitioned into the bottom and the floating fractions, with no difference between transgenic mice and non-transgenic controls (Figure 62.2). In this assay, soluble proteins remained in the bottom fractions, and the synaptophysin-positive floating fractions contained synaptosomes (Jensen et al., 1998; Kahle et al., 2000b). The same distribution of αSYN and βSYN was found in freshly processed cortical lobotomy samples of epilepsy patients (Kahle et al., 2000b).

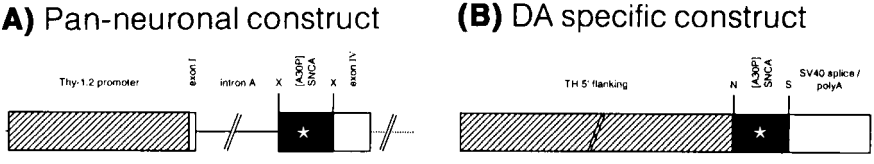

Figure 62.1. Schematic of the transgenic constructs (not drawn to scale). (A) Thy1 construct. Hatched box: mouse Thy1.2 promoter region. Open boxes: Thy1 exonic sequences. The (truncated) exon IV contains the polyadenylation signals. Solid line: Thy1 intron A. Start and stop codons of the open reading frame for h[A30P]αSYN (filled box; *: A30P mutagenesis site) are directly flanked by *Xho* I restriction sites (X). Dashed line: 3′ region of the Thy1 gene. (B) TH construct. Hatched box: 4.5 kB flanking DNA from the rat TH gene. Open boxes: SV40 small T antigen splice donor/acceptor site and SV40 polyadenylation site. Start and stop codons of the open reading frame for h[A30P]αSYN (filled box; *: A30P mutagenesis site) are directly flanked by *Nhe* I (N) and *Spe* I (S) restriction sites

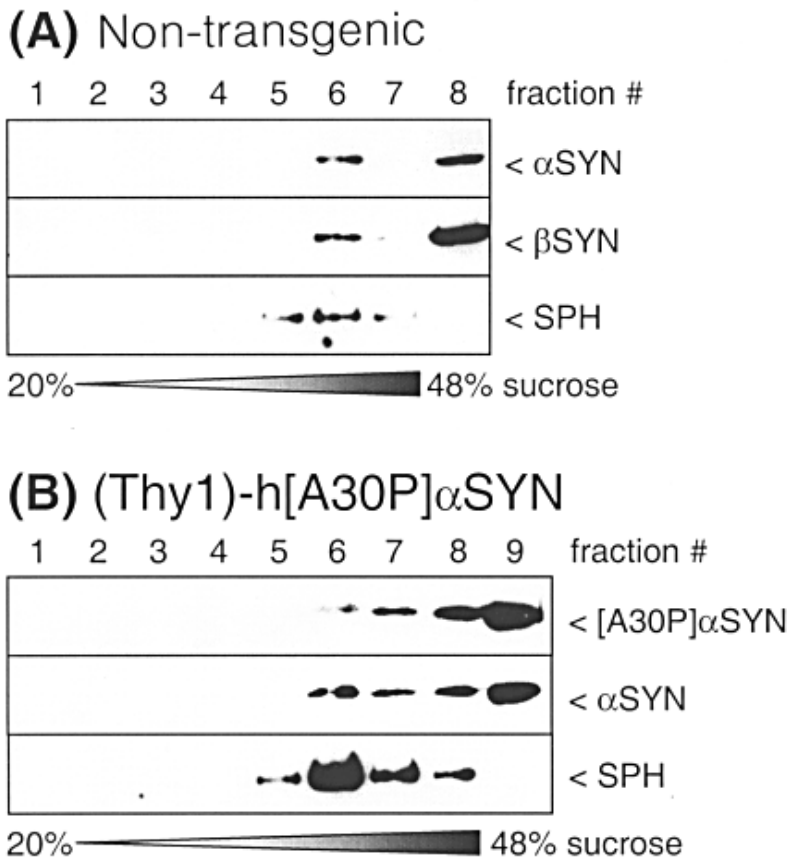

Figure 62.2. Synaptosomal localization of SYNs in mouse brain. Sucrose gradient (20–48%) flotation assays were performed with 200 µl post-nuclear supernatant from non-transgenic (A) and (Thy1)-h[A30P]αSYN (B) mice. Fractions were separated by SDS-PAGE (12.5%), and Western blots sequentially probed for αSYN, βSYN, and synaptophysin, as indicated

Immunohistochemical analysis of striata of (TH)-h[A30P]αSYN mice revealed positive staining with human-specific anti-αSYN (Figure 62.3A). Thus, the mutant protein was transported to the striatal DA terminals. Interestingly, h[A30P]αSYN was found to accumulate in somal compartments of DA neurons (Figure 62.3B,C). Moreover, strongly h[A30P]αSYN-immunoreactive neurites were frequently observed (Figure 62.3B). Such neuritic profiles were abnormally swollen or beaded.

In addition to the (normal) presynaptic synuclein staining of molecular layers and neuropil throughout the brain (examples from the cerebellar cortex and a deep cerebellar nucleus are shown in Figure 62.4), somal and neuritic

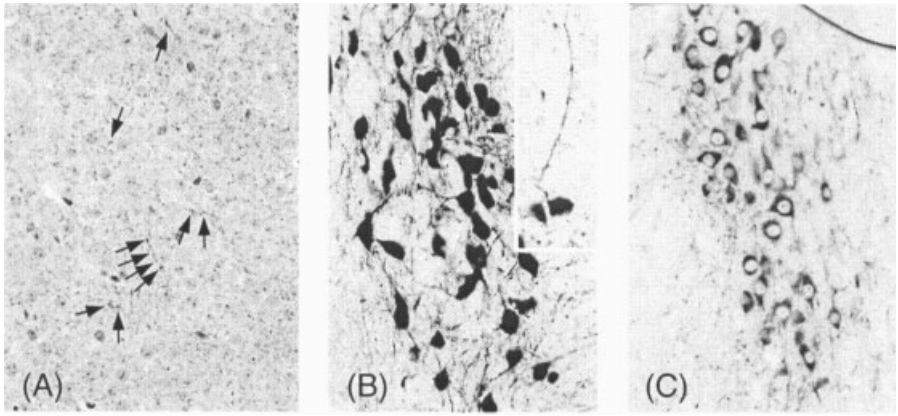

Figure 62.3. Presynaptic and somal/neuritic localization of h[A30P]αSYN specifically expressed in DA neurons. (A) H[A30P]αSYN-specific immunoreactivity associated with synaptic structures (arrows) was detected in the striatum. (B,C) Substantia nigra pars compacta stained with human-specific anti-αSYN (B) and anti-TH (C). h[A30P]αSYN was brightly stained in the cell bodies of most, if not all, TH-positive neurons. Moreover, bulbous h[A30P]αSYN-positive neurites could be traced up to several hundred μm (B, insert)

accumulations of the transgenic protein were also observed in (Thy1)-h[A30P]αSYN mice (Figure 62.4A,C). In striking contrast, endogenous mouse αSYN was not detected in these abnormal profiles (Figure 62.4B). The bulged appearance of h[A30P]αSYN-immunoreactive neurites was reminiscent of those found in Lewy body disease (Figure 62.4D). The occasional appearance of SDS-resistant h[A30P]αSYN-immunoreactive dimers in highly expressing transgenic mice (Figure 62.5) further indicated aberrations similar to early α-synucleinopathy in patients with PD and related diseases.

DISCUSSION

Synaptosomal partitioning and presynaptic staining of h[A30P]αSYN was found in transgenic mice. When expressed under control of the DA neuron-specific TH promoter, h[A30P]αSYN was correctly sorted to the striatum. Thus, the PD-associated A30P mutation did not abolish anterograde transport to the synaptic compartments. However, some perturbation of its axonal transport may be indicated by the accumulation of transgenic αSYN in neuronal cell bodies and neurites. Endogenous mouse αSYN was not retained in the pathological cell bodies and neurites. Thus, somal and neuritic

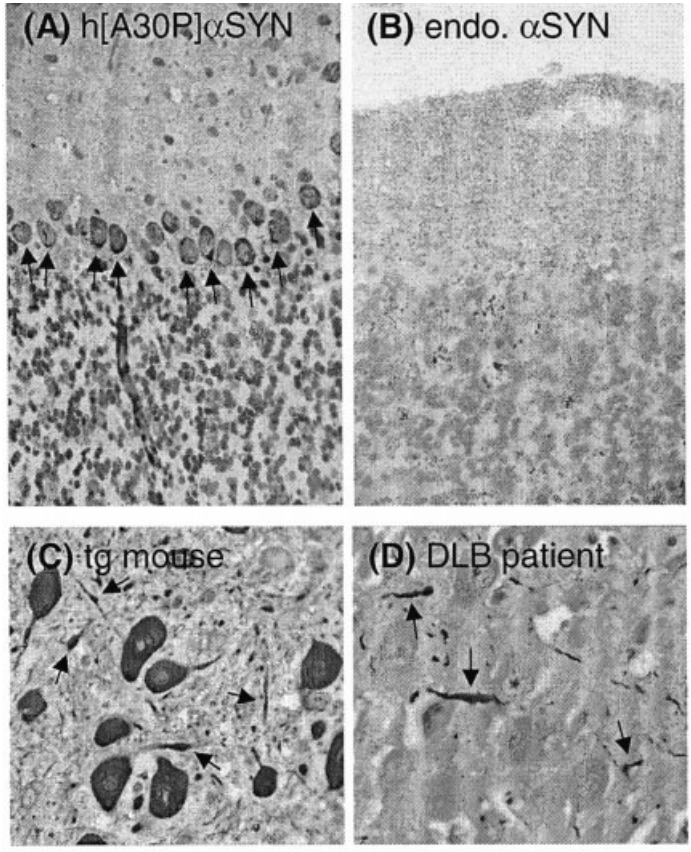

Figure 62.4. Presynaptic and somal/neuritic localization of pan-neuronally expressed h[A30P]αSYN. Human-specific anti-αSYN showed strong labeling of the molecular layer and cerebellar glomeruli in (Thy1)-h[A30P]αSYN mice (A). In addition, strong cytosolic immunoreactivity accumulated in Purkinje cells (arrows). In contrast, only the normal synaptic staining pattern of endogenous αSYN with labeling of the molecular layer was observed with the mouse-specific anti-αSYN (B). Bulbous, human αSYN-immunopositive neurites (arrows) in the cerebellar dentate nucleus of (Thy1)-h[A30P]αSYN mice (C) resembled Lewy neurites (arrows) in the hippocampal CA2/3 region of a human patient (D) stained with the same antibody

accumulation is a specific feature of transgenic human αSYN, and not simply due to an overload of the machinery transporting synaptic vesicle proteins.

Human αSYN (both wild-type and PD-mutant) expressed in transgenic *Drosophila melanogaster* was demonstrated to form the 7–10 nm fibrils which are characteristic for human Lewy bodies (Feany and Bender, 2000). An age-dependent decline in climbing behavior was found in transgenic flies. Transgenic mice expressing human wild-type αSYN under control of

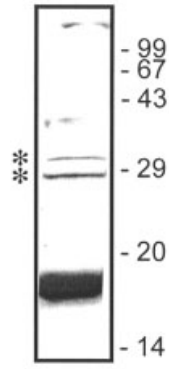

Figure 62.5. Whole brain cytosol (600 µg) from a (Thy1)-h[A30P]αSYN mouse (high expresser) was subjected to 15% SDS-PAGE. Western probing with human-specific antibody revealed the expected 19 kDa monomeric band and a double band with apparent molecular mass of an αSYN dimer (asterisks)

pan-neuronal promoters were presented very recently. These animals showed strong somal αSYN accumulations, which were sometimes ubiquitinated (Masliah et al., 2000; van der Putten et al., 2000). Decreases in dopaminergic markers and locomotor performance were reported for an exceedingly high expresser (Masliah et al., 2000). Our (Thy1)-h[A30P]αSYN and (TH)-h[A30P]αSYN mice showed no gross movement disability. Ubiquitination was not observed in the somal and neuritic abnormalities shown here. The pathological accumulation and dimerization of overexpressed h[A30P]αSYN might represent early stages of pathological abnormalities, which could finally lead to a PD-like phenotype. It remains to be shown if additional cofactors are required to induce fibril formation and generation of Lewy body-like deposits in vertebrate brain.

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63 *Ex Vivo* Transmission of Mouse-Adapted Prion Strains to N2a and GT1-7 Cell Lines

SYLVAIN LEHMANN, HUBERT LAUDE, DAVID A. HARRIS, RICHARD I. CARP, DIDIER VILETTE, SHIGERU KATAMINE, JEAN-YVES MADEC AND NORIYUKI NISHIDA

Transmissible spongiform encephalopathies (TSEs), or prion diseases, including Creutzfeldt–Jakob disease (CJD) and Gerstmann-Sträussler syndrome (GSS) in humans, and scrapie and bovine spongiform encephalopathy (BSE) in animals (Prusiner et al., 1998), are fatal neurodegenerative disorders. Human TSEs are unique in that they occur in infectious, sporadic or genetic forms. Although the nature of the infective agents, termed 'prions (Prusiner, 1982), is not fully understood, the conversion of the normal cellular prion protein, PrP^{C}, to an abnormal protease-resistant isoform, PrP^{Sc}, is a key event in the pathogenesis of all TSEs (Prusiner et al., 1998).

The *ex vivo* propagation of infectious agents causing TSEs presents, from both a fundamental and applied point of view, obvious advantages, including: (i) the ability to analyze in depth, at both the molecular and cellular levels, the nature of the infectious agent and the factors governing its propagation; (ii) detection and quantification of its infectivity without resorting to animal inoculation; and (iii) screening of drugs with potential therapeutic value. During the past two decades, considerable efforts have been made to set up tissue culture models supporting TSE agent replication. To date, very few propagating cell systems are available and their use is restricted to a small number of strains (Race et al., 1987; Rubenstein et al., 1984; Schatzl et al., 1997). We recently developed new models using the mouse cell lines GT1-7 and N2a (Nishida et al., 2000). The latter model utilizes transfected cells that overexpress the prion protein and have an increased sensitivity to the infectious agent. We report here attempts to infect GT1-7, N2a, and transfected MoPrP N2a cells with additional mouse-adapted prion strains of sheep, bovine, and human origin. Some strains were successfully propagated

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and in some cases with a high sensitivity, whereas other strains did not transmit in any of the cell line tested. Taken together, our results address the issue of the cell and strain tropism of prions.

GENERAL PROCEDURE OF *EX VIVO* TRANSMISSION EXPERIMENT

Our general method for *ex vivo* transmission is illustrated Figure 63.1. Brains from terminally ill mice infected with the different prion strains were

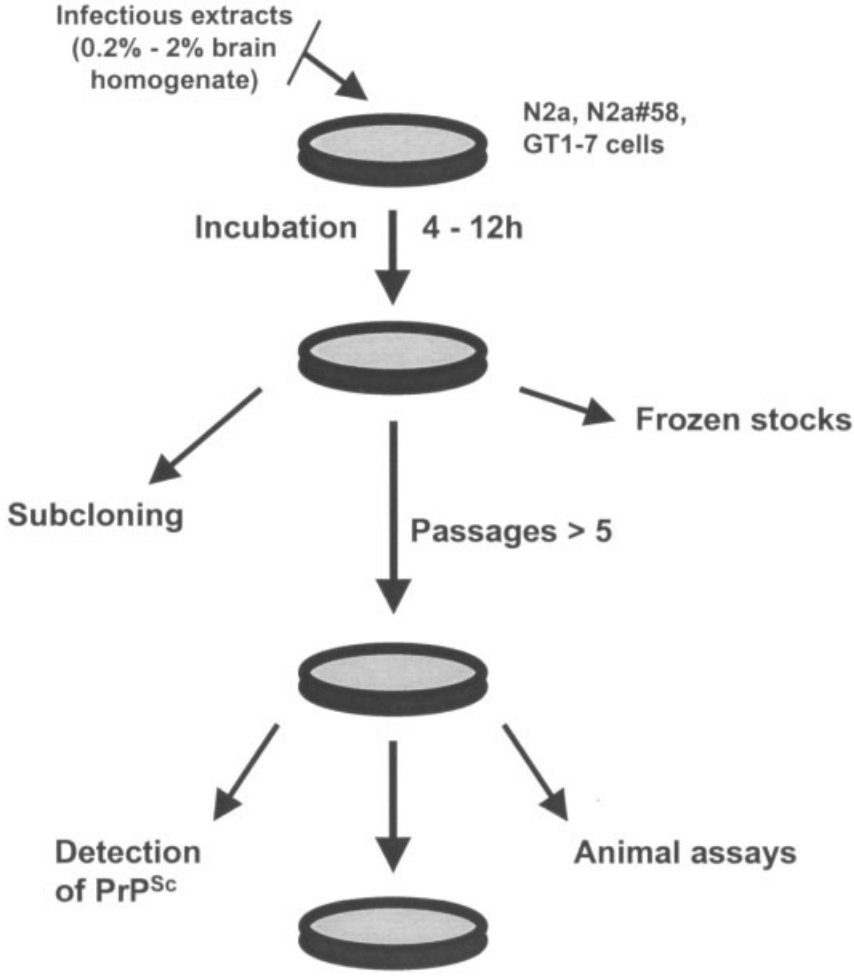

Figure 63.1. General procedure of *ex vivo* transmission experiments

homogenized (10% w/v) in cold phosphate buffer saline (PBS) containing 5% glucose. Cells were plated in six-well plates at an appropriate dilution for the culture to reach 60% of confluence after two days. The cells were then incubated for 4 to 12 hr with 1 ml of 0.2% or 2% brain homogenate diluted in Opti-MEM (in some cases the 2% homogenate showed a significant toxic effect on the cultures). After incubation with the homogenate, 1 ml culture medium was added and the cells were incubated for an additional day. The medium was then removed and the cells cultured as usual. At each passage of the cells, the presence of proteinase K (PK) resistant PrP was assessed by Western blotting as described (Nishida et al., 2000). We monitored the presence of PrP^{Sc} after five and ten passages of the cells to avoid false positive results linked to the detection of PrP^{Sc} from the inocula. Importantly, PK digestion must be optimized for each cell line in order to completely digest PrP^{C} from control, noninfected cells. Subcloning of the infected cells was sometimes performed, preferably at the first passage after inoculation to maximize the chance of getting infected clones. Frozen stocks were made at different times to prevent the loss or the contamination of infected cultures. In addition, it is essential after at least ten passages to demonstrate by animal assay that the cells not only generated PrP^{Sc} but actually carried the infectivity.

TRANSMISSION OF MOUSE-ADAPTED STRAINS TO N2A AND GT1-7 CELLS

We extended our first study (Nishida et al., 2000) on the transmission of Chandler, 22A, 22L, 139A, and 87V strains (see Figure 63.2) to three additional mouse-adapted prions (Table 63.1), as follows. The first one, ME7, is a well-known mouse-adapted scrapie strain established in *prnp–a* allele mice (Kascsak et al., 1985). The second one, Mo-BSE, has been

Table 63.1. *Ex vivo* transmission results

Name	Chandler (Ch)	22A	22L	87V	139A	ME7	Mo-BSE	Fukuoka-1
Mouse strain	CD-1	IM	C57/BL6	IM	C57/BL6	C57/BL6	C57/BL6	ddY
prpn alleles	aa	bb	aa	bb	aa	aa	aa	aa
N2a	0/8*	ND†	2/2	ND	ND	ND	ND	0/3
N2a moPrP	10/10	0/3	3/3	0/3	1/1	0/2	0/1	0/3
GT1-7	4/4	0/4	4/4	0/3	1/1	0/2	0/1	3/4

*Success/attempts.
†ND, not done.

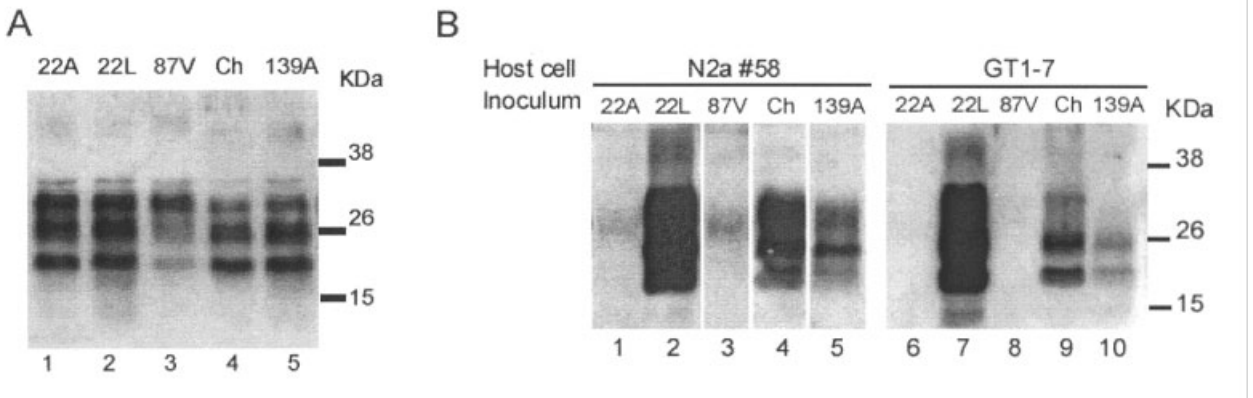

Figure 63.2. *Ex vivo* transmission of mouse-adapted strains to N2a#58 and GT1-7 cells. (A) Detection of PrP^{Sc} in brain extracts. Protein extracts (3 mg/ml) from brains of mice inoculated with various prion strains, 22A, 22L, 87V, Chandler (Ch), and 139A, were digested with proteinase K (100 μg/ml) and analyzed by Western blotting (Nishida et al., 2000). PrP^{Sc} was detected in all samples (15 μg/lane) but differences existed among the glycosylation patterns of the bands. The positions of molecular size marker proteins are designated in kDa. (B) Infection of transfected N2a and GT1-7 cell lines with different prion strains. GT1-7 and N2a#58 cells were infected with a 0.2% brain homogenate of 22A, 22L, 87V, Chandler, and 139A strains. After five passages (P5), the presence of PrP^{Sc} was analyzed by Western blotting, after PK digestion. Only 22L, Chandler, and 139A homogenates lead to the production of PrP^{Sc} by the infected cell lines. Reproduced from Nishida et al. (2000) by permission of the American Society for Microbiology

generated by inoculating BSE extract to C57/BL6 mice as described by Lasmezas et al. (1997). Finally, Fukuoka-1 strain was originally established from a GSS patient carrying the P102L mutation in the *prnp* gene (Tateishi et al., 1984). This strain was passaged many times in ddY mice.

As reported earlier (Nishida et al., 2000) (Figure 63.2), Chandler, 139A, and 22L were transmitted to both GT1-7 and N2a#58 cells while 22A and 87V were not. One possible explanation for the lack of transmission of the two latter strains was that they were generated in *prnp–b* allele mice while the cells are expressing the *prnp–a* allele. However, the fact that ME7, also generated in *prnp–a* allele mice, was incapable of infecting the cells suggests now that PrP polymorphism was not the only possible determinant for the transmission. Untransfected N2a cells were not infectable by Chandler in our hands, while the 22L strain was transmitted but with a low amount of PrP^{Sc} generated (not shown). These results are in agreement with our hypothesis that PrP overexpressing cells such as the N2a#58 clone are more susceptible to the agent.

The possibility of replicating the novel BSE strain in culture is of great interest for research focused on this disease and on vCJD (Collinge, 1999). Unfortunately, our transmission experiment using the mouse-passaged agent has been unsuccessful thus far (Table 63.1). On the other hand, the Fukuoka-1 strain of human origin was successfully transmitted to GT1-7 cells but not to N2a#58 cells. This suggests that strain propagation can be determined by cell specific factors other than PrP genotype.

EVALUATION OF THE SENSITIVITY OF THE *EX VIVO* TRANSMISSION EXPERIMENTS

In an attempt to evaluate the sensitivity of our *ex vivo* transmission experiments, N2a#58 cells were challenged with serial dilutions of Chandler brain extract (Table 63.2). The success of the experiment was assessed by the

Table 63.2. *Ex vivo* transmission of diluted Chandler extract in N2a#58 cell lines

Chandler inoculum* $10^{7.4}$ LD_{50}/g	µg of brain protein equivalent (1 ml)	LD_{50} equivalent	Success/attempts
2610^{-1}%	2000	5610^{4}	14/14
2610^{-2}%	200	5610^{3}	2/2
2610^{-3}%	20	500	2/2
2610^{-4}%	2	50	2/2
2610^{-5}%	0.2	5	0/2

Diluted inoculum (1 ml) was used to infect the cells. Positive transmission was initially assessed after five passages by the detection of PrP^{Sc} in the cultures but eventually confirmed after five additional passages, indicating that positive signal did not come from the original inoculum.

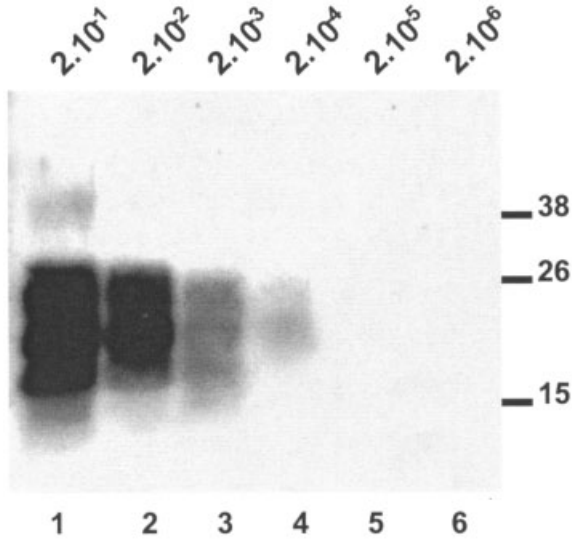

Figure 63.3. Test of the susceptibility of N2a#58 cells to serial dilution of Chandler brain homogenate. Western blot detection of PrP^{Sc} in mouse PrP transfected N2a cells line (#58) (Nishida et al., 2000), five passages after the infection using serial dilution (2610^{-2} to 2610^{-7}) of a 10% Chandler brain homogenate. Similar results were obtained using the GT1-7 cell line (not shown). The positions of molecular size marker proteins are designated in kDa

presence of PrP^{Sc} after five passages of the cells (Figure 63.3) and eventually confirmed after five additional passages (not shown). The transmission was positive for a dilution up to 2610^{-4} of the original 10% brain extract. Since 1 ml of the dilution was used to infect the cells, successive transmission was achieved using the equivalent of 2 μg of brain (Table 63.2). Titration of the original brain extract gave us an estimate of $10^{7.4}$ ID_{50} indicating that our cell model was able to detect around 50 ID_{50}. It is possible that the high sensitivity of this model results from the fact that transmission of the agent from cell to cell was detected in the cultures, as evidenced by the presence of infectivity in the culture medium (Nishida et al., 2000; Schatzl et al., 1997). An additional gain of sensitivity of the model could be achieved by scaling down the experiment to use less than 1 ml of inoculum, by increasing the time of contact of the culture with the agent, or by improving the detection of PrP^{Sc}.

CONCLUSION AND PERSPECTIVES

Our results showed clearly that one cell line can be infected by several prion strains. Conversely, it appeared that one strain can infect several different cell

lines. However, it is noteworthy that the replication of the agent in culture is less efficient than in animals, at least for the mouse-adapted strains used in this work. An important issue regarding this work is represented by the differences in susceptibility between cell lines. In addition to the level and the type of PrP molecules expressed by the cells, several other factors may also account for this phenomenon. First, it is possible that the cell lines differed by the expression of accessory molecules important for the replication of the agent (chaperon, protein X) (DebBurman et al., 1997; Telling et al., 1995). Second, the general trafficking of PrP in the different lines, as well as its cellular environment in detergent-resistant domains, can play a role (Caughey et al., 1991; Kaneko et al., 1997; Mange et al., 2000; Taraboulos et al., 1994). Along the same lines, it is possible that the processing of PrP itself, in particular its endogenous cleavage and degradation pathways, may be involved (Caughey et al., 1991; Chen et al., 1995). Finally, the possibility that post-translational modifications of PrP such as its glycosylation could modulate the conversion cannot be neglected (Dearmond et al., 1999; Lehmann and Harris, 1997).

Another conclusion of our work concerns the development of cell lines that are highly susceptible to TSEs agents. From our findings, it seems possible to envision the use of particular *ex vivo* transmission models as a tool for the detection of the infectious agents of TSEs that is both sensitive, relatively rapid, and does not rely on the use of laboratory animals. We are currently working towards establishing cell lines expressing high levels of bovine or human PrP that can be expected to be susceptible to the BSE and vCJD agents.

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64 *In Vivo* Perturbation of Lysosomal Function Promotes Neuro-degeneration in the $PS1_{M146V}/APP_{K670N,M671L}$ Mouse Model of Alzheimer's Disease Pathology

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The lysosomal system is a family of acidic vesicular organelles, which includes lysosomes, late endosomes, Golgi-derived vesicles containing newly synthesized hydrolases, and residual bodies containing indigestible materials such as lipofuscin. As the terminal degradative compartment of cells, lysosomes metabolize complex cellular and extracellular constituents to monomers, which can then be utilized for biosynthesis. Lysosomal hydrolysis in other compartments of the system, such as the early endosome, may carry out limited proteolysis generating new and potentially functionally important proteins or peptides (Cataldo et al., 1995). Genetic evidence strongly links dysfunction of the lysosomal system to progressive neurodegeneration of the cerebral cortex. In most of the 30 or more inherited human disorders involving defects in the synthesis, sorting and transport of lysosomal enzymes, extensive neurodegeneration is a prominent, if not the dominant, morphologic feature.

In Alzheimer's disease (AD), marked activation of the neuronal lysosomal system develops at a very early stage of the disease and intensifies as neurons become evidently compromised. This response, which occurs in virtually all neurons within cell populations susceptible to AD, is characterized by

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lysosome proliferation, elevated gene expression and synthesis of lysosomal acid hydrolases, and increased lysosomal protease (cathepsin) levels and activities (Callahan et al., 1999; Cataldo et al., 1991, 1995, 1996). One consequence of lysosomal system upregulation, an increased trafficking of 'lysosomal' proteases to early endosomes, accelerates beta-amyloid (Aβ) generation when this abnormality is modeled in transfected cells (see Chapter 41).

While calpains and caspases receive the greatest attention as executioners of cell death phenomena, cathepsins, particularly cathepsin D (Cat D), have recently been implicated as key agents of death in some cultured cell systems (Deiss et al., 1996; Heinrich et al., 1999; Isahara et al., 1999; Shibata et al., 1998). Lysosomal-mediated cell death is also initiated by the endocytosis of oxidizable substrates (e.g. Aβ, low-density lipoprotein; LDL), which selectively injure lysosomal membranes, cause leakage of cathepsins, and induce apoptosis or a mixed apoptotic/necrotic pattern (Brunk et al., 1997; Roberg and Ollinger, 1998; Roberg et al., 1999; Yang et al., 1998).

In this study, we investigated the relationship of lysosomal system function and neurodegeneration in transgenic mice expressing a familial AD-causing mutant form of human amyloid precursor protein, $APP_{K670N,M671L}$, and presenilin 1, $PS1_{M146V}$. Aβ begins to deposit in PS/APP mice at 10–12 weeks of age and progressively accumulates as plaque-like lesions throughout the animal's life span, reaching levels exceeding those in human AD brain (Holcomb et al., 1998; McGowan et al., 1999; Takeuchi et al., 2000). Several other features of the human disease are also seen, including neuritic dystrophy and associated local inflammation within the plaques (McGowan et al., 1999; Takeuchi et al., 2000); however, neurofibrillary tangle formation is absent and neuronal cell loss is below the limits of detection (Takeuchi et al., 2000). The lysosomal system is only modestly activated compared to that seen in Alzheimer brain (Mathews et al., 1999). To determine the effects of increasing lysosomal dysfunction in PS/APP mice and age-matched non-transgenic mice, we perturbed the lysosomal system with intraventricular administration of the cysteine protease inhibitor leupeptin over a 1-month period. Previous studies have shown that leupeptin elevates the levels and activity of Cat D (Bednarski and Lynch, 1996), presumably by inhibiting its degradation, and also causes ceroid and lipofuscin to accumulate within lysosomes (Ivy et al., 1984). These and other effects of leupeptin resemble alterations of the lysosomal system seen during normal brain aging (Bednarski et al., 1997; Ivy et al., 1989; Matus and Green, 1987; Nakanishi et al., 1997), which are accentuated in AD. Our results demonstrate synergistic effects between these aging-related alterations of the lysosomal system and the PS/APP phenotype in promoting neurodegeneration, supporting the view that the lysosomal alterations seen in AD are relevant to the mechanism of neurodegeneration.

METHODS

The PS/APP transgenic mouse has been previously described (Duff et al., 1996). For *in vivo* inhibition of cysteine proteases, ALZET osmotic pump brain infusion kits were stereotaxically implanted into the lateral ventricle of PS/APP mice and non-transgenic littermates. The attached model 2004 mini-osmotic pump (0.25 μl/h delivery rate) was loaded with 10 mg/ml leupeptin, 20 mM HEPES pH 7.4 or, for vehicle control mice, 20 mM HEPES alone. Mice were sacrificed 28 days later by CO_2 inhalation. One hemisphere from each dissected brain was quick frozen for biochemical analysis while the remaining hemisphere was immersion fixed in aldehyde for histological and immunocytochemical examination. Vibratome sections (30 μm) were stained in cresyl violet (Nissl) for inspection of cytoarchitecture. Serial adjacent sections were processed for immunocytochemical studies as previously described (Cataldo et al., 1990) using a commercial rabbit polyclonal antibody (Dako Corporation, Carpinteria, CA) raised to Cat D isolated from human liver. Western blot analysis of mouse brain and human tissue for Cat D and other lysosomal proteases was done and quantitated as previously described (Compaine et al., 1995). Cat D activity was determined using ^{14}C-methemoglobin as substrate as previously described (Nixon and Marotta, 1984).

RESULTS

Six-month-old PS/APP mice exhibit increased Aβ levels and moderate levels of Aβ deposition. These changes are associated with modestly elevated Cat D levels compared to control mice but no evident atrophy or loss of neurons (Figure 64.1A,B). Groups of PS/APP mice and age-matched non-transgenic mice were infused intraventricularly with leupeptin (10 mg/ml; 6 μl/day) or vehicle alone for one month through permanently implanted ALZET minipumps. Neither group of mice exhibited gross behavioral changes. In each of the leupeptin-infused groups, we observed lysosomal system effects similar to those previously described in rats (Ivy et al., 1989). Lipopigment accumulated substantially in pyramidal neurons in the neocortex and hippocampus and certain other cell populations that normally display minimal lipofuscin accumulation at this age in mice (Figure 64.2B,C). Markedly elevated Cat D activity was accompanied by commensurately increased levels of Cat D protein determined by Western blot analysis (Figure 64.2E). These alterations were comparable to those observed in human cortex from aged individuals (Figure 64.2A,D). The activities of two leupeptin-sensitive cysteine proteases, cathepsins B and L, were reduced despite an increase in levels of cathepsin B protein (data not shown).

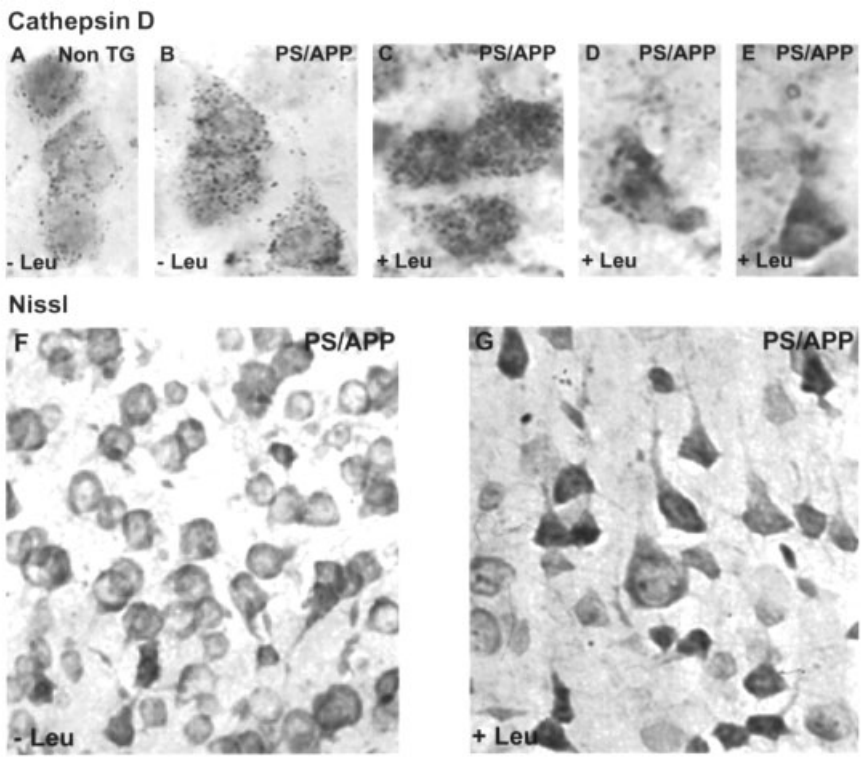

Figure 64.1. At 3 months of age, PS/APP mice (B) showed some lysosomal system upregulation as demonstrated by Cat D immunoreactivity when compared to non-transgenic mice (A). Lysosomal system upregulation, represented by increased numbers of hydrolase-positive lysosomes, was greater in PS/APP mice of the same age infused with the cysteine protease inhibitor, leupeptin (C). In PS/APP mice, the leupeptin-induced lysosomal response was further accentuated with age (D, 6 months; E, 9 months), with enlarged lysosomal profiles (lysosome fusion) and abnormal accumulation of Cat D immunoreactivity within the cell body and processes becoming apparent. Cresyl violet stained sections of the cingulate cortex from a leupeptin-infused PS/APP mouse (G) showed many of the same morphologic changes typical of aging human brain including lipopigment accumulation (not shown), increased neuronal sclerosis and decreased cellularity compared with buffer vehicle infused age-matched PS/APP mice (F)

Although leupeptin is able to inhibit calpain activity, we detected no change in the pattern of breakdown products of spectrin and MAP2, which would reflect a significant change in the activity of this proteolytic system (Fischer et al., 1991; Siman, 1990).

