RESEARCH AND PERSPECTIVES IN ALZHEIMER'S DISEASE

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Biological Markers of Alzheimer's Disease

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Preface

This volume presents the proceedings of the symposium held in Toulouse on April 24, 1989, on the topic "Biological Markers of Alzheimer's Disease." This symposium was the fourth of a continuing and successful series of Colloques Médecine et Recherche organized by the Fondation IPSEN pour la Recherche Thérapeutique, addressing various aspects of contemporary research in the field of Alzheimer's disease (AD). The series started in September 1987 with "Immunology and Alzheimer's Disease," followed 6 months later in Paris by "Genetics and Alzheimer's Disease" and in September 1988 in Montpellier by "Neuronal Grafting and Alzheimer's Disease."

The present symposium was organized for the purpose of gathering the most current ideas concerning biological markers of AD. The papers presented at this symposium may be roughly subdivided into three groups. The first deals with the markers of AD at the level of the brain itself. These markers are studied either through the cerebrospinal fluid or through techniques such as nuclear magnetic resonance (NMR) – approaches which respectively aim at demonstrating the cerebral changes indicated by the debris resulting from the disease, or studying the possible neurochemical abnormalities that occur in the earlier stages of AD. The second group of papers deals with possible extraneuronal changes in the blood, in the skin, or in other organs of AD patients, while the third group addresses the most current research on the genetic abnormalities which may be found in AD subjects. We think that the papers gathered in this volume will provide a unique view of the state of the art of these aspects of AD and their relation to both the pathogenesis and the in vivo diagnosis of the disease.

The city of Toulouse where this symposium was held has a long tradition of research in neuroscience, neurology and geriatrics. We wish to thank the Dean of the Faculty of Medicine, Professor Guiraud-Chaumeil, who generously provided hospitality in his prestigious insitution. We also wish to express our gratitude to the Institut National de la Santé et de la Recherche Médicale (INSERM) for kindly agreeing to sponsor this meeting. We also wish to thank Mrs Mary Lynn Gage for her editorial assistance, Mrs Jacqueline Mervaillie for the organization of the meeting, and Yves Agid for his collaboration as chairman of the meeting.

The next meeting was organized in Lille on October 16, 1989 and was devoted to "Imaging, Cerebral Topography and Alzheimer's Disease."

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Cerebrospinal Fluid Neurochemical Markers in Alzheimer's Disease

J. R. Atack

Summary

Measurement of the concentration of neurotransmitter markers in lumbar CSF offers a relatively non-invasive method for evaluating CNS function. Consequently, we have measured the concentrations of a number of neurotransmitter markers in the CSF of patients with probable AD as indices of the integrity of these systems in the brain. We observed relatively modest (14%–33%) reductions in the activity of the cholinergic marker enzyme AChE, the dopaminergic metabolite HVA and the neuropeptides somatostatin, CRF, ACTH and α-MSH. The concentrations of the serotonergic metabolite 5-HIAA, the noradrenergic metabolite MHPG and neuropeptide Y did not differ from those in age-matched healthy control subjects.

When these data were analysed further, we were able to identify neurochemically distinct subgroups of AD. Thus, reduced HVA concentrations observed in the AD group as a whole (33% reduction) were related to reduced HVA concentrations in subgroups of AD patients with extrapyramidal signs or myoclonus; HVA concentrations in AD without movement disorders did not differ from control values. A dopaminergic deficit in patients with extrapyramidal signs or myoclonus was further indicated by the fact that biopterin, a cofactor for tyrosine hydroxylase, was also reduced in these patients when compared to patients without movement disorders. In addition, in both extrapyramidal and myoclonic AD subgroups, 5-HIAA concentrations were also reduced relative to patients with no movement disorders, suggesting that, in addition to a dopaminergic deficit, extrapyramidal signs and myoclonus in AD are also associated with a serotonergic deficit.

There was also heterogeneity in AD patients without movement disorders. Thus, AChE activity and HVA concentrations were significantly reduced compared to control subjects in patients with early (65 years or less) but not late (over 65) age at onset, indicating more severe involvement of cholinergic and dopaminergic systems in patients younger at onset.

The relatively modest decrements in neurochemical marker concentrations in lumbar CSF of AD patients, along with the considerable overlap between AD and control groups, make it unlikely that the measurement of a single neurochemical parameter will be of diagnostic use. Nevertheless, the measurement of CSF neurochemical parameters offers a valuable insight into the functioning of the CNS in AD, particularly if repeated measurements are taken longitudinally during the progression of the disease.

Introduction

The cerebrospinal fluid (CSF) bathes the brain and spinal cord and in man has a volume of approximately 140 ml, of which the spinal subarachnoid fluid represents about 30 ml (Davson et al. 1987). The CSF acts as a mechanical support for the brain and greatly reduces the acceleration and deceleration of the brain produced by rapid head movement. It also provides a cushion between the delicate neural tissue and the hard skull and spinal column. Furthermore, CSF acts as a "third circulation" (Milhorat 1975) by removing products of metabolism as well as serving to circulate substances intracerebrally.

CSF is produced at a rate of 0.3–0.4 ml per minute in man via two routes (Fig. 1): the choroid plexus and extrachoroidal sites (Milhorat and Hammock 1983). Although it has been estimated that the contributions to total CSF production of choroidal and extrachoroidal pathways are of the order of 70% and 30%, respectively (Wood 1980b), the precise relative contribution of these two pathways remains controversial.

The rapid distribution of dye throughout the brain following intraventricular injection suggests that there is no significant diffusion barrier between the central nervous system (CNS) extracellular fluid and CSF (Goldmann 1913). Since no diffusion barrier exists between the extracellular fluid and CSF, the concentration of neurotransmitters not capable of permeating the blood-brain barrier and their related metabolites in extrachoroidal CSF is a function of their neuronal secretion into extracellular fluid. Presumably, therefore, altered secretion of such substances by degenerating neurotransmitter systems is reflected by changes in CSF

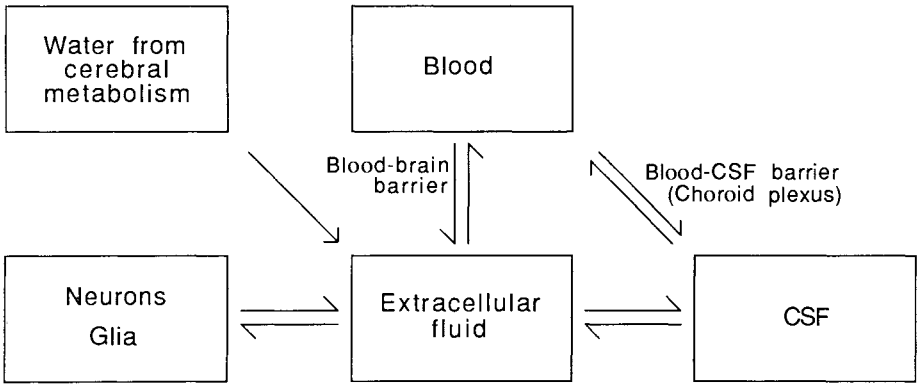

Fig. 1. Schematic representation of CSF production. CSF is produced at either the choroid plexus by ultrafiltration of plasma through the blood-CSF barrier or at extrachoroidal sites by passage of extracellular fluid into CSF. The relative contributions of choroidal and extrachoroidal sites to total CSF production have been estimated at 70% and 30%, respectively (Wood 1980b). The composition of extracellular fluid (and therefore extrachoroidal CSF) is related to secretion of CNS elements (neurons, glia), ultrafiltration of plasma constituents through the endothelial cells of the CNS vasculature which constitute the blood-brain barrier, and water produced during cerebral metabolism (Rapoport 1976). Ideally, a neurochemical marker should not cross the blood-brain or blood-CSF barriers, in which case its concentration in CSF is a function of its concentration in extrachoroidal CSF, which in turn is a function of its rate of neuronal secretion into extracellular fluid

concentrations. This is the fundamental assumption underlying the study of CSF neurochemical parameters in Alzheimer's disease (AD) as markers of degeneration of specific neurotransmitter systems in the brain.

As is discussed in more detail below, the measurement of lumbar CSF concentrations of neurochemicals and their metabolites and related enzymes as markers of brain dysfunction is dependent upon: a) the markers originating within the CNS (i.e. the marker should not cross the blood-brain or blood-CSF barrier) and b) the markers originating primarily from brain, so that alterations in secretion from the brain are not masked by normal secretion from the spinal cord.

CSF:Plasma Protein Ratios as Indices of Blood-Brain Barrier Integrity

Before further describing the use of CSF neurochemical markers in AD, it is perhaps pertinent to discuss the use of CSF:plasma protein ratios as indices of blood-brain barrier integrity. Thus, although some proteins (e.g. acetylcholinesterase AChE), dopamine-β-hydroxylase, neuropeptides) are undoubtedly secreted from cells of the CNS into the extracellular fluid (and thereby the CSF), the vast majority of CSF total protein is of plasma origin (Rapoport 1983). Consequently, the ratio of protein concentration in CSF and plasma (normally in the region of 1:200) has been used as an index of either disruption of the blood-brain or blood-CSF barrier (e.g. trauma, tumour formation) or intrathecal synthesis of proteins (e.g. multiple sclerosis); in both cases an increase in the CSF:plasma protein ratio is observed.

Since blood-brain barrier disruption has been suggested as a possible pathogenic mechanism for AD (Wisniewski and Koslowski 1982), we used measurements of CSF and serum albumin and IgG concentrations to assess the blood-brain barrier integrity in AD. Our results showed no evidence of intrathecal IgG production and no significant differences between CSF:serum albumin of IgG ratios between AD and age-matched healthy control subjects (Kay et al. 1987), suggesting, in agreement with others (Eikelenboom et al. 1988), that blood-brain barrier permeability is normal in AD. Similarly, no gross disruption in the blood-brain barrier permeability in AD could be detected using positron emission tomography (PET) with [^{68}Ga]EDTA (Schlageter et al. 1987).

Neurochemical Changes in Alzheimer's Disease Brain Tissue

Although a number of neurotransmitter systems have been implicated in the pathogenesis of AD (Hardy et al. 1985), the most consistent neurochemical deficit reported to date in AD brain tissue is a degeneration of the cholinergic projections from the basal forebrain to the neocortex and hippocampus (Perry 1986). Although less consistent than changes in the cholinergic basal forebrain projections, degenerative changes have nevertheless been reported to occur in cortically projecting neuronal systems of the noradrenergic locus coeruleus, serotonergic raphe nucleus and dopaminergic ventral tegmental area (Ishii 1966; Tomlinson

et al. 1981; Cross et al. 1983; Iversen et al. 1983; Curcio and Kemper 1984; Marcyniuk et al. 1986; Bondareff et al. 1987; Mann et al. 1987; Palmer et al. 1987a, b). With respect to neuropeptide systems, pathological changes in the cortical somatostatin, neuropeptide Y and corticotropin-releasing factor (CRF) systems have all been reported in AD (Davies et al. 1980; Beal et al. 1985, 1986a, b; Bissette et al. 1985; DeSouza et al. 1986). In addition, the amino acid neurotransmitters glutamate and GABA have also been reported to be involved in the pathogenesis of AD (Rossor et al. 1984; Palmer et al. 1986; Chu et al. 1987; Hardy et al. 1987a, b; Lowe et al. 1988; Procter et al. 1988).

In Vivo Evaluation of Neurotransmitter Systems in Alzheimer's Disease

Most neurochemical studies of the brain in AD have relied on studies of post-mortem tissue, which is generally obtained from severely demented patients in whom it is not unusual for the dementia to have existed for some 10–15 years. Consequently, in such subjects it is difficult to determine the extent to which neurochemical changes reflect the primary disease process or are a less specific consequence of chronic neurodegeneration.

Obviously, it would be advantageous to study neurotransmitter systems in vivo and evaluate their status during progression of the disease. The status of neurotransmitter systems in AD may be evaluated in vivo using either: a) neurochemical changes in lumbar CSF as indices of brain neurotransmitter systems, b) biopsy brain tissue, c) PET or d) neurochemical changes in blood.

With respect to lumbar CSF, the extrapolation between changes in lumbar CSF and brain tissue is confounded by a number of factors, not least of which is the extent to which neurochemical markers in lumbar CSF are derived from the brain (see below). On the other hand, brain biopsy offers a direct means of evaluating brain neurotransmitter function. However, biopsies are rarely performed and do not offer the possibility of longitudinal follow-up in individual patients. PET permits direct visualization and quantitation of neurotransmitter-related binding sites in the brain along with the potential of longitudinal follow-up. However, PET technology is very expensive and is only available at a limited number of research centers. Alternatively, blood is readily available, but although neurochemical parameters have been evaluated in various elements of the blood, it is often difficult to reconcile changes in the blood to changes in the brain. Neurochemical measurements in lumbar CSF therefore represent the only relatively non-invasive, inexpensive method of evaluating in vivo brain neurotransmitter function longitudinally.

Uses of Neurochemical Markers in Alzheimer's Disease

The definitive diagnosis of AD can currently be made post-mortem only on the basis of numerous cortical senile plaques and neurofibrillary tangles (McKhann et al. 1984; Khatchaturian 1985). Consequently, in the absence of a specific diag-

nostic test to discriminate reliably between the clinical diagnosis of AD and other forms of dementia, considerable effort has been expended in the search for antemortem markers of AD (Hollander et al. 1986). Since neurochemical deficits in the AD brain have been relatively well described, and the CSF bathes the brain and contains markers of the involved neurotransmitter systems, CSF neurochemical markers may be used in AD to answer the following questions: a) Are the neurochemical changes observed post-mortem also seen in vivo, are they progressive, and do they correlate with dementia severity? b) Are CSF neurochemical changes diagnostic, i.e. for any given demented subject is it possible to confidently diagnose AD on the basis of a CSF neurochemical measurement? And c) is there neurochemical heterogeneity among AD subjects?

Although the measurement and comparison of neurochemical markers in control and AD CSF are relatively straightforward propositions, interpretation of the data is complicated by a number of factors including the extent to which lumbar CSF composition reflects brain neurotransmitter status and the influence of a number of factors (e.g. environment, drugs, diet, physical activity, time of day and time of year) that should, as far as possible, be controlled for.

Do Lumbar CSF Concentrations Reflect Integrity of Brain Transmitter Systems?

An important factor in relating lumbar CSF neurochemical measurements to the integrity of the corresponding neurotransmitter systems in the brain is the extent to which the neurochemical marker crosses the blood-brain and blood-CSF barriers. For example, when a substance that is readily permeable across the blood-brain and blood-CSF barriers is secreted by neurons, it enters the extracellular fluid where it may enter either the CSF via the extrachoroidal route or the blood through the blood-brain barrier. This results in a less predictable relationship between neuronal secretion and extrachoroidal CSF concentration than for a substance that does not cross the blood-brain barrier. Moreover, in such a case, CSF may contain a significant contribution of peripherally derived neurochemical marker from plasma.

Although most monoamine neurotransmitters and their metabolites as well as neuropeptides do not to cross the blood-brain barrier (Wood, 1980c; Pardridge, 1983), there is a significant correlation between concentrations of 3-methoxy-4-hydroxyphenylethyleneglycol (MHPG, the major noradrenergic metabolite) in CSF and plasma (Fig. 2), suggesting a plasma contribution to lumbar CSF MHPG. This is compatible with the blood-brain barrier being permeable to MHPG, and consequently CSF concentrations must be corrected for the plasma contribution by subtracting a value equivalent to 90% of plasma MHPG concentrations (Kopin et al. 1983).

Having established that a neurotransmitter marker is primarily of central rather than peripheral origin, it is important next to determine to what extent the marker measured in lumbar CSF originates from the brain or spinal cord. There are two indirect methods for doing this. One is the measurement of rostrocaudal concentration gradients of the neurotransmitter marker (Wood 1980a). Concentrations

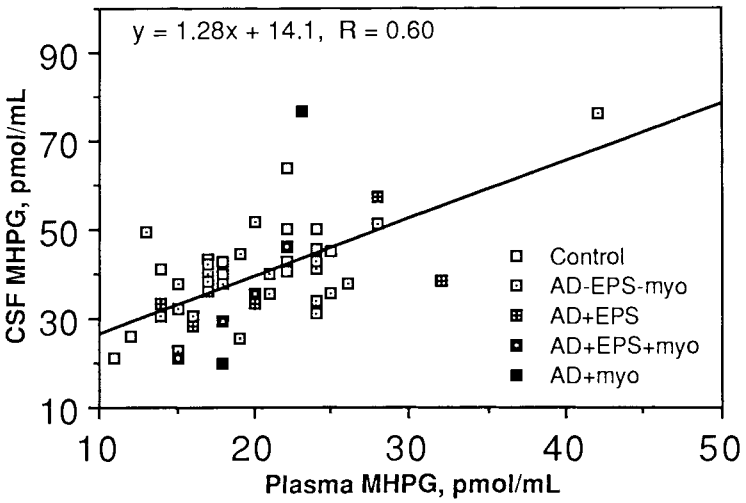

Fig. 2. Correlation between CSF and plasma MHPG concentrations. The significant correlation ($r = 0.60$, $n = 48$, $p < 0.0001$) is consistent with MHPG freely crossing the blood-brain barrier, and measured CSF MHPG concentrations should consequently be corrected for the plasma contribution (corrected MHPG = CSF MHPG − (plasma MHPG × 0.9); Kopin et al. 1983). NB When the single high plasma MHPG data point (plasma MHPG = 42 pmol/ml) was omitted, the correlation between CSF and plasma MHPG concentrations remained significant ($r = 0.47$, $n = 47$, $p < 0.0001$). *EPS*, Extrapyramidal signs; *myo*, myoclonus

may be measured either in simultaneously obtained ventricular and lumbar CSF (samples of which are not available from normal subjects and are generally available only from neurological patients with, for example, normal pressure hydrocephalus) or, more usually, in sequential fractions of lumbar CSF. Higher concentrations in more rostral CSF fractions indicate primarily a brain origin (e.g. homovanillic acid, or HVA; Fig. 3) whereas less pronounced or non-existent gradients (e.g. AChE, somatostatin, neuropeptide Y; Fig. 3) reflect a more diffuse (brain and spinal cord) origin. The other method is measurement of concentrations in lumbar CSF of patients with blockage of the spinal canal (Post et al. 1973a). If a neurochemical marker is derived primarily from the brain, its concentration in lumbar CSF of subjects with spinal canal blockage should be much lower than in subjects without blockage. On the other hand, if the marker originates primarily from the spinal cord, then its concentrations should be similar in subjects with and without blockage.

In general, HVA in lumbar CSF originates primarily from the brain whereas the major serotonergic metabolite 5-HIAA (5-hydroxyindoleacetic acid) has both a brain and a spinal contribution. Although CSF MHPG is derived in part from plasma, lumbar CSF concentrations nevertheless seem to reflect, to a more limited extent than HVA and 5-HIAA, central neurotransmission with both a brain and spinal component being present. The lack of rostrocaudal gradients for AChE and a number of neuropeptides (Fig. 3; Berrettini et al. 1988) is consistent with a relatively diffuse (i.e. brain and spinal cord) origin within the CNS.

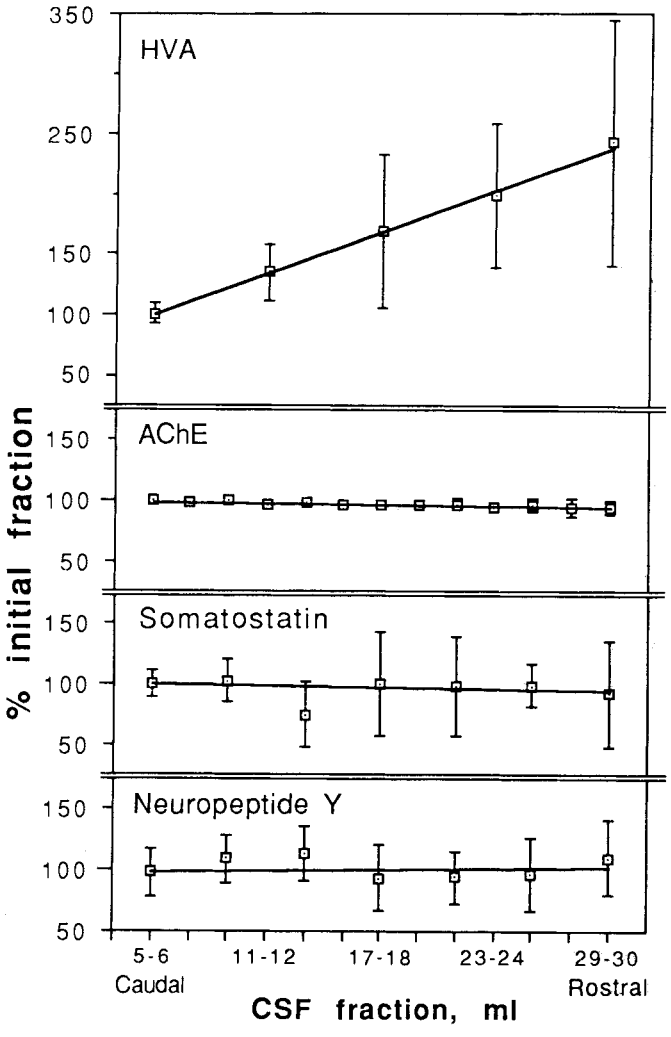

Fig. 3. CSF AChE activity and HVA, somatostatin and neuropeptide Y concentrations in sequential fractions of lumbar CSF obtained from healthy volunteers. Higher HVA in more rostral fractions suggests that lumbar CSF HVA originates primarily from the brain whereas the relatively flat gradients of AChE, somatostatin and neuropeptide Y are consistent with a relatively diffuse origin (i.e. brain and spinal cord) of these substances. Values shown are means (± SD) of values obtained in CSF from 14 healthy subjects in the case of HVA, AChE and neuropeptide Y or from 7 subjects for somatostatin. (For AChE, the variance in the data was relatively small and consequently the error bars were smaller than the size of most of the data points.)

Selection of Control and Alzheimer's Disease Subjects

When studying CSF neurochemical changes in AD, the selection of appropriate control subjects is very important. Although we are fortunate to have access to CSF from extensively screened healthy subjects (Duara et al. 1983) who are free from significant medical complaints and drug therapy and admitted as inpatients undergoing lumbar puncture under carefully controlled conditions, such a control group is not the norm. More usually CSF is obtained from neurological controls undergoing lumbar puncture for a variety of disorders. If control and AD CSF is not collected under similar conditions using standardized procedures, there may be differential effects between groups of a number of factors, including environ-

ment (Guthrie et al. 1986; Karlsson et al. 1988; Widerlov et al. 1989), physical activity (Post et al. 1973b), diet (Teff et al. 1989), time of day (Nicoletti et al. 1981; Sorensen et al. 1987) and time of year (Brewerton et al. 1988; Csernansky et al. 1988).

The selection of probable AD patients (McKhann et al. 1984) is of equal importance, and again we have been able to obtain lumbar CSF from AD patients free of other significant medical complaints, all of whom were drug free for a period of 2–4 weeks prior to lumbar puncture. If drug-free subjects are not available, drug effects may be of particular importance if there is a difference between drugs taken by AD and control subjects since drugs can alter brain and CSF neurochemical compositions. For example, neuroleptics increase CSF HVA concentrations (Scheinin 1985) while decreasing brain and CSF somatostatin and opioid-peptide levels (Tang et al. 1983; Beal and Martin 1984; Doran et al. 1989).

CSF Cholinergic, Monoaminergic and Peptidergic Markers in Alzheimer's Disease

In our AD patients, we observed reductions in the activity of AChE (16%) and concentrations of HVA (33%), somatostatin (26%), CRF (30%), corticotropin (ACTH; 14%) and α-melanocyte-stimulating hormone (α-MSH; 31%) relative to age-matched control subjects (Table 1). However, there were no significant correlations between any of these neurotransmitter markers and dementia severity assessed using the Mini-Mental State Exam (MMSE; Folstein et al. 1975). No significant differences in either 5-HIAA, MHPG, biopterin or neuropeptide Y were observed between groups (Table 1).

Table 1. Neurochemical markers in CSF of patients with Alzheimer's disease

Parameter	Age-matched control	Probable Alzheimer's disease
Age	68.0 ± 10.8 (14)	67.8 ± 8.2 (38)
Men/women	11/3	18/20
AChE (nmol ml^{-1} min^{-1})	21.5 ± 5.6 (13)	18.0 ± 4.8 (37)[b]
HVA (pmol/ml)	202 ± 100 (14)	133 ± 83 (38)[b]
5-HIAA (pmol/ml)	91 ± 51 (14)	99 ± 46 (38)
CSF MHPG (pmol/ml)	39.8 ± 12.7 (14)	39.2 ± 12.0 (38)
Plasma MHPG (pmol/ml)	19.3 ± 4.7 (13)	20.5 ± 5.9 (35)
cMHPG (pmol/ml)[a]	24.5 ± 7.9 (13)	20.8 ± 10.4 (35)
Biopterin (pmol/ml)	17.9 ± 5.6 (11)	14.1 ± 5.4 (31)
Somatostatin (pg/ml)	23.1 ± 8.2 (13)	17.2 ± 4.9 (37)[c]
Neuropeptide Y (pg/ml)	38.2 ± 12.8 (13)	36.6 ± 11.6 (37)
CRF (pg/ml)	35.2 ± 10.0 (13)	24.8 ± 9.6 (33)[c]
ACTH (pg/ml)	30.6 ± 5.5 (12)	26.4 ± 6.3 (33)[b]
α-MSH (pg/ml)	21.8 ± 10.0 (12)	15.0 ± 8.3 (35)[c]

Values shown are mean ± SD. Figures in parentheses show sample size. Data derived from May et al. 1986; Atack et al. 1988a, b; Kaye et al. 1988a b; Rainero et al. 1988b.

[a] CSF MHPG corrected for plasma contribution [cMHPG = CSF MHPG − (0.9 × plasma MHPG); Kopin et al. 1983]

[b] $p < 0.05$

[c] $p < 0.01$

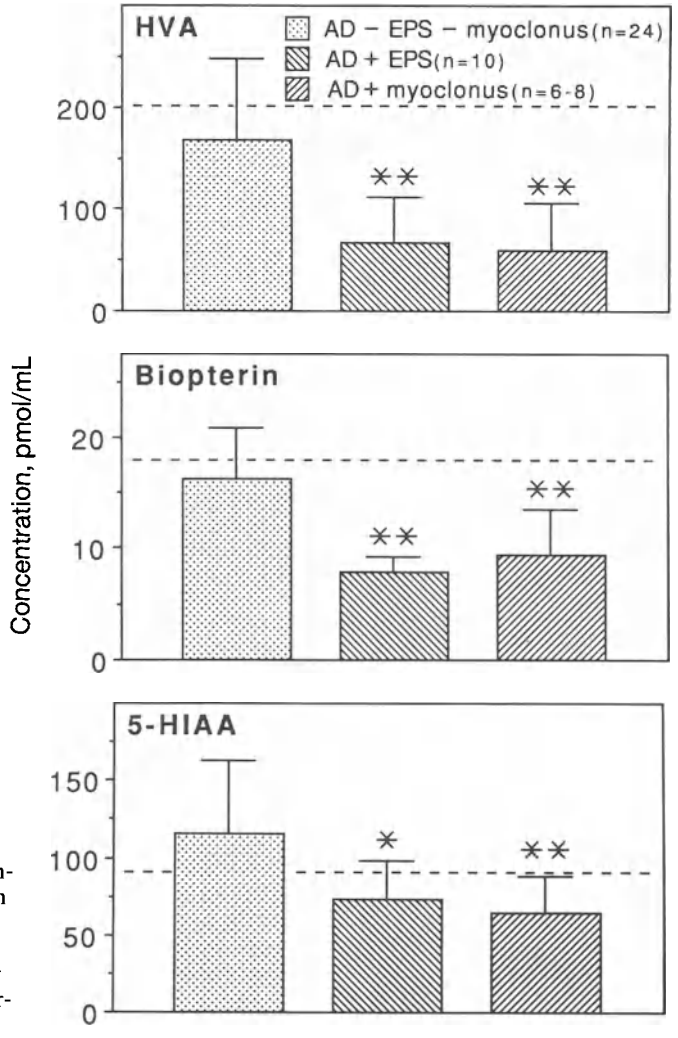

Fig. 4. Lumbar CSF concentrations of HVA, biopterin and 5-HIAA in subgroups of AD patients recognized on the basis of presence or absence of movement disorders. Values shown are means and error bars show standard deviations; *, $p < 0.05$; **, $p < 0.01$ compared to AD patients without movement disorders; *dashed lines,* mean values in age-matched controls (Table 1). In patients without movement disorders ($n = 24$), with extrapyramidal signs *(EPS);* ($n = 10$) or with myoclonus ($n = 6–8$), respectively, mean values (± SD) were: age = 70.4 ± 7.2, 64.8 ± 7.1 and 60.4 ± 7.5 years; age at onset = 63.8 ± 6.4, 58.2 ± 7.3 and 54.0 ± 7.1 years; durations of dementia = 6.6 ± 2.9, 6.6 ± 2.2 and 6.0 ± 2.8 years; MMSE = 14.8 ± 7.8, 5.1 ± 7.3 and 4.3 ± 5.4; HVA = 168 ± 80, 68 ± 44 and 60 ± 46 pmol/ml; biopterin = 16.1 ± 4.7; 7.8 ± 1.4 and 9.4 ± 4.1 pmol/ml; and 5-HIAA = 116 ± 47, 73 ± 25 and 64 ± 24 pmol/ml. In addition to reduced HVA, biopterin and 5-HIAA concentrations, AD patients with either EPS or myoclonus also had significantly ($p < 0.05$) earlier ages at onset and dementia severity (despite comparable durations of dementia) than AD patients without movement disorders. [It should be noted that in a previous analysis (Kaye et al. 1988b), a non-significant reduction in 5-HIAA concentrations was reported in AD patients with extrapyramidal subjects when compared to AD subjects without extrapyramidal signs. However, this latter group contained subjects with myoclonus, and when these individuals were omitted from the comparison group (i.e. AD patients with no movement disorders), the decrement in 5-HIAA in extrapyramidal AD patients achieved levels of significance.]

Of the 38 AD patients, 10 had extrapyramidal signs and 8 had myoclonus (4 had both extrapyramidal signs and myoclonus), and 24 had no movement disorders. Despite similar durations of disease (mean, 6.0–6.6 years, see legend Fig. 4), both the extrapyramidal and myoclonic subgroups had a significantly younger mean age at onset (58.2 ± 7.3 and 54.0 ± 7.1 years, respectively) and were more demented (MMSE, 5.1 ± 7.3 and 4.3 ± 5.4, respectively) than AD subjects without movement disorders (age at onset, 63.4 ± 6.4 years; MMSE, 14.8 ± 7.8), suggesting a more aggressive disease process in AD patients with movement disorders.

Compared to AD patients without movement disorders, HVA concentrations in AD patients with extrapyramidal signs or myoclonus were reduced by 66% or 70%, respectively (Fig. 4). This dopaminergic deficit in AD with movement disorders is further emphasized by the fact that biopterin, a cofactor for tyrosine hydroxylase (which catalyses the rate-limiting step in the dopamine synthetic pathway – the hydroxylation of tyrosine to L-dopa), was also significantly reduced by 52% in extrapyramidal patients and 42% in myoclonic patients relative to AD patients without movement disorders. In addition to a dopaminergic deficit, the 37% and 45% reductions in 5-HIAA concentrations in AD with extrapyramidal signs or myoclonus, respectively, relative to AD without movement disorders suggest that extrapyramidal signs and myoclonus in AD are also accompanied by a serotonergic deficit. None of the other parameters studied (AChE, 5-HIAA, cMHPG, somatostatin, neuropeptide Y, CRF, ACTH, α-MSH) differentiated AD patients with extrapyramidal signs or myoclonus from patients without movement disorders.

In addition to analysing data according to the presence or absence of movement disorders, we have also compared neurochemical parameters according to age at onset (Fig. 5). To eliminate bias due to monoamine deficits in subjects with movement disorders (who had younger age – at onset), analyses were confined to subjects with no movement disorders. We found that AChE activity and HVA concentrations were significantly reduced in early – compared to late – onset AD (Fig. 5).

To determine whether the decrements in AChE activity and somatostatin concentrations were progressive, we also measured levels of AChE and somatostatin (as well as neuropeptide Y) longitudinally in 5 control and 18 AD patients (Atack et al. 1988a, b). However, there was no tendency for any of these parameters to decrease systematically over a period of up to 2.5 years, although it should be emphasized that this period of time may be too short compared to the duration of AD (up to 10–15 years) to show progression of CSF neurochemical changes.

Discussion

Despite the greater than 50% reductions in AChE activity generally reported to occur in AD brain tissue, the decrement that we observed in the AD group as a whole was much more modest (16%). This discrepancy may be due to differences in severity in dementia between the patients used for post-mortem studies (usually severely demented) and CSF studies (generally less severe). However, the fact that a cholinergic deficit is observed in biopsy tissue of AD patients even in the

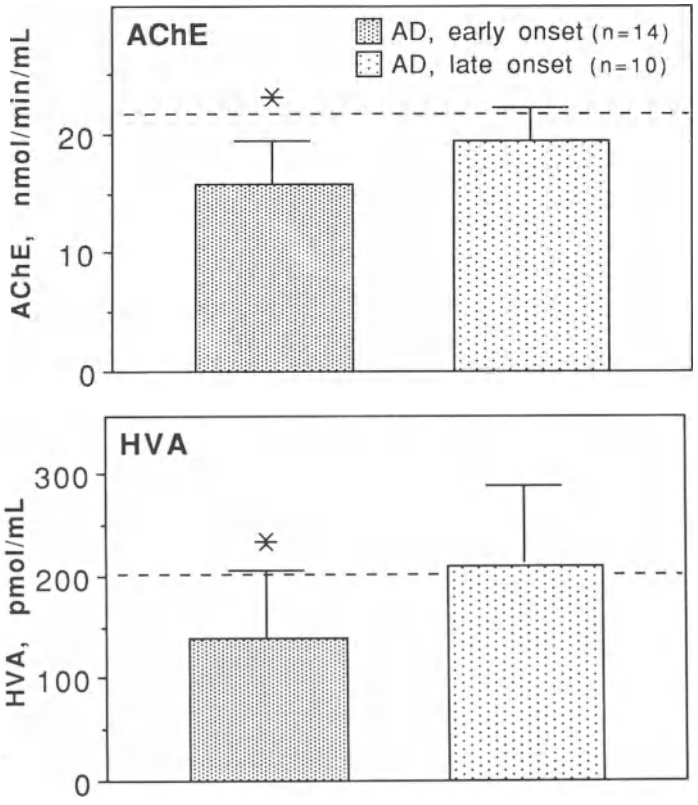

Fig. 5. AChE and HVA concentrations in early- ($\leq$ 65 years) and late-onset ($>$ 65 years) AD subjects with no movement disorders (i.e. no extrapyramidal signs or myoclonus). Values shown are means, and error bars show standard deviation; *, $p < 0.05$ compared to late-onset patients; *dashed lines*, mean values in age-matched controls (Table 1). In early-, and late-onset AD, the respective values were 15.8 $\pm$ 3.7 and 19.5 $\pm$ 2.7 nmol ml^{-1} min^{-1} for AChE activity and for HVA concentrations were 139 $\pm$ 67 and 209 $\pm$ 80 pmol/ml

relatively early stages of the disease process (Francis et al. 1985) suggests that the smaller reduction in CSF AChE compared to post-mortem brain tissue is probably not solely due to differences in dementia severity. It is more likely, however, that since AChE has a relatively diffuse origin within the CNS (Fig. 3), normal secretion of AChE from regions of the CNS relatively spared by AD (e.g. caudate nucleus, cerebellum and spinal cord) masks reduced cortical secretion of AChE.

Reductions in cortital somatostatin concentrations (Davies et al. 1980; Beal et al. 1986b) also appear to be reflected, to a limited extent, by reduced levels in lumbar CSF, although, as with AChE, the relatively diffuse central origin of somatostatin (Fig. 3) probably results in less affected regions of the CNS partially masking reduced cortical somatostatin secretion. Although neuropeptide Y has been reported to be reduced in AD brain tissue (Beal et al. 1986a), lumbar CSF concentrations were normal. However, neuropeptide Y involvement in AD is less consistent than is the case for somatostatin and may be found only in more severe

cases (Dawbarn et al. 1986; Foster et al. 1986). Consequently, normal lumbar CSF neuropeptide Y concentrations in the present study may be consistent with relatively normal brain neuropeptide Y concentrations in these subjects.

Interestingly, whereas reduced lumbar CSF CRF concentrations presumably reflect changes in brain CRF (Bissette et al. 1985; DeSouza et al. 1986), relatively little is known of the neurobiological basis for reduced ACTH concentrations in AD, although the interaction between CRF and ACTH could explain these findings. Similarly, the basis for reduced α-MSH concentrations is uncertain. However, we found a significant negative correlation between lumbar CSF α-MSH and HVA concentrations (Rainero et al. 1988b) which, along with the increased lumbar CSF α-MSH concentrations in Parkinson's disease (Rainero et al. 1988a), suggests an interaction between α-MSH and the dopaminergic system, the nature of which remains to be determined.

Although the similar dopaminergic and serotonergic deficits observed in AD patients with extrapyramidal signs or myoclonus may be partly related to that fact out of a total of ten extrapyramidal and eight myoclonic patients, extrapyramidal signs and myoclonus coexisted in four individuals, these data nevertheless suggest that the clinical heterogeneity of AD (Mayeux et al. 1985; Roth 1986; Folstein et al. 1988) has a neurochemical substrate.

The neurochemical heterogeneity of AD is further emphasized by the observations that AChE activity and HVA concentration were significantly lower in early – compared to late – onset AD. Similarly, neurochemical changes in post-mortem brain tissue tend to be greater in younger compared to older AD cases (Whitehouse et al. 1983; Rossor et al. 1984; Francis et al. 1985; Rossor and Iversen 1986), and two subtypes of AD can be identified on the basis of locus coeruleus cell counts (Bondareff et al. 1987).

In summary, decrements in a number of neurochemical markers reported to be reduced in brain tissue were also observed in lumbar CSF of AD subjects. However, AChE activity and somatostatin concentrations remained relatively stable in repeat lumbar punctures with no tendency for either to decline with progression of the disease. Moreover, none of the parameters studied correlated with dementia severity, and the relatively modest nature of these decrements (about 14%–33%), along with the considerable overlap between AD and control groups, suggests that none of the parameters alone is of diagnostic use.

Neurochemically distinct subgroups of AD patients were also identified. Thus, in AD patients the occurrence of extrapyramidal signs or myoclonus was associated not only with a younger age at onset but also reduced concentrations of dopaminergic and serotonergic markers. Furthermore, in AD without movement disorders, early-onset patients had lower AChE activity and HVA concentrations than late-onset patients. These data are therefore consistent with AD being a heterogeneous disease process with patients of an early age at onset being more likely to develop dopaminergic and serotonergic deficits associated with extrapyramidal signs or myoclonus or, in the absence of movement disorders, having greater involvement of central cholinergic or dopaminergic systems.

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Potential Diagnostic Markers for Alzheimer's Disease

P. Davies

Introduction

After a decade of intensive research it is now clear that there are several abnormalities in the protein composition of brains of patients who have died with Alzheimer's disease. At least some of these abnormalities are likely to be detectable in the cerebrospinal fluid, and perhaps in the blood, of living patients. This work raises the possibility that biologic tests to help in the diagnosis of this condition could be developed, and indeed there are claims being made that such tests will be available in the very near future. This chapter examines the potential sensitivity and specificity of some possible diagnostic markers for Alzheimer's disease.

Amyloid

A very prominent feature of senile plaques in the brains of patients with Alzheimer's disease is the deposition of an amyloid, either in the form of amorphous deposits or sometimes as a dense plaque core. Extensive amyloid deposition in cerebral blood vessels is also common in such cases. The composition of this amyloid is becoming clear, following the first successful isolation and sequencing studies of Wong and Glenner (Wong et al. 1985; Masters et al. 1985). The major component appears to be a peptide of 42 or 43 amino acids which is apparently deposited following cleavage of a larger precursor protein. The precise nature of this precursor is not yet established; the amyloid peptide sequence is apparently contained within at least three proteins, all of which are the product of the same gene (Goldgaber et al. 1987; Kang et al. 1987; Kitaguchi et al. 1988; Ponte et al. 1988; Tanzi et al. 1987). This gene, which has been assigned to chromosome 21, can produce at least three different messenger RNAs differing in length, and hence three proteins of slightly different size. Which of the three is cleaved to produce the amyloid peptide is not yet clear. There is some evidence that at least one of the possible precursors is heavily glycosylated, and it has been known for some time that some glycoprotein or glycopeptides are present in amyloid deposits.

Given that the deposition of amyloid involves the cleavage of at least one of the tentatively identified precursor molecules, it seems possible that some fragment or fragments of the precursor might be released into the cerebrospinal fluid. Because of the extensive vascular amyloid deposits found in most cases of Alzheimer's dis-

ease, it also seems possible that fragments of the precursor may be detectable in blood. There are no publications to support this proposal, but there can be little doubt that this is a very active area of investigation.

The single largest problem with the use of amyloid-related diagnostic testing will probably be the frequency of amyloid deposition in nondemented elderly people. Senile plaques and amorphous amyloid deposits are found with very high frequency (over 75% in most series) in patients over the age of 70 without evidence of intellectual or functional impairment (Tomlinson et al. 1968; Davies et al. 1988; Katzman et al. 1988). Vascular amyloid deposits are also found with high frequency in this population. It is possible that cleavage of precursor molecules to give rise to amyloid deposits occurs by different mechanisms in aging as compared to Alzheimer's disease, but as yet there is no indication that this is the case. Unless some fragment of the precursor can be found that will allow discrimination of Alzheimer patients from the nondemented elderly, efforts to develop diagnostic tests for amyloid deposition may be of limited usefulness, perhaps restricted to studies of younger patients with dementia, in whom amyloid deposition is rare in the absence of disease.

α-1-Antichymotrypsin (ACT) has also been detected in plaques (Abraham et al. 1988). This protein is normally present in only very small amounts in brain, and thus detection of this molecule in cerebrospinal fluid might be proposed as a potential diagnostic. However, as with amyloid, ACT seems to be present in plaques of both Alzheimer's disease patients and the nondemented elderly, and discrimination of these two populations may be problematic.

Both the putative amyloid precursors and ACT are probably relatively abundant proteins in tissues other than brain. The presence and possible processing of these proteins in nonneural tissues will need to be considered in attempts to develop diagnostic markers.

Tangle Proteins

The second hallmark lesion of Alzheimer's disease is the neurofibrillary tangle, and this structure has come under intensive biochemical scrutiny over the past few years. Definitive evidence for the presence of two proteins within the tangle has been obtained, and indirect evidence for the presence of at least three other proteins has been presented. The two proteins that have been isolated from tangles are tau, a microtubule-associated protein (Kosik et al. 1986; Goedert et al. 1988; Wischik et al. 1988), and ubiquitin (Mori et al. 1987; Perry et al. 1987), probably a marker added to abnormal proteins to signal that they should be degraded. Neurofilament proteins, other microtubule-associated proteins, and the amyloid precursors have been suggested to contribute material to the tangle (Goldman and Yen 1986; Cork et al. 1986; Yen et al. 1987), but the presence of these proteins or fragments of them has not yet been definitively shown.

All of the above proteins are normally present in brain tissue, most of them being specifically found in neurons. Presumably they are all subjected to some modification process during incorporation into the tangle. The molecular details of this modification process are far from clear, although aberrant phosphorylation

of tau and neurofilament proteins may occur. The basic strategy being employed to attempt to develop diagnostic tests is to use antibodies that react with some component of the tangle and to search for cross-reactive material in cerebrospinal fluid and/or blood. One report of such a study appeared some years ago (Mehta et al. 1985), and further data were presented at a recent IPSEN foundation meeting (Wisniewski and Iqbal 1988). Several potential problems with this approach have been identified. First, because the tangle seems to be composed of normal brain proteins, any condition that involves neuronal destruction seems likely to release these proteins into the cerebrospinal fluid. Thus great care would have to be taken to select antibodies that specifically reacted with the modified forms of these proteins that become incorporated into tangles. As mentioned above, these modifications appear to render normally soluble proteins insoluble in most aqueous media; whether or not modified forms of tangle proteins exist in cerebrospinal fluid is yet to be established.

Perhaps the most serious problem in the use of tangle-related antibodies for the diagnosis of Alzheimer's disease is the observation that many elderly demented individuals have only a few tangles in the hippocampus, and very few in other brain regions, despite the presence of numerous senile plaques. Terry and his colleagues (Terry et al. 1987; Hansen et al. 1988; Katzman et al. 1988) have discussed this point at length, and it is notable that the NINCDS/ADRDA criteria for the pathologic diagnosis of Alzheimer's disease in the elderly patient (Khachaturian 1985) does not require the presence of tangles in the neocortex. Furthermore, tangles are present in significant numbers in several disorders apart from Alzheimer's disease, including progressive supranuclear palsy and dementia pugilistica (Goldman and Yen 1986). Even if technical problems can be overcome, it does not seem likely that methods for detection of tangle-related elements in cerebrospinal fluid can provide completely specific diagnoses of Alzheimer's disease.

Alz-50 and A68

In 1986, Wolozin et al. (1986) reported that a monoclonal antibody which they called Alz-50 reacted with a protein termed A68 that was greatly enriched in the brains of patients dying with Alzheimer's disease. Subsequent studies reported the presence of the protein in the cerebrospinal fluid of at least some living patients (Wolozin and Davies 1987), and several groups have confirmed that A68, if present at all in the normal adult, is very difficult to detect (Hyman et al. 1988; Tabaton et al. 1988). Transient expression of an A68-like molecule during the first 2 years of human life has been reported (Wolozin et al. 1988), but it is essentially undetectable after age 2 years.

Direct and clear demonstration of the presence of A68 has been possible only in brain tissue from patients with Alzheimer's disease and elderly Down's syndrome individuals. Using sensitive immunocytochemical methods, Alz-50 reactivity has been found in Pick's disease, progressive supranuclear palsy, and a few other, very rare neurologic conditions (Wolozin and Davies 1987; Tabaton et al. 1988). However, in none of these diseases is the amount of immunoreactivity sufficient to

determine whether it is due to A68 or a similar cross-reactive molecule. Thus the presence of large amounts of A68 appears to be found only in individuals with Alzheimer's disease and older (over 40 years) individuals with Down's syndrome.

