Vol. 2, No 3
July
September 2002

BRAIN AGING

INTERNATIONAL JOURNAL

www.brainaging.org

EONIA

Perspectives for a Healthy Brain Aging

„Ana Aslan“ International Academy of Aging

Editorial office:
office@brainaging.org
Tel. / fax: +40 21 312 77 21
PO Box 19-64
Bucharest 3, Romania

Roxana Nat aslan@pcnet.ro
Gunilla Johansson
Gunilla.Johansson@neurotec.ki.se

Publishing manager: Laurenþiu Nat
eonia@pcnet.ro

Cover: Gheorghe Popescu

ISSN 1582-8352 (printed)
ISSN 1582-8360 (on-line)

Brain Aging International Journal®
Ana Aslan International Academy of Aging

The accepted abbreviation for **Brain Aging International Journal** for bibliographic citation is **Brain Aging**

Reviews

Articles

Scientific board

Treatment Strategies in Alzheimer's Disease

Vesna Jelic[#], Bengt Winblad

Karolinska Institutet, Dept of Neurotec, Division of Geriatric Medicine, Huddinge University Hospital, Huddinge, Sweden

Abstract

Intervention strategies in dementia and more specifically Alzheimer's disease (AD) are based on results from epidemiological studies on risk factors and experimental research on molecular pathogenesis of the disease and its biological substrates. Currently established treatment for AD with acetylcholinesterase inhibitors is symptomatic and cannot reverse the disease process or ameliorate neuropathological changes in the brain. Visionary interventions target more proximal events of the pathogenetic cascade of the disease like amyloid processing, tau hyperphosphorylation, apoptosis and oxidative stress. Findings from epidemiological studies have expanded our knowledge on risk as well as possible neuroprotective factors and given means to develop preventive strategies based on treatment of co-morbidity, like hypertension, or on therapy with antihyperlipidemic drugs – statins, anti-inflammatory agents or still controversial hormone replacement therapy.

Keywords: Alzheimer's disease, treatment

Increasing elderly population across the world is making operative one of the major risk factors for dementia, particularly its most common form, Alzheimer's disease. In EU estimated incidence of dementia is 824 000 persons per year[1].

Consequently, this highly prevalent chronic disease will challenge health care system and society to allocate additional funds to support not only affected individual but also caregivers. Natural course of the disease is seen as a functional deterioration over a series of clinical milestones starting with a mild cognitive impairment (MCI), progressing through the middle stages with a gradual loss of instrumental and later basic activities of daily living, emergence of behavioural problems leading to end-stage in a nursing home with a severe disability. In Sweden moderately and severely demented made up approximately 67% of the total number of dementia cases and represent 94 % of the total costs utilized for dementia[2]. There is no need to put additional emphasis on the importance of intervening early during the course of the disease in order to delay or halt progression to this severe end-stage that requires expensive institutionalization.

Rationale for present and future treatments

Expanding knowledge on the etiopathogenesis of the disease and its neurobiological substrates has given means to develop treatment strategies for each level of disability, figure. 1, and changed the attitude towards the disease treatment and prognosis from a passive and nihilistic towards active and optimistic.

Cascade of molecular events leading to the disease specific pathology and typical clinical expression of the disease are recognized as therapeutical targets and summarized in figure. 2.

[#]**Correspondence:** Vesna Jelic, MD, PhD, Karolinska Institutet, Dept of Neurotec, Division of Geriatric Medicine, Huddinge University Hospital, 141 86 Huddinge, Sweden

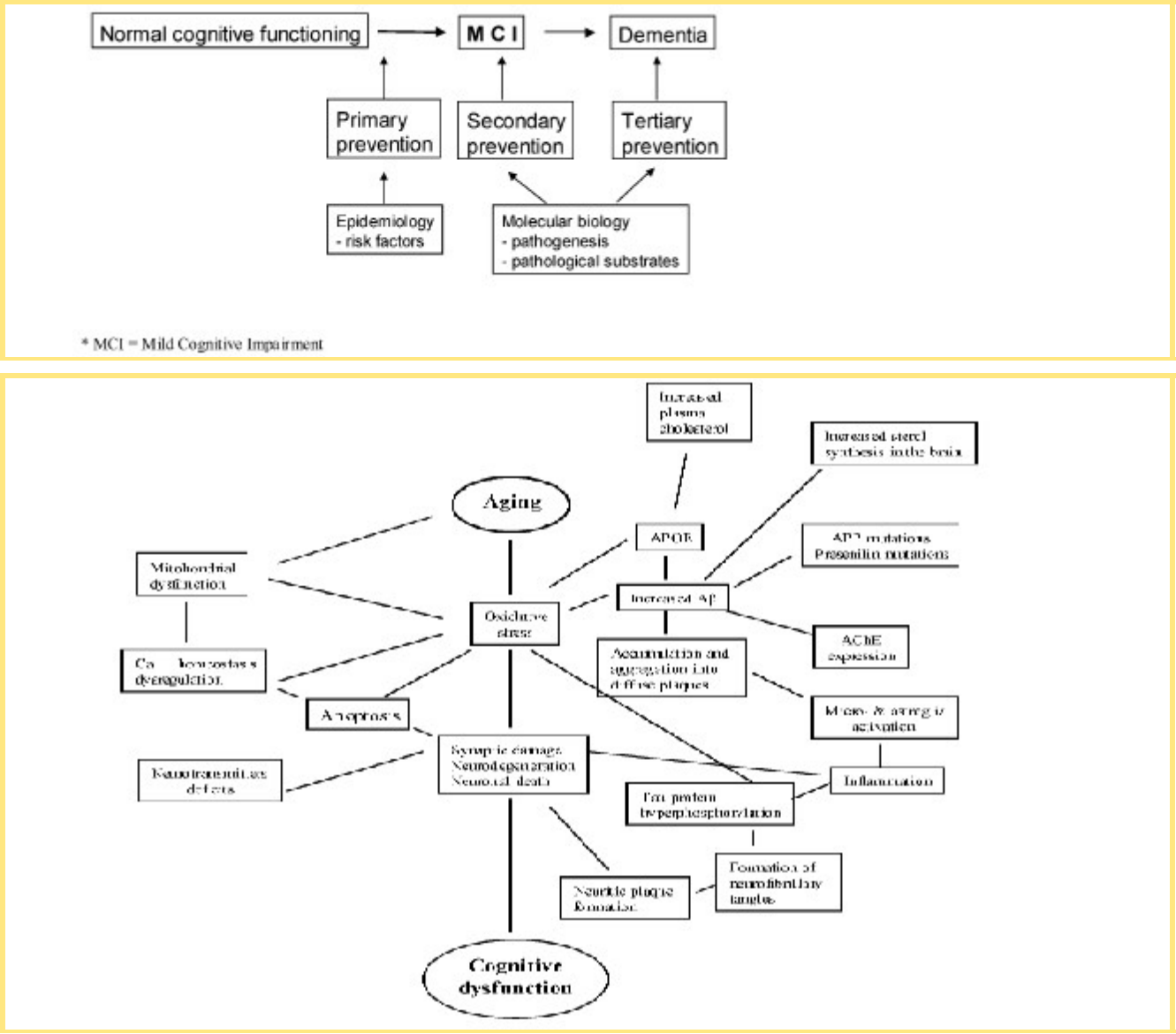


Figure 1: Intervention strategies in dementia.