While leupeptin infusion had similar effects on the lysosomal system in PS/APP and non-transgenic mice, the consequences for neurons of PS/APP mice

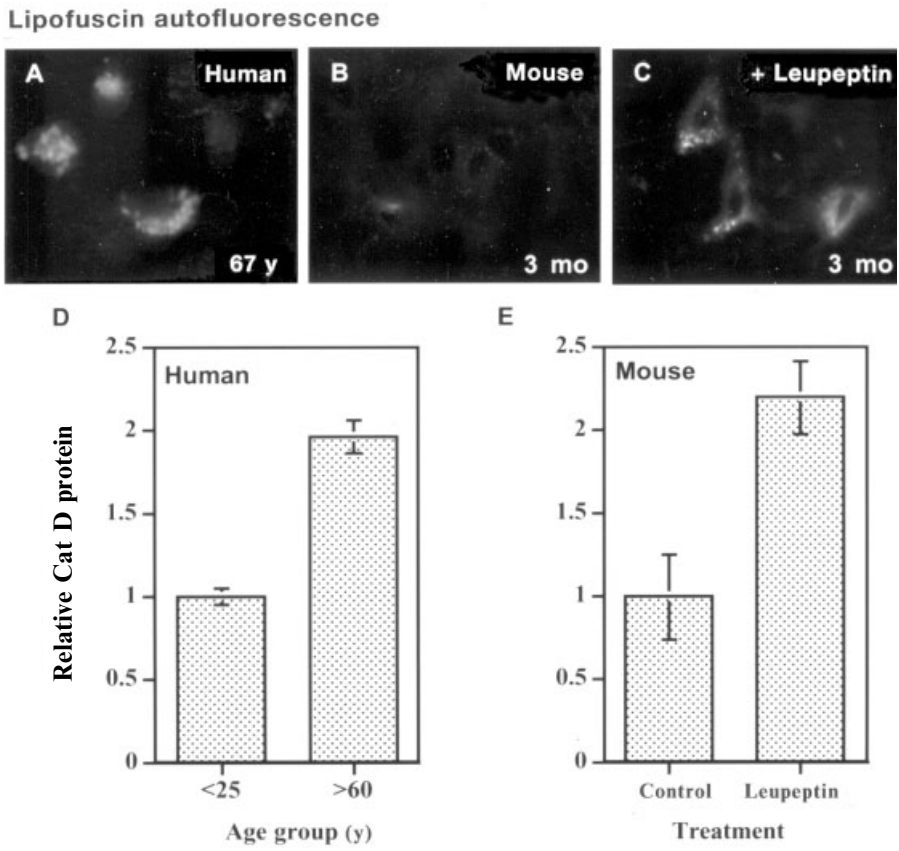

Figure 64.2. Abundant autofluorescent lipofuscin granules were seen in neurons from aged humans (A). Intraventricular infusion of leupeptin into a 3-month-old mouse resulted in a pattern of neuronal lipofuscin accumulation similar to that seen in an aged human (C) whereas a control mouse showed little autofluorescence (B). Quantitation by Western blot analysis showed a doubling of Cat D protein levels in brain when young individuals (14 –25 years of age) were compared to aged individuals (60–79) (D). A similar increase in Cat D protein increase was seen in mice following a one-month intraventricular infusion of leupeptin (E)

were much more grave. Hippocampal and neocortical neurons, immunolabeled with Cat D antibodies, contained larger and more numerous lysosomes and lipofuscin granules laden with Cat D, more closely resembling neurons in affected populations in the AD brain (Figure 64.1C). Some neurons at end stages of degeneration were identified based on their atrophic appearance and on the condensation of intracellular Cat D immunoreactivity into very large amorphous vesicular structures (Figure 64.1D,E). In tissue

sections stained with cresyl violet, neuronal sclerosis (Figure 64.1G) was obvious and resulted in a reduced thickness of the cortical mantle (data not shown). Visual inspection suggests that the number of neurons in the cerebral cortex of leupeptin-infused PS/APP mice was significantly diminished (Figure 64.1G). By contrast, loss or atrophy of neurons was not evident in leupeptin-infused non-transgenic mice (Figure 64.1F).

DISCUSSION

Our findings provide *in vivo* evidence for a close relationship between altered lysosomal function and the onset of neurodegeneration in a transgenic mouse model of AD pathology. These results are in accord with growing *in vitro* evidence suggesting that primary injury to lysosomes and the actions of specific cathepsins can initiate and mediate apoptosis or apoptotic/necrotic patterns of cell death. Cat D, which was markedly elevated in our studies, has been considered particularly important in this regard. Cat D has been shown in several laboratories to mediate cell death in response to various apoptotic stimuli (Deiss et al., 1996; Heinrich et al., 1999; Shibata et al., 1998). Cat D overexpression promotes apoptosis induced by trophic factor withdrawal independently of caspase actions, supporting other evidence that cathepsin actions may be upstream of caspase activation or independent of caspase activity (Heinrich et al., 1999; Isahara et al., 1999; Shibata et al., 1998).

Our findings suggest synergy between lysosomal system perturbations and the PS/APP phenotype in promoting neurodegeneration. A basis for this synergy is suggested by several observations. Neuronal lysosomal system activation is accentuated in PS1-linked human familial AD beyond that seen in sporadic AD (Mathews et al., 1999) and preliminary evidence indicates that, when expressed in transgenic mice or transfected cells, mutant PS1 activates the lysosomal system, in part by stimulating the autophagic pathway. Moreover, Aβ is one of several oxidizable substrates taken up by cells and delivered to lysosomes where it may cause direct oxidative injury to lysosomal membranes and further stimulate Cat D expression (Yang et al., 1998).

Normal brain aging is an essential requirement for AD development. The lysosome is a prominent target in normal cellular aging and multiple proteases have long been considered critical to the aging process. Certain cathepsins, notably the aspartyl protease Cat D, become substantially more active in the aging brain (Matus and Green, 1987; Nakanishi et al., 1997) while lysosomal cysteine proteases such as cathepsin B, which degrades Cat D, become less active. These and other aging-related changes of the lysosomal system are considerably more marked in the brains of humans than in lower mammals (unpublished observations). In PS/APP mice, leupeptin treatment, which causes a cathepsin imbalance similar to the human aging-related imbalance, accentuated pre-existing lysosomal system abnormalities to the level seen in

human AD brain and induced neuronal atrophy and neurodegeneration in the neocortex and hippocampus. The minimal 'lysosomal aging' seen in old mice when compared to humans may partly explain why transgenic models of AD pathology exhibit mild neurodegenerative phenotypes. Most importantly, these studies provide *in vivo* evidence for a close relationship between altered lysosomal function and neurodegeneration, and implicate lysosomal mechanisms in AD pathogenesis.

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65 Changes in Cognitive Characteristics of Tg(APP)CRND8 Mice at Early Stages of Immunization with Beta-Amyloid Peptide

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The progressive cognitive dysfunction of Alzheimer's disease (AD) is accompanied by a series of neuropathological changes including parenchymal and blood vessel deposition of beta-amyloid (Aβ) peptide in the human brain. Many lines of evidence indicate that abnormal processing of Aβ peptide (a proteolytic derivative of the Aβ precursor protein, βAPP) plays a central role in initiating the pathogenesis of AD (Steiner et al., 1999). There is also strong support for the positive correlation between Aβ plaques and cognitive impairment in AD patients (Cummings et al., 1996; Haroutunain et al., 1998; Kanne et al., 1998). However, there are gaps in our knowledge of the pathogenesis of even the 'simple' genetic forms of AD. Thus there has been a need for an animal model which develops some or all aspects of this uniquely human disease in a reproducible fashion to decipher and stratify crucial pathogenic events. Such animal models would also, of course, be useful for testing therapies.

Current transgenic (Tg) mouse models of AD fall short of recapitulating all of the pathological changes seen in this disease, or have confounding neuroanatomical perturbations, or have rather evanescent behavioral phenotypes. Nonetheless, some Tg mouse models of AD, such as the Tg2576 or PDAPP Tg, do develop incontrovertible AD-like mature amyloid deposits and

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researchers have used these to venture into 'second-generation' experiments to assess candidate therapies. One recent breakthrough in this field investigated the use of immunization against the Aβ peptide. These studies documented significant reduction in the Aβ plaque burden in the Tg PDAPP mouse brain (Schenk et al., 1999). The follow-up report proved that antibodies against Aβ peptide, when given peripherally to mice via passive administration, were sufficient to reduce amyloid burden via induction of clearance of pre-existing amyloid (Bard et al., 2000). In addition to pioneering a particular therapeutic regimen, these studies were important in that they established that AD amyloid deposits, long regarded as insoluble and metabolically inert, are in fact labile, as now confirmed by the use of other experimental paradigms (Nakagawa et al., 2000). However, the effect of immunization with Aβ peptide on learning and memory in a mouse Tg model was not addressed in the above studies. Evidence that Aβ immunization also reduces cognitive dysfunction in murine models of AD would strongly support the hypothesis that abnormal Aβ processing is critical to the pathogenesis of AD, and would encourage the development of strategies directed at intervening at various points in the 'amyloid cascade' (Steiner et al., 1999).

The experiment evaluating the effect of Aβ immunization on cognition in a Tg mouse model has to incorporate longitudinal administration of an immunogen (Schenk et al., 1999) and the behavioral paradigm should take into account the following characteristics of behavioral deficits in AD patients. First, in AD there are defects in multiple spheres of cognitive function (several types of memory, judgment, executive planning, motor praxias, cognitive processing speed etc). Thus, AD is not just a disease of learning or of memory (Katzman et al., 1999). Second, learning is not completely absent in AD patients until very late in the disease when there has been massive cell loss and the patients are bedridden. To replicate this, a potential animal model should therefore show slower rate of learning but not its complete absence. Third, the experimental design should mimic designs used in human clinical trial designs and should follow longitudinal administration of the immunogene.

Since the main purpose of the present behavioral experiment was to test the hypothesis that immunization with the Aβ42 peptide alleviates the cognitive impairment in transgenic APP mice a longitudinal design was chosen in order to provide a detailed overall characterization of alterations in cognitive performance in the same mice during overall immunization regimen. The choice of the design followed the premise that it is unlikely that AD patients should ever be treated continuously but evaluated only once. Moreover, and in contrast to cross-sectional, one time-point designs, the longitudinal design exposes the study to the type of confounds likely to be encountered in the real world of clinical trials. These confounds include: potential loss of power due to interference by intercurrent death, re-test (Martin and Bateson, 1996) effects, and the reality that the untreated phenotype progresses with time.

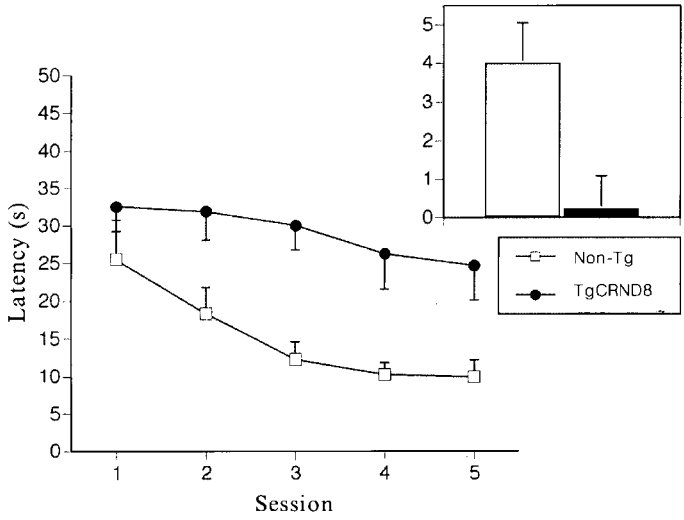

Figure 65.1. The TgCRND8 mice show significant impairment in spatial learning and spatial memory (insert) assessed in the water maze test. Experimentally naïve, non-immunized TgCRND8 mice ($n = 10$) and their non-Tg littermates ($n = 7$) were trained in the water maze throughout five sessions (days) with four trials per session at 11 weeks of age. Although all mice showed a significant improvement during training ($F(4,60) = 3.3$, $p < 0.02$), the TgCRND8 mice had significantly longer escape latencies ($F(1,15) = 17.98$, $p < 0.001$) to the hidden platform than non-Tg mice (genotype×session interaction was not significant). The insert shows an annulus crossing index during the probe trial administered after the learning acquisition phase. The index represents the number of platform site crosses in the target quadrant during the probe trial adjusted for site crossings in other quadrants (index = TQ−(AR+AL+OP)/3). A highly positive index indicates selective spatial memory for the spatial platform position. TgCRND8 mice showed significant impairment in spatial memory assessed by their search for the platform position during the probe trial

We have created a line of Tg mice denoted TgCRND8 encoding a compound mutant form of the human amyloid precursor protein, βAPP_{695} (K670N/M671L and V717F in *cis*), under the regulation of the Syrian hamster prion promoter on a C3H/B6 strain background (Chishti et al., in preparation). By ~12 weeks of age, these mice show significant spatial learning deficits (Figure 65.1) which are accompanied by increasing numbers of cortical Aβ-containing amyloid plaques and by increasing levels of SDS-soluble Aβ (Chishti et al., in preparation).

To assess the effect of $A\beta_{42}$ immunization on the learning deficits of TgCRND8 mice, age and sex-matched cohorts of TgCRND8 mice ($n = 21$) and of non-Tg littermates ($n = 39$) were repeatedly vaccinated, following the regimen used by Schenk and co-workers (Schenk et al., 1999), at 6, 8, 12, 16, and 20 weeks with either $A\beta_{42}$ or islet associated polypeptide (IAPP) peptide.

IAPP was selected as the control immunogen because it has similar biophysical β-sheet properties, but is associated with a non-CNS amyloidosis. The mice cognitive behavior was repeatedly evaluated in the hidden platform version of the Morris water maze test at 11, 15, 19, and 23 weeks of age. At each age of behavioral testing, mice were trained for five days with four trials per day and the hidden platform was placed in a different quadrant of the pool. The water maze was chosen because it allows evaluation not only of spatial learning and memory but also evaluates locomotor and exploratory abilities of mice, and development of appropriate search strategies for a spatial position. Behavioral pilot studies in non-Tg mice established that immunization with Freund's adjuvant + phosphate buffered saline, Freund's + $A\beta_{42}$, Freund's + IAPP, IAPP alone, or $A\beta_{42}$ alone all had no specific effect on locomotor performance or perceptual systems of mice in the Morris water maze test. To assess the effect of the vaccination on behavior, performance in the water maze for the entire 11–23 week experimental period was analyzed using a mixed model of factorial analysis of variance (ANOVA) with immunogen ($A\beta_{42}$ vs. IAPP) and genotype (TgCRND8 vs. non-Tg) as between-subject, and age-of-testing (11, 15, 19, 23 weeks) as within-subject factors.

The main analysis revealed significant immunogen × genotype ($p < 0.01$) and immunogen × genotype × age ($p < 0.05$) interactions, as well as significant main factor effects for immunogen, genotype, and age-of-testing ($p < 0.01$ for all), indicating that although all mice improved their performance during the experiment the response significantly depended on the type of immunogene and genotype of mice. Consecutive analysis of simple effects revealed that overall, the $A\beta_{42}$-immunized TgCRND8 mice performed significantly better than IAPP-immunized TgCRND8 mice (immunogen effect within TgCRND8 mice: $p < 0.05$) within 11–23 week experimental period. Figure 65.2 shows the age effect as the average escape latency of all five training sessions at each age of testing. In the conclusion, that $A\beta_{42}$ immunization ameliorates the cognitive deficit of TgCRND8 mice, was robust regardless of whether the analysis assessed latency to reach the hidden platform or swim path length (a measure less sensitive to swim speed and floating). Furthermore, $A\beta_{42}$ immunization had a relatively large effect because 31% of the variance in the performance of immunized TgCRND8 mice was attributable to the effects of the immunogen. However, although $A\beta_{42}$ immunization improved behavior in TgCRND8 mice, it did not fully restore it to the level of non-Tg mice (genotype effect within $A\beta_{42}$-immunized mice was significant: $p < 0.01$; Figure 65.2).

The performance of mice for individual test periods (11, 15, 19, and 23 weeks) is presented in Figure 65.3. *Post hoc* analyses revealed significant immunogen × genotype interactions at 11 and 23 weeks ($p < 0.02$ and $p < 0.001$, respectively). The subsequent analysis of simple effects revealed that $A\beta_{42}$ immunization markedly improved spatial learning in TgCRND8 mice relative to IAPP mice at 11 weeks ($p < 0.05$) and 23 weeks ($p < 0.01$).

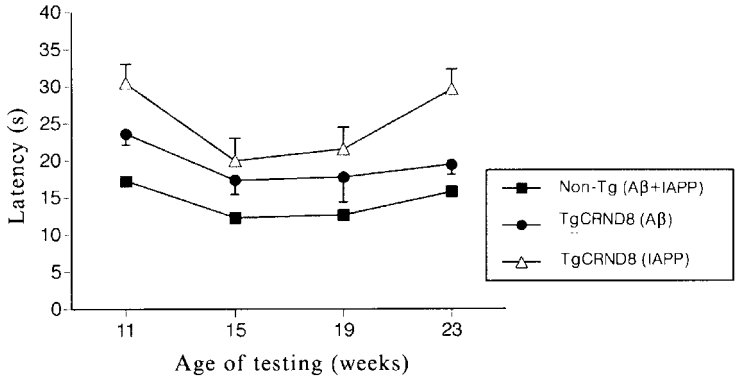

Figure 65.2. The overall effect of immunization with $A\beta_{42}$ peptide on TgCRND8 mice spatial learning during the 11–23 week experimental period. Escape latencies were averaged across all five training sessions at each age of longitudinal testing. The performance of $A\beta_{42}$ immunized TgCRND8 mice was similar to that of non-Tg littermates. However, their performance did not reach the level of non-Tg mice. In contrast, IAPP immunized mice showed longer latencies when compared to $A\beta_{42}$ immunized TgCRND8 mice ($p < 0.05$) and their non-Tg littermates ($ps < 0.001$)

Furthermore, $A\beta_{42}$ immunization accounted for large portions of the variance in performance at both age periods ($\omega^2 = 19\%$ at 11 weeks; $\omega^2 = 42\%$ at 23 weeks). Analyses at 15 and 19 weeks did not show statistically significant effects. This lack of significant immunization effect at 15 and 19 weeks, and its reappearance at 23 weeks, may reflect a carry-over effect and progression of the phenotype.

In addition to reducing the behavioral deficits, $A\beta_{42}$ immunization caused significant ($\sim 50\%$) reductions in the number and size of dense-cored plaques containing fibrillar, β-sheet forms of Aβ in the hippocampus and cerebral cortex of TgCRND8 mice (Figure 65.4).

A simple explanation of the results is that cerebral amyloid plaques are the toxic moiety, and that the modest ($\sim 50\%$) reduction in dense-cored, mature amyloid plaques caused by $A\beta_{42}$ vaccination is sufficient to prevent or reverse the behavioral deficits. However, a more likely alternative explanation is that immunotherapy is active against either a particular conformational species of Aβ (e.g. fibrillar or protofibrillar forms), or Aβ in a restricted compartment. Several lines of evidence favor the former option. First, recent *in vitro* studies have suggested that small, oligomeric assemblies of Aβ ('protofibrils') are the most neurotoxic conformational species (Hartley et al., 1999, Pike et al., 1993, Walsh et al., 1999). Second, these diffusible, toxic forms of Aβ, which comprise only a small proportion of the total Aβ in the brains of AD patients, are the most accurate predictors of neurodegeneration (McLean et al., 1999).

Although producing a strong effect, $A\beta_{42}$ immunization did not fully reverse the behavioral deficits and neuropathology in TgCRND8 mice. This might

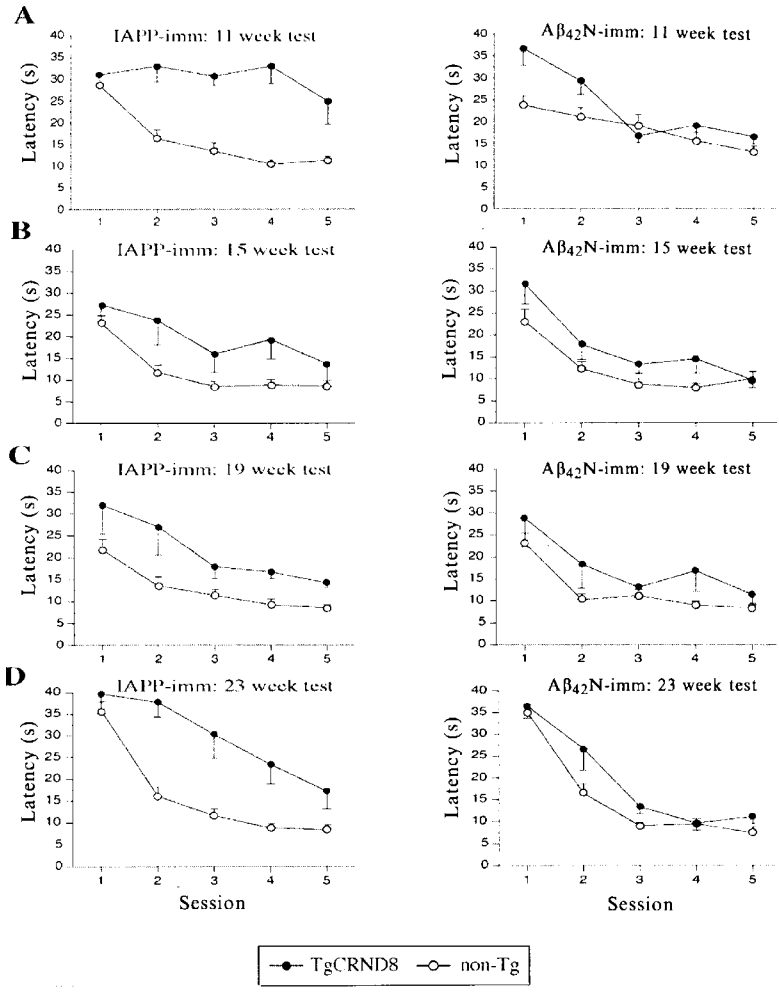

Figure 65.3. Water maze test performance of TgCRND8 mice at each age of testing. At 11 weeks of age, IAPP immunized TgCRND8 mice ($n = 12$) show cognitive impairment relative to non-Tg controls ($n = 8$) (A, left), whereas the performance of $A\beta_{42}$ immunized TgCRND8 mice ($n = 9$) (A, right) approaches that of non-Tg littermates ($n = 19$). At 15 weeks of age, the IAPP immunized TgCRND8 mice ($n = 6$) (B, left) showed significant ($p < 0.01$, $\omega^2 = 36\%$) impairment compared to non-Tg littermates ($n = 16$), but were not different from the $A\beta_{42}$ immunized TgCRND8 mice ($n = 7$) (B, right). At 19 weeks of age, the IAPP immunized TgCRND8 mice ($n = 6$) (C, left) also showed significant ($p < 0.01$, $\omega^2 = 34\%$) impairment compared to non-Tg littermates ($n = 15$), but similar performance to $A\beta_{42}$ immunized TgCRND8 mice ($n = 6$) (C, right). At 23 weeks of age, the IAPP immunized TgCRND8 mice ($n = 6$) (D, left) showed significant ($p < 0.001$, $\omega^2 = 65\%$) impairment from non-Tg littermates ($n = 15$), but were also significantly impaired relative to $A\beta_{42}$ immunized TgCRND8 mice ($n = 6$) (D, right) ($p < 0.01$). Vertical bars represent $\pm$SEM

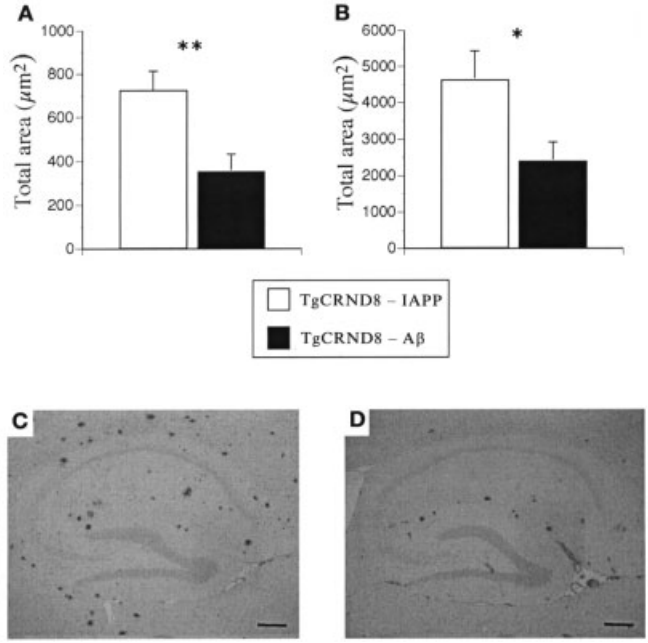

Figure 65.4. At the end of experiment (25 weeks of age) the Aβ plaque load of TgCRND8 mice immunized with $A\beta_{42}$ and IAPP peptides was quantified in the hippocampus and in the cerebral cortex. The total area (μm^2) of plaques in Aβ immunized mice was reduced two-fold, in the hippocampus (A, $p < 0.01$), and in the cortex (B, $p < 0.05$), as compared to Tg mice immunized with control IAPP peptide. Comparison of plaque counts between IAPP and Aβ immunized mice yielded a similar two-fold reduction in number of plaques in the hippocampus (20.9 ± 1.7 vs. 11.6 ± 1.6 for IAPP and Aβ immunized mice, $p < 0.01$) and in the cortex (119.7 ± 14.6 vs. 71.4 ± 10.8 for IAPP and Aβ immunized mice, $p < 0.05$). (C,D) Representative images of the distribution of Aβ plaques in the hippocampal region in IAPP and $A\beta_{42}$ immunized mice, respectively. Vertical bars represent SEM, * $p < 0.05$, ** $p < 0.01$. Scale bars: 100 μm

reflect either the inefficient ingress of antibodies to the CNS (estimated at 0.1% of serum levels; Bard et al., 2000), or the fact that other βAPP-proteolytic fragments may also be involved in the pathogenesis of AD (Lu et al., 2000). Nonetheless, the strong effect of immunization discerned in this first series of experiments (i.e. without a prior series of experiments to optimize the procedure) hints at the potential utility of this intervention. Our data imply that either very small reductions in the levels of $A\beta_{42}$ and Aβ plaques are sufficient to affect cognition, or, that vaccination need only modulate the activity/abundance of a small subpopulation of toxic fibrillar $A\beta_{42}$ species. Overall, these findings have positive implications not only for further iterations of the immunization procedure, but also for therapies directed against other points in the Aβ cascade.

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66 Galantamine, a Novel Treatment for Alzheimer's Disease: A Review of Long-term Benefits to Patients and Caregivers

PIERRE TARIOT AND BENGT WINBLAD

INTRODUCTION

Alzheimer's disease (AD) is the most frequent cause of dementia (Fratiglioni, 1993), accounting for at least 50–60% of all dementia cases (Henderson, 1994). Estimates suggest that 7–25 million people worldwide have AD (Ritchie and Kildea, 1995) and that the incidence increases from 6% in people over 65 years to 30% or more in people over 85 years (Bachman et al., 1993; Ritchie, 1995). AD is estimated to cost more than $100 billion annually in direct and indirect costs in the USA (Ernst and Hay, 1994; Schumock, 1998; Wimo et al., 1998). The world's population is aging, and the resulting increase in prevalence of AD has become a major public health issue (Jeste et al., 1999).

AD is characterized by a progressive decline in patients' cognitive and functional abilities (Gauthier et al., 1997), and most patients will go on to develop behavioral disturbances (Lyketsos et al., 2000). The emergence of behavioral problems places a significant emotional and psychological burden on carers, whose inability to cope is one of the major factors contributing to nursing home placement or institutionalization of patients.

Although many neurotransmitter abnormalities occur in AD, deficits in the cholinergic nervous system are the most pronounced. Cholinergic deficits are thought to contribute to many of the symptoms of AD, particularly cognitive and behavioral symptoms (Bartus et al., 1982; Coyle et al., 1983; Cummings, 2000). The most effective strategy to date for the symptomatic treatment of AD has been the use of acetylcholinesterase (AChE) inhibitors, which inhibit AChE, the enzyme that breaks down acetylcholine (ACh) in the synaptic cleft (Nordberg and Svensson, 1998). The three AChE inhibitors currently available in the USA, tacrine, donepezil, and rivastigmine, have been shown to have beneficial effects on cognitive and global ratings of dementia severity relative to placebo in patients

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with mild-to-moderate AD in randomized, controlled studies of 6 months' duration (Knapp et al., 1994; Rogers and Friedhoff, 1998; Rösler et al., 1999). There are some data to suggest that AChE inhibitors may have favorable effects on patients' activities of daily living (ADLs) (Rösler et al., 1999), although it is not clear whether the benefit extends to all members of this class of drug (Pryse-Phillips, 1999). With the exception of one metrifonate study (Morris et al., 1998), there is an absence of prospectively collected data from randomized, placebo-controlled clinical trials, suggesting that AChE inhibitors have favorable effects on behavioral symptoms. However, no prior trials have specifically addressed this issue in an *a priori* manner. Results from open-label studies suggest that donepezil and tacrine may have some beneficial effects on behavioral symptoms (Kaufer et al., 1996; Mega et al., 1999), but these findings need to be confirmed in double-blind studies.

There are few published data about the long-term effects of AChE inhibitors. One group of investigators found that donepezil maintained cognitive function for 38 weeks, at which point levels deteriorated below baseline, as measured by the Alzheimer's Disease Assessment Scale-cognitive subscale (ADAS-cog) (Rogers and Friedhoff, 1998).

Galantamine is a novel treatment for AD that inhibits AChE and allosterically modulates nicotinic receptors. Galantamine has been approved for the treatment of mild-to-moderate AD in Austria, Belgium, Brazil, Denmark, Greece, Finland, France, Spain, Luxembourg, Switzerland, Thailand, the UK, Ireland, Sweden, Norway and Iceland. Additionally, galantamine is pending approval in the USA. The main aim of this chapter is to review galantamine's long-term effects on cognitive, functional, behavioral, and caregiver outcomes in AD.

GALANTAMINE: A NOVEL TREATMENT FOR ALZHEIMER'S DISEASE

Galantamine increases the availability of ACh in the cholinergic synapse by competitively inhibiting AChE (Thomsen et al., 1991). In contrast to tacrine, but similar to donepezil, galantamine is more selective for AChE than butyrylcholinesterase (Nordberg and Svensson, 1998), which may partly account for galantamine's favorable tolerability profile (Tariot et al., 2000a).

Galantamine also potentiates cholinergic neurotransmission by positively modulating the response of the nicotinic acetylcholine receptor (nAChR) to ACh (Albuquerque et al., 1997; Schrattenholz et al., 1996). Because galantamine acts at a site on the nAChR that is different from the ACh binding site, it is referred to as an allosteric potentiating ligand (Figure 66.1) (Maelicke, 2000; Schrattenholz et al., 1996). Galantamine appears to enhance both pre- and postsynaptic nAChR function by making nAChR more sensitive to available ACh (Figure 66.2) (Albuquerque et al., 1997; Maelicke, 2000; Schrattenholz et al., 1996).

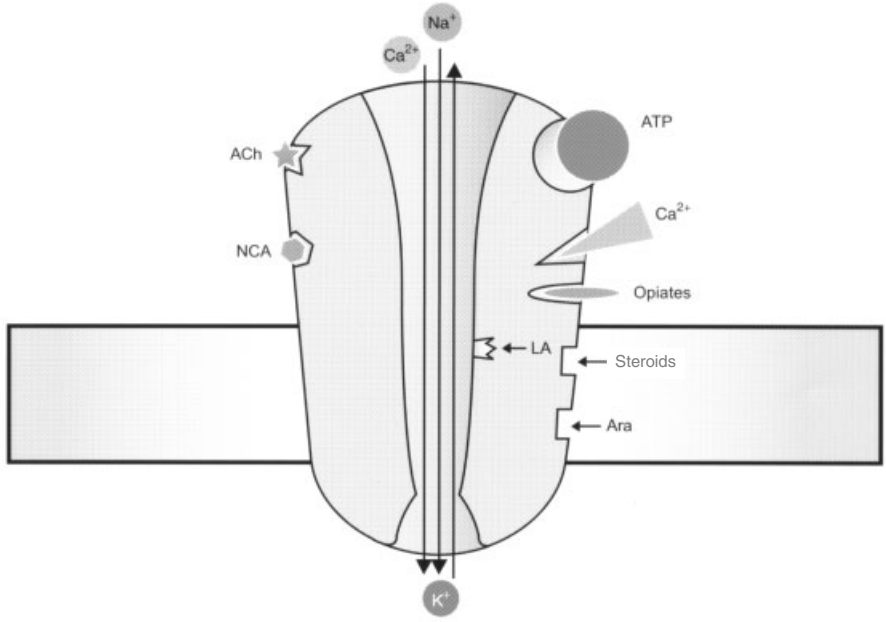

Figure 66.1. Galantamine and ACh bind to different sites on nAChR. LA, local anesthetics; ACh, acetylcholine; NCA, noncompetitive agonists including galantamine; Ara, arachidonic acid. Reproduced from Albuquerque et al. (1997) by permission of ASPET

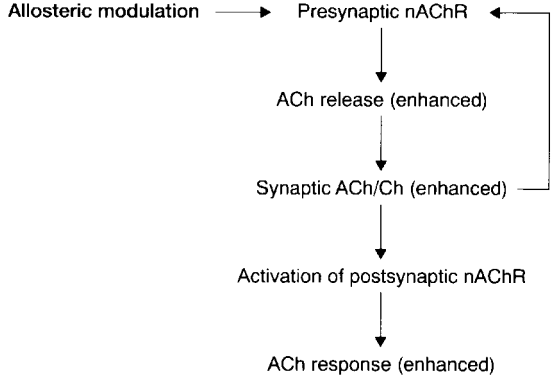

Figure 66.2. Allosteric nicotinic modulation amplifies the effect of ACh at nicotinic receptors. ACh is released presynaptically from a cholinergic neuron and, after diffusion through the synaptic cleft, interacts with postsynaptic nAChR. At higher levels of synaptic activity, some ACh and/or, after hydrolysis by AChE, some Ch will also reach the presynaptic membrane and interact with presynaptic nAChR, resulting in additional release of ACh. The enhanced level of released ACh will produce an increased postsynaptic response. ACh, acetylcholine; nAChR, nicotinic acetylcholine receptors; AChE, acetylcholinesterase; Ch, choline

Enhancing neurotransmission via nAChR holds hope as a promising treatment strategy in AD (Paterson and Nordberg, 2000), although the clinical relevance of allosteric modulation of nAChR is not established at this juncture. Blocking nAChR may lead to cognitive impairment (Newhouse et al., 1992), and it has been suggested that nicotinic cholinergic drugs may be of therapeutic value in some patients with AD (Maelicke and Albuquerque, 2000; Newhouse, et al., 1997; Paterson and Nordberg, 2000). It is thought that the α-4-β-2 nAChR subtype might be the most vulnerable in AD and therefore a possible target for therapeutic strategies (Warpman and Nordberg, 1995). Several lines of evidence suggest that modulatory effects on nAChR could reduce the symptoms of AD via several mechanisms:

- Activation of presynaptic nAChR augments the release of a number of neurotransmitters including ACh, glutamate, and monoamines (Wonnacott, 1997). Deficiencies in these neurotransmitters might contribute to the symptoms of AD (Nordberg, 1992).
- Activation of nAChR may protect against beta-amyloid toxicity (Kihara et al., 1998).
- In the early phase of AD, the symptoms were not associated with a loss of cholinergic function according to one report (Davis et al., 1999). Therefore, to the extent that this is true, mechanisms such as modulation of nAChR that enhance the function of noncholinergic systems may be an effective strategy for combating early cognitive decline (Davis et al., 1999).

It is worth emphasizing that allosteric modulators by themselves have little effect on nAChR, and may therefore avoid the desensitization of nAChR and the subsequent development of tolerance and loss of clinical efficacy that can occur with agonists (Maelicke, 2000). This distinction between agonists and receptor modulators may prove to be important.

In the end, provided clinically meaningful effects of modulation of nAChR can be proven, galantamine may well be clearly differentiated from available agents lacking this property.

GALANTAMINE: EFFECTS ON THE COURSE OF ALZHEIMER'S DISEASE SYMPTOMS

NATURAL DECLINE IN COGNITIVE AND DAILY FUNCTIONING IN UNTREATED ALZHEIMER'S DISEASE PATIENTS

A useful model for the natural decline in symptoms of AD over time is provided by the deterioration in cognitive and daily functioning that occurs in placebo-treated patients. Two 12-month, placebo-controlled studies (SAB-INT-12 and SAB-USA-25) using an investigational drug, sabeluzole, have been carried out in patients with mild-to-moderate AD, using ADAS-cog and

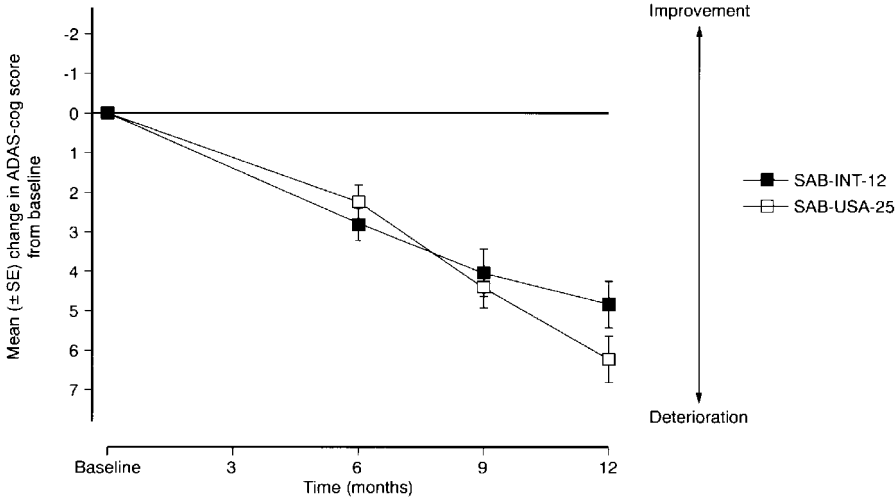

Figure 66.3. Mean change from baseline ADAS-cog score over 12 months in two historical placebo groups (OC analysis)

the Disability Assessment for Dementia (DAD) scale as outcome measures (Torfs and Feldman, 2000).

The placebo groups of SAB-INT-12 and SAB-USA-25 experienced a significant and progressive decline in ADAS-cog of 5–6 points over 12 months (Figure 66.3) (Torfs and Feldman, 2000). These placebo groups also experienced a significant and progressive decline in DAD of 11–13 points from baseline over 12 months (Figure 66.4) (Torfs and Feldman, 2000). The

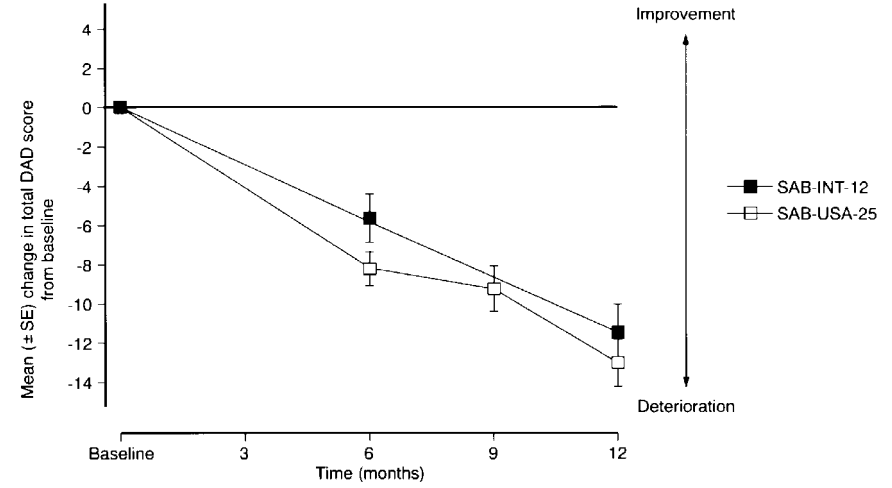

Figure 66.4. Mean change from baseline DAD score over 12 months in two historical placebo groups (OC analysis)

declines in ADAS-cog and DAD scores were somewhat greater in patients with moderate AD (Mini-Mental State Examination [MMSE] $\leqslant 18$) than in those with mild AD (MMSE > 18) at all time points investigated (6, 9, and 12 months). Slowing or postponing this expected decline obviously become meaningful outcomes in AD trials, and agents that achieve this would provide very important clinical benefits to patients suffering this relentlessly progressive disease.