The above would seem to suggest that detection of A68 in cerebrospinal fluid might have the potential to assist in the diagnosis of Alzheimer's disease. Much further work is needed before this potential can be realized. Samples of brain tissue from many more cases of both Alzheimer's disease and other neurologic disorders need to be examined to determine whether preliminary results hold up for a very large series of cases. Assays for A68 need to be improved – made more sensitive and more quantitative; so far it has been possible only to determine the presence or absence of the protein in the spinal fluid of patients, and not all the patients with a clinical diagnosis of Alzheimer's disease have tested positive. Whether the samples from patients that do not show the presence of A68 are errors in clinical diagnosis or are the result of assay unreliability needs further investigation.

DNA Markers

There are families in which Alzheimer's disease is clearly an inherited disorder, and progress appears to be being made in the use of linkage analysis with DNA probes to find the location of the gene defect causing the disease. In four families with very early onset of the disease, the gene appears to be on chromosome 21 (St George-Hyslop et al. 1987). With the development of DNA probes closer to the gene itself, screening to determine which members of this family are likely to develop the disease should be possible. How generally useful such probes will be is impossible to determine at this time.

DNA markers are unlikely to be useful in diagnosis for the vast majority of cases of Alzheimer's disease, in which there is either no clear pattern of inheritance or no predictability in the age of onset. Even if a gene determining susceptibility to Alzheimer's disease were identified, knowing that a particular individual patient carried this gene would not rule out the presence of dementia due to other diseases. Without a clear-cut pattern of disease inheritance, dementia could not be attributed to the presence of a particular gene without, as at present, ruling out other possible causes.

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Cerebrospinal Fluid-Based Laboratory Test for Alzheimer's Disease*

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Summary

Mouse monoclonal antibodies to specific paired helical filament (PHF) antigens and a sensitive immunoassay were used to evaluate the presence of PHF antigens in cerebrospinal fluid (CSF) of patients with Alzheimer's disease (AD) and non-AD controls. Our results showed higher levels of PHF antigens in CSF of AD patients than in CSF of non-AD controls. Although the range of values overlaps with those of non-AD controls, the higher values of PHF antigens in CSF of AD patients may be useful to the diagnosis of AD. Our data also showed the presence of beta-protein in CSF of patients with AD. Further evaluation of the concentrations of both PHF antigens and beta-protein in CSF of AD patients and their correlation with the degree of dementia are needed to determine their usefulness in the diagnosis of AD.

At present a conclusive diagnosis of Alzheimer's disease (AD) can be made only by correlating clinical findings (cognitive deficiency) and neuropathological studies of postmortem tissue. The leading neuropathological and biochemical changes correlated with the diagnosis of AD are: 1. amyloid deposits in the form of neuritic (senile) plaques and diffuse amyloid infiltrates and 2. paired helical filaments (PHF) which accumulate in neuronal perikarya, neurites, the neuritic plaques, and neuropil threads in nerve cell processes. An extensive loss (over 40%) of large and medium-sized nerve cells is seen in association with a deficiency in several neurotransmitters. Many other pathological changes are also found in AD (Wisniewski and Terry 1973). However, they have not been considered of critical importance in the diagnosis of AD. At this point we would like to stress that the same changes are found in the aged, but otherwise normal brain. However, there are many more lesions in the AD brain and they are more widely distributed in the CNS than in the normal aged brain. Recent studies (Ogomori et al. 1989) show that, indeed, in comparison with normal aging the expanded distribution of AD neuropathology is the most characteristic feature of AD.

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As indicated above the difference between AD and aged normal brain appears to be quantitative, not qualitative. The question then arises: Is AD a disease or part of the normal aging process? From a biological point of view aging should be considered a developmental process and is the stage when the cell, organ, or organism, after reaching a stage of *maximal* functional performance, will start to show evidence of functional decline. Differences in life span between various animal species and the existence of syndromes associated with premature aging (Brown and Wisniewski 1983) point to the existence of aging genes. From studies of Down syndrome (DS) and familial AD, we also know that gene expression influences the deposition of beta-protein amyloid. Topographical and individual susceptibility to PHF formation also points to the role of local gene(s) expression in the formation of these lesions.

Since AD appears to be a cerebral form of amyloidosis (Wisniewski et al. 1988), it is possible that local ectoantigens (toxic, bacterial, or viral) may play an essential role in AD amyloidosis, as has been observed in systemic and experimental amyloidosis. We consider AD a disease because the intensity of the pathology far exceeds that which is seen in a majority of normal *old-aged* individuals. Because there are no new lesions in AD, in comparison to age-matched controls, we have to assume the existence of a preclinical form of AD (Wisniewski et al., in press). This preclinical form of AD would be a condition in which the number of characteristic AD lesions (plaques and tangles or plaques with few, if any, tangles) would be equal to the neuropathological criteria for AD. From recent clinicopathological studies it is evident that the number of senile plaques by and large does not distinguish between demented and nondemented subjects (Dickson et al. 1988). However, the presence of many plaques and tangles correlates well with dementia. From these observations, it has become apparent that the presence of neurofibrillary changes has greater impact on the function of the brain than amyloid deposits. Tangles made of PHF occur in many, unrelated pathological conditions. In diseases where there are many PHF-bearing neurons, dementia is present, i.e., parkinsonism-dementia complex, dementia pugilistica, and sporadic cases with many neurons with neurofibrillary degeneration (Wisniewski et al. 1979). On the other hand, the conditions with beta-peptide amyloid deposits present are not common, i.e., AD, DS, normal aging, and Dutch familial vascular amyloidosis. Because PHF tangle formation is a result of neuronal responses to different "noxious" agents, individuals in whom many neurons show neurofibrillary changes without plaques are, therefore, not AD patients. However, patients with many plaques, but few, if any, neurons with neurofibrillary changes should be diagnosed as having AD. A great majority of AD patients have both plaques and tangles in the neocortex. In very old people with AD, however, there may be few tangles in the neocortex, but they are present in the hippocampus (Katzman et al. 1988). Evidence is accumulating that deposits of beta-peptide amyloid fibers may precede formation of neuronal perikarya localized neurofibrillary tangles.

The abnormal form of tau, a microtubule-associated protein shown to be part of the PHF, has also been shown by recent immunohistological studies to exist in a nontangle form (Bancher et al. 1989). We have used this information to develop our PHF-protein diagnostic test which is based on the existence (1) of a soluble PHF-protein present in the CSF, which is in a ubiquitinated, unpolymerized form,

and (2) on the observation that there is also an antigen in the CSF which reacts with anti-beta-peptide antibody.

Using a monoclonal antibody to PHF in an enzyme-linked immunosorbent assay (ELISA), CSF from groups of AD and control patients from different AD clinics was examined for the presence of PHF antigens. In our initial experiments, CSF specimens from 18 patients were collected by Dr. Leon Thal (Albert Einstein College of Medicine, Bronx, NY, USA). These included nine patients with AD (mean age ± SD, 63.9 ± 9.7 years) and nine non-AD controls (mean age ± SD, 55.6 ± 19 years). The non-AD controls consisted of patients with stroke, seizures, multiple sclerosis, and other neurological conditions. Only Alzheimer's disease/ senile dementia of the Alzheimer type (AD/SDAT) patients meeting research diagnostic criteria for the diagnosis of SDAT (Eisdorfer and Cohn 1980; McKhann et al. 1984) were included. The specimens were coded and the code was broken by Dr. Thal after completion of CSF analysis. The PHF antigen concentration was determined by a two-step competitive inhibition ELISA (Mehta et al. 1985). The mean concentration of PHF antigen in CSF from patients with AD was significantly increased compared with that of the non-AD controls ($P < 0.05$, one-tailed "t" test). Eight of the nine AD CSF specimens contained over 0.2 units of PHF antigen(s), whereas only three of nine controls exceeded this level (Fig. 1).

In another experiment, 40 CSF specimens were obtained from 14 patients with AD (mean age ± SD, 63.0 ± 8.2) and 26 non-AD controls (mean age ± SD, 51.2

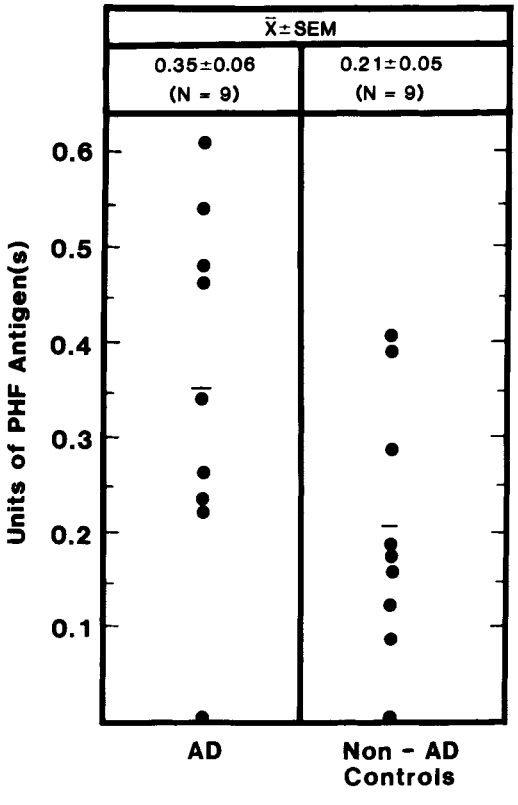

Fig. 1. Relative concentration of PHF antigen(s) in CSF of patients with AD and a group of non-AD controls. A unit was defined as the inhibition of ELISA obtained with 180 ng protein of PHF suspension

± 18.5). The latter included patients with multiple sclerosis, seizures, migraine, vasculitis, stroke, and other neurological disorders. Using a modified competitive inhibition ELISA assay, the CSF specimens from the AD patients showed a higher percentage inhibition of binding (mean ± SD, 72.6 ± 30.6) between PHF and the monoclonal antibody to PHF antigens than the control specimens (mean ± SD, 47.9 ± 21.8). This difference was statistically significant ($P < 0.005$, one-tailed t-test). Again, even though the difference was significant, some degree of overlap was observed between these groups (Fig. 2). The reason for this overlap is not clear. It is possible that the several non-AD controls which exhibited "false-positive" values may belong to a preclinical group of AD.

Comparison of monoclonal antibody 5-25 reactivity with PHF, ubiquitin (from bovine red blood cells, Sigma, St. Louis, MO, USA) and synthetic beta-peptide by direct ELISA revealed identical titers for PHF antigen and ubiquitin. There was no reactivity to the beta-peptide. However, it required 20-fold more ubiquitin to reach the same titer obtained against the PHF antigen. These findings are consistent with those of Grundke-Iqbal et al. (1988), who found that monoclonal anti-

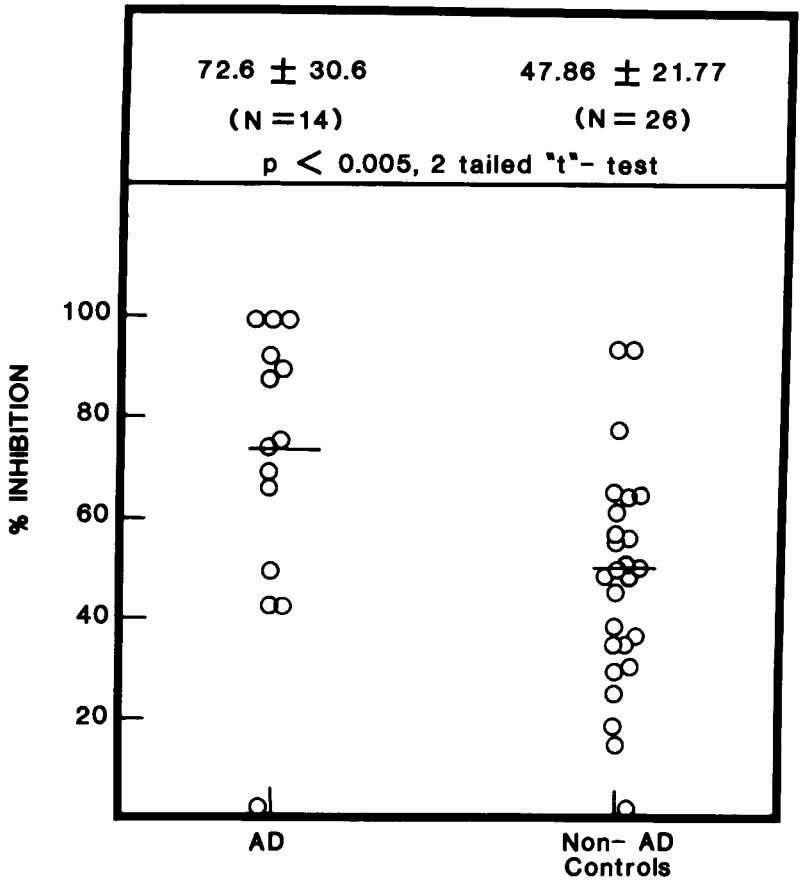

Fig. 2. Percent inhibition binding of AD and non-AD control CSF with monoclonal antibody to PHF

body 5-25 reacts with both PHF and ubiquitin in an immunoblot and dot blot assay. These observations suggest that the antibody 5-25 recognizes a ubiquitinated form of tau. They also indicate that ubiquitin can be used as a standard in the ELISA assay. One of the main advantages of using ubiquitin instead of a PHF suspension as the standard is that it is commercially available, and thus variability between assays will be minimal.

Because the AD neuropathology is also characterized by the presence of amyloid deposits, we also examined CSF for the presence of both PHF and beta-peptide antigens. Coded CSF specimens from 19 patients were provided by Dr. Barclay (Psychiatry Service, Westchester Medical Center, Cornell University, NY, USA). The specimens included five patients with AD (mean age ± SD, 73.8 ± 5.81) in late stage of the disease and 14 non-AD controls (mean age ± SD, 65.7 ± 13.14). The controls included CSF from patients with multi-infarct dementia, unspecified dementia, and CNS tumor. PHF antigens were quantitated by a two-step competitive inhibition ELISA using monoclonal antibody 5-25 with ubiquitin as the standard. When the code was broken, CSF from AD patients had significantly higher ($P < 0.025$) reactivity with the monoclonal antibody than did the 14 non-AD controls (Fig. 3).

To assay for beta-peptide antigen we used a monoclonal antibody raised against a synthetic peptide corresponding to the first 24 amino acid residues (Kim et al. 1988). The antibody selectively stains both vascular and neuritic plaque beta-peptide amyloid, but does not stain PHF or tau. Using a sandwich ELISA, we quanti-

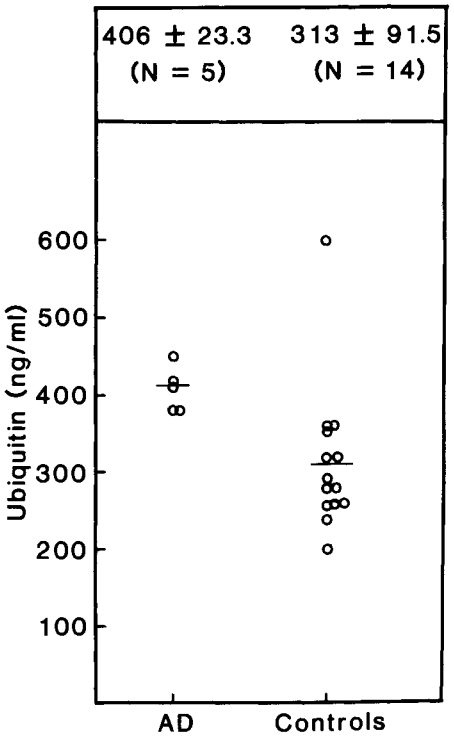

Fig. 3. Concentration of ubiquitin in CSF of AD and non-AD controls

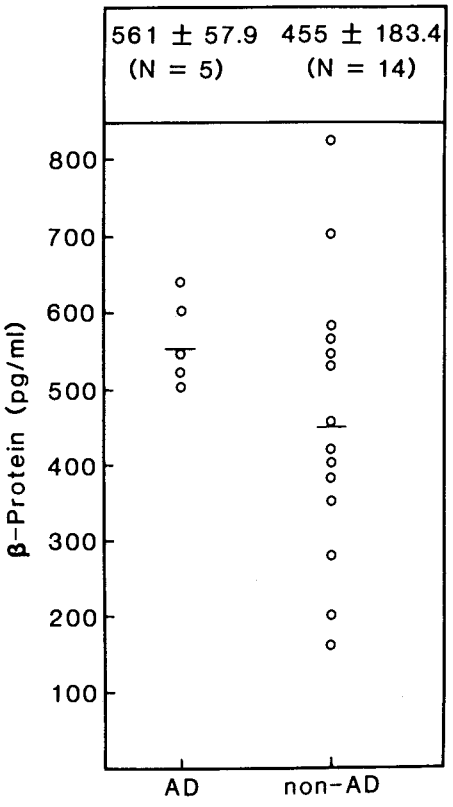

Fig. 4. Concentration of beta-protein in CSF of AD and non-AD controls

tated beta-protein antigen concentrations in the same 19 CSF specimens in a blind fashion. Although beta-peptide antigen was detected in both the AD patients and the controls, the concentrations did not differ significantly (Fig. 4). Thus, CSF specimens from AD patients have consistently had a higher concentration of PHF antigens than those of non-AD controls, but beta-protein levels (as measured in this series) were similar for both groups. These findings appear to be consistent with neuropathological data showing that, in patients with AD in late stage, the number of neurons with neurofibrillary changes was more closely associated with the degree of dementia than the number of plaques (Barcikowska et al., in press; German et al. 1987; Katzman et al. 1988).

In three separate analyses the concentration of PHF antigens was elevated in the CSF of AD patients. Although the range of values overlaps with those of other neurological diseases, the higher values may serve as a useful adjunct to the diagnosis and therapeutic response when combined with appropriate clinical neuropsychological findings. Because of the complex nature of the pathology, it is possible that no single biochemical marker will be specific for the diagnosis of AD. Our preliminary finding that amyloid beta-protein is present in AD CSF will permit the correlation of the concentrations of both PHF antigens and beta-protein in CSF of AD patients. The correlation of these measurements with neuropsychological

data and neuropathological findings is needed to determine their usefulness in the clinical diagnosis of AD.

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Probes for the Molecular Components of Plaques and Tangles Point to a Broadening View of Alzheimer Pathology

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Summary

The use of new antibody and cDNA probes has broadened our view as to the anatomical loci of the lesions in Alzheimer's disease. Amyloid is deposited in many locations in the central nervous system, including areas that are not clinically affected by the disease process. Likewise, the neurofibrillary lesions are seen in locations beyond the classical distribution of NFT; however, these lesions do remain restricted to regions such as the hippocampus, cerebrocortical association areas, and certain limbic and brainstem nuclei that are affected clinically. Associated with the neurofibrillary lesions are some of the light microscopic stigmata of a growth or regenerative response. The stimulus for this response may arise as a result of the deposition of β-amyloid protein. Regenerative-type neurites are present in neurons undergoing degeneration, and therefore these processes occur concurrently in the same cell. A major component of the neuritic response is the microtubule-associated protein tau, which we have shown to undergo developmentally regulated splicing. One of the previously reported sequences obtained directly from paired helical filament preparations corresponds to an immature tau isoform. The presence of an immature isoform in the paired helical filament may occur as a recapitulation of development, and its presence within a mature cellular milieu may be associated with the assembly of tau into filaments.

Introduction

Even a superficial look at the research into Alzheimer's disease (AD) reveals a broadening of the dichotomy between neurofibrillary tangles (NFT) and senile plaques, the two classical hallmarks of the disease. However, the commonly framed format of the debate concerning the fundamental importance of one or the other structure to the disease process is not nearly so useful as elucidating the relationship between these two lesions. The following observations suggest some of the hazards and complexities of arriving at a basic disease mechanism by considering either plaques or tangles in isolation. Neither NFT nor senile plaques are completely specific for AD. NFT without senile plaques occur in a number of pathological entities, such as subacute sclerosing panencephalitis (Wisniewski et al. 1979), dementia pugilistica (Corsellis et al. 1973), and the Guamanian Parkinson dementia complex (Wisniewski et al. 1970), to name only a few (Wis-

niewski et al. 1979) [Whether in each of these diseases the ultrastructural correlates of NFT are Alzheimer-type paired helical filaments is not completely resolved (Wischik and Crowther 1986).] While senile plaques are considerably more specific for AD, the amyloid protein is also deposited in hereditary Dutch disease (Van Duinen 1987). Senile plaques can also occur, even in numbers sufficient to diagnose Alzheimer's disease, without any clinical evidence of dementia (Katzman et al. 1988). Furthermore, amyloid precursor protein deposits diffusely in the cerebellum and spinal cord of some Alzheimer patients without any apparent clinical dysfunction localized to these tissues (Yamaguchi et al. 1989). Diffuse plaques were detected by the Bielschowsky silver stain in earlier studies of AD brain, but they have only recently received attention due to their labeling with antibodies to the β-amyloid protein (Ulrich 1985; Tagliavini et al. 1988; Yamaguchi et al. 1988, 1989; Mann and Isiri 1988; Joachim et al. 1988; Struble et al. 1988; Kitamoto et al. 1987).

These diffuse plaques are not likely to contain β-amyloid protein in a β-pleated sheet conformation since they are not detected by Congo red or thioflavin. A feature of diffuse plaques is that they do not appear to be associated with a neuritic response.

While it is possible that deposition of the amyloid precursor protein may be an early manifestation of the disease process, the observed clinical impairment may more closely parallel the neurofibrillary lesions, the compaction of amyloid into senile plaques, or ultimately the neuronal loss. If this scenario is correct, it raises the question as to why certain brain regions, such as the cerebral cortex and regions of the limbic system, develop a neurofibrillary reaction to the amyloid, whereas other regions, such as the cerebellum and spinal cord, do not.

Tau Immunocytochemistry

Because the NFT has nearly become a household word, it is worth briefly describing how the concept of this lesion has grown from its original description using silver stains to its recent immunohistochemical analysis with highly specific antibodies. The perceived scope of pathological features encompased by the neurofibrillary lesions has been limited by equating this aspect of the pathology solely with NFT. However, it has become quite clear that NFT represent only one aspect of a host of neurofibrillary-type lesions. NFT have been well visualized by antibodies to the microtubule-associated protein, tau (Brion et al. 1985; Wood et al. 1986; Grundke-Iqbal et al. 1986b; Kosik et al. 1986; Delacourte and Défossez 1986; Nukina and Ihara 1986; Yen et al. 1987). Tau protein is of interest to investigators from strikingly diverse vantage points. As a microtubule-binding protein it serves as a means of probing microtubule function and dynamics. As a microtubule-associated protein that is compartmentalized within neurons, it is a window upon neuronal polarity, the establishment of axonal identity, and possibly the selective sorting of molecules or organelles to the axon. As a protein that is developmentally regulated in the expression of its isoforms, it serves as a starting point for the identification of regulators of brain development and morphology. As a phosphoprotein it leads to the characterization and function of brain kinases.

And finally, as a component of the Alzheimer NFT, it clearly has a role in brain pathology.

In addition to labeling NFT, tau antibodies label a massive neuritic dystrophy that is more pervasive than either the NFT or the senile plaques (Braak et al. 1986; Kowall and Kosik 1987). The dystrophic neurites extend well beyond the neuritic portion of the senile plaque, which surrounds the amyloid core, to regions such as layer I of the cerebral cortex, where senile plaques are rare. The identification of these structures as either axons or dendrites has been difficult. In favor of a dendritic origin is the fact that occasionally these neurites are observed in contiguity with the apical dendrite of pyramidal cells (McKee et al. 1989), and that they are observed in dendritic fields, particularly the region around the layer II entorhinal star cell clusters. These clusters of neuronal somata are surrounded, under normal conditions, by a rich dendritic plexus that is readily demonstrated with antibodies to MAP2. The clusters and their dendritic surrounds are separated by axonal bundles. Unlike control tissue, in AD the dendritic fields are readily labeled by the tau antibody in a dystrophic pattern. Since we have observed that tau consistently labels axons and has failed to label neuronal somata and dendrites, the presence of tau-immunoreactive NFT in the somatodendritic region of the neuron represents an aberrant localization of the antigen, and tau reactivity in distal dendritic fields as dystrophic neurites is even more displaced. One possibly unifying hypothesis is that the abundant neuritic dystrophy of AD represents ectopic axonal growth from dendrites.

Many of these neurofibrillary features appear related to a widespread regenerative process, in that one observes supernumerary basilar dendrites on pyramidal cells, projections from tangle-bearing cell somata of filopodial- and lamellipodial-like structures, and the proliferation of the apical dendritic tree in the form of dystrophic neurites or "curly fibers" (McKee et al. 1989). While these features might not strike one as typical of regeneration, dendritic growth is, in fact, a type of primary response that a vertebrate neuron can undergo. The "close axotomy" in the lamprey (Hall and Cohen 1983, 1988a, b; Hall et al. 1989), a system ideally suited for the in situ observation of regeneration, demonstrates a possibly analogous pattern. In this model close axotomy results in ectopic axonal regeneration from the dendrites. The more familiar axonal sprouting, which results from a distal axotomy, has also been observed in AD (Geddes et al. 1985) but may not contribute as massively to the neurofibrillary pathology. Our observations in cerebrocortical neuronal cell cultures suggest that during regeneration of neurites after plating tau is transiently present in dendrites (Kosik and Finch 1987). We have also observed that a subset of the curly fibers are labeled by a ubiquitin monoclonal antibody raised in our laboratory, and thus the presence of ubiquitin conjugates within these structures suggests the possibility of ongoing simultaneous degeneration and regeneration within the same cellular locus.

Polarity and the Compartmentation of Tau Protein

The basic mechanisms by which neurons establish, maintain, and reestablish polarity after injury clearly arise, directly from AD pathology. In this area the

work of Banker has been seminal. Although limited to cultured hippocampal pyramidal cells at low density on a polylysine substrate, his work has demonstrated specific sorting of molecules to axons and dendrites, and, along with other workers, he has suggested that a basis for selective sorting lies in the orientation of microtubules. While microtubules have their plus ends directed distally in the axon, they appear to orient in both directions in the mature dendrite (Baas et al. 1988). Perhaps more germane to the scientific underpinnings of this proposal is Banker's finding of the intrinsic plasticity in neurites, such that after a close axotomy any of the dendrites can undergo a transformation to an axon (Dotti and Banker 1987). The potential traffic problems encountered to direct appropriate sorting pari passu with the establishment of axonal or dendritic identity are staggering, in that molecules and organelles are being rearranged while certain microtubule-based transit systems are becoming uniform in their orientation.

Where does tau fit with regard to questions concerning neuronal polarity? Although not universally agreed upon, a significant body of data, including our own, suggest that tau protein is most abundant in neurons, and within neurons it is enriched in the axonal compartment. Our data are based on immunocytochemical patterns with various tau monoclonal antibodies in both tissue (Kowall and Kosik 1987) and in culture (Kosik and Finch 1987), Northern blots of various tissues (Neve et al. 1986), and in situ hybridization (Kosik et al. 1989b) from which a consistent picture emerges. The axonal localization of tau raises a problem commonly encountered by the neuron – that is, since the axon is devoid of ribosomes beyond the axon hillock, how does the protein become compartmentalized? For some proteins, particularly those transported within vesicles, a potential answer lies in the orientation of the microtubule, since vesicle translocator molecules, e.g., kinesin and dynein, are defined in terms of their movement either toward or away from the plus end of microtubules. For tau, one theroretically possible way in which some degree of compartmentation might be obtained is a positioning of the tau ribosomal population such that their synthetic products can be directly launched into the axon. In fact, this is not the case, since we have shown, by in situ hybridization using probes to two discrete nonoverlapping regions of the tau sequence, that the population of tau mRNAs is present throughout the cell body and extends into the proximal dendrite. The dendritic extent of hybridization was comparable to a ribosomal RNA probe. Two possibilities that might explain the localization are: (a) tau is only immunocytochemically detectable in the axon because its antigenic presentation changes upon entry into the axon; (b) tau moves through the somatodendritic compartment by diffusion so that its levels are low, but once entering the axon it moves by slow transport and thus accumulates. The presence of NFT-related tau immunoreactivity in the somatodendritic domain and tau immunoreactivity in more distal dendritic fields suggests a highly aberrant localization of the protein in AD. The site of NFT deposition, in fact, more closely resembles the distribution of the tau ribosomal population than the tau protein (Kosik et al. 1989b).

Tau Splicing

Recently we have discovered that differentially spliced forms of tau are developmentally regulated (Kosik et al. 1989a). Mature tau involves the insertion of at least two distinct sequences, one of which is a 31 amino acid sequence homologous to the carboxy-terminal repeated sequences (Lee et al. 1988) that have the property of microtubule binding (Aizawa et al. 1988; Lewis et al. 1989; Lee et al. 1989). Using insert and junctional probes to rodent brain RNA extracted at defined developmental time points, a very clear isogene switching event occurs at approximately postnatal day 8. The internal homology of these repeats and their homology to the microtubule-binding domain in MAP2 (Lewis et al. 1988) is shown in Fig. 1. Tau repeat 2 is the inserted sequence.

The two reported tau sequences derived from the paired helical filament and described as type I and type II (Goedert et al. 1988) can now be assigned to developmentally distinct isotypes. The immature tau mRNA is either shut off or down-regulated to nondetectable levels after approximately postnatal day 8. At this time the brain is undergoing synaptogenesis, and the isogenic switch to a four repeat binding domain may relate to the more highly targeted transport required of microtubules once synaptic contact is made. The presence of the immature tau sequence within the paired helical filament suggests either a reexpression of the immature isoform, accumulation of low-level continued constitutive expression of an immature isoform through adult life, or significant expression of the immature isoform in neuronal subpopulations vulnerable to the development of NFT. Emergent reexpression may be a concomitant of the observed regenerative response and its known tendency to recapitulate developmental events (Hoffman and Cleveland 1988). An immature tau isoform within an adult milieu may have decreased affinity for microtubules and/or may be an inappropriate substrate for developmentally regulated kinases. (Phosphorylation is thought to decrease the affinity of microtubule-associated proteins for microtubules.) One potential example of inappropriate phosphorylation could be via calcium/calmodulin-dependent kinase which, we have found, does not get expressed until the isogenic

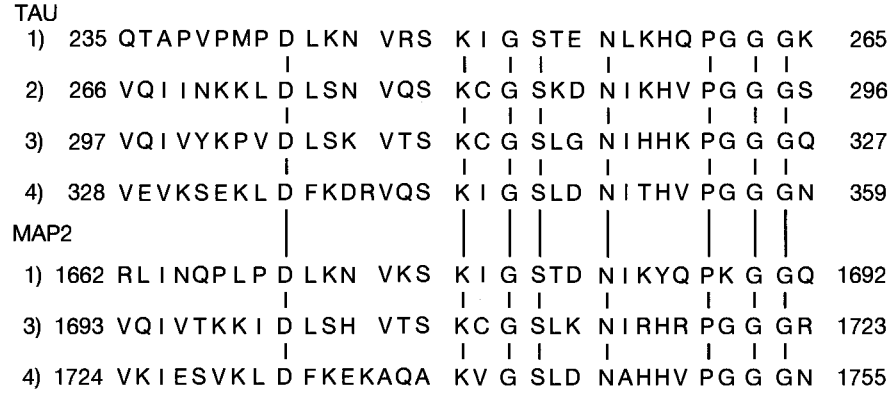

Fig. 1. Sequences of tau and MAP2 proteins

switch of tau to its mature form occurs (Kosik et al. 1989a). These events might lead to the accumulation of tau unassociated with microtubules in certain neurons and the precipitation of these proteins into insoluble filaments or paired helical filaments.

The clearly distinct forms of tau mRNAs that are differentially present during development poses the question as to the site at which this regulation occurs. Both *cis* and *trans* controls that become operationally active at postnatal day 8 are possible sites. While many examples of alternative splicing of mRNA precursors have been documented, clearly demonstrated examples of regulation entirely by splice site selection and, by implication, *trans*-acting factors, are less numerous.

Tau Phosphorylation

Two features appear to predominate in studies of the posttranslational modifications of tau. One is that phophorylation is the only known posttranslational modification of tau. A second feature is that conformational changes, perhaps linked to phosphorylation events, must be considered in the design of protein level tau probes, and these changes are likely to have both biological and pathological implications. Regarding tau phosphorylation the following observations are found in the literature. Tau is a phosphoprotein; however, the degree to which it is phosphorylated under various conditions and the in vivo sites of the phosphates are not known. Tau appears to exist in at least two distinct phosphorylation states (Lindwall and Cole 1984); however, its microheterogeneity by two-dimensional gels has raised the possibility of multiple phosphorylation states (Butler and Shelanski 1986). Tau can serve as an in vitro substrate for three kinases: calcium/calmodulin-dependent protein kinase (Schulman 1984; Yamamoto et al. 1983), protein kinase C (Hoshi et al. 1987; Baudier et al. 1987), and an independent kinase (Ishiguro et al. 1988). Crude tau preparations can incorporate phosphate stoichiometrically in the presence of calcium/calmodulin-dependent protein kinase (Schulman 1984; Yamamoto et al. 1983), and recently conditions have been described by which purer tau preparations can incorporate up to 100% phosphate (mole/mole; Baudier and Cole 1987). Tau does not appear to be a good in vitro substrate for any of the cyclic AMP-dependent kinases, including that which is associated with MAP2, nor for any of several tyrosine kinases tested. Phosphorylated forms of tau have been detected in NFT, and the suggestion has been made that the phosphorylation of tau in Alzheimer tangles is aberrant (Grundke-Iqbal et al. 1986a; Wood et al. 1986; Ihara et al. 1986; Saitoh and Dobkins 1986). One of the experimental bases for this suggestion is the tau monoclonal antibody designated tau-1. This antibody is specific for tau but, unlike other tau antibodies, recognizes NFT only after they have been treated with alkaline phosphatase (Grundke-Iqbal et al. 1986a; Wood et al. 1986). In physiologic terms phosphorylation of microtubule-associated proteins has been reported to decrease their ability to promote microtubule polymerization.

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Tau 64 and Tau 69: Two Early Biochemical Markers of Neurofibrillary Degeneration

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Summary

Tau proteins are the major antigenic component of PHF that accumulate in degenerating neurons in the brains of patients with AD. An abnormal phosphorylation of tau proteins has been suggested, but this is still under discussion. However, the mechanisms of incorporation of these normal cytoskeletal proteins in the PHF structure remain unknown. To obtain clues about the means by which tau proteins are modified during AD, we compared the tau immunodetection profile on immunoblots of brain homogenates from numerous Alzheimer patients versus numerous controls, using well-characterized antisera against human tau and against PHF.

The results of this study are that two pathological tau variants, having a higher MW than the normal set of tau proteins, are systematically found in homogenates of Alzheimer brain areas showing NFT and SP while they are never detected in control brains. These proteins, called tau 64 and tau 69, were also found in a biopsy, suggesting that they may be early markers of neurofibrillary degeneration. Tau 64 and tau 69 have a high MW due to their abnormal phosphorylation, as shown by the alkaline phosphatase treatment which provoked a decrease in their MW.

A parallel study was performed on CSF from controls and from probable AD patients. Even if tau 64 and tau 69 were never found, we observed that CSF contained a heat-stable material which cross-reacted with anti-PHF, anti-human tau and other antibodies raised against tau. Nevertheless, there was not a significant correlation between the detection of this "tau-like" material and the Alzheimer pathology.

We suggest that tau 64 and tau 69, which are early markers of neurofibrillary degeneration, might be used to establish experimental models for the study of the degenerating process in AD.

Introduction

Alzheimer's disease (AD) is a progressive dementing illness accompanied by characteristic neuropathological changes in the association cortical areas and areas anatomically connected to it (Mann 1988a). The changes consist of (a) severe loss of cortical neurons (Duyckaerts et al. 1985; Hansen et al. 1988), (b)

accumulation of amyloid filaments in the extracellular domain, and (c) accumulation of paired helical filaments (PHF) in neurofibrillary tangles (NFT), at the periphery of extracellular amyloid deposits (neuritic plaques; Hansen et al. 1988), in degenerating neurites scattered in the parenchyma (Braak and Braak 1988), and sometimes around pathological blood vessels (Peers et al. 1988).

Many observations of cross-reactions between antibodies raised against PHF and tau proteins have shown that microtubule-associated tau proteins are probably likely the major structural proteins of PHF (Brion et al. 1985; Delacourte and Défossez 1986; Grundke-Iqbal et al. 1986a, 1988; Kosik et al. 1986; Wischick et al. 1988). It has also been suggested that tau proteins are abnormally phosphorylated in AD, following the observation that the staining of NFT with a monoclonal anti-tau antibody named tau-1 is enhanced by pretreatment of tissue sections with phosphatase (Wood et al. 1986; Grundke-Iqbal et al. 1986b), and that the immunolabeling of NFT is increased with antibodies against phosphorylated tau proteins (Ihara et al. 1986). Surprisingly, a more detailed analysis of tau proteins with tau-1 and Alz-50 has suggested that tau proteins in brain affected by AD are not abnormally phosphorylated (Ksiezak-Reding et al. 1988).

We summarize here the results of our various analyses of tau proteins in the different brain areas of patients with AD compared to those of controls using well-characterized antisera raised against PHF and normal human tau proteins. Our results show that two abnormal tau variants are systematically present in human brain areas having the characteristic lesions of neurofibrillary degeneration of the Alzheimer type. The analysis of these two proteins demonstrates that they are abnormally phosphorylated. The potential use of detecting pathological tau proteins for diagnosis is also examined.

Materials and Methods

Clinical data

Ten patients with definite AD (McKhann et al. 1984), aged between 55 and 85 years (mean, 70), were compared to ten age-matched individuals aged 53–98 years (mean, 74) who had died without clinical or histopathological evidence of neurological disease. Control brains and AD brains were obtained 3–7 h after death (mean, 5 h).

Immunoblot studies

Biochemical and histochemical studies on the different brain areas were performed simultaneously as previously described (Delacourte and Défossez 1986). Brain tissue was homogenized in the Laemmli sample buffer (Laemmli 1970) 1:10 weight/volume, with or without β-mercapto-2-ethanol (βME) or dithiotreitol (DTT), and then heat treated. The brain proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), using a 5%–20% acrylamide gradient and 14 × 14 cm slab gels. Resolved proteins were

then transferred electrophoretically to nitrocellulose paper for tau immunodetection, as described by Parent et al. (1988).

The anti-human tau immuneserum was obtained by immunizing a rabbit with tau proteins. These were isolated from a heat-stable tau protein preparation from a normal human brain and were further purified by a preparative SDS-PAGE. The anti-human tau was used at a dilution of 1/10 000, and the anti-PHF was used at 1/200.

Alkaline Phosphatase Treatment

Tau preparations from Alzheimer brains were incubated either with alkaline phosphatase from *Escherichia coli* type III S (Sigma) at 10 units/ml or with calf intestine alkaline phosphatase (Boehringer Mannheim) at 100 units/ml. The enzyme action was stopped by heating after addition of an equal volume of the Laemmli solution to the sample. Since proteases may be present in the phosphatase alkaline preparation, the phosphatase was also inhibited by the addition of sodium pyrophosphate at 100 mg/ml and used as a control (Flament et al. 1989b).

Immunoabsorption

The anti-tau serum and anti-PHF serum were incubated with different protein amounts of control human brain homogenates (protein concentrations were 0.0125 g/ml−1 g/ml) or with Tris buffered saline alone overnight at 4°C. The next morning, the serum samples were centrifuged at 30 000 *g* for 30 min. The supernatants were collected and filtered through 0.22-μm pore size filters. Then nitrocellulose sheets with resolved brain proteins from controls were reacted with the absorbed antisera to estimate the protein concentration needed to annul the immunoreactivity. The anti-PHF at 1/200 was also absorbed with brain homogenates from Alzheimer patients. Thereafter, both antisera were immunochemically and immunocytochemically tested.

CSF Treatment

The CSF study was performed on 12 controls aged 54−81 years (mean, 67 ± 7.5). These were patients with lumbar disk hernias in whom a lumbar puncture was performed for radiculography. They were free of any neurological disease. Each had a score higher than 28 on the Mini-Mental State Exam (Folstein et al. 1975). The study was performed with the approval of the ethics committee. The 12 probable Alzheimer patients (diagnosed according to NINCDS-ADRDA classification) were aged between 38 and 88 years (mean, 66.7 ± 15.1). CSF samples were stored frozen at −20°C immediately after the sampling. In order to analyze the tau protein contents immunochemically, the CSF was treated as follows: one volume of the Laemmli sample buffer was added to one volume of CSF, and the sample was heat treated at 100°C for 10 min. For heat stability, NaCl 0.75 *M* was added to several CSF samples and after storage for 1 h at 4°C these samples were boiled (100°C, 5 min). The samples were then centrifuged at 10 000 *g* for 15 min, and supernatants and pellets were collected and heat treated after the addition of the

Laemmli sample buffer (1 volume added to 1 volume of supernatant; pellets were resuspended in 2 volumes) for electrophoresis and immunoblot analysis.

Properties of Anti-PHF and Anti-Tau

We report below a detailed analysis of the tau immunoblot profile, using immunesera prepared against a pathological structure, the PHF, and against a preparation of native tau proteins of human origin. Before describing the modifications of the tau profile in Alzheimer brains, it is important to recall the main features of these antibodies that are currently known.

Anti-PHF and Anti-Tau Immunocytochemical Properties

At the optical level, on Alzheimer tissue sections the anti-PHF serum labels the neurofibrillary degeneration exclusively – the NFT, the degenerating neurites around senile plaques (SP) and numerous degenerating neurites scattered in the parenchyma. Sometimes, blood vessels with amyloid angiopathy are surrounded by a sleeve of degenerating neurites immunolabeled with these antisera (Delacourte et al. 1987; Peers et al. 1988). On the same tissue sections, using the elution technique (Delacourte and Défossez 1986) or on adjacent semithin sections embedded in araldite (Défossez et al. 1988), anti-PHF and anti-tau label the same structures. At the electron microscopic level, the double immunogold-labeling technique performed on the ultrathin section adjacent to the previous semithin sections showed that anti-PHF and anti-tau detect PHF structures exclusively (Défossez et al. 1988).

During the course of the neurodegenerative process, the immunological properties of tau proteins are modified. First, we observe a strong tau immunoreactivity of the NFT which are not labeled with thioflavine, a marker of the amyloid substance. Second, after the neuron death, the neurofibrillar material of the NFT becomes extracellular since the membrane and the nucleus of the dead neuron have disappeared. These NFT ("ghost tangles") are no longer stained with tau antibodies, but they are strongly stained with thioflavine. All transition phases between these two stages are observed (Défossez and Delacourte 1987). The anti-PHF and anti-tau sera also stain the tangles of the nervous tissue of patients with Down's syndrome (Mann et al. 1989) and with Pick bodies (unpublished results). No immunological cross-reactions are observed between these antibodies and antibodies against the β-protein amyloid A4 (Behrouz et al. 1989a, b).

Immunoblot Study

Upon Western blot analysis the antiserum against PHF specifically detects the tau profile among the total protein extracts of normal brains. In these extracts and on enriched preparations of cytoskeletal proteins, anti-PHF does not detect neurofilament protein, tubulin, or MAP2. Anti-PHF detects tau proteins in the brain of

all the vertebrates. It detects fetal and adult tau proteins, with the adult tau profile in brains from normal individuals aged 20–80 years being identical (Parent et al. 1988). The anti-native human tau proteins possess roughly the same characteristics, but there are minor differences, such as its higher affinity for tau proteins. We used a dilution of 1/10 000. In control brain homogenates, the binding of both antibodies was strong in the gray matter of cortical areas while it was weak in the cortical white matter or in brain regions rich in axons, such as spinal cord or corpus callosum. These results suggest that tau proteins are major components of the somato–dendritic domain (Delacourte et al. 1988a) and are abundant in Alzheimer brain regions where NFT are found (Delacourte and Défossez 1986).

Results

Immunoblot Detection of Tau Proteins in Autopsied Human Brain

In Different Regions of a Normal Brain and an Alzheimer Brain

In the control brain, the anti-PHF and anti-tau sera detected the same profile of tau proteins: four main components with MW ranging from 45 kDa to 62 kDa. In the Alzheimer brain, two higher tau proteins of MW 64 and 69 kDa were detected in addition to the four tau variants present in control brain (Flament et al. 1989a). These proteins which we call tau 64 and tau 69, were never found in areas of the control brain. In the Alzheimer brain, both were always present in regions which contained NFT and SP – temporal, frontal, parietal, and occipital – but they were absent from regions not affected by the neurofibrillary degeneration – caudate nucleus, cerebellum, spinal cord, and thalamus.

Control experiments showed that tau 64 and tau 69 were not detected if the first antibody (anti-PHF or anti-tau) was omitted, or if the anti-PHF or anti-tau was preincubated with a tau preparation from an Alzheimer patient. Antitubulin, anti-neurofilament proteins, and anti-MAP2 did not stain tau 64 or tau 69.