Review of intervention strategies

Treatment strategies are according to their modes of action as well as their efficacy classified into:

- symptomatic, meaning modification of the brain function without change of the disease progression;
- disease modifying, meaning intervention in the central pathogenetic event;
- preventive, meaning affecting the disease occurrence.

a. Currently available

Current treatments operative at the clinic are mainly symptomatic and substitute transmitter deficits, i.e. acetylcholine. Efficacy of acetylcholinesterase inhibitors (AchEI) has been evaluated across three key symptom domains of AD – cognition, bahaviour and activities of daily living (ADL) and short symptomatic improvements up to one year have been reported[3]. Possibilities of long-term effects via modification of amyloid precursor protein metabolism (APP) have also been considered[4] and studies designed to measure long-term efficacy of (AchEI) are ongoing[5]. Additional combination with neuroprotective agents like vitamins with antioxidant properties seem plausible, since oxidative stress is a central pathogenetic mechanism in various neurodegenerative diseases, including AD[6,7]. There is also more hope for the patients in moderately severe and severe stages of the disease since an antiglutamatergic drug, memantine, has been approved recently in Europe for this indication[8]. These approaches are summarized in the table 1.

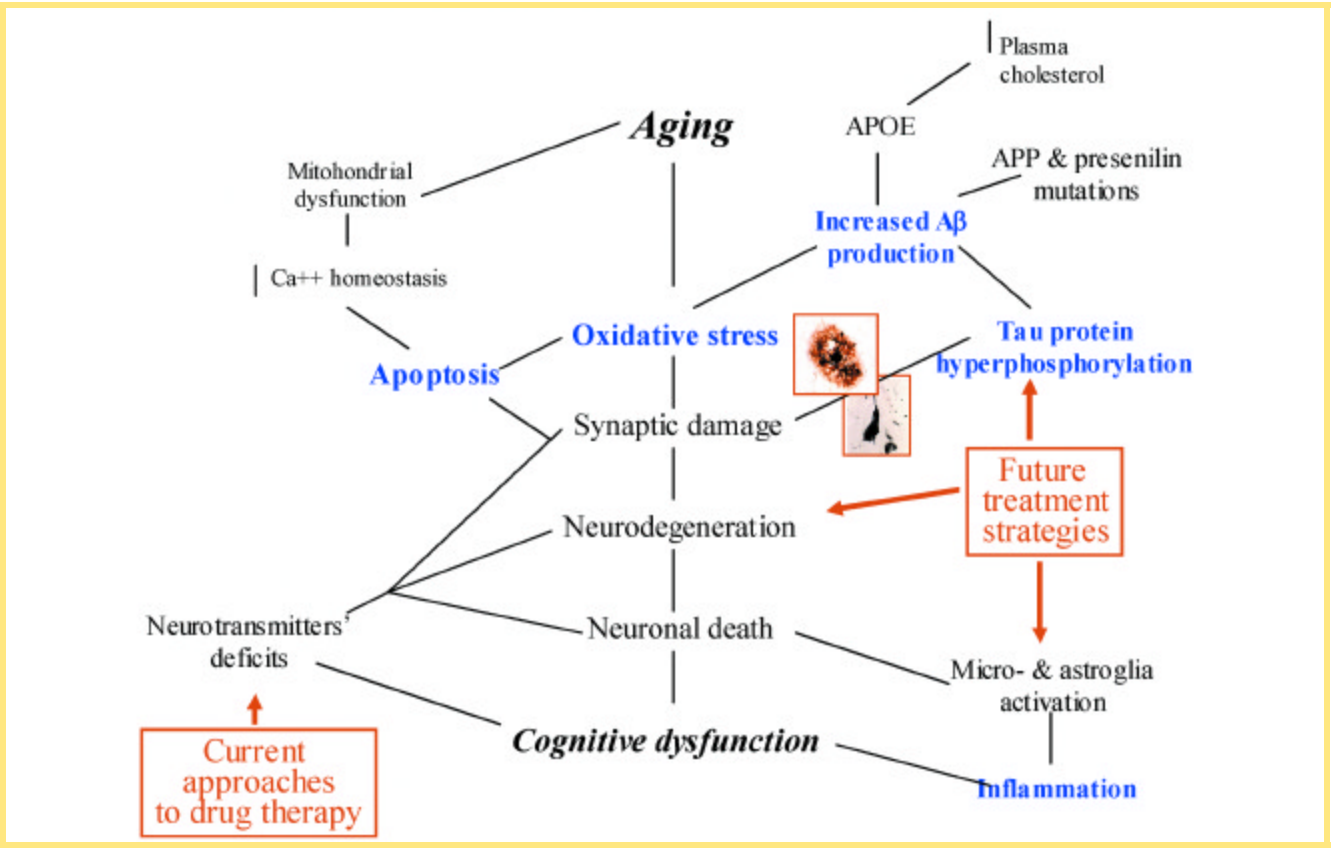


Figure 2: Molecular biology of Alzheimer's disease. Possible targets for therapeutical interventions.

b. Visionary

Novel treatment approaches under development or at the early stages of clinical trials are targeting central pathogenetic events that lead to the production of key pathological features of the disease: amyloid production, accumulation or aggregation; tau hyperphosphorylation with formation of neurofibrillary tangles, and apoptosis, also summarized in table 1. Nerve growth factor and neurotrophines are alternative neuroprotective approaches which promise to regulate neuronal plasticity by controling neurotransmission, connectivity and neuritic outgrowth. Growth factor gene therapy is currently in a phase I clinical trial and seems to be an effective way of delivering therapeutical substance to the most vulnerable brain regions via transplantation of patient's fibroblasts modified to secrete NGF[13].

Epidemiological studies have expanded our knowledge on risk factors for dementia, namely AD and suggested neuroprotective interventions applicable on a large scale.