EFFECTS OF GALANTAMINE ON COGNITIVE AND FUNCTIONAL DECLINE

Design of a 12-month galantamine study

The long-term efficacy, safety, and tolerability of galantamine have been assessed in a 12-month study (GAL-USA-1/3), which consisted of an initial 6-month, randomized, placebo-controlled, double-blind phase study (GAL-USA-1), followed by a 6-month, open-extension phase (GAL-USA-3) (Raskind et al., 2000). Patients received either galantamine 24 mg/day, galantamine 32 mg/day or placebo during the double-blind phase and then, after a 3-week dose-escalation phase, all patients received galantamine 24 mg/day in the open-extension phase (Figure 66.5). Of the 636 patients who entered the double-blind phase, 353 patients were eligible to enter the open-extension phase; 268 remained in the study after 12 months (Raskind et al., 2000).

At study entry, patients had a diagnosis of probable AD (defined according to the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association [NINCDS–ADRDA] criteria), mild-to-moderate dementia (defined as an MMSE score of 11–24 and an ADAS-cog score of $\geqslant 12$), and no clinical evidence of another cause of cognitive impairment. Outcome measures included the ADAS-cog and DAD scale. The memory component of the ADAS-cog (ADAS-cog-mem) was analyzed separately.

The **ADAS-cog** scale was designed specifically to assess cognitive function in AD, and has been shown to be valid and reliable (Rosen et al., 1984). It assesses various cognitive abilities including attention, memory, orientation, and language ability. The score range is 0–70, with higher scores indicating poorer functioning.

The **DAD** scale is a validated measure of ADLs, designed specifically for use in patients with AD. It regards functional impairment as a core symptom of AD and assesses 11 areas of daily functioning, including both basic and more complex (instrumental) ADLs (Gelinas et al., 1999). The score range is 0–100, with higher scores indicating better functioning.

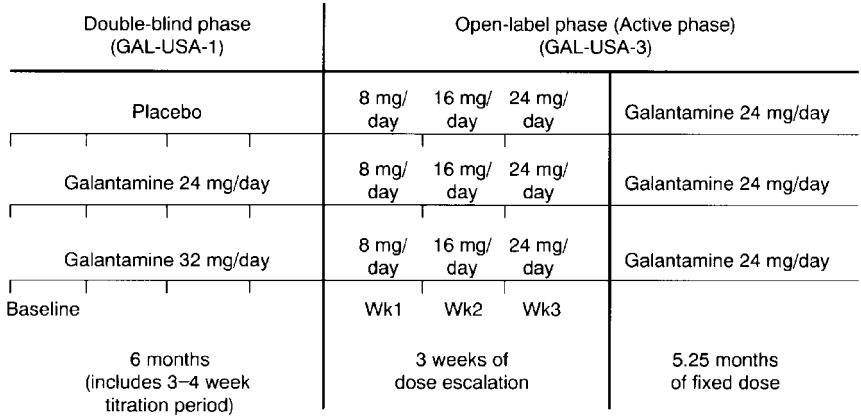

Figure 66.5. Design of GAL-USA-1/3

LONG-TERM COGNITIVE BENEFITS OF GALANTAMINE

Cognitive function was preserved for 12 months in the group of patients who received galantamine 24 mg/day throughout the study, as indicated by a mean ADAS-cog score that was not significantly different from baseline level at 12 months. Patients who switched from galantamine 32 mg/day in the double-blind phase to 24 mg/day in the open-extension phase showed a small deterioration in ADAS-cog relative to baseline (1.8 points) (Raskind et al., 2000).

Given the absence of a 12-month placebo-control group in galantamine trials, the long-term data from Raskind et al. (2000) have been compared with the 12-month placebo-control group from SAB-USA-25 in order to better examine the effects of treatment. The groups of subjects being compared had similar characteristics. The treatment difference between the group receiving galantamine 24 mg/day and those receiving placebo on the ADAS-cog was large (6 points) and highly significant ($p < 0.001$) after 1 year (Figure 66.6). The finding that galantamine 24 mg/day appears to postpone the decline in general cognitive function for at least 12 months is clinically important, particularly as patients with AD usually survive only 8 years from the time of diagnosis (Barclay et al., 1985). In addition, a significant improvement in memory (specifically as measured by the ADAS-cog-mem score) relative to baseline was observed in patients who had received galantamine 24 mg/day for 12 months ($p < 0.05$) (Raskind et al., 2000).

The design used in GAL-USA-1/3, known as a staggered start design, has been proposed as a potential method for identifying whether a treatment

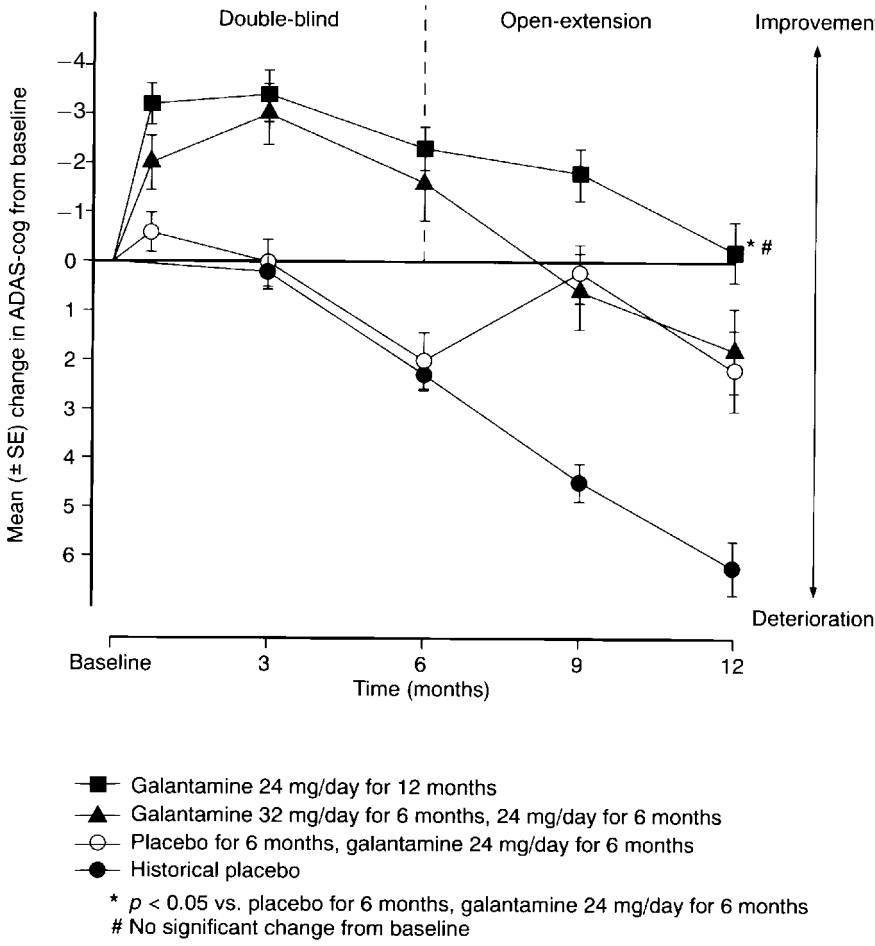

Figure 66.6. Mean change from baseline in ADAS-cog scores over 12 months (OC analysis)

influences the course of AD (Leber, 1996). If a treatment has only symptomatic effects, placebo-treated patients who are then placed on the active drug might be expected to approach the performance of those who were taking the drug from the beginning of the trial (Grundman and Thal, 1998; Leber, 1996). After 12 months patients who received galantamine 24 mg/day for the duration of the study had a significantly better cognitive outcome, as assessed by ADAS-cog, than those who had received placebo for 6 months prior to receiving galantamine ($p = 0.03$) (Figure 66.6). Cognitive function improved once patients were changed from placebo to galantamine, but it did

not reach the level of those who had started galantamine 6 months earlier. These results suggest, but do not firmly establish, that early treatment with galantamine may optimize long-term cognitive outcome. Further clinical, basic, and preclinical studies are required to evaluate whether galantamine may in fact modify the disease process, since a clinical trial by itself is unlikely to distinguish protective from symptomatic effects.

Long-term Functional Benefits of Galantamine

Uncertainty exists as to whether improvements in cognition necessarily translate into improvements in ADLs (Gelinas and Auer, 1996). Therefore, optimal clinical trials in AD should include a separate measure of daily functioning.

In the study reported by Raskind et al. (2000), functional ability was preserved for 12 months in patients who received galantamine 24 mg/day throughout the study, as indicated by a mean DAD score that was not significantly different from baseline at 12 months. A DAD cluster analysis showed that both instrumental and basic ADLs were preserved at 12 months. In contrast, patients who received placebo in the double-blind phase before being changed to galantamine experienced a significant decline in ADLs at 12 months (8.1 points, $p < 0.001$ vs. baseline) (Raskind et al., 2000). The historical placebo group (from SAB-USA-25) deteriorated by 13 points on DAD over 12 months (Torfs and Feldman, 2000). The difference between this historical placebo group and the group receiving galantamine 24 mg/day on the DAD scale (11.4 points) was highly significant ($p < 0.001$). To date, the duration of functional benefit seen with galantamine has not been published with other cholinergic treatments (Imbimbo et al., 1999; Rogers and Friedhoff 1998).

Deterioration in daily functioning contributes to patients' dependence on family members and other caregivers. In the early stages of AD instrumental ADLs are affected (e.g. housework, leisure, managing finances), developing to a progressive loss in the ability to undertake basic ADLs in later stages (e.g. eating, dressing, personal hygiene). Galantamine's stabilizing effects on instrumental and basic ADLs for at least 12 months would therefore be expected to be an important benefit for patients and caregivers.

GALANTAMINE'S EFFECT ON THE DEVELOPMENT OF BEHAVIORAL SYMPTOMS

Behavioral symptoms tend to manifest in a more variable fashion than the progressive decline in function and cognition (Devanand et al., 1998; Mohs et al., 2000). However, it is likely that behavioral symptoms will eventually emerge in patients at some point during the illness. Furthermore, data suggest that improvements in cognitive function do not always produce benefits in behavioral symptoms (Cummings, 2000). Therefore, it is important to assess

behavioral symptoms separately as an outcome measure in clinical trials in patients with AD.

The safety and efficacy of galantamine were evaluated in a 5-month, randomized, double-blind, placebo-controlled study carried out in the USA (GAL-USA-10) (Tariot et al., 2000a). Patients had a diagnosis of probable AD (according to NINCDS–ADRDA criteria) and mild-to-moderate dementia (MMSE score of 10–22 and an ADAS-cog score $\geqslant 18$) (Tariot et al., 2000a). Patients were randomized to either placebo ($n = 286$), galantamine 16 mg/day ($n = 279$) or galantamine 24 mg/day ($n = 273$), and the dose of galantamine was increased over a slow dose-escalation phase of 4–8 weeks. The Neuropsychiatric Inventory (NPI) scale was used to assess the effects of galantamine on behavioral symptoms (Tariot et al., 2000b).

The **NPI** is a validated and reliable tool that measures the frequency and severity of behavioral and psychological symptoms. The 10 behavioral domains that were assessed in galantamine trials are delusions, hallucinations, agitation/aggression, dysphoria, anxiety, euphoria, apathy, disinhibition, irritability/lability, and aberrant motor behavior. The score range is 0–120, with higher scores representing more severe behavioral disturbances (Cummings et al., 1997).

At the end of the study galantamine 16 mg/day and 24 mg/day produced a significantly better outcome on behavioral symptoms than placebo ($p < 0.05$ for both doses). The NPI scores at 5 months for galantamine 16 mg/day and 24 mg/day were not significantly different from baseline values (0.1-point improvement for both doses), but NPI scores in the placebo group declined significantly (2.3 points, $p < 0.05$ vs. baseline) (Figure 66.7). These data suggest that galantamine delays the expected development of behavioral symptoms in patients with AD.

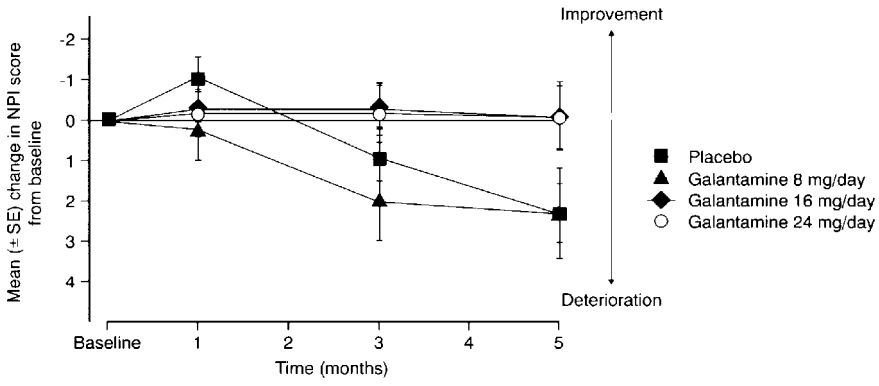

Figure 66.7. Mean change from baseline in total NPI scores over time (OC analysis)

Behavioral symptoms cause a great deal of distress to families and caregivers (Kaufer et al., 1998) and often necessitate a change in patients' level of care as patients become increasingly dependent on family members or assisted living (Brodaty et al., 1993; Canadian Study of Health and Aging, 1994; Teri et al., 1989). Behavioral symptoms are the most common reason for referral to a specialist and placement in a nursing home (Steele et al., 1990). Therefore, postponing the emergence of these symptoms is an important treatment goal, which would be expected to benefit patients and their families, and possibly delay nursing home placement (Knopman et al., 1996).

DO GALANTAMINE'S CLINICAL BENEFITS REDUCE CAREGIVER BURDEN?

As patients lose their independence more responsibility is placed on family members and other caregivers, and they are put at increased risk of physical and psychological illness (Alspaugh et al., 1999). Around 36% of patients with moderate or severe dementia are cared for in the community (Melzer et al., 1997) and require constant care and supervision, mostly by their spouses and children (Melzer et al., 1997). Two factors that have a major impact on caregiver burden are the emergence of behavioral symptoms and the loss of ability to perform daily activities (Canadian Study of Health and Aging 1994; Kaufer et al., 1998; Teri et al., 1989).

BENEFITS OF GALANTAMINE ON CAREGIVER BURDEN

Caregiver Distress Due to Behavioral Symptoms

In the 5-month placebo-controlled study the total NPI distress score was used as a method of assessing whether favorable effects on behavioral symptoms reduced caregiver distress (Tariot et al., 2000b). The total NPI distress score was significantly better for galantamine 24 mg/day compared with placebo ($p < 0.05$) and approached significance for galantamine 16 mg/day ($p < 0.1$ vs. placebo). This intriguing result suggests that the beneficial effects achieved in the galantamine 24-mg/day group may have translated into reduced caregiver distress. Data of this nature does not appear to have been presented before in an antidementia trial.

Caregiver Time

In a 6-month, double-blind, placebo-controlled, international study (GAL-INT-1) caregiver burden was assessed using a Caregiver Time Questionnaire. Patients were randomly assigned to galantamine 24 mg/day or 32 mg/day. Patients had a diagnosis of probable AD (according to the NINCDS–

ADRDA criteria) and mild-to-moderate AD (MMSE score of 11–24 and an ADAS-cog score $\geqslant 18$) (Wilcock and Lilienfeld, 2000).

The total **NPI** distress score was calculated by asking caregivers to rate the amount of distress each behavior caused them. The sum of the individual scores for each behavior was the total NPI distress score.

The **Caregiver Time Questionnaire** was used in GAL-INT-1 to assess (i) time spent supervising patients, and (ii) time spent assisting patients with ADLs including hygiene, dressing, feeding, medication, housekeeping, toileting, finances, and transport. The clinician interviews the caregiver to determine the time spent supervising and assisting patients (measured in minutes and hours).

Galantamine reduced the time spent by caregivers caring for and assisting patients with AD. In contrast, the carers of placebo-treated patients experienced a significant increase in time spent assisting patients with ADLs (increase of 23 min/day; $p = 0.027$ vs. baseline) and time spent supervising patients (an increase of 2 h/day; $p < 0.001$ vs. baseline). Carers of patients receiving galantamine 24 mg/day spent 1 hour less per day assisting patients with ADLs than carers of placebo-treated patients (Wilcock and Lilienfeld, 2000).

The long-term benefits of galantamine on caregiver time were confirmed in GAL-INT-3, the 6-month open-extension phase of GAL-INT-1. Caregiver time spent on patients who received galantamine 24 mg/day throughout the 12 months was maintained at baseline level (Lilienfeld and Gaens, 2000). Caregiver exhaustion is strongly correlated with caregiver distress and nursing

Table 66.1. Number (%) of patients with adverse events occurring at least 5% more often during treatment with any galantamine dose than with placebo (Tariot et al., 2000a)

Adverse event	Placebo (n = 286)	Galantamine 16 mg/day (n = 279)	Galantamine 24 mg/day (n = 273)
Nausea	13 (4.5)	37 (13.3)	45 (16.5)
Vomiting	4 (1.4)	17 (6.1)	27 (9.9)
Anorexia	9 (3.1)	18 (6.5)	24 (8.8)
Diarrhea	17 (5.9)	34 (12.2)	15 (5.5)
Any adverse event	206 (72.0)	206 (73.8)	219 (80.2)
Any serious adverse event	31 (10.8)	28 (10.0)	35 (12.8)
Deaths	4 (1.4)	3 (1.1)	3 (1.1)

home placement, therefore galantamine's stabilizing effects on caregiver time provide additional evidence that galantamine produces clinically important outcomes in AD.

TOLERABILITY AND SAFETY OF GALANTAMINE

ADVERSE EVENTS AND DISCONTINUATION RATES

Galantamine is well tolerated. The most common adverse events, usually gastrointestinal, are those expected from cholinergic stimulation (Table 66.1). No clinically relevant effects on vital signs and liver function were observed (Tariot et al., 2000a). Using a dose-escalation period of up to 8 weeks, the discontinuation rates due to adverse events in patients who received galantamine 16 mg/day and 24 mg/day were low (7% and 10%, respectively) and comparable with the discontinuation rate in the placebo group (7%) (Tariot et al., 2000a). These data suggest better tolerability than seen in rivastigmine and tacrine studies (Knapp et al., 1994; Rösler et al., 1999), and are roughly comparable with tolerability data from donepezil studies (Burns et al., 1999; Rogers and Friedhoff, 1998). However, no studies have directly compared these agents and comparisons of separate studies must be viewed with caution.

GALANTAMINE'S BENEFICIAL EFFECTS ARE NOT OFFSET BY SLEEP DISTURBANCES

Some AChE inhibitors have been shown to adversely affect sleep in a limited number of patients (Burns et al., 1999; Rogers and Friedhoff, 1998). To address this issue in galantamine's clinical development program, galantamine's effects on patients' quality of sleep were assessed in a 3-month, randomized, double-blind, placebo-controlled, multicenter study, which evaluated the efficacy and tolerability of a flexible dosage regimen of galantamine 24–32 mg/day (GAL-INT-2) (Wilkinson et al., 2000). Patients had probable AD (according to the NINCDS–ADRDA criteria) and mild-to-moderate dementia (MMSE score of 11–24 and ADAS-cog score $\geqslant 12$). Quality-of-sleep pattern was assessed by the Pittsburgh Sleep Quality Index (PSQI) (Buysse et al., 1989).

> The **PSQI** measures sleep quality in various domains including sleep onset latency, subjective sleep quality, sleep duration, habitual sleep efficiency, sleep disturbances, use of medication for sleep, and daytime functioning (Buysse et al., 1989).

After 3 months there was no significant difference between the galantamine- and placebo-treated patients in mean PSQI scores (Rockwood

and Kershaw, 2000). These results indicate that galantamine maintains sleep quality and that the benefits of galantamine do not occur at the expense of drug-induced sleep disturbance (Rockwood and Kershaw, 2000).

CONCLUSION

Galantamine is a new and effective agent for the long-term treatment of AD. It is a competitive AChE inhibitor with the additional effect of allosteric modulation of nicotinic receptors. Galantamine delays the inevitable cognitive decline seen in AD for at least 1 year, slows functional decline, and postpones the emergence of behavioral symptoms in patients with mild-to-moderate AD. These clinical benefits would be expected to help patients maintain independent living, and reduce the psychological and physical burden for carers.

These data provide evidence to support the concept that postponing the progression of symptoms, an important goal in AD therapy, can now be achieved.

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67 Benefits of Donepezil on Cognition, Function and Neuropsychiatric Symptoms in Patients with Mild and Moderate Alzheimer's Disease over One Year

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INTRODUCTION

Alzheimer's disease (AD) is predominantly a disease of the elderly: its incidence is reported to increase from approximately 3% of those aged 65 years to almost 50% in people over 85 years of age (Eggert et al., 1997). Furthermore, the prevalence of AD is rising exponentially as the longevity of the population increases (Mayeux and Sano, 1999). Thus, the management of AD is becoming increasingly recognized as a major healthcare challenge.

AD is a chronic, irreversible neurodegenerative disease, characterized clinically by a progressive decline in cognitive function, which can result in the inexorable decline in functional ability as the disease progresses (Galasko et al., 1995, 1997; Schmeidler et al., 1998; Green et al., 1999). In the early to mid-stages of the disease, functional decline is signaled by a hierarchical loss of ability to perform instrumental activities of daily living (ADLs) such as managing finances and using the telephone (Galasko et al., 1997). As the disease progresses, basic ADLs such as washing, toileting, and feeding also become affected. Moreover, patients often develop neuropsychiatric and behavioral symptoms, such as depression, agitation and delusions, that further impair their functional ability (Teri et al., 1990).

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The pathogenesis of AD is not understood entirely at present. However, the pathology of AD is characterized by the presence of beta-amyloid (Aβ) plaques, neurofibrillary tangles, and nerve cell loss within the brain. The widespread loss of cholinergic innervation in the cerebral cortex and subcortical structures is also a prominent and well-known feature of the disease (Davies and Maloney, 1976; Bartus et al., 1982). Indeed, this cholinergic deficit has been shown to correlate positively with the cognitive deterioration in AD (Perry et al., 1978; Lawrence and Sahakian, 1995) and the presence of the senile plaques (Perry et al., 1978, Näslund et al., 2000). In addition, reduced cholinergic function could also be involved in the expression of some of the neuropsychiatric and behavioral symptoms in AD patients (Cummings and Back, 1998). Thus, the central cholinergic deficit in AD has become a major focus for the development of pharmacological treatments for AD.

More recently, there has been a resurgence of interest in the cholinergic hypothesis of AD and, more specifically, attempts to correlate the degree of the cholinergic deficit with clinical symptoms at a variety of disease stages. For example, one recent report has suggested that the cholinergic deficit is not significant until relatively late in the course of AD (Davies, 1999). However, another recent study suggests that the cholinergic deficit coincides with the earliest histopathological stages of AD (Beach et al., 2000). Nevertheless, the most successful strategy for ameliorating the cholinergic deficit has proved to be the use of cholinesterase inhibitors (ChEIs). ChEIs that have been approved for the treatment of mild to moderate AD include donepezil (a piperidine-based, specific, and reversible inhibitor of centrally-active acetylcholinesterase [AChE]; Rogers et al., 1991), rivastigmine (a pseudo-irreversible, carbamate-based ChEI; Corey-Bloom et al., 1998; Rösler et al., 1999), and galantamine (a reversible ChEI; Raskind et al., 2000; Tariot et al., 2000).

Donepezil hydrochloride, for example, received approval in the USA in 1997 and has since been approved in over 50 countries for the symptomatic treatment of patients with mild to moderate AD. Donepezil has a long terminal elimination half-life (approximately 70 hours), allowing simple once-daily dosing, and displays a low potential for drug–drug interactions (Tiseo et al., 1998a, b, c, d; Nagy et al., 2000). Pivotal phase II and III studies in patients with mild to moderate AD have consistently shown that donepezil is well tolerated and gives rise to significant improvements in cognition and benefits in global function and ADLs for at least 6 months (Rogers and Friedhoff, 1996; Rogers et al., 1998a, b; Burns et al., 1999). To date, the efficacy and tolerability of the ChEIs in AD patients has been assessed in double-blind, randomized, placebo-controlled clinical trials for up to 6 months (Knapp et al., 1994; Corey-Bloom et al., 1998; Rogers et al., 1998a, b; Burns et al., 1999; Rösler et al., 1999). Most studies submitted for regulatory approval of ChEIs are based on analyses of patients with mild to

moderate AD. At present, therefore, the use of ChEIs is restricted to patients with mild to moderate AD. However, a recent trial has demonstrated that donepezil treatment also provides benefits in cognitive (Panisset et al., 2000), functional (Gauthier et al., 2000), and behavioral symptoms in moderate to severe AD patients (Hecker et al., 2000).

Due to the ethical constraints on performing long-term clinical trials in patients with dementia, evidence of the longer-term benefits of ChEIs in AD has been limited to observations from open-label extension trials. Such studies with donepezil have followed some patients for up to 4.5 years (Pratt et al., 1999; Rogers et al., 2000) and have shown less deterioration in cognition in donepezil-treated patients compared with untreated patients followed in observational studies. However, while such studies have provided important information on the sustained and continued efficacy of donepezil, it is recognized that open-label studies are not optimal for full evaluation of drug effects due to the absence of a placebo treatment arm which necessitates the use of historical controls for comparative purposes.

This study represents the first multinational, double-blind, randomized, placebo-controlled trial to investigate the efficacy and safety of a ChEI over a period of 1 year. The results of a number of subanalyses from this study, including the response of mild to moderate AD patients to donepezil with regard to cognitive, functional and behavioral symptoms, are described.

METHODS

PATIENTS AND STUDY DESIGN

Men and women with diagnostic evidence of mild to moderate, possible or probable AD consistent with DSM-IV (American Psychiatric Association, 1994), NINCDS–ADRDA (McKhann et al., 1984) criteria, with a Mini-Mental State Examination (MMSE; Folstein et al., 1975) score of $\geqslant 10$ and $\leqslant 26$, and who fulfilled the inclusion and exclusion criteria (Winblad et al., in press), were recruited into the trial. Patients were enrolled from 28 sites in five Northern European countries (Denmark, Finland, Norway, Sweden and the Netherlands), and were randomized to receive either donepezil ($n = 142$; 5 mg/day for 28 days, and then 10 mg/day, as per the clinician's judgment) or placebo ($n = 144$) for 1 year. If required, a dose reduction back to the 5 mg/day level was permitted.

ASSESSMENTS

Efficacy measures included assessments of global function (Gottfries–Bråne–Steen [GBS] scale; Gottfries et al., 1982; Bråne et al., 2001), cognition (MMSE; Folstein et al., 1975), ADLs and quality of life (Progressive

Deterioration Scale [PDS]; DeJong et al., 1989), and behavior (10-item Neuropsychiatric Inventory [NPI]; Cummings et al., 1994). All efficacy assessments were performed at baseline, and at weeks 4 (GBS only), 12, 24, 36, and 52. Safety was assessed throughout the study through monitoring treatment-emergent adverse events (AEs), physical examinations, clinical laboratory tests, and evaluation of general health and well-being.

STATISTICAL ANALYSIS

For all efficacy parameters, an analysis of covariance (ANCOVA) model was used for estimating and testing treatment effects. All statistical tests were two-sided and performed at the 0.05 significance level. Both observed cases (OC) and last observation carried forward (LOCF) analyses were conducted on the intent-to-treat (ITT) population. The results of the OC analyses are reported here, together with the week 52 LOCF analysis (endpoint).

Patients with Mild versus Moderate AD

An analysis examining the effect of donepezil treatment in patients with mild AD (MMSE score $\geqslant 21$ and $\leqslant 26$) vs. moderate AD (MMSE score $\geqslant 10$ and $\leqslant 20$) was conducted using the mean change from baseline in MMSE scores.

Responder Analysis

The proportion of responders to donepezil treatment was evaluated. Response was defined as the percentage of patients demonstrating a stabilization (i.e. no change or improvement from baseline) or improvement from baseline. Changes in cognition (MMSE), ADLs (PDS) and/or behavior (NPI) were assessed for both the placebo and donepezil treatment groups at weeks 24 and 52.

RESULTS

Overall, the donepezil ($n = 142$) and placebo ($n = 144$) treatment groups were comparable at baseline (Table 67.1). A high proportion of patients in both the donepezil (82%) and placebo (88%) treatment groups had at least one abnormal medical history finding or co-morbid illness prior to or at screening. The most common were non-psychotic mental disorders (donepezil 30.3%; placebo 27.8%), miscellaneous symptoms such as cough, flatulence or rash (donepezil 28.9%; placebo 27.1%), and hypertensive disease (donepezil 14.1%; placebo 16.7%). Most patients (120 [91%] with donepezil and 132 [92%] with placebo) took at least one concomitant medication over the course of the trial.

Table 67.1. Patient baseline characteristics

	Donepezil (n = 142)	Placebo (n = 144)
Age in years		
Mean (SD)	72.1 (8.6)	72.9 (8.0)
Range	49–86	51–88
Number (%) of female patients	99 (69.7)	85 (59.0)
Race, white (%)	142 (100)	144 (100)
Baseline MMSE score		
Mean (SD)	19.37 (4.37)	19.26 (4.54)
Range	10–26	10–26

Table 67.2. Patient disposition

Number of patients (%)	Donepezil (n = 142)	Placebo (n = 144)
Completed	95 (66.9)	97 (67.4)
Total discontinued	47 (33.1)	47 (32.6)
Withdrawal due to:		
Withdrawal of consent	20 (14.1)	18 (12.5)
AE related to study drug	5 (3.5)	6 (4.2)
AE unrelated to study drug	5 (3.5)	3 (2.1)
Insufficient clinical response	4 (2.8)	6 (4.2)
Death	4 (2.8)	3 (2.1)
Other	9 (6.3)	11 (7.6)

In total, 66.9% of donepezil-treated and 67.4% of placebo-treated patients completed this 1-year study, and the numbers of patients discontinuing treatment for any reason were similar in both groups (Table 67.2). The ratio of mild (MMSE $\geqslant 21$ and $\leqslant 26$) and moderate AD (MMSE $\geqslant 10$ and $\leqslant 20$) patients was approximately 1:1 at baseline for both treatment groups (60 and 75 patients in the donepezil group compared with 61 and 76 patients in the placebo group had mild and moderate AD, respectively).

EFFICACY

Statistically significant differences in the least squares (LS) mean change from baseline in global function (GBS scores) in favor of donepezil over placebo were observed at weeks 24 ($p = 0.046$), 36 ($p = 0.012$), and 52 ($p = 0.014$), and this benefit was maintained at endpoint ($p = 0.054$) (Winblad et al., in press). At baseline, the mean MMSE total scores were 19.37 ± 4.37 and 19.26 ± 4.54 for the donepezil and placebo groups, respectively. Statistically

significant differences in the LS mean change in MMSE scores from baseline in favor of donepezil over placebo were observed at weeks 24, 36, 52, and endpoint ($p < 0.02$). Donepezil-treated patients were also favored over placebo-treated patients in their ability to perform ADLs, as assessed by the PDS, with statistically significant differences for LS mean change from baseline at week 52 ($p = 0.042$) and endpoint ($p = 0.011$). At all evaluations donepezil-treated patients showed a greater improvement (weeks 12, 24, and 36) or less decline (week 52 and endpoint) in LS mean change from baseline in NPI total scores than placebo-treated patients. However, these treatment differences were not significant. The above results are discussed in more detail elsewhere (Winblad et al., in press).

Changes in Patients with Mild versus Moderate AD

At baseline, the mean MMSE total scores for patients with mild AD (MMSE $\geqslant 21$ and $\leqslant 26$) were 23.3 ± 1.8 and 23.5 ± 1.69 for the donepezil and placebo groups, respectively. For patients with moderate AD (MMSE $\geqslant 10$ and $\leqslant 20$), the mean MMSE total scores at baseline were 16.2 ± 3.0 for the donepezil group and 15.8 ± 2.9 for the placebo group. Significant treatment differences in MMSE scores favored donepezil compared with placebo in patients with mild AD at week 36 ($p = 0.029$) and endpoint ($p = 0.049$) and at weeks 24 ($p = 0.002$), 52 ($p = 0.003$), and endpoint ($p = 0.002$) for patients with moderate AD. Donepezil-treated patients with mild AD showed improvement on the MMSE at week 52, whereas untreated patients declined: this treatment difference approached statistical significance ($p = 0.065$; Figure 67.1). At week 52, donepezil-treated patients with moderate AD showed a significantly smaller deterioration in their MMSE scores than the corresponding group of placebo-treated patients ($p = 0.003$; Figure 67.1).

Responder Analysis

A significantly higher percentage of donepezil-treated patients (72%) were improved from baseline on the MMSE and/or the PDS scores compared with placebo-treated patients (53%) at week 24 ($p < 0.01$; Figure 67.2). Similarly, a total of 81% of donepezil-treated patients were rated as stabilized according to changes from baseline in the MMSE and/or the PDS scores compared with 63% of placebo-treated patients at week 24 ($p < 0.01$; Figure 67.2). At week 52, the percentage of donepezil-treated patients improved from baseline on the MMSE and/or the PDS scores was 59% compared with 33% in the placebo-treated patients ($p < 0.001$). At week 52, 63% of donepezil-treated patients compared with 37% of placebo-treated patients were rated as stabilized on the MMSE and/or the PDS ($p < 0.001$; Figure 67.2).

When the NPI results were included in the responder analysis, 92% of donepezil-treated patients were categorized as stabilized compared with 79%

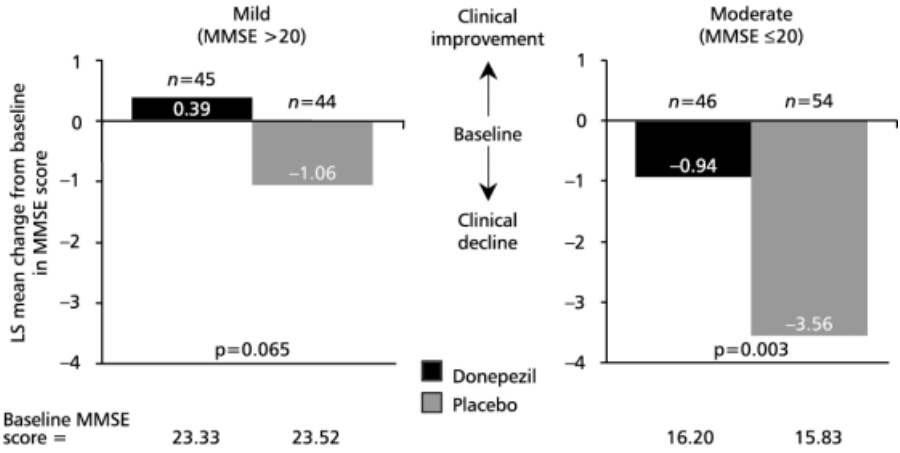
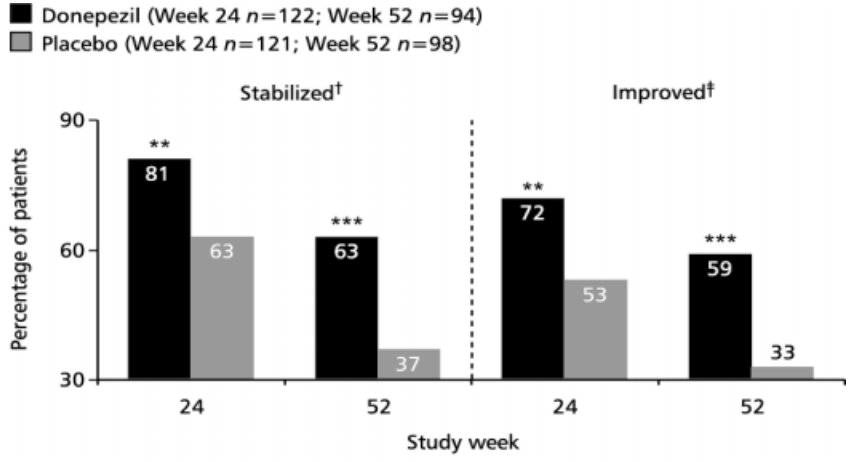
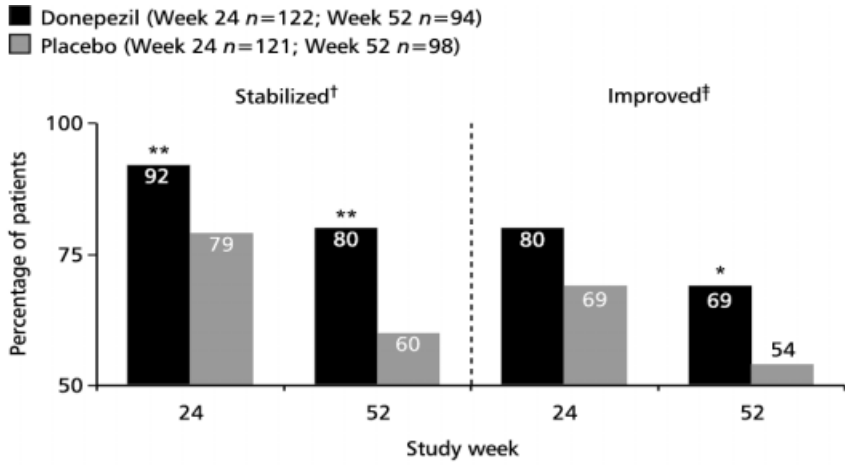


Figure 67.1. Least squares (LS) mean change from baseline in MMSE score in donepezil- and placebo-treated patients with mild and moderate Alzheimer's disease at week 52 (observed case analysis)



Stabilized is defined as no change or improvement from baseline

of placebo-treated patients at week 24 ($p < 0.01$; Figure 67.3). At week 52, a significantly higher proportion of donepezil-treated patients compared with placebo-treated patients were categorized as stabilized (80% vs. 60%; $p < 0.01$) or improved (69% vs. 54%; $p < 0.05$; Figure 67.3).