In Different Alzheimer Brains

The cortical areas of ten Alzheimer brains and ten control brains were compared. Tau 64 and tau 69 were never found in control cortices (Fig. 1, C1), even in a very old (98-year-old) patient. In fact, there was only one case in which tau 64 and tau 69 were found: the hippocampus of an 80-year-old man (Fig. 1, C2). Immunohistochemical study of this tissue revealed the presence of SP and NFT in smaller amounts than in Alzheimer brains (Fig. 1, C2). In Alzheimer brains, the two variants were always detected, and the immunoreactivity was stronger in regions in which the Alzheimer pathology (presence of NFT and SP) was more developed (Fig. 1, Alz). In the cortex, the tau 64 and tau 69 detection generally decreased from the temporal and frontal cortex to the occipital cortex and was absent in the occipital pole (not shown). Their labeling seemed stronger in Alzheimer brains having an early age of onset. Tau 64 and tau 69 were also detected by several antisera raised against bovine tau proteins – one described in Delacourte and Défossez (1986) and the other a generous gift of Dr. J. Baudier (not shown). In addition,

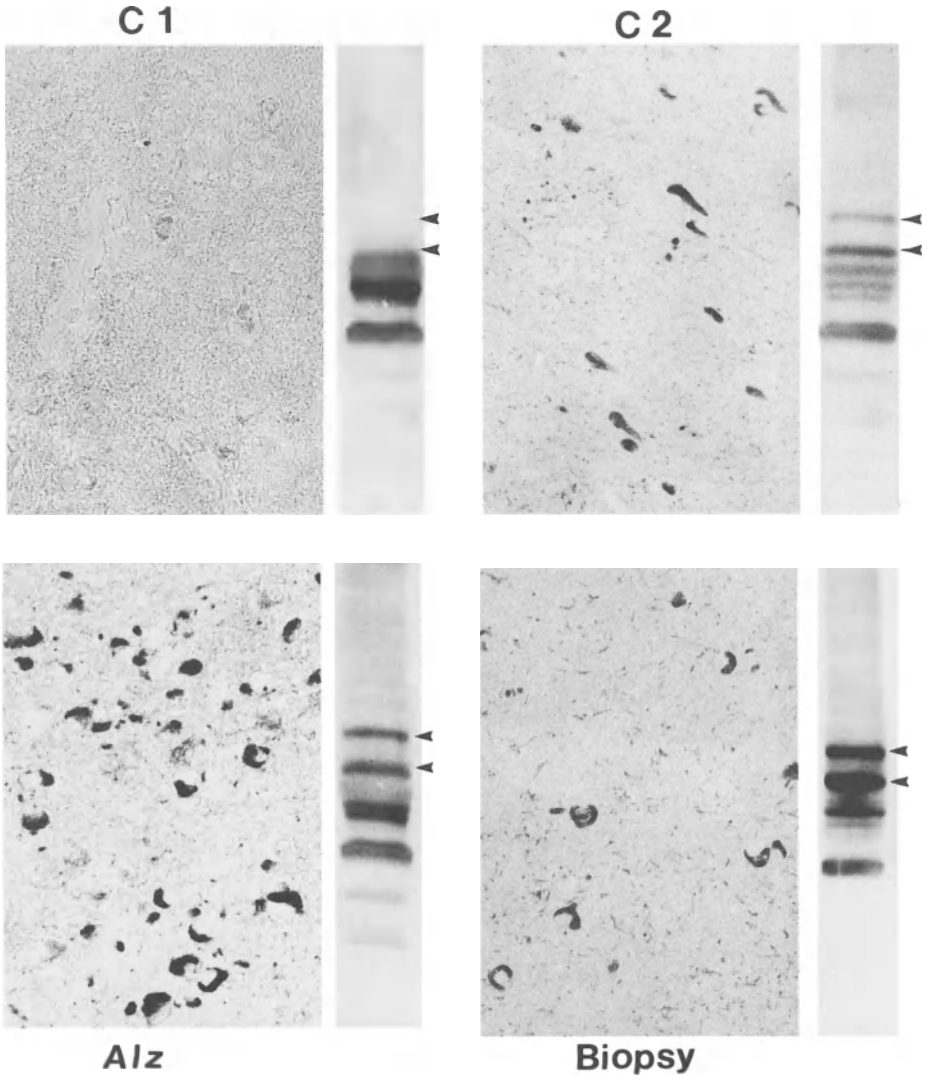

Fig. 1. Tau 64 and tau 69 and neurofibrillary degeneration. Tissue sections and corresponding immunoblots stained with anti-PHF. *C1*, temporal cortex from a 52-year-old control; *C2*, hippocampus from an 80-year-old control; *Alz*, frontal cortex from a 73-year-old Alzheimer patient; *Biopsy*, frontal biopsy from a 58-year-old patient who began to develop an unusual dementia. In autopsy pieces, tau 64 and tau 69 *(arrowheads)* are more strongly detected in areas which contain a much higher density of NFT and SP. In the frontal biopsy tissue, tau 64 and tau 69 are more strongly detected than in the frontal autopsy tissue although the density of NFT and SP is not higher (compare *Alz* and *Biopsy*). Therefore, it seems that the phosphorylation of tau proteins might appear before their incorporation in PHF, and that tau 64 and tau 69 are reliable early markers of neurofibrillary degeneration

smears along migrating tracks always accompanied proportionally the presence of tau 64 and tau 69.

In Different Regions of the Same Alzheimer Brain

It would be interesting to know how tau proteins are modified at the beginning of the process of neurofibrillary degeneration, and in regard to their phosphorylation whether tau 64 appears before tau 69. To explore this possibility, we observed the tau staining pattern in homogenates from many cortical areas of the same Alzheimer brain, in the hope of finding areas which had very recently been affected by neurofibrillary degeneration. The patient was a 78-year-old woman with clinically and histopathologically diagnosed AD. In the 30 cortical brain areas that we studied, the situation was similar to that observed in all the brain homogenates which had previously been studied. Indeed, only two patterns were found: either tau 64 and tau 69 were both absent or they were present together, with the intensity of their immunodetection varying from one area to another. There was no transitional case showing only one of these pathological tau variants (tau 64) which might have corresponded to a precursor present at early stages of neurofibrillary degeneration.

Immunoabsorption Experiments

On immunoblots, the immunostaining of normal human tau proteins with the two antisera was tested after their immunoabsorption with control brain homogenates. The immunoreactivity of the anti-tau was completely abolished when the antisera were preincubated with control brain proteins at 0.15 g/ml. This absorbed anti-tau was unable to stain either tau proteins from controls or those from Alzheimer brains, including tau 64 and tau 69. This complete loss of immunoreactivity was immunocytochemically confirmed. Absorption of the anti-PHF with normal brain proteins (0.025 g/ml) enhanced the loss of immunoreactivity towards normal tau proteins. By contrast, the staining of tau proteins from Alzheimer brains by the same absorbed anti-PHF was not abolished; even if the immunoreactivity decreased, tau 64 and tau 69 were still detected, as well as the heaviest normal tau variants. The addition of increasing amounts of normal brain proteins did not result in eliminating the immunoreactivity of anti-PHF toward tau 64 and tau 69.

On the other hand, however, it was possible to abolish the detection of tau 64 and tau 69 by anti-PHF using Alzheimer brain extracts. These results suggest that tau 64 and tau 69 possess at least two types of epitopes. The first type is probably due to the primary and secondary structures of tau proteins, and therefore, these epitopes are shared by all tau proteins, including tau 64 and tau 69. The immunodetection of these epitopes may be abolished using normal brain proteins, which explains the elimination of the immunoreactivity of the absorbed anti-tau towards tau 64 and tau 69 as well as the decrease in immunoreactivity of the absorbed anti-PHF towards tau 64 and tau 69. The second type of epitope is specific to the pathology and may be due to a tertiary structure with numerous abnormal phosphorylated sites. These epitopes are detected exclusively by anti-PHF, and their immunodetection can only be absorbed with Alzheimer brain extracts.

Characterization of Tau 64 and Tau 69

State of Reduction of the Protein Samples

The pathological tau variants have a heavier MW than normal tau proteins. This may be due to their binding to a small protein. To verify this hypothesis, we compared the tau staining pattern from the same sample heat treated after addition of different amounts of reducing agents (βME up to 5%, DTT up to 1%, or no reducer at all). Immunodetection of tau 64 and tau 69 was independent of the state of reduction of the samples.

Alkaline Phosphatase Treatment

To determine whether these abnormal tau variants were the result of a pathological phosphorylation of normal tau proteins, we dephosphorylated a solution of tau proteins extracted from an Alzheimer brain cortex. After 4 h of incubation with alkaline phosphatase at 37°C, we observed a decrease in the MW of the two pathological tau variants. As a control, the solution of Alzheimer tau proteins were incubated with the same amount of phosphatase in the presence of sodium pyrophosphate at 100 mg/ml; the two tau variants were still labeled, and their MW remained unchanged, even after overnight incubation. This control test shows that the decrease in the MW of tau 64 and tau 69 is not due to their proteolysis but is provoked by their dephosphorylation. Nevertheless, it is also possible that our antisera were unable to detect tau 64 and tau 69 once they were in a dephosphorylated state. To test this hypothesis, we incubated the heat-stable fraction once separated by SDS-PAGE and electroblotted on a nitrocellulose paper with the same amount of enzyme for 4 h at 37°C. After this experiment, our antibodies were still able to stain dephosphorylated tau 64 and tau 69 (Flament et al. 1989b).

Diagnosis of the Presence of Neurofibrillary Degeneration in Brain Tissue

From Brain Biopsies

Very recently, we had the opportunity to analyze a biopsy from the frontal cortex, performed on a 58-year-old patient with unusual clinical frontal signs (Flament and Delacourte 1989). The two pathological tau variants were detected at a much higher level than in all the autopsied regions (compare Biopsy and Alzheimer in Fig. 2), suggesting that the brain sample was affected by neurofibrillary degeneration. Our presumption was corroborated by the neuropathological study which demonstrated the presence of SP and NFT (Fig. 1, Biopsy), which were not present at a much higher density than in autopsied pieces (compare Alz and Biopsy in Fig. 1).

From Cerebrospinal Fluid

Since tau 64 and tau 69 are reliable biochemical markers of neurofibrillary degeneration of the Alzheimer type, we were interested to determine whether they are detected at the peripheric level, for example in the CSF of patients. Such a detec-

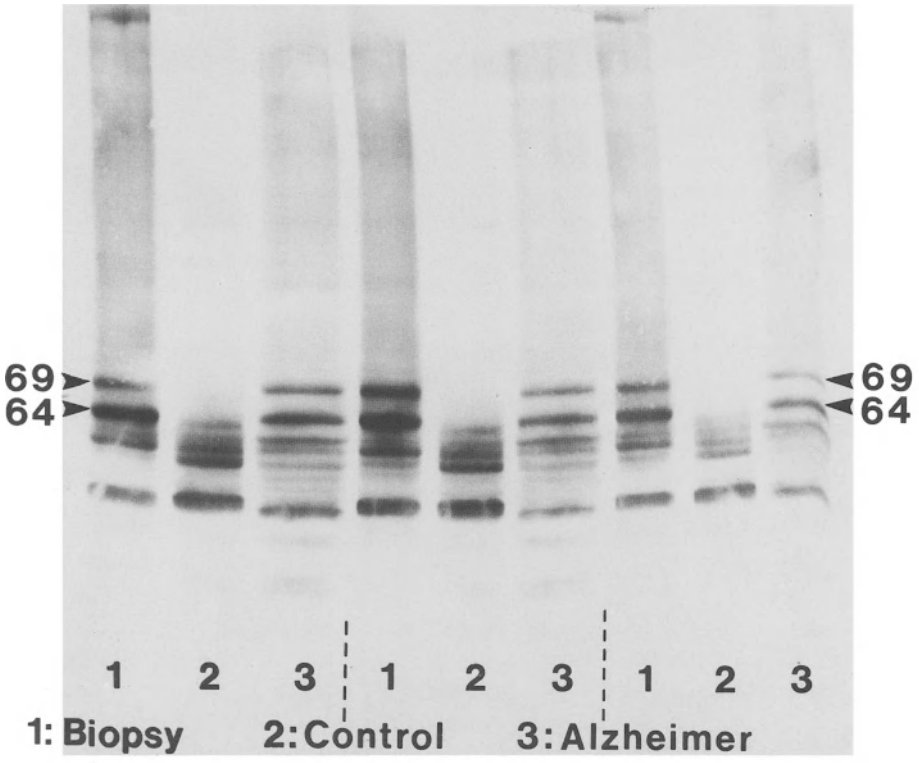

Fig. 2. Tau 64 and tau 69 at an early stage of the illness. Frontal cortex homogenates from a 58-year-old patient (biopsy, *1*), a 52-year-old patient (control, autopsy, *2*), and a 72-year-old patient with AD (autopsy, *3*). *Left,* dilution 1; *middle,* dilution 1/2; *right,* dilution 1/4. Tau 64 and tau 69 are more strongly detected in the frontal biopsy *(1)* than in a corresponding autopsy piece *(3)*. Therefore, tau 64 and tau 69 are more strongly detected at early stages of the illness, which might be due to the fact that they appear before the PHF structure. The standard molecular weights are bovine serum albumin (68 kDa) and ovalbumin (43 kDa)

tion of these pathological tau proteins would permit establishment of the diagnosis of neurofibrillary degeneration in a nontraumatic way. Once the electrophoretic transfer was performed, the staining of nitrocellulose sheets with Ponceau red revealed the protein profile of CSF. Two bands were detected: a fine band, sometimes present at 70 kDa, and another band, often large and diffuse, present between 60 and 65 kDa. These two proteins are present in different amounts and in different ratios for each patient. When anti-tau or anti-PHF was applied on immunoblots of CSF proteins, a band, often large and diffuse, was detected at 55 kDa (Fig. 3); another band was sometimes weakly immunolabeled at 65 kDa. Nevertheless, this immunodetection profile was similar to neither a normal tau profile nor an Alzheimer tau profile (Fig. 3, B). To confirm that these proteins were really related to tau proteins, different anti-tau antibodies were used: each of them also stained the two bands. Then, the heat-stable properties of these proteins were assayed, and we observed that this immunoreactive material was heat stable.

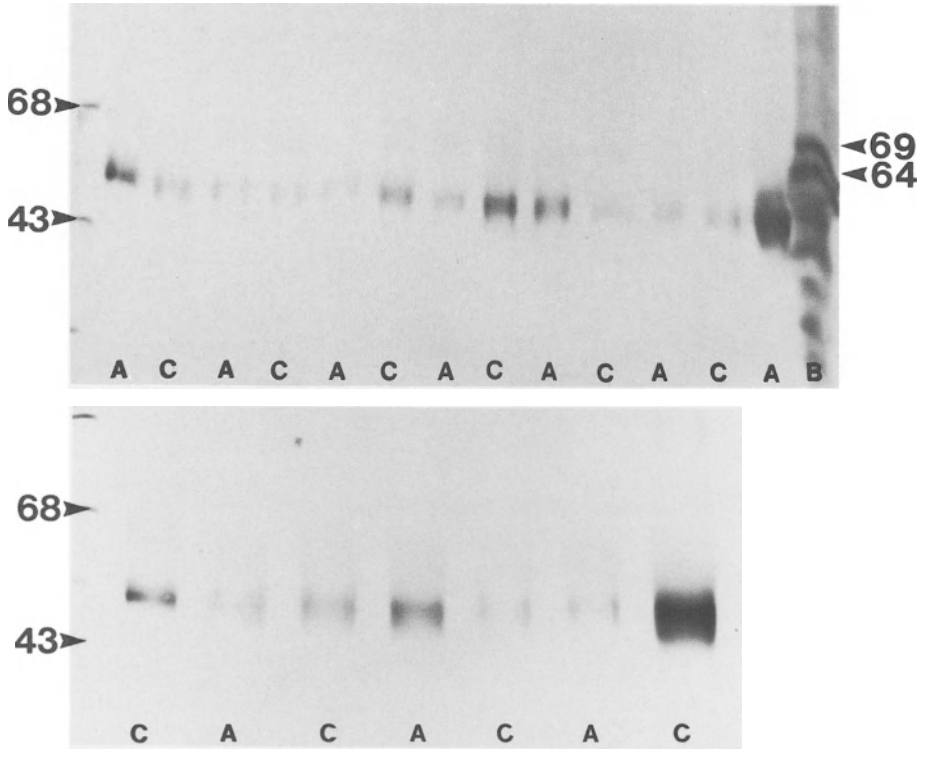

Fig. 3. Tau 64 and tau 69 in cerebrospinal fluid. The binding of anti-tau on immunoblots of CSF from 20 patients aged 54–88 years: ten patients with probable Alzheimer's disease *(A)* and ten age-matched controls *(C)*. All samples were identically prepared: one volume of CSF was treated by an equal volume of the Laemmli sample buffer and heat treated; 25 µl *(top)* or 50 µl *(bottom)* of this solution was loaded per well at the top of 10%–20% polyacrylamide gels. Proteins were electrophoretically resolved and then transferred onto a nitrocellulose sheet before addition of anti-tau at 1/10 000. Samples are organized from the youngest *(left)* to the oldest patient *(right)*. The immunostaining in CSF is quite different from usual tau profiles which are observed in brain homogenates (compare with the brain homogenate from the 72-year-old Alzheimer patient, *B)*. However, tau 64 and tau 69 *(arrowheads)* are never detected. In addition the intensity of immunostaining was not significantly correlated with the pathology

The "tau-like" proteins were found in varying amounts in CSF from probable Alzheimer patients (Fig. 3, A) and from controls (Fig. 3, C), but there was no significant correlation between the amount of "tau-like" material and the Alzheimer pathology (*U*test of Mann and Whitney, not significant).

Discussion

The anti-PHF and the anti-tau sera used in this study have already been shown to be very sensitive tools for the immunohistochemical detection of neurofibrillary

degeneration. Both antisera specifically label neurons containing PHF structures in Alzheimer brain sections. In addition, they detected tau proteins exclusively and identically when used on the same blot of resolved proteins from a brain homogenate, showing that both are suitable for tau immunodetection.

Tau 64 and Tau 69 are Biochemical Markers of Neurofibrillary Degeneration

The findings of this study using polyclonal antibodies are that two abnormal variants of tau proteins, tau 64 and tau 69, are present in Alzheimer brain homogenates. These results corroborate our preliminary report showing the presence of two additional higher MW tau proteins in cortical areas of an Alzheimer brain (Parent et al. 1988). They were detected only in regions showing an Alzheimer pathology and their staining intensity increased in regions that are strongly affected during the disease (temporal cortex). The detection of tau 64 and tau 69 was proportional to the smears, which is probably due to the partial dissociation of tau aggregates in PHF (Delacourte and Défossez 1986, Grundke-Iqbal et al. 1988). Both were often more strongly detected in homogenates from Alzheimer brains having an early age of onset. They were found in neither normal brain homogenates aged up to 98 years nor in Alzheimer brain extracts from regions without NFT and neuritic plaques (caudate nucleus, cerebellum, spinal cord). The only exception in our study was the presence of tau 64 and tau 69 in the hippocampus from one elderly control. The neuropathological study of this brain region revealed the presence of NFT.

A blind study was also performed with J.-J. Hauw and C. Duyckaerts of Salpetrière Hospital, Paris. We quantified tau 64 and tau 69 in different samples whose neuropathology was known only by J. J. Hauw. The results of this study show that there is a strong correlation between the presence of tau 64 and tau 69 and the presence of neurofibrillary degeneration (manuscript in preparation).

Since their detection is correlated with the neurofibrillary degeneration observed in AD, tau 64 and tau 69 may be considered as reliable biochemical markers of the Alzheimer degenerative process. These tau 64 and tau 69 proteins have not been detected by other researchers. A possible explanation for this is that many authors have worked on tau preparations after extraction in Mes buffer, according to the general protocol for tau protein preparation isolated from young vertebrates (Fellous et al. 1977). However, human tau proteins are essentially insoluble, especially in autopsy pieces, and we observed that a high percentage of tau was lost during the extraction. For this reason we worked with SDS extracts that solubilized all the forms of tau proteins except those previously incorporated in the PHF structure.

Monoclonal antibodies tau-1 and Alz-50 were not able to detect such pathological tau variants in brain homogenates (Ksiezak-Reding et al. 1988). This is probably because these monoclonal antibodies react with specific epitopes which are not present in tau 64 and tau 69. On the other hand, all the polyclonal antibodies against tau proteins that we have studied so far have reacted with tau 64 and tau 69. Tau 64 and tau 69 are probably not related to the 68-kDa component detected by Alz-50 (Flament et al. 1989b; Wolozin et al. 1986; Nukina et al. 1988).

Tau 64 and Tau 69 are Abnormally Phosphorylated Proteins

To determine the nature of these pathological tau variants, we analyzed the samples in different reducing conditions to see whether tau 64 and tau 69 were bound by disulfide bridges to a small protein such as protein S100 (Baudier and Cole 1988). The MW remained unchanged in extreme conditions of reduction (5% βME, 1% DTT), suggesting that tau 64 and tau 69 are not bound to such a protein.

We also analyzed the state of phosphorylation of these proteins, since an abnormal phosphorylation of tau during AD has been suggested (Wood et al. 1986; Grundke-Iqbal et al. 1986; Ihara et al. 1986). In addition, it has been shown that the phosphorylation of bovine tau proteins by the Ca^{2+}/calmodulin-dependent protein kinase enhanced a shift in the electrophoretic mobility of the set of tau proteins (Baudier and Cole 1987). The incubation of a heat-stable preparation of Alzheimer brain tau proteins with alkaline phosphatase for 4 hours dramatically changed the staining pattern of tau proteins; the two pathological variants disappeared, and the staining of the bands from 45 to 62 kDa increased. When sodium pyrophosphate was added to the alkaline phosphatase to inactivate the enzyme without removing the potential protease activity that might contaminate the preparation, we did not observe the disappearance of tau 64 and tau 69. Thus, the decrease in MW of tau 64 and tau 69 after incubation with the alkaline phosphatase is not due to their proteolysis. Therefore, we conclude that the high MW of tau 64 and tau 69 is due to a dysregulation in their phosphorylation. Nevertheless, this phosphorylation seems to be different from that which is observed with the Ca^{2+}/calmodulin-dependent protein kinase. Indeed, after alkaline phosphatase treatment the two pathological tau variants rejoin the normal set of tau proteins whereas the MW of the normal tau variants do not decrease. Whether the phosphorylation leading to the appearance of tau 64 and tau 69 is due to another protein kinase or to an extreme phosphorylation has yet to be determined.

Up to now, the theory of the abnormal phosphorylation of tau proteins during AD was most often based on the fact that a stronger immunolabeling of Alzheimer tau proteins on immunoblots as well as of NFT on tissue sections from Alzheimer brains, was observed after dephosphorylation (Grundke-Iqbal et al. 1986; Wood et al. 1986). (The immunostaining of normal brain homogenates or sections was unchanged when the same treatment was applied.) Nevertheless, to our knowledge no other researchers using immunoblots of brain homogenates have reported the tau protein profile from patients with AD to be so different from that of controls. Therefore the discovery of tau 64 and tau 69, which are two abnormal tau proteins having a high MW due to their abnormal phosphorylation, constitutes new and direct evidence for the presence of an abnormal phosphorylation of tau proteins in AD.

The Diagnostic Value of Tau 64 and Tau 69

We were able to detect tau 64 and tau 69 in a frontal biopsy performed on a patient who began to develop an unusual dementia. The detection of the pathological tau variants was stronger than in all the autopsy pieces which were studied up to that

time. Based on this tau 64 and tau 69 detection, we diagnosed the presence of neurofibrillary degeneration. The histopathological analysis of the tissue revealed the presence of NFT and SP, confirming our diagnosis. Therefore, tau 64 and tau 69 appear to be reliable tools for the detection of neurofibrillary degeneration in brain tissue.

PHF antigens and PHF cross-reactive antigens had already been found in CSF (Mehta et al. 1985). This PHF immunoreactivity, detected by the enzyme-linked immunosorbant assay (ELISA), was shown to be present at higher concentrations in CSF from AD patients than from non-AD controls. Nevertheless, there was a considerable overlap between the detection of this antigen in CSF from patients and age-matched controls, suggesting that this test was not reliable enough to diagnose AD. The ELISA method described by Mehta et al. (1985) provides quantitative data. We employed the Western blotting method, with which qualitative data can be obtained, and were able to observe the electrophoretic profile of the CSF immunoreactive material as well as its approximative MW (it is well known that tau proteins are quite a heterogeneous family). We found that CSF contained material which cross-reacted with the anti-tau and anti-PHF antisera. This immunoreactive material consisted of a major band, often diffuse, with MW ranging from 50 to 60 kDa, accompanied sometimes by a minor band at 65 kDa. The electrophoretic profile was therefore different from the usual tau profiles: it was similar to neither a control tau profile nor to an Alzheimer tau profile. This material was related to tau proteins, as shown by the cross-reaction obtained with all the other anti-tau antibodies which were tested. In addition the "tau-like" material had heat-stable properties. However, tau 64 and tau 69 were never found in the CSF. Therefore, this test does not allow diagnosis of the presence of neurofibrillary degeneration, as done previously for biopsy pieces. Furthermore, even if the tau immunoreactive material was present in different amounts from one patient to another, we were not able to establish significant correlations between the quantity of this material in the CSF of different individuals and the extent of their dementia.

In conclusion, tau 64 and tau 69 appear to be early markers of neurofibrillary degeneration. They allowed us reliably to diagnose the presence of neurofibrillary degeneration in brain tissue in biopsy samples, but this diagnosis could not be established from CSF.

The Sequence of Events that May Provoke Neurofibrillary Degeneration

To understand the pathological mechanisms at the origin of AD it is important to dissect chronologically the sequence of events that lead to the destruction of the central nervous system. To determine the primary cause of the illness, it is necessary to know which areas degenerate first during the course of the disease, and in these regions which lesions are observed first.

The determination of structural components of the lesions has permitted the development of immunological tools which are more sensitive than the conventional histochemical techniques. Two main groups of antibodies have been prepared, the first against the β-protein A4, which is the main component of the

straight 6- to 9-nm amyloid filaments found in the extracellular domain, and the second consisting of antibodies raised against tau proteins which are the major antigenic components of the PHF found in degenerating neurons. Based on these and conventional neuropathological tools, there is some consensus that, during the course of the disease, amyloid deposits of βPA4 first appear in areas such as the temporal cortex, the amygdaloid nucleus, and the hippocampus (Mann 1988b). Later, the degenerating process spreads to other areas, and degenerating neurons with bundles of PHF appear (Duyckaerts et al. 1988, Mann 1988b). It has also been shown that dystrophic peptidergic neurites found at the periphery of neuritic plaques precede the PHF formation (Lenders et al. 1989).

Amyloids deposits of βPA4 may therefore be responsible for the disease, provoking a toxification of neuronal cells. However, these deposits are also found, in varying amounts, located in the parenchyma (Davies et al. 1988; Tagliavini et al. 1988) and/or vessel walls (Masuda et al. 1988) in aged control brains with absolutely no signs of dementia. Some of these patients with preamyloid deposits might be in a preclinical stage of AD. However, the amyloid deposits in cortical blood vessels were present in 50% of the very old normal population, and the amyloid deposits in the nervous parenchyma were present in 80% of the old normal population, indicating that the prevalence of amyloid deposits was different from the prevalence of AD (Davies et al. 1988; Masuda et al. 1988). Consequently, some other factors may act in synergy with the presence of the amyloid deposits to provoke the neurodegenerative process (environmental or genetic factors, for example). Nevertheless, even if neurofibrillary degeneration is a late consequence of the disease, it is the resulting neuronal loss which must be responsible for dementia.

This is the reason that we have focused our interest on the factors that lead to neuronal death, and that may be responsible for the illness. Our results describing new biochemical markers of neurofibrillary degeneration show that an abnormal phosphorylation of tau proteins occurs during the course of neurofibrillary degeneration. In our opinion, since tau 64 and tau 69 are always detected in conditions which are not able to solubilize PHF, and since they are more strongly detected in biopsy pieces at the beginning of the disease than in autopsy pieces, they must be early markers of neurofibrillary degeneration. Thus, it seems that tau proteins might be abnormally phosphorylated before their incorporation into insoluble PHF structures. At present, the reason for such a dysregulation of the phosphorylation-dephosphorylation system is still unknown.

Towards the Elaboration of an In Vitro Model for the Study of Neurofibrillary Degeneration

An in vitro model of AD may help to determine the factors able to provoke the degenerating process. Tau 64 and tau 69 may be helpful in elaborating such a model. For instance, it will be interesting experimentally to induce the production of tau 64 and tau 69 by attempting to phosphorylate normal human tau protein preparations using different kinases or different ratios of kinases and phosphatases. We will thus obtain clues about the enzymes involved in neurofibrillary degenera-

tion. It will also be interesting to know how many phosphate groups are incorporated in each of these pathological tau proteins. Then, given such data about the phosphorylation of tau 64 and tau 69, we could attempt to induce as precisely as possible the formation of tau 64 and tau 69 in neuronal cell cultures and to verify the different etiopathogenic hypotheses which have been proposed. For example, we could test whether toxins such as glutamate, aluminum salts, or other components have an effect on the phosphorylation of tau proteins.

It will be interesting to determine if tau 64 and tau 69 are produced in transfected cells with the gene of pre-A4 (Salbaum et al., this volume) or the gene of superoxide dismutase (Delacourte et al. 1988b), or in transgenic animals with these genes. These models may help to identify the first biochemical events which are responsible for the abnormal physiology of the neuron enhancing the abnormal phosphorylation of tau proteins and, later, their accumulation into insoluble PHF structures leading ultimately to neuronal death.

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A cDNA Encodes Epitopes Shared Between Microtubule-Associated Protein MAP2 and Alzheimer Neurofibrillary Tangles: In Situ Hybridization and Immunocytochemistry*

J. P. Brion, A. M. Couck, M. E. Cheetham, D. Hanger, B. H. Anderton, and *J. Flament-Durand*

Summary

Neurofibrillary tangles in Alzheimer's disease are composed of abnormal filaments whose biochemical composition is partly resolved. Cytoskeletal proteins are major components of these abnormal fibers, the microtubule-associated protein tau being the main constituent demonstrated in neurofibrillary tangles. Other cytoskeletal proteins or fragments of them which have been immunologically detected in tangles are the microtubule-associated protein MAP2 and neurofilament 150- and 200-kDa polypeptides. We have isolated a cDNA encoding epitope shared between MAP2 and neurofibrillary tangles, as demonstrated by immunocytochemistry with an antiserum raised against a fusion protein. In situ hybridization with this cDNA on tissue sections of the hippocampus in Alzheimer's disease did not show increases in the hybridization signal in tangle-bearing neurons. These results suggest that the accumulation of MAP2 immunoreactivity in neurofibrillary tangles is not directly related to an overexpression of this protein. This also strengthens the hypothesis implicating post-translational modifications of cytoskeletal proteins as mechanisms of neurofibrillary tangles formation.

Introduction

Neurofibrillary tangles and senile plaques are characteristic neuropathological lesions of Alzheimer's disease, the most common cause of dementia in aging. Quantitative studies have shown that the number of neurofibrillary tangles and plaques is correlated with the severity of dementia (Wilcock and Esiri 1982; Duyckaerts et al. 1987). Unraveling the molecular composition and the mechanisms of formation of these lesions can thus provide important insights into the etiology of this disease.

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Neurofibrillary tangles are made of bundles of abnormal filaments (paired helical filaments, PHF) accumulating in neurons. Although these abnormal filaments are ultrastructurally different from the normal intraneuronal fibers (microtubules, neurofilaments, and microfilaments), they have been shown to share antigenic cross-reactivity with several cytoskeletal proteins. Immunocytochemical (Brion et al. 1985; Kosik et al. 1986; Delacourte and Défossez 1986; Nukina and Ihara 1986; Wood et al. 1986) and biochemical studies (Grundke-Iqbal et al. 1986a; Goedert et al. 1988; Wischik et al. 1988; Kosik et al. 1988b) have demonstrated that these abnormal filaments contain tau proteins, a group of four to six closely related microtubule-associated proteins (MAP). These MAP play an important role in the stability of microtubules in situ and induce the polymerization of tubulin in microtubules. Other cytoskeletal epitopes which have been detected in neurofibrillary tangles concern MAP2 (Kosik et al. 1984; Ksiezak-Reding and Yen 1987; Yen et al. 1987) and neurofilament 150- and 200-kDa polypeptides (Anderton et al. 1982; Perry et al. 1985; Sternberger et al. 1985; Miller et al. 1986). Abnormalities of microtubule proteins in tangle-bearing neurons could explain the collapse of microtubule networks in these cells, with probable severe impairment of neuroplasmic flow (Flament-Durand and Couck 1979; Dustin and Flament-Durand 1982). Ubiquitin, a polypeptide involved in an extralysosomal proteolytic pathway, has also been demonstrated in tangles (Mori et al. 1987; Perry et al. 1987; Brion et al. 1989); this ubiquitination of tangles could reflect an attempt by affected neurons to remove these abnormal fibers.

The senile plaques are composed of abnormal neurites, containing neurofibrillary tangles, which surround an extracellular deposit of amyloid material. This amyloid deposit is composed mainly of a 4-kDa polypeptide (A4 protein; Masters et al. 1985); A4 polypeptide derives from a large precursor which could be a membrane protein (Kang et al. 1987). Amyloid A4 and neurofibrillary tangles do not share immunological cross-reactivity (Selkoe et al. 1986).

The molecular mechanisms leading to the accumulation of cytoskeletal proteins in neurofibrillary tangles are unknown. It is not known whether this is associated with an overexpression of these proteins, a decrease in their catabolism, or no change in their turnover. Some aspects of these questions can be investigated by in situ hybridization, and we report here results obtained with a cDNA encoding epitope shared between a MAP and neurofibrillary tangles.

Isolation of a cDNA Encoding MAP epitope

Total RNA was prepared from adult rat brain by the guanidinium thiocyanate extraction procedure. Polyadenylated RNA was obtained by oligo(dT)-cellulose chromatography, and 10 μg polyadenylated RNA was used to prepare cDNAs which were inserted into the *Eco*RI site of the bacteriophage expression vector λ gt11 (Huynh et al. 1985) and yielded a library of 10^6 recombinant phages. A total of 200 000 plaques were screened with a rabbit polyclonal anti-tau serum diluted 1/100 (stripped from anti-*Escherichia coli* antibodies), using a protein A alkaline phosphatase detection method. This serum was obtained by immunization with rat tau proteins contained in a slice of a preparative 5%−15% gradient sodium

dodecyl sulfate (SDS) polyacrylamide gel on which thermostable MAP were run. On Western blots of thermostable MAP, this serum strongly labels tau proteins but also shows a weak reactivity with MAP2. The same MAP2 band was labeled on blots of total brain proteins (10^5 g supernatant; Fig. 1, A). This serum also strongly labels neurofibrillary tangles.

Five clones were isolated (Brion et al. 1987). One 2.2-kb cDNA, called pt6, was subcloned into bluescript vector (Stratagene). Northern blot analysis indicated that in rat brain this cDNA hybridized mainly to a 6-kb RNA. No signal was detected in kidney, liver, and muscle RNA.

E. coli Y1089r-containing a lysogen recombinant phage corresponding to pt6 was used to prepare a β-galactosidase fusion protein. Bacterial homogenates were run on a preparative 5% SDS-polyacrylamide gel, and the high molecular weight

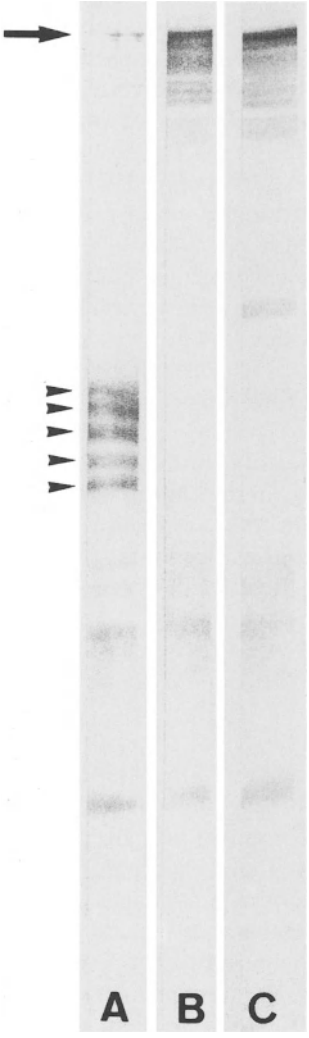

Fig. 1. Immunoblots of total bovine brain proteins (10^5 g supernatant) separated on a 10% polyacrylamide gel (SDS-PAGE), labeled with: *A*, the anti-tau serum used to screen the λgt 11 library – tau proteins *(arrowheads)* and also, weakly, MAP2 *(arrow)* are labeled; *B*, an anti-MAP2 serum; *C*, the serum raised against the pt6 fusion protein. MAP2 is strongly labeled in *B* and *C*

(>200 kDa) fusion protein, clearly separated from bacterial proteins, was sliced out from the gel. This gel slice was homogenized with Freund complete adjuvant and injected in rabbits. The serum was tested by Western blotting and observed to label high molecular weight bands in total brain proteins, the most intense migrating at the same level as MAP2 (Fig. 1, B and C), as judged from adjacent blots labeled with a polyclonal anti-MAP2 (Brion et al. 1988). The fusion protein was labeled on Western blots by the anti-fusion serum, the anti-MAP2, and the anti-tau used to screen the library (not shown).

The cDNA pt6 Encodes Epitopes Present on Neurofibrillary Tangles

The anti-fusion protein was used to perform immunolabeling on paraffin sections of hippocampus in Alzheimer's disease. Many neurofibrillary tangles were observed to be labeled (Fig. 2A); this labeling was strongly reduced by preabsorption of the serum with rat brain thermostable MAP. Immunolabeling was also performed in electron microscopy on PHF in situ and after isolation in the presence of SDS, as previously reported (Miller et al. 1986; Brion et al. 1989). Approximately 15%–20% of isolated PHF were labeled (Fig. 2B).

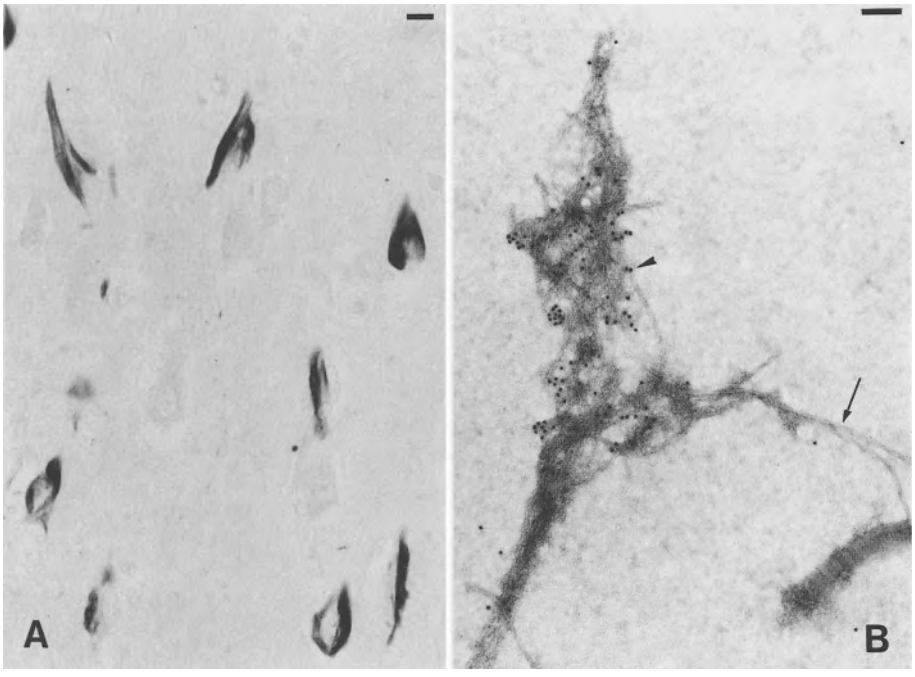

Fig. 2. A Paraffin section of the hippocampus in a case of Alzheimer's disease. Immunolabeling (PAP method) with the serum raised against the pt6 fusion protein. Several neurofibrillary tangles are strongly labeled. *Scale bar,* 10 μm. **B** Electron microscopy. Immunolabeling on isolated PHF (immunogold staining method) with the serum raised against the pt6 fusion protein. *Arrowhead* points to gold particles; *arrow* shows an unlabeled PHF; *Scale bar,* 200 nm

In Situ Hybridization with pt6

In situ hybridization was performed on rat and human brain sections with pt6 cDNA labeled with [^{35}S-]dATP using the random primed method (Feinberg and Vogelstein 1983). Cryostat sections were fixed for 15 min in 4% paraformaldehyde, digested with proteinase K, prehybridized for 2 h in 5 × SSC, 50% formamide, 100 μg/ml salmon sperm DNA, 5 × Derhnardt solution, 100 μg/ml poly(A+), and 200 m*M* dithiotreitol and then hybridized overnight at 42 °C in the same solution added to the denatured probe (2.10^4 cpm/section, specific activity of 5.10^8 cpm/μg) and 10% dextran. Sections were then washed for 2 h in 2 × SSC and for 2 h in 0.1 × SSC at room temperature. After dehydration they were dipped in K5 Ilford emulsion and exposed for 1 week. Control sections were pretreated with ribonuclease (50 μg/ml, 12 h at 37 °C).

On rat and human brain sections, the hybridization signal was observed on neurons; this signal was especially strong in the hippocampus on granule cells and pyramidal neurons (Fig. 3). In Alzheimer's disease, the hybridization signal was also observed on granule cells and on pyramidal neurons in the Ammon's horn. A counterstaining with Congo red was performed to visualize simultaneously neurofibrillary tangles. The hybridization signal in tangle-bearing neurons was similar or decreased by comparison with adjacent neurons without neurofibrillary tangles (Fig. 4).

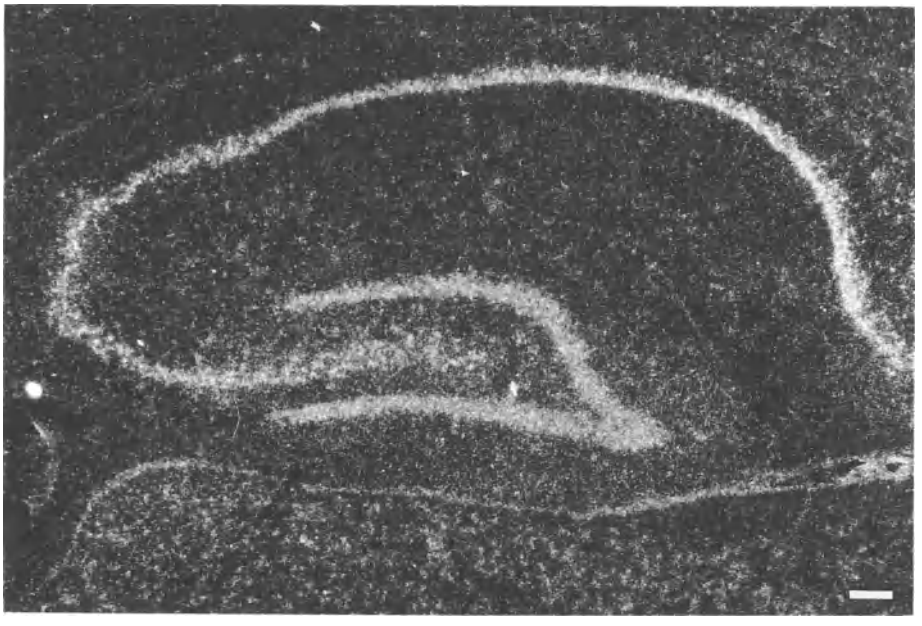

Fig. 3. In situ hybridization with pt6 cDNA labeled with ^{35}S-dATP, on a section of adult rat brain. Dark-field photography, showing the strong hybridization signal on granule cells and on pyramidal neurons in the hippocampus. *Scale bar,* 200 μm

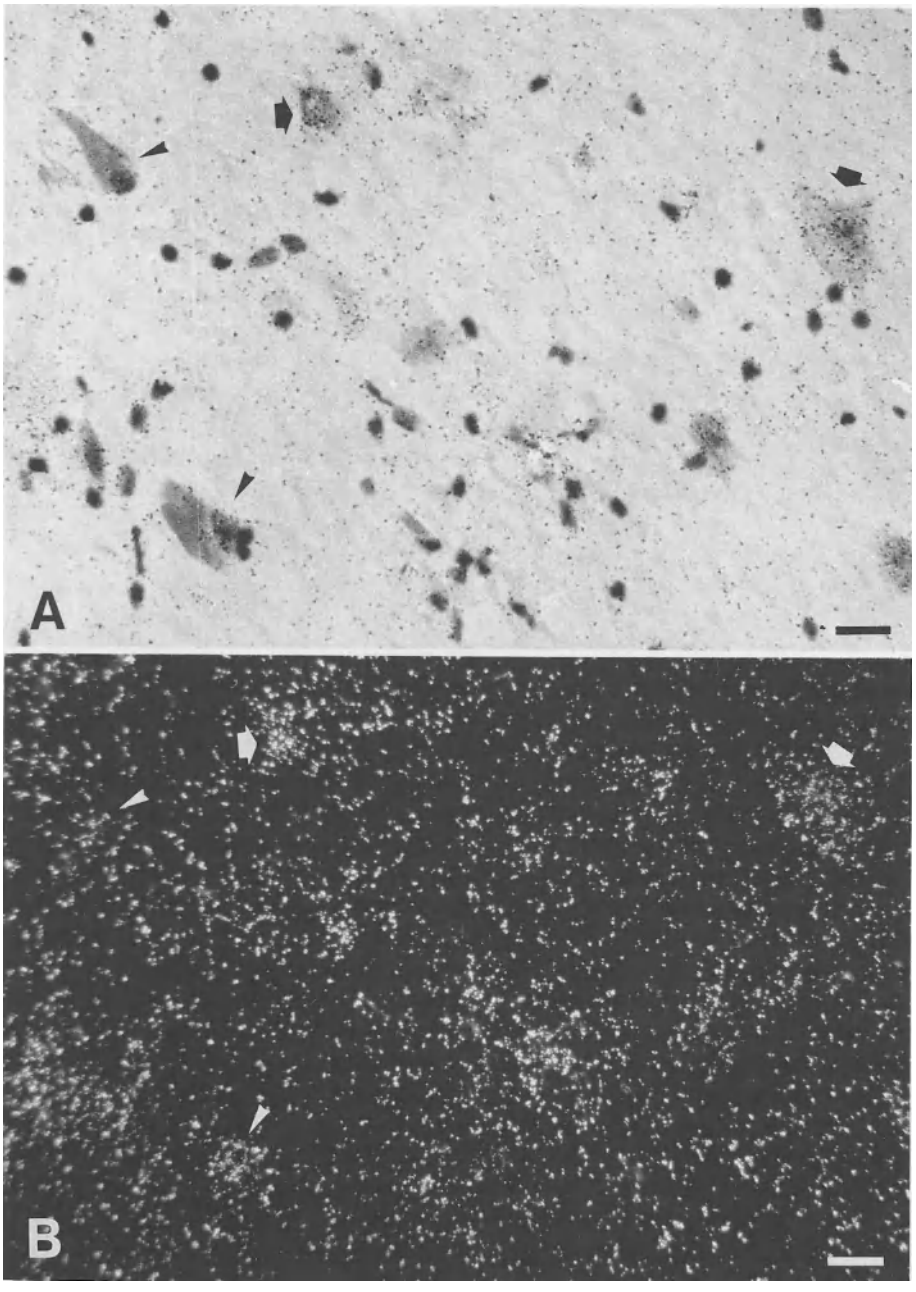

Fig. 4 A, B. In situ hybridization with pt6 cDNA labeled with ^{35}S-dATP, on sections of Ammon's horn in a case of Alzheimer's disease. **A** Area examined under half-crossed polarization filters after Congo red staining. *Arrowheads* point to neurofibrillary tangle-bearing neurons and *arrows* to neurons without tangles. **B** Dark-field photography of the same area, showing the same hybridization-positive neurons with *(arrowheads)* and without *(arrows)* neurofibrillary tangles. *Scale bar,* 20 μm

Discussion

A cDNA encoding epitope shared between MAP and neurofibrillary tangles was used for in situ hybridization studies on Alzheimer's disease sections of the hippocampus, to appreciate the level of corresponding messenger in affected cells. The hybridization signal was not observed to be increased in tangle-bearing neurons relative to adjacent neurons. These results suggest that simple overexpression of this MAP is not directly related to the formation of neurofibrillary tangles.