Midlife vascular risk factors like high cholesterol levels and high systolic blood pressure were shown to influence late life development of cognitive impairment[14]. Therefore, treatment of co-morbid conditions could modify clinical expression of dementia, as was suggested in large retrospective epidemiological studies which showed reduced risk of developing dementia among statin users[15,16,17]. Similar reduction of AD incidence has been found in subjects exposed to anti-inflammatory drugs[18] and prevention trial with selective COX-2 inhibitor is ongoing[19]. Estrogen replacement therapy is a plausible hypothesis built on experimental data[20], but prospective observational studies were inconclusive[21]. The most recent report from Women Health Initiative on intervention trial of estrogen plus progestin therapy in prevention of chronic vascular diseases shows that overall risks exceed benefits[22].

Basic research is focusing on the development of more specific selective estrogen receptor modulators.

Implications

If proved to be successful, beneficial effects of antidementia treatments are seen as manifold. Successful interventions in early, presymptomatic stages of the disease will decrease the disease prevalence and help society to more efficiently cope with a problem and allocate more funds for multifaceted support to both patients and caregivers.

Table 1. Current and hypothetical treatment approaches in Alzheimer's disease.

Symptomatic	Disease modifying	Preventive
Acetylcholinesterase Inhibitors	Antioxidants (Vitamin E, Gingko Biloba)	Anti-inflammatory drugs Antihypertensive therapy
Antiglutamatergic drugs (NMDA antagonists)	NGF (gene-therapy) neotrophines?	Antihyperlipidemic agents (statins)
	Interventions in β-amyloid processing (Active* and passive** Aβ–immunization, γ & β secretase inhibitors, SAP binding inhibitors***)	Estrogen replacement therapy??
	Interventions in tau-hyperphosphorylation (glycogen synthase kinase 3β inhibitors)	

*Trial suspended, new options of immunization with a nontoxic/nonfibrillar amyloid-β are under development.[9,10]

**Tested on animal model, subchronic passive immunization using the monoclonal anti-Aβ antibody.[11]

***Serum amyloid P component.[12]

In addition, possible success of neuroprotective and disease modifying treatments will validate the existing theories on disease pathogenesis. Efficacy of similar treatments in different forms of dementia could influence clinical practice and our thinking about the neurodegenerative diseases as processes with common genetic and molecular biological background.

References

1. Launer L.J., Hofman A., Frequency and impact of neurologic diseases in the elderly of Europe: A collaborative study of population-based cohorts. *Neurology* 2000; 54(Suppl 5): S1-S3
2. Wimo A., Karlsson G., Sandman P.O., Corder L., Winblad B., Costs of illness due to dementia in Sweden. *Int J Ger Psychiatry* 1997; 12: 857-861
3. Winblad B. K., Engedal, H. Soininen, A 1-year, randomized, placebo-controlled study of donepezil in patients with mild to moderate AD. *Neurology* 2001; 57: 489-495
4. Giacobini E. Cholinesterase inhibitor therapy stabilizes symptoms of Alzheimer's disease. *Alzheimer Dis Assoc Disord*. 2000;14 Suppl 1: S3-10.
5. Sramek J.J., Veroff A.E., Cutler N.R., The status of ongoing trials for mild cognitive impairment. *Exp. Opin. Invest. Drugs* 2001; 10: 741-752
6. Rosler M, Retz W, Thome J, Riederer P. Free radicals in Alzheimer's dementia: currently available therapeutic strategies. *J Neural Transm Suppl* 1998; 54: 211-219
7. Sano M., Ernesto C., Thomas R.G., Klauber M.R., Schafer K., Grundman M., A controlled trial of selegiline, alpha-tocopherol, or both as treatment for Alzheimer's disease. *N Eng J Med* 1997; 336: 1216-22
8. Winblad B., Poritis N., Memantine in severe dementia, results of the M-best study (benefit and efficacy in severely demented patients during treatment with memantine). *International Journal of Geriatric Psychiatry* 14[2], 135-146: 1999
9. Senior K., Dosing in phase II trial of Alzheimer's vaccine suspended. *Lancet Neurology* 2002; 1: 3
10. Sigurdsson E.M., Scholtzova H., Mehta P.D., Frangione B., Wisniewski T., Immunization with a nontoxic/nonfibrillar amyloid-β homologous peptide reduces Alzheimer's disease associated pathology in transgenic mice. *Am J Pathology* 2001;159: 439-447
11. Dodart J.C., Bales K.R., Gannon K.S., Immunization reverses memory deficits without reducing brain Aβ burden in Alzheimer's disease model. *Nature Neuroscience* 2002; 5: 452-457
12. Pepys M.B., Herbert J., Hutchinson W.L., Targeted pharmacological depletion of serum amyloid O component for treatment of human amyloidosis. *Nature* 2002; 417: 254-259
13. Tsuzynski M.H., Growth-factor gene therapy for neurodegenerative disorders. *Lancet Neurology* 2002; 1: 51-56
14. Kivipelto M., Helkala E.-L., Laakso M., Midlife vascular risk factors and Alzheimer's disease in later life: longitudinal, population based study. *BMJ* 2001; 322: 1447-51
15. Jick H., Zornberg G.L., Jick S.S., Seshardi S., Drachman D.A., Statins and the risk of dementia. *Lancet* 2000; 356: 1627-1631
16. Rockwood K., Kirkland S., Hogan D.B., Use of lipid-lowering agents, indication bias, and the risk of dementia in community-dwelling elderly people. *Arch Neurol* 2002; 59: 223-227
17. Yaffe K., Barret-Connor E., Lin F., Grady D., Serum lipoprotein levels, statin use, and cognitive function in older women. *Arch Neurol* 2002; 59: 378-384
18. Int´Veldt B.A., Ruitenberg A., Hofman A., Nonsteroidal anti-inflammatory drugs and the risk of Alzheimer's disease. *N Eng J Med* 2001; 345: 1515-21
19. Breitner J.C., Welsh K.A., Helms M.J., Delayed onset of Alzheimer's disease with nonsteroidal anti-inflammatory and histamine H2 blocking drugs. *Neurobiol Aging.* 1995;16(4):523-30.
20. Behl C., Manthey D., Neuroprotective activities of estrogen: an update. *J Neurocytology* 2000; 29: 351-8
21. Seshadri S., Zornberg G.L., Derby L.E., Myers M.W., Jick H., Drachman D.A., Postmenopausal estrogen replacement therapy and the risk of Alzheimer's disease. *Arch Neurol* 2001; 58: 435-440
22. Writing Group for the Women's Health Initiative Investigators. Risks and benefits of estrogen plus progestin in healthy postmenopausal women. *JAMA* 2002; 288: 321-333

"Ana Aslan" International Academy of Aging

First Announcement

The First International Conference of Brain Aging

Bucharest, Romania
October 6-10, 2003

Topics:

Brain Aging and Dementia
Genes and Therapy
New Dementia Therapies
The Receptor Mediated Amyloid Reduction
Cholinesterase Inhibitors Treatment and Long Term Clinical Effects
Imaging and Detection of MCI
Treatment Strategies in Vascular Dementia
Treatment of Behavioral Disturbances
Lipid Lowering Agents: Prevention or Treatment?