Stabilized is defined as no change or improvement from baseline

SAFETY

AEs were observed in 81.7% of donepezil- and 75.7% of placebo-treated patients and were mostly of mild or moderate intensity. AEs that occurred in at least 5% of the donepezil group and twice the rate of the placebo group were vertigo, asthenia, and syncope. A total of 13 patients had syncope recorded as an AE (nine with donepezil and four with placebo), but syncope was not considered related to study drug in any of these patients and all continued taking the study drug. Only one patient in the donepezil group reported a serious AE (moderate nausea) considered to be related to the study drug and this patient continued taking the drug. The number of patients discontinuing the study due to AEs was comparable in the donepezil- (7%) and placebo-treated (6.3%) patients. There were no clinically relevant changes in vital signs, hematological or biochemical laboratory values in either treatment group throughout the study.

DISCUSSION

This 1-year, multinational, double-blind, randomized, placebo-controlled trial with donepezil represents the first such study of a ChEI in the treatment of AD. These data extend observations from previous shorter-term, double-blind studies of donepezil and confirm the beneficial effects of donepezil on

global function, cognition, and ADLs in patients with both mild and moderate AD (Rogers et al., 1998a, b; Burns et al., 1999). In addition, discontinuation rates due to AEs were comparable between the donepezil and placebo groups over the course of 1 year, further supporting the favorable tolerability profile of donepezil. Indeed, the high completion rates observed in this study are consistent with those reported in the previous shorter-term clinical trials of donepezil (Rogers et al., 1998a, b; Burns, et al., 1999).

Overall, less deterioration in global function as assessed by the GBS scale was observed at all visits for donepezil- compared with placebo-treated patients, with significant treatment benefits observed in the donepezil group at all evaluation points from week 24 onwards to the completion of the study. These results provide further support for the benefits of donepezil treatment in global function in AD patients as demonstrated in earlier trials (Rogers et al., 1998b; Burns et al., 1999) using the Clinician's Interview-Based Impression of Change—plus version (Knopman et al., 1994). A more detailed description of these results and the use of the GBS scale in this study are reported elsewhere (Bråne et al., 2001; Winblad et al., in press).

Donepezil treatment was also associated with post-baseline improvements on the MMSE at weeks 12 and 24 with the mean MMSE score remaining at or close to baseline levels in donepezil-treated patients over the entire 52 weeks of the study. In contrast, the mean MMSE score in the placebo group deteriorated by 2.2 ± 0.5 points throughout the course of the study (Winblad et al., in press). This figure corresponds to the deterioration of 2–4 points per year on the MMSE reported previously in untreated patients with mild to moderate AD (Salmon et al., 1990; Brooks et al., 1993; Han et al., 2000). The mean MMSE score demonstrated improvement from baseline until approximately 9 months into this study. This demonstrates remarkable consistency with both of the previously reported phase II and III open-label extension studies of donepezil where mean scores on the Alzheimer's Disease Assessment Scale-cognitive subscale (ADAS-cog) (Rosen et al., 1984) in donepezil-treated patients demonstrated improvements from baseline until approximately 9 months into therapy (Pratt et al., 1999; Rogers et al., 2000). After that point, and as expected in a progressive illness with a symptomatic treatment, all patients eventually showed deterioration from baseline, although this was less in the donepezil-treated patients compared with that observed in the placebo-treated patients over the course of 1 year. This again was consistent with expected rates of decline noted in untreated patients in observational studies (Kramer-Ginsberg et al., 1988; Stern et al., 1994).

The beneficial effects of donepezil on ADLs were confirmed by the results for the PDS scale in this 1-year study. The PDS scale evaluates a patient's ability to perform both instrumental and basic ADLs, and may also provide an indication of the patient's quality of life (Rabins and Kasper, 1997). In this study, the ability to perform ADLs showed less deterioration in the donepezil-treated patients compared with the placebo-treated patients (Winblad et al.,

in press). Further evidence concerning the longer-term beneficial effects of donepezil on ADLs in AD patients was recently provided in another 52-week, prospective, placebo-controlled study performed in the USA in patients with mild to moderate AD, which demonstrated that the time to a clinically evident decline in function was at least 72% longer in patients treated with donepezil compared with placebo over a 1-year period (Mohs et al., 1999).

Benefits in behavioral symptoms, as assessed by the NPI scale, were observed during the course of this study, although these failed to reach statistical significance. The lack of a significant treatment effect on behavioral symptoms may be due to the fact that the patients enrolled in this trial had only relatively mild behavioral symptoms at baseline. Furthermore, the use of certain psychotropic medications, which might further reduce the incidence of behavioral symptoms, was permitted in this trial. Two recent studies have demonstrated that donepezil provides significant improvements in behavioral symptoms (Kaufer et al., 1998; Hecker et al., 2000), particularly in patients with more advanced AD where such symptoms are likely to be more prevalent.

The benefits of donepezil treatment relative to placebo in this trial, in terms of cognitive functioning, were demonstrated for patients with either mild (MMSE $\geqslant 21$ and $\leqslant 26$) or moderate (MMSE $\geqslant 10$ and $\leqslant 20$) AD as assessed by the MMSE. Thus, it may be hypothesized that these data support the assertion that patients with mild as well as moderate AD benefit from donepezil treatment thereby supporting the early diagnosis and management of AD.

The responder analyses demonstrated that cognition, ADLs, and/or behavioral symptoms were more likely to have improved or stabilized in donepezil-treated patients compared with those administered placebo over 1 year. The results of the responder analysis also suggest that as the duration of donepezil treatment increased, the gap between the donepezil- vs. placebo-treated patients improving or stabilizing (according to cognitive, functional and/or behavioral assessments) increased from week 24 to 52 (Figures 67.2 and 67.3).

The results of this study also confirm and extend the good tolerability profile of donepezil observed in previous trials (Rogers et al., 1998a, b; Burns et al., 1999). Donepezil was well tolerated throughout the study with the overall incidence of AEs and withdrawals due to AEs being similar in the donepezil and placebo groups.

CONCLUSIONS

The results of this first 1-year, multinational, double-blind, placebo-controlled study of an AChE inhibitor confirm the beneficial effects of donepezil on cognition, ADLs and behavioral symptoms over 1 year in the

treatment of patients with mild to moderate AD. Indeed, the data are consistent with those from the previously reported pivotal studies that led to the widespread marketing approval of donepezil. Furthermore, cognitive benefits were observed in patients with both mild and moderate AD, and stabilization or improvement in cognition, ADLs, and/or behavioral symptoms were demonstrated in a significantly higher proportion of donepezil-treated patients than placebo-treated patients. In addition, the proportion of donepezil-treated patients demonstrating these benefits increased over the course of 1 year compared with untreated patients. Taken together, these data suggest that to maximize the benefits of donepezil, treatment should begin as early as possible, ideally in patients with mild AD. Further, the determination of the benefits of therapy should be made by the physician and caregiver in conjunction with the patient, if possible, on the basis of improvement, stabilization, or a slowing of the deterioration in symptoms in any of the three areas commonly affected with AD: cognition, ADLs, and behavior.

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68 Characterization of Alzheimer's β-Secretase Protein BACE: Processing and Other Post-translational Modifications

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INTRODUCTION

Beta amyloid (Aβ), the main component of the hallmark amyloid plaques of Alzheimer's disease (AD) is generated by proteolytic cleavage of the large amyloid precursor protein (APP). Two distinct proteolytic activities, termed β-secretase and γ-secretase, cleave to release the amino- and carboxy-termini, respectively, of the 39–42 amino acid Aβ peptide from its precursor protein. According to the amyloid cascade hypothesis, Aβ plays an early and critical role in AD (Hardy and Allsop, 1991). Consequently, inhibition of Aβ formation could be useful in preventing or treating AD. β- and γ-secretases appear as the most tractable targets in the Aβ formation pathway. Therefore, many groups have tried to identify these enzymes for several years. In 1999 we demonstrated that a novel transmembrane aspartic protease, BACE1, has all the properties of β-secretase (Vassar et al., 1999). Four subsequently published studies arrived at the same conclusion using different methods (Hussain et al., 1999; Sinha et al., 1999; Yan et al., 1999; Lin et al., 2000). The identification of BACE1 as β-secretase and its relevance as a drug target have sparked interest in a detailed study of the biology of BACE1. For a drug development campaign, one would ideally like to completely understand the cell biology of BACE1, know all its substrates, and have high resolution

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structural information. As a first step in that direction, we have analyzed the post-translational modifications of BACE1, in particular the processing of the pro-enzyme to the mature form.

RESULTS AND DISCUSSION

Sequence analysis of the BACE1 protein immediately suggested that it is a type 1 transmembrane protein. The ectodomain contains four potential N-glycosylation sites and six cysteines which could form disulfide bonds (Vassar et al., 1999). We had also shown that mature BACE1 starts at E46, suggesting N-terminal processing (Vassar et al., 1999). We set out to analyze these post-translational modifications in detail.

BACE1 IS A GLYCOSYLATED INTEGRAL MEMBRANE PROTEIN

To prove that BACE1 is indeed an integral membrane protein, we immunoprecipitated BACE1 from stably expressing 293 cells after overnight labeling. BACE1 immunoreactivity was only found in membrane fractions and could not be removed by NaCl or Na_2CO_3 washes, which release peripheral membrane proteins into the wash phase.

We had shown before that mature BACE1 migrates on gels at ~70 kDa, a higher molecular mass than predicted from the amino acid sequence (Vassar et al., 1999). When we treated the immunoprecipitate with N-glycosidase F, we could reduce the molecular weight to ~50 kDa, indicating that BACE1 is indeed N-glycosylated (Haniu et al., 2000).

A more detailed analysis of BACE1 glycosylation required generation of significant amounts of BACE1 for biochemical studies. We had shown before that a soluble form of BACE1, termed BACE-IgG, consisting of the first 460 amino acids of BACE1 (the entire ectodomain) fused to the Fc portion of human γ-immunoglobulin, retains enzymatic activity (Vassar et al., 1999). This form is secreted from cells and can be readily purified to homogeneity. Because it is enzymatically active, we assume that the structure of the BACE1 ectodomain is not compromised in a major way. Purified BACE-IgG was treated with N-glycosidase F, O-glycanase, sialidase, and sialidase + O-glycanase. Only N-glycosidase F treatment changed the apparent molecular weight on SDS gels. We conclude that BACE1 shows N-glycosylation, but insignificant O-glycosylation. To determine whether all four potential N-glycosylation sites are occupied, we digested purified BACE-IgG with pepsin and subjected the resulting peptic peptides to mass spectrometry and amino acid sequencing. N-glycosylation was determined by no detection of Asn at the corresponding sequencing cycle. Using this method, we demonstrated that all four potential N-glycosylation sites are indeed occupied by

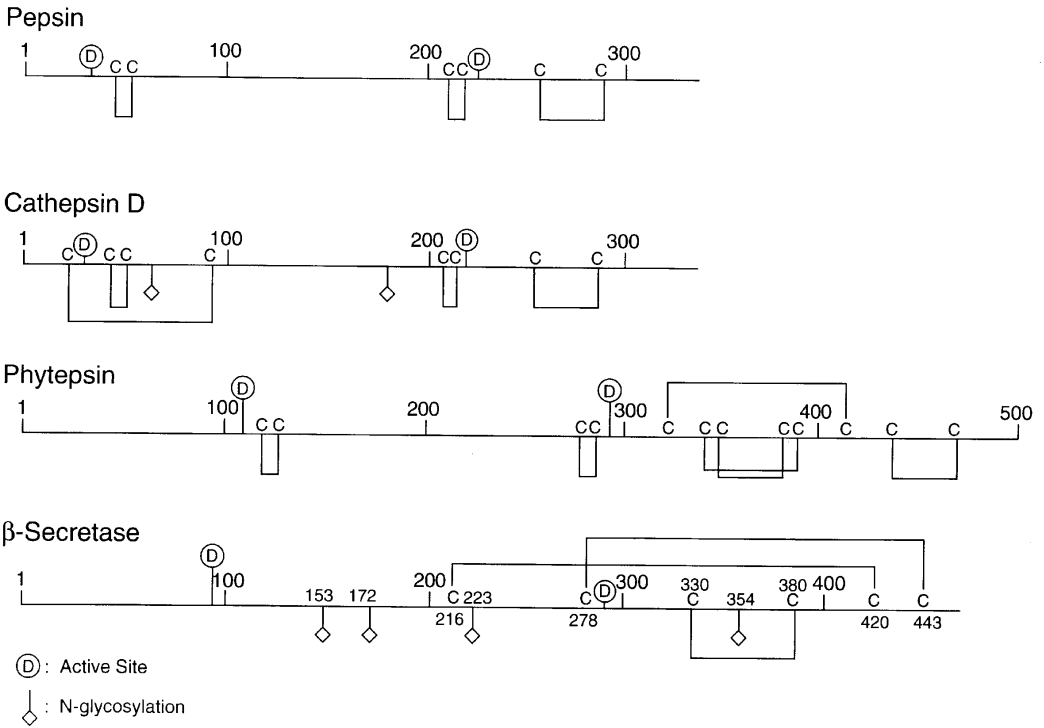

Figure 68.1. Comparison of disulfide motif and N-glycosylation sites in aspartic proteases. The ectodomain of BACE1 has full enzyme activity and contains the three intramolecular disulfide bonds as determined here. In comparison with other aspartic proteases like pepsin and cathepsin D, BACE contains a different disulfide connectivity. Active site Asp (D) residues are circled. N-glycosylation sites are indicated. (Reproduced from Haniu et al., 2000 by permission of the American Society for Biochemistry and Molecular Biology)

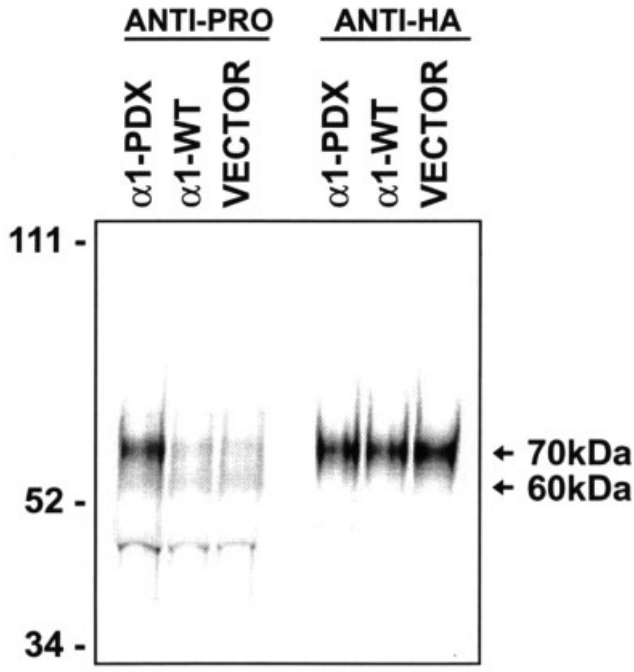

Figure 68.2. Inhibition of BACE propeptide processing with α-1-PDX. 293 cells expressing BACE1 were transiently transfected with α-1-PDX, α-1-WT (wild-type antitrypsin which does not inhibit furin), or vector alone. After metabolic labeling, cell lysates were made and equivalent amounts were immunoprecipitated with either an anti-pro antibody or an antibody to the C-terminus of BACE (tagged with hemagglutinin; HA). Immunoprecipitates were run on NuPAGE 7% Tris-acetate gels, and visualized on a phosphorimager. Molecular weight markers (left) and the ~60 kDa and ~70 kDa BACE species (right) are indicated. (Reproduced from Bennett et al., 2000 by permission of the American Society for Biochemistry and Molecular Biology)

carbohydrate moieties. The fact that glycopeptides with the same amino acid sequence were separately eluted on HPLC suggests that the N-glycosylation sites may have carbohydrate heterogeneities. However, the exact carbohydrate structure could depend on the particular cell line and also be influenced by the choice of soluble vs. membrane-bound form (Haniu et al., 2000).

BACE1 FORMS THREE INTRAMOLECULAR DISULFIDE BONDS

BACE-IgG was examined for the presence of free sulfhydryl residues using fluorescein-5-maleimide (FM) labeling. The FM-labeled protein was digested

with proteases, but no FM-labeled peptides could be detected, suggesting that all six cysteine residues in the BACE1-ectodomain form disulfide bonds. To determine the disulfide bonds, BACE-IgG was digested with trypsin+ endoproteinase Asp-N. The resulting peptides were subjected to mass spectrometry and sequence analysis. Digestion products containing disulfide linked peptides gave two sequences. BACE-IgG was also digested with pepsin under acidic conditions. Using mass spectrometry and sequence analysis on the resulting peptides we could definitively assign the disulfide bonds in the ectodomain: Cys216–Cys420, Cys278–Cys443, Cys330–Cys380. We conclude that in comparison with other aspartic proteases like pepsin and cathepsin D, BACE1 contains a different disulfide bond connectivity (Figure 68.1) (Haniu et al., 2000).

BACE IS N-TERMINALLY PROCESSED IN THE GOLGI APPARATUS

To analyze the turnover of BACE1 in a stable 293 cell line, we performed pulse-chase experiments followed by immunoprecipitation of BACE1 using a C-terminal antiserum or a propeptide antiserum. Using the C-terminal antiserum we detected at time 0 immediately after labeling an ~60 kDa band, representing immature N-glycosylated BACE1. After 3 h this band had disappeared, and less than half of the original material was recovered as the mature glycosylated ~70 kDa form, which is degraded slowly. Using the propeptide antibody the same ~60 kDa form was detected at time 0 as with the C-terminal antibody; however, by 2 h chase time most of the signal had disappeared. These results indicate that the BACE1 protein undergoes constitutive N-terminal processing and that the N-terminal processing occurs in temporal proximity with the trimming/adding of carbohydrate residues, i.e. in the Golgi apparatus. This conclusion is confirmed when the cells are treated with Brefeldin A, which blocks both N-terminal processing and further glycosylation of the immature ~60 kDa form. It is noteworthy that processing of BACE1 appears to be very efficient, even under overexpression conditions (Haniu et al., 2000).

BACE1 PROPEPTIDE CLEAVAGE DOES NOT APPEAR TO BE AUTOCATALYTIC

Pepsin and other aspartic proteases require proteolytic processing of the inactive proprotein to a mature enzyme. This process involves the autocatalytic removal of the propeptide domain (Tang and Wong, 1987). To determine whether BACE1 propeptide cleavage may be autocatalytic as well, we generated an inactive form of BACE1 by mutating the active-site amino acid Asp93 to Ala. This mutation inactivates BACE1 but it does not affect processing of BACE1, as demonstrated by radiosequencing of the

mature mutant protein. Thus, BACE1 propeptide cleavage does not appear to be autocatalytic, but instead seems mediated by another protease (Bennett et al., 2000).

FURIN IS REQUIRED FOR BACE1 PROPEPTIDE PROCESSING IN CELLS AND CLEAVES BACE1 AT THE E46 SITE *IN VITRO*

Two lines of evidence show that BACE1 processing is furin dependent. First, transfection of BACE1 expressing cells with α1-PDX, a potent and selective inhibitor of furin (Anderson et al., 1993), inhibits propeptide cleavage (Figure 68.2). Second, transfection of BACE1 into LoVo cells which do not express functional furin (Takahashi et al., 1993) results in accumulation of unprocessed pro-BACE1. Upon cotransfection of furin this species is completely processed (Bennett et al., 2000).

Secreted BACE1 derivatives are not completely processed to the mature form (Haniu et al., 2000). To directly demonstrate that furin cleaves the BACE1 propeptide domain *in vitro*, a soluble form of BACE1 was purified to homogeneity and aliquots were digested with recombinant furin *in vitro*. Only 50–70% of the originally purified soluble BACE1 are processed to start at E46; in contrast, after the furin digestion 100% of the resulting material starts at E46, directly demonstrating that furin cleaves the propeptide domain only at the known start site of mature BACE1 (Bennett et al., 2000).

CONCLUSIONS

Our results indicate that BACE1 is an N-glycosylated transmembrane protein. All four N-glycosylation sites are occupied and no O-glycosylation could be detected. The six cysteines within the ectodomain are involved in the formation of three intramolecular disulfide bonds which do not show conservation to known aspartic proteases. These structural differences may affect the substrate specificities of the enzymes. BACE1 is processed from the pro-enzyme to the mature form in the Golgi apparatus. Furin is required to cleave the BACE1 propeptide. While we cannot formally rule out a role for PC5B, this enzyme is not significantly expressed in brain (Nakagawa et al., 1993). Likewise, other protein convertases may participate with furin to cleave the BACE1 propeptide in specific tissues. Because furin is ubiquitously expressed and critical *in vivo* (Molloy et al., 1999) inhibition of BACE1 propeptide cleavage does not appear as a viable therapeutic strategy to indirectly lower Aβ production.

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69 Androgen Treatment Reduces Cognitive Deficits in Female apoE4 Transgenic Mice

JACOB RABER, ANTHONY LeFEVOUR AND LENNART MUCKE

INTRODUCTION

Apolipoprotein E (apoE) plays an important role in the metabolism and redistribution of lipoproteins and cholesterol (Mahley, 1988). In the brain, apoE has been implicated in development, regeneration, neurite outgrowth, and neuroprotection (Buttini et al., 1999; Gordon et al., 1995; Masliah et al., 1995; Nathan et al., 1994; Poirier et al., 1995; Sun et al., 1998). The three human apoE isoforms, encoded by distinct alleles (e2, e3, and e4), differ in their effect on Alzheimer's disease (AD). Compared with apoE2 and E3, apoE4 increases the risk of cognitive impairments and of developing AD (Corder et al., 1993, 1995; Farrer et al., 1997; Roses, 1996; Yaffe et al., 1997). ApoE4 appears to interact with female gender, further increasing AD risk and diminishing responsivity to treatments in women (Farlow, 1997; Farrer et al., 1997; Reed et al., 1994; Yaffe et al., 2000).

Understanding apoE–gender interactions may be important for the development of AD treatments. To assess how interactions between gender and apoE isoforms affect cognition, we study female and male mice lacking mouse apoE (*apoe*$^{-/-}$) and expressing human apoE isoforms in the brain. As they age, female, but not male, apoE4 (*apoe*$^{-/-}$) mice develop progressive impairments in spatial learning and memory in the water maze, compared with age- and sex-matched wild-type, *apoe*$^{-/-}$, or apoE3 (*apoe*$^{-/-}$) mice (Raber et al., 1998, 2000). These cognitive impairments are independent of the cellular source of apoE in the brain as they were observed in female *apoe*$^{-/-}$ mice expressing apoE4 in neurons or astrocytes. We have now begun to test the hypotheses that sex steroids contribute to the gender-dependent behavioral alterations in apoE4 mice and that androgens can antagonize apoE4-induced cognitive deficits.

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RESULTS AND DISCUSSION

AGE-, GENDER-, AND ISOFORM-SPECIFIC EFFECTS OF APOE ON SPATIAL LEARNING AND MEMORY IN THE WATER MAZE

The behavioral and neuropathological characteristics of the neuron-specific enolase (NSE)-apoE model have been described previously (Buttini et al., 1999, 2000; Raber et al., 1998; Raber et al., 2000). In this mouse apoE-deficient model, apoE3 or apoE4 are expressed at similar levels in the brain and can be detected in the cerebrospinal fluid; thus all central nervous system (CNS) cell types are exposed to the transgene-derived apoE isoforms.

Spatial learning and memory are severely impaired in AD and can be assessed readily in experimental animals. The Morris water maze test provides a good measure of spatial learning and retention of spatial memory. Mice are first trained to locate a visible platform (cued training) and then to locate a hidden platform (acquisition). Subsequently, the hidden platform is removed (probe trial) to measure retention of spatial memory (Figure 69.1). At 6 months of age, male and female *apoe*$^{-/-}$, NSE-apoE3, and NSE-apoE4 mice learned to locate both the visible and the hidden platform, and there were no significant differences in the learning curves. At 18 months of age, only *apoe*$^{-/-}$ (p50.01) and NSE-apoE3 (p50.05) females learned to locate the hidden platform, whereas NSE-apoE4 females did not (see Table 69.1). In the probe trial, 6-month-old male *apoe*$^{-/-}$, NSE-apoE3, and NSE-apoE4 mice, and female *apoe*$^{-/-}$ and NSE-apoE3 mice showed an increased preference to search the quadrant where the hidden platform was located previously (target quadrant), indicating retention of spatial memory, whereas NSE-apoE4 females did not, confirming the age-dependent development of significant cognitive impairments (Table 69.2).

Endogenous apoE is detected primarily in astrocytes in rodents and in astrocytes and neurons in humans (Bao et al., 1996; Han et al., 1994; Metzger et al., 1996; Xu et al., 1999). Neuronal apoE levels increase after CNS injuries in humans and rodents (Horsburgh and Nicoll, 1996; Kida et al., 1995). Female mice in which apoE4 expression was targeted to astrocytes by the glial fibrillary acidic protein (GFAP) promoter showed cognitive impairments similar to those observed in the NSE-apoE4 mice (unpublished data). These results suggest that the apoE4-induced deficits are independent of the cellular source of apoE (Table 69.3) and validate the findings obtained in the NSE-apoE model.

ApoE4-induced learning deficits could be detected even earlier with a modified version of the water maze test (Figure 69.2), in which the platform location is hidden from the start and changed daily (Raber et al., 1998). In this paradigm, NSE-apoE4 females showed clear learning impairments by 6 months of age (Figure 69.2). However, even with this more sensitive test, no spatial learning deficits could be detected in NSE-apoE4 males (age range: 3–18 months).

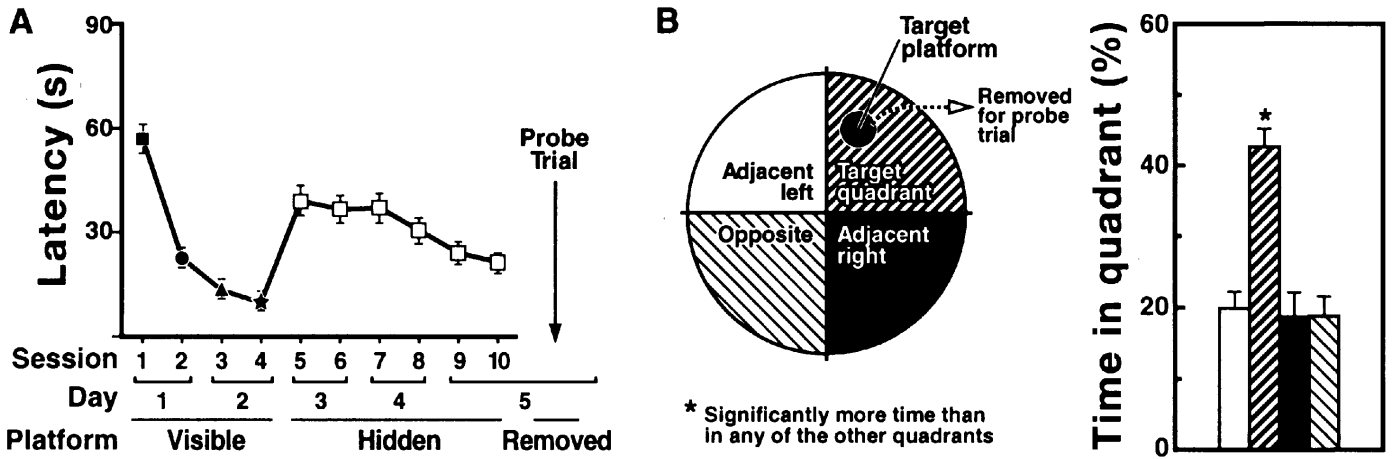

Figure 69.1. Learning curve (A) and probe trial (B) in the Morris water maze test. (A) In this version of the water maze, mice are first trained to locate a visible platform (days 1–2), and then a hidden platform (days 3–5) in two daily sessions 3.5 h apart, each consisting of three trials (10 min intertrial intervals). The pool (122 cm in diameter) is divided into four quadrants. During the visible platform training, the platform is moved to a different quadrant for each session. During the hidden platform training, the platform location is hidden under the surface of the opaque water and its location is kept constant for each mouse. The starting point at which the mouse is placed into the water is changed for each trial. Mice that fail to find the platform within 1 min are put on it for 15 s. (B) Retention of spatial memory is measured in the probe trial, in which the hidden platform is removed. Mice with spatial memory retention preferentially search the quadrant where the hidden platform was located previously (target quadrant). The time to reach the platform (latency), path length, time in quadrant, and swim speed are recorded with a video tracking system (EthoVision, Noldus Instruments)

Table 69.1: Age-dependent cognitive impairments in NSE-apoE4 female mice

	3 Months	6 Months	18 Months
Learning curve	Normal	Normal	;
Memory retention	Normal	;	;

Table 69.2. Deficits in spatial memory retention (probe trial) in 6-month-old apoE4 mice are gender-dependent

Model	Males, p	Females, p
apoe$^{-/-}$	50.01*	50.05*
NSE-apoE3	50.01*	50.05*
NSE-apoE4	50.01*	40.05

*Significantly more time in target quadrant than in any other quadrant.

Table 69.3. Cognitive impairments in 6-month-old apoE4 female mice are independent of cellular source of apoE

Model	Source of apoE	p
apoe$^{-/-}$	–	50.05*
NSE-apoE4	Neurons	40.05
GFAP-apoE4	Astrocytes	40.05

*Significantly more time in target quadrant than in any other quadrant

Interestingly, although NSE-apoE4 mice clearly develop age-dependent neurodegenerative alterations (Buttini et al., 1999, 2000), the morphological changes cannot fully explain the behavioral deficits we observed in this model. As they age, NSE-apoE4, but not NSE-apoE3, mice lose a significant portion of immunolabeled presynaptic terminals and neuronal dendrites in the neocortex and hippocampus. However, the neurodegenerative alterations in NSE-apoE4 mice are of similar magnitude in males and females, and they are similar to those observed in age-matched *apoe*$^{-/-}$ mice (Buttini et al., 1999; Masliah et al., 1995). Less obvious biochemical or neurophysiological alterations may account for the gender-dependent cognitive deficits present in apoE4 mice and absent in *apoe*$^{-/-}$ controls. We hypothesize that sex steroid-dependent pathways play an important role.

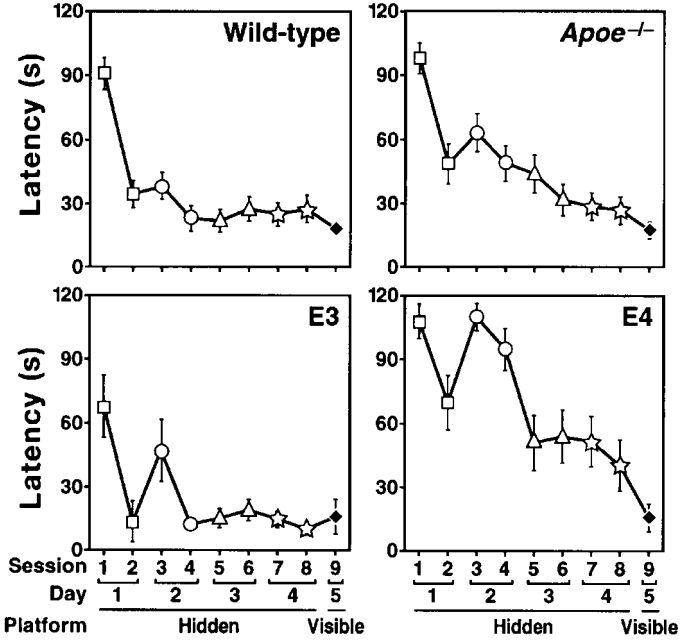

Figure 69.2. Differential effects of human apoE3 and apoE4 on water maze performance in 6-month-old female mice in a modified water maze test. This version of the water maze test was selected based on pilot experiments which showed it to be sensitive to the *in vivo* CNS effects of apoE3 and apoE4. In this version, mice are first trained to locate a hidden platform (days 1–4) in two daily sessions 2 h apart, each consisting of two trials (30 min intertrial interval) and then to locate a visible platform (day 5). The platform location was changed between days. To find the platform, mice must relate their position in the pool to constant extra-maze cues and quickly store, retrieve, and utilize information on where the platform is located and where it is not. Mice that fail to find the platform within 2 min are put on it for 15 s. The time required to locate the platform (latency) was compared among wild-type ($n = 16$), knock-out ($n = 16$), NSE-apoE3 ($n = 6$), and NSE-apoE4 ($n = 8$) mice. The average latency recorded for each mouse in two successive trials per session was used to calculate group means $\pm$SEM. Mice that failed to locate the platform were assigned a latency value of 120 s. Comparison of genotypes on individual days by Tukey–Kramer post hoc test revealed that NSE-apoE4 mice differed significantly from NSE-apoE3 (p50.01) and wild-type (p50.05) mice on day 1 and from all genotypes other than NSE-apoE4 on day 2 (p50.01). In contrast, male mice of corresponding genotypes showed no significant differences in this test, indicating that female gender significantly increases the susceptibility to apoE4-dependent impairments in spatial learning. Differences among female groups similar to those described above for latencies were also observed for path lengths. Age- and sex-matched mice of all genotypes swam continuously at similar speeds and in similar patterns, and there were no significant periods of passive floating. On day 5 of the training cycle, the ability of the mice to locate a clearly visible platform was tested to exclude basic deficits in vision, motivation, motor strength, or coordination. No significant differences in time to locate the visible platform were identified. The pool diameter was 61 cm. We also successfully used this version with a 122 cm pool diameter

SEX STEROIDS AND COGNITION

Sex steroids cause sex differences in brain organization (Beatty, 1979) and in behaviors in adulthood (Joseph et al., 1978). Male rodents castrated at birth have learning curves in the water maze resembling those of females, and administration of testosterone to newborn female rodents produces learning curves resembling those of males (Joseph et al., 1978). Because testosterone can be converted to 17-β estradiol by aromatase, the results of testosterone treatments can be difficult to interpret. This problem can be addressed by comparing the effects of testosterone and 17-β estradiol or by using dihydrotestosterone, which cannot be aromatized to 17-β estradiol. For example, testosterone, but not 17-β estradiol, enhances spatial memory (Janowsky et al., 1994; MacLusky et al., 1987), suggesting an androgen-specific effect.

Clinical trials are being conducted in patients with AD to evaluate the benefit of estrogen in females and of testosterone in males (Birge 1997a,b; Brinton and Yamazaki, 1998; Crawford, 1998; Cummings et al., 1998; Farlow, 1997; Haskell et al., 1997; Henderson, 1997; Henderson et al., 1994; Paganini-Hill and Henderson, 1996; Van Duijn 1999). The results from clinical studies of estrogen treatments have been difficult to interpret due to differences in the ages of the women studied, duration of estrogen treatment (continuous or intermittent), form of estrogen preparation used, and educational levels of women who do or do not choose to use estrogen (Shaywitz and Shaywitz, 2000). While estrogen treatment seems unable to slow disease progression (propagation phase), some, but not all, studies suggest that it may delay or prevent the onset of AD (initiation phase) (Barrett-Conner and Kritz-Silverstein, 1993; Costa et al., 1999; Haskell et al., 1997; Henderson, 1997; Jacobs et al., 1998; Johnson et al., 1998; Kampen and Sherwin, 1994; Kawas et al., 1997; Matthews et al., 1999; Mulnard et al., 2000; Paganini-Hill and Henderson, 1996; Phillips and Sherwin, 1992; Resnick et al., 1998; Shaywitz et al., 1999; Sherwin 1994; Steffens et al., 1999; Tang et al., 1996).

In a recent study, estrogen had a beneficial effect on cognitive function in healthy elderly women who lack the e4 allele, whereas it had no beneficial effect in those carrying the e4 allele or in women with AD (Yaffe et al., 2000). Although estrogen might enhance verbal memory skills, it does not appear to improve performance in visuospatial memory tasks (Kampen and Sherwin, 1994). In fact, lower estrogen levels may enhance spatial cognitive performance in humans (Janowsky et al., 1994). Lastly, testosterone, but not estrogen, levels in serum correlated positively with cognitive performance in older women (Barrett-Connor and Goodman-Gruen, 1999), and androgen therapy improved cognition in surgically menopausal women (Sherwin, 1988). The above observations suggest that both estrogens and androgens could affect cognitive performance in AD and normal aging. However, the

precise interaction between apoE and sex steroids may be difficult to dissect in human patients due to the many variables involved in clinical studies. It is therefore interesting to further assess this interaction in experimental models.

EFFECTS OF ANDROGENS ON SPATIAL LEARNING AND MEMORY DEFICITS IN APOE4 FEMALE MICE

To test whether androgens can antagonize gender-dependent apoE4-induced cognitive deficits, 6-month-old NSE-apoE4 female mice were anesthetized and Silastic capsules filled with testosterone or dihydrotestosterone were implanted subcutaneously; controls received placebo capsules. One week later, the mice were tested in the water maze (shown in Figure 69.1). While there was no difference between the learning curves of testosterone-, dihydrotestosterone-, and placebo-treated mice during the visible platform sessions, testosterone increased the ability of the mice to learn the hidden platform location compared with placebo-treated controls. In the probe trial (platform removed), placebo-treated mice showed no spatial memory retention. In contrast, the testosterone- and dihydrotestosterone-treated mice showed retention of spatial memory reflected in strong preferential searching in the target quadrant.

These results are encouraging as they demonstrate that even brief treatment with androgens can improve the spatial learning and memory deficits in adult NSE-apoE4 female mice.

ANDROGEN RECEPTORS

Testosterone and dihydrotestosterone exert androgenic effects by interacting with androgen receptors (ARs) (Couse and Korach, 1998). To determine whether AR-dependent pathways protect against apoE4-induced cognitive deficits, 6-month-old male NSE-apoE3 and NSE-apoE4 mice received daily subcutaneous injections of the AR antagonist hydroxyflutamide (Malgor et al., 1998) or vehicle alone, starting three days before behavioral testing. Male NSE-apoE4 mice, which do not show cognitive deficits at baseline, developed striking impairments in learning and retention of spatial memory after AR blockade, whereas male NSE-apoE3 mice did not. Thus, AR-dependent pathways may protect against the detrimental effects of apoE4 on cognition.

CONCLUSIONS

Our results confirm that the effects of apoE4 on brain function are critically influenced by gender and suggest that AR-dependent pathways may protect against apoE4-induced cognitive impairments. Even relatively brief periods of androgen treatment were able to reduce apoE4-associated cognitive deficits.

Understanding the relationship between apoE isoforms and sex steroids could help predict and improve the response of people with different *APOE* genotypes to treatments with sex steroids, which are currently used to prevent manifestations of menopause, improve sports performance, and treat AD.