This cDNA most probably encodes a fragment of rat MAP2. By Western blotting of soluble brain proteins, the corresponding anti-fusion protein labels a high molecular weight protein which migrates at the same level as MAP2. Sequencing data (manuscript in preparation) indicate that this cDNA shows a high homology with a published sequence of MAP2 (Lewis et al. 1988). By Northern blot analysis, this cDNA detects messengers which are brain specific (Brion et al. 1987); the stronger signal is seen for a size of 6 kb, but a 9-kb signal has been observed in primary cultures of embryonic neurons (unpublished data). The reported size for MAP2 messenger is 9 kb in adult mouse (Lewis et al. 1986), but a 6-kb messenger is also abundantly present in the developing and the neonatal brain, although it can be detected in adult (Garner and Matus 1988). This 6-kb MAP2 messenger encodes a 70-kDa MAP2 species (MAP2c), also abundant in the developing and the neonatal brain (Couchie and Nunez 1985; Garner et al. 1988).

Two hundred amino acids at the carboxyl end of MAP2 share a high homology with tau proteins in mouse (Lewis et al. 1988). Several reports have already indicated that MAP2 and tau share immunological cross-reactivity with some antibodies (Ksiezak-Reding and Yen 1987; Yen et al. 1987). The anti-tau serum that we used to screen the library also shows a cross-reactivity with MAP2, and it labels the fusion protein corresponding to pt6 cDNA on Western blot. This could explain why this anti-tau serum identified a MAP2 clone.

Some anti-MAP2 antibodies label neurofibrillary tangles (Kosik et al. 1984; Yen et al. 1987). Similarly, our anti-fusion protein labels tangles in situ, which suggests that pt6 cDNA encodes similar epitopes shared between MAP2 and neurofibrillary tangles. The labeling of neurofibrillary tangles by this antiserum is also strongly decreased by preabsorption with rat MAP. Only a portion of isolated, SDS-extracted PHF was labeled by this anti-fusion protein; these epitopes could thus be lost during the extraction procedure. The isolation of MAP2 cDNA using antibodies labeling neurofibrillary tangles has been reported by other authors (Neve et al. 1986; Dammerman et al. 1988; Kosik et al. 1988a); we show here that a serum raised against a MAP2 fusion protein corresponding to a cDNA directly labels neurofibrillary tangles.

For tau proteins, no differences in the level of corresponding messengers have been reported between normal and Alzheimer's disease cases, by Northern analysis, or by in situ hybridization (Goedert et al. 1988, 1989). The present results suggest that this also holds true for MAP2 in tangle-bearing neurons. The pathological events leading to the formation of neurofibrillary tangles could thus involve primarily posttranslational modifications of MAP rather than simple overexpression of them. Abnormal phosphorylation (Grundke-Iqbal et al. 1986b) and

ubiquitination (Mori et al. 1987; Perry et al. 1987; Brion et al. 1989) are examples of such posttranslational modifications.

Changes in the stability and the translational efficiency of MAP messengers in Alzheimer's disease would not be appreciated directly by Northern analysis and in in situ hybridization studies. cDNA probes like the one we have isolated are also interesting tools to investigate these aspects of MAP metabolism in Alzheimer's disease.

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Epitopes Characteristic of Paired Helical Filaments Demonstrated in Microglial Cells and Macrophages of the Meninges: A Possible Laboratory Diagnosis of Alzheimer's Disease from Cerebrospinal Fluid?

J. Ulrich, S. Ipsen, A. Probst, and *J. P. Brion*

Summary

In AD microglial cells were known for a long time to be present in the senile plaque. Here we demonstrate that in AD a minority of the microglial cells in the neocortex and in the hippocampus can be immunostained with an antiserum to paired helical filaments. Some of them are localized within or in the vicinity of senile plaques, and some have no obvious relationship to plaques. They are more numerous in brains from patients with AD than from those who have only occasional changes of Alzheimer type. Occasional macrophages immunostained for paired helical filaments can also be observed in the cerebrospinal fluid. Therefore it is conceivable that laboratory tests for Alzheimer dementia based on cerebrospinal fluid cytology could become useful.

Introduction

Paired helical filaments (PHF) are the ultrastructural element of Alzheimer's neurofibrillary tangles (Kidd 1963). Recently several groups have prepared antisera which stain these structures very specifically (Brion et al. 1985; Joachim et al. 1987; Bancher et al. 1987; Perry et al. 1987; Défossez et al. 1988). The most important epitopes to which these sera bind are phosphorylated tau proteins (Grundke-Iqbal et al. 1986).

If one looks at histological brain sections from patients with Alzheimer's disease (AD) immunostained with one of these sera, one discovers that, in addition to the neurofibrillary tangles, the processes of tangle-afflicted neurons (so-called "threads"; Braak and Braak 1988) and PHF-containing neurites of senile plaques are stained. Furthermore, when screening the sections carefully, one can also discover immunostained microglia within the central nervous system and in macrophages of the leptomeninges.

The observation of an immunoreactivity thought to be characteristic of PHF in mesenchymal cells might be of considerable interest for research on the pathogenesis of AD. Therefore we investigated these cells more closely. Among other considerations, the possibility of using the immunostained cells as diagnostic markers was kept in mind.

Materials and Methods

In all immunostains used for this study the antiserum to PHF prepared and described by Brion et al. (1985) was used as a primary antiserum.

A total of 380 sections previously immunostained with the antiserum in diagnostic work and in unpublished studies was reevaluated. On most of these sections the immunostains had been used in conjunction with an antibody-alkaline-phosphatase complex, using fuchsin as a chromophore.

For the quantitative study sections from neocortex and hippocampus of ten cases, i.e., five Alzheimer dementias and five controls, were immunostained using Mason's immunoalkaline-phosphatase technique (Mason and Sammons 1978), as recommended by Dakopatts. Briefly, the sections were deparaffinized and soaked in Tris buffer and then exposed for 30 min to the primary antiserum at a dilution of 1 : 600. After washing with Tris buffer the sections were exposed to a goat anti-rabbit immunoglobulin G conjugated to alkaline phosphatase at a dilution of 1 : 40 for 30 min. After washing in buffer the sections were reacted with a substrate containing naphthol AS-Bi phosphatase, propaniol-levamisol, dimethylformamide, sodium nitrate, and new fuchsin. With this procedure, the immunoreactive structures stained red. They were photographed in dark green illumination, which made them appear dark gray or black in the photographic prints.

On each section the total number of immunostained cells with a microglial morphology in the gray matter was counted. The cut surface of gray matter was measured, and the number of microglial cells per square centimeter was calculated. Only cells with a clearly visible nucleus were counted.

Results

Reexamination of Stained Sections Previously Stained for PHF

In addition to senile plaques and Alzheimer tangles (Fig. 1a), microglia-like cells stained with the antiserum to PHF were observed (Fig. 1b). These cells were sometimes present within plaques, usually in contact with the central amyloid core (Fig. 1d). Similar cells without obvious relationship to plaques were also seen. Furthermore, some of these immunostained cells were present in the perivascular spaces (Fig. 1c), in perineuronal position and within the meninges (Fig. 2a).

As these sections were counterstained with hematoxylin, it was possible to identify not only the mature types of plaques by their amyloid core but, occasionally, also some more primitive forms, such as those observed by Probst et al. (1987). About 50 plaques of this type with a central microglial cell could be identified. In this particular type of plaque the central cells were never observed to contain any material reactive with the anti-PHF serum.

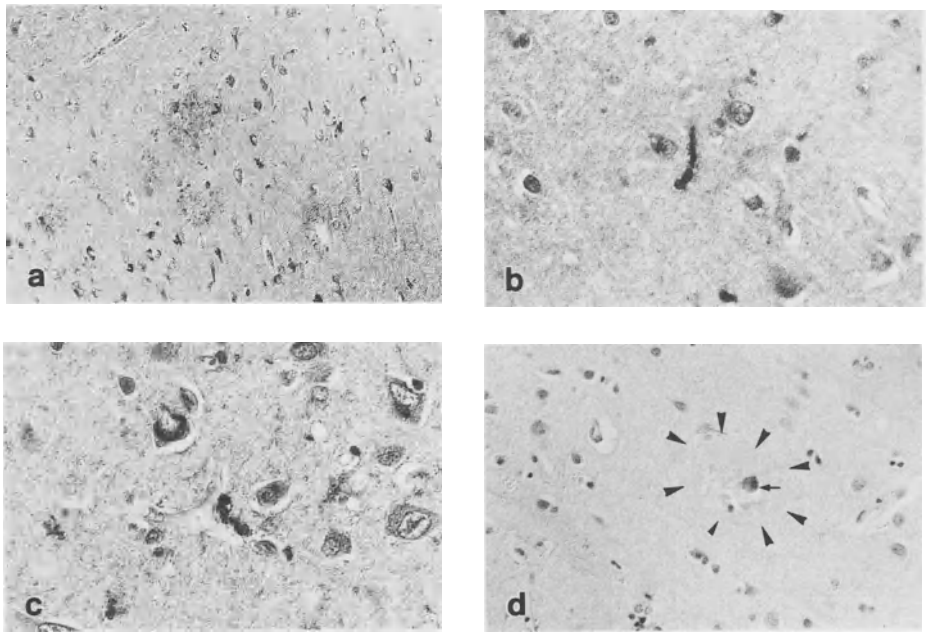


Fig. 1a–d. a Plaques and Alzheimer tangles immunostained with anti-PHF serum (× 80). **b** Microglial cell in the hippocampal neuropil on the same section as **a** (× 320). **c** Microglial cell in contact with a capillary, same section as **a** and **b** (× 320). **d** Microglial cell *(arrow)* in close relationship to a senile plaque (× 320). The photograph was taken through a green filter, which makes the immunostained structures in the tissue (red) appear black

New Sections Stained with the AAP Method

No microglial cells were stained in any of these sections when PBS was used instead of the anti-PHF serum. In the nondemented control cases, occasional neurones with neurofibrillary tangles and their processes ("threads") (Braak and Braak 1988) were seen. A few immunostained mesenchymal cells were also observed. They were clearly less numerous, however, than in the demented cases. No positively stained cells in the meninges were present. Cases with Alzheimer's dementia, as expected, showed many immunostained neurofibrillary tangles, threads and abnormal neurites within senile plaques (Fig. 1a). Furthermore, there was a considerable number of immunostained microglial cells. Some of these were in obvious relationship to plaques. As in the sections mentioned above, such cells were seen in the immediate vicinity of the amyloid core or between altered immunostained neurites of the plaque. It should be stressed, however, that the majority of all microglial cells, both within and outside of the plaques, were *not* reactive with our anti-PHF serum. Occasional, very primitive plaques (Probst et al. 1987) could be identified because of the altered texture of the neuropil and because of their central microglial cell. None of these showed any positive staining

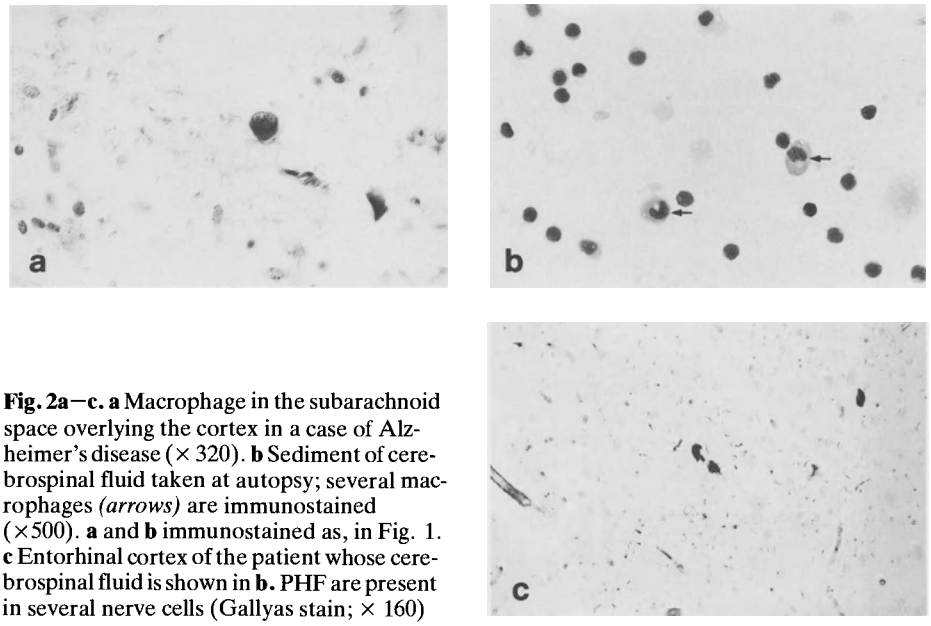


Fig. 2a–c. a Macrophage in the subarachnoid space overlying the cortex in a case of Alzheimer's disease (× 320). **b** Sediment of cerebrospinal fluid taken at autopsy; several macrophages *(arrows)* are immunostained (×500). **a** and **b** immunostained as, in Fig. 1. **c** Entorhinal cortex of the patient whose cerebrospinal fluid is shown in **b.** PHF are present in several nerve cells (Gallyas stain; × 160)

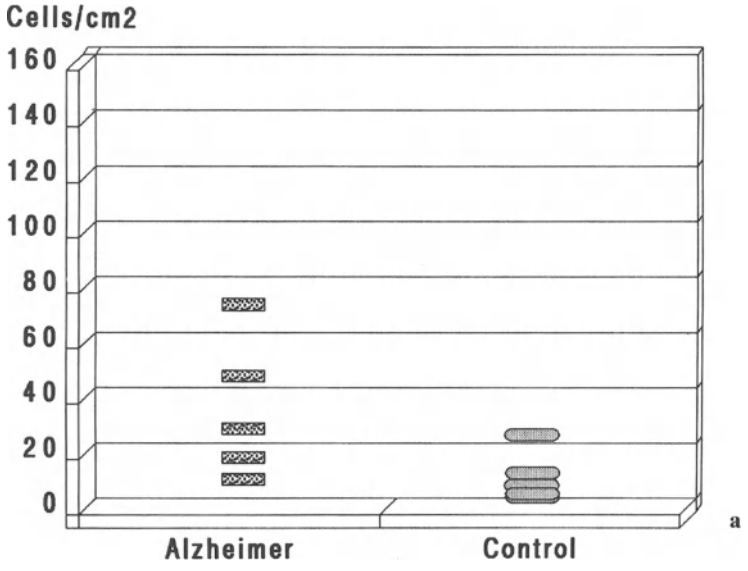
with the anti-PHF serum. A considerable number of immunostained microglial cells without obvious relationship to senile plaques was also observed (Fig. 1b). As with the similarly stained cells in the previously stained sections, some of them were in contact with vessels and perivascular spaces (Fig. 1c), some were in satellite position to neurones and their processes, and some were present in the neuropil without any obvious relationship to other structures. Occasional positively stained cells were also present in the leptomeninges (Fig. 2a). These meningeal cells were not in contact with vessels.

The positively stained microglial cells in gray matter were counted, and their number per square centimeter of gray matter was calculated. They were found to be more numerous in cases of Alzheimer's dementia than in the controls (Fig. 3). The difference was statistically significant in the hippocampus ($p < 0.05$) but not in the cortex.

Discussion

Microglial cells are present both in normal and pathological central nervous tissue. They can be identified morphologically by their elliptic nucleus rich in chromatin, their scanty cytoplasm, and their slender, rarefied cytoplasmic processes stainable with the silver carbonate method of del Rio Hortega. In electron micrographs, their cytoplasm is of distinctive high density (Peters et al. 1976).

In AD microglial cells have been known for a long time to be present in the senile plaque, where they were considered to be a kind of reactive glia (Wisniewski



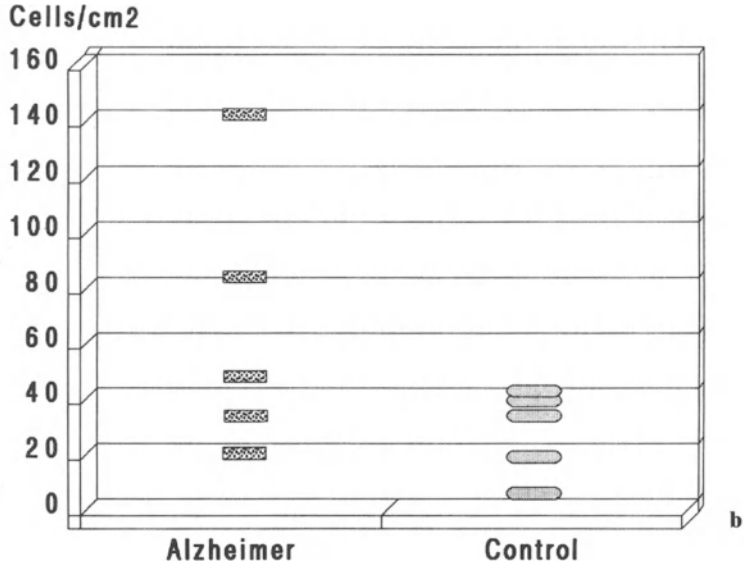


Fig. 3a, b. Number of immunostained microglial cells per square centimeter in hippocampus **(a)** and cortex **(b)** of patients with Alzheimer's disease and normal controls

and Terry 1973). Their development from normal microglia has not been studied so far. It is known, however, that in AD there are microglial cells present in the cortical tissues which present type II histocompatibility antigens (McGeer et al. 1987), and that in some types of senile plaques they take a central position, which argues for an important role in the formation of the plaque (Probst et al. 1987).

They can be stained with various markers, such as the Ricinus communis agglutinin (Mannoji et al. 1986).

In the present study the microglial cells and macrophages were not identified by immunocytochemical markers but by morphology. The majority did not stain for PHF antigens. In a minority, however, as first observed by chance, PHF antigens can be demonstrated. In cortex and hippocampus such immunostained cells occur much more frequently than in tissue of healthy controls. Both in clinically healthy cases with some Alzheimer-type changes and in cases with AD, they were seen in relation to senile plaques, in perivascular and perineuronal position or in the neuropil without any obvious relationship to normal or pathological structures, and in the meninges. Their number was roughly proportional to the number of immunostained neurofibrillary tangles and neuronal processes inside and outside of the plaques. It is probable, therefore, that they phagocytose fragments of cells containing PHF. It is conceivable, however, that they express pathological epitopes related to AD by themselves.

The fact that immunostained microglial cells were not observed in the simpler types of plaques which do not contain neurites with PHF (Probst et al. 1987) suggests the presence of PHF epitopes in phagocytosed material. As macrophages immunoreactive for PHF were also observed in the meninges, it should be possible to discover them in the cerebrospinal fluid. This was actually possible in cerebrospinal fluid drawn at autopsy from a patient with moderate Alzheimer-type changes (Fig. 2b, c).

The present investigation is oriented to the PHF-reactive microglial cells. These might, however, not be representative of the complete population of microglial cells. More complete investigations into these cells are planned.

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Relationship Between Tau, Paired Helical Filaments, Amyloid and the Intellectual Deterioration in the Neocortex in Normal Aging and in Senile Dementia of the Alzheimer Type

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Summary

The mental status of 15 women over 75 years, either intellectually normal of affected by senile dementia of the Alzheimer type at various degrees of severity, was assessed prospectively by Blessed's test score. The pattern and density of tau immunoreactivity were studied in temporal neocortex area 22, and compared with those of PHF immunoreactivity and with the density of amyloid revealed by thioflavin S.

Anti-tau labelled a neuropil meshwork only in cases in which it also labelled senile plaques. The density of this meshwork increased with the severity of the disease. It was higher in layers II, III and V. The number and size of tau positive fibres within the senile plaques also increased with the intellectual deficit.

The densities of senile plaques and of NFT were correlated with the severity of the disease regardless of the staining methods used – anti-tau, anti-PHF or thioflavin S. The three methods revealed a systematically different number of changes. The pathological changes best linked to dementia were those in NFT revealed by tau antiserum. A higher proportion of amyloid-rich plaques was noted in the least affected cases, suggesting that tau and PHF epitopes appeared secondarily. Tau and PHF epitopes were observed simultaneously in the more affected cases. However, tau-positive NFT were statistically more numerous than PHF-positive NFT and increased by a higher factor in the most demented patients. This suggests that PHF accumulate later than tau epitopes in the course of the disease. Another explanation would be that the changes of phosphorylated tau are more directly linked to the dementing process.

Introduction

Tau proteins, which are normally associated with tubulin in microtubules, are considered a major constituent of paired helical filaments (PHF; Brion et al. 1985a; Kosik et al. 1986; Wischik et al. 1988). They are abnormally phosphorylated in these changes (Ihara et al. 1986; Nukina and Ihara 1986). Little is known about the pathological accumulation of tau proteins in relationship with PHF according to the severity of dementia in Alzheimer's disease. Moreover, the relationship

between neurites and amyloid remains open to question. Amyloid may appear first and then be surrounded by neurites, or it may occur secondarily to the degenerating neurites. There is as yet no model to study the development of lesion components in Alzheimer's disease.

A prospective study was undertaken to select intellectually normal elderly subjects and patients of the same age range with senile dementia of the Alzheimer type (SDAT). The test score of Blessed et al. (1968; BTS) was used to quantify the severity of the mental impairment. Clinical criteria were chosen so as to obtain all possible degrees of deterioration and to discard all known causes of dementia other than SDAT. The use of prospectively assessed cases and of morphometric methods allowed us to correlate the severity of the mental dysfunction with neuropathological changes. After death, one hemisphere (randomly right or left) was formalin-fixed. The other was frozen for biochemical studies. Fifteen brains were studied. Macroscopic and microscopic data have been reported previously (Duyckaerts et al. 1985, 1986, 1987, 1988). Anti-tau serum, anti-PHF serum and thioflavin S were used to stain contiguous sections of the temporal neocortex (area 22) of the same series of 15 cases (Delaère et al. 1989).

Anti-Tau Immunostaining

Three major neuropathological lesions are immunolabelled by various anti-tau antibodies in Alzheimer's disease: neurofibrillary tangles (NFT), senile plaques (SP; Brion et al. 1985b; Nukina and Ihara 1986; Delacourte and Défossez 1986;

Table 1. Densities of SP, NFT and tau-positive fibres dispersed in the neuropil

Case No.	BTS	SP			NFT		Neuropil.
		Tau	PHF	Thio	Tau	PHF	Tau fibres
2634	28	0	0	9.25	0	0	0
2944	27	0	0	2.4	0	0	0
2809	26	0	0	3.48	0	0	0
2739	26	0	0	5.81	0.54	0.59	0
2857	19	0	0	0.36	0	0	0
2916	16	0	0	0	0.32	0	0
2943	13	1.07	3.17	24.67	2.97	0.32	+
2813	10	0	0	0	0	0	0
2825	8	7.63	1.90	21.69	24.24	11.98	++
2722	6	0.71	1.77	11	2.15	3.55	+
2942	6	4.62	3.15	12.14	9.53	6.30	++
2812	4	14.82	9.52	29.1	54.08	40.29	++++
2782	2	2.19	1.48	2.54	18.22	6.24	++
2704	2	13.06	8.71	27.59	23.23	11.25	+++
2971	0	1.40	0	27.44	34.53	16.21	++

BTS, Test score of intellectual status (Blessed et al. 1968) prospectively assessed in each case the most affected cases having the lowest values; tau and PHF, densities (n/mm^2) of SP and NFT immunostained with antisera against tau proteins and against PHF – labelling was revealed by peroxidase anti-peroxidase; Thio, SP density (n/mm^2) after staining by thioflavin S; neuropil tau fibers, semiquantitative evaluation of the density of fibers dispersed in the neuropil, immunolabelled with antiserum raised against tau proteins, from a few (+) to very abundant (++++)

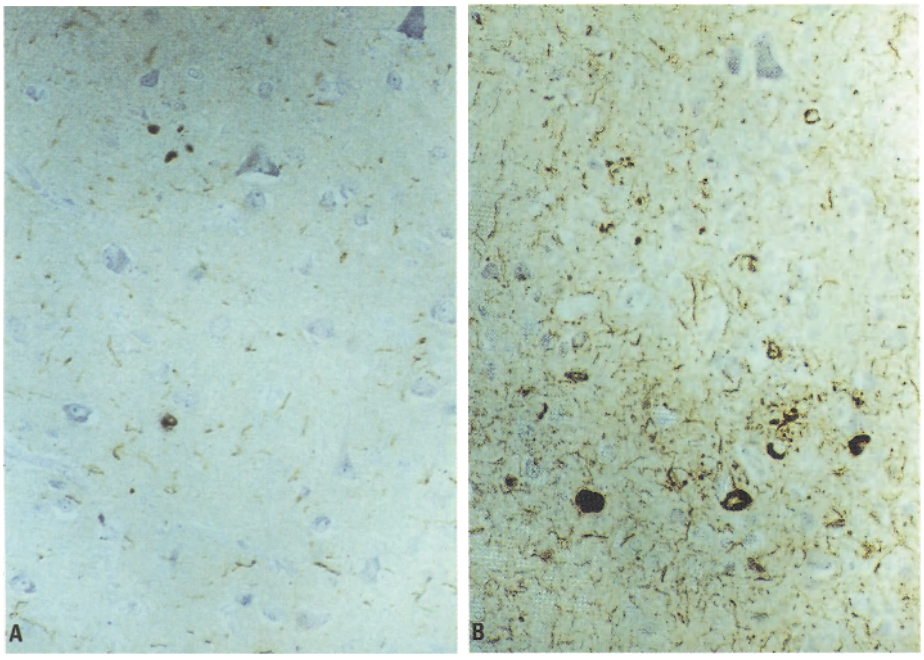

Fig. 1A, B. Tau immunoreactivity in the neuropil of SDAT temporal neocortex (revealed by peroxidase anti-peroxidase, counterstained by cresyl violet). **A** Tau-positive fibres (dark) were rare and very thin in the neuropil of mildly affected patients. **B** Tau-positive fibres were numerous and thick in the neuropil of severely affected patients. (× 25)

Yen et al. 1987) and an abnormal meshwork in the neuropil (Joachim et al. 1987; Kowall and Kosik 1987). The antiserum that we used was shown to be specific in a series of studies (Brion et al. 1985a,b, 1986). Anti-tau labelled an abnormal meshwork only in cases where it also labelled SP (Table 1), i.e. in the demented cases and not in the intellectually normal subjects. The density of this meshwork (Fig. 1) was linked to the number of SP. It was higher in layers II, III and V. This abnormal accumulation of tau proteins could result in a disorganization of the cortical connective network. Recent data obtained after Bodian silver impregnation on the same series of 15 cases indicated that the density of the small, tortuous and abnormally oriented fibres in the same neocortical area was statistically linked to the severity of the dementia (Duyckaerts et al. 1989). The number and size of tau-positive fibres within the SP also increased with the intellectual deficit (Table 2; Fig. 2). Features of tau-positive NFT varied: weak, fibrillary, dense, globular or torch-like, and sometimes reaching the proximal dendrite segments (Fig. 3).

Localization of SP and NFT in the Neocortical Thickness

As revealed by techniques other than immunocytochemistry, SP have a prominent distribution in layers II and III (Pearson et al. 1985; Rogers and Morrison 1985;

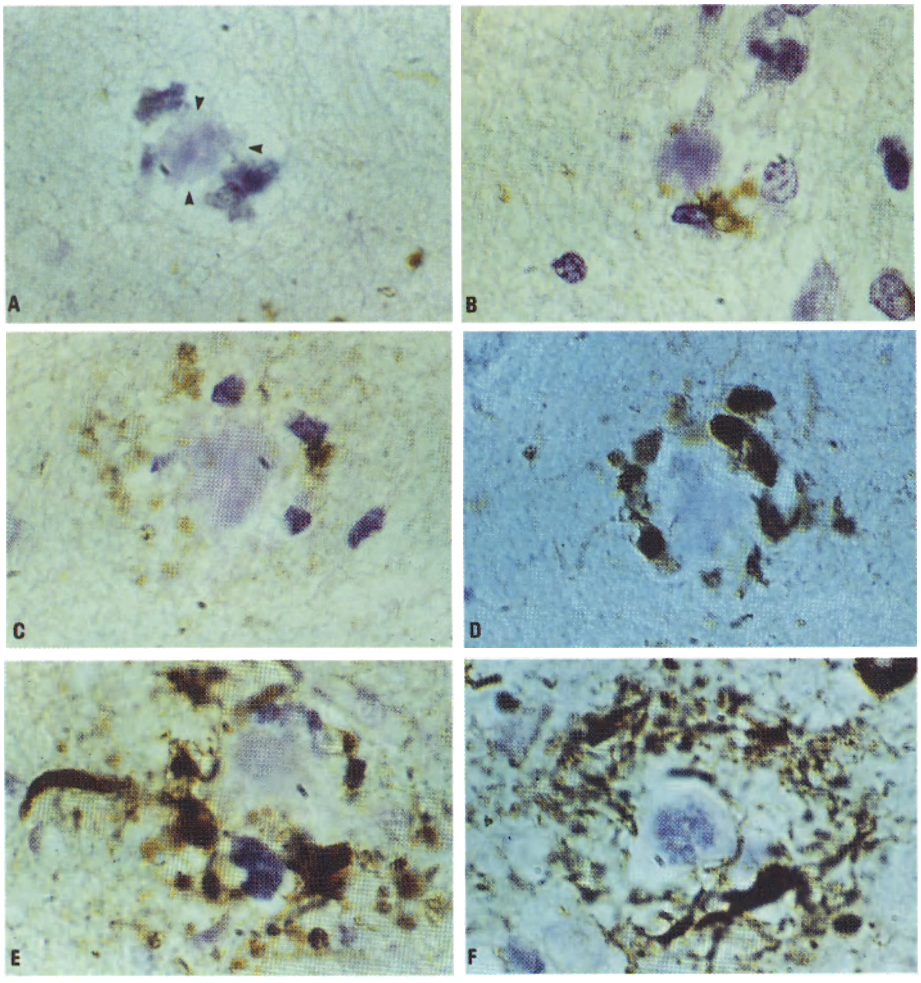

Fig. 2A–F. Tau immunoreactivity in SDAT temporal neocortex showing various aspects of senile plaques (revealed by peroxidase anti-peroxidase, counterstained by cresyl violet). **A** Amyloid core *(arrows)* stained by cresyl violet without tau immunoreactivity. **B, C** A small number of tau-positive neurites (dark) within the SP. **D, E** A large number of tau-positive neurites, sometimes thick, within the SP. **F** A large number of tau-positive neurites embedded in a dense tau-positive matrix. (× 100)

Duyckaerts et al. 1986), and NFT are more numerous in layers III and V (Pearson et al. 1985). According to our morphometric data, the distribution of SP and NFT in the thickness of the neocortex was the same after anti-tau, anti-PHF and thioflavin S staining (Figs. 4, 5). This suggested that the methods which we used stained the same SP and the same NFT, since the latter have the same laminar topographies.

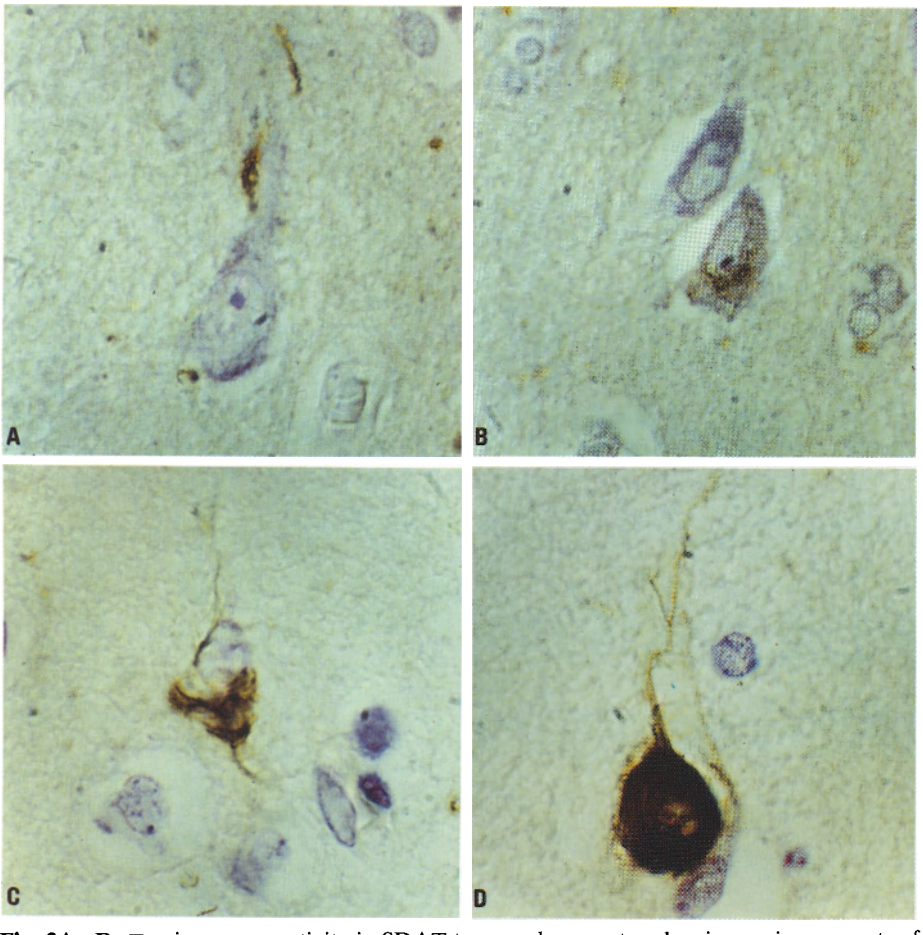

Fig. 3A–D. Tau immunoreactivity in SDAT temporal neocortex showing various aspects of neurofibrillary tangles (revealed by peroxidase anti-peroxidase, counterstained by cresyl violet). **A** Small weak labelling *(dark)* in the proximal dendritic process but not in the perikaryon. **B** Weak labelling in the perikaryon. **C** Dense fibrillar labelling in the perikaryon and in proximal dendritic processes. **D** Dense torch-like labelling which fills all the perikaryon and extends into a dendritic process. *Bar,* 25 μm

Densities of SP and NFT after Anti-Tau, Anti-PHF and Thioflavin S

The density of silver-stained cortical SP is linked to the severity of dementia (Blessed et al. 1968; Wilcock and Esiri 1982; Duyckaerts et al. 1986). The densities of SP and NFT revealed by each of the three staining methods (Table 1) were negatively correlated with the BTS (Table 3); the density of each lesion increased with the intellectual deficit. These correlations were not significantly different from one another. Nevertheless, the pathological changes best linked to dementia were those in NFT revealed by anti-tau.

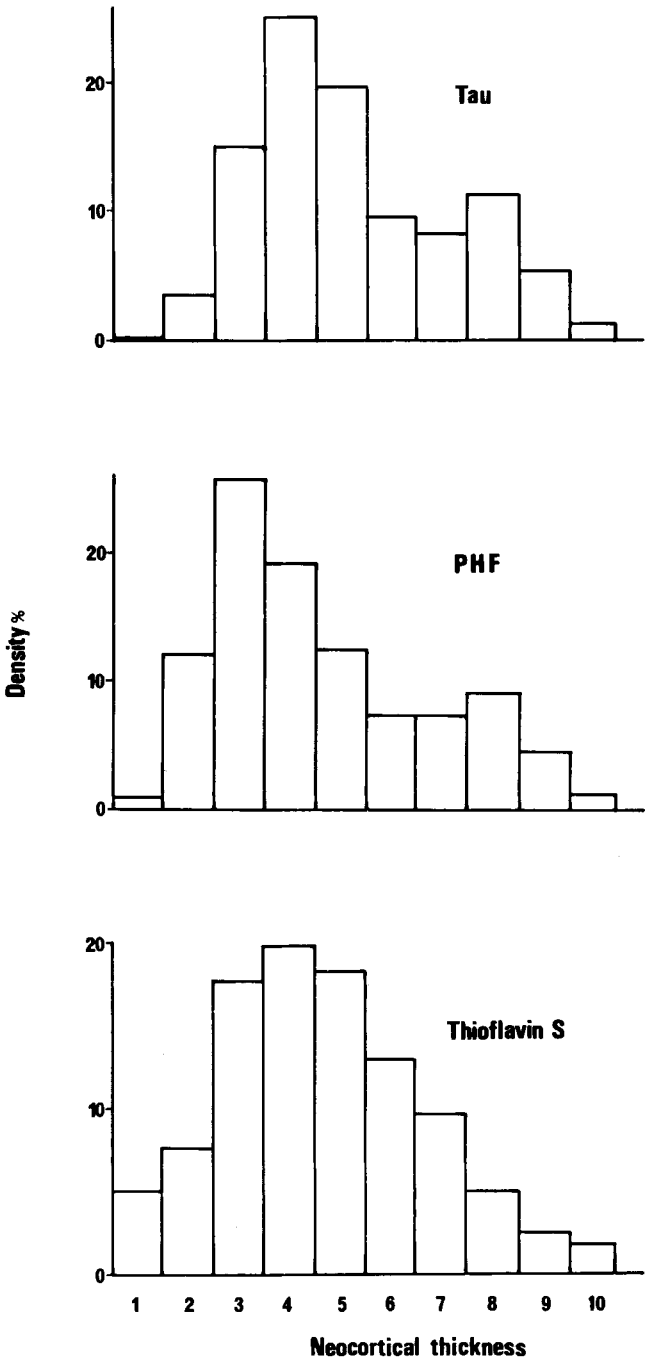

Fig. 4. Density of the SP as a function of their localization in the thickness of the neocortex. Neocortical thickness expressed on a standardized 10-level scale (1, under the pial surface; 10, bordering the white matter). Density, relative frequency; tau, SP immunolabelled by anti-tau; PHF, SP immunolabelled by anti-PHF; thioflavin S, staining method for amyloid

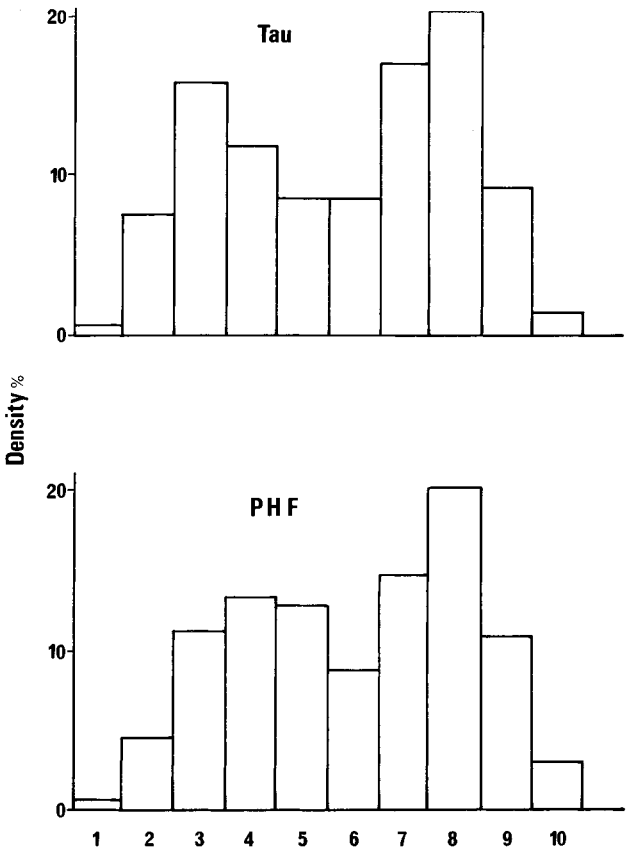

Fig. 5. Density of the NFT as a function of their localization in the thickness of the neocortex. Neocortical thickness expressed on a standardized 10-level scale (1, under the pial surface; 10, bordering the white matter). Density, relative frequency; tau, NFT immunolabelled by anti-tau; PHF, NFT immunolabelled by anti-PHF

Table 2. Assessment of neurites within SP immunolabelled by anti-tau according to the severity of the disease

Case No.	BTS	Number of SP	% of tau-positive SP with					
			Few neurites		Many neurites			Dense matrix
			Small	Large	Small	Large	Very large	
2943	13	25	48	24	16	8	4	–
2825	8	26	12	46	19	19	4	–
2722	6	14	7	21	36	29	7	–
2942	6	27	15	29	15	26	15	–
2812	4	30	–	3	13	50	17	17
2782	2	30	10	13	30	34	13	–
2704	2	29	–	4	24	41	21	10
2971	0	30	3	10	30	50	7	–

BTS, Test score of intellectual status (Blessed et al. 1968) prospectively assessed in each case, the most affected cases having the lowest values; % of tau-positive SP, percentage of SP within which anti-tau have labelled a variable number of neurites; few neurites, <10 neurites in a SP; many neurites, ≥10 neurites; small, tau-positive SP neurites with an approximate diameter of 1 μm; large, approximate diameter of 2.5 μmm; very large, approximate diameter of 5 μm; dense matrix, SP with tau-positive neurites embedded in a tau-positive dense matrix.

Table 3. Correlation of intellectual status (BTS) with the densities of SP and NFT assessed by three staining methods

	SP			NFT	
	Tau	PHF	Thio	Tau	PHF
BTS	−.56[a]	−.54[a]	−.57[a]	−.67[b]	−.57[a]

Pearson correlation coefficients using the least squares method. BTS, test score of intellectual status (Blessed et al. 1968), the most affected cases having the lowest values − this explains the negative correlation between BTS and the densities of senile changes; tau, PHF, immunostaining with antibodies against tau proteins and paired helical filaments (revealed with peroxidase anti-peroxidase); Thio: staining of amyloid with thioflavin S.
[a] $p < 0.05$
[b] $p < 0.002$

Amyloid, Tau and PHF Epitopes: Development According to the Severity of Dementia

The density values varied systematically according to the stain. This could be explained by various sensitivities of the staining methods. It may also have been the consequence of a change in the composition of the lesions.

Neocortical SP in the least affected cases contained amyloid revealed by thioflavin S but not tau- and PHF-positive neurites (Table 1). Moreover, SP with amyloid were statistically more numerous than SP immunolabelled by anti-tau and by anti-PHF. These results suggest that amyloid appeared not only before PHF-positive neurites, as previously shown (Duyckaerts et al. 1988), but also before tau-positive neurites.

Tau- and PHF-positive lesions were lacking in the temporal neocortex of the least affected cases. They were observed simultaneously in the more affected cases (Table 1). These results suggest that the presence of tau and PHF epitopes is strongly related to the changes. However, tau-positive NFT were statistically more numerous than PHF-positive NFT and increased by a higher factor in the most demented patients. This suggests that PHF accumulate later than tau epitopes in the course of the disease. According to our data and those from other studies (Joachim et al. 1987; Bancher et al. 1989), tau epitopes could be present early, and PHF accumulate later in NFT formation. Another explanation would be that the changes of phosphorylated tau are more directly linked to the dementing process.

Conclusion

These results suggest that, in the neocortex, amyloid is present in normal aging while abnormal intracytoplasmic accumulation of tau proteins and PHF is more characteristic of SDAT. The accumulation of tau protein in the neocortical connective network, in SP and in NFT is linked to the severity of the dementia. Tau

and PHF epitopes are strongly related within Alzheimer's changes, but tau protein aggregation seems to be an early stage in PHF formation (Delaère et al. 1989). These results suggest also that the changes in tau proteins are more directly linked to the dementing process. Recent results (Flament et al. 1989) support the assumption that an abnormal phosphorylation of tau proteins leads to their incorporation into PHF (Grundke-Iqbal et al. 1986). The earlier appearance of amyloid in the neocortex over abnormally phosphorylated tau proteins does not necessarily mean a direct or constant relationship of causality. Even in the brain of centenarians, in spite of constant amyloid accumulation, labelling of abnormal neurites by Bodian silver impregnation makes it possible to discriminate easily two groups of cases – those with and those without Alzheimer's changes. In addition, the prevalence of AD is not markedly higher in this group of centenarians than in the 80- or 90-year-old groups (Hauw et al. 1986). This suggests that SDAT is not necessarily an unavoidable consequence of aging and of amyloid deposit. Transcriptional events (Palmert et al. 1988), including modulation of the production of amyloid from the precursor protein (Tanzi et al. 1989) or decrease of amyloid resorption by circulating monocytes/macrophages, microglial cells (Probst et al. 1982) and perhaps astrocytes, are possible candidates for the regulation of amyloid deposit in the brain (Hauw et al. 1988). The factor responsible for the abnormal phosphorylation of tau protein remains open to question.

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Molecular Insights into Alzheimer's Disease

J. W. Pettegrew

Summary

Recent in vitro and in vivo ^{31}P NMR studies demonstrate increased levels of PME and PDE in Alzheimer's brain. The PME levels have an inverse correlation with the numbers of SP and are thought to represent an early molecular event in the pathogenesis of the disease. Similar high levels of PME are observed normally in the developing brain, especially during the period of dendritic proliferation. Cytological studies demonstrate the elaboration of dendritic processes in AD. The levels of PDE have a positive correlation with the numbers of SP and are thought to represent markers of neuronal degeneration. Fluorescence spectroscopy studies reveal that PME and PDE can alter membrane molecular dynamics. Solid-state ^{31}P NMR studies reveal that PME but not PDE can alter the conformation of synthetic phospholipid model membranes and induce transformations from the bilayer phase to the hexagonal II and micellar phases. Similar transformations in vivo could induce the formation of vesicles (micellar) and the fusion of membranes (hexagonal II) with important biological consequences. Computer modeling studies demonstrate the PME to have striking conformational similarities with the neurotransmitters NMDA and L-glutamate. Recent studies reveal the PME to be neuromodulators at L-glutamate receptors in hippocampal CA1 pyramidal cells. This finding suggests possible molecular mechanisms for memory loss and the degenerative features of the disease. The elevated levels of PME also could reflect enhanced phospholipase C activity, which could stimulate protein kinase C activity. Enhanced protein kinase C activity could lead to many diverse biological effects, including the hyperphosphorylation of proteins such as the ADAP and microtubule-associated tau proteins, resulting in altered posttranslational processing of these proteins. These findings provide insights into the molecular pathology of AD which could guide future therapeutic and preventive strategies.