Organizing Committee:

Bengt Winblad (Sweden)
Khalid Iqbal (USA)
Luiza Spiru (Romania)

http://www.brainaging.org
office@brainaging.org
aslan@pcnet.ro

http://www.anaaslan.org
http://www.brainaging.org

aslan@pcnet.ro
office@brainaging.org
Gunilla.Johansson@neurotec.ki.se

"Ana Aslan" International Academy of Aging

Welcome to the Academy!

My colleagues and I are delighted to welcome you to membership in "Ana Aslan" International Academy of Aging. You will join people from more than 50 countries all over the world, who are members in this organization, committed to medical science, education, culture and art.

The Academy proves itself to be committed to medicine, especially through creating excellent educational and clinical centers for elderly, in accordance with the agreements of World Health Organization, European Union, International and European Consortium in the field of Ageing.

You will join a society of scientists, which was founded in 2000. We will invite you to all the conferences, symposia, carried out yearly, which cover all medical, social and educational disciplines.

"Brain Aging" International Journal issues are available free of charge this entire year (2002). You may also visit our web site or our on-line journal web site.

We are looking forward to your joining our Academy!

Bengt Winblad **Luiza Spiru**

Long-term Efficacy of Cholinesterase Inhibitors

Serge Gauthier[#]
McGill Centre for Studies in Aging, Quebec, Canada

Abstract

Alzheimer's disease (AD) is a progressive neurodegenerative disorder, and the aim of therapy should be to stabilize symptoms and slow progression over the long term. This review addresses the challenges associated with long-term studies in AD, the importance of continuing treatment over time, and available clinical data on the long-term efficacy of donepezil, rivastigmine and galantamine. The cholinesterase inhibitors have been shown to provide sustained symptomatic benefits in patients with AD, potentially reducing the overall burden of the disease to patients, caregivers and society. Physicians should aim to keep their patients on active treatment over the long term, and should consider switching between the agents in cases of intolerability or failing efficacy. In the absence of direct comparative studies, it is difficult to compare the different cholinesterase inhibitors, but available data suggest that the differential pharmacological properties of these agents may drive important differences in clinical efficacy. For example, rivastigmine, which is a dual inhibitor of acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) may provide broader and more sustained efficacy, and may have a greater potential to delay disease progression, compared with AChE-selective inhibitors. Moreover, the rapidly reversible actions of donepezil and galantamine, associated with upregulation of AChE levels over the long term, could lead to tolerance and loss of benefit. The results of ongoing randomized studies evaluating potential differences between these drugs over a long-term therapeutic perspective will be of great importance.

Introduction

Alzheimer's disease (AD) is characterized by the degeneration of cortical neurons and the presence of amyloid plaques and neurofibrillary tangles[1]. These pathological changes, which begin in the entorhinal cortex, gradually but unremittingly spread across the brain, destroying the hippocampus and neocortex. The progressive loss of cholinergic neurons, and the resulting decline in levels of acetylcholine (ACh), correlates with the cognitive decline in AD[2].

Impaired memory is the clinical hallmark of AD, but attentional, fronto-executive and language functions are also affected[1]. An annual decline in untreated patients on cognitive scales, such as 2–4 points the Mini-Mental State Examination (MMSE)[3,4] or 4–6 points on the Alzheimer's Disease Assessment Scale – cognitive subscale (ADAS-cog)[4,5] may be expected in the mild to moderate stages of AD. Initially, instrumental activities of daily living (ADL) such as leisure and housework are impaired, and there is a progressive loss of basic ADL such as dressing and toileting in later stages[6]. In addition, emotional and neuropsychiatric manifestations are particularly notable in later stages of the disease[7].

The cholinesterase inhibitors donepezil, rivastigmine and galantamine provide the best available symptomatic treatment for patients with AD[8,9]. Each demonstrated symptomatic efficacy in large, 6-month, double-blind, placebo-controlled studies before they were approved by regulatory authorities. However, AD is a progressive neurodegenerative disorder, and the aim of therapy should be to stabilize symptoms and slow progression over the long term (at least 1 year), rather than to achieve short-term, transient symptomatic improvements[5].

It may be possible to differentiate between 'slowing' and 'delaying' disease progression. All cholinesterase inhibitors have demonstrated short-term symptomatic effects which provide actively-treated patients with a higher baseline from which they subsequently decline. This effect results in *delaying* patient decline, as the line is shifted to the right (Figure 1a). However, ideally, agents should also *slow* decline – demonstrated by a widening gap over time between the courses of treated and untreated patients (Figure 1b). The

[#]**Correspondence:** Dr. Serge Gauthier, FRCPC McGill Centre for Studies in Aging
6825 LaSalle Boulevard, Verdun, Quebec, Canada H4H 1R3, Tel: +1 514 766 2010;
Fax: +1 514 888 4050; Email: serge.gauthier@mcgill.ca

latter effect could provide some evidence of disease modification, which would need additional support from surrogate outcomes such as slowing of brain atrophy on serial volumetric measurements using magnetic resonance imaging (MRI)[10]. There are a number of mechanisms that could produce such effects. For example, cholinesterase inhibitors may protect against neuronal degeneration by modifying the formation of amyloidogenic compounds or increasing the production of neuronal growth factors[11].

This paper reviews the challenges associated with long-term studies of cholinesterase inhibitors. The importance of continuing treatment over the long term is discussed, and available clinical data on the long-term efficacy of cholinesterase inhibitors are reviewed for discussion. Tentative comparisons between these agents are made in the absence of direct comparative studies.

The challenge of performing long-term clinical studies

Randomized, double-blind, placebo-controlled studies offer the gold standard in clinical trial design. However, the long-term use of placebo in a progressive condition such as AD is associated with ethical problems[12], and it is difficult to recruit patients to these trials when symptomatic available agents are available. Therefore, most long-term studies of cholinesterase inhibitors have been open-label. However, in the absence of a placebo-group, evaluation of treatment effects presents a challenge.