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70 Studies with the Memory-Enhancing Drug AIT-082 in PC12 Cells

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INTRODUCTION

A significant loss of presynaptic cholinergic neurons and decreased levels of acetylcholine and choline acetyltransferase are the major characteristics of Alzheimer's disease (AD) (Becker et al., 1997). In order to maintain levels of the neurotransmitter acetylcholine, which plays a key role in memory and cognition, the cholinesterase inhibition strategy has been developed to treat AD patients (Giacobini, 2000). However, current FDA-approved anti-cholinesterase drugs such as tacrine, donepezil, and rivastigmine only moderately improve symptoms in AD, and these drugs are not believed to delay biological progression of the disease. It is clear that there is need for more effective treatments for AD patients. Therefore, therapeutic approaches that are based on increasing neuronal protection and improving other biochemical abnormalities associated with the disease are being investigated. Currently AIT-082 (Neotrofin^{TM}), a unique hypoxanthine derivative, is being studied as a treatment of AD (NeoTherapeutics Inc., CA). AIT-082 has been previously reported to induce neurite formation in primary hippocampal neuron, induce nerve growth factor (NGF) and other neurotrophic factors *in vivo* and *in vitro*, and promote recovery from a variety of neurodegenerative conditions (Rathbone et al., 1998). Our first objective was to study the effects of AIT-082 on cellular toxicity and neuronal differentiation in PC12 cells.

AD is also characterized by extracellular depositions of beta-amyloid (Aβ), which is derived from a large integral membrane glycoprotein, Aβ precursor protein (APP) (Selkoe, 2000). APP is processed by three proteases, termed α-, β-, and γ-secretases. The Aβ domain is bisected within APP by a constitutively expressed α-secretase enzyme that results in the release of carboxyl-truncated soluble derivatives (sAPP) in conditioned

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media of cells. The α-secretase pathway precludes Aβ formation and thus is non-amyloidogenic. The processing of APP by α-secretase is altered by growth factors (Checler, 1995) and activation through M1 and M3 cell surface receptors (Nitsch et al., 1992). These agents increase sAPP secretion and also reduce Aβ production in some cell types. The stimulation of sAPP secretion by growth factors is partly mediated by protein kinase C (PKC) and partly by tyrosine kinase activities. Our second objective was to study the effects of AIT-082 on levels of sAPP in neuronal cells.

EXPERIMENTAL PROCEDURES

MATERIALS

AIT-082 was a gift from NeoTherapeutics (Irvine, CA). Basic fibroblast growth factor (bFGF) and NGF were procured from Life Technologies (Gaithersburg, MD). Media and sera were obtained from Life Technologies. Other chemicals were of high purity and purchased from Sigma (St Louis, MO).

CELL CULTURE AND TREATMENT CONDITIONS

Rat pheochromocytoma cells (PC12) (ATCC) ($5–7\times10^6$) were seeded onto collagen-coated plates (100 mm) in the RPMI 1640 medium containing 10% horse serum (HS) and 5% fetal bovine serum (FBS) and grown in the presence of 5% CO_2. Before adding the drug, the cells were fed with low serum media (LSM) that contained 0.5% FBS. The cells were differentiated in the presence of either NGF (10-50 ng/ml) or bFGF (50 ng/ml) as described previously (Lahiri et al., 2000b). Cells were also incubated with AIT-082 (10 nM–100 μM). After incubation for a certain period of time, conditioned media from plates were collected before and after differentiation. The cell pellet was sonicated and centrifuged at 11 000 g for 20 min at 4 °C in IP buffer as described previously (Lahiri et al., 1998). Proteins of the supernatant solution (cell lysate) and conditioned media were measured.

DIFFERENTIATION EXPERIMENT

PC12 cells were first grown to 80–90% confluence in the regular medium. A day prior to the experiment, PC12 cells were subcultured uniformly onto the plate with minimum cellular aggregation/clumping to approximately 1×10^6 cells per 60 mm plate. On the day of treatment (day zero), the medium was changed to LSM. The PC12 cells were then subjected to treatments with either AIT-082 or NGF or both for three days (see Figure 70.2). Quantification of neurite bearing cells was done as follows. Five different

regions were selected from each plate in which cells were well separated from each other and which did not contain more than 150 cells. Within each region the total number of living cells was counted (trypan blue exclusion method) and the number of neurites bearing cells was counted. Neuritic length should be more than two times the cell diameter. The proportion of cells bearing neurites was calculated by dividing the number of differentiated cells by the number of viable cells under our conditions. All data were evaluated using a one-way ANOVA using the SPSS statistical analysis software. All statistics were evaluated at a significance level of $p < 0.05$.

ASSAY OF LDH TO MEASURE TOXICITY

The release of the enzyme lactate dehydrogenase (LDH) was measured in the conditioned medium samples using a commercial kit (Sigma). If the cells are lysed prior to assaying the medium, there is a resulting decrease in cell number, with a concomitant change in the amount of substrate converted. This indicates the degree of cytolysis or membrane damage (cytotoxicity) caused by the test material when the assay is done in the linear range. Cell-free aliquots of medium from cultures subjected to different treatments were assayed and the amount of LDH activity was used as an indicator of relative cell viability as a function of membrane integrity (Lahiri et al., 1998).

ANALYSIS OF PROTEINS BY PAGE AND WESTERN IMMUNOBLOTTING

Total proteins from the cell lysate or conditioned media were analyzed on a 12% polyacrylamide gel containing SDS (SDS-PAGE), and Western blot analysis was performed in the Mini-PROTEAN II system of Bio-Rad, Hercules, CA as described previously (Lahiri et al., 1998). Levels of sAPP were detected using the 22C11 (Boehringer Mannheim, Indianapolis, IN). A biotinylated secondary antibody, horse anti-mouse (Boehringer Mannheim), was also used. The detection system was based on the avidin-biotinylated complex (Vector Laboratories, Burlingame, CA) and the enzymatic color reaction.

RESULTS

EFFECT OF AIT-082 ON CELLULAR TOXICITY

We measured the toxic effect of AIT-082 in cell culture by counting the viable cells after staining with a vital dye, and observed that there was no significant change in the number of cells after drug treatment for 24 h. This suggests that AIT-082 had no effect on the viability of the cells. We also employed a

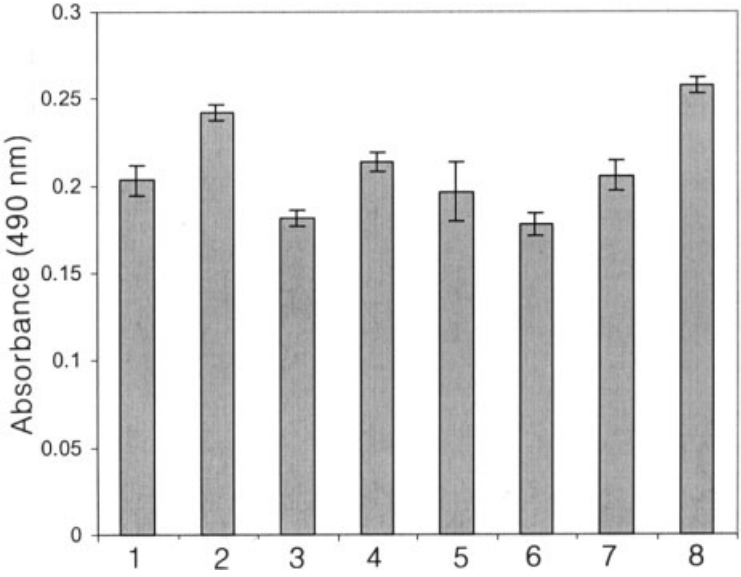

Figure 70.1 Effect of AIT-082 on toxicity of the cells. PC12 cells were cultured in the presence of the following agents, each in a separate plate: (1) untreated (no drug), (2) NGF (10 ng/ml), (3) AIT-082 (300 nM), (4) AIT-082 (1 μM), (5) AIT-082 (10 μM), (6) AIT-082 (100 μM), (7) AIT-082 (300 μM), and (8) AIT-082 (100 μM) +NGF (10 ng/ml). LDH assay was performed in the conditioned media samples collected after 24 h drug treatment as described in the text. A change in absorbency at 490 nm reflects the amount of LDH released: the higher the absorbency, the greater the LDH release, membrane damage and hence cellular toxicity (n = 4)

sensitive LDH assay to measure membrane integrity. Our results indicated that there was no significant change in levels of LDH enzyme released in conditioned media of drug-treated cultured cells from the control during 24 h treatment (Figure 70.1). For example, the absorbency from the control sample was 0.25, which was almost unchanged when the cells were treated with AIT-082 for 24 h from a dose of 300 nM to 300 μM. However, under the same conditions we noted approximately 20% increase in absorbency when the cells were treated with either NGF alone (10 ng/ml) or NGF (30 ng/ml) plus AIT-082 (100 μM).

EFFECT OF AIT-082 ON THE DIFFERENTIATION OF PC12 CELLS

We compared the neuronal-differentiating ability of AIT-082 and NGF in PC12 cells. At day 1, neuronal differentiation had started appearing only in NGF-treated cells (data not shown). At days 2 and 3, the treatment of PC12

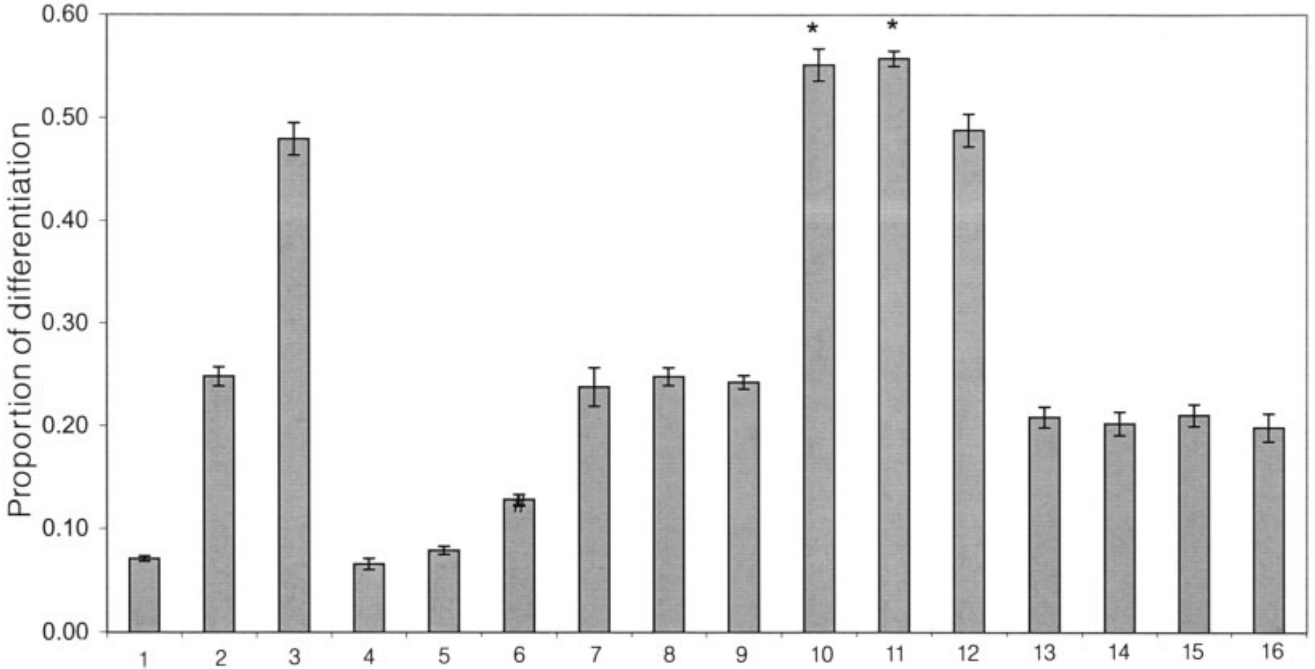

Figure 70.2. Effect of AIT-082 on the differentiation of PC12 cells. PC12 cells were cultured in the presence of the following agents under the conditions described in the text: (1) untreated, (2) NGF (10 ng/ml), (3) NGF (30 ng/ml), (4) AIT (10 μM), (5) AIT (100 μM), (6) AIT (300 μM), (7) NGF (10 ng/ml) + AIT (10 μM), (8) NGF (10 ng/ml) + AIT (100 μM), (9) NGF (10 ng/ml) + AIT (300 μM), (10) NGF (30 ng/ml) + AIT (10 μM), (11) NGF (30 ng/ml) + AIT (100 μM), (12) NGF (30 ng/ml) + AIT (300 μM), (13) FGF (30 ng/ml), (14) FGF (30 ng/ml) + AIT (10 μM), (15) FGF (30 ng/ml) + AIT (100 μM), and (16) FGF (30 ng/ml) + AIT (300 μM). Cell counting for the differentiated cells and total number of cells was done as described in the text

cells with both low- (10 ng/ml) and high-dose (30 ng/ml) NGF resulted in an induction of characteristic neuronal differentiation. Results from day 3 are presented in Figure 70.2. At day 2, the treatment of PC12 cells with different doses of AIT-082 (10–300 μM) failed to induce neuronal differentiation (data not shown). However, at day 3 the treatment of PC12 cells with only the highest dose of AIT-082 (300 μM) resulted in a significant amount of neuronal differentiation, but still much lower than that observed with NGF-treated cells (Figure 70.2). The co-treatment of PC12 cells with NGF (30 ng/ml) and AIT-082 resulted in a significantly greater extent of neuronal differentiation than the treatment of NGF alone. Under the same conditions, the treatment of PC12 cells with basic fibroblast growth factor (30 ng/ml) induced a modest level of neuronal differentiation. However, the co-treatment of PC12 cells with bFGF (30 ng/ml) and AIT-082 resulted in no greater extent of neuronal differentiation than the treatment of bFGF alone (Figure 70.2).

EFFECT OF AIT-082 ON THE SECRETION OF APP INTO THE CONDITIONED MEDIA OF CELLS

In a denaturing polyacrylamide gel, an equal amount of total protein was loaded from each of the conditioned medium samples and the efficiency of transfer was verified by staining the membrane with Ponceau stain. When the immunoblot was probed with an antibody against N-terminal epitope of APP, we detected distinct bands of 100 and 110 kDa, which correspond to soluble APP derivatives (sAPP) arising from alternate forms of APP and/or their post-translationally modified derivatives. With the NGF treatment, a significant increase in the secretion of sAPP was observed (data not shown). With the bFGF treatment, a slight increase in the secretion of sAPP was observed from the control. When the PC12 cells were incubated with different doses of AIT-082, an increase of sAPP similar to bFGF treatment was observed from the control. When the cells were simultaneously treated with AIT-082 and NGF, a significant increase of sAPP release was observed, which was more than treating cells with either NGF or AIT-082 alone. A similar but smaller synergistic effect of bFGF and AIT-082 treatments was observed (data not shown).

DISCUSSION

Here we studied the effect of AIT-082 on PC12 cells. First, we determined the toxicity profile of AIT-082 in cell culture system and observed that the drug is non-toxic to cells at a wide range of dosages (nM to mM levels) when measured by trypan blue and a sensitive LDH assay. However, under the same conditions we observed significant membrane damage when the cells

were treated with either NGF alone or NGF plus AIT-082. This increase with NGF was probably due to the observation that PC12 cells became elongated with neuritic projections on differentiation and thus they became delicate and sensitive to physical handling. These data indicate that the control and AIT-082-treated cells did not significantly differ in LDH release under our conditions. Thus the overall membrane integrity of the cells was not compromised with AIT-082 treatment at the dose studied. We then examined whether AIT-082 has the ability to induce differentiation of PC12 cells into sympathetic neuronal features under our conditions. This possibility was based on earlier observations that AIT-082 treatment induced NGF secretion in rat astrocytes (Rathbone et al., 1999). Our morphological evaluation suggests that, unlike NGF treatment, AIT-082 alone cannot fully differentiate PC12 cells to sympathetic neuronal phenotypes. This is consistent with our recent observations suggesting that AIT-082 treatment did not increase steady-state levels of endogenous NGF in conditioned media of PC12 cells under our conditions (D.K. Lahiri and J. Connors, unpublished data). However, AIT-082 treatment can promote differentiation that was already initiated by NGF, but not by bFGF, suggesting specificity for the drug's action. In short, in PC12 cells, AIT-082 lacks an independent neuronal-inducing property while it can promote NGF-induced differentiation. In primary hippocampal neurons, AIT-082 treatment alone can induce neurite formation (Juurlink and Rathbone, 1998; Bitner et al., 1999).

In addition to these morphological studies, we have studied the effect of AIT-082 on the level of APP in PC12 cells, which were treated with NGF and/or AIT-082 at different doses. Levels of sAPP in samples from conditioned media were measured by Western immunoblotting. As expected, we detected APP as 100–110 kDa protein bands in Western blots. When the PC12 cells were treated with NGF, there was a significant increase in sAPP levels, which is consistent with a previously published report (Refolo et al., 1989). Treating PC12 cells with AIT-082 also caused an increase in sAPP levels. The effect of co-treatment of NGF and AIT-082 on sAPP levels was found to be approximately additive. These results suggest that AIT-082 may enhance the α-secretase pathway and thereby could potentially decrease the amyloidogenic (amyloid formation) pathway. However, this is yet to be confirmed. Throughout this study untransfected cells were used in order to avoid alterations induced by overexpression of APP. These data may indicate a non-amyloidogenic and potentially neurite-promoting effect of AIT-082 in PC12 cells. Thus, these cell culture experiments provide a strong impetus to a thorough study of the effects of AIT-082 on intracellular APP, Aβ, and other AD protein markers both in neuronal cells and in samples from drug-treated AD subjects. In addition to the effect of AIT-082 on sAPP levels, we have recently shown that treating PC12 cells with AIT-082 increased the levels of synaptophysin (Lahiri et al., 2000a). Since synaptophysin protein is actively involved in exocytosis at synaptic cleft (Scheller, 1995), the treatment of

AIT-082 may enhance the neurotransmitter release at the presynaptic terminal. Taken together, these results suggest that the effects of AIT-082 on APP, synaptophysin and synaptic neurotransmission may potentially benefit the cognitive impairment seen in AD subjects.

CONCLUSION

One of the hallmarks of AD is the extracellular deposition of Aβ, which is derived from a large glycosylated membrane-bound Aβ precursor protein. The α-secretase enzyme cleaves APP within Aβ (between residues 16 and 17) to the secreted derivative sAPP and prevents Aβ formation. A new drug, AIT-082, which is a purine hypoxanthine derivative, is under clinical trial for the treatment of AD. The toxicity profile of AIT-082 in cell culture systems indicates that the drug is non-toxic at a wide range of dose (nM to mM levels) when measured by a sensitive LDH assay. Differentiation studies suggest that, unlike NGF, AIT-082 lacks an independent neuronal-inducing property in PC12 cells but can promote NGF-induced differentiation. In addition, we have determined that AIT-082 can regulate levels of APP in a cell culture system. We measured sAPP levels by Western blotting in PC12 cells that were treated with AIT-082 and observed an increase in the α-secretase pathway. These data suggest a potentially non-amyloidogenic and neurodifferentiation-promoting effect of AIT-082 in PC12 cells.

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71 Generation of Auto-antibodies toward Alzheimer's Disease Vaccination

BEKA SOLOMON AND DAN FRENKEL

INTRODUCTION

β-amyloid (Aβ) formation is a complex kinetic and thermodynamic process (Barrow et al., 1992) and the reversibility of amyloid plaque growth *in vitro* suggests a steady-state equilibrium between Aβ peptide (AβP) in plaques and in solution (Maggio and Mantyh, 1996). The dependence of AβP polymerization on peptide–peptide interactions to form a β-pleated sheet fibril and the stimulatory influence of other proteins on the reaction suggest that amyloid formation may be subject to modulation.

Site-directed monoclonal antibodies towards the N-terminal region of the AβP bind to preformed Aβ fibrils, leading to disaggregation into an amorphous state and inhibition of amyloid neurotoxic effect (Solomon et al., 1997). The effect of these antibodies to modulate Aβ formation *in vitro* (Solomon et al., 1996, 1997) and *in vivo* (Bard et al., 2000) was found to be epitope-dependent.

We found that the epitope EFRH (Frenkel et al., 1998), which is located at the soluble tail of the human N-terminal region of AβP, acts as a regulatory site controlling both the solubilization and the disaggregation process of the AβP molecule. Locking of this epitope by appropriate antibodies may modulate the dynamics of conformational changes occurring in the whole peptide, preventing aggregation.

Preparation of antibodies against 'aggregation epitopes' in the N-terminal region of AβP lead to both prevention as well as disaggregation of the pathological Aβ fibrils.

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IMMUNIZATION AGAINST Aβ USING PHAGE DISPLAY TECHNOLOGY TOWARD VACCINATION AGAINST AD

The generation of antibodies against peptides usually involves the chemical synthesis of the peptide. Moreover, such small synthetic peptides, consisting of antibody epitopes, are generally poor antigens and need to be coupled to a large carrier, but even then they induce a low-affinity immune response. Injection of AβP or toxic Aβ fibril in the presence of adjuvant leads to a slow immune response (Schenk et al., 1999).

Filamentous bacteriophages offer an obvious advantage over other protein carriers. Filamentous bacteriophages have been used extensively in recent years for the 'display' on their surface of large repertoires of peptides generated by cloning random oligonucleotides at the 5′-end of the genes coding for the phage coat proteins (Scott and Smith, 1990; Scott, 1992; Greenwood et al., 1993). Filamentous phages are long thread-like single-stranded DNA phages which infect bacteria via sex pili. The best known, the Ff phages, are a group of three phages (M13, fd, and fl). The phage protein that is responsible for binding to the pilus tip, pIII, is present in four copies, encoded by gene 3. If foreign DNA that encodes a peptide or protein is inserted downstream of the leader sequence of gene 3, it will be translated and exposed at the N-terminus of the mature pIII without compromising the ability of pIII to mediate infection via the F pilus (Marvin, 1998). Protein pIII and the major coat protein pVIII are the proteins that have been most widely used for phage display. The main difference between pIII and pVIII is the copy number of the displayed protein: while PIII is present in four copies, the pVIII is found in 2700 copies (Figure 71.1).

Parenteral administration of filamentous phages in animals induced a strong immunological response to the phage proteins in all animals tested (Willis et al., 1993; Motti et al., 1994; Meola et al., 1995; Delmastro et al., 1997). Mimotopes displayed on filamentous phages could be used as immunogens and form the basis for developing a wide range of vaccines.

The recombinant filamentous phage approach to obtain specific peptide antigens has a major advantage over chemical synthesis, as the products obtained are the result of the biological fidelity of translational machinery and are not subject to the 70–94% purity levels common in the solid phage synthesis of peptides. The phage represents an easily renewable source of antigen since more material can be obtained easily by growth of bacterial cultures.

We developed a novel immunization procedure for the production of effective anti-aggregating Aβ antibodies, using filamentous phages displaying the only four amino acids EFRH found to be the main regulatory site for amyloid modulation. To select the phages containing EFRH peptide epitope we screened the phage-epitope library with biotinylated antibody (Frenkel et al., 1998). Immunization with the EFRH-phage raises the high concentration

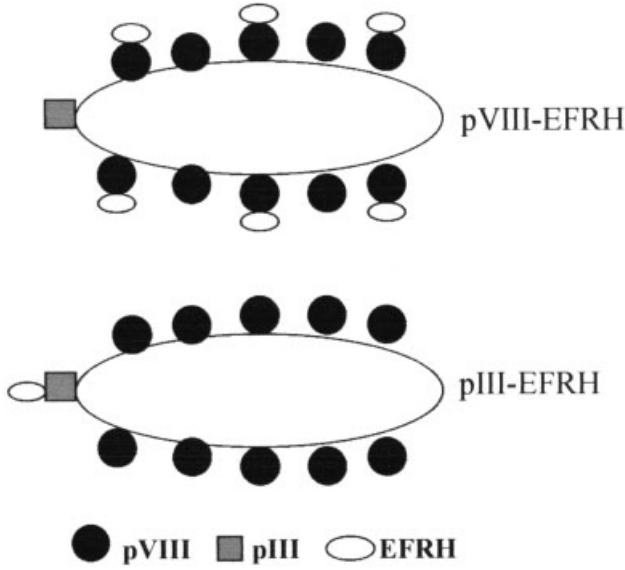

Figure 71.1. Scheme of epitope exposed on filamentous phage coat proteins pIII and pVIII

of high affinity (IgG) anti-aggregation antibodies in a short period of time (a few weeks).

The high immunogenicity of filamentous phages enables the raising of antibodies against self-peptides or antigens. Immunization of guinea-pigs with EFRH-phage as antigen, in which the AβP sequence is similar to that in humans, resulted in the production of high-affinity (IgG) antibodies against self-peptides in a short period of time (a few weeks) (Frenkel et al., 2000a). Doses of 10^{11} phages per injection were used to immunize subcutaneously (sc) at 14-day intervals. Highly specific antibodies (IgG) against the AβP (1:5000) were received after the fourth injection of EFRH-phage (Figure 71.2). Similar results were obtained after immunization with 10^{11} phages per intranasal injection without the adjuvant with an additional administration of antigen.

The antibodies resulting from EFRH-phage immunization are similar regarding their immunological properties to monoclonal antibodies previously studied and to the antibodies raised by direct injection with fibrillar toxic Aβ. These antibodies recognize the full beta-peptide (1–40) as antibodies raised against whole peptides and/or Aβ (Frenkel et al., 2000a).

Sera raised against EFRH peptide exhibits a protective effect in preventing Aβ-mediated neurotoxicity toward pheochromocytoma PC12 cells and disrupted the fibril structure of Aβ, with extensive deterioration of fibril morphology compared to unrelated serum used as control, which did not inhibit fibril formation and neurotoxicity through neuron (Frenkel et al.,

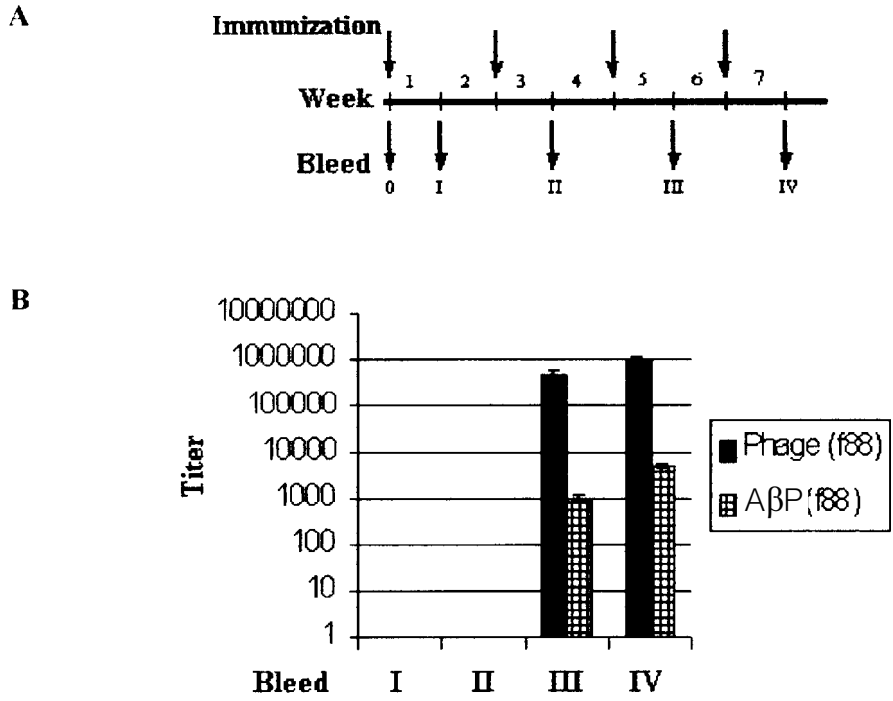

Figure 71.2. Immunization with f88 filamentous phage displaying EFRH epitope. (A) Immunization schedule. (B) Serum IgG titer of guinea-pigs from animals immunized with EFRH-phage through sc administration was measured after the third and the fourth administration. Each experimental marker represents the mean data obtained from three different experiments, with standard deviation varying between 4% and 7%. (Reproduced by permission of Elsevier Science)

2000a,b). Such antibodies are able to sequestrate the peripheric AβP, thus avoiding the passage to the blood–brain barrier (BBB) (Schenk et al., 1999). Moreover, use of transgenic mice as AD antibodies to overcome the BBB and dissolve already formed Aβ plaques (Bard et al., 2000) confirmed previous *in vitro* data (Solomon et al., 1997).

Filamentous phages are normally grown using a laboratory strain of *Escherichia coli*, and although the naturally occurring strain may be different, it is reasonable to assume that delivery of phage into the gut will result in infection of the natural intestinal flora (Zuercher et al., 2000). The presence of filamentous phages displaying epitopes in the guts of the immune animals two months after the last administration was detected by the ability of the mature phage found in the animal feces to infect laboratory *E. coli* strain, and by dot blot for the epitope EFRH detection on the phage envelope (Frenkel

et al., 2000a). Indeed, immunization through phage-carrying epitope was found to be long-lasting. No toxic effect due to autoimmune response against EFRH self-epitope was detected in the challenged animal sections, including liver, kidney, spleen, lung, and brain.

The authors have found that UV-inactivated phages are as immunogenic as their infective counterparts.

Application of this new approach in the development of Aβ anti-aggregating antibodies by injection of phage-EFRH as antigen in AD models of transgenic mice is now under investigation.

SUMMARY

We previously showed that epitope EFRH, sequenced 3–6 within the human AβP, acts as a regulatory site controlling both the solubilization and disaggregation process of the Aβ fibril. Locking of this epitope by highly specific antibodies affects the dynamics of the entire AβP molecule, preventing self-aggregation as well as enabling resolubilization of already formed aggregates. Production and performance of such antibodies obtained by repeated injections of toxic human Aβ fibrils into transgenic mice suggests the feasibility of vaccination against AD. Here we report the development of a novel immunization procedure that is quick, specific, and non-toxic for the production of effective anti-aggregating Aβ antibodies. This procedure is based on using filamentous phages displaying EFRH peptide as an antigen. Due to the high antigenicity of the phage no adjuvant is needed to obtain high affinity anti-aggregating IgG antibodies, which are achieved after a very short immunization period. Effective auto-immune antibodies are obtained by EFRH-phage administration in guinea-pigs, which exhibit human identity in the AβP region. Availability of these antibodies may help devise new approaches toward the development of an efficient and long-lasting vaccination for the prevention and treatment of AD.

CONCLUSIONS

The advantages of phage-EFRH antigen in raising anti-aggregating Aβ antibodies vs. whole Aβ can be summarized as follows:

1. The high immunogenicity of the phage enables production of high titer of IgG antibodies in a short period of weeks without the need for adjuvant administration. Self-expression of the antigen led to long-lasting immunization.

2. The key role of EFRH epitope in Aβ formation and its high immunogenicity leads to anti-aggregating antibodies which recognize whole AβP, substituting the use of whole toxic Aβ fibrils.

This novel approach model has demonstrated for the first time that a recombinant bacteriophage displaying a self-epitope can be used as a vaccine to induce auto-antibodies for AD treatment.

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72 Toward the Identification of γ-Secretase: Using Transition State Analog Inhibitors

WILLIAM P. ESLER, W. TAYLOR KIMBERLY, BETH L. OSTASZEWSKI, WEIMING XIA, DENNIS J. SELKOE AND MICHAEL S. WOLFE

INTRODUCTION

Current evidence implicates the beta-amyloid (Aβ) protein as the principal culprit in the pathogenesis of Alzheimer's disease (AD) (Selkoe, 1999). Its biogenesis results from the sequential proteolytic cleavage of the amyloid precursor protein (APP) by two enzymes designated β- and γ-secretase. As a result, intense interest has focused on identifying the proteins responsible for these activities, since they are key targets for therapeutic intervention. Recently, the identity of β-secretase (BACE1, memapsin 2) was reported by several groups independently (Hussain et al., 1999; Sinha et al., 1999; Vassar et al., 1999; Yan et al., 1999; Lin et al., 2000). BACE1 and its homolog BACE2 (Saunders et al., 1999; Farzan et al., 2000) have been demonstrated to fulfill the characteristics of bona fide β-secretase, although only BACE1 is appreciably expressed in adult brain (Bennett et al., 2000). On the other hand, comparatively little is known about the protein(s) that constitute γ-secretase activity. Despite the lack of identity of the protein components, less direct studies have revealed substantial information about its activity. Indeed, pharmacological studies revealed that γ-secretase exhibits properties of an aspartyl protease (Wolfe et al., 1999a). Mutagenesis near the substrate cleavage site in APP suggested that γ-secretase is an intramembrane-cleaving enzyme with low sequence specificity (Lichtenthaler et al., 1999). Furthermore, protease activity is highest at acidic to neutral pH, which contrasts with the more acidic optimum of most other prototypical aspartyl proteases such as cathepsin D, but is similar to that of the aspartyl protease renin (Li et al., 2000). Although γ-secretase is a highly unusual protease without an identity, two proteins—presenilin 1 and 2 (PS1 and PS2)—have emerged as candidates.

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The PS proteins were first discovered as genes responsible for a majority of the autosomal dominant cases of AD (Levy-Lahad et al., 1995; Rogaev et al., 1995; Sherrington et al., 1995). Shortly after their discovery, mutations in PS1 and PS2 were found to alter subtly the heterogeneity of the C-terminus of Aβ, favoring an increased production of the more amyloidogenic 42-amino acid form (Scheuner et al., 1996; Citron et al., 1997). However, based on a modest degree of homology to a putative trafficking protein called Spe-4, the PS proteins were speculated to exert their effect indirectly, through the regulation and trafficking of γ-secretase (Sherrington et al., 1995; Naruse et al., 1998). Interestingly, however, the absence of PS1 in cells derived from knock-out embryos led to a sharp decrease in γ-secretase activity (De Strooper et al., 1998). Subsequent work identified two conserved aspartates located within transmembrane (TM) domains 6 and 7 of PS that are critical for γ-secretase function (Wolfe et al., 1999b). Since two (and only two) aspartates are required for an aspartyl protease active site, this observation provided provocative evidence that PS could be the active site of γ-secretase. However, reasonable alternative explanations remained (Haass and De Strooper, 1999). Therefore, in an attempt to use an unbiased approach to identify the active site of γ-secretase, we have modified one of our substrate-based γ-secretase inhibitors to convert it into an affinity probe.

By attaching a bromoacetyl group on the N-terminus and a biotin group to the C-terminus of one of our peptide analog γ-secretase inhibitors (compounds directed toward the active site of this aspartyl protease), we have identified two bands of ~21 and ~31 kDa that were specifically labeled by the inhibitor. Furthermore, we have confirmed that these bands correspond to the PS1 N-terminal and C-terminal fragments. Similar results were obtained for PS2. Therefore, we conclude that the active site of γ-secretase consists of the two conserved TM aspartates in the PS proteins. These two residues coordinate to form the active site of γ-secretase and catalyze the unusual transmembrane hydrolysis of several transmembrane substrates, including APP and Notch.

METHODS

The methods used in this study have been described elsewhere (Capell et al., 1998; Wolfe et al., 1999b; Esler et al., 2000).

RESULTS

DESIGN OF A γ-SECRETASE AFFINITY REAGENT

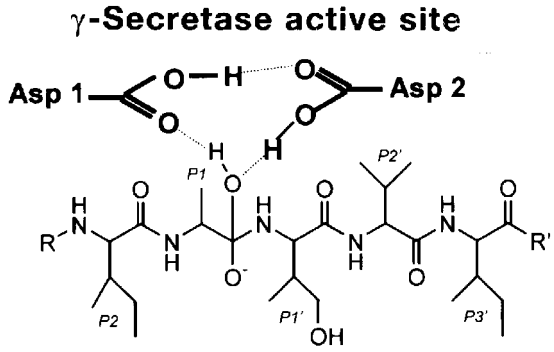
Cleavage of the scissile bond by an aspartyl protease proceeds through a transient gem-diol intermediate, or transition state, in the substrate. In the

enzymatic cleavage mechanism, the two active-site aspartates are believed to coordinate with this substrate transition state. The design of inhibitors to known aspartyl proteases such as renin and HIV protease has relied on mimicking this transition state to generate highly specific and potent inhibitors. X-ray crystal structures of such inhibitors (closely related to those used in this study) complexed with their target enzyme have shown that the transition state analog does indeed coordinate with the two active-site aspartates (James et al., 1992; Parris et al., 1992; Veerapandian et al., 1992; Silva et al., 1996).

To rationally design an inhibitor to interact directly with the active site of γ-secretase, we wished to closely mimic the $A\beta_{42}$ γ-secretase cleavage site in C99. The γ-cleavage site for production of $A\beta_{42}$ is shown in Figure 72.1A, with the predicted gem-diol transition state between P1 and P1′ coordinated with the two active site aspartates of an aspartyl protease. The class of compounds developed by our research group employs a difluoroalcohol transition state analog (Wolfe et al., 1999a). This functionality was originally developed in the design of inhibitors of the aspartyl protease renin (Thaisrivongs et al., 1986). While our initial γ-secretase inhibitors of this class closely mimicked the $A\beta_{42}$ cleavage site, structure activity studies indicated that installment of a large substituent such as a sec-butyl group at the P1 position improved inhibitor potency (Moore et al., 2000). The inhibitor shown in Figure 72.1B represents the end product of an effort to rationally design a γ-secretase inhibitor through relatively conservative and iterative alterations of a substrate-based transition-state analog. This molecule contains the difluoroalcohol transition state analog (outlined) which replaces the P1′ side chain. The P2′ and P3′ substituents are indistinguishable from those positions in the substrate, the P1 side chain in the substrate has been replaced with a large substituent in the inhibitor to improve compound potency, and the side chain at position P2 was altered conservatively from that found in the substrate to improve inhibitor potency. This compound inhibited Aβ production with an IC_{50} of $\sim 10\,\mu M$ in intact living cells (Esler et al., 2000).