Introduction

The histological hallmarks of Alzheimer's disease (AD) have long been considered to be neurofibrillary tangles (NFT) and neuritic or senile (SP) plaques distributed throughout the allo- and neocortices (Tomlinson and Corsellis 1984). However, recent reports suggest that AD does not necessarily require the presence of NFT (Terry et al. 1987), and the cytopathological changes may be limited to the hippocampus (Ball et al. 1985). In addition, NFT have been demonstrated in some

24 neurological disorders (Halper et al. 1986), and SP occur with normal aging without associated dementia (Tomlinson 1972). Therefore, NFT and SP are not specific to AD and could represent end-stage markers. More recent studies, such as event-related evoked potentials (Fletcher and Sharpe 1986, 1988), positron emission tomography (PET; Cutler et al. 1985; Duara et al. 1986; Jagust et al. 1987; Haxby et al. 1987; Beradi et al. 1987; Berent et al. 1987; Friedland et al. 1987; Horwitz et al. 1987; Rapoport et al. 1986) and phosphorus 31 nuclear magnetic resonance (^{31}P NMR) spectroscopy (Pettegrew et al. 1984; 1987a, b, 1988a, b; Gorell et al. 1988; Gdowski et al. 1988) have demonstrated physiological and metabolic abnormalities in areas of association neocortex, such as the prefrontal and inferior parietal cortices, that are not always the site of numerous NFT or SP. The PET studies demonstrate alterations in glucose uptake, and the ^{31}P NMR studies demonstrate alterations of membrane phospholipid metabolism in the association cortex of AD patients. Our ^{31}P NMR studies demonstrate elevations in the levels of phosphomonoesters (PME) early in the course of AD which progress to elevations in the levels of phosphodiesters (PDE) as the disease progresses (Pettegrew et al. 1988a, b).

Central Hypothesis

The central hypothesis guiding our studies is that the primary molecular/metabolic abnormalities of AD start in the neo- and allocortices and produce secondary retrograde changes in subcortical nuclei such as the nucleus basalis of Meynert, septal nucleus, locus coeruleus, and dorsal raphe nucleus (Pettegrew et al. 1988b). The retrograde changes are simple atrophy of neurons and astrocytosis that occurs with transsynaptic degeneration under other circumstances, for example, dorsomedian neuronal atrophy in the thalamus after frontal leukotomy (Cowan 1970; Duchen 1984). As such, the earliest molecular/metabolic changes in AD occur in the neo- and allocortices, resulting in cellular degeneration and death in these cortical areas and retrograde transsynaptic degeneration in subcortical nuclei. A type of degeneration of cholinergic neurons in the basal nucleus secondary to cerebral cortical damage has been demonstrated in the rat (Sofroniew et al. 1983; Sofroniew and Pearson 1985). Others have suggested that a similar retrograde degeneration may occur in AD (Pearson et al. 1983a, b; Pearson and Powell 1987). Our studies suggest that the earliest molecular/metabolic changes in AD result in elevated levels of PME in the neocortex and allocortex. Cortical and subcortical elevations of PDE occur later in the course of the disease and reflect cellular degeneration and death (Pettegrew et al. 1984, 1987a, b, 1988a, b).

Methodology

Information Contained in the ^{31}P NMR Spectrum

The ^{31}P NMR spectrum of mammalian brain can be conveniently separated into three regions (Glonek et al. 1982): (a) orthophosphate (5 to -1.5 ppm), (b)

guanidophosphate (−3.5 to −5 ppm), and (c) polyphosphate (−5 to −23 ppm). The orthophosphate region can be further subdivided into PME (5 to 1.5 ppm) and PDE regions (1.5 to −1.5 ppm). The polyphosphate region can be further subdivided into ionized ends (−5 to −8 ppm), esterified ends (−8 to −14 ppm), and middles (−18 to −23 ppm).

Contributing to the PME region are hexose 6-phosphates, triose phosphates, pentose phosphates, phosphoethanolamine, phosphocholine, inorganic orthophosphate (P_i), anomeric sugar phosphates, and several signals that have not been characterized as to the source phosphate. Contributing to the PDE region are glycerol PDE (primarily glycerol 3-phosphoethanolamine and glycerol 3-phosphocholine), a broad resonance from phosphorylated glycolipids and glycoproteins, and several uncharacterized resonances. The guanidophosphate region contains resonances from phosphocreatine (P_{Cr}) and phosphoarginine.

In the polyphosphate part of the spectrum, the ionized ends region contains resonances from the γ-phosphate of nucleotide triphosphates and the β-phosphate of nucleotide diphosphates. The esterified ends region contains resonances for the α-phosphate of nucleotide triphosphates, the α-phosphate of nucleotide diphosphates, the nicotinamide adenine dinucleotides, and the uridine diphospho sugars (galactose, glucose, mannose). The only resonance that makes a contribution to the middles region is the β-phosphate of nucleotide triphosphates. In mammalian brain, the predominant contributors to the nucleotide triphosphate and nucleotide diphosphate resonances are adenoside triphosphate (ATP) and adenosine diphosphate (ADP) (Fig. 1).

From a metabolic viewpoint, the ^{31}P NMR spectrum contains information about the energy status of the brain from the resonances for P_{Cr}, ATP, ADP and P_i. Resonances related to phospholipid metabolism are contained in the PME and PDE regions (Pettegrew et al. 1987a, b). In mammalian brain, the PME region contains resonances predominantly from α-glycerol phosphate, phosphoethanolamine and phosphocholine, with smaller contributions from sugar phosphates such as hexose 6-phosphates, fructose 1,6- diphosphate, inositol phosphates, and nucleoside phosphates such as ribose 5′-phosphate, inosine 5′-

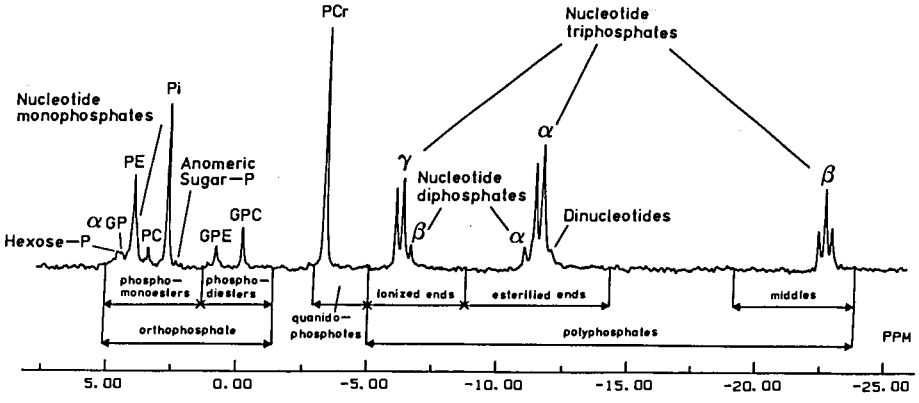

Fig. 1. High-resolution ^{31}P NMR spectrum of 24-month-old Fischer 344 rat brain perchloric acid extract

monophosphate, adenosine 5′-phosphate and NADP 2′-phosphate. In normal mammalian brain, α-glycerol phosphate, phosphocholine, and phosphoethanolamine are found predominantly in the anabolic pathway of membrane phospholipid metabolism. However, a recent study demonstrates that the Ha-*ras* oncogene specifically activates phospholipase C hydrolysis of phosphatidylcholine (PtdC) and phosphatidylethanolamine (PtdE), giving rise to elevated levels of diacylglycerol and the PME phosphocholine and phosphoethanolamine (Lacal et al. 1987). Other biological activators of phospholipase C also could produce elevated levels of PME. The PDE region contains predominantly the resonances of glycerol 3-phosphoethanolamine and glycerol 3-phosphocholine which, in mammalian brain, are catabolic breakdown products of phospholipids due to phospholipase A_1 + A_2 activity (Dawson 1985; Porcellati and Arienti 1983). Therefore the steady-state turnover of brain phospholipids (anabolism/catabolism) can be assessed by ^{31}P NMR spectroscopy (Pettegrew et al. 1987a, b). Since neural membrane (especially synaptosomal) structure, dynamics and function are of vital importance to normal neurochemical, neurophysiological, and neuropharmacological function, ^{31}P NMR has the potential to provide important insights into normal and altered brain function. ^{31}P NMR spectra of post-mortem brain contains resonances only from the PME, PDE and P_i. Therefore, ^{31}P NMR studies on post-mortem brain predominantly allow assessment of antemortem phospholipid metabolism and possible biological activation of phospholipase C and phospholipase A_1 + A_2 through the PME and PDE resonances, respectively.

In Vitro ^{31}P NMR Studies

The in vitro ^{31}P NMR studies provide chemical conditions more favorable to ^{31}P NMR analysis than occur in the living brain and, therefore a greater sensitivity and resolution are achieved as compared to in vivo analytical approaches. The enhanced sensitivity and resolution of in vitro extract studies permit the characterization and quantitation of many different phosphorus-containing compounds. Previous in vitro ^{31}P NMR studies demonstrated a remarkable correlation with more classical assay procedures and, in addition, revealed previously uncharacterized metabolites and unrecognized metabolic relationships (Pettegrew et al. 1982a, b, 1983, 1984, 1985, 1986a, b, 1987a, b; Glonek et al. 1982; Cohen et al. 1984).

To correctly interpret ^{31}P NMR spectra, the identities of the individual resonance signals must be carefully verified through the use of appropriate biochemical and spectroscopic procedures. The importance of this verification was recently demonstrated for a prominent ^{31}P NMR resonance at 3.84 ppm in mammalian brain which has now been definitively identified as phosphoethanolamine (Pettegrew et al. 1986a). The identification was based on ^{1}H and ^{31}P NMR findings (including pH titrations) at 4.7 and 14.1 Tesla, as well as thin-layer chromatography studies. The ^{31}P NMR studies are in agreement with earlier studies that demonstrated a relative abundance of phosphoethanolamine in developing rabbit brain (Cohen and Lin 1962). A relatively prominent PME resonance exhibiting the appropriate ^{31}P chemical shift has been reported in human neonatal brain

(Cady et al. 1983; Younkin et al. 1984) and childhood neuroblastoma (Maris et al. 1985) using an in vivo ^{31}P NMR surface coil technique.

In Vivo ^{31}P NMR Methodology

We recently have obtained in vivo spectra under resting conditions (lying quietly, eyes open, ears unoccluded) from the dorsal prefrontal cortex of 41 subjects with an age range of 12–81 years. In vivo ^{31}P NMR spectroscopy was conducted on a General Electric Signa 1.5 Tesla whole body system equipped with the standard spectroscopy accessory. The spectra were acquired using a depth-resolved pulse sequence employing an H_1 field gradient and concentric 8- and 3-in. surface coils A ^1H image was initially obtained using the MRI body coil as the ^1H transmitter and the 3-in. surface coil as the ^1H receiver. The images obtained were from 15–20 cm^3 of the dorsal prefrontal cortex (Fig. 2). The H_0 field was then shimmed on the H_20 ^1H signal to a line width of approximately 0.1 ppm, and ^{31}P spectra were obtained which were easily phased. The individual resonances were deconvoluted into component Lorentzian peaks and integrated to yield quantitative results.

The in vivo results compare quite favorable with results obtained from extracts of freeze-clamped animal cerebral tissue. The resonances of PME, P_i, PDE, ionized ends, esterified ends, and middles are easily distinguished (Fig. 3). The PME, P_i, PDE, and P_{Cr} resonances of brain are not completely resolved in the

Fig. 2. ^1H labeled brain image of a normal volunteer demonstrating that the surface coils used for ^{31}P NMR spectroscopy observe signals only from the dorsal prefrontal cortex

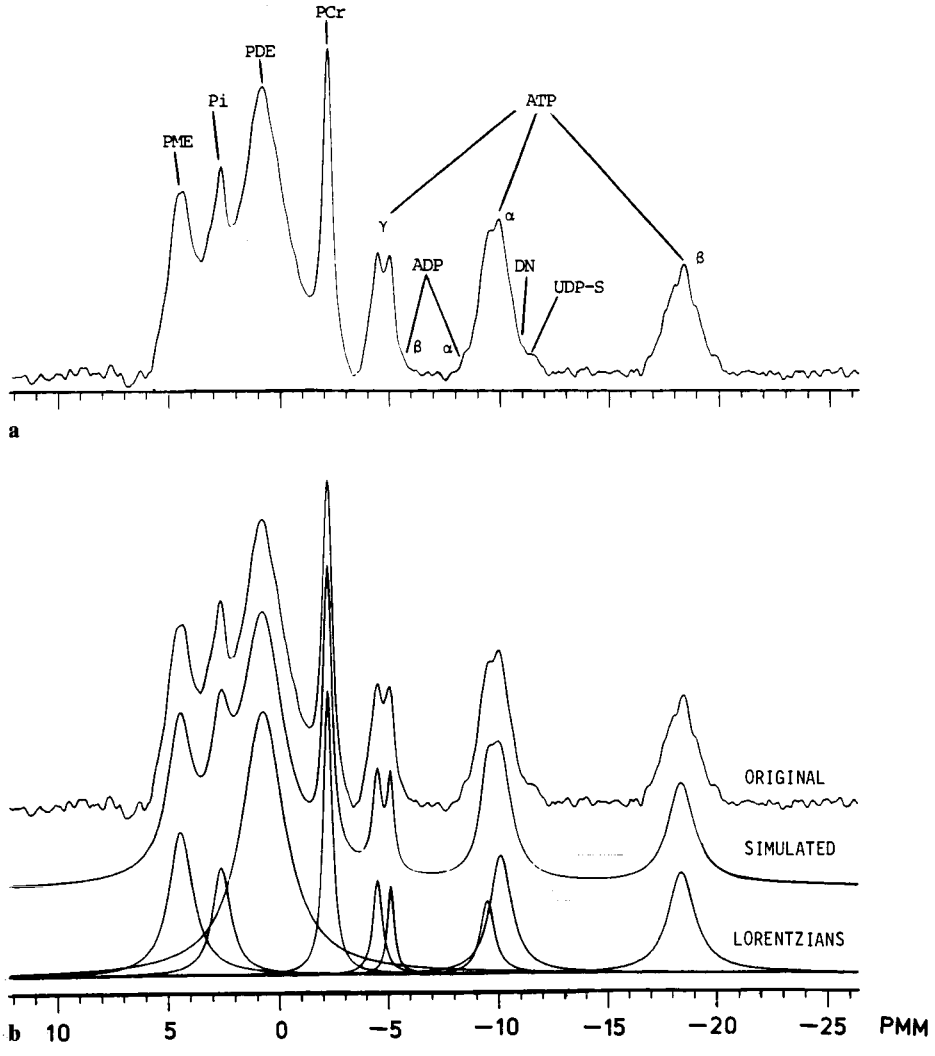

Fig. 3a, b. a In vivo ^{31}P NMR spectrum from the dorsal prefrontal cortex of a normal elderly volunteer. **b** The unprocessed *(top)*, simulated *(middle)*, and component Lorentzian *(bottom)* spectra are shown

unprocessed in vivo ^{31}P NMR spectrum obtained at 1.5 Tesla. This is probably due to a combination of at least three factors. (a) The NMR natural line widths for these brain chemical species in vivo are probably broader than for the same chemical species in solution. (b) There is decreased resolution of these resonances at 1.5 Tesla compared to H_0 fields of 4.7 Tesla or higher. (c) The H_0 field homogeneity across the human head is not as good as that attainable across smaller sample (5–20 mm) diameters.

Our in vivo results in human subjects demonstrate that we not only can detect the PME and PDE resonances as well as the resonances for P_i, P_{Cr}, ATP, ADP, and nucleoside diphospho-derivates (NDP-D), but that we also can accurately quantify the levels of these metabolites and the intracellular pH (Pettegrew et al. 1988c). We have determined test-retest (n = 10) and interrater reliability (n = 10) in young and elderly controls and the above-mentioned patient groups for all of these metabolites. The test-retest results demonstrate a standard deviation of less than 5% of the mean, and the interrater reliability results also demonstrate a standard deviation of less than 5% of the mean.

Results

In Vitro ^{31}P NMR Studies of Autopsy Brain

Recent ^{31}P NMR studies from this laboratory have demonstrated alterations of membrane phospholipid metabolism in AD brain obtained at autopsy and biopsy (Pettegrew et al. 1984, 1985, 1987a, b, 1988a, b; Miotto et al. 1986). The alterations do not correlate with the duration of the postmortem interval and are thought to reflect antemortem metabolic changes. AD brains contain significantly elevated levels of PME ($p < 0.001$), which are precursors to membrane phospholipids or products of phospholipase C activity, without significant elevations of PDE, which are phospholipase $A_1 + A_2$ mediated breakdown products of membrane phospholipids. In contrast, non-AD diseased control brains contain significant elevations of PDE ($p < 0.01$). The areas of AD brain with PME elevations are the superior and middle frontal gyri and the inferior parietal cortex. These same cortical areas of AD brain have decreased glucose utilization and abnormal electrophysiological responses to event-related evoked potentials (Cutler et al. 1985; Duara et al. 1986; Jagust et al. 1987; Haxby et al. 1987; Beradi et al. 1987; Berent et al. 1987; Friedland et al. 1987; Horwitz et al. 1987; Rapoport et al. 1986; Fletcher and Sharpe 1986, 1988). Correlative ^{31}P NMR and morphological studies have been conducted on 32 samples obtained from the middle frontal and superior temporal gyri of 11 AD brains. A significant negative correlation was found between the levels of PME and the numbers of SP ($p = 0.05$; $r = 0.76$) and a significant positive correlation between the levels of PDE and the numbers of SP ($p = 0.01$; $r = 0.89$). No correlations were found between the numbers of NFT and the levels of PME or PDE. These findings suggest that elevations in the levels of PME precede the appearance of SP, and that elevations in the levels of PDE coincide with the appearance of SP.

In Vivo ^{31}P NMR Studies

Effect of Normal Aging

Studies to date investigated the levels of PME, P_i, PDE, P_{Cr}, ATP, NDP-D (uridine diphospho-sugars (UDP-S) and cytidine diphospho-derivatives such as

CDP-choline and CDP-ethanolamine) and intracellular pH in the dorsal prefrontal cortex of normal young adult ($n = 10$, 27 ± 2 years) and normal elderly ($n = 6$, 72 ± 3 years) individuals. All subjects were screened for and determined to be free of medical, neurological, and psychiatric disorders.

Compared to young adults (27.6 ± 1.7 years), normal elderly subjects (72.3 ± 3.1 years) have decreased levels of PME ($p = 0.05$) and NDP-D ($p = 0.0002$) and increased levels of PDE ($p = 0.0004$) without changes in P_{Cr}, ATP, or intracellular pH. The decreased levels of PME and increased levels of PDE suggest decreased synthesis and increased breakdown of membrane phospholipids in normal elderly human cerebral cortex. The decreased levels of NDP-D suggest decreased glycosylation of membrane proteins and lipids (UDP-S) or decreased synthesis of membrane phospholipids (CDP-choline). Neither of these aging-related changes in membrane metabolism is surprising given the histological evidence for changes in dendritic spines in aged animal and human brain (Connor et al. 1980, 1982; Flood et al. 1986; Hinds and McNelly 1977, 1981; Rogers et al. 1984). However, for the first time these changes can be noninvasively followed in living human subjects. These findings provide molecular insights into normal brain aging processes not available with other in vivo techniques.

In Vivo ^{31}P NMR Studies in Alzheimer's Disease

These studies examined high-energy phosphate and membrane phospholipid metabolism in the dorsal prefrontal cortex of AD patients by in vivo ^{31}P NMR and correlated the NMR findings with the degree of dementia as determined by the Mattis dementia rating scale. To date we have studied six probable AD patients (ADRDA-NINCDS criteria) and six normal age-matched elderly controls. Mattis dementia rating scales were performed on all subjects within 6 months of the NMR studies. The results demonstrate a negative correlation between the levels of PDE and the Mattis scores ($R^2 = 0.97$; $p = 0.01$) and a positive correlation between the P_{Cr} levels and the Mattis scores ($R^2 = 0.95$; $p = 0.01$). The PME levels did not have a significant correlation with the Mattis scores ($R^2 = 0.61$). There were no within-group or between-group differences in the intracellular pH. Therefore, the P_{Cr} changes were not due to a pH-induced alteration in the creatine kinase equilibrium.

These in vivo findings are consistent with our PDE findings in AD autopsy brain and suggest that the alterations in PME perhaps antedate the onset of cognitive changes and, therefore, are an early molecular alteration. The correlation between PDE and Mattis scale suggests that the cognitive decline is closely associated with neuronal degeneration which occurs later in the course of the disease. The correlation between P_{Cr} and Mattis scale suggests either decreased synthesis or increased utilization of P_{Cr} as the disease progresses. These metabolic insights taken together could prove valuable in advancing our understanding of the molecular basis of AD. In vivo ^{31}P NMR also could prove valuable in noninvasively following metabolic responses to therapeutic interventions. Recently an independent in vivo ^{31}P NMR spectroscopy study also demonstrated increased levels of PME in the temporoparietal cortex of probable AD patients as compared

to patients with subcortical multi-infarct dementia and demented patients with Parkinson's disease (Gdowski et al. 1988; Brown et al. 1989).

Possible Explanations for Increased PME

The findings of increased PME in AD brain cannot be explained simply by degeneration of brain tissue; with degeneration, the PDE should be elevated and not PME. Increased turnover of membrane phospholipids should result in elevations of both PME and PDE as observed in normal aging in Fischer 344 rats (Pettegrew et al. 1985). The PDE elevations in non-AD diseased brain could be consistent with increased phospholipid turnover or degeneration of brain tissue. The finding of increased PME in AD brain suggests one or more of the following mechanisms:

a) increased synthesis of membrane phospholipids (Pettegrew et al. 1987a, b, 1988a, b);
b) a relative metabolic block in the synthetic pathway (Pettegrew et al. 1987a, b, 1988a, b);
c) a decreased breakdown of PME secondary to decreased phospholipase D activity in AD brain (Kanfer and McCartney 1987); or
d) stimulation of phospholipase C by neuromodulators and growth factors (Nishizuka 1986) or oncogenes (Lacal et al. 1987).

Elevated levels of PME are observed normally in the immature, developing brain (Pettegrew et al. 1986a, b, 1987a, b, 1988a, b), particularly during the period of elaboration of dendritic processes. Our findings of elevated PME in AD brain could, therefore, suggest an increase in membrane phospholipid synthesis as occurs during the elaboration of complex membranous structures such as dendritic processes. Cytological evidence for the elaboration of dendritic processes has been previously reported in AD (Scheibel 1979; Geddes et al. 1985) and normal aged brain (Buell and Coleman 1979, 1981). In addition, similar regenerative attempts have been observed in Huntington's disease brain (Graveland et al. 1985), another "degenerative" neurological disorder in the behavioral and neuropathological senses. However, other studies have been interpreted as evidence of failed compensatory dendritic growth in the parahippocampal gyrus and the hippocampal dentate gyrus of AD brain (Flood et al. 1986; Flood and Coleman 1986). Neither of these structures has been studied to date by ^{31}P NMR, and the ^{31}P NMR findings in cerebral cortex may not be the same as for the parahippocampal gyrus and the hippocampal dentate gyrus. In fact, studies in the rat have demonstrated regional variability for age-related dendritic changes (Hinds and McNelly 1977, 1981; Connor et al. 1980, 1982; Rogers et al. 1984). Finally, a recent study of AD platelets has demonstrated increased proliferation of platelet internal membranes (Zubenko et al. 1987a), suggesting a generalized increase in the synthesis of membranes in AD.

An elevation of the PME phosphoethanolamine and phosphocholine also could occur if there existed a relative metabolic block at the enzyme cytidine triphosphate (CTP):phosphoethanolamine (or phosphocholine) cytidyltransferase (EC 2.7.7.15), which is the rate-limiting enzyme in phospholipid synthesis (Dawson

1985). An enzymatic block at this step could result in elevated levels of PME as assayed by ^{31}P NMR and decreased levels of the phospholipids, PtdC and PtdE. Recent high performance liquid chromatography (HPLC) studies of AD brain phospholipids do demonstrate a reduction in AD brain phospholipids as expressed as micromoles of phospholipid per gram wet weight of brain (Pettegrew et al. 1988d). As compared to non-AD diseased controls (60 samples, 9 brains), AD brain (131 samples, 17 brains) has decreased levels of PtdC ($p = 0.001$), PtdE ($p = 0.003$), and cholesterol ($p = 0.01$) and small but nonsignificant decreases in the levels of phosphatidylserine (PtdS). The levels of PtdC, PtdE, PtdS, and cholesterol were correlated with the number of SP per × 200 magnification between cortical layers II and IV in the same brain regions. This revealed a nonlinear correlation for PtdC which peaked around 10 SP/× 200 magnification and declined with increasing SP numbers ($R^2 = 0.9$; $p = 0.0001$). Linear negative correlations were observed for PtdE ($R^2 = 0.6$; $p = 0.2$) and PtdS ($R^2 = 0.8$; $p = 0.003$) and no correlation for cholesterol ($R^2 = 0.8$; $p = 0.5$). These findings suggest an increase in PtdC synthesis early in the pathogenesis of AD at a time when PME levels are high. In theory, the augmented synthesis of PtdC could shunt available choline from acetylcholine synthesis, producing a functional cholinergic deficit which is observed clinically (Bartus et al. 1982). As the disease progresses, membrane degradation assumes prominence. It must be emphasized that these findings are based on HPLC analysis which uses UV detection at 203 nm as the method of quantitation. Therefore the number of fatty acid double bonds that are present determine the absorbance. One cannot assume the fatty acid double bond composition for any given phospholipid to be the same in a human disease state as it is in the nondiseased state from which the phospholipid standards are derived. For this reason an independent analytical method is essential to confirm or refute the HPLC findings. A recently described ^{31}P NMR method for the direct qualitative and quantitative determination of phospholipids on the basis of the phosphorus atom is such a method (Meneses and Glonek 1988). We are presently pursuing these ^{31}P NMR phospholipid studies.

The increased levels of PME early in the pathogenesis of AD also could reflect altered levels of, or responses to, neuromodulators, growth factors, or oncogenes. It is therefore of interest that CTP:phosphocholine cytidyltransferase activity has been demonstrated to be decreased under conditions that favor phosphorylation of proteins, such as stimulation of protein kinase systems (Pelech et al. 1985). There is other evidence for possible elevated protein kinase activity in AD brain. The microtubule-associated tau proteins have been shown to be hyperphosphorylated in paired helical filaments found in AD brain (Grundke-Iqbal et al. 1986). The hyperphosphorylation of the tau proteins could secondarily result in defective microtubule assembly and altered axoplasmic flow in AD brain (Iqbal et al. 1986). The hyperphosphorylated tau proteins also might be abnormally metabolized, leading to the formation of paired helical filaments found in NFT. A recent report demonstrates that the AD amyloid precursor protein (ADAP) is phosphorylated by protein kinase C and Ca^{2+}/calmodulin-dependent protein kinase II (Gandy et al. 1988). Phosphorylation of ADAP potentially could interfere with membrane insertion resulting in cleavage to β-amyloid protein which is deposited intracellulary and in SP in AD brain. It is conceivable that the elevated levels of

PME early in the course of AD reflect increased synthesis of membrane phospholipids, but that later in the course of the disease a metabolic block develops at the rate-limiting enzyme step which further increases PME levels. The metabolic block could arise secondary to activation of receptors linked to phospholipase C and protein kinase C activity such as the L-glutamate and *N*-methyl-D-aspartate (NMDA) receptors. A recent study of protein kinase C levels in AD autopsy brain (Cole et al. 1988) is consistent with a compensatory down-regulation of protein kinase C after overactivation early in the disease process. It is also of interest that a 28 amino acid fragment (β1−28) of the β-amyloid precursor protein has been demonstrated to have neurotrophic activity in cultures of hippocampal pyramidal neurons (Whitson et al. 1989). What physiologic role if any the β1−28 fragment has in AD is unknown at this time.

Effects of PME and PDE on Membrane Properties

In an effort to study the possible consequences of elevated levels of PME and PDE on membrane molecular properties, we have performed fluorescence spectroscopy on intact normal human erythrocytes and platelets in the absence and presence of varying concentrations of the PME (phosphocholine, phosphoethanolamine, L-phosphoserine, and combinations thereof) and PDE (glycerol 3-phosphocholine and glycerol 3-phosphoethanolamine). The fluorescence spectroscopy studies reveal that PME can significantly alter membrane molecular dynamics in normal human erythrocytes and platelets (Pettegrew et al., manuscript in preparation). These findings provide an explanation for the recent platelet findings in AD (Zubenko et al. 1984, 1987a, b). The platelet findings could reflect the alterations in PME and PDE levels that are occurring in the brain of AD patients.

In an effort to study the consequences of the elevated levels of PME and PDE on plasma membrane phase and conformation, we performed solid-state ^{31}P NMR spectroscopy on model membranes in the presence of 1.0 m*M* PME or PDE. It was observed that the PME do not affect the powder pattern spectrum of the phospholipid palmitoryloleoylphosphatidylcholine (POPC), whereas significant alterations were observed in palmitoryloleoylphosphatidyl-ethanolamine (POPE) and bovine brain PtdS. These results demonstrate alterations in the head group orientation and motion in POPE and PtdS but not in POPC. PDE had no effect on the line shape of any of the lipids (Pettegrew et al., in preparation). It should be noted that POPC is predominantly on the external face, and POPE and PtdS are the major components of the cytoplasmic face of cell membranes. Should similar changes occur in plasma membranes in vivo, significant functional changes can be expected. Calcium and Al^{3+} induce very similar alterations in membrane phase and conformation (Pettegrew and Panchalingam 1989). These studies taken together strongly suggest that in the presence of elevated levels of Ca^{2+}, Al^{3+}, or PME, the phospholipids located on the cytoplasmic face of membranes can be transformed from a normal bilayer phase to micellar and hexagonal II phases. The micellar phase could facilitate the formation of vesicles providing a packaging of the PME and other neurotransmitters such as L-glutamate. The formation of the

hexagonal II phase could facilitate the fusion of the vesicles to the cytoplasmic face of the membrane for transmembrane transport to the extracellular space. These functional alterations could affect membrane receptors, ion channels, and structural and enzymatically active proteins such as those involved in the second messenger molecular cascade. Such alterations could have significant clinical implications.

Computer Molecular Modeling Studies

Some consideration should be given to the possibility that the elevated PME could have a neurophysiological role in AD beyond merely reflecting membrane abnormalities. Available evidence now suggests that the elevated PME (phosphoethanolamine, phosphocholine, and L-phosphoserine) have other functions, for example, as false neurotransmitters. This possibility was stimulated by our observation that the PME have chemical structural similarities to the known neurotransmitters L-glutamate and NMDA. To investigate the possibility of conformational similarity, minimum energy conformations were computed by

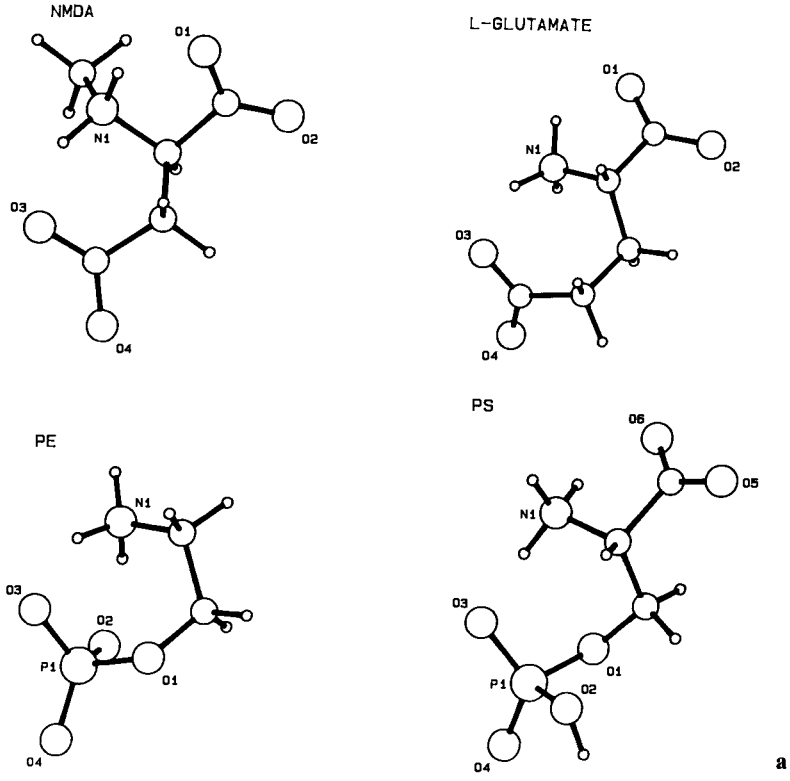

Fig. 4a–c. Computer calculated minimum potential energy conformations. **a** NMDA, L-glutamate, phosphorylethanolamine *(PE)* and L-phosphoserine *(PS)*. **b** Phosphorylcholine *(PC)* and AP_5. **c** Two different views of the same conformation of IP_3 in which the phosphates are axial

molecular mechanics calculations for L-glutamate, NMDA, and the PME phosphocholine, phosphoethanolamine, and L-phosphoserine, as well as for inositol 1,4,5-triphosphate (IP_3 and 2-amino-5-phosphonopentanoic acid (AP_5). The molecular mechanics calculations are based on the AMBER force fields of Kollman (Weiner et al. 1984) using the Macro Model computer program. The calculated minimum potential energies (in kilo joules/mole) are: L-glutamate (−233.40), NMDA (−203.05), phosphocholine (−78.18), phosphoethanolamine (−262.90), L-phosphoserine (−292.60), IP_3 (axial +199.95) and AP_5 (−59.47). The conformations corresponding to these minimum potential energies reveal definite simarilities for NMDA, L-glutamate, phosphoethanolamine, and L-phosphoserine when shown in stereo projections (Pettegrew et al. 1988a) (Fig. 4). Phosphocholine and AP_5 have conformational similarities, and IP_3 has a different conformation from all the others. Phosphocholine has a conformation intermediate between L-glutamate and the L-glutamate antagonist AP_5.

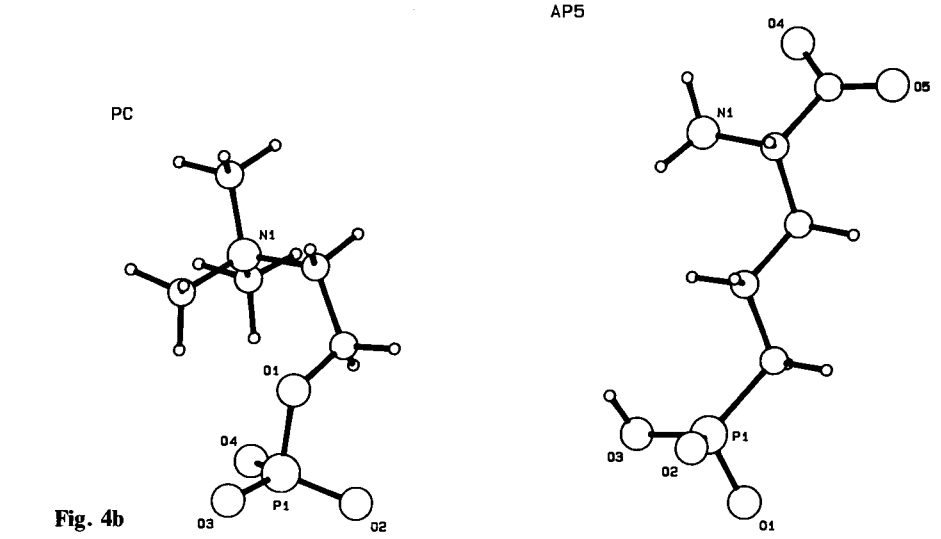

Fig. 4b

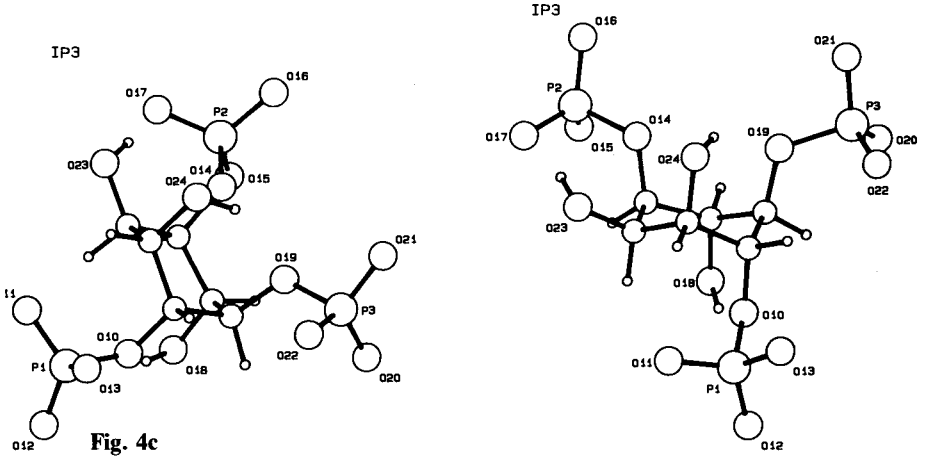

Fig. 4c

Possible Electrophysiological Effects of PME

Recently we found that PME have profound effect on extracellular population excitatory postsynaptic potential (EPSP) responses evoked by stimulating the

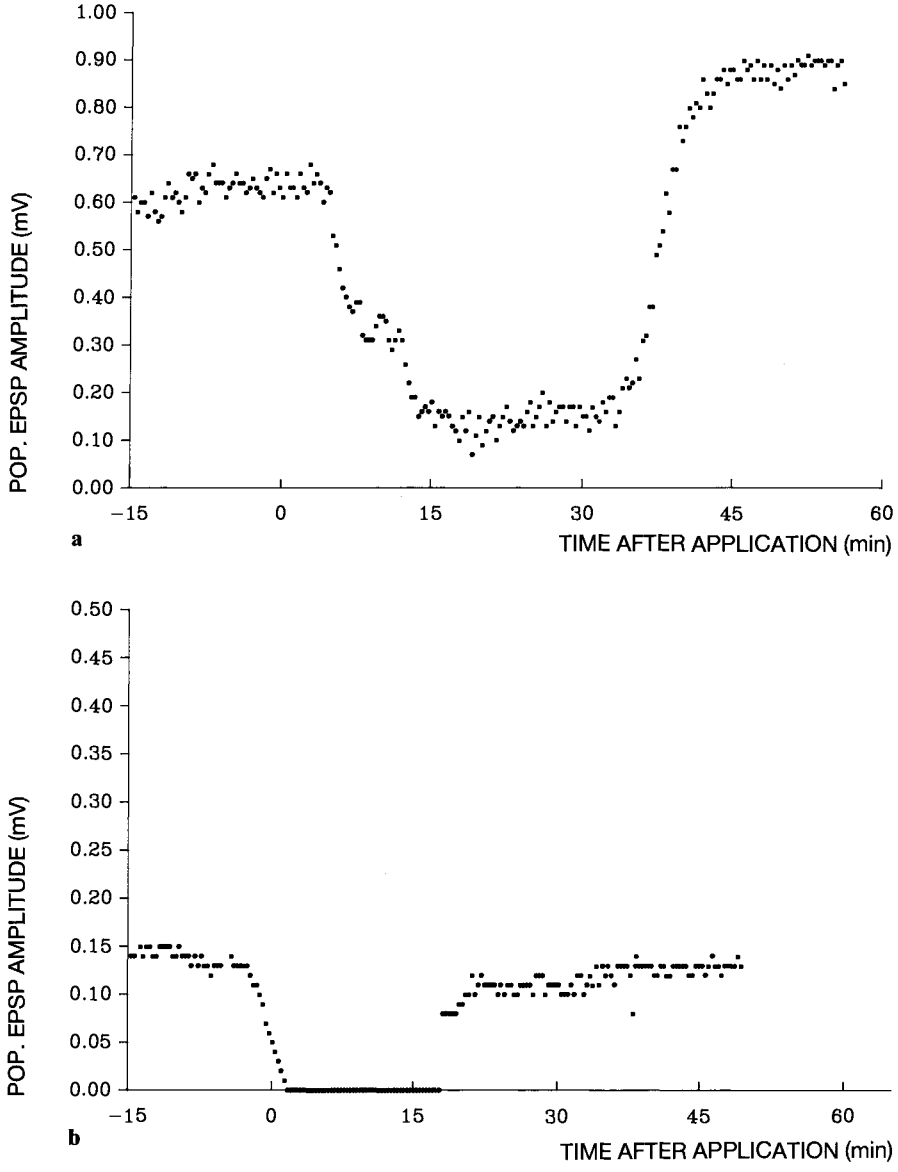

Fig. 5a–d. Extracellular population EPSP recorded from rat hippocampal slice CA1 pyramidal cell layer during stimulation of the Schaffer collaterals. Depicted are the EPSP before, during, and after perfusing either phosphorylethanolamine *(PE)* or phosphorylcholine *(PC)* at the indicated concentration. **a** 100 μ*M* PE. **b** 1 m*M* PE. **c** 100 μ*M* PC. **d** 1 m*M* PC

Schaffer collateral/commissural input to field CA1 (Barrionuevo et al. 1988). These studies of superfused rat hippocampal brain slices demonstrate that phosphoethanolamine and phosphocholine both depress the amplitude of the EPSP of CA1 neurons in a dose-dependent fashion at 10 μ*M* and 100 μ*M* concentrations. However, at 1 m*M* concentration phosphocholine greatly enhances the amplitude of the population EPSP of CA1 neurons, whereas phosphoethanolamine and L-phosphoserine continue to depress the amplitude of the EPSP (Fig. 5). ^{31}P NMR spectroscopy studies conducted on hippocampal slices in the presence of varying

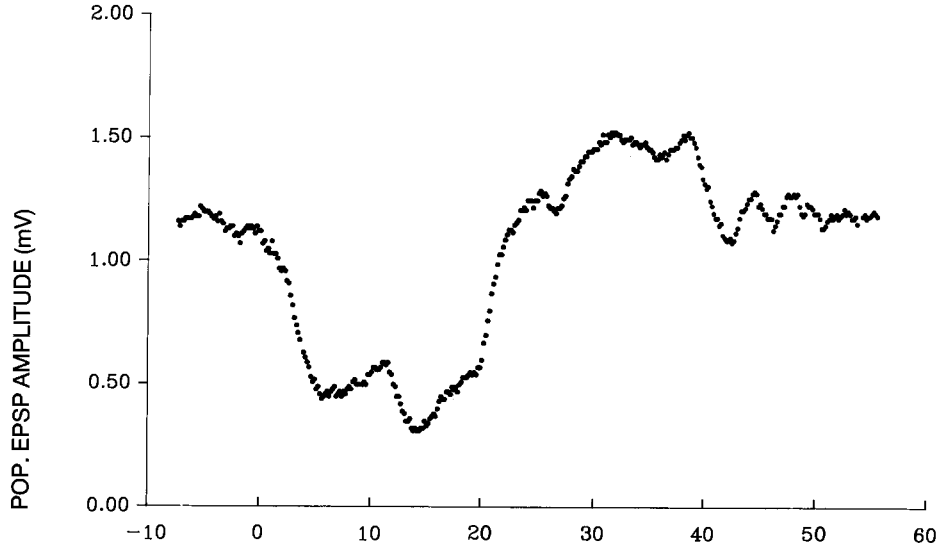

Fig. 5c

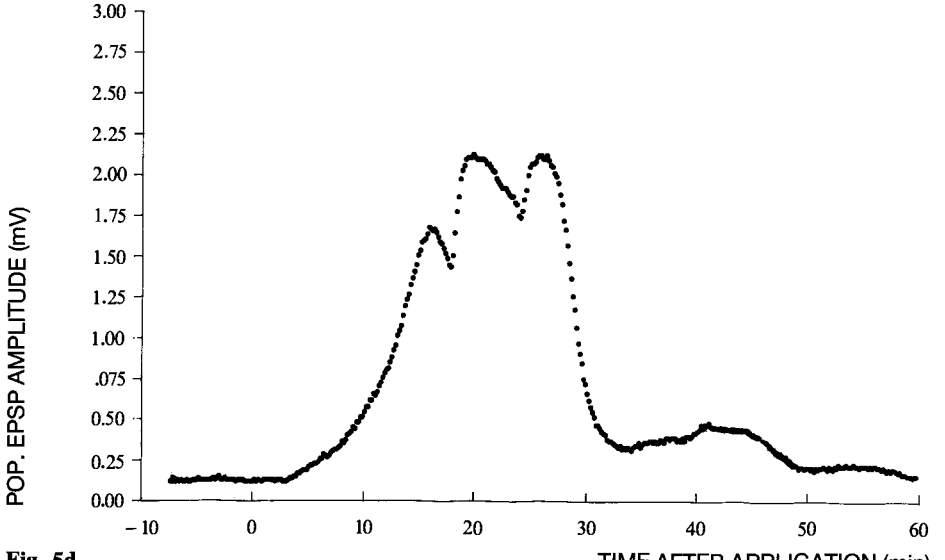

Fig. 5d

concentrations (10 μM–1.0 mM) of PME demonstrate normal high-energy phosphate metabolism (Pettegrew et al. 1988e). These results suggest that the effects of PME on the CA1 population EPSP represent a specific interaction between the PME and some aspect of the L-glutamate receptor complex. These findings lead us to suggest that low concentrations of PME could block L-glutamate receptors in the hippocampus and might be important in the memory impairment characteristic of AD. However, at higher concentrations, phosphocholine may have the characteristics of an excitatory neurotoxin resulting in cellular degeneration and death. The levels of PME that we detect in AD brain could be in the 5–7 mM range in individual affected cells.

Recent studies, a combination of extracellular recordings and current- and voltage-clamp techniques, were conducted to examine the effects of phosphoethanolamine on the biophysical properties of CA1 neurons (Bradler et al., in preparation). As in previous studies, recordings were made from rat hippocampal slices (3-month-old Fischer 344) prepared in the conventional manner. Extracellular and intracellular EPSP as well as EPSC amplitudes were monitored prior to and during a 30-min bath application of 1 mM phosphoethanolamine. Based on these measurements, two groups of CA1 cells were defined. In one group ($n = 6$), synaptic responses decreased in amplitude with a peak effect within 10–15 min exposure to phosphoethanolamine (mean depression, 63%; range, 25%–98%). Recovery was observed in only one of five cells in which recordings were obtained during the wash-out period. In three cells, amplitudes of synaptic responses rebounded during the wash, exceeding preexposure baseline. In the remaining cell, no recovery was detected. In three cells of this group, action potential duration (both orthodromic and current-evoked) decreased in the presence of phosphoethanolamine by 15%, 19%, and 7%. In the other group ($n = 7$), synaptic responses increased within the first 5 min of phosphoethanolamine exposure and continued to increase throughout the exposure (mean increase, 124%; range, 11%–345%). Recovery was observed in only one of five cells in which recordings were obtained during wash-out. In three cells there was no recovery, and in one cell the amplitude of synaptic responses rebounded below original baseline levels. In six cells of this group, action potentials increased by 13.8%, with a range of 8%–18%. In six of the previously mentioned 13 cells, the after-hyperpolarization measured in hybrid clamp was reduced or abolished. No recovery was seen in any case, even in recordings obtained after 40 min of wash.