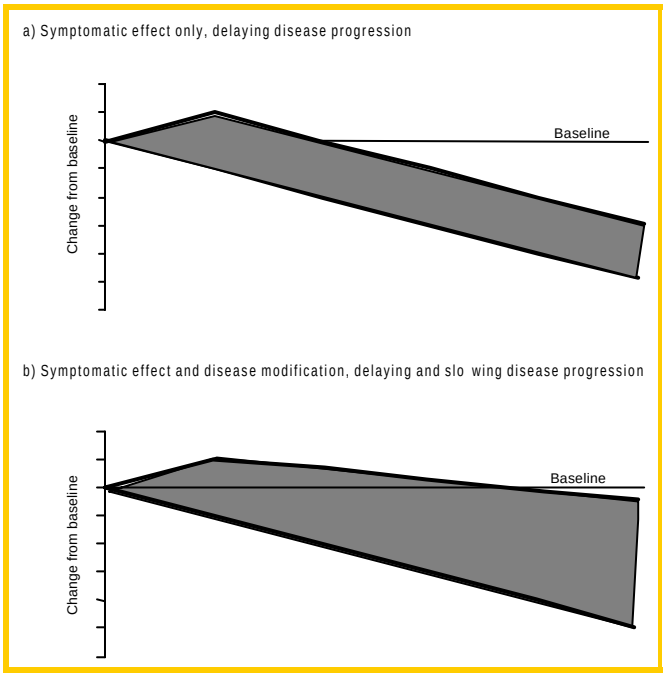


Figure 1. The difference between delaying and slowing disease progression.

In addition, it is important to consider the types of symptoms assessed. Most long-term studies of cholinesterase inhibitors in patients with AD have focussed on cognition, yet the emergence of behavioural problems and decline in ADL may have more impact on prognosis. It is important to collect data on domains other than cognition to ensure that the results are clinically relevant.

Some investigators have used 'delayed-start' designs to evaluate cholinesterase inhibitors over the long term. These are approximated by open-label extensions analysed together with their preceding placebo-controlled studies[13,14]. Patients entered large, randomized trials during which they received placebo or active treatment for 26 weeks. All patients were then eligible to enter open-label studies for a further 26 weeks or more, during which they received recommended doses of the test drugs. Thus, by the end of the full study, some patients had been receiving the test drugs for at least 52 weeks (during the double-blind and open-label phases) while some had only received cholinesterase inhibitors for the open-label phase. Comparisons between curves of patient decline with regard to whether patients receiving placebo for the first 6 months 'catch up' with those receiving active treatment provide interesting data concerning potential effects on disease progression.

Historical data from untreated patients are sometimes used to estimate the long-term benefits of cholinesterase inhibitors. However, it is difficult to match baseline characteristics and demographics of historical placebo and treated groups. Alternatively, long-term efficacy may be evaluated by comparisons with baseline values, although this approach does not consider placebo or other unspecific effects. For example, in one study, ADAS-cog and
t for Dementia (DAD) scores of
antamine 24 mg/day remained near
ths[14], and this was interpreted as
ition and ADL, respectively. No
parators were provided at 12 months.
placebo data were available and they
difference between DAD scores in
acebo groups in that particular study.
ility that the 12-month ADL data,
ear baseline levels, may also have
ver placebo, if a placebo group had

that some kind of comparator is
tations of treatment effects. One
e use of sophisticated statistical
that project the expected natural
lved in drug trials, had they remained
r example, the Stern *et al*[15] multiple
ay be used to calculate projected
og. The use of such formulae means
eristics of treated and 'untreated'
patients are matched, and assumes that no external factors affect outcomes.

Table 1. Factors to consider when using a model to project decline in placebo-treated or untreated AD patients.

Modelling techniques	Specific issues
Disease progression should be projected using a published regression model (e.g. Stern *et al* 1994)	Early withdrawals should be recorded and analysed appropriately
Baseline values of patients involved in the study should be entered into the model, to ensure characteristics of treated and 'untreated' patients are perfectly matched	For long-term AD studies, which aim to slow or delay decline from baseline, OC analyses provide a more conservative estimate than ITT/LOCF analyses
Data validity should be confirmed by comparing the model-predicted decline with placebo decline in a preceding randomized clinical trial	

Table 2. Pharmacological properties of commonly-used cholinesterase inhibitors.

	Donepezil	Rivastigmine	Galantamine
Chemical class	Piperidine	Carbamate	Phenanthrene alkaloid
Cholinesterase inhibition	AChE	AChE and BuChE	AChE
Type of inhibition	Rapidly reversible	Slowly reversible (pseudo-irreversible)	Rapidly reversible
Preferential selectivity for AChE isoforms	None	G1	None
Metabolism route	CYP_{450} isoenzymes	Target enzymes (AChE and BuChE)	CYP_{450} isoenzymes

Finally, when reviewing long-term data from various studies, attention must be paid to the types of analyses that have been performed and how early withdrawals are handled in the analysis (Table 1). When treatment leads to symptomatic improvements, intention-to-treat (ITT) analyses using the last observation carried forward (LOCF) provide conservative estimates of treatment effects. In such cases, premature withdrawals may lead to incomplete drug efficacy, and carrying forward this result will lead to underestimation of the drug effect. In extreme circumstances, had a patient never been treated, carrying forward the baseline value would show no effect of the drug. However, the long-term use of AD drugs is generally not to provide symptomatic improvements, but rather to delay and to slow the natural course of the disease. Carrying forward the last observation would in fact lead to overestimations of the drug effect since the natural course of the disorder is relentless decline. In extreme circumstances in this case, carrying forward the baseline value would incorrectly give the best possible result of no change. Thus, for long-term AD studies, observed case (OC) analyses may be preferable.

Currently-available cholinesterase inhibitors

Four cholinesterase inhibitors are currently available in most countries. Tacrine is no longer widely prescribed due to an association with liver toxicity[16], therefore this paper focuses on donepezil, rivastigmine and galantamine. The suggestion that pharmacological properties of individual cholinesterase inhibitors (Table 2) may drive differential clinical profiles has been discussed extensively. The salient points are summarized here.

Inhibition of AChE and BuChE

All three agents inhibit acetylcholinesterase (AChE), the main enzyme responsible for the hydrolysis of ACh in the normal brain[17]. Rivastigmine also inhibits butyrylcholinesterase (BuChE)[17]. The clinical significance of this pharmacological difference remains hypothetical in the absence of large, randomized, direct comparative studies, but since both AChE and BuChE play important roles in both the normal and Alzheimer brain[18], it has been proposed that dual cholinesterase inhibition may provide greater, broader

and more sustained effects[17,19-21]. In addition, it has been suggested that the elevations in cerebrospinal fluid (CSF) activities of AChE seen following long-term treatment with the rapidly reversible inhibitory agents donepezil and galantamine[22] may be due to upregulation of AChE gene expression that may eventually lead to tolerance[21] (Figure 2). However, upregulation is not seen with slowly reversible (pseudo-irreversible) inhibitors such as rivastigmine[21-23].