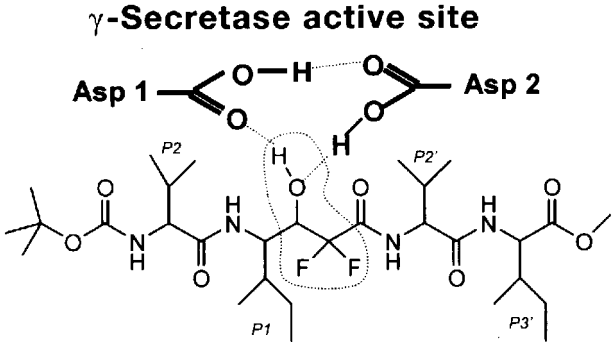
Toward the goal of an affinity reagent, we sought to derivatize the γ-secretase inhibitor shown in Figure 72.1B to contain: (i) a reactive crosslinking group to allow covalent attachment to the active site of γ-secretase, and (ii) a molecular tag to facilitate subsequent isolation and detection of candidate γ-secretase proteins. These needs were met by incorporation of a reactive bromoacetyl functionality on the N-terminus and a biotin tag on the C-terminus. Bromoacetamides are relatively stable compounds that are susceptible to nucleophilic attack, and similar derivatization of other bioactive compounds has provided affinity-labeling reagents for target receptors and enzymes (Atlas et al., 1976; Kohrle et al., 1990; Smar et al., 1992). Thus, we reasoned that while the difluoroalcohol moiety of the probe should interact with the γ-secretase residues directly

A



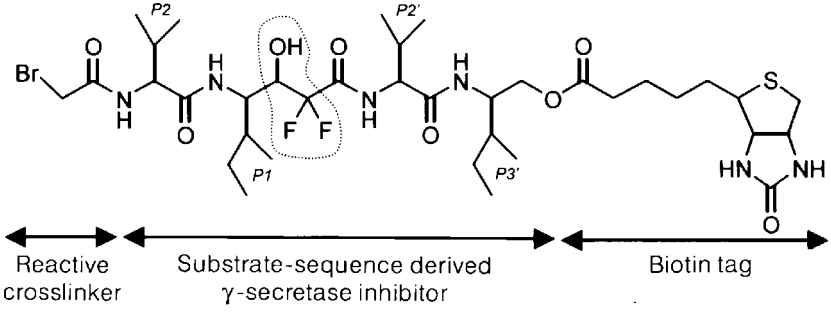
$A\beta_{42}$ γ-Secretase cleavage transition state

B



Peptidomimetic transition state analog

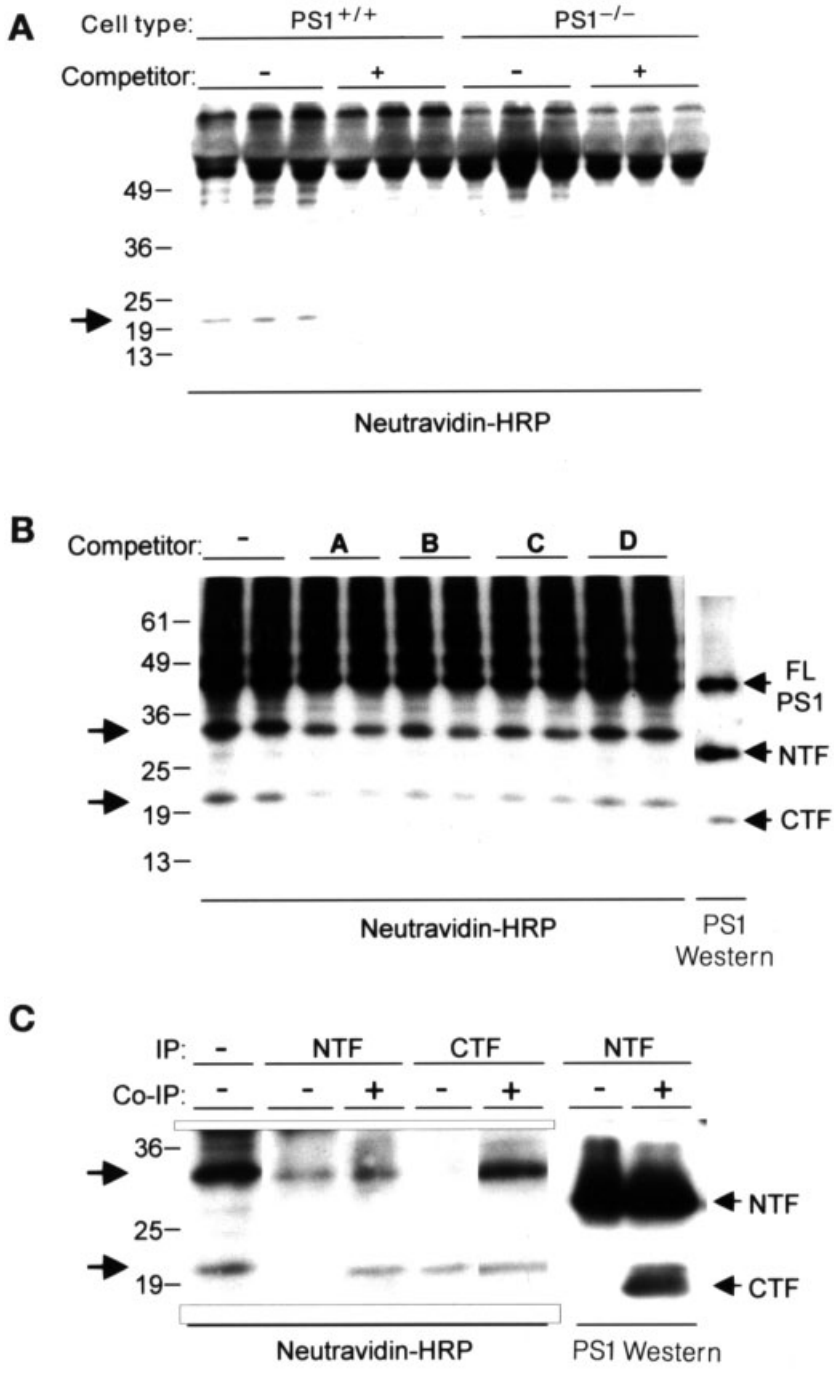
C Rational design of a γ-secretase affinity label



involved in catalysis (i.e. its two aspartates; see Figure 72.1), the bromoacetamide group would be available for attack by any nearby nucleophilic residues (e.g. serine, threonine, cysteine), allowing covalent attachment to the enzyme. The resulting affinity reagent is shown in Figure 72.1C. Incorporation of the biotin tag or the bromoacetamide group did not alter compound potency. As was observed for the parent compound, the affinity reagent raised levels of the γ-secretase substrates C83 and C99 in a dose-dependent manner (Esler et al., 2000).

Because the parent compound was designed from first principles both to mimic the γ-secretase cleavage site in APP and to coordinate with the active site aspartates of γ-secretase, we utilized the resulting affinity reagent in an unbiased search to identify the enzyme. To identify proteins covalently bound to the γ-secretase affinity probe, wild-type murine fibroblasts were treated with this compound. Because bromoacetimides can non-specifically crosslink common proteins that have surface-exposed nucleophiles, we utilized displacement with a non-biotinylated γ-secretase inhibitor (i.e. competition) to identify specific interactions. Following pretreatment in the presence or absence of a non-biotinylated γ-secretase inhibitor, the fibroblast lysates were treated with the affinity reagent. The samples were then electrophoresed on SDS-PAGE and blotted onto PVDF; any biotinylated proteins were detected using neutravidin-horseradish peroxidase conjugate (neutravidin-HRP) (Figure 72.2A). A biotinylated band at ~21 kDa was detected after treatment with the affinity reagent, and this band was markedly reduced in amount by pretreatment of the lysates with the non-biotinylated γ-secretase inhibitor. Because γ-secretase activity is dependent on PS expression, we also examined the ability of the affinity reagent to tag proteins in lysates from PS1-deficient murine fibroblasts (Figure 72.2A). Following treatment with the affinity reagent, the biotinylated ~21 kDa protein detected in the wild-type lysates was dramatically reduced in the knock-out murine fibroblast lysates. These results show that specific labeling of the ~21 kDa protein by a transition-state analog inhibitor of γ-secretase is dependent on the presence of PS1, consistent with the requirement of PSs for γ-secretase activity.

Figure 72.1. Design of a γ-secretase affinity reagent. The $A\beta_{42}$ cleavage site transition state for an aspartyl protease cleavage mechanism is depicted with the substrate gem-diol coordinated to the active site aspartates (A). A rationally designed γ-secretase inhibitor is shown in (B). This compound closely mimics the $A\beta_{42}$ cleavage site using a transition-state analog and was developed to coordinate with the catalytic aspartates in a manner similar to the substrate-transition state. It would thus inhibit Aβ production by interacting with the active site of the enzyme (B). To convert this inhibitor to an affinity reagent, it was derivatized to contain a reactive crosslinking moiety that would covalently attach the inhibitor to the enzyme and a biotin tag to facilitate isolation and detection of an enzyme:inhibitor complex (C). The transition-state mimic, which should coordinate with the active site aspartates, is outlined


A
Cell type:
PS1+/+
PS1−/−
Competitor:
-
+
-
+
49–
36–
25–
19–
13–
Neutravidin-HRP
B
Competitor:
-
A
B
C
D
61–
49–
36–
25–
19–
13–
FL PS1
NTF
CTF
Neutravidin-HRP
PS1 Western
C
IP:
-
NTF
CTF
NTF
Co-IP:
-
-
+
-
+
-
+
36–
25–
19–
NTF
CTF
Neutravidin-HRP
PS1 Western

To examine a more physiologically relevant model system for candidate γ-secretase proteins, intact microsomes prepared from APP-transfected Chinese hamster ovary (CHO) cells were treated with our affinity probe. This system has the advantage that we can search for candidate γ-secretase proteins under *in vitro* conditions known to preserve γ-secretase enzymatic activity (i.e. Aβ generation) (Xia et al., 2000). Indeed, microsomal generation of Aβ was blocked by our γ-secretase inhibitors in a dose-dependent manner closely similar to that seen in whole cells. Isolated microsomes from CHO cells were therefore treated with the affinity probe under these same conditions with or without competitors and then lysed with 2% CHAPS detergent. Electrophoresis by SDS-PAGE, transfer to PVDF, and probing with neutravidin-HRP revealed the biotinylated band at ~21 kDa seen in cell lysates, as well as an additional biotinylated band at ~31 kDa (Figure 72.2B). The amounts of both labeled bands were decreased by competitor analogs in correlation with their ability to block γ-secretase activity: compound A is a more potent inhibitor than compound B, which is more potent than compound C. Compound D is an inactive control lacking the transition-state analog. As seen previously in lysates, these observations demonstrate the specificity of covalent binding of our affinity reagent to these particular proteins in microsomes. While a number of higher molecular weight biotinylated proteins were also observed, the binding of these proteins to the affinity reagent did not appear to be specific, i.e. was not competed by the unbiotinylated analogs.

The ~21 kDa biotinylated protein was similar in size to that reported for the PS1 CTF (~18 kDa) and the ~31 kDa protein was similar in size to the PS1 NTF (~28 kDa) (Figure 72.2B), with covalent modification by the affinity probe possibly accounting for the difference in electrophoretic mobility. We therefore tested the ability of PS1-specific antibodies to immunoprecipitate and co-immunoprecipitate these biotinylated proteins from affinity probe treated CHO microsomes. After incubation with the affinity probe, microsomes from CHO cells were treated with 2% CHAPS detergent in Tris buffer (conditions reported to enable co-immunoprecipitation of PS1 NTF and CTF) (Capell et al., 1998). Subsequent immunoprecipitation with the PS1 C-terminal specific antibody 4627

Figure 72.2. Specific, covalent binding of a γ-secretase affinity probe to PS1. (A) Biotinylation of proteins in cell lysates of $PS1^{+/+}$ and $PS1^{-/-}$ mouse fibroblasts upon binding of affinity probe and competition by a non-biotinylated γ-secretase inhibitor. (B) Biotinylation of proteins in microsomes with the affinity probe and competition by non-biotinylated analogs. The last lane shows the migration of PS1 NTF, CTF, and holoprotein (no compound treatment) in these microsomes by IP/Western. (C) Proteins in isolated microsomes tagged by the γ-secretase affinity probe were brought down with antibody 4627 to the PS1 CTF or antibody Ab14 to the PS1 NTF under normal immunoprecipitation conditions or under co-immunoprecipitation conditions

brought down the biotinylated protein at ~21 kDa, as well as the biotinylated ~31 kDa protein (Figure 72.2C, lane 5). Conversely, immunoprecipitation under these conditions with antibody Ab14 to the N-terminus of PS1 resulted in detection of the biotinylated protein at ~31 kDa plus co-precipitation of the ~21 kDa protein (Figure 72.2C, lane 3). When the immunoprecipitations were performed under normal (not co-immunoprecipitation) conditions (NP40, not CHAPS, as the detergent), only the ~31 kDa protein came down with PS1 NTF antibody Ab14, and only the ~21 kDa protein came down with PS1 CTF antibody 4627 (Figure 72.2C, lanes 2 and 4). These results provide strong evidence that the biotinylated proteins are authentic PS NTFs and CTFs and that these two proteins are able to bind transition-state analogs targeted to the active site of γ-secretase when they are physiologically associated with each other.

DISCUSSION

Although originally implicated in autosomal dominant, early-onset forms of AD, the PS and APP genes appear to play a paramount role in a general AD pathogenic mechanism (Selkoe, 1999). While PS was discovered more than five years ago, the detailed nature of the functional relationship between APP and PS has remained unclear. Our data now provide strong evidence for the most parsimonious model of that relationship: PS and APP proteins are enzyme-substrate partners in the generation of Aβ. The APP C-terminal stub C99 is a substrate for γ-secretase (Selkoe, 1999). Our data suggest that PS forms the active site of the enzyme that cleaves C99. We have shown that transition state analog inhibitors, modified to contain a reactive side group and a biotin tag, specifically bind to PS heterodimers. Since the inhibitor was designed to be directed to the active site of γ-secretase, we conclude that the heterodimers form the catalytic center. Substrates such as C99 would therefore enter the multipass TM PS protein, exposing them to the two TM aspartates that catalyze the hydrolysis reaction (depicted in Figure 72.3).

In fact, several lines of evidence indicate that the PS proteins (and the two TM aspartates in particular) form the active site of γ-secretase (Table 72.1). Such evidence includes pharmacological studies indicating that γ-secretase is an aspartyl protease (Wolfe et al., 1999a). The mutagenesis of two key TM aspartates in PS1 or PS2 abrogates γ-secretase activity (Wolfe et al., 1999b; Kimberly et al., 2000), a phenotype which is mimicked by the complete absence of PS1 or PS2 in mouse embryonic cells (Herreman et al., 2000; Zhang et al., 2000). γ-Secretase activity can also be co-immunoprecipitated with PS-specific antibodies (Li et al., 2000) and finally, γ-secretase-specific inhibitors bind to PS (this study).

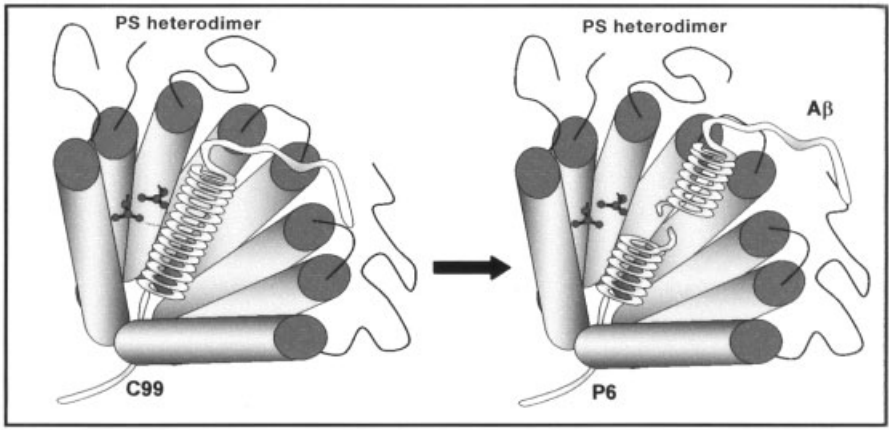


Figure 72.3. Hypothetical model of PS cleaving C99 intramembranously to release Aβ and P6

Are the PS proteins the only protein components of γ-secretase? Since PS levels are tightly regulated (Thinakaran et al., 1997), it is likely that there are several additional proteins that contribute to the enzyme complex. In fact, it appears that PS is a part of a large multimeric complex (Capell et al., 1998; Yu et al., 1998). Although the evidence presented here argues strongly that PS forms the active site, the nature and identity of this complex should emerge with its biochemical purification and reconstitution in artificial membranes.

Table 72.1. Evidence that presenilins are γ-secretases

FAD mutations in PS alter γ-secretase activity
PS knock-out cells produce no detectable Aβ
γ-Secretase is an aspartyl protease: PS contains two transmembrane aspartates and mutation of either of these aspartates blocks Aβ production
Antibodies to PS1 co-immunoprecipitate CD99 and C83. This co-immunoprecipitation is enhanced in aspartate mutant cell lines
PS is found in partially purified γ-secretase preparations and γ-secretase activity can be immunoprecipitated from these systems using PS antibodies
Transition-state analog γ-secretase inhibitors bind directly to PS1 and PS2
PS shares sequence homology in the vicinity of one of the presumed active-site aspartates with a known family of multi-transmembrane acid proteases

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73 Photoactivated, Active-Site-Directed γ-Secretase Inhibitors Covalently Label Presenilin 1

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INTRODUCTION

Amyloid precursor protein (APP) is proteolytically clipped by the β- and γ-secretases to generate the N- and C-termini, respectively, of the beta-amyloid (Aβ) peptides $A\beta_{40}$ and $A\beta_{42}$ (Selkoe, 1998). The identity of γ-secretase has proven to be elusive. Several pivotal studies have documented a close association between γ-secretase activity and the presenilins (PS1 and PS2), which are polytopic membrane spanning proteins. Key supportive evidence includes: (i) mutations in PS1 and PS2 that are associated with early-onset familial Alzheimer's disease (AD) (Levy-Lahad et al., 1995; Sherrington et al., 1995) increase production of Aβ42 (Borchelt et al., 1996; Duff et al., 1996; Scheuner et al., 1996), the more amyloidogenic peptide; (ii) γ-secretase activity that participates in the formation of secreted Aβ is reduced in neuronal cultures derived from PS1-deficient mouse embryos (De Strooper et al., 1998) and virtually abolished in the corresponding cells from PS1/PS2-doubly deficient mouse embryos (Herreman et al., 2000; Zhang et al., 2000); (iii) directed mutagenesis of two conserved transmembrane-situated aspartates in PS1 inactivates γ-secretase catalyzed processing within the transmembrane domain of APP (Wolfe et al., 1999a); and (iv) PS1 and γ-secretase are linked in the detergent solubilized state (Li et al., 2000a). The

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logical conclusion that PS1 is γ-secretase has been widely challenged because the presenilins bear little or no homology to any known protease. We embarked upon a campaign to elucidate the identity of γ-secretase using photoreactive derivatives of a potent active-site-directed γ-secretase inhibitor. The proteins that are covalently modified by these photoactivated γ-secretase inhibitors are PS1 and PS2.

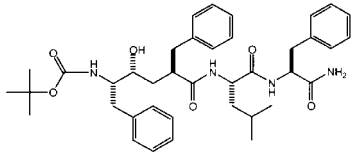
METHODS

The methods are described in detail in Li et al. (2000b).

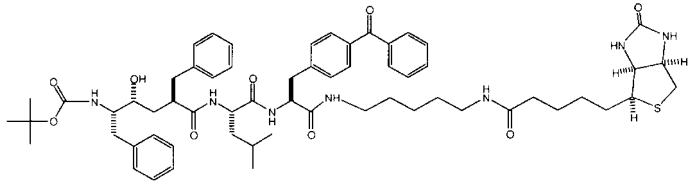
RESULTS

L-685,458 (Figure 73.1) is a potent γ-secretase inhibitor (Shearman et al., 2000) that contains a hydroxyethylene dipeptide isostere which should serve as a transition state analog targeting the inhibitor to the active site of an aspartyl protease (Rich, 1986; Wolfenden, 1972). L-685,458 was shown to display an IC_{50} value of approximately 0.3 nM when tested with a novel *in vitro* γ-secretase assay that uses a recombinant APP-related substrate, C100Flag and solubilized γ-secretase (CHAPSO-solubilized HeLa cell membranes) (Li et al., 2000a). We propose that L-685,458 is an active-site-directed transition state analog inhibitor for an aspartyl protease since its inhibitory potency is sensitive to the configuration of the hydroxylic carbon atom and its peptidic analog is cleaved by γ-secretase and binds much more weakly to the enzyme (Li et al., 2000b). It was claimed elsewhere that γ-secretase is an aspartyl protease based on inhibition of cellular γ-secretase activity by other aspartyl protease transition state analogs (Wolfe et al., 1999b).

The benzophenone-containing compounds L-852,505, L-852,631, and L-852,646 (Figure 73.1) were synthesized in order to generate photoreactive derivatives of L-685,458 that are also directed to the active site of γ-secretase. The photoreactive benzophenone group was placed at the N- or C-terminus of the L-685,458 template structure (in L-852,505 or L-852,646, respectively) in an attempt to label different domains within the active site of γ-secretase. L-852,631 contained benzophenone groups at both of these positions. A biotin moiety was incorporated in each photoaffinity probe to facilitate the isolation and identification of the target proteins. L-852,505, L-852,631, and L-852,646 each display excellent inhibitory potency (IC_{50} $\sim$1 nM) toward solubilized γ-secretase in the absence of photoactivation. These benzophenone-containing compounds were photoactivated in the presence of solubilized γ-secretase to covalently label the active site of their aspartyl protease targets. After photoactivation, the reaction mixtures were



L-685,458



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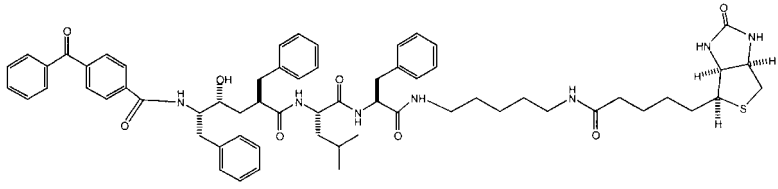

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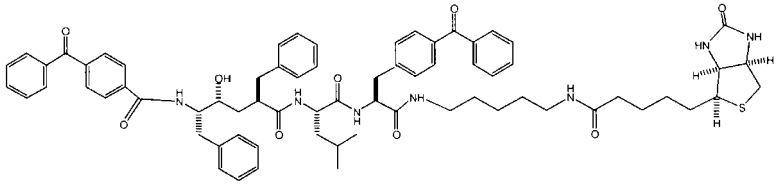

L-852,631

Figure 73.1. Chemical structures of the γ-secretase inhibitor L-685,458 and its photoreactive/biotinylated derivatives, L-852,505, L-852,646, and L-852,631

treated with RIPA buffer (50 mM Tris HCl, pH 8.0, 150 mM NaCl, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) to increase the accessibility of the biotin group in the photolabeled proteins to streptavidin. The photolabeled proteins which were then decorated with biotin were isolated from the reaction mixture by absorption on streptavidin–agarose beads,

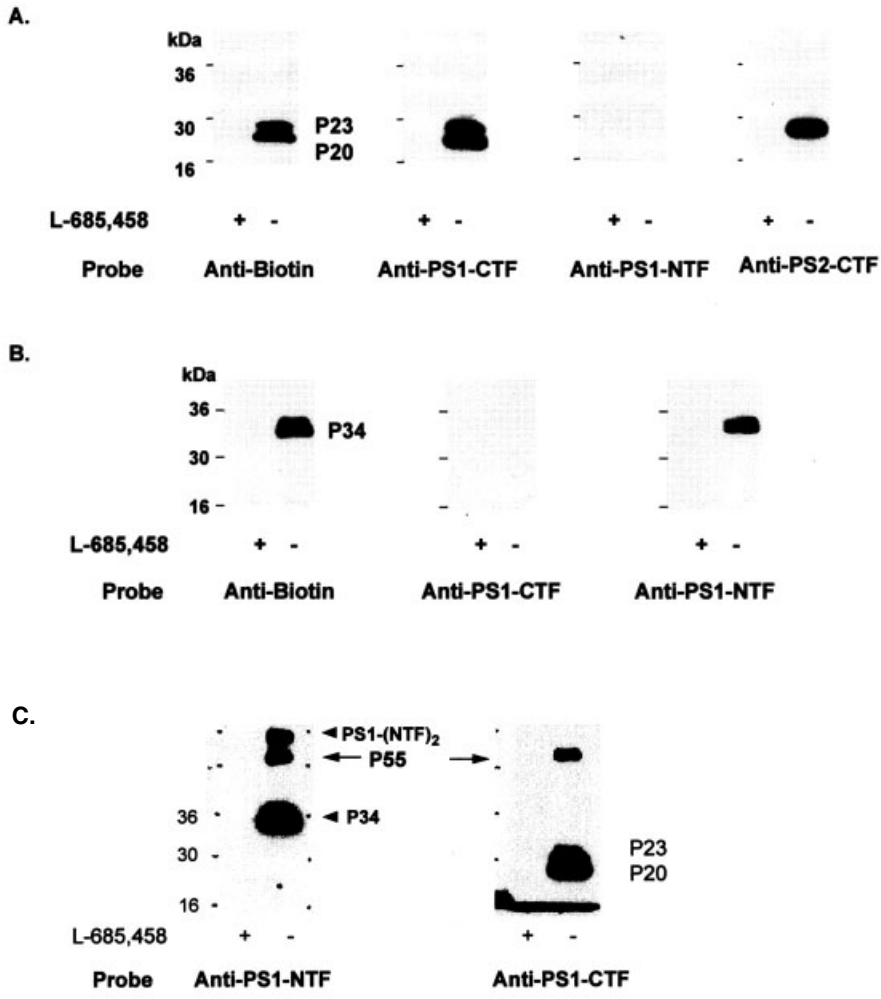

Figure 73.2. Covalent labeling of PS1 by photoreactive active-site-directed γ-secretase inhibitors. L-852,505, L-852,646 or L-852,631 (part A, B or C, respectively) was photoactivated with CHAPSO-solubilized HeLa cell membranes in the absence or presence of L-685,458. The sample was diluted with RIPA buffer. Biotinylated proteins were captured with streptavidin–agarose and probed by SDS-PAGE/immunoblotting using anti-biotin antibody (anti-biotin), antibody vs. the C-terminus of PS1-CTF (anti-PS1-CTF), antibody vs. the N-terminus of PS1-NTF (anti-PS1-NTF) or antibody vs. the C-terminus of PS2-CTF (anti-PS2-CTF). The mobilities of the P20, P23, P34, and P55 species as well as molecular weight standards are indicated. Also shown in part C is an additional species, PS1-$(NTF)_2$, that is tentatively assigned as a PS1-NTF dimer that is not disrupted during SDS-PAGE. (Parts A and B reproduced by permission from Li et al. *Nature*, **405**: 689–94. ' 2000 Macmillan Magazines Ltd)

eluted from the beads, subjected to SDS-PAGE and visualized by immunoblotting using an anti-biotin antibody.

Photoactivation of L-852,505 in the presence of solubilized γ-secretase resulted in the generation of two major biotinylated species, P20 and P23, that displayed apparent relative molecular weights (M_r) of 20 000 and 23 000, respectively (Figure 73.2A). The appearance of both P20 and P23 are blocked when the photoactivation is performed in the presence of L-685,458 (Figure 73.2A). Similar results are obtained when L-852,505 is photoactivated in the presence of intact HeLa cell membranes (data not shown). Photoactivation of L-852,646 in the presence of solubilized γ-secretase results in the covalent labeling of a species (P34) with an apparent M_r of 34 000 (Figure 73.2B). The appearance of P34 is blocked when the photoactivation is performed in the presence of L-685,458 (Figure 73.2B). Treatment of solubilized γ-secretase with L-852,505 or L-852,646 without photoactivation failed to generate any covalently labeled biotinylated species.

The accumulating evidence that γ-secretase and PS1 are closely linked (De Strooper et al., 1998; Li et al., 2000a; Wolfe et al., 1999a) coupled with the striking similarity between the M_rs of the biotinylated species and the PS1 fragments (PS1 is proteolytically cleaved to yield a non-covalent heterodimer comprised of an ~30 kDa N-terminal fragment (PS1-NTF) and an ~20 kDa C-terminal fragment (PS1-CTF) (Thinakaran et al., 1996)) prompted a test of whether PS1 is the target for these photoreactive γ-secretase inhibitors. The biotinylated species in the solubilized γ-secretase preparations treated with L-852,505 or L-852,646 were evaluated by immunoblot analysis using antibodies against PS1. The biotinylated species in L-852,505-treated solubilized γ-secretase, P20 and P23, both reacted with the antibody against the C-terminus of PS1-CTF (Figure 73.2A). The appearance of the immunoreactive PS1-CTF species is blocked when photoactivation of L-852,505 is performed in the presence of the non-photoreactive derivative, L-685,458 (Figure 73.2A). The PS1-NTF fragment is not observed when L-852,505-treated, biotinylated proteins are probed with the anti-PS1-NTF antibody. The biotinylated P34 species in L-852,646-treated solubilized γ-secretase reacted with antibody vs. the PS1-NTF subunit (Figure 73.2B). Formation of this immunoreactive species is abolished when photoactivation with L-852,646 is performed in the presence of L-685,458 (Figure 73.2B). No immunoreactive bands are observed when the L-852,646-treated, biotinylated proteins are probed with antibody vs. the PS1-CTF subunit. We conclude that photoactivated L-852,505 and L-852,646 covalently label PS1-CTF and PS1-NTF, respectively. The P23 species is most likely a phosphorylated form of PS1-CTF (Seeger et al., 1997). Dissociation of the PS1 heterodimer by the detergent-containing RIPA buffer, as previously shown with Triton X-100 (Cappell et al., 1998), probably accounts for our inability to capture and visualize both PS1-CTF and PS1-NTF when one (but not both) subunit is covalently labeled with a biotinylated photoaffinity probe.

Treatment of solubilized γ-secretase with L-852,631 resulted in the generation of a set of biotinylated proteins including P34, which reacted with antibody vs. PS1-NTF, and both P20 and P23, which reacted with the antibody vs. PS1-CTF (Figure 73.2C). This result shows that L-852,631, the 'double warhead' compound, displays the binding and labeling characteristics exhibited by each of the 'single warhead' photoreactive γ-secretase inhibitors. Remarkably, another biotinylated species, P55, was evident that displayed M_r 55 000 and reacted with PS1 antibodies vs. both the CTF and NTF fragments. The mobility of P55 coupled with the coexistence of epitopes for both PS1 fragments indicates that photoactivated L-852,631 crosslinks the PS1 fragments.

The only biotinylated species that are generated by the photoactivated γ-secretase inhibitors were P20, P23 or P34; hence, we conclude that only PS1 or proteins co-migrating with the PS1 fragments are labeled with the photoaffinity probes. The growing evidence that PS2 also displays γ-secretase activity (Zhang et al., 2000; Herreman et al., 2000) prompted us to test whether the photoaffinity probes also bind to PS2. We showed with an antibody vs. the C-terminus of PS2-CTF that photoactivation of L-852,505 in the presence of solubilized γ-secretase also leads to covalent labeling of PS2-CTF (Figure 73.2A). The covalent labeling of PS2-CTF by photoactivated L-852,505 was blocked in the presence of L-685,458, the non-photoreactive derivative. These observations demonstrate that PS2, like PS1, contains the active site of a γ-secretase.

We aimed to determine whether intact, single-chain PS1 also served as a target protein for the photoreactive γ-secretase inhibitors. There are vanishing low amounts of full-length PS1 in the solubilized γ-secretase preparation from HeLa cells. However, heterologous expression of PS1 in mammalian cells leads to accumulation of unprocessed PS1 (Thinakaran et al., 1996). We thus prepared solubilized γ-secretase from murine N2a neuroblastoma cells that express human PS1 (N2a/PS1 solubilized γ-secretase). Immunoblot analysis of N2a/PS1 solubilized γ-secretase using anti-PS1-NTF and anti-PS1-CTF antibodies confirmed that both the intact and heterodimeric forms of PS1 were present (Figure 73.3A). Treatment of N2a/PS1 solubilized γ-secretase with photoactivated L-852,505 yields a biotinylated protein that co-migrates with PS1-CTF and reacts with anti-PS1-CTF antibody. The appearance of this biotinylated protein is blocked when the photoactivation is performed in the presence of excess L-685,458. Full-length PS1, which is clearly evident in the immunoblot of N2a/PS1 solubilized γ-secretase, is not labeled by photoactivated L-852,505. L-852,505 is a transition state analog; hence, it is expected to bind selectively to active forms of γ-secretase. Thus, the most parsimonious explanation of why L-852,505 does not covalently label intact (wild-type) PS1 is that this form of PS1 is a zymogen.

We also explored the binding of L-852,505 to a PS1 variant, PS1ΔE9, which is associated with early-onset familial AD (Perez-Tur et al., 1995).

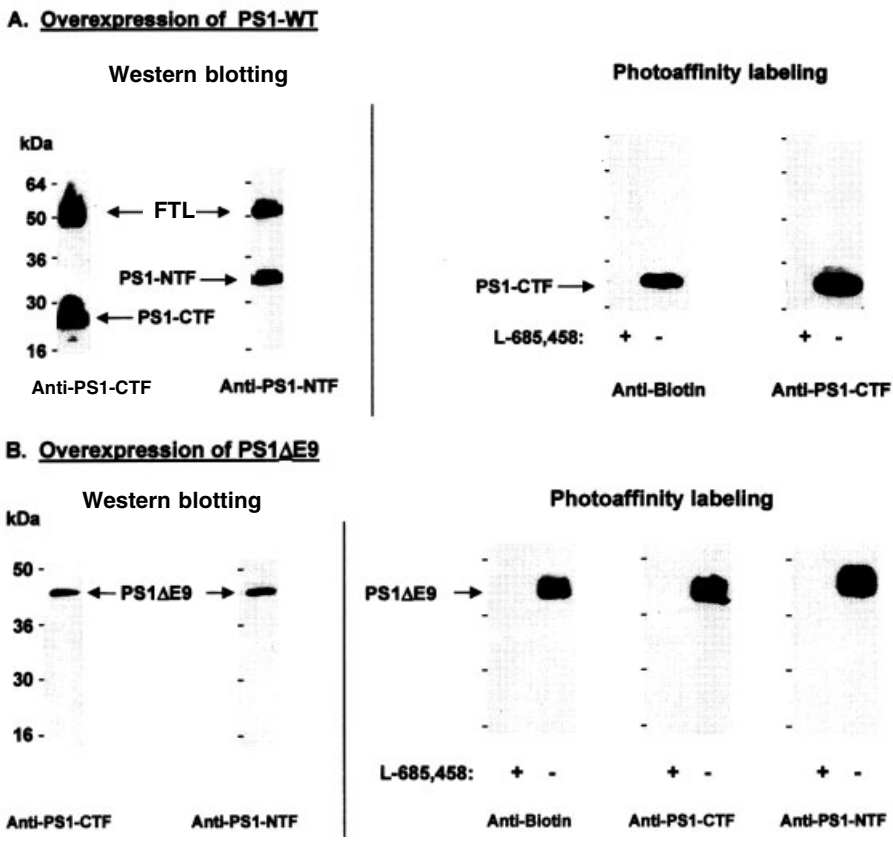

Figure 73.3. Photoactivated γ-secretase inhibitor does not bind covalently to intact wild-type PS1 but reacts with the PS1-FAD ('familial Alzheimer's disease') Δexon 9 mutant. Membrane extracts were prepared from (A) N2a neuroblastoma cells stably transfected with PS1 and (B) N2a neuroblastoma cells stably transfected with the PS1-FAD Δexon 9 mutant. The membrane extracts were analyzed by SDS-PAGE/immunoblotting using anti-PS1-NTF and anti-PS1-CTF antibodies. L-852,505 was added to the membrane extracts (in the absence or presence of excess L-685,458) and subjected to photoactivation. The ensuing biotinylated proteins were diluted with RIPA buffer, captured with immobilized streptavidin and analyzed by SDS-PAGE/immunoblotting using anti-biotin, anti-PS1-CTF, and anti-PS1-NTF antibodies. The mobilities of intact wild-type PS1 (FL), PS1-NTF, PS1-CTF and PS1-FAD Δexon 9 (PS1ΔE9) as well as molecular weight standards are shown. (Parts A and B reproduced by permission from Li et al. *Nature*, **405**: 689–94. '2000 Macmillan Magazines Ltd)

PS1ΔE9 lacks the purported cytosolic loop encoded by exon 9 which contains the endoproteolytic cleavage site in PS1 (which gives rise to PS1-NTF and PS1-CTF); hence, PS1ΔE9 is a stable single-chain derivative (M_r ~43 000). Immunoblot analysis of solubilized γ-secretase prepared from N2a cells that overexpress PS1ΔE9 (N2a/PS1ΔE9 solubilized γ-secretase) with anti-PS1-

NTF and anti-PS1-CTF antibodies confirmed the presence of PS1ΔE9 (Figure 73.3B). The absence of PS1 immunoreactivity at the positions expected for PS1-NTF or PS1-CTF reflects the so-called 'replacement' phenomenon in which overexpression of human PS1ΔE9 blocks accumulation of the murine PS1/PS2 heterodimers (Thinakaran et al., 1997). Treatment of N2a/PS1ΔE9 solubilized γ-secretase with photoactivated L-852,505 yields a covalently labeled biotinylated protein that co-migrates with PS1ΔE9 and reacts with both the anti-PS1-NTF and anti-PS1-CTF antibodies (Figure 73.3B). This biotinylated protein is not observed when the photoactivation is performed in the presence of excess L-685,458. These results suggest that PS1ΔE9 also binds a transition-state analog aspartyl protease inhibitor and is thus catalytically competent. Consistent with this view, N2a/PS1ΔE9 solubilized γ-secretase displays activity in the *in vitro* γ-secretase assay and L-685,458 blocks this activity with a potency similar to that observed toward solubilized γ-secretase from HeLa cells (data not shown). The fact that single-chain PS1ΔE9 exhibits catalytic activity, whereas intact single-chain wild-type PS1 is inactive (as deduced from its failure to bind the photoaffinity probes) suggests that the putative conformational change triggered by endoproteolytic cleavage of intact wild-type PS1 may be mimicked by loss of the cytosolic loop in PS1ΔE9.

Our studies with N2a/PS1ΔE9 solubilized γ-secretase have also provided important insight regarding the selectivity of the L-852,505 photoaffinity probe. Biotinylated species that comigrate with P20 or P23 are not observed after treatment of N2a/PS1ΔE9 solubilized γ-secretase with photoactivated L-852,505 (Figure 73.3B). This result suggests that treatment of wild-type solubilized γ-secretase with photoactivated L-852,505 results in labeling of PS1-CTF and PS2-CTF only but not an additional co-migrating protein—

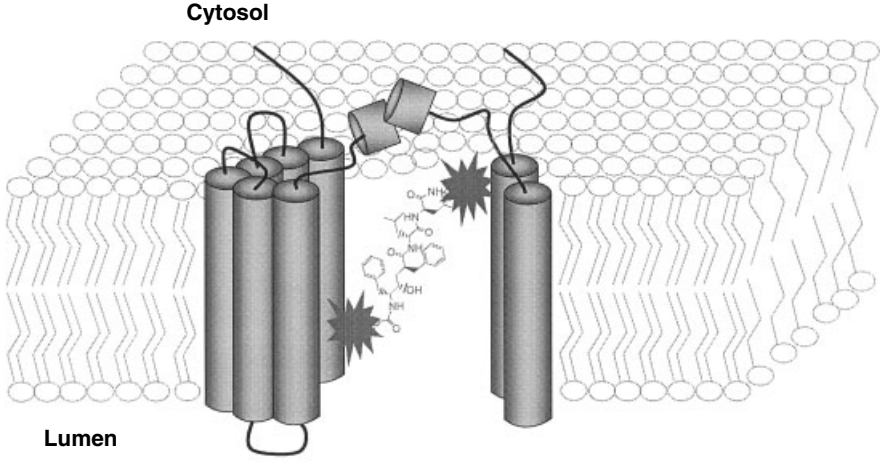

Figure 73.4. Schematic representation showing the docking of the photoreactive derivatives of L-685,458 to the PS1 heterodimer, which is situated in the lipid bilayer

assuming that overexpression of PS1ΔE9 only suppresses (via the 'replacement' phenomenon) the appearance of the PS1 and PS2 fragments.