A potential neurotoxic role for L-glutamate in AD has been hypothesized by Maragos et al. (1987). In addition, a recent report demonstrated a normal density and distribution of NMDA receptors in AD hippocampus (Geddes et al. 1986), which contrasts with previous reports that claimed decreased NMDA receptors in AD cortex (Greenamyre et al. 1985) and hippocampus (Young and Greenamyre 1986). The contrasting results of these two laboratories could be due to differences of the severity of the disease in the two studies, or could reflect a difference in the methods used. If the density and distribution of NMDA receptors are normal in AD brain until cell loss occurs, then cells with NMDA receptors could be vulnerable to the potential neurotoxic effects of elevated levels of PME. In addition, the lower levels of PME which would occur in the earlier stages of the disease could produce memory problems by blocking NMDA receptors.

Conclusions

Recent studies provide substantial evidence for elevated levels of PME early in the course of Alzheimer's disease, perhaps even prior to the onset of clinical symptoms, although this remains to be demonstrated. Similar high levels of PME are observed normally in the developing brain, especially during the period of dendritic proliferation. Along with the elevations of PME in AD brain, transient increases in the levels of PtdC occur, suggesting increased synthesis of PtdC. In theory increased synthesis of PtdC could shunt available choline away from acetylcholine synthesis and produce a functional cholinergic deficit. As the disease progresses, the elevated levels of PME decline and are replaced by elevated levels of PDE and decreasing levels of brain phospholipids.

The elevated levels of PME also could reflect enhanced phospholipase C activity which would stimulate protein kinase C activity. Enhanced protein kinase C activity could lead to many diverse biological effects, including the hyperphosphorylation of proteins such as ADAP and microtubule-associated tau proteins. The hyperphosphorylation of these proteins could alter their metabolism, including membrane insertion of ADAP, leading eventually to β-amyloid and paired helical filament deposition. The hyperphosphorylated β-amyloid and tau proteins would provide chelation sites for Ca^{2+} and Al^{3+}, leading to further cross-linking of the individual polymers and sequestration of these cations. The enhanced phospholipase C activity also could elevate cytoplasmic levels of IP_3, leading to mobilization of intracellular Ca^{2+} and further exaggerating the above processes.

The PME phosphocholine, phosphoethanolamine, and L-phosphoserine have now been demonstrated to share striking conformational similarities with the neurotransmitters NMDA and L-glutamate. Hippocampal brain slice extra- and intracellular recordings demonstrate that these PME are neurophysiologically active in CA1 cells which contain L-glutamate receptors and appear to inhibit the slow Ca^{2+}-activated K^+ channel (Bradler et al., in preparation). In addition, the PME have been demonstrated to alter the structure and dynamics of erythrocyte and platelet membranes and the phospholipid head group region in synthetic model membranes. These alterations could have in vivo biological significance for the packaging and release of the PME and other neurotransmitters such as L-glutamate. These mechanisms are summarized in Fig. 6. An important unanswered question is what turns this whole molecular process on in AD? Since the findings early in the course of AD appear to resemble events normally occurring in the developing brain, abnormal levels of, or responses to, growth factors, neuromodulators or oncogenes should be pursued.

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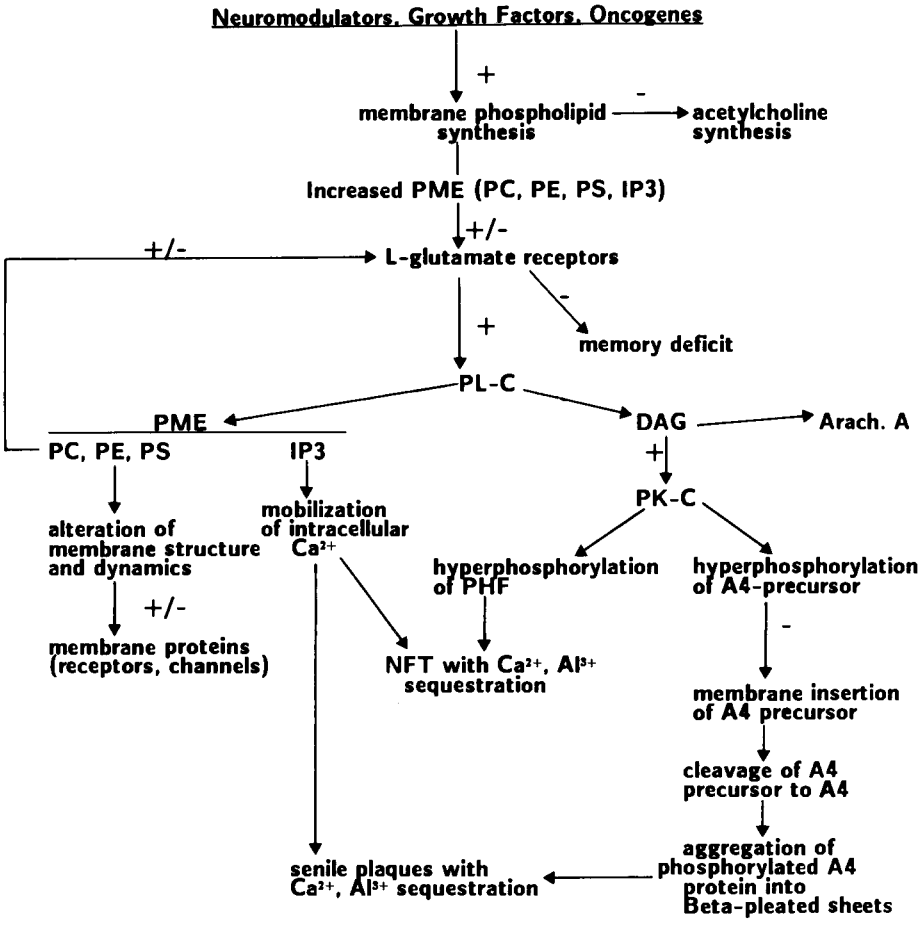

Fig. 6. Possible interactions between neuromodulators, growth factors, oncogenes, and membrane receptors giving rise to changes in the intracellular levels of PME, PDE, Ca^{2+}, and Al^{3+} resulting in memory impairment, membrane changes, neurotoxicity, and Ca^{2+} and Al^{3+} deposition in Alzheimer's disease

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Altered Protein Kinase and Amyloid β-Protein Precursor in Alzheimer's Disease: Which Comes First?*

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Summary

AD is a neurodegenerative disease characterized by the formation of neuronal inclusions, termed NFT, and extracellular amyloid deposits surrounded by dystrophic neurites, collectively referred to as NP. NP are specific to AD, whereas NFT are found in many other neurodegenerative diseases. The purpose of this report is to present a hypothesis regarding the biochemical basis for these pathologic features of AD – neurodegeneration and NFT and NP formation.

Among the three AD pathologies, neurodegeneration is central to the disease. The reason for the premature death of neurons in AD brains is unknown. Because neuronal survival requires the combined effect of growth factors, it is possible that one or more growth factors are missing in the AD brain. Alternatively, the intracellular machinery responsible for the function of growth factors may be deficient in the disease. One form of machinery important for growth factor-mediated cell maintenance is the battery of protein kinases, in particular, PKC. PKC mediates many functions of nerve growth factor, the best-characterized neurotrophic factor. Both the kinases, including PKC, and the phosphorylation levels are altered in AD brains, whereas levels of nerve growth factor are not.

There are questions to be answered. Is aberrant phosphorylation in AD specific to this disease? Is aberrant phosphorylation intrinsic to AD or secondary to neurodegeneration? Is PKC reduction relevant to NFT and NP formation? Why is phosphorylation aberrant in AD?

The abnormality in phosphorylation may be related to the expression of some biochemical characteristics that correlate with NFT formation. Furthermore, phosphorylation may be involved in the regulation of the processing of ABPP, which constitutes a major portion of the NP core. Conversely it is possible that ABPP is involved in the regulation of phosphorylation in neurons. Because ABPP is aberrantly processed in AD brain, this protein may be a candidate as the cause of abnormal phosphorylation and eventual neurodegeneration in AD.

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Introduction

In the brain tissue of Alzheimer's disease (AD) patients, many proteins are affected. It is possible that some biochemical changes are primarily involved in the pathogenesis of the disease, although many are the result of neurodegeneration or neuronal malfunctioning. Because a key issue of AD pathology is the malfunction and death of neurons, it is worth investigating molecules involved in the maintenance of neuronal survival and activity. We have been studying protein phosphorylation and protein kinases in AD brain, hoping that the study will bring us to an understanding of mechanisms crucial to neuronal degeneration. We have found that several protein kinases and phosphoproteins are altered in the disease. In this chapter, we summarize these findings and speculate as to the hierarchy of these alterations. Furthermore, we propose a hypothesis for the initial event which triggers the aberrant protein phosphorylation in AD.

Phosphorylation of Altered Proteins in AD Brain

Many biochemical alterations have been documented in homogenates of AD brains (see Terry and Katzman 1983, for a review). Several of these biochemical abnormalities have been found in various protein kinases and phosphoproteins. Because protein phosphorylation mediates the effect of second messengers generated by the activation of receptors for growth factors, and because it plays a pivotal role in regulating cellular metabolism (Greengard 1978; Krebs and Beavo 1979), these findings may provide a clue to the cause of neuronal death in AD.

Aberrant phosphorylation in AD was first reported in 1985. Sternberger et al. (1985) discovered that, among the many monoclonal antibodies that react with epitopes of neurofilaments, one that recognizes phosphorylated epitopes reacts with all neurofibrillary tangles (NFT) and neuritic plaques (NP). The other monoclonal antibodies react with many normal axonal projections, but with few NP and NFT. These data suggest that there is an aberrant posttranslational processing of neurofilament such that one or several sites become phosphorylated in AD, sites that are not normally phosphorylated in the cell body. Furthermore, alkaline phosphatase treatment of sections abolishes the immunostaining of NFT and NP but does not completely abolish staining in normal neuronal tissue. This suggests that normal neurofilaments have phosphorylated epitopes that are inaccessible or insensitive to phosphatase treatment. Finally, the specific phosphorylation of neurofilaments in AD also suggests that there is a specific kinase that is operating in AD.

The second cytoskeletal phosphoprotein identified to be aberrant in AD brain was a microtubule-associated protein, tau (Grundke-Iqbal et al. 1986; Ihara et al. 1986). In normal neurons, the tau protein functions to promote tubulin polymerization and thus microtubule assembly (Cleveland et al. 1977). In AD, however, the tau protein is overphosphorylated and is located on the NFT; all tau antibodies react with NFT, and many paired helical filament (PHF) antibodies react with tau (Brion et al. 1985; Kosik et al. 1986; Wood et al. 1986). Also, recent sequencing work by Goedert et al. (1988) has shown that tau is an integral part of the PHF and

comprises at least 10% of its composition. It is known that overphosphorylated tau, as seen in AD, is no longer able to promote tubulin assembly (Hoshi et al. 1987; Lindwall and Cole 1984). In these same NFT-bearing neurons, the microtubules are disorganized. The tubulin seems to be normal because, given the right polymerization conditions, the tubulin forms microtubules (Iqbal et al. 1986). Therefore, it is reasonable to assume that the overphosphorylated tau in the NFT-bearing neurons is unable to promote microtubule assembly and thus contributes to the destabilization of microtubules.

The kinase responsible for the tau phosphorylation in AD is currently unknown. There are two modes of tau phosphorylation, one which changes the electrophoretic mobilities of tau (mode I) and another which does not (mode II; Lindwall and Cole 1984). Protein kinase C (PKC) dependent phosphorylation does not change the mobility of tau on sodium dodecyl sulfate polycrylamide gel electrophoresis (SDS-PAGE; Baudier et al. 1987). The overphosphorylated tau associated with NFT in AD has slower mobility on SDS-PAGE than normal tau, and it has been proposed that this mode I phosphorylation is catalyzed by a calcium/calmodulin-dependent kinase (Baudier and Cole 1987). Yet other evidence indicates that calcium/calmodulin-dependent protein kinase is not responsible for the tau phosphorylation reported in AD. The tau-1 monoclonal antibody does not recognize overphosphorylated tau from AD brains although it recognizes the phosphatase-treated tau from AD. Phosphatase treatment of tau shifts the mobility of AD tau on SDS-PAGE (Grundke-Iqbal et al. 1986) to that of tau from normal individuals. The overphosphorylated tau protein generated using calcium/calmodulin-dependent protein kinase is reactive with the tau-1 monoclonal antibody and, thus, does not have the same conformation as the tau seen in AD. Therefore, there is another protein kinase that phosphorylates a different residue on tau and is responsible for changing its conformation to mask the tau-1 epitope, as well as to change the electrophoretic mobility.

Ishiguro et al. (1988) have discovered a tubulin-dependent protein kinase that phosphorylates tau according to mode I phosphorylation and thus alters tau mobility on SDS-PAGE. Furthermore, the phosphorylation of tau with the tubulin-dependent kinase forms an epitope on tau that is recognized by anti-PHF antibodies. Thus, the tubulin-dependent protein kinase may be responsible for overphosphorylating tau in AD. This kinase is not activated by known kinase activators and has a molecular mass of 30 kDa. This distinguishes it from kinases such as PKC, casein kinase I and II (CK-I and -II), and cAMP-, cGMP-, and Ca^{2+}/calmodulin-dependent protein kinase. This enzyme, however, may not be involved in tau phosphorylation in AD because this kinase is activated under conditions which stimulate microtubule formation. Since the microtubules are disassembling rather than forming in AD, it would suggest that this would inactive the kinase rather than activate it during the AD pathogenesis.

It is important to determine which kinase is responsible for tau phosphorylation in AD because there are two pieces of evidence that tau is overphosphorylated before NFT assembly (Bancher et al. 1989), and this could be an early stage in the neurodegenerative process seen in AD. Previous work by Hyman et al. (1988) has shown Alz-50 immunoreactivity prior to NFT formation. Alz-50 recognizes an epitope of tau (Nukina et al. 1988; Ksiezak-Reding et al. 1988) which is preferen-

tially expressed in AD. Since this epitope is not expressed in tau artificially synthesized in bacteria (Kosik et al. 1988), it is likely that this epitope is a posttranslational modification. We obtained evidence that the Alz-50 epitope is also phosphorylated. Therefore, it is possible that tau is phosphorylated by several protein kinases in the AD brain and, as mentioned below, CK-II may be one of these kinases.

Aberrant In Vitro Phosphorylation in AD Brain

Another aberrant phosphorylation found in AD was that of a M_r 60000 protein (P60; Saitoh and Dobkins 1986a). In in vitro phosphorylation experiments of cytosolic fractions, P60 was phosphorylated 250% more in the AD brains than in the control samples. This increased phosphorylation may be due to a greater concentration of the P60 or the kinase responsible for the P60 phosphorylation in AD brains. Another possibility is that the P60 has a lower phosphorylation state in AD than in normal brains, such that more sites are available for in vitro phosphorylation. Regardless, the in vitro phosphorylation of P60 is altered in AD. Furthermore, the increased in vitro phosphorylation of this protein is correlated with the number of NFT, but not with the quantity of NP present (Saitoh et al. 1988) This indicates that the P60 phosphorylation might be a marker for the insult which causes the formation of the NFT. The association of the P60 with the NFT, if any, must be a loose one at best, because as previously mentioned, P60 is a cytosolic protein whereas the PHF is an insoluble structure that would be found in the particulate fraction.

The nature of the P60 protein is not well elucidated because it has not yet been purified. P60 phosphorylation seems to be an autophosphorylation event because the rate of phosphorylation is not affected by increased sucrose concentrations, suggesting that an intramolecular reaction is occurring. Also, the kinase and phosphoprotein cannot be separated by protein purification on several chromatography columns. The phosphorylation of this protein is not dependent on cAMP, Ca^{2+}/calmodulin, or Ca^{2+}/phospholipid (Saitoh and Dobkins 1986a). Furthermore, this phosphorylation is not inhibited by heparin (data not shown). Therefore, the kinase which phosphorylates P60 is not cAMP-dependent kinase, Ca^{2+}/calmodulin-dependent protein kinase, or CK-II. It will be important to determine the nature of the kinase which acts on this phosphoprotein, and therefore it will be important to purify P60 and determine its structure and function. By doing so, we will gain a better appreciation of the link between P60 and the biochemical abnormalities seen in AD.

It may be useful to point out here the possibility that P60 is not specific to AD or NFT-bearing diseases. First, after ischemic insult on rabbit spinal cord, we observed a phosphoprotein of M_r 63 000 in the cytosolic fraction (Kochhar et al. 1989). At the moment, there is no conclusive evidence that this is a homologue of the AD P60, although some biochemical characters, such as localization in cytosol, heat and alkali stability, and isoelectric point, indicate that they are homologous. Second, we observed higher levels of P60 phosphorylation after prolonged treatment of human neuroblastoma cells with phorbol esters (Saitoh et al.

1989). Again, circumstantial evidence indicates that this is a homologue of the AD P60. The concentration of the phorbol ester used was destructive to neurons (Mattson et al. 1988) and effectively down-regulated PKC. Therefore, this experiment may indicate the hierarchy of two altered molecules in AD. Reduced levels of PKC precede altered P60, which is detected as its increased in vitro phosphorylation.

Another phosphorylation which is altered in the AD brain is the M_r 86 000 protein (P86) phosphorylation (Cole et al. 1988). The P86 is a substrate of PKC, and in in vitro phosphorylation experiments the P86 phosphorylation is reduced in AD as compared to control samples. The P86 protein is one of two major proteins phosphorylated by PKC in an in vitro assay of human cortex homogenate (Saitoh and Dobkins 1986b). It is possible that the reduced level of P86 phosphorylation in AD brain is due to a reduced level or activity of PKC and/or P86 in AD. Yet, it is also possible that the phosphorylation state of P86 is higher in AD brains than in controls, and therefore it would be less phosphorylated in an in vitro assay. The reduced P86 phosphorylation in AD brains is specific to AD, and not a general response in neurodegeneration. P86 phosphorylation was unchanged in Pick's disease, Parkinson's disease, and Binswanger's disease. How the altered P86 phosphorylation is related to the pathogenesis of AD is not known at this point and awaits further investigation.

Protein Kinases in AD Brain

The phosphorylation of several proteins seems to be affected in AD. How is the altered phosphorylation brought about? There are several possibilities. The state of phosphorylation is determined by the extent of forward and backward reactions. In other words, the balance between protein kinases and phosphoprotein phosphatases determines the state of phosphorylation. The other possibility is the accessibility of substrate proteins to kinases and phosphatases. Compartmentalization of enzymes and substrate proteins may be an important factor for determining phosphorylation state. One additional factor is the structure of the substrate. For example, it is possible that a nonexposed phosphorylation site in the soluble tau may be exposed after tau becomes denatured or aggregated into a pathological structure. Here, we consider only the altered protein kinases in AD, because the other aspects of altered phosphorylation in AD have not been studied.

The activity of PKC, as determined by its ability to phosphorylate histone, is reduced in AD brain particulate fractions. The activity of PKC in cytosolic fractions, however, shows a slight, though not significant, increase in AD (Cole et al. 1988). It is known that activating PKC shifts free PKC to its membrane-bound form. The results might indicate, therefore, that there is less active PKC in AD brains.

One way to quantify PKC is to use a radioactive activator of PKC such as [^{3}H]-phorbol, 12,13 dibutyrate (PDB). Because the interaction is stoichiometric, the quantity of PKC can be determined by counting the radioactivity bound to the enzyme. The comparison of [^{3}H]-PDB binding in AD samples to age-matched and postmortem time-matched controls demonstrated that the [^{3}H]-PDB binding

activity in the total or particulate fraction from AD cases was about half of that in the control. Thus, we can assume that AD tissue contains fewer PKC molecules, and therefore less PKC activity.

There are at least seven different isoforms of PKC which differ slightly in their amino acid sequence, the conditions required for activation, and in the types of tissues and cells in which they are found (Nishizuka 1988). There is no reason to suspect that all of the isoenzymes are affected in AD. Preliminary immunoquantification of several of the isoenzymes by Western blot analysis indicates that some of the isozymes are not reduced in AD. Thus, the lower level of PKC in AD may be confined to a few isoforms. Furthermore, PKC is immunolocalized in the NP in AD. Therefore, not only are PKC activity and concentration altered in AD, but its localization is also aberrant. Its presence in the NP indicates that PKC is directly associated with the pathological process of AD. Whether NP formation is linked to cellular death or is part of a neurite regeneration is debatable, and the answer to this question may be determined in part by the role that PKC plays in the NP.

Another protein kinase which is altered in AD is CK-II. Immunoquantification by Western blot analysis revealed that CK-II is 60% lower in AD cortex than in control cortex. These data are supported by immunocytochemical data showing a 15%–30% decrease in CK-II immunoreactivity in non-NFT-bearing neurons. The CK-II activity was measured by its ability to phosphorylate casein and is reduced, though not significantly, in AD. As a result, the specific activity of the CK-II is higher in AD brains. Therefore, there seems to be a compensatory mechanism in the brain for the lower amounts of CK-II present. Furthermore, the localization of CK-II seems to be altered. Immunohistochemical data show that CK-II is associated with the NFT. Since the overphosphorylated tau protein is also located in the NFT, and since tau has several sites that can be phosphorylated by CK-II, this kinase may be responsible for overphosphorylating tau and may play a role in NFT formation. Rabbit anti-CK-II also stains the neuritic component of senile plaques whose adjacent sections are positive for anti-PHF. This would support a CK-II association with some component of the PHF.

There are protein kinases which are apparently not affected in AD. They include cAMP-dependent protein kinase (Meier-Ruge et al. 1984) and casein kinase I (Iimoto and Saitoh 1988). Therefore, the aberrant protein kinases described hitherto are relatively specific changes relevant to neurodegeneration. Nevertheless, it should be stressed that these protein kinase alterations are not entirely specific to AD. For example, PKC was found to be involved in the neurodegeneration caused by ischemia (Kochhar et al. 1989). On the other hand, CK-II was found to be involved in many neurodegenerative diseases such as Pick's disease, multi-infarct dementia, and Binswanger's disease (Iimoto et al. 1989).

Protein Phosphorylation in AD Fibroblast

In the brain tissue of AD, many biochemical changes are secondary to neurodegeneration. To obtain insight about primary changes in the disease, it may be useful to study tissues which do not undergo pathological changes.

Although AD manifests itself most severely in the brain, it is thought that the disease is also systemic, and that some of the biochemical abnormalities in the brain may also be seen in fibroblasts (Blass and Zemcov 1984). For example, cultured AD fibroblasts are more sensitive to X rays and alkylating reagents (Li and Kaminskas 1985), and the Ca^{2+} concentration in AD fibroblasts is altered (Peterson and Goldman 1986). Also, secretion of a cholinergic differentiation factor is reduced in AD fibroblast (Kessler 1987), and AD fibroblasts spread less (Peterson et al. 1986) and adhere less to a plastic substratum (Uéda et al. 1989) In addition to the above abnormalities, one phosphoprotein, a M_r 79000 protein (P79), and one protein kinase, PKC, have been found to be abnormal in AD fibroblasts (Huynh et al. 1989).

P79 seems to be the major cytosolic endogenous substrate for PKC in fibroblasts, as is P86 in brain. Similar to P86 in brain, the in vitro phosphorylation of P79 is reduced in both sporadic AD fibroblasts and, to a greater extent, in the familial AD fibroblasts. The greater reduction of P79 phosphorylation in familial AD fibroblasts, as opposed to sporadic AD fibroblasts, parallels the severity of the disease seen in familial AD cases. Thus, reduced P79 phosphorylation in the fibroblast may be a good marker for AD.

In addition to the altered phosphoprotein, PKC is affected in AD fibroblasts. The levels of PKC, as determined by immunoreactivity of goat anti-PKC on a Western blot, were lower in the cytosolic fraction of fibroblasts for both familial and sporadic AD cases than for those of controls. The activity of PKC in fibroblasts, however, was unaltered in both sporadic and familial cases. As a result, the specific acitivity of PKC is higher in the AD fibroblasts than in the control samples. This indicates that there may be a compensatory mechanism for the lower PKC levels in the affected cells. This compensatory mechanism may well take place in fibroblasts but not in neuronal tissue, because the AD brain tissue shows both lower PKC activity and concentration in the particulate fraction. This could partially explain why the pathology of AD manifests itself in brain more than elsewhere.

Altered Protein Phosphorylation and Neurodegeneration

To determine the effects of the altered protein kinases and phosphoproteins and how they might interrelate with each other, we formulated a working model based on existing evidence that can be modified as new information arises. We propose such a model for altered kinases and their substrate proteins in AD (see Fig. 1).

It is conceivable that one reason that a neuron would start to die is the formation of an abnormal structure, such as PHF, that places stress on normal cellular function. During this process the nutrient transport system, composed of microtubules, breaks down, hastening the death of the neuron. Feedback mechanisms, which are not yet understood at the molecular level, mobilize various trophic factors to compensate for this neuronal degeneration. In the process, neurite outgrowth takes place, generating NP.

On a biochemical level, several proteins, including CK-II and tau, are abnormally regulated at transcriptional, translational, and posttranslational levels. The

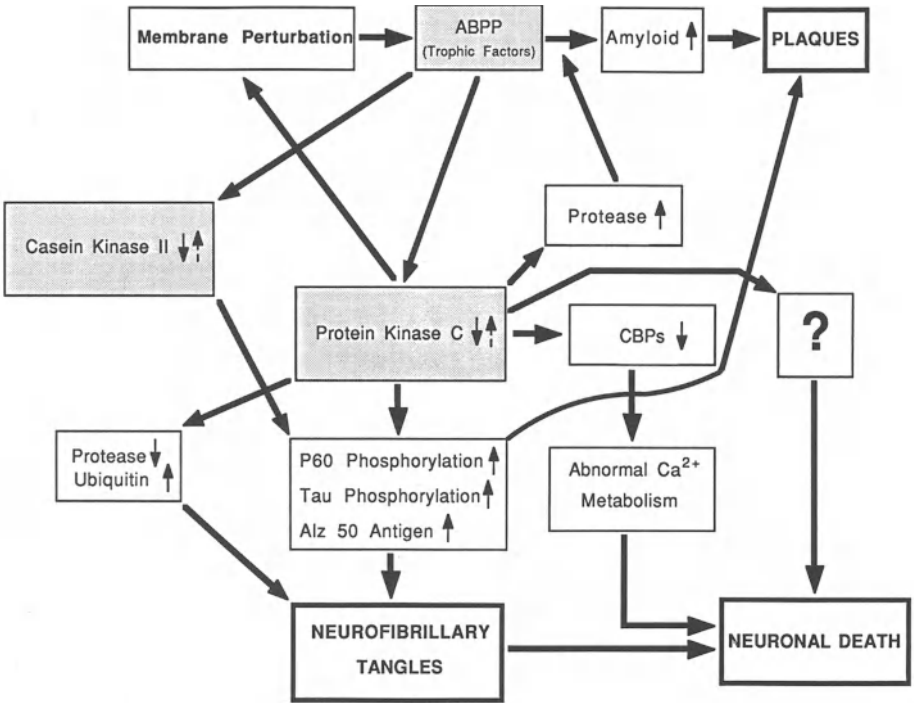

Fig. 1. A hypothesis for the neuronal death in Alzheimer's disease. The primary deficit in Alzheimer's disease is postulated to be in the deregulation of a trophic factor, such as amyloid β-protein precursor *(ABPP)*, which is linked to protein kinase C (PKC). The resulting aberrant PKC reactions induce many abnormal biochemical reactions, including membrane perturbation, increased ATP-dependent proteolysis (which may be involved in the plaque formation), decreased expression of Ca^{2+}-binding proteins (which leads to abnormal Ca_{2+}-metabolism), increased P60 phosphorylation (which may be involved in tangle formation), and decreased Ca^{2+}-dependent proteolysis (which is involved in tangle formation). Concurrent with the PKC abnormality, casein kinase II becomes aberrant and may be involved in the aberrant tau phosphorylation. Neuronal death is postulated to be brought about by the combination of aberrant gene expression, abnormal Ca^{2+} metabolism, and altered cytoskeletons. The *?* denotes some proteins with altered expression; these may include IMCAL, calpain I and II, ubiquitin, A68, P86, P60 GAP-43, PKC, CK-II, and ChAT

reason that these proteins may be among the first affected is the following. In pathologically affected regions of the brain there is a decrease in CK-II immunoreactivity in non-NFT-bearing neurons, suggesting that the morphologically normal-appearing neurons are undergoing biochemical changes. In addition, in non-NFT-bearing neurons, Bancher et al. (1989) have shown that tau protein is aberrantly phosphorylated. This work is supported by evidence that there are many Alz-50 (a tau antibody) positive neurons without NFT formation (Hyman et al. 1988). CK-II levels in non-NFT-bearing neurons are reduced in AD, and CK-II is localized to the NFT. Whether CK-II is responsible for phosphorylation tau through a colocalization event is not known. Another, as yet unidentified,

kinase could also phosphorylate tau. For example, because P60 phosphorylation increases proportionally to the number of NFT in AD, it is possible that the kinase phosphorylating P60 may also phosphorylate tau.

Based on altered phosphorylation, the disintegration of the microtubule transport system and the formation of PHF might be explained. First, CK-II phosphorylates microtubule-associated protein 1B and enhances its ability to promote microtubule assembly from tubulin monomers (Diaz-Nido et al. 1988). Also, CK-II is responsible for phosphorylating β-tubulin in its polymerized form and, therefore, for maintaining its structure (Serrano et al. 1987). It has also been shown that CK-II phosphorylation of tubulin may be related to neurite outgrowth (Serrano et al. 1987). Thus, the reduction in CK-II may contribute to the destabilization of microtubules in the axon. Second, it has been shown that overphosphorylated tau, as seen in AD, is unable to promote microtubule assembly (Iqbal et al. 1986). This then leaves a smaller population of normal tau which is probably below the critical concentration to promote microtubule assembly. Thus, with these two factors plus other unknown factors, the microtubules may begin to disassemble, and the disassembly of microtubules may lead to neurite degeneration.

At the same time, PHF formation may take place. Since overphosphorylated tau has been shown to be present in non-NFT-bearing neurons, it is likely that this form of tau is involved in early PHF development. It is known that the turnover of tau occurs when the unassembled tau becomes ubiquinated and then degraded. Ubiquitin normally binds to proteins targeted for removal by the cell. Because ubiquitin has also been immunolocalized to NFT, it is possible that ubiquitin binds to the phosphorylated tau on some other aberrant component of the NFT, such as the neurofilament. The different conformation of these aberrant proteins may make them resistant to proteolytic degradation even if they were ubiquitinated. Alternatively, it is possible that these aberrant proteins are not degraded because they have only a single ubiquitin molecule associated with them; it has been shown that many ubiquitin molecules must be associated with proteins for their degradation. Hence, these abnormal proteins could continue to aggregate and form insoluble structures that form NFT.

It is then thought that the degenerating neurites attract astrocytes to the area. This response may be one of the feedback mechanisms in the brain to promote neurite outgrowth. There is evidence that in a brain injury, such as an infarct, resting astrocytes become reactive and surround the injured area (Birecree et al. 1988). It is conceivable that these reactive astrocytes are secreting trophic factors which are responsible for the sprouting of degenerating neurons.

In fact, within NP of AD brain, the degenerating neurites that are positive for PHF are positive for epidermal growth factor (EGF) receptors (Birecree et al. 1988), a finding that is compatible with the idea that these aberrant neurites are regenerating axons responding to EGF secreted by reactive glial cells. The deposition of the amyloid β-protein may be interpreted in the context of a feedback mechanism to generate axonal outgrowth. The amyloid β-protein is thought to be a result of aberrant processing of the amyloid β-protein precursor (ABPP). PKC phosphorylates ABPP (Gandy et al. 1988) and it is known that PKC is aberrant in AD and is colocalized with the ABPP in NP. The significance of phosphorylation of ABPP by PKC is not known, although it is possible that aberrant PKC activity

and localization may induce the abnormal processing of ABPP, causing the amyloid deposition. Whitson et al. (1989) have evidence that the amyloid β-protein may have a limited effect in maintaining the viability of hippocampal neurons in vitro. Because they used only a portion of the total amyloid β-protein to test for trophic effects, it is possible that the entire amyloid β-protein in the NP might induce a better trophic response and may be involved in the process of neuritic regeneration. It is also known that the NP cores contain heparan sulfate proteoglycans which also have been shown to have trophic activity (Snow et al. 1988).

This neurite regeneration attempt eventually fails because the neuron is unable to digest the PHF and clear the abnormal proteins from the intracellular space. Eventually the neurites completely degenerate and the neuron dies, leaving a burned out plaque and a ghost tangle. Although not proven, it is not difficult to imagine that the neurons containing NFT send axons to NP.

The Initial Event in Aberrant Protein Phosphorylation

Since abnormal protein phosphorylation may be a key event in neuronal death, it is imperative to determine the initial event leading to this alteration. It is known that growth factors play an important role in regulating kinase activity. For example, CK-II activity in fibroblasts can be stimulated by adding insulin or an insulin-like growth factor (Klarlund and Czech 1988) and, therefore, any alteration affecting these growth factors would affect CK-II activity. It is tempting to speculate that CK-II synthesis is also under the control of these growth factors. PKC is also regulated by growth factors, and thus any alteration of the growth factors responsible for regulating PKC activity may alter its activity. It is interesting to note that the levels of PKC are under the control of PKC activity. For example, hyperactivating PKC by phorbol esters can down-regulate PKC by accelerating its degradation. The reason for the lower PKC levels in AD must be elucidated. Thus far, nerve growth factor has been shown to be unaltered in AD (Goedert et al. 1986). However, many other growth factors which act on neuronal tissue have not been studied. These include fibroblast growth factor, EGF, insulin, insulin-like growth factors, and others.

Whatever is affecting the aberrant phosphorylation events seen in AD is also affecting cells in the peripheral tissue. Again, growth factors may be responsible for these changes. In addition to classical growth factors listed above, a previously unknown growth factor may be involved in causing aberrant protein phosphorylation. Recently, Whitson et al. (1989) reported that amyloid β-protein is neurotrophic to some extent. It is reasonable to speculate that its precursor, ABPP, also has trophic activity. This idea fits in with the finding that ABPP is secreted from cells such as PC12 and fibroblasts (Schubert et al. 1988, 1989; Uéda et al. 1989). It is conceivable that an insult that affects the ability of a neuron to synthesize, posttranslationally modify, or secrete ABPP, may alter its trophic effect on cells for which it is targeted. This alteration as a trophic factor may affect the phosphorylation cascade in AD. Importantly, a few reports have appeared that described aberrant processing of ABPP in AD at the transcriptional level (Neve et al. 1988; Tanaka et al. 1988; Uéda et al. 1989; Clark et al. 1989) or at the post-

translational level (Cole et al. 1989). Therefore, at present, ABPP is a strong candidate which may trigger aberrant phosphorylation cascade in AD.

One other, although more remote, possibility is that some transmissible agent or environmental factor enters neuronal tissue which may be especially vulnerable to it. The viral or environmental factors may then interfere directly with the genetic processing that leads to aberrant protein synthesis. As a result, this could alter protein kinase levels and the entire phosphorylation cascade mechanism.

In conclusion, in AD, there are a few phosphoproteins and protein kinases that are altered. PKC and CK-II, both under regulation by trophic factors, are severely affected in this disease. It is conceivable that an aberrant trophic factor is causally involved in the generation of the altered protein kinase cascade. It will be important to pinpoint the cause of the altered phosphorylation because this may bring us closer to the primary insult responsible for causing AD.

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The Amyloid Gene of Alzheimer's Disease and Neuronal Dysfunction*

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The pathological hallmark of Alzheimer's disease (AD) is the deposition of fibrillar amyloid aggregates in the brain (Glenner and Wong 1984; Masters et al. 1985). The major constituent of these depositions is a protein that is now commonly termed the βA4 protein, according to its proposed secondary structure of beta pleated sheets and its relative molecular weight of 4 kDa. Protein sequencing of amyloid from brains of patients with AD as well as Down's syndrome revealed the βA4 protein to have a length of 42 to 43 residues (Kang et al. 1987). The βA4 amyloid arises from a much larger precursor protein, PreA4. Elucidation of the mechanism leading to βA4 deposition is crucial in understanding AD.

The precursor of the amyloid βA4 protein is encoded by the precursor of amyloid in Alzheimer's disease and Down's syndrome (PAD) gene on chromosome 21. Analysis of the structure of the PAD gene has so far revealed 18 exons (Lemaire et al. 1989; Kitaguchi et al. 1988). Exon 1 contains the 5′-untranslated region, the translation start, and the signal peptide. It extends two codons beyond the cleavage site of the signal peptidase. Exon 7 encodes a protein sequence with homology to the Kunitz family of serine protease inhibitors (Kitaguchi et al. 1988; Tanzi et al. 1988; Ponte et al. 1988). The 19 amino acids of exon 8 show homology to the MRC OX-2 antigen. Exons 7 and 8 are subject to alternative splicing. Recently, the presence of an additional exon, 13A, was reported between exons 13 and 14 (DeSauvage et al. 1989). Exon 17 contains 11 amino acids of the extracellular domain, the transmembrane sequence of 24 residues, and 14 residues of the cytoplasmic domain of the precursor protein. The rest of the cytoplasmic domain and the 3′-untranslated region are covered by exon 18. The region coding for the βA4 amyloid is extended over two exons, supporting the idea that βA4 is not a functional protein entity. The N-terminal 17 amino acids of the β4A amyloid protein are encoded by the 3′-part of exon 16, whereas the further 26 residues could be located in the 5′-part of exon 17. The last 15 residues of βA4 belong to the membrane spanning region of the precursor protein. Aberrant splicing was proposed to be a mechanism to generate βA4. However, a splicing event between exons 1 and 16 would lead to a shift in the translation frame since exon 16 does not start on a full codon. The consequence of that frameshift would be premature termination at a TGA codon at $PreA4_{770}$ cDNA position 1965. Thus, the structure of

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exons 1, 16, and 17 rules out the possibility that the βA4 amyloid is the product of an aberrant alternative splicing event (Lemaire et al. 1989).

The analysis of genomic DNA from the 5′-end of the PAD gene revealed the structure of the PAD gene promotor (Salbaum et al. 1988). The promoter has the characteristic features of promoters of housekeeping genes. It lacks a typical TATA box, has a high GC content, and transcription initiates at multiple sites. Sites for DNA methylation are clustered in the region up to −400 bp from the transcription start. The concept of a housekeeping gene is consistent with the ubiquitous expression of the PAD gene. However, the term does not imply constitutive expression as the PAD gene does respond to regulatory events (Mobley et al. 1988). The DNA sequence at the 5′-end leads to the identification of 26 sequence elements which may be involved in the transcriptional regulation of the PAD gene. The sequence of the elements and their position relative to the strongest transcription start site are shown in Table 1. Acute phase elements in the distal part of the promoter may be involved in regulation of the PAD gene during a response to inflammatory conditions (Tsuchiya et al. 1987). Cellular stress conditions may act on the heat shock element (HSE) in the proximal promoter region. These two groups of elements could well interact in their effect on the PAD gene during stress responses. The binding sites for the Hox1.3 protein presumably have a function in regulation of the PAD gene during development and, possibly,

Table 1. Putative elements of the PAD promoter DNA

Position	Sequence	Putative element
−3314	CAGTGGGAT	Acute phase element
−2986	GAGTGGGAG	Acute phase element
−2887	AGTGA	Acute phase element
−2655	AGTGA	Acute phase element
−2598	GATAATCATC	Hox1.3 binding site
−2310	AGCTGGGAC	Acute phase element
−2278	GCTAATTTT	Hox1.3 binding site
−2092	CGGTGGGAG	Acute phase element
−1898	AGCTGGGAT	Acute phase element
−1799	TGACCTCA	CRE
−1762	TGCTGGGAT	Acute phase element
−1716	AATAATGTAA	Hox1.3 binding site
−1555	AGTGA	Acute phase element
−1471	GTTCATTAGC	Hox1.3 binding site
−1436	AATAATAGTA	Hox1.3 binding site
− 913	TATAATTCAG	Hox1.3 binding site
− 762	CCCCCCGCCCCG	SP-1 site
− 350	TGATTCA	AP-1 site
− 317	CTCGACTTTTCTAG	HSE
− 198	GGGAGCGGA	GC-rich box
− 187	GGGCGCGTG	GC-rich box
− 178	GGGTGCAGG	GC-rich box
− 146	GGGCGCGGG	GC-rich box
− 123	CGGCGCGAG	GC-rich box
− 113	GGGCGCAGT	GC-rich box
− 45	TGACTCG	AP-1 site

regeneration. Hox1.3 is a transcription factor which interacts with DNA via a homeobox motif. Homeobox proteins are involved in gene regulation during development (Gehring 1987). The binding site consensus sequence for the transcription factor SP-1, as well as the cluster of six copies of the GC-rich element, may be involved in the ubiquitous expression of the PAD gene, but could also modulate the level of expression in different tissues. The cyclic AMP responsive element (CRE) relates the PAD gene to receptor signal transduction events. The two consensus binding sites for the transcription factor AP-1 make the PAD gene subject to regulatory events after an "immediate-early response." This involves the products of the proto-oncogenes c-*fos* and c-*jun,* which form the AP-1 complex. The transcriptional induction of the PAD gene by nerve growth factor or phorbol esters (Mobley et al. 1988) is presumably mediated by the AP-1 sites. AP-1 binding sites and CRE can be included in common regulatory circuits (Karin 1989), and it remains to be established whether the AP-1 sites of the PAD promoter can also respond to cyclic AMP.

The single copy PAD gene gives rise to a number of protein products. Alternative splicing of exons 7 and 8 leads to primary translation products of 770, 751, or 695 amino acids, respectively. These proteins are typical transmembrane proteins which span the lipid bilayer once (Dyrks et al. 1988). The presence of the recently described exon 13A generates much shorter transcripts due to the termination of transcription and use of an alternative polyadenylation site. Exons 14 through 18 are missing. The encoded proteins are secreted as a consequence of the absence of exon 17, which encodes the transmembrane domain (De Sauvage et al. 1989). These proteins are nonamyloidogenic, and their biochemistry has yet to be worked out.

The amyloidogenic protein products of the PAD gene have been studied extensively. In vitro translation studies monitored membrane insertion and the removal of the 17 amino acid signal peptide from the precursor during translation. The exact site of cleavage was determined by radiosequencing. The signal peptide cleavage is followed by N-linked glycosylation (Dyrks et al. 1988). Monoclonal antibodies against a recombinant $PreA4_{695}$ protein allowed us to identify the PreA4 proteins in cells and tissues and to follow their biogenesis. The precursor proteins undergo multiple posttranslational modifications. Following N-glycosylation, we were able to demonstrate O-glycosylation, sulfation of tyrosine residues, and proteolytic cleavage. This cleavage dissects the extracellular domains of the PreA4 proteins from the transmembrane and cytoplasmic domain. The proteolysis takes part in the Golgi apparatus and leads to secretion of C-terminally truncated, nonamyloidogenic proteins, which are also found in serum and CSF. Both forms, transmembrane as well as secretory PreA4 proteins, coexist in cells, whereas only the secretory form is detectable in conditioned medium (Weidemann et al. 1989). The half-life of the transmembrane proteins in cells is 20 to 30 min (Weidemann et al. 1989). The posttranslational modifications that have been demonstrated and the molecular weights of the corresponding proteins are compiled in Table 2.

The fact that amyloid precursor proteins are proteolytically processed further advances the idea that βA4 amyloid is generated by proteolysis and not by aberrant splicing, as outlined before. The release of the extracellular domain by cleav-

Table 2. Biogenesis of βA4 amyloid precursor proteins

Protein	$PreA4_{695}$	$PreA4_{751}$	$PreA4_{770}$
Primary translation product	91 kDa	101 kDa	103 kDa
Signal peptide removal (17 AA), N-glycosylation	91 kDa	101 kDa	103 kDa
O-glycosylation	110 kDa	128 kDa	130 kDa
Tyrosine sulfation	110 kDa	128 kDa	130 kDa
Secreted form, due to intracellular proteolysis	93 kDa	111 kDa	113 kDa

age leaves a peptide in the membrane that spans the transmembrane and the cytoplasmic domains of the precursor proteins. The exact site of cleavage is not known. The A4CT protein, which starts at the methionine residue preceding the βA4 protein and extends to the C-terminus of the precursor, could resemble the peptide that is left in the membrane after precursor processing. In vitro expression of A4CT revealed the aggregational properties of this protein. Proteinase K treatment of A4CT aggregates results in a protein with the same electrophoretic mobility as βA4, suggesting a precursor–product relationship (Dyrks et al. 1988). A proteolytic cleavage step that is part of the normal metabolism of the PreA4 proteins could be the first step towards βA4 amyloid deposition.

The precursor proteins were identified in neurons (Shivers et al. 1988; Schubert et al., submitted). Laser scanning light microscopy as well as electron microscopy using monoclonal anti-PreA4 antibodies showed the presence of the precursor proteins at synaptic sites in brain and muscle. Colocalization with synaptophysin could be demonstrated (Schubert et al., submitted). These results suggest a function of these proteins in synaptogenesis. Accordingly, their expression might be linked to synaptic turnover and neuronal plasticity. Amyloid deposition initiated in neurons or between synapses would occur at sites relevant for impairment of intellectual functions. It might thereby interfere with neuronal and synaptic function either by depletion of precursor proteins or massive buildup of amyloid depositions. This may represent the proximal cause of the clinically detectable impairment of AD.

We have now learned that the deposition of these characteristic pathological hallmarks may predate clinical expression of AD by three decades. This became evident from postmortem studies of brains from patients with AD, normal controls, and individuals with trisomy 21 (Davies et al. 1988; Rumble et al. 1989).