Inhibition of both AChE and BuChE may also slow the formation of amyloidogenic compounds[19-21]. Animal studies have shown that amyloid precursor protein (APP) processing is modulated by cholinergic activity[24]. Increased ACh stimulates the production of soluble APP, probably through the ?-secretase-mediated pathway via the protein kinase C cascade as a result of muscarinic ACh receptor activation[25]. In humans, BuChE appears to participate in the transformation of inert plaques into malignant ones associated with neuronal degeneration and dementia[26], and amyloid plaque density increases proportionally with BuChE activity in cortical areas of the AD brain[27]. AChE has also been shown *in vitro* and *in vivo* to accelerate the formation of insoluble, neurotoxic β-amyloid[28,29]. This could have important implications for agents that upregulate AChE gene expression, as higher levels of the enzyme could potentially accelerate plaque formation.

Selectivity for different isoforms

AChE exists in three globular forms – G1, G2 and G4. In normal subjects, G1 is present in the brain, G2 is present in the skeletal muscle, and G4 is present in the brain and in the neuromuscular endplate[17]. All three isoforms are found in the heart, although G4 activity appears to be predominant[30]. In the AD brain, G1 activities progressively increase while G4 activities decline[31]. Therefore, an affinity for G1 may contribute to targeted actions in the brain and sustained efficacy over the course of AD[21], while a lower affinity for G2 or G4 may lead to improved safety over the course of AD as predominant forms of the enzyme in the periphery remain unaffected[32]. Moreover, since the G1 forms of both AChE and BuChE are associated with amyloid plaques and neurofibrillary tangles[27], an affinity for G1 may lead to a greater potential for disease modification[21].

Therefore, pharmacological differences may influence the clinical profiles of cholinesterase inhibitors, potentially leading to important implications with regard to the different agents' breadth and duration of efficacy over the course of AD, their potentials for slowing disease progression, and long-term tolerability and safety[21,32].

Importance of continuing treatment

The aim of long-term AD treatment is to slow disease progression. As such, patients, caregivers and physicians will still see some clinical decline on cholinesterase inhibitors after a period of stabilization, but this could be slower and later than that which would be expected if the patients were untreated. Therefore, expectations of treatment must be realistic (e.g. caregivers should not expect increasing improvements over the long-term) to ensure that individuals make the effort to comply with beneficial treatment regimens and that they do not simply give up on treatment when they think it is not working. Continued treatment is very important because, following the interruption of some cholinesterase inhibitor therapy, the

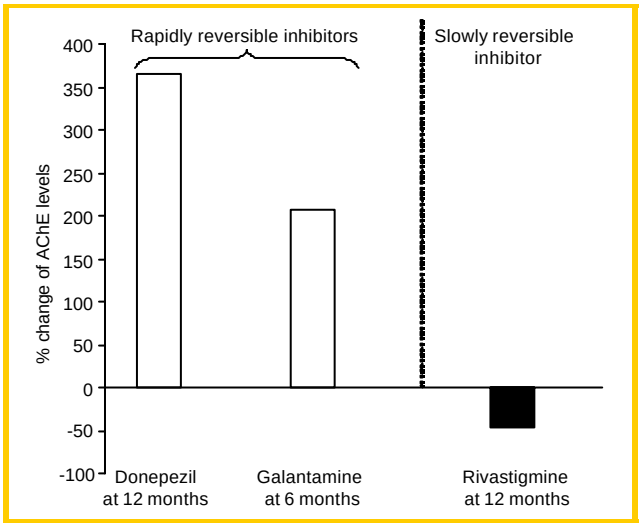


Figure 2. Rapidly reversible inhibition leads to elevations in CSF activities of AChE following 12 and 6 months of treatment with donepezil and galantamine, respectively, while slowly reversible inhibition does not increase levels of this target enzyme after 12 months[22,23].

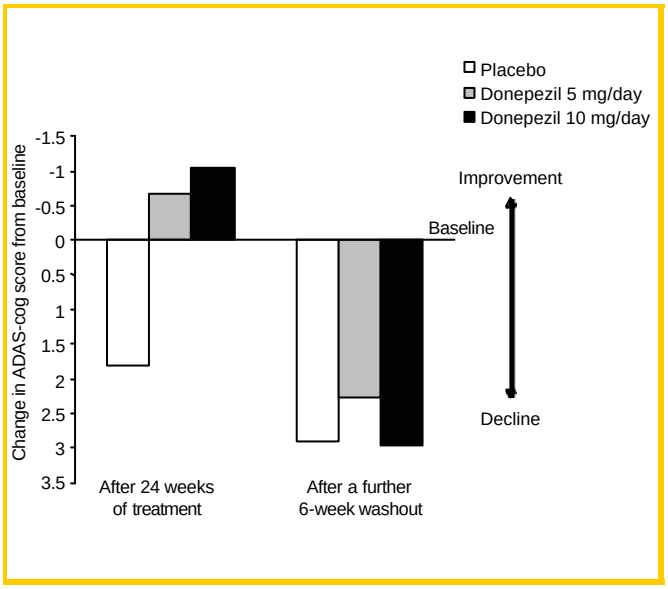


Figure 3. Benefits of 24 weeks of previous treatment may be lost within 6 weeks if treatment is interrupted[33].

benefits of previous treatment on cognition or global function may be completely lost within 6 weeks[33] (Figure 3).

The loss of benefits following the withdrawal of donepezil, which is sometimes referred to as a 'crash', may reflect underlying mechanisms similar to those observed following benzodiazepine withdrawal. Tolerance develops rapidly to benzodiazepines and, following cessation of treatment, withdrawal symptoms are frequently observed[34]. Similarly, in patients receiving donepezil, the upregulation of AChE levels following long-term treatment may lead to high activities of the enzyme in the brain that may be maintained while treatment is continued but, if treatment were to be stopped, this now high level of AChE would not be inhibited. This may lead to a precipitous lowering of ACh and a considerable worsening of the patient's symptoms.