CONCLUSION

The binding of the photoreactive active-site-directed γ-secretase inhibitors to PS1 and PS2 (shown schematically in Figure 73.4) provides compelling biochemical evidence that the presenilins are proteases responsible for γ-secretase activity. A key presumption in this study is that the activated photoreactive probes bind to and covalently label γ-secretase rather than bind to γ-secretase and label a contiguous protein. The validity of this presumption is supported by the finding that two different photoaffinity analogs, L-852,505 and L-852,646, both covalently label the same protein—PS1. The binding of L-852,505 and L-852,646 to dissimilar PS1 fragments indicates that the active site of γ-secretase may be shared between the two PS1 subunits. The crosslinking of the PS1 fragments by photoactivated L-852,631, which possesses both benzophenone groups, is concordant with this hypothesis. Moreover, the active site of PS1 was previously predicted to be shared between the fragments on the basis of a PS1 mutagenesis study, suggesting that the putative active-site aspartates in PS1 are situated on dissimilar subunits (Wolfe et al., 1999a). It is important to note that whereas the presenilins appear to contain the active site of γ-secretase, it does not exclude involvement of other protein co-factors as determinants of γ-secretase activity. Our recent observations suggesting that γ-secretase activity is catalyzed by a PS1-containing macromolecular complex is consistent with the existence of such putative co-factors (Li et al., 2000).

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74 Functional Analysis of β-Secretase Using Mutagenesis and Structural Homology Modeling

GWEN TATSUNO, JOHN ANDERSON, JIN HONG, DAVID A. AGARD, NOBUYUKI OTA, SUKANTO SINHA, GURIQBAL BASI AND LISA McCONLOGUE

INTRODUCTION

Beta-amyloid (Aβ) peptide, the major component of Alzheimer's disease (AD) plaques, is generated by proteolytic cleavage of the amyloid precursor protein by β- and γ-secretases. The recently identified β-secretase and its close homologue BACE2 (Vassar et al., 1999) form a new subclass of aspartic acid proteases, containing an ectodomain showing a high degree of homology to the pepsin family of proteases. Very little is known about this new subfamily. It displays distinct features from the pepsin family in that it contains intracellular and transmembrane domains, inserted sequences within the pepsin homology domain, and cysteines in the ectodomain that do not share homology with other pepsin family proteases. We have used a combination of mutagenesis and structure homology modeling to investigate pro-protease processing and the role of disulfide bonds in β-secretase.

RESULTS AND DISCUSSION

PROCESSING OF β-SECRETASE FROM THE PRO-ENZYME TO THE MATURE ENZYME

There are two catalytic aspartic acid residues in all known aspartyl proteases. β-secretase contains homologous aspartic acid residues (Asp 93 and Asp 289; the numbering for all residues begins with the initiating methionine of the 501 amino acid protein) which have been shown to be critical for β-secretase

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activity in a whole-cell assay (Hussain et al., 1999). These active-site aspartates were individually mutagenized to alanines, expressed in HEK293T cells, and assayed for *in vitro* β-secretase activity using a highly sensitive enzymatic assay on an extended substrate (Sinha et al., 1999). No activity was seen, as would be expected if these were the critical catalytic residues. We next looked at protein production and the processing of the pro-enzyme to form mature β-secretase in these mutants and compared it to that of the wild-type enzyme. If β-secretase processes its own pro-region, overexpressed protein should be less efficiently processed in the mutant. Wild-type enzyme and active-site aspartate mutants were overexpressed in HEK293T (Edge Biosystems, SV40 large T antigen transfected HEK293) cells by transient transfection. One of the mutant forms, Asp 289, bound specifically to a previously described affinity column, allowing purification by a combination of cation exchange flow-through and affinity chromatography, as was used to purify wild-type β-secretase (Sinha et al., 1999). In contrast, the other mutant did not bind to the affinity column. Its purification was accomplished by cation exchange flow-through, anion exchange chromatography, and SDS polyacrylamide gel electrophoresis (PAGE). For both mutants, protein production levels were within three-fold of normal, indicating that the mutations did not prevent protein folding (data not shown). Homogeneity of purified protein was assessed by SDS-PAGE followed by Coomassie and silver staining. The protein was subjected to N-terminal sequencing and amounts of the different sequences found were determined by averaging yields of 10 cycles of Edmund degradation.

As reported previously (Sinha et al., 1999), all of the purified β-secretase from brain initiates at the beginning of the mature protease (ETDEEP). Wild-type and active-site Asp mutants both show equivalent amounts of pro-region processing: ~80% of total enzyme processed to maturity, with ~20% remaining as pro-protease (Figure 74.1), despite the fact that the inactive β-secretase is overexpressed 700-fold beyond endogenous levels in 293HEKT cells (data not shown). This strongly suggests that β-secretase does not process itself.

The processing site of β-secretase contains a motif, RXXR, that is typical of a furin family processing protease. To test this specificity, the arginine in the P1 position (Arg45) was mutated to proline, which should block cleavage. As before, expressed protein was purified by anion and affinity chromatography, and subjected to N-terminal sequencing. Although Western analyses showed an increase in the amount of pro-region containing β-secretase in the mutant enzyme (Figure 74.2A), sequencing of the purified protein showed that, surprisingly, the majority of the protein is still processed to remove its pro-region (Figure 74.2B). However, from the sequencing data, it was clear that this mutation fully blocked processing at the normal site and shifted the cleavage site of the most abundant product three amino acids upstream (Figure 74.2B). While this cleavage site contains one arginine at P1, it does

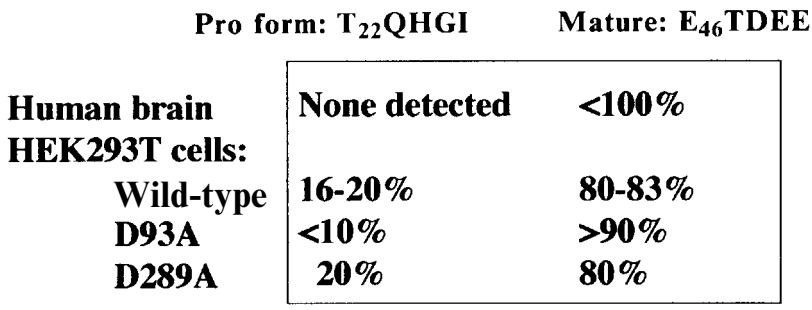

Figure 74.1. Processing of β-secretase containing active-site catalytic residues. cDNAs encoding wild-type and mutant forms of β-secretase were inserted into a mammalian expression vector containing the CMV promoter to direct expression and SV40 large T antigen to drive plasmid replication. These expression cassettes were transfected into human HEK293T cells using fugene reagent (Boehringer Mannheim). Cells pellets were harvested 48 hours post-transfection, and β-secretase forms were purified to homogeneity. The D93A mutant was purified by cation exchange flow-through, anion exchange chromatography, and SDS-PAGE. The D289A mutant was purified by cation exchange flow-through and affinity chromatography (Sinha et al., 1999). Homogeneity of the purified proteins was determined by SDS-PAGE followed by Coomassie and/or silver staining. The N-terminal sequences of the purified proteins as determined by Edmund degradation are shown

not have the arginines at P2 or P4 that are typical of furin family protease cleavage sites. This suggests that either an unusual protease is responsible for this processing, or that multiple enzymes are capable of removing the pro-region from β-secretase.

FUNCTION OF ECTODOMAIN CYSTEINE RESIDUES

Unlike the majority of pepsin family proteases, which have six conserved cysteines involved in three disulfide bonds, the ectodomain of β-secretase has only three of those conserved cysteines (Cys278, Cys330, and Cys380; Figures 74.3 and 74.4) and it also contains a cysteine in the ectodomain C-terminal to the region of aspartyl acid protease homology (Figure 74.3, Cys443). There are two novel cysteines in the aspartyl acid homology domain (Cys 216, Cys 420, Figures 74.3 and 74.4), yielding a total of six cysteines in the ectodomain of β-secretase. Through careful examination of the functional consequences of mutagenesis of cysteine residues, it may be possible to determine disulfide boding patterns (Zingler et al., 1995). To probe the role that these individual cysteines play in the folding and function of β-secretase, each of the six single Cys→Ala mutants were made, transfected into HEK293T cells, and tested for expression levels and enzymatic activity. Western analyses (Figure 74.5) with pro-region- or C-terminal region-specific

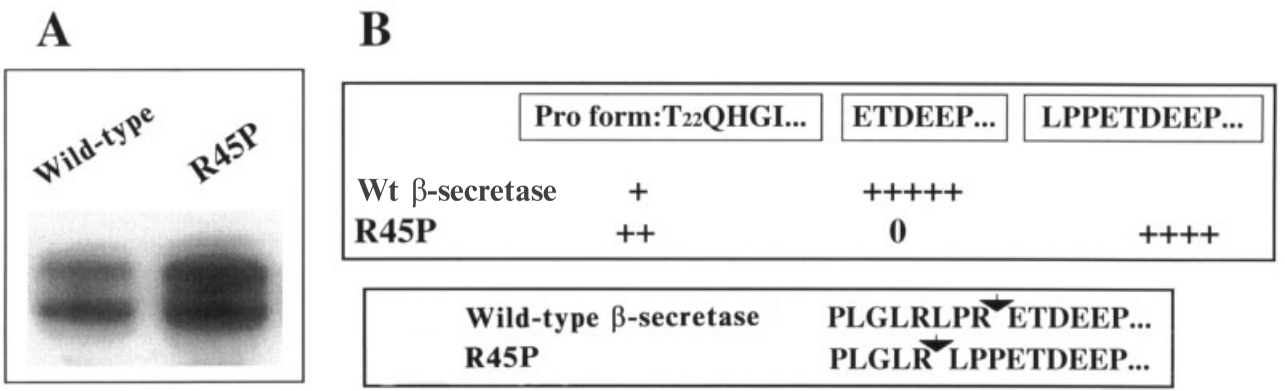

Figure 74.2. Processing of β-secretase containing a pro-region mutant. (A) Wild-type and R45P mutant forms of β-secretase were expressed as described in Figure 74.1 and Western analysis performed on crude cell lysates using a pro-region-specific antibody (mouse monoclonal antibody 9H10, generated to residues 21–33 of β-secretase). (B) Wild-type and R45P mutant protein were purified and sequenced as described for Figure 74.1

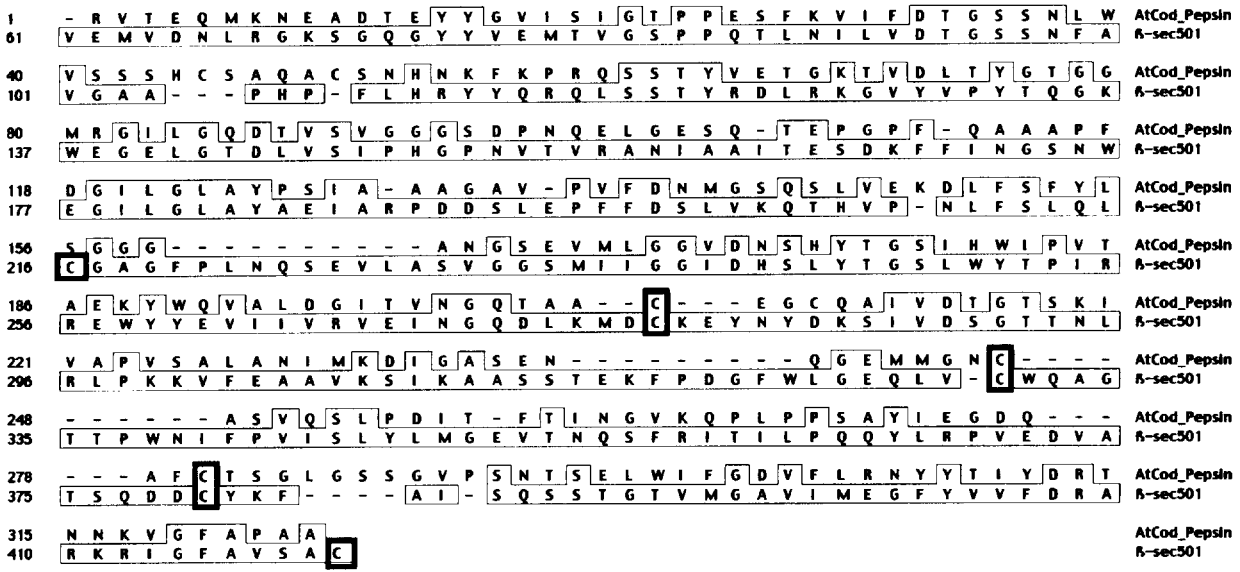

Figure 74.3. Alignment of the aspartyl protease domain of β-secretase sequence to Atlantic cod pepsin. The alignment generated by the LOOK software package (Molecular Dynamics) was minimally adjusted to align Cys330 and Cys380 to the conserved cysteines in other pepsin family members. Other alignment algorithms (DNAstar, Megalign software package) with select aspartyl family members will align these residues without adjustment. β-Secretase numbering begins at the initiating methionine of the 501 amino acid protein. Atlantic cod pepsin numbering starts at the N-terminus of the mature protease used for generating the crystal structure in the pdb database (pdb|1AM5|). The Cys residues present in the aspartyl protease homology domain of β-secretase are boxed in heavy lines. The residues showing identity to β-secretase are boxed

antibodies showed that although the overall expression of the Cys→Ala mutant forms is lower than that of wild-type enzyme, the expression levels of all six mutants are equivalent to one another. Furthermore, although the pro-region processing efficiency is reduced in all of the cysteine mutants compared to wild-type, processing efficiency is also comparable among all six mutants. Therefore, any activity differences observed between these mutant forms is not due to differential expression or pro-region processing but rather due to folding and/or structural alterations. The enzymatic activity of the expressed β-secretase was tested in crude cellular lysates as described above, and normalized to the protein levels as measured by Western analysis. All six cysteine residues are required for full β-secretase activity (Figure 74.5). Cys330 and Cys380, a pair homologous to cysteines involved in a highly conserved and critical disulfide bond in the pepsin family members (see below), are each absolutely required for activity. Thus, this pair forms a critical disulfide bond similar to that in other pepsin family members. The only remaining conserved cysteine, Cys278, has lost its normal binding partner (Figure 74.3) and therefore must be bound to one of the novel cysteine residues in β-secretase. Mutating Cys278, Cys216, and Cys420 resulted in intermediate levels of activity consistent with involvement of two of these in a disulfide bond and leaving the third to be bonded to Cys443. Surprisingly, Cys443 is more critical for enzymatic activity than any of its possible disulfide bond partners (Cys216, Cys278, and Cys420). This suggests that Cys443 may play a role in enzyme function in addition to its role in forming the disulfide bond.

HOMOLOGY MODELING OF β-SECRETASE STRUCTURE ELUCIDATES THE ROLE OF DISULFIDE BONDS IN ANCHORING AN EXTENDED SUBSTRATE BINDING POCKET

To further determine the assignment of individual cysteines in disulfide bonds and look at the role of disulfide bonds in β-secretase, a predicted three-dimensional structure of the protease was generated by homology modeling. The pepsin-like aspartic acid protease family members show both homology of primary sequence and remarkable structural similarities. We therefore generated a homology-modeled structure for β-secretase using LOOK software (Molecular Applications Group) based on the structure of its closest homologue in the PDB database, Atlantic cod pepsin (pdb|1AM5|). This algorithm determines the initial positions of the alpha carbons based on the position of Atlantic cod pepsin, and further refines the structure based on other proteins in the PDB database showing higher local homology in a segmental piecewise manner, followed by energy minimization. Proper sequence alignment to Atlantic cod pepsin is crucial to accuracy of the final predicted structure, and the alignment used is shown in Figure 74.3. The

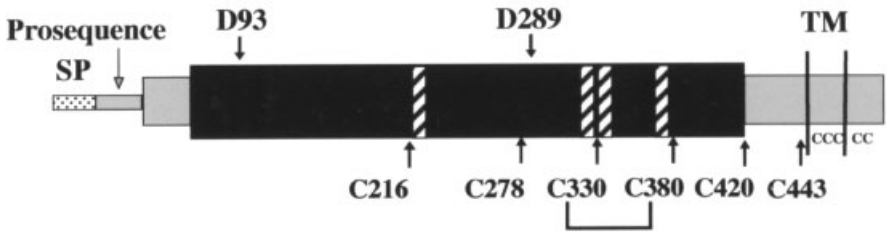

Figure 74.4. Schematic of primary structure of β-secretase. The structural domains of β-secretase are shown as separate rectangles. The aspartic acid homology domain is colored black. Cysteine residues are indicated by 'C', with the position of the ectodomain cysteine residues indicated. The two active-site aspartates are indicated by 'D', again with the position indicated. The conserved disulfide bond predicted between Cys330 and Cys380 is indicated. Hatched regions indicate the inserted sequences in the aspartyl protease domain of β-secretase showing no homology to pepsin family members

critical feature of this alignment is the matching of Cys330 and Cys380 with the conserved Cys247 and Cys280, respectively, of the pepsin family. Although not all alignment algorithms will show this pairing, we believe this is correct for several reasons. It aligns a pair of cysteines (380 and 330 of β-secretase), which are crucial for β-secretase activity (see above), to a pair of cysteines (C247 and C280 of pepsin) involved in a conserved disulfide bond crucial for activity in the pepsin family members. Furthermore, this pairing of Cys330 and Cys380 in a disulfide bond has subsequently been validated by mass spectrometry analyses (Haniu et al., 2000).

When the structure is modeled using such an alignment, an interesting feature is apparent. Adjacent to Cys216, Cys330, and Cys380 are inserted sequences showing no homology to the pepsin family members (Figure 74.4). Since there is no homology to known structures for these inserts, they are visualized as loops in the modeled structure. In the modeled structure these inserted sequences form loops extending from and encircling the end of the active-site cleft. These loops are placed on the side of the active-site cleft that binds the N-terminal end of the substrate (Figure 74.6; see Plate IV), indicating that β-secretase contains an unusual extended substrate binding pocket.

This extended domain is tethered by the conserved disulfide bond between Cys330 and Cys380, and also by a novel disulfide bond predicted by this homology modeled structure between Cys216 and Cys 420. The intimate association of these disulfide bonds with the extended active site is consistent with the crucial role for enzyme folding and activity demonstrated by the mutagenesis studies above. The conserved Cys278 that has lost its normal binding partner must be bound to the only remaining cysteine in the ectodomain, Cys443, since both Cys are required for full enzymatic activity. Since the region of homology to pepsin family members extends only to

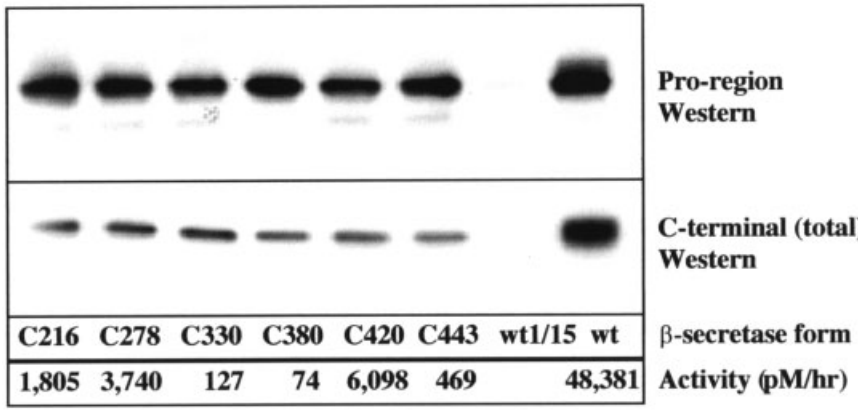

Figure 74.5. Activity of Cys mutations in the ectodomain of β-secretase. Each of the indicated ectodomain cysteines was individually mutated to Ala and expressed in HEK293T cells as described in Figure 74.1. Cell lysates were subjected to Western analysis using antibodies specific to the pro-region (mouse monoclonal antibody 9H10, generated against peptides from positions 21–33 of β-secretase) or to the C-terminus (mouse monoclonal antibody 38.20.13, generated against peptides from positions 489–501 of β-secretase) of β-secretase. The activity of each form was determined in the crude cell lysates as described (Sinha et al., 1999)

position 419, the structure of the region from 420 to Cys443 could not be accurately predicted. However, given the length of this region it could certainly wrap around the back of the molecule, opposite to the active site, forming a disulfide bond between Cys443 and Cys278. This suggests a role for this disulfide bond in pinning together the entire molecule and is consistent with the fact that these cysteines are both required for properly folded and functional enzyme.

SUMMARY

Mutations of the catalytic aspartic acids result in mutant β-secretases which are processed efficiently to the mature protease, indicating that β-secretase does not process itself from pro- to mature forms. Western analyses of the processing of the R45P mutant were consistent with similar analyses of an R45A mutant β-secretase (Capell et al., 2000). However, our additional sequencing analyses indicate that the pro-form is still a minor portion of the total protein. Thus in the R45P mutant form of β-secretase, pro-region processing is still highly efficient but is altered so that cleavage takes place after R43.

All of the ectodomain cysteines are required for full β-secretase activity, indicating that they all play a critical role in disulfide bonding. Interestingly, Cys443 is even more crucial for β-secretase activity than its disulfide bonded partner, suggesting that it plays an additional functional role, perhaps in mediating folding intermediates. Homology modeling and activity data of the six Cys→Ala mutations suggests disulfide bonding of C330–C380, C278–C443, C216–C420. This bonding has recently been validated by mass spectroscopic analyses (Haniu et al., 2000).

The homology modeled structure most strikingly shows that inserted sequences in β-secretase form an extended substrate-binding pocket on the side of the active-site cleft that binds the N-terminus of the substrate. The newly identified close homologue of β-secretase, BACE2 (Vassar et al., 1999) also known as Asp1 (Hussain et al, 1999; Yan et al., 1999), shows similar insertions and an analogous extended substrate-binding pocket in a homology modeled structure (data not shown). This is a highly unusual feature of this subfamily, since pepsin-type aspartic acid proteases usually have smaller substrate-binding domains. The model further suggests that the disulfide bonds in β-secretase play a critical role in anchoring, positioning, and integrity of this extended binding domain.

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75 Therapeutic Approaches to Prion Diseases: *In Vitro* Studies with Tetracycline Compounds

TAZEEN AWAN, GIANLUIGI FORLONI, ENZIO RAGG, SELINA IUSSICH, GIACOMINA ROSSI, LAURA COLOMBO, LAURA GIROLA, TANIA MASSIGNAN, ORSO BUGIANI, MARIO SALMONA AND FABRIZIO TAGLIAVINI

INTRODUCTION

Prion diseases are a group of fatal neurodegenerative disorders of humans and animals which may be sporadic or inherited in origin and can be transmitted. In animals these diseases include scrapie of sheep and spongiform encephalopathy of cattle (BSE), while in humans the most frequent form is Creutzfeldt–Jakob disease (CJD). A common characteristic of these diseases is the conversion of the normal cellular prion protein (PrP^{C}) into a disease-specific isoform, termed PrP^{Sc} (Prusiner, 1991). This process involves a profound conformational change of PrP^{C} resulting in decreased α-helical structure and striking increase in β-sheet content (Caughey et al., 1991; Pan et al., 1993; Safar et al., 1993). Such conformational change is accompanied by the acquisition of abnormal chemicophysical properties, including insolubility in non-denaturing detergents, partial resistance to protease digestion, and a high tendency to form aggregates and amyloid fibrils (Prusiner et al., 1998). Amyloid formation occurs to the highest degree in Gerstmann-Sträussler–Scheinker (GSS) disease. Evidence indicates that interaction between the neoformed PrP^{Sc} and PrP^{C} causes the normal prion protein to convert into the abnormal isoform (Kocisko et al., 1994; Bessen et al., 1995). This conversion could therefore be considered the key event in the pathophysiology of prion diseases and be used to design therapeutic

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strategies. One such strategy could be to interfere with the PrP^{Sc} and PrP^{C} interaction, thus hindering the conversion process, or bind to PrP^{Sc} destabilizing its structure. Although several compounds have been studied, to date no suitable pharmacological therapy is available. Here we identify a group of compounds that take advantage of destabilizing the PrP^{Sc} structure.

ANTI-PRION AGENTS

Compounds that have been found to possess an antagonizing effect on prion propagation *in vitro* and/or *in vivo* include sulfated polyanions (Kimberlin and Walker, 1983, 1986; Ehlers and Diringer, 1984; Farquhar and Dickinson, 1986; Caughey and Raymond, 1993), amphotericin B (Pocchiari et al., 1987; Xi et al., 1992; Adnjou et al., 1995), Congo red (Caughey and Race, 1992; Ingrosso et al., 1995), iododoxorubicin (Tagliavini et al., 1997), tetrapyrroles (Caughey et al., 1998; Priola et al., 2000), branched polyamines (Supattapone et al., 1999), and modified PrP peptides (Soto et al., 2000). These molecules are structurally heterogeneous and their mechanism of action is largely unknown. However, some have common structural features in that they contain an extended hydrophobic core formed by aromatic moieties. It is conceivable that this characteristic allows a strong interaction with PrP^{Sc}, since PrP^{C} to PrP^{Sc} conversion is accompanied by an increase in hydrophobicity resulting from solvent exposure of lipophilic residues. This interaction has already been demonstrated for Congo red and iododoxorubicin, which bind to amyloid fibrils of different biochemical composition, including PrP amyloid (Prusiner et al., 1983; Merlini et al., 1995; Tagliavini et al., 1997).

TETRACYCLINE COMPOUNDS

Based on structural analogies with both Congo red and iododoxorubicin, we hypothesized that tetracyclines might be able to interact with PrP^{Sc} and PrP amyloid. These compounds have several advantages such as relatively low toxicity and availability of many congeners with well-characterized pharmacological and pharmacokinetic properties, some of which are able to cross the blood–brain barrier (Sande and Mandell, 1991). To verify possible tetracycline–PrP interactions and their effects, we used synthetic peptides homologous to residues 82–146 and 106–126 of human PrP. The former corresponds to the amyloid protein purified from GSS brain, while the latter is the major determinant of the physicochemical properties of PrP82–146. These peptides recapitulate central features of the protease-resistant core of PrP^{Sc} such as tendency to adopt β-sheet structure and aggregate into amyloid fibrils, partial resistance to proteinase K (PK) digestion, neurotoxicity, and ability to activate glial cells *in vitro*.

TETRACYCLINE PREVENTS AGGREGATION OF PrP PEPTIDES

We first determined the effects of tetracycline on PrP peptide aggregation. PrP82–146 and PrP106–126 solutions in deionized water (1 mM concentration) were incubated in the absence or presence of equimolar concentration of tetracycline for 1 and 4 h, respectively. Phosphate buffer, pH 7.0, was then added to the samples to a final concentration of 200 mM, and aggregation was determined by variation of the optical density of peptide suspension at 600 nm. The analysis showed that PrP106–126 and PrP82–146 progressively formed aggregates, which reached a plateau after 48 h incubation. Pre-incubation of samples with tetracycline almost abolished the aggregation ability of the peptides (Figure 75.1).

TETRACYCLINE REDUCES PROTEASE RESISTANCE OF PrP PEPTIDES

PrP peptide aggregation is accompanied by acquisition of protease resistance. To verify whether tetracycline affected this property, PrP peptides were incubated for 18 h in the absence or presence of the compound (peptide-to-drug

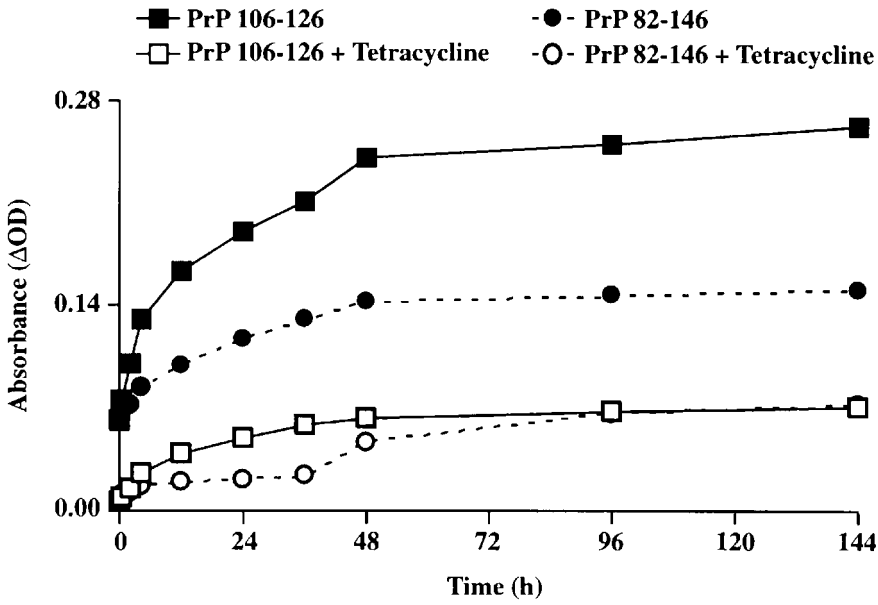

Figure 75.1. Time-course of PrP106–126 and PrP 82–146 aggregation in the absence or presence of tetracycline as determined by the variation of optical density of peptide suspensions

molar ratio ranging from 4:1 to 16:1) and then subjected to PK digestion (1:100 w/w enzyme-to-substrate ratio). After incubation at 37 °C for 10–30 min, the digestion was terminated and the samples were centrifuged at 13 000 **g** for 10 min. The pellets were dissolved in 10% formic acid containing 0.1% trifluoroacetic acid and analyzed by reverse-phase HPLC. The study showed that tetracycline caused a dose-dependent increase in protease sensitivity of PrP peptides (Figure 75.2). Similar results were obtained with PrP82–146 and PrP^{Sc} partially purified from CJD brains (Tagliavini et al., 2000).

TETRACYCLINE COMPOUNDS HAVE DIVERSE EFFECTS ON PROTEASE RESISTANCE OF PrP PEPTIDES

The family of tetracyclines consists of a large number of congeners that differ in their hydrophilic side chains. Since such differences could result in diverse physiochemical properties, we selected four compounds with substantially different side chains (tetracycline hydrochloride, metacycline, 4-epichlorotetracycline or anhydrotetracycline) to identify whether these differences influenced their ability to interact with the synthetic peptides. The peptides

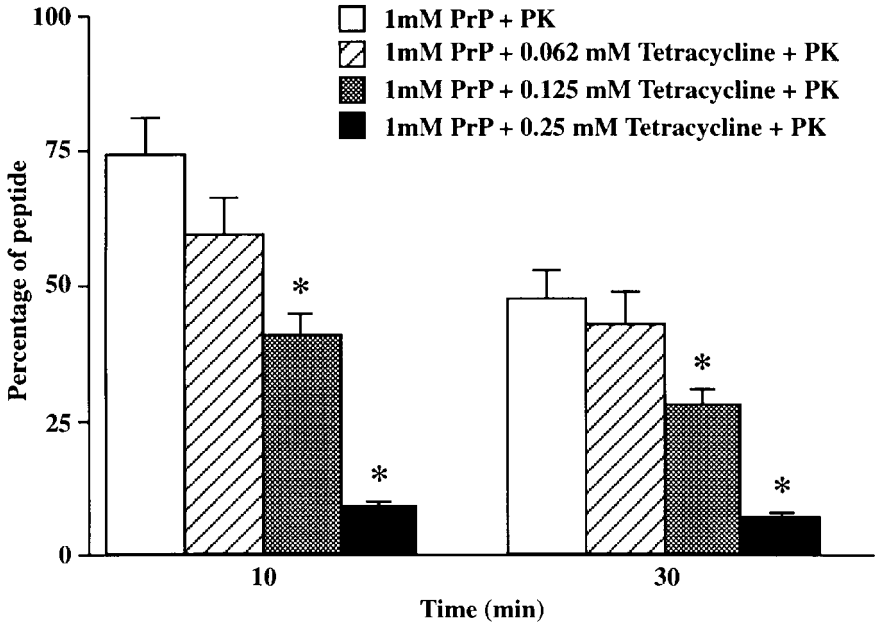

Figure 75.2. Dose–response decrease of protease resistance of PrP106–126 caused by tetracycline after a 10 or 30 min digestion with proteinase K. Data are the mean ±s.d. of five experiments. *p50.001 vs. control group (Student's *t* test)

were incubated alone or with the compounds at 8:1 molar ratio for 18 h at 37 °C. The samples were then subjected to PK digestion (1:100 w/w, enzyme-to-substrate ratio) for 10–30 min at 37 °C, and the extent of proteolysis analyzed as described above. This study demonstrated that the most active compound was tetracycline hydrochloride followed by 4-epichlorotetracycline, while the other two molecules failed to show a significant effect (Figure 75.3).

TETRACYCLINE DISRUPTS AMYLOID FIBRILS GENERATED BY PrP PEPTIDES

We then investigated whether tetracycline was able to affect amyloid fibrils generated by the highly fibrillogenic PrP106–126. The peptide was suspended in 20 mM Tris-HCl, pH 7.0, at a concentration of 0.5 mM in the absence or presence of equimolar concentration of tetracycline. Following 1-, 24- and 72-h incubation at 37 °C, aliquots were analyzed by electron microscopy after negative staining with 5% uranyl acetate. While the peptide alone readily formed a dense meshwork of straight, unbranched, 8 nm

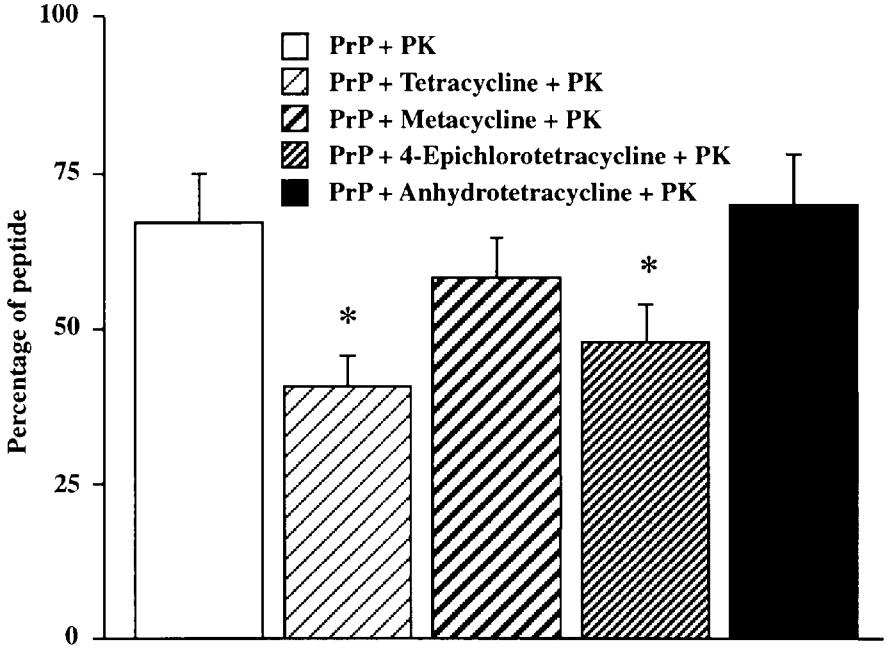

Figure 75.3. The effect of distinct tetracycline derivatives on the protease resistance of PrP106–126 after a 10 min incubation with proteinase K. Data are the mean ± s.d. of five experiments. *p50.001 vs. control group (Student's *t* test)

diameter fibrils, co-incubation with tetracycline resulted in progressive disarrangement and fragmentation of the fibrils, which were no longer detectable after 72 h (Figure 75.4).

PrP-TETRACYCLINE INTERACTION SITE AS DEFINED BY ^{1}H-NMR SPECTROSCOPY

The interaction between PrP106–126 and tetracycline in water solution was analyzed by 2D-NMR nuclear Overhauser experiments (NOESY) performed at 600 MHz. The NOESY spectra performed on tetracycline alone (Figure 75.5A) were characteristic of small molecules with fast molecular tumbling (τ_c50.4 ns) and allowed for a rapid assignment of the resonances, confirming the published data (Williamson and Everett, 1975; Wüthrich, 1986). The addition of PrP106–126, at either pH 3.5 or 7.0, caused a dramatic change in sign for the intermolecular NOESY interactions (Figure 75.5B). This indicated an increase in the tetracycline molecular tumbling, with an estimated correlation time τ_c value greater than 1 ns, a value consistent with an intermolecular complex formation (Wüthrich, 1986). Other intermolecular NOESY interactions were observed, indicating the existence of through-space interactions with PrP106–126 side chains, located at the peptide C-terminus.