The ultimate cause or start of pathology, i.e., the primary lesion, is presumably not represented by βA4 amyloid deposition. The primary lesion may lie in processes such as PAD gene dosage, PAD gene expression, alternative splicing, membrane damage, incorrect membrane insertion of amyloid precursor proteins, and pathological degradation of precursor proteins to βA4 amyloid protein. The genetic predisposition to the primary lesion are represented by the conditions of Down's syndrome and familial AD. Which of the aforementioned mechanisms are triggered by Down's syndrome or the familial AD gene remains to be shown.

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Cloning of Different Amyloid Peptide Precursors from Brains of Patients with Alzheimer's Disease

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Introduction

Alzheimer's disease (AD) is a progressive degenerative disorder of the central nervous system characterized by neuronal loss and by brain lesions including senile plaques and neurofibrillary tangles (Roth et al. 1966; Terry et al. 1981; Whitehouse et al. 1982; Glenner 1983). Recent studies have provided an amino acid sequence for a major polypeptide of the cerebral amyloid found in AD, the amyloid β-protein (Glenner and Wong 1984) or A4 protein (Masters et al. 1985).

The sequencing data permitted the isolation of related cDNAs and characterization of a larger A4 amyloid peptide precursor (A4 APP; Kang et al. 1987; Tanzi et al. 1987a; Goldgaber et al. 1987; Robakis et al. 1987). The A4 APP resembles a transmembrane protein (Kang et al. 1987), and its mRNA is preferentially expressed by large pyramidal neurons (Bahmanyar et al. 1987). The gene coding for the A4 APP is located on chromosome 21 (Kang et al. 1987; Tanzi et al. 1987a; Goldgaber et al. 1987; Robakis et al. 1987) and may be responsible for the amyloid deposits in Down's syndrome and AD. However, the A4 APP gene is different from the genetic defect on chromosome 21 responsible for familial AD (Tanzi et al. 1987b; Van Broeckhoven et al. 1987) and is not duplicated in sporadic or familial AD (Tanzi et al. 1987c; St George-Hyslop et al. 1987; Podlisney et al. 1987).

Alternate forms of the A4 APP mRNA have been found and are different from the originally described form by an internal domain with extensive homology to a class of protease inhibitors (Tanzi et al. 1988; Ponte et al. 1988; Kitaguchi et al. 1988). While the presence or absence of the inserts could explain the difference between the two major RNA forms found as 3.2- and 3.4-kb bands in Northern blot analysis, our results show instead that the doublet corresponds to the use of two polyadenylation sites.

The A4 APP can be detected in two forms using immunoblotting performed with polyclonal antibodies directed against synthetic peptides corresponding to various portions of the precursor: a membrane-bound form which can be stained with both the antibodies to the N-terminal and the C-terminal portions of the A4 APP, and a soluble form lacking the C-terminus including the A4 protein, detected only with the N-terminal antibodies (Abraham and Potter 1989). The soluble form seems to be derived from the transmembrane precursor by proteolytic cleavage (Weidemann et al. 1989). However, we have recently isolated a clone from a human brain cDNA library that contained the structural sequence to

encode an amyloid precursor devoid of the transmembrane domain and the A4 peptide, which could correspond to a secreted form of the A4 APP.

The Use of Two Polyadenylation Signals in the 3′ Untranslated Region of the A4 APP mRNA

A λ GT11 AD brain cDNA library was screened with a 1-kb cDNA probe (positions 1796–2850 in the Kang sequence). Among 12 isolated clones, two showed an unusual pattern when restricted with *Eco*RI. Sequencing data indicated that these clones use the first polyadenylation site, present at position 2934, and that a poly(A) chain starts at position 2950 (Fig. 1). Consequently, this cDNA sequence has 258 fewer nucleotides, compared to the Kang sequence (Kang et al. 1987).

An anti-sense riboprobe, encompassing 50 nucleotides between the two polyadenylation sites (Fig. 1), was used for Northern blot analysis. The results, presented in Fig. 2, indicate that this 50-nucleotide riboprobe recognizes only the 3.4-kb band and not the two bands recognized by the riboprobe corresponding to the cDNA probe which was used for screening of the cDNA library. These results indicate that the 3.2- to 3.4-kb mRNA doublet expressed in normal brain and in

A : The short 3'UTR.

CTGCAGGATGATTGTAGAGAATCATTGCTTATGACATGATCGCTTTCTACACTGTATTAC 2930
PstI

ATA AATAAA TTAAATAAAAT-poly(A)tail

B: The long 3' UTR

CTGCAGGATGATTGTAGAGAATCATTGCTTATGACATGATCGCTTTCTACACTGTATTAC 2930
PstI

ATAAATAAATTAAATAAAATAACCCCGGGCAAGACTTTTCTTTGAAGGATGACTACAGAC 2990

ATTAAATAATCGAAGTAATTTTGGGTGGGGAGAAGAGGCAGATTCAATTTTCTTTAACCA 3050

GTCTGAAGTTTCATTTATGATACAAAAGAAGATGAAAATGGAAGTGGCAATATAAGGGGA 3110

TGAGGAAGGCATGCCTGGACAAACCCTTCTTTTAAGATGTGTCTTCAATTTGTATAAAAT 3170

GGTGTTTTCATGTA AATAAA TACATTCTTGGAGGAGC-poly(A)tail

Fig. 1a, b. Nucleotide sequence of the two possible 3′ UTR sequences of the A4 APP. **a** The short 3′ UTR. **b** The long 3′ UTR. The positions corresponding to the Kang sequence and the PstI site used for the constructs are indicated. The two polyadenylation sites used are *boxed,* and the sequence synthesized to obtain a riboprobe between the two polyadenylation sites is *underlined*

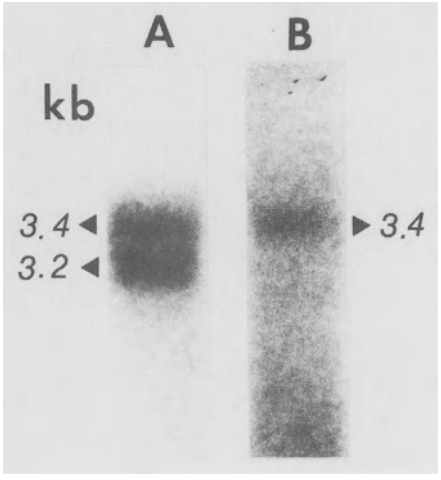

Fig. 2. Northern blot analysis of human brain RNA. The anti-sense riboprobes used for hybridization were the A4 APP riboprobe *(A)* or the 50-nucleotide riboprobe encompassing the sequence between the two polyadenylation sites *(B)*

other tissues is due to alternative use of two polyadenylation sites at the 3′ noncoding sequence of the A4 APP mRNA.

We have studied the influence of the sequence contained within the two polyadenylation sites used in the 3′ untranslated region (UTR) of the A4 APP on mRNA translation. The two possible 3′ UTR sequences of the A4 APP mRNA were subcloned into an SP64 plasmid downstream of the sequence coding for the chicken lysozyme, which corresponds to a mRNA highly translated in *Xenopus* oocytes. When the chimeric messengers, obtained by in vitro transcription, were translated for 6 h in *Xenopus* oocytes, the mRNA containing the long 3′UTR sequence had synthesized more protein than the mRNA containing the short 3′UTR sequence (Fig. 3).

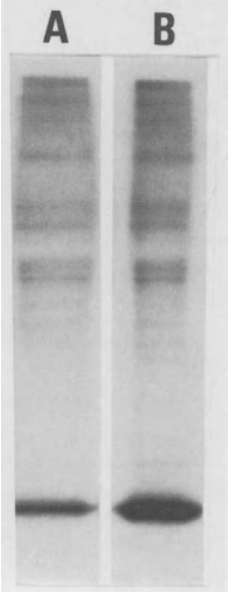

Fig. 3. Autoradiograms obtained after 6 h of in vivo translation of the lysozyme mRNA construct. The labeled proteins obtained after translation of the mRNA construct containing the short *(A)* or the long *(B)* 3′ UTR sequence of the A4 APP mRNA were immunoprecipitated by an anti-lysozyme antibody and analyzed by SDS-PAGE

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T :  PRPRHVFNML KKYVRAEQKD RQHTLKHFEH VRMVDPKKAA QIRSQVMTHL
     410                  430                  450
S :  PRPRHVFNML KKYVRAEQKD RQHTLKHFEH VRMVDPKKAA QIRSQVMTHL

T :  RVIYERMNQS LSLLYNVPAV AEEIQDEVDE LLQKEQNYSD DVLANMISEP
     460                  480                   500
S :  RVIYERMNQS LSLLYNVPAV AEEIQDEVGA VAHACNSSIL GGQGGQMT*