Guidelines produced by the UK National Institute of Clinical Excellence (NICE)[8] recommended that cholinesterase inhibitor treatment should be withdrawn in severely demented patients with MMSE scores below 12. This was based on the fact that, at the time of their assessment, only data on patients with mild to moderate AD were available. However, there is evidence that the effect of rivastigmine may actually be stronger in patients with more severe disease – improvements in ADAS-cog scores of 4.9 points were reported in patients with mild to moderate AD[35], compared with improvements of 8.1 points in a subset of patients with moderately severe AD[36]. Similarly, less decline has been reported in ADL in patients with more severe AD receiving rivastigmine[37]. Donepezil has also demonstrated significant benefits over placebo in a 6-month double-blind study involving patients with moderate to severe AD (baseline standardized [s]MMSE scores 5–17) on the sMMSE, the Severe Impairment Battery, the DAD and the NeuroPsychiatric Inventory (NPI)[38]. In an exploratory analysis of this study, the significant differences between donepezil treatment and placebo were seen to arise in moderately ill patients (MMSE 10–17), whilst in patients with severe AD (MMSE 5–9), despite positive trends in favour of donepezil there were no significant differences between treatment groups on any of the study assessments at week 24 (LOCF results)[39]. Moreover, in another double-blind, placebo-controlled study, donepezil was not shown to be effective in nursing home patients[40]. Therefore, further research is required to ascertain the effects of cholinesterase inhibitors before the guidelines are revised for use in severe patients.

Nevertheless, continued use is further justified by a recent observational study that demonstrated that cholinesterase inhibitor (tacrine, donepezil or rivastigmine) use delayed admission to a nursing home (34% fewer patients over 1 year), although time to death was unaffected[3]. The fact that these agents delay time to institutionalization but do not affect physical survival means that patients are able to maintain relatively normal ADL for a longer period of time, but they do so without significantly prolonging life[3].

However, the average duration of treatment is not optimal in all cases. For example, it was recently reported that at least 25% of 163 patients taking part in an audit study in the USA withdrew from donepezil within the first year of treatment[41], while an audit of 113 AD patients in the UK showed that 34% withdrew from donepezil treatment within 3 months[42]. These data are supported by an ongoing persistency and compliance study, which indicates that about 30% of patients starting donepezil or rivastigmine treatment in 2000 remained on treatment a year later, less than 20% were on treatment at 18 months, and about 15% were on treatment at 21 months[43]. Treatment withdrawal may be due to disappointing efficacy or poor tolerability of the initial treatment, as well as secondary efficacy failure or adverse effects emerging during the maintenance phase. In such cases, pharmacological differences between available cholinesterase inhibitors provide a good rationale to switch to another drug in the same class[44].

Two studies[45,46] have indicated that a substantial proportion of patients who fail to tolerate or are perceived as failing to benefit from treatment with donepezil may experience improvement in their symptoms after being switched to rivastigmine. In both studies, about half of those switching due to lack of efficacy with donepezil improved when receiving rivastigmine, and up to two thirds of those suffering from intolerable side effects with donepezil benefited from the switch to rivastigmine. Moreover, the experience of several studies suggests that an immediate (overnight) switch from patients who have received a suitable trial of donepezil treatment to rivastigmine is well tolerated and is not associated with safety or tolerability problems[44,47].

Clinical evidence for the benefits of switching to galantamine comes from a *post hoc* analysis of a 5-month clinical trial, for which outcomes were evaluated according to previous exposure to cholinesterase inhibitors (following at least 90 days' washout). Patients with or without prior anticholinesterase therapy had similar efficacy outcomes following 5 months' treatment with galantamine, indicating that discontinuation of prior cholinesterase inhibitors did not affect the efficacy or tolerability of galantamine[48], i.e. patients who presumably could not tolerate or did not respond to another cholinesterase inhibitor could still draw benefit from galantamine.

There are no published reports of patients switching from rivastigmine or galantamine to donepezil. However, on the basis of available data, it appears that patients not tolerating or not responding to one particular cholinesterase inhibitor may still draw benefits upon switching to another.

Long-term efficacy of cholinesterase inhibitors

In the absence of direct comparative studies, it is difficult to compare the efficacy profiles of individual cholinesterase inhibitors. However, currently-available data on the long-term efficacy of these agents appear to support the hypothesis that pharmacological differences may influence their clinical profiles. Most importantly, there is evidence that the agents may have different potentials to modify the underlying disease process and slow disease progression.

Donepezil

Donepezil is a rapidly reversible AChE-selective inhibitor. As mentioned previously, rapidly reversible inhibition leads to elevations in CSF activities of AChE, possibly due to upregulation of the AChE gene expression. Increases in AChE levels of 366% have been seen in the CSF of AD patients after 12 months of donepezil treatment[22] (Figure 2).

In a placebo-controlled, 52-week study in 286 patients with mild to moderate AD (mean baseline MMSE 19.3), donepezil 5–10 mg/day provided initial symptomatic benefits that appeared to wane over time[49]. Efficacy analyses were performed on the LOCF population. The shortcomings of such analyses are discussed in a previous section of this review – such techniques may overestimate the effects of long-term treatment. Donepezil provided benefits in cognitive performance, as demonstrated by initial improvements in MMSE scores which stayed above baseline until the 36-week time-point, following which cognition steadily declined, roughly in parallel with the placebo group. The primary outcome measure in this study, the Gottfries-Bråne-Steen (GBS) scale, was statistically significantly different to placebo at weeks 24, 36 and 52, but did not reach statistical significance at the study endpoint. Less deterioration was observed in ADL, as assessed using the Progressive Deterioration Scale (PDS) in the donepezil group compared with the placebo group at week 52 (Figure 4)[49]. No significant differences in favour of donepezil were observed on behavioural symptoms (using the GBS behaviour scale or the NPI) at any time point.

In another placebo-controlled, 52-week survival study involving 431 patients with moderate AD (mean baseline MMSE 17.1), most benefits of donepezil were observed early on in the study and appeared to fade over time[50]. Total scores on the MMSE, the AD Functional Assessment and Change Scale (ADFACS; baseline scores not provided) and the Clinical Dementia Rating – Sum of Boxes (CDR-SB; mean baseline score 6.8 points) were superior to placebo in the LOCF analysis at study endpoint for all patients. Donepezil 5–10 mg/day delayed the median time to clinically evident decline by 5 months, and patients receiving donepezil were 38% less likely than those receiving placebo to deteriorate over the 1-year study period[50].

The effects of long-term donepezil treatment on behaviour were further assessed in an open-label study involving 25 patients with probable or possible AD[51]. Donepezil (up to 10 mg/day) transiently improved behavioural and psychological symptoms at 3–4 months (especially depression and behavioural dysregulation), as assessed using the CERAD Behavior Rating Scale for Dementia (CBRSD) scale. However, CBRSD scores faded over time and had returned to baseline values by week 52, when they were little different from a historical reference group that had received no cholinesterase inhibitor treatment[51].