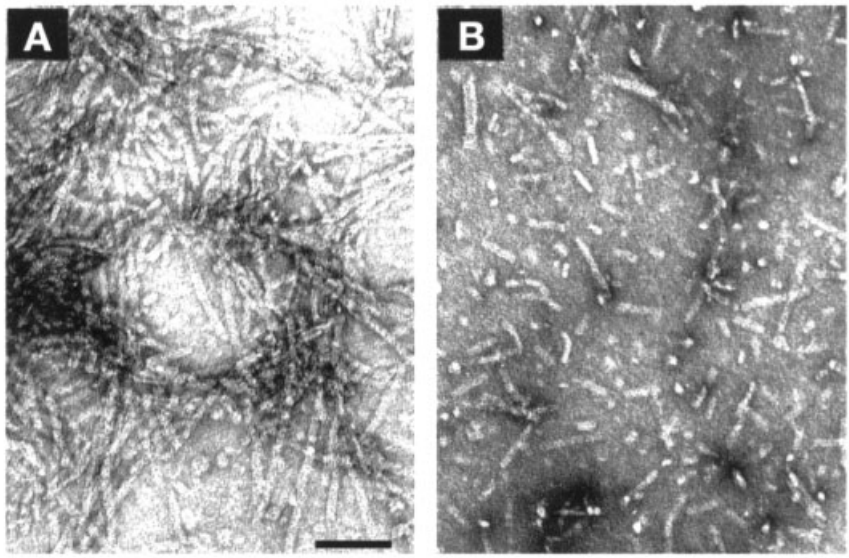

Figure 75.4. Electron micrographs of PrP106–126 assemblies after a 24-h incubation in the absence (A) or presence (B) of tetracycline. Scale bar = 100 nm

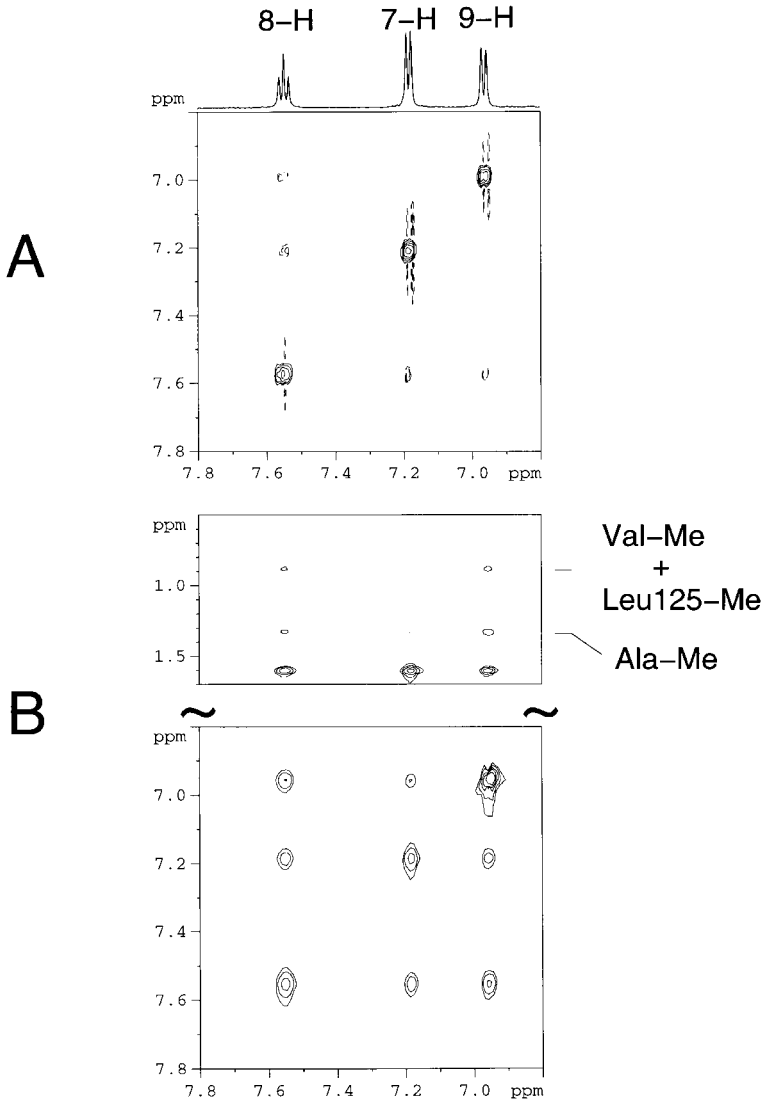

Figure 75.5. (A) 2D-NMR NOESY spectrum (600 MHz, $t_{mix} = 3$ s) of 3 mM tetracycline in D_2O, pH 7. Positive diagonal peaks and negative cross-peaks are drawn with continuous and dashed lines, respectively. The expansion shows the intermolecular interactions involving tetracycline aromatic protons. The 1D-NMR spectrum, together with the assignments, is drawn on top for reference. (B) NOESY spectrum (600 MHz, $t_{mix} = 3$ s) of 3 mM tetracycline, in D_2O, pH 7, in the presence of 1.5 mM PrP106–126. All peaks are positive. Upper panel: high-field region with interactions between Ala, Val, and Leu Me groups with tetracycline aromatic protons. Lower panel: same expansion as in (A)

The strongest interactions involved PrP methyl groups of Val121 or Val122 and of Leu125 with the tetracycline aromatic protons, as well as 6-Me, 5-H, 4-H, and 4-NMe_2 (Figure 75.5B). The high resonance overlapping in the high-field region of the NMR spectrum prevented a detailed assignment of the NOE interactions; however, the number of the observed interactions was sufficient to allow the construction of a molecular model, with the explicit inclusion of water molecules. The model was refined by a standard cycle of energy minimization, 40 ps Molecular Dynamics at 300 K, and a final energy minimization, with the peptide conformation constrained in the previously determined α-helix conformation between residues 113 and 125 (Ragg et al., 1999). The resulting molecular model, with the solvation molecules omitted for clarity, is depicted in Figure 75.6. Tetracycline, with its concave shape due to the *cis*-junction between rings C and D, nicely fits into the groove of the peptide α-helix at the level of Val121–Leu125 segment, with the hydrophilic groups of ring D directed towards the solvent and the hydrophobic part interacting with Val121/122 and Leu125 side chains. The positively charged 4-NMe2 group points towards the terminal Gly126 carboxylate, thus providing an electrostatic attractive interaction. In addition, the same group is involved in a stabilizing hydrophobic interaction involving the methyl groups situated on Val122. The Val121–Leu125 region was proved to be inherently unstable (Ragg et al., 1999) and may be the origin of the conformational instability of the peptide. Thus, the stabilization of the helical conformation in this region may be one of the molecular determinants for the antifibrillogenic properties of tetracycline.

TETRACYCLINE INHIBITS NEURONAL DEATH AND ASTROGLIAL PROLIFERATION INDUCED BY PrP PEPTIDE

We previously showed that PrP106–126 has a toxic effect on neuronal cell cultures and stimulates astrocyte proliferation *in vitro* (Forloni et al., 1993, 1994). To investigate whether these effects were antagonized by tetracycline, primary cultures of rat hippocampal or cortical neurons and astrocytes were exposed for 7 days to micromolar concentrations of PrP106–126 in the absence or presence of the compound at 1:0.5 molar ratio. The study showed that tetracycline reduced the neurotoxic effect of PrP106–126 by 50% and abolished glial cell proliferation.

CONCLUSION AND PERSPECTIVE

The emergence of a new variant of CJD in the UK, which is proposed to be causally linked to BSE, has increased the urgency to identify and develop therapeutic compounds due to fear of a possible future epidemic (Collinge et al., 1996; Will et al., 1996; Bruce et al., 1997). In this study we showed that

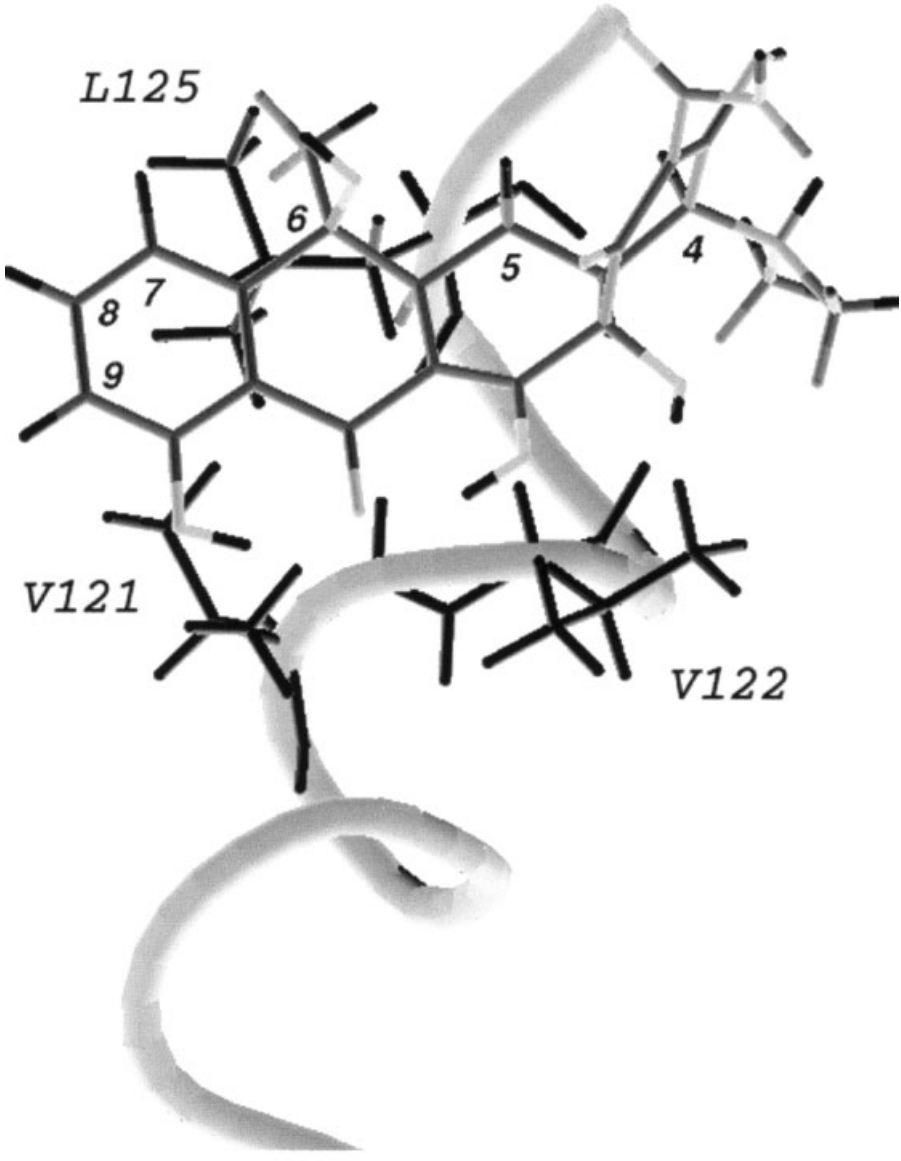

Figure 75.6. Molecular model of tetracycline–PrP106–126 complex. The peptide is represented as a ribbon

tetracycline prevents aggregation and acquisition of protease resistance of PrP peptides, disrupts PrP peptide aggregates, and abolishes the neurotoxicity and astroglial proliferation induced by PrP peptides *in vitro*. These properties make tetracycline a prototype of compounds with the potential of inactivating the pathogenic forms of PrP, impeding PrP^{C} to PrP^{Sc} conversion, and hindering the development and progression of prion disease. *In vivo* studies with tetracycline in experimental scrapie are already under way. Preliminary data are proving to be very promising, where Syrian hamsters inoculated intracerebrally with 263K scrapie-infected brain homogenates that had been pre-incubated with the compound showed a significant delay of onset of clinical signs of disease, accumulation of PrP^{Sc} in the central nervous system, and related neuropathological changes.

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VII Psychosocial Care

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76 Influence of a Memory Training Program on Attention and Memory Performance of Patients with Dementia

TANJA BERNHARDT, KONRAD MAURER, AND LUTZ FRÖLICH

INTRODUCTION

Only a few results of controlled studies concerning the training of cognitive functions in association with dementing illnesses have been published. Usually cognitive training programs do not consider different cognitive abilities of the participants and training tasks can not be generalized to daily living.

Most frequently, memory training for older people with dementia aims at the improvement of learning and can be subdivided into three different areas: repetitive exercising of recall tasks, training of internal memory techniques, and the use of external memory aids. Those techniques are based on psychological theories of learning. External memory aids, e.g. the use of diaries and calendars, employ compensatorial strategies, because memory performance can not be improved. Internal techniques try to structure the storage and recall of information. Verbal techniques summarize information in sentences and stories, while so-called imagery techniques store verbal information through figurative imagination. The use of these mnemonic techniques can help to manage mild cognitive impairments.

There are several disadvantages of these memory strategies (Cornoldi, 1988; Denney, 1989): they are not developed especially for older people with dementia, they can not be generalized to daily tasks, and the training effects are not long lasting.

Assumed reasons for their restricted use in daily living are the following: most mnemonic techniques are cognitively very elaborate (e.g. method of loci) and use very specific learning material (e.g. number sequences); intensive training can show short-term effects, but cannot be generalized to

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daily living; participants cannot realize any context with daily tasks. A reason for the short-term nature of the effects is probably that training tasks cannot be assimilated into the natural repertoire of behavior (Poon 1984; Stigsdotter and Bäckman, 1989).

Our program investigated the influence of a memory training program on attention and memory performance of patients with dementia. We tried to develop a program for participants with different cognitive abilities that can be generalized to daily tasks and exercises short-term memory, long-term memory, and activities of daily living.

MATERIAL AND METHODS

SAMPLE AND DESIGN

Participants of our study were 26 elderly subjects aged 72 to 93, recruited from a retirement home in Frankfurt. All participants had a diagnosis of mild cognitive impairment according to Petersen et al. (1999) or a diagnosis of dementia according to DSM-IV/ICD-10-criteria (American Psychiatric Association, 1994; World Health Organization, 1992). No further etiological diagnoses were available, but all subjects were regularly visited by a physician of the retirement home.

After examination with the Mini-Mental State Test (MMST; Folstein et al., 1975), the participants were randomly assigned to either the control group or the experimental group. Our groups showed no significant differences in age, gender, marital status, education and profession. The experimental group consisted of 13 subjects aged 74 to 93 years ($\chi = 83.54$; $sd = 6.4$), and the control group included 13 subjects aged 72 to 91 ($\chi = 84.54$; $sd = 5.53$) (Table 76.1).

In both groups MMST scores varied between 23 and 29 points with a mean of 22.32 and a standard deviation of 1.4. Thus, there were subjects with different stages of dementia or mild cognitive impairment distributed in both groups.

Before and after the treatment we tested attention and memory performance with the Short Cognitive Performance Test (SKT; Erzigkeit, 1989) and the severity of dementia with the Brief Cognitive Rating Scale (BCRS; Reisberg and Ferris, 1988). The SKT was applied by the trainer of the memory program in form A (pre-test) and B (post-test) to prevent learning effects. The ten subscales of the BCRS were applied by a staff member of the retirement home, who was not aware of the group assignment. In addition, participants of the experimental group were asked about the cognitive training via questionnaire after treatment conditions (Table 76.2).

Table 76.1 Group characteristics before treatment

		Experimental group (n = 13)	Control group (n = 13)
Age		Mean = 83.54 sd = 6.4 Range 74–93	Mean = 84.54 sd = 5.53 Range 72–91
Gender	Male/female	1/12	0/13
Marital status	Single/married/widowed	3/2/8	2/1/10
Education	9/10/13 Years of schooling	9/3/1	9/3/1
Profession	Semi-skilled/qualified	5/8	5/8
Cognitive status	MMST	Mean = 22.31 sd = 1.42	Mean = 22.38 sd = 1.32

TRAINING PROGRAM

Subjects of the experimental group participated in the cognitive training in 12 lessons for a period of 6 weeks. We had two training groups, one with 6 and one with 7 participants, who met two times a week for a one hour training session.

In our memory training program we tried to create playful and understandable exercises for subjects with different cognitive abilities that can be generalized to daily tasks. A further aim was to increase motivation and support independence of our participants. One problem in the training of independence is that people who are living in a retirement home lose parts of their independence automatically, because they do not have to buy their food or prepare meals etc. For this reason training of independence and daily requirements is limited.

Table 76.2 Design

Week	Control group (discussion circle)	Experimental group (cognitive training)
0	MMST	
1	Testing (BCRS, SKT Form A)	
2–7	Treatment	
8	Testing (BCRS, SKT Form B)	

Short-term memory, which shows disturbances of remembrance and concentration in the early stages of dementia, was trained with letter, word, and calculation games, e.g. searching for fruits with the letter E, guessing an unfinished proverb, or after consecutive presentation of two pictures of objects, participants had to find out what is missing on the second picture (Oswald and Rödel, 1995; Stengel, 1984).

The long-term memory was mainly trained with color slides and audio cassettes (e.g. slides of fruit and vegetables from the market to talk about ingredients and preparation of meals; slides from animals in the zoo to remember names of animals and talk about own pets; slides from sights of Frankfurt to remember well-known places). During the listening exercises participants were asked to guess sounds of animals, musical instruments, and music pieces (Stengel, 1993). In addition we talked about holidays and customs and tried to remember fairy tales.

Training of daily tasks referred to the fields of clothing and personal hygiene, shopping, eating, traffic, communication and orientation of the activities of daily living scales (e.g. in the field of eating, participants were asked to explain how to set the table, where to put the cutlery, what are the differences between breakfast, lunch and dinner time; in traffic we looked for the way to the supermarket, post office and bank on a city map, talked about what is important for pedestrians and the meaning of traffic signs; in communication we did a role play of how to make an appointment with a physician on the phone).

After a short welcome, every lesson started with a warm-up exercise to introduce the topic of the day. Between the topic of the day and the final exercise we had a short break with a relaxation exercise (Klampfl-Lehmann, 1986). At the end of every lesson the participants received homework, which was discussed in the next training session.

CONTROL CONDITION

The controls participated in a weekly one hour discussion circle and were distributed to two groups with 6 and 7 subjects as well. We discussed daily life in the retirement home, planned events, celebrations and day trips. Former topics were news from the daily paper and the district.

STATISTICAL PROCEDURES

Statistical analysis was performed with SPSS (Statistical Package for the Social Sciences) and SigmaPlot (Jandel Scientific Software). Because of the small sample size and because the data were not normally distributed, all statistical analyses were performed with the Wilcoxon matched pairs signed rank test and a level of significance with p less than 0.05.

RESULTS

SHORT COGNITIVE PERFORMANCE TEST

The SKT is a neuropsychological test battery developed by Erzigkeit (1989) which measures attention and memory performance. The test score varies between 0 and 27 points, with higher scores representing increasing memory impairment. The test is divided into nine fields and every field is scored from 0 to 3 points.

After treatment, the control group showed no significant difference in attention and memory performance (median = 15 vs. = 14). Within the experimental group, we experienced a significant improvement in the test performance with $p = 0.0046$ (median = 13 vs. = 8). Data between the control and the experimental group showed significant differences in the SKT ($p = 0.0376$) (Figure 76.1).

BRIEF COGNITIVE RATING SCALE

The BCRS is a rating scale developed by Reisberg and Ferris (1988) which assesses the severity of dementia across 10 different domains. Every domain is assessed by a scale from 1 to 7, in which one point stands for the absence of a

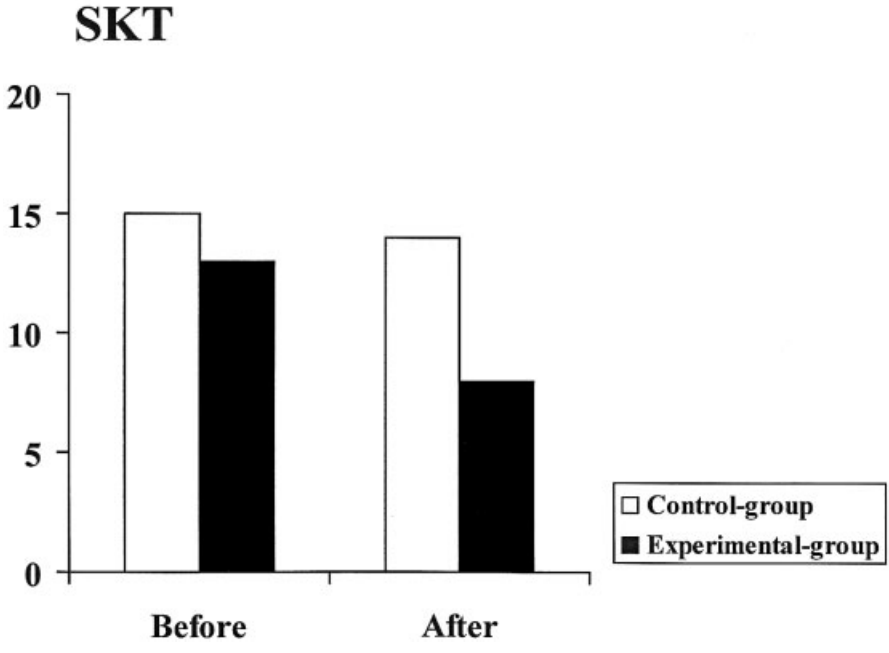

Figure 76.1. Short Cognitive Performance Test (SKT)

cognitive disturbance. This scale is divided into 10 fields and every field is scored from 1 to 7 points.

After treatment conditions, the control group showed significant impairments in the severity of dementia ($p = 0.0033$; median $= 2.1$ vs. $= 2.2$). In comparison, the experimental group showed significant improvements ($p = 0.0033$; median $= 3.4$ vs. $= 3.1$). We found no significant differences in the BCRS scores between the control and the experimental group (Figure 76.2).

QUESTIONNAIRE

After treatment conditions, 11 participants of the experimental group filled in a questionnaire about the cognitive training. All participants had fun in the 12 training sessions, all were satisfied with the choice of the topics, all felt well in their group, and all of them would participate in training again.

DISCUSSION

Cognitive abilities, including memory and attention measured with SKT and BCRS, can be improved by a memory training as outlined in our study, at

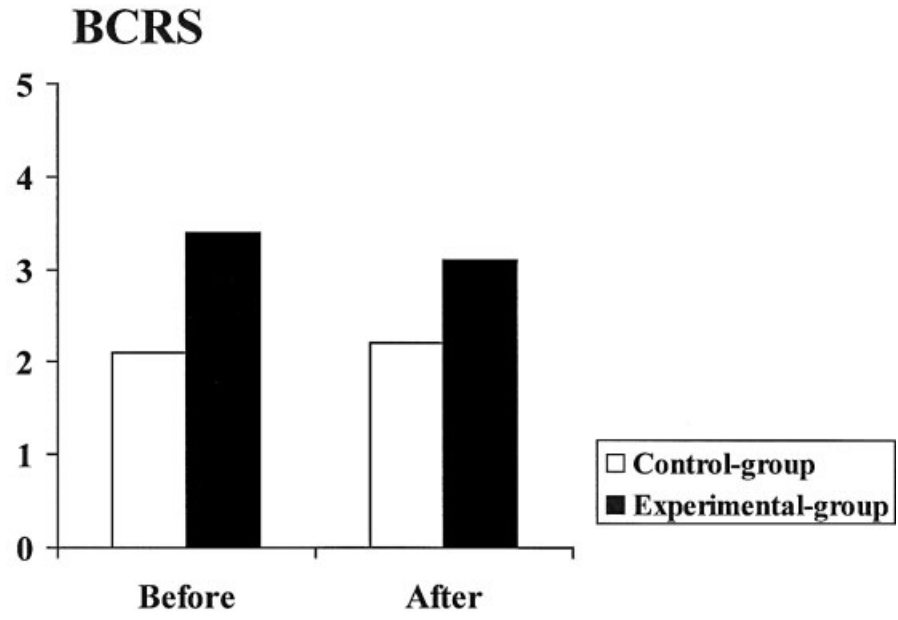

Figure 76.2. Brief Cognitive Rating Scale (BCRS)

least in the short term. The positive results of the questionnaire point to an improvement of the perception of quality of life.

There are few results of controlled studies about psychosocial interventions concerning the training of cognitive functions in dementing illnesses.

Some studies found short-term improvements for patients with the diagnosis of dementia within training conditions that disappeared after training (Zarit et al., 1982). A combined training program consisting of attention, memory, and concentration tasks showed nearly no change in cognitive performance of patients with Alzheimer's disease or vascular dementia (Beck et al., 1988). The face–name method, which connects the name of a person with concise features of his face, only showed an improvement in the memory performance of one participant in a study with eight demented patients (Bäckman et al., 1991). Other studies showed that cognitive training shows improvements only in mild dementias (Yesavage et al., 1981; Yesavage, 1982). Because of retardation and impairment of cognitive processing, the use of mnemonic techniques is not suitable for progressive dementias (Jorm, 1986; Salthouse, 1980). To obtain significant memory improvements, Wilson (1989) recommends design of an individual training program for every patient that exercises mnemonic techniques dependent on cognitive status and severity of dementia.

In contrast to those studies we designed a training program for participants with different cognitive abilities that can be generalized to daily tasks and that exercises short-term memory, long-term memory, and activities of daily living.

Our results confirm the hypothesis that cognitive training can improve the memory performance of patients with dementia, if the training considers individual cognitive abilities and practices activities of daily living. During our investigation we noticed that even confused older people can manage new or unfamiliar situations through training of their specific impairment.

The aim of this pilot project was to elucidate the possibility of improving cognitive abilities through application of our memory program. It did not assess whether these positive effects are long lasting nor if the effects can be generalized to performance of daily life. Further questions should address the influence on caregiver stress, quality of life, and activities of daily living with suitable test scales in a subsequent study.

These effects demonstrate the utility of psychological interventions in dementing illnesses. Motivation and support of independence appear to be important elements in the design of psychological training, as well as therapeutic components and social aspects like group activities.

SUMMARY

There are few results of controlled studies about psychological interventions, i.e. training of cognitive functions in dementing illnesses. Most frequently

very specific memory strategies are trained which cannot be generalized to activities of daily living. However, it is the consensus that dementia patients have difficulties in generalizing learnt material into activities of daily living.

Thus, a memory training program was developed considering the different cognitive abilities of the participants and which was meant to be generalized to daily tasks.

Short-term and long-term memory were trained as well as activities of daily living in 12 sessions for a period of 6 weeks in a group of 26 elderly dementia patients divided into an experimental and a control group. In this pilot study we can show significant improvements of attention and memory performance in our experimental group in comparison to the control group.

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Alzheimer's Disease: Advances in Etiology, Pathogenesis and Therapeutics
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77 Prediction of Use of Emergency Community Services by Cognitively Impaired Seniors who Live Alone: Preliminary Findings of a Prospective Study

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Two important trends have begun to converge in industrialized nations: there are more older people who live alone and there are more people with Alzheimer's disease (AD) and related dementias (Chappell, 1991; Webber et al., 1994). Those with cognitive impairment or dementia who live alone are more frequently placed in residential care facilities than those who reside with another person (Cohen et al., 1988). Family caregivers' decisions to institutionalize their elders are often based on their perception of their elders' needs for more care and their own difficulty coping with caregiving. Rarely are they based on their elders' actual risk of harm or their elders' cognitive or physical condition (Colerick and George, 1986). This situation is compounded by the fact that primary health care providers often cannot determine those with the greatest likelihood of experiencing harm, thus limiting their ability to focus scarce resources on those at greatest risk (Webber et al., 1994). Consequently, cognitively impaired elderly people who want to remain in their own home may be inappropriately or prematurely institutionalized because of difficulties anticipated by concerned relatives and because they receive insufficient assistance from community agencies.

Our purpose was to determine whether we could accurately predict an individual's probability of needing emergency community services based on cognitive, behavioral, and demographic characteristics. We were specifically interested in use of emergency services required as the result of negligence due to cognitive impairment.

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METHOD

PARTICIPANTS

Physicians, community agencies, and other health care professionals in a large urban center were asked to identify their patients and clients who were 65 years of age and older, lived alone (i.e. no one remained overnight in their home regularly) were suspected of having cognitive impairment, were fluent in English, and were medically stable. Patients were excluded if they had a history of bipolar disorder, schizophrenia or a progressive neuromuscular disease. All who scored <131 on the cognitive screening examination, the Dementia Rating Scale (DRS) (Mattis, 1973), were asked to give us the name of someone familiar with them who would serve as their study informant.

MEASURES

Diagnostic Assessment for Dementia

Participants were seen for neuropsychological assessment and then by a geriatrician for a medical assessment. Based on the joint decisions of the neuropsychologist and geriatrician, participants were then diagnosed as meeting DSM-IV criteria for dementia or not, and the etiology of the dementia or cognitive impairment was specified according to accepted criteria (American Psychiatric Association, 1994; McKhann et al., 1984).

Research Battery of Neuropsychological Tests

Ten test scores based on performance on the following tests were used to compare those who did or did not use emergency services (the results of these tests were kept separate from the diagnostic process):

- Rey Auditory Verbal Learning Test (Lezak, 1995): 5th trial recall, short delay recall, recognition
- Wechsler Memory Scale–Mental Control subtest (Wechsler and Stone, 1973): raw scores
- Trail Making Test Parts A and B (TMT-A and B) Intermediate Forms (Reitan, 1977): time to complete each part
- WAIS-R Comprehension and Similarities subtests (Wechsler, 1981) raw scores
- Controlled Oral Word Association Test (Spreen and Benton, 1969): total number of words generated for letters FAS
- Boston Naming Test odd/even version (Kaplan et al., 1983): total correct.

Behavioral Measure

The Behavioral Problems Checklist (BPC) (Niederehe, 1988) was selected because it is a valid and reliable 52-item measure developed for community-residing people with mild dementia. It has six subscales that measure self-care deficits, instrumental activities of daily living deficits, dysphoric mood, acting-out behavior, cognitive symptoms, and inactivity/withdrawal. Study informants indicated whether a behavioral problem occurred over the last week.

Social Resources

Study informants and family physicians provided information about the amount and frequency of formal services currently available in the home (i.e. Home Care, Meals on Wheels, homemaking, personal care, any professional services, and use of day care or respite services). We also documented the amount and frequency of unpaid caregiving from friends or family, including assistance with meals, housekeeping, bathing, dressing, transportation, and assistance with medication.

Outcome Measure

We defined our outcome measures as an event or circumstance that occurred because of a participant's cognitive impairment that required an emergency intervention. Study informants were asked at baseline and then every three months for a total of 18 months to recount any events that involved the use of emergency medical, police or fire department services and the circumstances surrounding them. We also asked them whether they believed negligence due to their participant's cognitive impairment contributed to any of these events. We asked family physicians the same questions. Next, we obtained all available documents pertaining to ambulance use, hospital emergency visits, police and fire department records. We attempted to interview as many people as possible to obtain a judgment about the contribution of the participant's cognitive impairment to the event.

All interview information pertaining to an event together with any records obtained from other sources were reviewed independently by three raters (a physician, a psychologist, and a trained research assistant) who judged whether there was sufficient evidence that negligence due to the participant's cognitive impairment caused the event and that emergency medical, fire or police services were used. All disagreements were resolved by consensus. Raters were blind to participants' neuropsychological, behavioral, and diagnostic information.

RESULTS

PARTICIPANTS

A total of 149 individuals volunteered to participate in this study. Family physicians referred 26 participants, 5 were referred from hospital emergency rooms (ER), 33 from a hospital geriatric rehabilitation unit, 5 from an acute care hospital ward, 10 from the outpatient clinic of a regional service provided by geriatricians (Toronto Regional Geriatric Program), 58 from Toronto-area community agencies servicing older people, and 12 from family members. Of the above, 21 had not yet completed their 18-month follow-up period, 8 were placed or died before the follow-up started, and 6 refused to complete significant portions of the neuropsychological test battery, leaving 114 for these preliminary analyses.

The demographic characteristics of these 114 participants, their scores on the DRS and the Mini-Mental State Examination (MMSE) (Folstein et al., 1975), and frequency of dementia diagnoses are presented in Table 77.1. Also noted in Table 77.1 is the average number of weeks during which the participant lived alone during the prospective 18-month period. (Participants did not live alone because of hospitalization, moving in with family temporarily etc.) The kinds of residences in which participants lived are described in Table 77.2. Government-subsidized apartments house low-income individuals. Supportive housing is also government subsidized for

Table 77.1. Demographic and other characteristics of participants

Characteristic	Mean (sd)	Range
Age, y	83.0 (7.1)	66–97
Education, y	11.0 (3.1)	2–21
MMSE	22.8 (3.6)	12–30
DRS	116.7 (9.3)	90–130
Weeks living alone in 18-month period (maximum 78 weeks)	60.7 (23.6)	3–78
Sex	76 F; 38 M	
Dementia diagnosis	70 ND; 44 D	

*ND, non-demented; D, demented.

Table 77.2. Number of participants living in each type of residence

Detached or semi-detached home	31
Independent apartment	58
Government-subsidized apartment	11
Supportive housing	14

people with physical limitations and provides independent self-contained apartment units with on-site assistance available on request.

REASONS FOR USE OF EMERGENCY SERVICES

Of the 114 participants, 28, i.e. 24.6%, for whom we have completed preliminary results, met our criteria of having at least one incident requiring the use of emergency services as a result of cognitive impairment over the 18-month prospective period. Of the 28 experiencing harm, 21 had only one incident, 5 participants had two incidents, and 2 had three. Table 77.3 describes the problems encountered as a result of cognitive impairment and the emergency services used. The most frequently occurring kind of negligence was failure to eat and drink, which occurred in nine cases. The second most frequently occurring problems, each occurring in six cases, were failure to keep oneself or one's environment clean and failure to recognize a familiar environment. The fourth most frequently occurring problem was failure to use medications properly, which occurred in five cases. Other incidents that occurred in three or fewer people are also listed in Table 77.3.

ROLE OF SOCIAL RESOURCES

We compared the frequency of formal and informal assistance for those who did or did not use emergency services due to cognitive impairment. The

Table 77.3. Problems occurring as a result of neglect due to cognitive impairment which resulted in use of emergency intervention services*

Negligence due to cognitive impairment (number of cases)	Emergency intervention (number of incidences)
Failure to eat and drink (9)	Ambulance and ER (7); police (1); ER (1)
Failure to keep oneself or one's living environment clean (6)	Ambulance and ER (1); urgent family physician visit (5)
Failure to recognize a familiar environment (6)	Ambulance and ER (3); ER (1); police (2)
Failure to use medications properly (5)	Ambulance and ER (1); ER (3); police (1)
Failure to report a medical condition (3)	Ambulance and ER (2); urgent family physician visit (1)
Failure to use assistive devices properly (3)	Ambulance and ER (2), ER (1)
Failure to find way home or gain access to home (2)	Police (2)
Failure to turn off electrical appliances (1)	Fire and ambulance (1)
Failure to judge fraudulent activity (1)	Police (1)
Failure to control aggressive impulses (1)	Urgent family physician visit (1)

*Some cases had more than one problem and some cases had multiple incidents of the same problem.

amount of informal assistance with meals, housekeeping, bathing, dressing, transportation, and medications showed no statistically significant differences between the groups. We also compared the two groups on frequency of use of Meals on Wheels, housekeeping services, personal assistance, nursing, other professional services, use of day hospital, respite care, or day centers. Again, none of the differences was statistically significant.

PREDICTION OF USE OF EMERGENCY SERVICES

Logistic regression analyses (forward stepwise procedure) were used to determine whether a model based on the cognitive, behavioral, and demographic variables could be identified that accounted for problems experienced. The dependent variable was categorized as those who experienced problems at least once during the 18-month period ($n = 28$) and those who had no problems during this period ($n = 86$). Only one of the six behavioral scales, the Self-Care Deficits Scale, met criteria for entering the forward-stepping logistic model, $\chi 2(1) = 6.36$, $p = 0.012$. Two of the neuropsychological tests, RAVLT recognition, $\chi 2(1) = 11.67$, $p = 0.001$, and TMT-B, $\chi 2(1) = 4.29$, $p = 0.038$, entered the next logistic model. The six demographic variables that were entered in the third logistic analyses were age, education, sex, dementia diagnosis, referral source, and number of weeks not living alone. The latter variable was included because not all participants were living alone for the entire 18-month period (see Table 77.1). The following variables emerged as significant: age, $\chi 2(1) = 4.002$, $p = 0.045$, and total time in study, $\chi 2(1) = 6.109$, $p = 0.013$. This sequential statistical process attempted to filter out a final model that would include risk factors from all three domains of behavior, cognition, and demographics. We believe such a model would be more generalizable than one that contained risk factors from a single domain.

When the five significant behavior, cognitive, and demographic variables were all entered into a logistic regression, the model was significant, $\chi 2(5) = 25.758$, $p = 0.001$. The direction of the coefficients suggested that poor self-care, poor recognition memory, longer time to complete TMT-B, older age, and less amount of time in study were associated with an increased probability of requiring emergency services due to problems associated with cognitive neglect.

We used the Receiver Operating Characteristics (ROC) curve to determine the optimal combination of sensitivity and specificity for this five-variable model. This analysis yielded a sensitivity of 86% and a specificity of 69%. The likelihood ratio of the positive test (LR+) of this model was 2.73, which represents a small but meaningful change in pre-test to post-test probability of harm (Jaeschke et al., 1994). This indicates that the pre-test odds for harm increase almost three-fold for a person testing positively. The likelihood ratio of the negative test (LR−) was 0.20, which represents a moderate change

from pre-test to post-test probability. The pre-test odds for harm are reduced to one fifth of the prior odds for a person testing negatively.

DISCUSSION

We found that the recognition test of the RAVLT, Trail Making Part B, the Self-Care Deficits Scale, age, and time in study were significant predictors of an individual's risk of requiring emergency community services due to cognitive impairment. Moreover, this five-variable model predicted outcome in several areas of competency, including, for example, nutrition, hygiene, and health care.

Our findings suggest that impairment in the neuropsychological abilities measured by RAVLT recognition memory and TMT-B may place an individual at risk for needing emergency services for problems due to cognitive impairment. Impairment on the RAVLT recognition test is considered to reflect deficits in storage due to deficient consolidation of new memory traces. Individuals who perform poorly on this test may not benefit from cues and prompts designed to aid recall of new information. Trail Making Test Part B is thought to measure executive function, requiring attention, complex visual searching, and sequencing, as well as cognitive flexibility.

Consider how impairment in these two neuropsychological abilities may lead to problems requiring emergency services for problems arising from malnutrition and dehydration. Ensuring that one avoids malnutrition and dehydration requires that one remembers to eat and drink, but also requires that one plans meals, prepares or orders them, and recognizes internal and external cues that it is time to eat. Similarly, the ability to take medications properly involves remembering to take them, but also involves planning to ensure that medications are taken in the proper sequence, at the proper time, and in the proper circumstances, as well as understanding the relevance of the medication(s) to maintaining current health. Thus, the ability to benefit from cues and prompts that will remind an individual to perform an important behavior is only one component of the skill set necessary to avoid harm. The ability to plan and organize the environment to ensure the stage is set to engage in healthy behaviors is another critical element necessary to maintain health and avoid harm.

The Self-Care Deficits Scale of the BPC includes 10 items asking questions about whether the participant has problems wandering, dressing, grooming, bathing, feeding, toileting, bowel or bladder incontinence, walking, and getting in and out of bed. The ability to perform these basic activities of daily living (ADL) may be a prerequisite for avoiding harm and the need for emergency services. The scales measuring instrumental ADL, cognitive symptoms, mood and withdrawal did not account for a significant amount of the variation in performance once the ADL scale entered the logistic model. It is interesting that the score on the Self-Care Deficits scale was an important

predictor of harm in these participants and yet there were no significant differences between the groups in the amount of care they were receiving from their primary caregivers or from formal service providers. Reasons for this lack of difference should be explored further as factors such as refusal to accept services, in addition to lack of availability, may account for the failure to find differences despite greater apparent need.

These findings provide important directions for assisting individuals who may be potentially at risk of needing emergency services. First, simply providing meals, memory books, drug dispensers, or other aids may not be sufficient to alleviate potential problems that are likely to arise in cognitively impaired people at greatest risk. What may also be necessary is to provide these forms of assistance in conjunction with help in organizing the older person's schedule and lifestyle around their use. Assistance, which can be provided either by family members or professional caregivers, in the form of telephone calls at specific times of the day to remind the older person to eat or take medication may be more effective. If these do not alleviate the problems, then more direct help in the form of assistance with bathing, medications or meals may be required. The results of these preliminary analyses indicate that we may be able to target those at highest risk of needing emergency services before they actually require them. Targeting only those at greatest risk will reduce the overall cost of assistance. Moreover, the benefits of pre-empting harm should far outweigh the cost of emergency services as well as premature and unnecessary institutionalization.

These findings are generalizable to a broad spectrum of older cognitively impaired people who live alone as the sample was recruited from many different referral sources, including family physicians, hospitals, and community sources. However, because our design required that we obtain data regarding use of emergency services from sources other than the participant, our findings may not be applicable to those people who do not have someone who could act as an informant or to those who do not have a family doctor. Highly isolated individuals who do not have any family, friends or professional contacts were not represented in this sample.

This study represents a first critical step in the identification of those who are at greatest risk for harm. The next step in this research is to confirm these preliminary findings with the 21 additional participants who have not yet completed their follow-up period. The changing demographics of our population will result in an increase in the prevalence of older people with cognitive impairment who live alone and thus it is critical that we begin to develop strategies to identify those at greatest risk of harm. If we can reduce the incidence of harm and use of emergency services in this growing segment of our society, this should have a resounding impact on the quality of life of seniors and their families as well as on the rate of hospitalization and institutionalization.

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