T :  RISYGNDALM PSLTETKTTV ELLPVNGEFS LDDLQPWHSF GADSVPANTE
     510                  530                  550

T :  NEVEPVDARP AADRGLTTRP GSGLTNIKTE EISEVKMDAE FRHDSGYEVH
     560                  580                  600

T :  HQKLVFFAED VGSNK GAIIG LMVGGVVIAT VIVITLVML K KKQYTSIHHG
     610              630                      650

T :  VVEVDAAVTP EERHLSKMQQ NGYENPTYKF FEQMQN*
     660                  680
```

Fig. 4. Amino acid sequence of the transmembrane *(T)* and the soluble form *(S)* of the A4 APP. The amino acid positions corresponding to the Kang sequence are indicated. The 20 novel amino acids of the soluble protein are *underlined,* and the proposed transmembrane region of the membrane receptor is *boxed*

The Soluble Form of the A4 APP

A 1.4-kb cDNA fragment (Vitek et al. 1988) was used for the screening of a λ GT11 cDNA library constructed from the cerebral cortex of a 54-year-old AD patient. Of several hybridizing clones, one clone was shown by DNA sequence analysis to contain the structural sequence to encode an A4 APP with a serine protease inhibitor domain in which the 208 amino acids at the carboxyterminal are replaced by 20 amino acids (Fig. 4) derived from nucleotide sequences bearing homology to the Alu repeat family. The existence of the novel mRNA was confirmed by enzymatic amplification of the cDNA region where divergence occurred. Whereas no amplification was observed on the DNA, a fragment of 0.5 kb was amplified using cDNA templates from normal and AD mRNA. The sequence of the amplified fragment confirmed the existence of the novel mRNA, and the absence of amplification on the DNA ruled out the possibility that the region homologous to the Alu sequences at the 3′ end of the A4 APP represents an unspliced intron.

The novel mRNA described here, devoid of the A4 peptide and the transmembrane sequences (Fig. 5), most likely corresponds to a secreted form of the A4

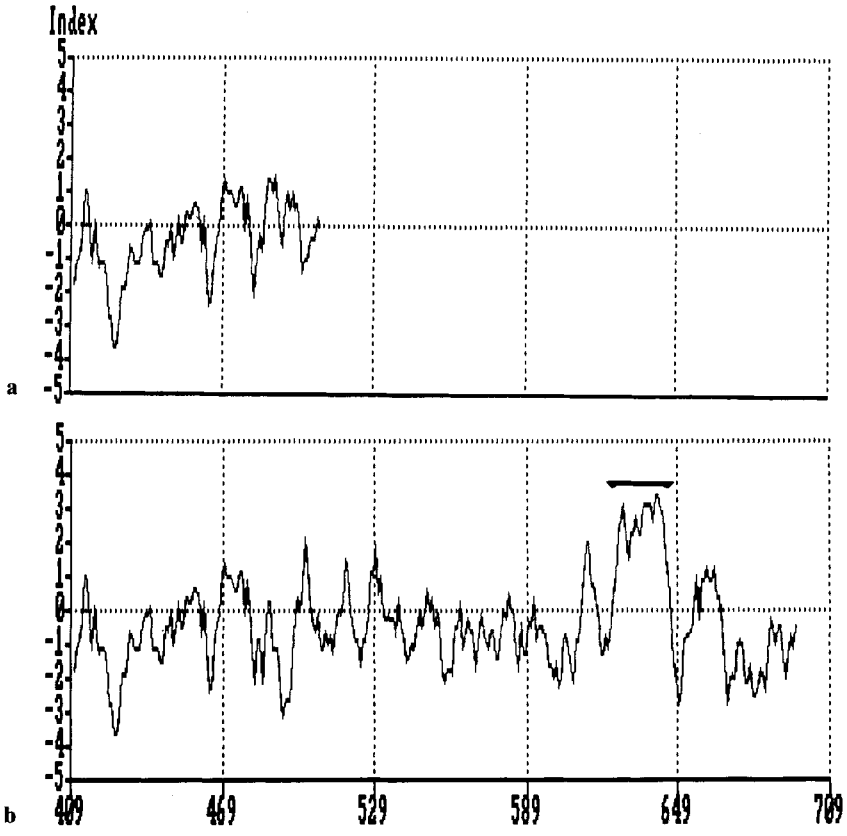

Fig. 5a, b. Hydropathy blot of the soluble **(a)** and the transmembrane **(b)** A4 APP. The Kyte hydropathy values for a window of six amino acid residues were plotted with respect to position along the Kang amino acid sequence. *Positive numbers,* hydrophobicity; *negative numbers,* hydrophilicity

APP and shows that the APP gene can produce not only transmembrane receptors but also soluble proteins which are not cleavage products of the transmembrane precursor.

Discussion

Sequence analysis of several clones encoding the A4 APP have provided insights into the biochemistry of the amyloid precursor. Analysis of a full-length clone showed the precursor of the A4 protein to be a 695 amino acid protein which resembles a cell surface receptor (Kang et al. 1987). The expression of the gene was found to be relatively widespread in the organism, and alternative spliced versions of the mRNA were found, with the longer ones encoding a region highly homologous to the Kunitz type protease inhibitors.

We report here that the 3.2- to 3.4-kb mRNA doublet expressed in normal brain and in other tissues is due to alternative use of two polyadenylation sites at the 3′ noncoding sequence of the A4 APP mRNA. Furthermore, the sequence contained within the two polyadenylation sites increases translation of the lysozyme reporter gene.

The A4 APP can be detected as a soluble form which seems to be derived from the membrane-bound form (Weidemann et al. 1989). The proteolytic cleavage of the transmembrane precursor is probably not the only way to obtain soluble precursor, as indicated by the cloning of a novel mRNA obtained by a new alternative splicing.

To get better insight into the normal function of the A4 APP, it is important first to identify the different precursors, since a transmembrane receptor could play a completely different role than a secreted protein with a protease inhibitor activity.

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Genetic Studies of the Alzheimer's Disease-Associated Amyloid β-Protein Precursor Gene and Familial Alzheimer's Disease

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Summary

Genetic linkage analysis of DNA markers from chromosome 21 in families with FAD should eventually lead to the isolation and characterization of the gene or genes responsible for autosomally transmitted FAD. Identification of the FAD gene should then help to elucidate the mechanism of pathogenesis in the sporadic form of AD. Parallel investigations of the process of amyloid formation should, in a retrograde manner, provide clues about other molecular factors involved in amyloidogenesis. These "factors" would not only act as candidates for the FAD gene defect but also provide new insights into the etiology of Alzheimer's disease with respect to their interactions with APP. Approaching the study of Alzheimer's disease from both ends should accelerate the understanding of the molecular changes underyling this disorder and, consequently, provide clues for new therapies aimed at ameliorating the effects of both sporadic and familial AD.

Introduction

The aggregation of a 4.2-kDa fragment (amyloid β-protein; ABP, or A4) in the form of amyloid plaques and cerebrovascular deposits (Glenner and Wong 1984) appears to be a key event in the pathogenesis of Alzheimer's disease (AD). ABP is derived from a much larger precursor protein (APP) encoded by a gene located on chromosome 21 (Tanzi et al. 1987a; Goldgaber et al. 1987; Kang et al. 1987; Robakis et al. 1987a). Physical mapping techniques employing somatic cell hybrid panels and in situ hybridization place the APP gene immediately proximal to the obligate Down's syndrome region at the border of bands 21q21.3 and 21q22 (Patterson et al. 1988). Others map APP to the more proximal region of band 21q21.1 (Korenberg et al. 1988; Robakis et al. 1987b). The localization of the APP gene on chromosome 21 suggests that the presence of β-amyloid deposits in the brains of patients with Down's syndrome (DS, trisomy 21) might be most easily explained by gene dosage due to a third copy of the APP gene. However, increased APP gene dosage in the germline does not appear to explain the situation in AD or familial AD (FAD), based on studies of brain and lymphocyte DNA (Tanzi et al. 1987b; St. George-Hyslop et al. 1987a; Podlisney et al. 1987). Recently, genetic linkage has been demonstrated between FAD and the anonymous DNA markers D21S1/D21S11 and D21S16 in four FAD kindreds (St. George-Hyslop et al.

1987b). This marker is also genetically linked to the APP gene, suggesting that APP and FAD reside in the same general vicinity of chromosome 21. Whether this is merely coincidental, or the two loci interact in some way at the transcriptional or translational levels, is unknown. As a first step to resolving this question, we have employed genetic linkage analysis of the APP and FAD genes in both man and mouse to define the genetic relationship between these two loci.

Genetic Linkage Analysis of the APP and FAD Loci

We have constructed a genetic linkage map of the long arm of chromosome 21 using multiple anonymous DNA fragments and the genes encoding superoxide dismutase 1 (SOD 1), the *ets*-2 oncogene, and the leukocyte adhesion molecule CF18 (Tanzi et al. 1988a). To determine the position of APP on this map, the segregation of three restriction fragment length polymorphisms (RFLP; two with *Eco*RI and one with *Ban*I) detected by the APP gene was traced through a large Venezuelan kindred employed as a reference pedigree for genetic linkage map construction. The results of these analyses indicate that the APP gene maps 4 cM above (centromeric to) the gene for SOD 1 (21q22.1) and 5 cM below (telomeric to) the anonymous DNA marker D21S1/D21S11 (Tanzi et al. 1988a). The latter marker has been previously demonstrated to be genetically linked to FAD in four extensively characterized pedigrees exhibiting a clear autosomal dominant inheritance of FAD with a relatively early age of disease onset (St. George-Hyslop et al. 1987a). Collectively, these data placed the APP gene in the same general vicinity on chromosome 21 as a locus for FAD.

If the APP gene actually harbored the defect leading to FAD, one would expect to observe no crossovers between the two loci in a direct two-point genetic linkage test. When tested against the four FAD families, six recombination events were observed between the APP and FAD loci (Tanzi et al. 1987c). Linkage analysis indicated that the APP gene was excluded from linkage with the FAD by a minimal genetic distance of 8 cM (approximately 8 Mb, assuming recombination to be random; Tanzi et al. 1987c). The presence of at least one crossover in each of the four families (detected with both *Eco*RI and *Ban*I RFLPs) implies that the APP gene is not the site of the defect in these pedigrees. It is still possible, however, that the APP gene is defective in cases of sporadic AD or, in the case of nonallelic heterogeneity, other FAD pedigrees. Preliminary analysis on 12 pedigrees and limited linkage disequilibrium analyses on 60 Caucasian sporadic cases, however, have not yet shown evidence to support this possibility (St. George-Hyslop et al. 1987a and unpublished data).

The approximate localization of the FAD locus on chromosome 21 represents the first step in molecular genetic strategies aimed at isolating the disease gene. Next, the precise location of the gene must be determined, and the possibility of genetic heterogeneity (other FAD loci on other chromosomes) must be addressed. Both of these tasks require the continuing isolation of novel informative DNA markers and the testing of additional FAD pedigrees. To provide a more exact location for the FAD gene, recombination events between FAD and linked DNA markers are used to direct the way to the gene. The possibility of genetic

heterogeneity is addressed by demonstrating the existence of two sets of disease pedigrees, one that shows *close* genetic linkage to a set of genetic markers, and another in which linkage can be excluded at the initial locus and can be demonstrated at a second locus. The latter is required since failure to discover linkage could result from analysis of familially aggregated but nongenetic sporadic AD.

Schellenberg et al. (1988) have suggested nonallelic heterogeneity based on the apparent exclusion of FAD from D21S1/D21S11 in a set of pedigrees of predominantly Volga-German descent. In their primary analysis, an exclusion of 17 cM was obtained assuming full penetrance of the FAD gene and an FAD gene frequency of .001. However, an analysis of the same families using only affected individuals, to eliminate the uncertainty of the correct penetrance value for FAD, reduced the exclusion value to 4–6 cM. This genetic distance is considerably smaller than either the current estimates of genetic distance separating D21S1/D21S11 and the FAD gene (15 cM) or the distance between D21S16 and D21S1/D21S11. Therefore, the second, more conservative analysis reported by these authors does not entirely exclude the possibility that the FAD gene may be located centromeric to both D21S1/D21S11 and D21S16. This possibility would be compatible with the very broad curve of the multipoint analysis in our original report of linkage (St. George-Hyslop et al. 1987b), and with the more recent positive data from Goate et al. (1989) and Van Broeckhoven et al. (1989) which both suggest a more centromeric location for FAD. Thus, while the Volga-German pedigrees may appear to support the notion of genetic heterogeneity in FAD, the uncertainty associated with parameters necessary for accurate LOD score analysis (e.g., mutation rate, penetrance, age of onset, frequency of the gene defect) argues for a conservative interpretation of this conclusion at the present time. Given this problem, unequivocal evidence for genetic heterogeneity will most likely require the discovery of positive linkage between FAD and loci on chromosomes other than chromosome 21.

Pericak-Vance et al. (1988) have also suggested the possibility of a second FAD locus based on a lack of linkage between D21S1/D21S11 in pedigrees manifesting late-onset FAD. However, the pedigree with the potentially most informative structure in this study showed strongly positive scores with both D21S1/D21S11 and D21S16. Since this very informative pedigree also had an early age of onset, these authors suggested that early-but not late-onset FAD is linked to DNA markers on chromosome 21. Since not all early-onset pedigrees in their study showed such strongly positive scores, another possibility should be entertained. It could be argued that whether a family displays positive or negative linkage depends more heavily on pedigree size and structure than on the disease phenotype (e.g., age of onset or ethnic origin). Most late-onset FAD pedigrees and some early-onset pedigrees are small nuclear pedigrees of limited informativeness because most family members die of other diseases before demonstrating signs of FAD. It is probably not coincidental, therefore, that larger, more informative, multigenerational FAD pedigrees are usually characterized by an early age of onset given such an inherent selection bias. Consequently, positive LOD scores between FAD and D21S1/S11 or D21S16 derived from early-onset pedigrees could well reflect the

fact that these pedigrees are usually larger, contain more affected individuals, and are thus more informative in linkage studies.

A number of additional circumstances associated with FAD linkage studies may tend to prevent the discovery of linkage even when it truly exists. First, the relatively high rate of misdiagnosis in the assessment of AD (10%–20%) and the occasional occurrence of sporadic AD in an otherwise genetic pedigree, or familial clusters of nongenetic AD, can lead to false crossover events. These false recombinant events may be more likely to occur in late-onset, small pedigrees where the differential diagnosis of sporadic versus familial AD and the confirmation of unambiguous genetic transmission are more difficult to obtain. False recombinants, in the absence of a sufficient number of informative meioses favoring cosegregation in these small pedigrees, results in negative LOD scores. A second problem stems from the location of the most informative marker, D21S1/S11, quite distant from the FAD gene (15 cM). Theoretical modeling of expected LOD scores generated from linkage analysis with markers distant (e.g., >10 cM or approximately 10^6 base pairs) from a disease gene indicates that smaller pedigrees individually provide primarily neutral or negative scores. The summation of these neutral to mildly negative LOD scores obtained from multiple smaller, nuclear pedigrees thus results in overall negative scores, thereby excluding linkage with the DNA markers previously shown to be linked to larger, early-onset families. It therefore remains unclear whether negative LOD scores obtained with smaller late-onset nuclear pedigrees and distantly linked markers reflect true nonallelic heterogeneity or simply reflect the lack of sufficient information to detect the existence of a distantly linked gene in the presence of the above confounding circumstances. Clearly, the issue of heterogeneity in FAD will only be ameliorated by the ascertainment of additional large, multigenerational pedigrees and highly informative, closely linked markers.

Given the above problems associated with FAD linkage analysis, we have focused subsequent analyses on additional pedigrees that meet the following criteria. First, the pedigree should be multigenerational, exhibit a clear autosomal dominant segregation of FAD, and include at least three affected individuals. Second, there should be at least one sampled or potentially fully reconstructable parent-to-child transmission of the disease. And, third, affected individuals should be diagnosed as such according to strictly specified criteria. Thirteen pedigrees, including the original four, have met these requirements and yield a two-point LOD score between FAD and D21S1/D21S11 of 3.25, at a distance of 15 cM, but still with a large confidence interval of 5–30 cM. This places FAD approximately 20 cM proximal of APP in a location close to the centromere.

The actual physical distance separating FAD and APP is not easily derived from the genetic studies. If recombination were perfectly random, the physical distance would be 25×10^6 bases of DNA (1 cM = approximately 10^6 base pairs). However, in this region of chromosome 21, a statistically significant higher frequency of recombination in women versus men has been observed, which implies that this region may contain a "hot spot" for recombination events (Tanzi et al. 1988a). Therefore, it is possible that the physical distance between the APP and FAD genes is somewhat smaller than 25 Mb.

To further analyze the genetic relationship between the APP and FAD loci, we have mapped them in a region of mouse chromosome 16 syntenic with the long arm of chromosome 21 (Cheng et al. 1988). Interestingly, in the mouse, *App* is very tightly linked to the mouse homologue of the anonymous DNA marker D21S16. As discussed above, this marker is at least 20 cM from APP in human but is the most closely-linked marker to date with FAD. Aged mice do not develop amyloid plaques and deposits. Whether this is related to the tight genetic linkage between mouse *App* and the putative FAD locus region, as indicated by D21S16, is not known. The mouse does, however, provide the opportunity to clone within a relatively small region, the genetic material between *App* and D21S16 that has been "expanded" in man, thereby providing a possible "shortcut" to the FAD gene.

A Serine Protease Inhibitor Domain in APP

We have recently reported an alternative transcript for the APP gene encoding a 751 amino acid polypeptide containing an additional 56 residue domain interrupting amino acid 289 of the originally cloned APP695 (Tanzi et al. 1988b; Ponte et al. 1988; Kitaguchi et al. 1988). This domain exhibits 50% homology with the Kunitz family of serine protease inhibitors. Another exon of 57 bases was also discovered (Kitaguchi et al. 1988) and is separated from the 168 base exon by approximately 3000 bases of intron DNA. Northern blot analysis reveals that the Kunitz protease inhibitor (KPI) containing transcript (APP751) is present in both neuronal and nonneuronal tissues, while the APP695 transcript, lacking the inhibitor, appears to be more closely associated, although not exclusively, with neuronal tissues (Tanzi et al. 1988b; Ponte et al. 1988). In addition, while transcripts for APP695 appear to be more abundant in fetal tissues, APP751 RNA is present at higher levels in adult tissues, implying that the precursor lacking the protease inhibitor may be required in higher amounts during development. This suggests the possibility that the protease inhibitor domain regulates some activity of APP that is more crucially needed during fetal development. The most parsimonious explanation would be that the protease inhibitor domain prohibits specific cleavages of APP or other proteins associated with plasma or intracellular membranes that are crucial during periods of neuronal plasticity.

It has recently been demonstrated that Cos cells transfected with APP751 DNA were able to inhibit trypsin in vitro (Kitaguchi et al. 1988). This result agrees with the observation that the reactive site in the KPI domain encoded APP751 consists of an arginine flanked by a cysteine and an alanine indicating a specificity for trypsin and trypsin-like proteases. The strongest homology between the KPI domain and a human protein is with the HI-30 portion of the serum protease inhibitor, inter-α-trypsin inhibitor (Kaumeyer et al. 1986). HI-30 is composed of two interconnected KPI domains (I and II) each with a relative molecular weight of 7 kDa, and two carbohydrate groups on the N-terminal domain I. The KPI domain in APP751 exhibits a greater degree of homology to Kunitz domain II of HI-20, which is highly selective for trypsin-like proteases (Wachter and Hochstrasser 1981). These data suggest that, in considering proteases that may be involved in

processing APP, special emphasis should be placed on trypsin-like serine proteases (e.g., cathepsin G and plasmin).

The presence of a functional protease inhibitor in APP carries profound implications for the process of amyloidogenesis in AD. Amyloid formation might be prevented by the KPI domain by inhibiting certain proteases capable of releasing ABP or its penultimate proteolytic intermediate. Alternatively, the KPI domain could potentially accelerate the generation of A4 by impeding specific proteases from degrading this peptide or its penultimate fragment. It is conceivable that the proteolytic intermediates of APP might differ appreciably depending upon the presence or absence of the KPI domain, and that only one of these sets proceeds toward subsequent modification into amyloid plaques and deposits. It is, therefore, an important task to determine whether APP695, 751 or 770 gives rise to amyloid fibrils.

Assuming that neuronally derived APP contributes to amyloid formation, it becomes important to determine which forms of APP transcripts (with or without the protease inhibitor) are prevalent in affected regions of AD brain. So far, preliminary results have been somewhat contradictory. Northern blot analysis indicates a selective reduction of APP695 (lacking the inhibitor) in the frontal cortex of patients with AD (Tanzi et al. 1988b; Johnson et al. 1988). One interpretation of this result is that the neurons that are most heavily affected in this region primarily express APP695, and that reduced levels of this transcript simply reflect the degree of neuronal cell death. On the other hand, in situ hybridization of APP cRNA probes to sections of affected brains of 11 Alzheimer's patients reveals increased levels of APP695 transcripts (Palmert et al. 1988). This increase was observed in the nucleus basalis of Meynert and in the locus coeruleus but not in the subiculum, basis pontis, or occipital cortex.

The results obtained from these neuronal mRNA studies do not exclude the alternative possibility that serum-derived APP may be the source of cerebrovascular and senile plaque amyloid. In fact, APP polypeptides have been observed in human sera (D. Selkoe, personal communication). Finally, although it is important to determine the actual source of amyloid (e.g., neuronal or systemic, APP695 or APP751), it may be of equal import to identify the common denominator(s) in specific regions of the brain where amyloidogenesis occurs. These factors might reside in the blood-brain barrier or the neuronal microenvironment and include proteases, inhibitors, or certain cell populations (e.g., microglial cells) that specifically play a role in amyloid formation.

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Alzheimer's Disease and Chromosome 21*

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Summary

Evidence for a genetic basis to Alzheimer's disease includes several families of early onset, where the disease appears to segregate as a fully penetrant autosomal dominant disorder. The familial Alzheimer's disease (FAD) locus can be localised to a region of chromosome 21 by cosegregation of anonymous DNA markers with the disease phenotype. New data confirming the original linkage is presented and in addition suggests the location of the Alzheimer's disease gene as being centromeric of markers S1/S11 on the proximal long arm of chromsome 21. Each of the six pedigrees used in the recent study have disease onset below 62 years of age, with affected individuals present in at least two generations. The inability of other groups to find linkage of FAD to chromosome 21 (Schellenberg et al. 1988; Pericak-Vance et al. 1988) is discussed and their data shown to be consistent with our finding in early onset Alzheimer's disease.

Introduction

The discovery of a genetic defect predisposing to Alzheimer's disease on the long arm of chromosome 21 by St. George-Hyslop et al. (1987) marked a major step towards our understanding and, ultimately, our treatment of this disorder. These authors reported that, in four large pedigrees in which Alzheimer's disease segregated as an autosomal dominant disorder with onset in the presenium, the disease was genetically linked to two polymorphic loci on chromosome 21. This immediately raised two challenges: to locate more precisely and eventually identify the genetic defect in these and similar families and to determine the proportion of cases of familial Alzheimer's disease that are caused by mutations at this locus.

* This research was supported by grants provided by the Medical Research Council, the Mental Health Foundation, and Research into Ageing.

Mapping the Genetic Defect on Chromosome 21 that Predisposes to Alzheimer's Disease

The original report of linkage placed the disease gene close to two genetic markers: D21S1/S11 and D21S16 (St. George-Hyslop et al. 1987). However, the orientation of these two loci was not known with certainty because D21S16 had not been put on the genetic map of chromosome 21 (Tanzi et al. 1988; Fig. 1). Furthermore, the linkage data given in the original report gave two possible positions of equal likelihood for the Alzheimer's disease locus – one on either side of D21S1/S11. Thus, there was a clear need to map D21S16 relative to other loci on chromosome 21 and to follow the segregation of Alzheimer's disease in other families to determine whether the report of linkage could be replicated, and the position of the disease locus could be mapped more precisely.

D21S16 is a relatively uninformative genetic locus; approximately 95% of chromosomes share the common allele (human gene mapping IX). It had not, therefore, been sufficiently informative in reference pedigrees to determine its precise genetic position, although it was known to be close to D21S1/S11. Therefore, we decided to use physical mapping techniques, specifically, pulsed field gel electrophoresis (Barlow and Lehrach 1987) and analysis of somatic cell hybrids, to locate D21S16 with respect to the other loci on the proximal long arm of chromo-

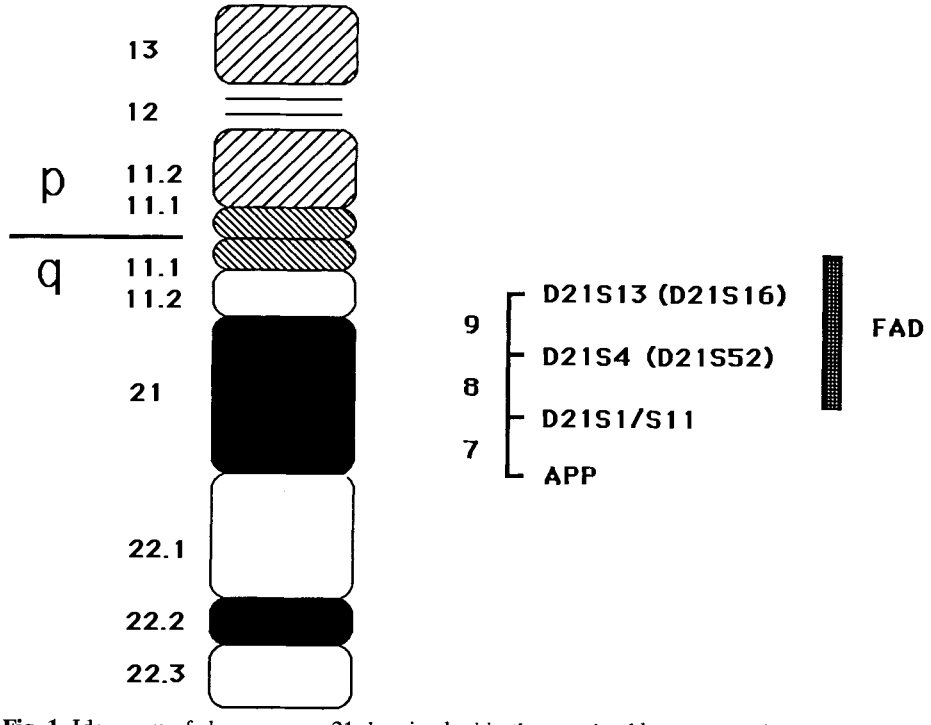

Fig. 1. Ideogram of chromosome 21 showing loci in the proximal long arm and the genetic distances between them in centimorgans. *Brackets,* loci which we have linked to genetically mapped loci by pulse field gel electrophoresis. (Modified from Tanzi et al. (1988) and Goate et al. (1989); see also Fig. 3)

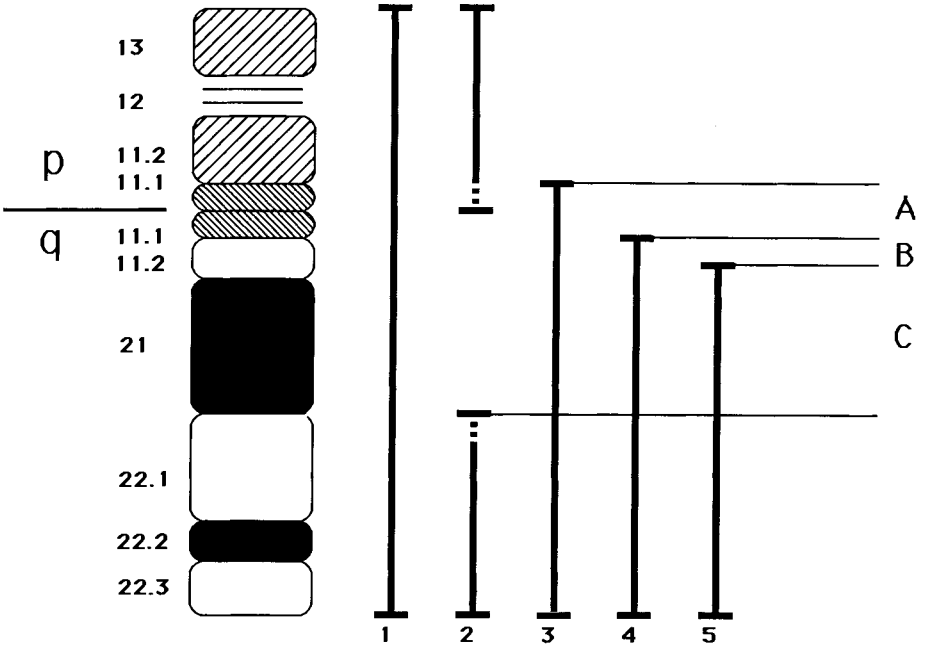

Fig. 2. Cell hybrids used to define sections *A*, *B*, and *C* of chromosome 21. *1*, WA17 and 72532X-6; *2*, ACEM-90; *3*, 153E7BX; *4*, 2Fur1; *5*, R50-3

some 21. The results of this study are given in Fig. 2 and 3 and in Table 1. In essence they show that D21S16 maps within 700 kbp centromeric to D21S13 and is the most proximal marker locus on the long arm of chromosome 21. Our results concur with those obtained by other groups (Van Broeckhoven et al. 1989; Gardiner et al. 1988) and allow the construction of a more complete map of this area of the chromosome. The identification and positioning of more polymorphic loci in the vicinity of D21S1/S11 and D21S16 mean that more meioses will be genetically informative in existing pedigrees multiply affected by Alzheimer's disease.

Table 1. Pattern of hybridization to hybrid cell lines

Probes	Hybrids						
	WA17	72532X-6	ACEM-90	153E7BX	2FU^{r}1	R50-3	Region[a]
D21S16	+	+	−	+	+	−	B
D21S48	+	+	−	+	+	−	B
D21S13	+	+	−	+	+	+	C
D21S46	+	+	−	+	+	+	C
D21S4	+	+	−	+	+	+	C
D21S52	+	+	−	+	+	+	C
D21S1	+	+	−	+	+	+	C
D21S11	+	+	−	+	+	+	C

[a] See Fig. 1

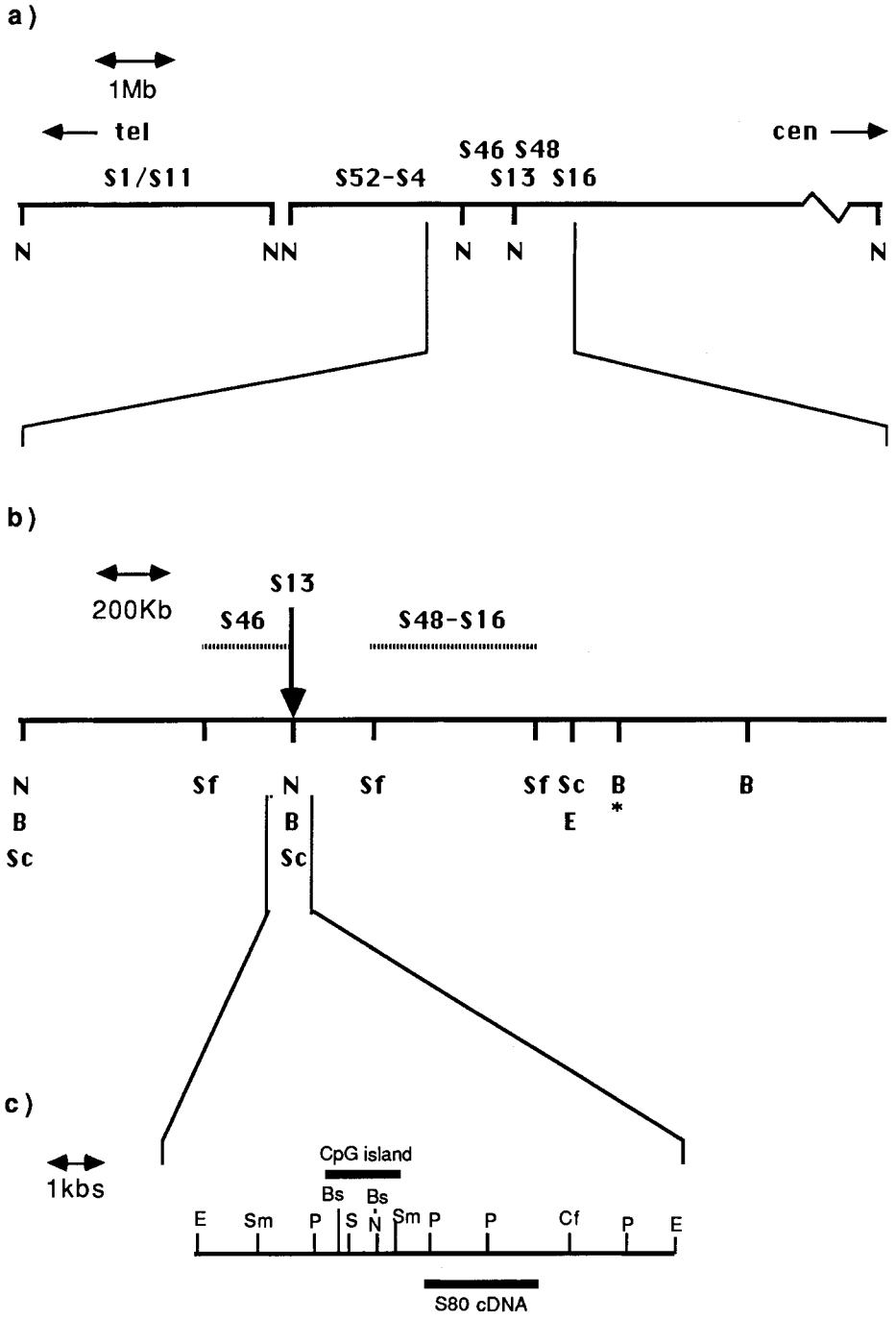

Fig. 3a–c. a, b Pulse field maps at different resolutions around D21S13 and neighbouring loci showing probe positions and rare cutter sites. *N, Not*I; *Sf, Sfi*I; *E, Eeg*I; *S, Sac*I; *B, Bss*HII. **c** Restriction map of D21S13 showing position of CpG island and position of D21S80 sequence. *E, Ecor*RI; *Sm, Sma*I; *P, Pvu*II; *S, Sac*I; *Bs, Bss*HII; *Cf, Cfi*I

This, together with the collection of more such pedigrees, will permit more accurate mapping of the Alzheimer's disease locus and the determination of the generality of this linkage.

Largely through the Alzheimer's Disease Society, we have identified over 130 families, multiply affected by dementia. Of these, 13 have pedigrees with three or more affected individuals in two or more generations with a mean age of onset of under 60 years and clinical histories consistent with a diagnosis of probable Alzheimer's disease. Several have had histological confirmation of this clinical diagnosis. So far, we have collected six of these families and carried out linkage analysis using probes on the proximal short arm of chromosome 21. The results of this linkage analysis are shown in Fig. 4 (see Goate et al. 1989 for full details). In essence, our results confirm and refine those reported by St. George-Hyslop et al. (1987). Thus, they confirm that this form of Alzheimer's disease is predisposed by a locus in this region of chromosome 21; however, they also suggest that the disease locus is some considerable distance centromeric of the locus D21S1/S11 and close to the loci D21S13 and D21S16. Van Broeckhoven et al. (1989) have obtained essentially similar results through the genetic analysis of two large, early-onset Belgian families. Thus, there are three reports which strongly suggest that early-onset disease is predisposed to by this locus.

Determining the Generality of the Chromosome 21 Linkage for Familial Alzheimer's Disease

None of the reports referred to above contain data which suggest that the disease is non-allelically genetically heterogeneous. In contrast, two reports (Schellenberg et al. 1988; Pericak-Vance et al. 1988) have failed to find evidence of a chromosome 21 linkage to Alzheimer's disease in the pedigrees that they have studied. However, these groups have used a more heterogeneous group of families for their analysis. Besides using early-onset families which conform to the criteria described above, they have also used pedigrees with a late age of onset (Pericak-Vance et al. 1988; Schellenberg et al. 1988) and pedigrees derived from a cultural isolate, the Volga Germans (Schellenberg et al. 1988). In both of these reports, the data derived from the early-onset, outbred families are consistent with a chromosome 21 location for the predisposing gene; they are thus in agreement with the three positive reports referred to above (St. George-Hyslop et al. 1987; Goate et al. 1989; Van Broeckhoven et al. 1989).

However, Schellenberg et al. (1987), Pericak-Vance et al. (1988) and our group (unpublished data) have found no evidence for linkage in families with a late age of onset of illness. Clearly, this may mean that the late-onset disease is caused by mutations at other loci. However, we are cautious about making this interpretation for two reasons. Firstly, because the genetic loci D21S13 and D21S16 are not very informative and are at the end of the current genetic map for the chromosome (human gene mapping IX; Tanzi et al. 1988), no group has been able to test the hypothesis that the late-onset disease is predisposed close to these loci or proximal of them. Yet our data (Fig. 4) and those of Van Broeckhoven et al. (1989), suggest that this is the most likely position for the locus leading to early-onset disease. Sec-

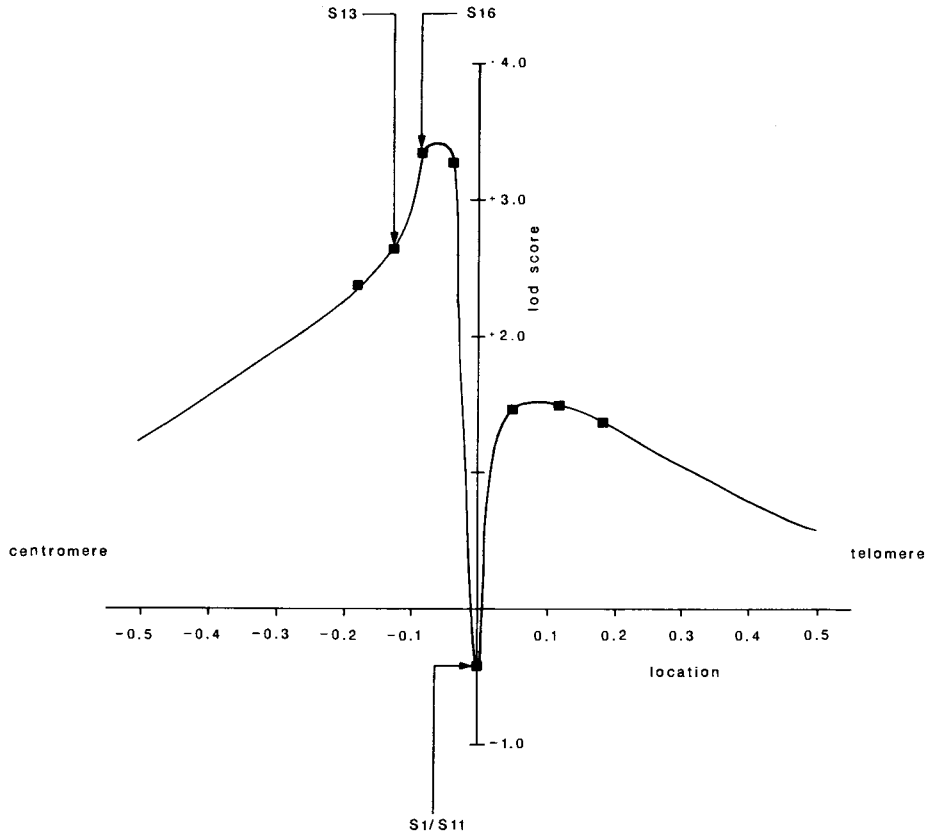

Fig. 4. Multipoint linkage map between early-onset Alzheimer's disease and loci on D21q1-q21. (From Goate et al. 1989)

ondly, as Alzheimer's disease is extremely common in the elderly, it is possible that some at least of the familial late-onset disease is merely happenstance and not related to genetic predisposition.

The data derived from the Volga German pedigrees (which are generally early-onset; Schellenberg et al. 1988) are equally difficult to interpret at present. Thus, it is possible that this disease is predisposed by a locus on another chromosome, as the authors suggest; however, it is equally possible that the disease locus in these pedigrees, as for other familial cases of early-onset, is close to, or proximal of, D21S13 and D21S16. These loci have not been analysed or have not been informative in the Volga German population. A further problem in the analysis of the Volga German pedigrees is that the gene frequency for disease is not known in this population and may be high. A high gene frequency would reduce the chance of observing genetic linkage because it increases the possibility that the disease can be inherited from more that one source in a particular pedigree.

In conclusion, data from all outbred early-onset Alzheimer's disease families are consistent with a predisposing locus on chromosome. Analyses of data derived from families with late onset are inconclusive; it remains possible that these, too, are predisposed to by the same locus, or by another locus, or are not genetic. The aetiology of the disease in Volga Germans also remains obscure.

Evidence for Allelic Heterogeneity in Early-Onset Alzheimer's Disease

The evidence reviewed above strongly suggests that the great majority, at least of familial early-onset (<60 years), Alzheimer's disease cases is caused by a locus on the proximal long arm of chromosome 21. However, many reports have demonstrated that the age of onset within a family is fairly constant, but that it differs between families (see Folstein et al. 1988). We have analysed the distribution of the age of onset within our population of families in which we have demonstrated linkage (Fig. 2; Goate et al. 1989). The results are illustrated in Fig. 5. We have confirmed in this population that the age of onset is more constant within families than between them (analysis of variance, $p < 0.01$). The simplest explanation of this observation is that different mutations at the same locus predis-

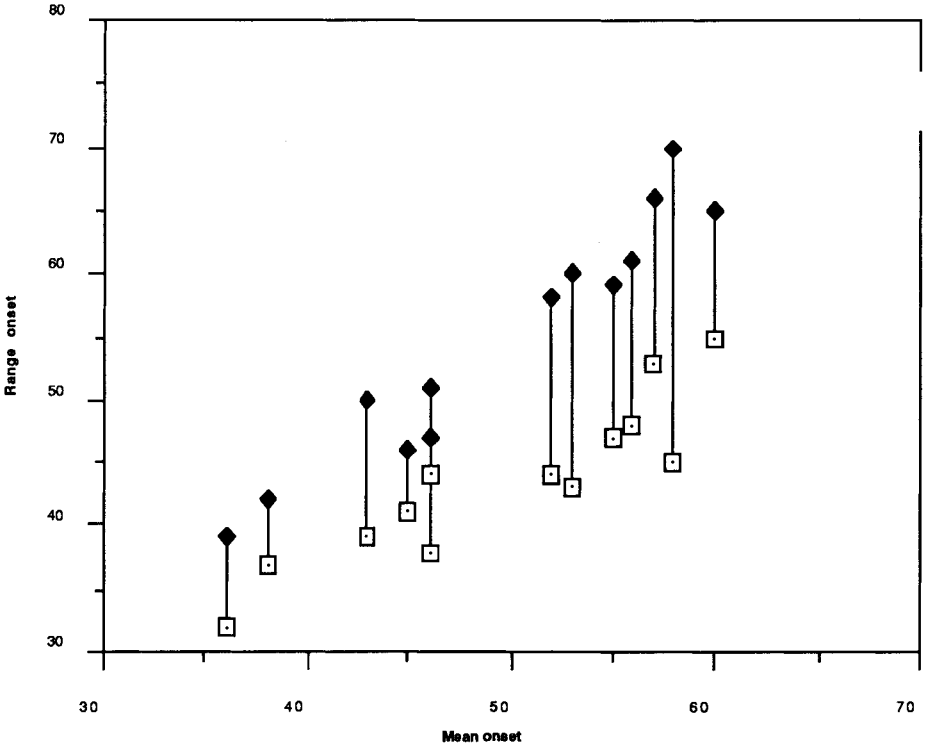

Fig. 5. Graph showing familiality of age of onset of chromosome 21 linked Alzheimer's disease through a plot of range of onset within a pedigree against mean age of onset within that pedigree (see also Folstein et al. 1988)

pose to disease of different onset age. This observation is likely to be important in deriving risk curves for familial Alzheimer's disease for use both in research and in clinical counselling.

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Calcium and the Cytoskeleton in Alzheimer's Disease*

M. L. Shelanski

Summary

Numerous studies in AD have shown the presence of a variety of cytoskeletal alterations within nerve cells. The presence of the microtubule-associated protein tau in the paired helical filaments has led to an increase of interest in this protein. The function of tau in vivo is still poorly understood, but knowledge of its primary structure has permitted us to better define its actions in vitro. AD appears to involve tissues other than the brain in a number of ways, including alterations of calcium homeostasis and cellular spreading.

Introduction

Although this conference focuses on markers for Alzheimer's disease (AD), it is difficult to avoid discussion of the function of the cytoskeleton. The paired helical filaments, one of the two major anatomical hallmarks of AD, is in part composed of the microtubule-associated tau and may also contain microtubule-associated protein 2 (MAP2; Brion et al. 1985; Kosik et al. 1986; Wischik et al. 1988; Yen et al. 1987). This may be a result of alterations in the cytoskeleton, or, conversely, the immobilization of tau may lead to cytoskeletal alterations. Tau normally plays a role in the regulation of the assembly of microtubules in vitro (Weingarten et al. 1975) and possibly in vivo (Drubin and Kirschner 1986). Other factors, especially the calcium ion, can also have profound effects on the form and function of the cytoskeleton. This chapter addresses two themes which are loosely interconnected by their relationship to the control of microtubular assembly. The first is the finding that the free cytosolic calcium concentration $[Ca^{2+}]_i$ is markedly lower and cell spreading is inhibited in the fibroblasts of patients with familial AD as compared to age-matched controls. The second theme concerns the mechanism by which normal tau protein promotes microtubule assembly.

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Alterations of $[Ca^{2+}]_i$ in Familial Alzheimer's Disease

The search for the cause of AD has concentrated on the brain. However, in many genetic diseases the results of the gene defect can be detected in all tissues of the body, even though clinical dysfunction is apparent in only one organ or organ system. For example, Tay-Sachs disease causes devastating damage to the central nervous system, but the deficiency in lysosomal hexosaminidase A which causes the disease can be detected in all tissues. Therefore, it is reasonable to assume that in familial AD, at least, the biochemical abnormalities which ravage the brain can also be detected in more readily sampled peripheral tissues. Such alterations may serve as useful markers of the disease, while the tissues themselves may allow antemortem studies of biochemical processes in AD. At this moment it is unclear what pathways may be affected or what appropriate markers might be. However, in this paper and in the contributions by Saitoh and Blass in this volume, evidence is presented that characteristic alterations can be detected in skin fibroblasts taken from patients with AD.

The study of calcium in fibroblasts from AD donors began with the observation that radiocalcium (^{45}Ca) transport into fibroblasts from AD donors was reduced as compared to age-matched controls (Peterson et al. 1983). Shortly thereafter, the total calcium content of these cells was shown to be elevated (Peterson and Goldman 1986). The decreased entry of calcium into the cell together with the excess accumulation of calcium in the cell pointed toward a defect in the calcium metabolism in AD. However, the physiologically important form of calcium in these cells is the ionic or free calcium in the cytoplasm, $[Ca^{2+}]_i$. This is the calcium responsible for the regulation of a wide range of cellular functions, including neurotransmitter release, cytoskeletal rearrangements, and the activation of a number of enzymes. Its normal concentration is below 100 n*M*, in contrast to the millimolar levels of total or bound calcium in normal cells. Measurement of ^{45}Ca uptake neither reveals the fate of calcium which enters the cell, nor does it reflect the amount of unlabeled calcium which may leave the cell during the same time period. Total calcium determinations are also incapable of informing us about free calcium levels. Until recently our ability to measure free calcium was limited to the use of ion-selective microelectrodes and the calcium-sensitive photoprotein aequorin. The former are too large to be utilized in most mammalian cells while the latter is relatively insensitive at concentrations under 100 n*M*. The development of calcium-sensitive fluorescent dyes based on dicarboxylic acid chelators (Tsien et al. 1982; Grynkiewicz et al. 1985) has greatly expanded our ability to make these measurements on a wide variety of cell types. Our laboratory has successfully used these dyes to study the role of $[Ca^{2+}]_i$ in the regulation of mitosis (Ratan et al. 1986; Ratan and Shelanski 1986), and we have applied these techniques to fibroblasts from familial AD and control donors.

In these studies, cells were labeled with either Quin2 or Fura2, and the ionic calcium was measured using a fluorescent microscope with UV-sensitive optics and a computer-controlled photometer (Maxfield et al. 1989). Measurements were made on six cell lines from familial AD patients, six age-matched controls, and six young normal controls. With Fura2, the values obtained were (Peterson et al.

1986): Young normals, 60 n*M;* age-matched controls, 40 n*M;* familial AD patients, 16 n*M* (values rounded to nearest nM).

The ability of the AD fibroblasts to respond to a variety of stimuli was also altered (Peterson et al. 1988). The results suggested that there are alterations both in the entry of calcium into the cells and in the release of calcium from intracellular reserves.

As suggestive as these results are, they must be interpreted with extreme caution. The data are limited to the familial form of AD and may not be typical of sporadic AD cases. Moreover, the number of cases that we have studied is very small and are the same cases that have been examined in the studies of ^{45}Ca uptake and total cell calcium (Peterson et al. 1983; Peterson and Goldman 1986). The number of cases must be expanded and other degenerative neurological diseases included to establish the specificity of the alteration. Nonetheless, data are beginning to appear from other laboratories which also point to abnormalities of calcium metabolism in aging and AD. For example, alterations in the response of aged CD4 lymphocytes to mitogens have been reported using the dye INDO-1 (Grossman et al. 1989). A report has also appeared showing a modest decrease in ^{45}Ca uptake into mixed lymphocyte populations from AD patients (Gibson et al. 1987).

Cell Shape and Motility in AD

When cells from the same lines that were used in the calcium studies were plated into tissue culture dishes, it was noted that almost all of the young normal cells were completely spread on the culture dish within 2 h. In contrast, only a small fraction of the AD cells spread in this time period. The age-matched controls were intermediate in value (Peterson et al. 1986). When the calcium was elevated in the AD cells using a calcium ionophore, normal spreading was observed, suggesting that the failure to spread may be the result of the low level of ionic calcium in the AD cells. Calcium transients have not been observed in the slow spreading of fibroblasts, but the more rapid spreading of neutrophils substrate is accompanied by a transient elevation of $[Ca^{2+}]_i$ (Kruskal et al. 1986).

Another indication of altered cytoskeletal function in AD cells comes from studies on the migration of neutrophils in a thermal gradient (Fu et al. 1986). In this case the migration of neutrophils from AD patients is severely reduced and closely resembles that of cells treated with colchicine, a microtubule-depolymerizing agent. While direct measurements of free cytosolic calcium have not been done, normal neutrophil chemotaxis is marked by an elevation of $[Ca^{2+}]_i$ in the leading portion of the cells as it approaches and phagocytoses its target (Marks and Maxfield 1989). Should changes in free calcium similar to those seen in fibroblasts be observed in AD neutrophils, rapid diagnosis from a simple venipuncture would be possible. Experiments are currently underway to evaluate this phenomenon.

The alterations in calcium and cell spreading do not prove a link between the levels of ionic calcium and the cytoskeleton. However, alterations in the regulation of cytoskeletal structures may well be involved in the evolution of AD.

Tau Structure and the Promotion of Microtubule Assembly

The control of cytoskeletal assembly and function depends on the complex interplay of a variety of ionic factors, posttranslation events, and interactions of the principal fibrillar elements with accessory proteins. It has recently been established that the MAP tau is a constituent of the paired helical filaments in AD (Brion et al. 1985; Kosik et al. 1986; Wischik et al. 1988). Tau is a complex of proteins with molecular weight in the range of 50–70 kDa, and whose members are generated by both splicing and posttranslational modification. The amino acid sequences of tau from mouse (Lee et al. 1988) and man (Kosik et al. 1986, 1989) have been deduced from cDNA clones. A remarkable feature of the sequence is the presence of three highly conserved but nonidentical 18 amino acid repeats in the amino-terminal portion of the molecule. In the adult an additional repeat of this type is also present. A similar repeat is present in MAP2 (Lewis et al. 1988) and in a 190-kDa MAP (Aizawa et al. 1988). All of these MAP show an ability to promote the assembly of pure tubulin. While it has been demonstrated that the triple-repeat region of MAP2 binds to tubulin (Lewis et al. 1988), it is unclear what role this region plays in the promotion of microtubule assembly. In an attempt to clarify this issue, our laboratory synthesized peptides corresponding to the three repeat regions: Tau-(187–204), Tau-(218–235) and Tau-(250–267). Each of these peptides was then tested for its ability to promote the assembly of phos-

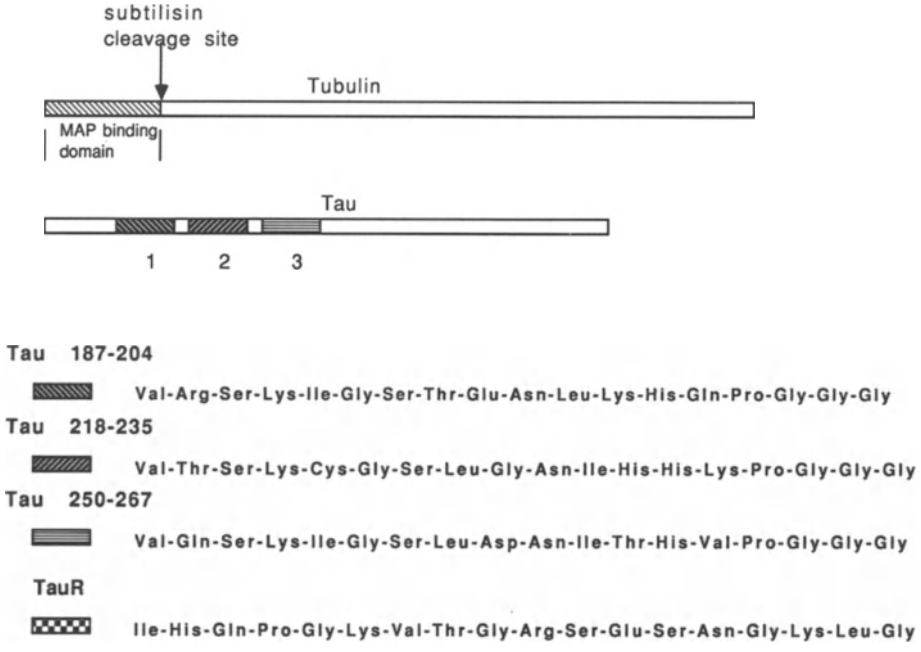

Fig. 1. Tau structure and the promotion of microtubule assembly. Both Tau-(187–204) and Tau-(218–235) are capable of promoting assembly of phosphocellulose-purified tubulin. Tau-(250–267) is without activity. A fourth peptide, TauR, which is composed of the same amino acids as Tau-(187–204) in a random arrangement, does not promote assembly by itself

phocellulose-purified tubulin. Both Tau-(187–204) and Tau-(218–235) were capable of promoting assembly at concentrations over 100 μ*M*. Tau-(250–267), on the other hand, was without activity (Ennulat et al. 1989). The calculated pI of each of the assembly-promoting peptides was 10.55, whereas the pI of the inactive peptide was 7.0. This raised the possibility that the effect was due purely to an ionic interaction between the basic peptide and the acidic MAP-binding region of tubulin (Fig. 1) (Maccioni et al. 1989). To test this possibility, we assayed a fourth peptide, TauR which was composed of the same amino acids as Tau-(187–204) in a random arrangement and had the same pI. TauR did not promote assembly by itself, but did inhibit assembly by Tau-(187–204). This suggested that the ionic interactions are important in binding, but that that promotion was sequence specific. The very high concentration of the peptides suggests that other portions of the tau molecule are critical to optimal tau activity.

There are two principal ways in which tau may act to promote microtubule assembly. It could act to cross-link tubulin molecules into oligomers which form the nucleus for tubule elongation, or it could induce a conformational change in individual tubulin molecules which in turn favors polymerization. The finding of multiple repeats in the tau molecule would be consistent with the oligomerization model. However, it is unlikely that the individual 18 amino acid peptides would serve such a function. The data favor an allosteric mechanism. The conformational change could then lead to oligomerization or directly to polymer elongation (Fig. 2).

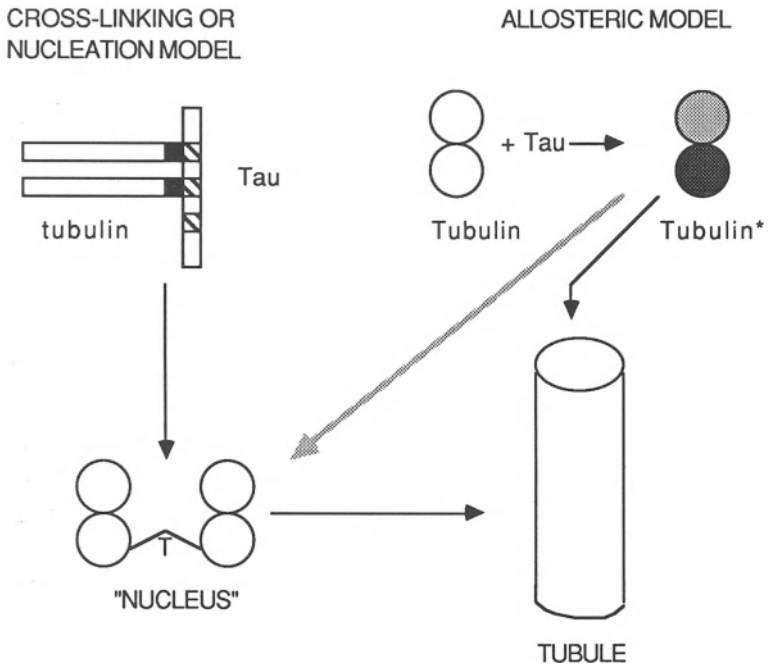

Fig. 2. Tau assembly models: the two principal ways in which tau may act to promote microtubule assembly

Acknowledgements. This work was carried out in collaboration with Drs. Rajiv Ratan, James Goldman, Christine Peterson, David Ennulat, and Frederick Maxfield.

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Use of Cultured Skin Fibroblasts in Studies of Alzheimer's Disease

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Summary

Since there are at least 14 reports of abnormalities in cultured skin "fibroblasts" from patients with Alzheimer's disease (DAT), studies were performed to test whether these cells cultured from skin could also express materials related to those associated with characteristic DAT lesions. When grown in a modified PC12 medium containing chick embryo extract or in a chemically defined medium, cells from both DAT patients and controls reacted both immunocytochemically and on Western blots with antibodies to neuron-specific enolase (NSE) and neurofilaments (NF). Both DAT and control cells reacted immunocytochemically to antibodies to paired helical filaments (PHF), but under appropriate culture conditions the proportion of DAT cells containing anti-PHF reactive materials was significantly higher than in the controls. Western blots revealed no anti-PHF reactive materials entering the gels, and ultrastructural studies revealed fascicles of 10 nm filaments, but no PHF in these cells. Although the nature of the immunochemically reactive materials requires further characterization, these data do suggest that cultured cells from skin are one of the useful systems for investigating the fundamental cellular abnormalities in DAT.

Introduction

A number of approaches are being taken in different laboratories toward identifying the etiology and pathophysiological mechanisms which lead to the premature dysfunction and death of neurons in Alzheimer's disease (DAT). One approach is to characterize the structures which accumulate in the neuropathological lesions characteristic of DAT, specifically amyloid and paired helical filaments (PHF). The precursor protein of the amyloid which accumulates in the plaque cores and in small blood vessels appears to be a normal gene product (Wong et al. 1985; Glenner 1988; Kang et al. 1987). It appears to be expressed in all mammalian tissues except nonnucleated red blood cells (Glenner 1988); excessive amounts of the protein can be found in the brains of aging humans who did not (at least, not by the time they died) suffer from dementia (Davies et al. 1988). PHF are not yet well characterized at the protein level, but the components which have been robustly identified are also normal gene products, namely tau proteins and ubiquitin (Grundke-Iqbal et al. 1986; Mori et al. 1987). Available evidence suggests that the

abnormal accumulation of amyloid and of PHF in DAT is less likely to result from abnormal transcriptional or translational mechanisms than from posttranslational modifications of the protein products of these genes, which have been proposed to be due to more fundamental cellular abnormalities (Glenner 1988; Grundke-Iqbal et al. 1986; Love et al. 1988). Abnormal kinase-mediated phosphorylation has been proposed to be a mechanism in the formation of PHF (Glenner 1988; Grundke-Iqbal et al. 1986; Baudier and Cole 1987), as well as ubiquitination (Glenner 1988; Mori et al. 1987). A second approach toward identifying etiological and mechanistic factors is epidemiological; epidemiological studies have reinforced evidence for familial clustering in DAT (Rocca et al. 1986) and raised a possible role for toxins, including aluminium (Birchall and Chappel 1988). A third approach is the systematic search of the genome in familial DAT, by restriction fragment length polymorphism (RFLP) analysis and then further studies to identify the specific gene(s). Results from this approach, including the suggestion of a pericentromeric RFLP on C21, remain controversial (Sinet et al. 1988). The possibility has been raised that DAT is genetically heterogeneous, and the question has been raised as to whether or not the currently available informative kindreds have enough living affected members (from whom DNA can be obtained) to allow confident identification of an RFLP if different genes are indeed responsible in different kindreds. A fourth approach is to identify cellular and molecular abnormalities in DAT cells and to trace their cause and their relationship to the pathophysiology of the brain disease. This approach is capable, in principle, of detecting pathophysiological mechanisms which are common to several genetic variants of the disease, as indicated by precedents in the study of inborn errors of metabolism (Stanbury et al. 1983). This fourth approach has involved the use of extraneural tissues as well as autopsy brain since a number of the abnormalities of interest, which could be linked by plausible hypotheses to the brain damage (Baker et al. 1988), are labile post mortem (e.g., membrane transport, signal transduction, cellular calcium homeostasis, or metabolism – including mitochondrial metabolism).

There are at least 40 reports of abnormalities in extraneural tissues in DAT patients (see Baker et al. 1988 for partial review), including at least 14 reports of abnormalities in "fibroblasts" cultured from their skin (Table 1). These observations suggest that cellular and molecular abnormalities in DAT may be expressed in, and therefore may be studied in cells cultured from the Alzheimer patients themselves.

As a model system, the cultured cells have the advantage that they contain the same genome (and, therefore, any abnormal genes) as in the patients. On the other hand, the clinical disabilities in DAT relate uniquely to damage to the brain, raising the question of the relevance to the brain disease of abnormalities in clinically unaffected tissues. Positive precedents exist in a number of other neurological disorders with major genetic components, such as the lipidoses, in which studies of cultured skin cells and other extraneural tissues have led not only to improved understanding of the cellular pathophysiology, but also to useful diagnostic laboratory tests (Stanbury et al. 1983). However, negative precedents also exist, for instance, in Huntington's disease (Pettegrew et al. 1979) where reports of abnormalities in cultured skin cells have not yet led to significant advances in

Table 1. Reports of abnormalities in cultured Alzheimer cells

Abnormality	Reference
Abnormal calcium homeostasis (uptake, levels, and response to agents which alter levels)	Peterson et al. 1985, 1986, 1988; Peterson and Goldman 1986
Excessive isoproterenol-stimulated cyclic AMP synthesis	McSwigan 1986; Hanson and Sheppard 1979; Malow et al. 1989
Decreased [U-^{14}C]glutamine oxidation	Peterson et al. 1986; Sims et al. 1987
Abnormal glucose utilization	Peterson et al. 1986; Sims et al. 1987
Abnormal protein kinase activities (PK-A, PK-C, and CaM)	Saitoh et al. 1988, Huyhn 1989; Peterson and Cotman 1987
Decreased secretion of a cholinergic growth factor	Kessler 1987
Decreased cell adhesiveness	Saitoh et al. 1988, Huyhn 1989
Decreased cell spreading	Peterson et al. 1988
Decreased mRNA for APP (only in familial form)	Saitoh et al. 1988, Huyhn 1989
Decreased secretion of APP	Saitoh et al. 1988, Huyhn 1989
Increased accumulation of materials which react with antibodies to PHF	Baker et al. 1988 (and see text)
Defective DNA repair	Robison et al. 1987; Lai and Kaminskas 1985
Decreased transketolase activity	Gibson et al. 1988

PK, protein kinase; APP, amyloid precursor protein; PHF, paired helical filaments

understanding the disorder, despite the identification of an RFLP associated with this disorder.

The approach taken by most researchers to the problem of whether or not abnormalities in cultured DAT cells (and in other extraneural tissues) provide significant information about the disease process has been to study intensively the mechanisms underlying the abnormalities, in order to determine whether or not any of them relate by compelling hypotheses to the brain damage (Baker et al. 1988; Malow et al. 1989; Sims et al. 1987; Gibson et al. 1988). The hypotheses generated to relate the abnormalities discovered in the cultured cells to the brain damage by researchers using this approach have been plausible, but none has been generally accepted as compelling (Baker et al. 1988; Peterson et al. 1986; Malow et al. 1989; Sims et al. 1987; Kessler 1987; Robinson et al. 1987; Gibson et al. 1988). An alternative approach is to try to investigate abnormalities which have a "face validity," in that they deal with materials which characteristically accumulate in DAT. The classical neuropathological markers for DAT are paired helical filaments (PHF) and Alzheimer amyloid (Wong et al. 1985; Glenner 1988; Love et al. 1988). At the molecular level, neither marker is entirely specific for DAT, but no more widely accepted molecular markers for the biological process of DAT have as yet been identified (Glenner 1988; Love et al. 1988). Recent observations suggest that molecules which react with antibodies raised to preparations of PHF ("anti-PHF reactive materials") can be studied in cells cultured from human skin.

Methods and Results

Fibroblasts cultured from human skin were obtained from the Coriell Institute Repository in Camden, NJ, USA. These cells were then grown in a modified PC12 medium. Cells were plated at $10^4/cm^2$ in DMEM containing 16% fetal calf serum (FCS) and incubated overnight to facilitate attachment. This medium was then carefully removed, with three rinses in Puck's saline A to remove all traces of FCS. The medium was then replaced with the modified PC12 medium (EGE medium). This EGE medium contained "low glucose" (1.0 g/l) DMEM, 5% chick embryo extract, 1 m*M* dibutyryl cyclic AMP, 100 ng/ml of nerve growth factor (NGF, 7S, from Collaborative Research), and 10 μg/ml gangliosides (mixed, from bovine brain, type II, Sigma), 1% penicillin-streptomycin and 1% fungizone. It was filtered through an 0.45 μ Nalgene filter before use. The cells survived for up to 4 weeks in the EGE medium, but were normally studied after 10–14 days. Medium was replenished with fresh medium, including fresh growth factors every 4th day.

After 10–14 days in the EGE medium, the morphology of the cells was altered compared with cells from the same culture grown in parallel, but in DMEM with 16% FCS. Of the cells in the EGE medium, 29.7% ± 9.3% had a spindle-shaped "fibroblastoid" morphology, while 70.3 ± 9.3% had a more symmetrical cell body; in cells from the same cultures, but grown in DMEM with FCS for the same time, 93.3 ± 2.0% of the cells were spindle shaped and 7.3 ± 2.3 were not (mean ± SEM for six cultures in each condition, at least 100 cells counted for each culture). The differences in the distribution of spindle-shaped versus nonspindle-shaped cells under the two culture conditions were significant ($P < 0.002$ by two-tailed t-test).

In recent experiments, in which cells have been cultured in a chemically defined medium, the cells have developed long processes although the cell bodies retain a flat morphology (Fig. 1). This chemically defined medium (CDMd) consists of low glucose DMEM, 1 m*M* dibutyryl cyclic AMP, 100 ng/ml of NGF, and 10 ng/ml of basic fibroblast growth factor (from bovine pituitary, Collaborative Research). Cells were plated in DMEM with 16% FCS at $10^3/cm^2$ polylysine-coated, glass-based two-chamber slides or polylysine-coated plastic petri dishes. The low plating density was chosen to prevent contact inhibition. After an overnight incubation to allow attachment of the cells, the medium was changed to CDMd. Medium was replenished on the 3rd day, and on the 4th day the cells were fixed or harvested.

Cells grown in EGE medium stained immunocytochemically for two conventional markers used to identify neurons in culture, namely neuron-specific enolase (NSE; polyclonal, Polysciences, Warrington, PA) and neurofilaments (Polyclonal kindly donated by Dr. Charles Marotta, Harvard University). Western blots of extracts of the cells confirmed the existence of materials immunoreactive with these antibodies and with the electrophoretic motility expected of NSE (Fig. 2) and of each of the three neurofilament polypeptides. In recent experiments, cultured skin cells grown in the CDMd medium (a chemically defined modification of the EGE medium) have been found to react immunocytochemically with the polyclonal antibody to NSE and with a total of three polyclonal and three monoclonal antibodies to neurofilaments. These included polyclonal and monoclonal antibodies to each of the three neurofilament subunits.

Fig. 1. Fibroblasts grown in chemically defined medium (CDMd). Cells from a control subject were cultured on glass-based, polylysine coated two-chamber slides in CDMd, exactly as described in the text, fixed in 4% paraformaldehyde in phosphate-buffered-saline (PBS) for 30 min, and viewed with Nomarski optics (× 138)

When grown in EGE medium, DAT cells have been found to contain materials which react immunocytochemically with antibodies raised to PHF ("anti-PHF reactive materials") significantly more frequently than did cells from control subjects (Fig. 3). Results have been consistent in studies of 19 patients with both familial and apparently sporadic DAT compared to 19 cognitively intact controls of comparable age and sex. Preliminary results suggest that when grown in the CDMd medium, cells from both Alzheimer and control subjects contain anti-PHF reactive materials, but the staining is again greater in the Alzheimer cells. Western blots of Alzheimer cells grown in the EGE medium revealed no anti-PHF reactive

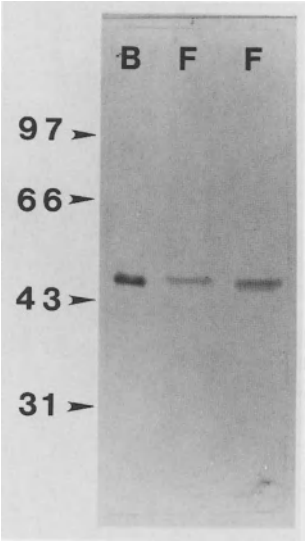

Fig. 2. Immunoblot for neuron-specific enolase. Cell extracts were prepared from the temporal cortex of a patient who died without neurological disease *(lane B)* or from fibroblasts *(lanes F)* grown in EGE medium. Brain and cells were homogenized in a solution containing proteinase inhibitors (10 m*M* Na-phosphate buffer (pH 7.2), 150 m*M* NaCl, 2 m*M* EDTA, 2 m*M* EGTA, 25 μ*M* leupeptin, 5 μl aprotinin, and 50 μl trypsin inhibitor). Blots were prepared and analyzed by published methods (Rittling et al. 1986; Sheu et al. 1988; Towbin et al. 1979)

materials entering the separating sodium dodecyl sulfate (SDS) gels; electron microscopy revealed fascicles of 10 nm filaments, but no structures identical to PHF (Baker et al. 1988).

Close attention to the details of tissue culture technique has proven critical to observing increased immunocytochemical staining with anti-PHF antibodies in Alzheimer compared to control lines. Experiments to explore the mechanisms leading to the expression of anti-PHF reactive materials in these cells have indicated that a variety of controlled insults can increase the proportion of control cells reacting immunocytochemically with anti-PHF antibodies to levels comparable to DAT cells. These include culturing control cells with CCCP, an uncoupler of mitochondrial oxidative phosphorylation (Baker et al. 1988), and with aluminum. These agents were chosen because mitochondrial uncoupling (Sims et al. 1983, 1987a, b) and aluminum toxicity (Balin et al. 1988) have been proposed as cellular mechanisms in DAT. Whether other agents also induce anti-PHF reactivity in this system is being studied; data so far are consistent with the hypothesis that accumulation of anti-PHF reactive materials is one of the ways cells can respond to metabolic insults (Love et al. 1988; Baker et al. 1988). Furthermore, preliminary observations suggest that anti-PHF reactivity increases even in cells from controls which are approaching the end of their life span in culture (i.e., close to phaseout). This observation is also of potential pathophysiological interest since it raises the possibility that the accumulation of these materials, which are typical of DAT, may represent an interaction of biological age with environmental factors as well as with genetic predisposition. From the viewpoint of the proposed experiments, however, they reinforce the necessity of comparing DAT and control cell lines under precisely comparable conditions and at closely comparable biological age in culture.

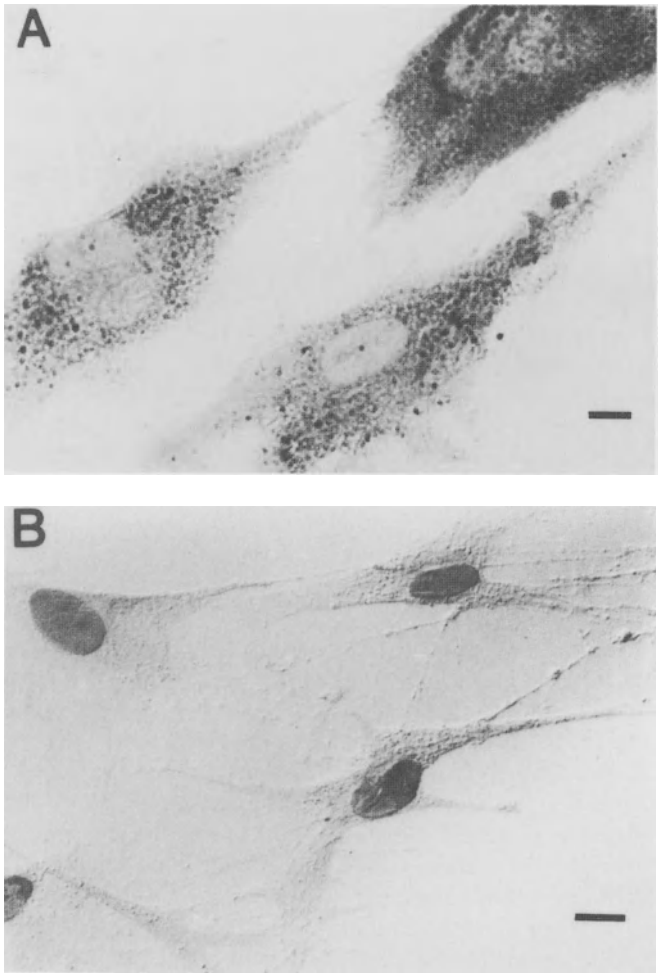

Fig. 3A, B. Immunocytochemical reactivity of skin cells with an anti-PHF antibody. Cells from an Alzheimer patient **(a)** and a control subject **(b)** were grown in EGE medium exactly as described in the text, fixed with 4% paraformaldehyde in phosphate-buffered saline for 30 min, and stained immunocytochemically with the commercial (ICN) polyclonal anti-PHF antibody (dilution 1 : 1000), using the SABC technique (Zymed). The nuclear staining in **b** reflects counterstaining with hematoxyclin

Growth properties and biological ages of DAT and control cultures have been compared in detail in a collaborative study between Burke and the Laboratory of Investigative Dermatology at Rockefeller University (Rittling et al. 1986). Four DAT lines were compared with six control lines from donors of comparable chronological age and sex in thymidine incorporation (Cristofalo index), DNA/cell, and protein/cell; cumulative population doubling levels (cPDL) and passages to phaseout were marginally, but statistically significantly higher in the DAT lines

($P < 0.05$). These data indicate that DAT cells do not show premature aging in culture. Others have noted no significant differences in the growth properties of DAT and control cells (Peterson and Goldman 1986; Peterson et al. 1986). Since growth properties and biological age in culture can have profound effects on the properties of cells cultured from skin, including the genes expressed (Sheu et al. 1988), this point is critical and requires reexamination in a larger population.

Discussion

The differences reported between cells from DAT and control lines have been quantitative and not so large as to appear "all-or-none." Their replicability over time and their confident interpretation require that DAT and control cultures be compared under as closely controlled and matched conditions as possible and in a large series. Abnormalities reported in cells from relatively small series of DAT patients and controls which do not replicate in a large series, including disease controls, are likely to be epiphenomena which do not call for intensive further research from the viewpoint of DAT.

Abnormalities which do replicate in a larger series or to characterize subgroups of DAT patients are likely to be interesting. They may provide useful laboratory aids to diagnosis, particularly for patients with atypical manifestations or other complicating disease processes. Such laboratory aids may be useful whether they turn out to be trait-dependent or state-dependent markers. They would be complimentary to RFLP or other markers at the DNA level since markers at the DNA level are presumptively trait-dependent markers and may be difficult to use if there is marked genetic heterogeneity (Sinet et al. 1988). Cellular abnormalities could turn out to relate to common pathophysiological mechanisms, which could, in principle, result from a number of related gene defects, and therefore be more useful diagnostic markers than studies at the DNA level; precedents for this possibility exist in a number of inborn errors of metabolism (Stanbury et al. 1983). Abnormalities which characteristically occur in cells from DAT or DAT subgroups may also be of interest by providing clues to the cellular pathophysiology. Such clues can then be used to guide further mechanistic research. If such mechanistic studies in relatively small series lead to interesting new observations, the clinical significance of those abnormalities will again have to be checked in a larger series.

The data available at the present time do suggest, however, that the use of extraneural tissues, including cultured cells from skin, may be one of the valid approaches to trying to unravel the cellular pathophysiology of DAT.

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Lactate Production and Glycolytic Enzymes in Skin Cultured Cells from Alzheimer's Disease Patients*

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Abstract

Changes in energy metabolism related enzymes have been reported in the brain of patients affected by Alzheimer's dementia. Recently an increase in lactate production was also observed in cultured cells from Alzheimer's patients. In this paper we report the results of an investigation of lactate production and of the activity of phosphofructokinase, lactate dehydrogenase and hexokinase in skin cultured fibroblasts from familial and sporadic Alzheimer's disease patients. The production of lactate and the activity of phosphofructokinase and lactate dehydrogenase were similar in all groups studied. However, the activity of hexokinase was deficient in some patients with the familial dominant form of Alzheimer's disease whereas it was normal in sporadic cases. These results suggest that Alzheimer's disease may be a heterogenous disorder and that a modification on the catalytic activity of the rate limiting glycolitic enzyme hexokinase may play an important role in the pathogenesis of the disease in at least a subgroup of patients.

Introduction

Abnormalities in cerebral metabolic rate, cerebral oxygen consumption, cerebral blood flow, and cerebral glucose utilization were among the earliest and best documented alterations in dementias (Quastel 1932; Sokoloff 1966). Some studies have also indicated modifications in activities of enzymes of energy metabolism in Alzheimer's disease (AD) and other dementias. Perry et al. (1980) and Sorbi et al. (1983) observed decreased activity of the pyruvate dehydrogenase complex (PDHC) in affected areas of brains from patients with AD and Huntington's disease. In an extensive study Bowen et al. (1979) observed that 19 of 37 neuroconstituents examined were reduced in postmortem AD brain. Among these the most marked decrease was in the activity of phosphofructokinase (PFK), a key enzyme in glycolysis that may be the rate-limiting enzyme in cerebral glucose utilization (Lowrey and Passanneau 1964). Iwangoff et al. (1980) also reported

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declines of PFK activity with age and a marked decrease (90% of age-matched control values) in Alzheimer cerebral cortex.

The abnormality in PFK observed in postmortem brain seems to persist, in at least some patients, in cultured skin fibroblasts even after several passages (Sorbi and Blass 1983). Studies on cultured skin fibroblasts have also shown abnormalities in PDHC and PFK activities in patients with trisomy C-21 (Down's) syndrome (Sorbi et al. 1983; Sorbi and Blass 1983), who are at very high risk to develop Alzheimer's changes if they live long enough. Recently Sims et al. (1985, 1987) observed increased lactate production and decreased CO_2 production in cultured skin fibroblasts from AD patients. Contrasting results, however, have been reported by Peterson and Goldman (1986), who failed to observe changes in lactate production in similar cell cultures from AD patients. Moreover, the study by Peterson and Goldman (1986) indicated an overall alteration in cell metabolism in cultured skin fibroblasts. They reported changes in glucose oxidation, calcium content, protein and DNA synthesis in aged and AD fibroblasts.

To further investigate the involvement of glycolysis in cultured cells from AD patients, we studied lactate production, PFK, hexokinase (HK), and lactate dehydrogenase (LDH) activities in cultured skin fibroblasts from patients affected by familial AD, their unaffected relatives, patients with sporadic AD, apparently normal controls, and pathological controls.

Materials and Methods

The subjects of this study included three patients with familial AD: two brothers and a cousin from the same pedigree (family 1). Skin fibroblasts were also studied from three nonblood relatives (spouses) of family 1 and six age-matched, nonrelated, apparently normal controls. Leukocytes were also used to study PFK and HK in three other patients with familial AD, five with sporadic AD, and five apparently normal age-matched controls.

Fibroblast cell cultures were obtained from forearm skin biopsy. Cells were grown in Dulbecco's modified Eagle's medium, supplemented with 20% fetal bovine serum and harvested at confluence, 5–7 days after a 3 : 1 subculture, by mild trypsinization. Periodic testing revealed no contamination by pleuropneumonia-like organism. Protein content and cell numbers were similar in all lines studied as well as passage numbers. All cell lines were coded and studied in triplicate in at least three different experiments. Leukocytes were obtained in 30 ml blood collected from patients who fasted overnight, using a plastic syringe containing heparin.

PFK activity was measured by the method of Racker (1947) as modified by Sorbi and Blass (1983), LDH by that of Clark and Nicklas (1970), HK by that of Racker (1947), and protein concentration by that of Lowry et al. (1951).

Results

The activities of fibroblast PFK and LDH were comparable in all groups studied (Table 1). Fibroblast HK activity was significantly decreased in the affected mem-

Table 1. Fibroblast PFK, LDH, and HK, in familial and sporadic Alzheimer's disease (nmol/kg protein per minute)

	PFK	LDH	HK
Controls ($n = 6$)	34.3 ± 11	997 ± 27	25.3 ± 6
Familial AD ($n = 3$)	39.6 ± 11	621 ± 12	19.3 ± 2[a]
Unaffected relatives ($n = 3$)	28.8 ± 12	486 ± 72	29.0 ± 5

[a] $p < 0.05$

bers of family 1 compared to the unaffected relatives. All affected members of this family had HK activity below the lowest of control values. Leukocyte PFK activity was normal in those with sporadic and those with familial AD (Table 2). Leukocyte HK activity was significantly decreased in patients with familial but not in those with sporadic AD. LDH production was comparable in cultured skin fibroblasts from affected members of family 1, their unaffected relatives, and three controls (Table 3).

Table 2. Leukocyte PFK and HK in familial and sporadic Alzheimer's disease (nmol/kg protein per minute)

	PFK	HK
Controls ($n = 5$)	27.11 ± 9	55.9 ± 12
Familial AD ($n = 3$)	20.9 ± 9	36.1 ± 5[a]
AD ($n = 5$)	40.5 ± 19	56.3 ± 24

[a] $p < 0.05$

Table 3. LDH production in familial Alzheimer's disease (nmol/kg protein per hour)

Familial AD ($n = 3$)	858 ± 401
Unaffected relatives ($n = 5$)	756 ± 126
Controls ($n = 3$)	676 ± 242

Discussion

In vivo studies of cerebral metabolic rates for oxygen and glucose and of cerebral blood flow have consistently disclosed significant reductions in the Alzheimer brain. Moreover, there is now evidence that metabolic changes associated with AD are not confined to the brain. The significance of the finding of abnormalities in peripheral tissues in AD is not yet clear. It is difficult to assess the role of metabolic changes in peripheral cells in the pathogenesis of AD. The finding of any abnormality in cultured cells even after several cell passages should indicate that such an abnormality is independent of exogenous factors and is linked, at least

broadly, to the cellular genome. Moreover, the finding of a consistent abnormality in peripheral cells may lead to a biological marker being used as a test for the diagnosis.

In this study we report evidence of an alteration in the catalytic activity of the glycolytic rate-limiting enzyme HK in patients affected with the familial form of AD. Enzyme values were decreased versus unaffected relatives and versus apparently normal, unrelated controls. HK activity was decreased in cultured skin fibroblasts and in leukocytes from these patients but was normal in cells from patients with sporadic AD. In patients with familial AD, LDH concentration was normal, in contrast to the results of Sims et al. (1986).

These results suggest a possible heterogeneity among AD patients, suggesting that at least a subgroup with the familial form has a modification in the activity of HK. Further studies are in progress to evaluate the frequency of this finding among patients with familial AD and to characterize this abnormality at a molecular level.

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