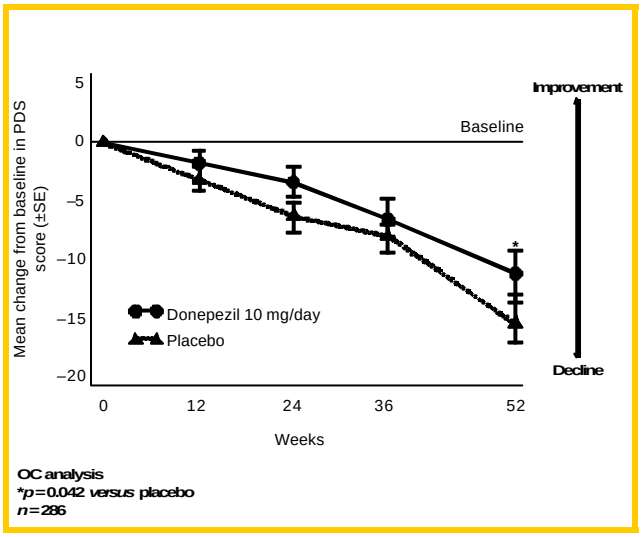


Figure 4. Effects of donepezil on ADL, as assessed using the PDS, over 52 weeks[49].

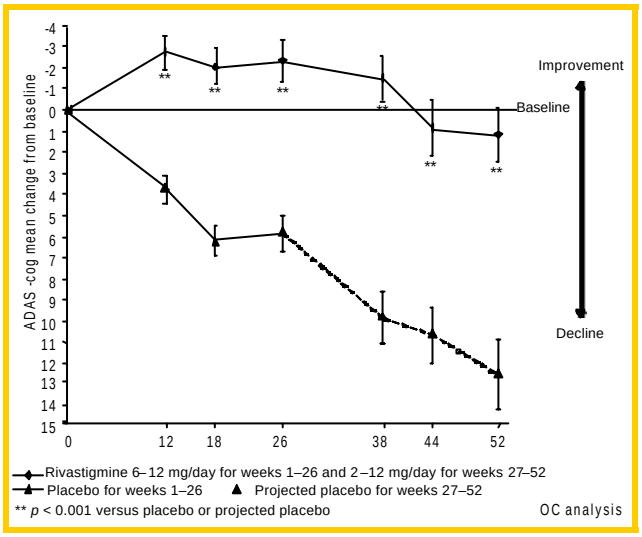


Figure 5. Mean change from baseline on the ADAS-cog in moderately severe AD patients (n = 158) receiving rivastigmine or placebo for 12 months (bars show standard error values)[36].

The results of a 144-week open-label extension of two US short-term, double-blind, placebo-controlled studies have been reported by Doody *et al*[52]. A total of 763 patients with mild to moderate AD (mean baseline MMSE 19.4) were enrolled from an original 15-week study comprising 12 weeks of treatment followed by a 3-week washout and a 30-week study comprising 24 weeks of treatment followed by a 6-week washout. For the open-label analysis, mean total scores for observed cases at each visit were used to calculate the mean changes from baseline scores, providing a conservative estimate of the drug's efficacy. The number of patients completing the 144-week extension study was reported to have halved over the first 108 weeks[52]. Donepezil 5–10 mg/day treatment resulted in improved ADAS-cog scores relative to the new baseline for 24 weeks, following which scores declined below baseline[52]. CDR-SB scores also remained improved compared with baseline scores for 24 weeks[52]. Scores declined progressively for the rest of the study (144 weeks). By the end of the study, subgroups of patients who had received placebo in the original two studies (and then donepezil during the open-label extension) had declined by 13–18 points on the ADAS-cog, while those who had originally received donepezil declined by approximately 10–12 points, respectively[52]. These declines in both groups are within the range expected in untreated patients – i.e., 4–6 points per year[5]. No data modelling was attempted and no comparison was made with historical findings from untreated patients.

Rogers *et al*[53] reported results of an open-label study that followed patients treated with donepezil for up to 4.9 years. This open-label study was an extension of a 14-week double-blind, placebo-controlled study, which involved 161 patients with mild to moderate AD (mean baseline MMSE 18.6). Of these, 133 patients enrolled in the open-label extension study, and the population fell to 18 patients by the time the study was terminated at week 254[53]. Therefore, the very long-term data were based only on a small number of patients and should be interpreted with caution. Improvements on the ADAS-cog relative to baseline were described until week 38, but by week 50 a gradual decline relative to baseline was observed. Rogers & Friedhoff[54] reported on an interim LOCF analysis of this study, showing that at week 98 (84 weeks of open-label treatment), ADAS-cog scores had deteriorated by 7 points compared with baseline. In the final publication of this study[53], a more appropriate OC type of analysis was used and the deterioration now seen in the ADAS-cog at week 98 was higher than previously reported by Rogers & Friedhoff[54] –about 11 points after two years. That is, some 4 points more than that shown by the earlier LOCF analysis. Cognitive deterioration over the full study period was fairly constant, with a mean annual decline of 6.07 points on the ADAS-cog[53]. Since untreated patients may be expected to decline by 4–6 points each year on the ADAS-cog[5], this suggests that, following initial symptomatic improvements, disease progression was unaffected (as in Figure 1a). Similarly, MMSE and CDR-SB scores remained close to baseline values until week 26, following which patients declined at a mean rate of 1.4 and 2.8 points per year, respectively.

Rivastigmine

Rivastigmine has been shown to inhibit both AChE and BuChE activities in the CSF of AD patients by up to 45% and 58%, respectively, after 12 months of treatment[23]. Further analyses demonstrated statistically significant correlations between improvement in cognitive scores and the inhibition of both AChE and BuChE activities[23], suggesting that rivastigmine provides clinically-significant inhibition of central enzymes for at least 12 months in AD patients. Evidence for the clinical benefits of this sustained inhibition come from a number of long-term studies involving patients with mild to moderate AD, moderately severe AD, Lewy body dementia or vascular dementia.

Farlow *et al*[13] reported the results of a 52-week 'delayed-start' study involving mild to moderate AD patients (n = 699; mean baseline MMSE 19.7). For the first 26 weeks, patients received placebo or rivastigmine 1–4 or 6–12 mg/day. All patients were then eligible to enter an open-label study (n = 533) for a further 26 weeks, during which they received individually optimized rivastigmine at doses of 2–12 mg/day. There was a significant treatment difference of 5.7 points with rivastigmine on the ADAS-cog at 52 weeks ($p < 0.001$; OC analysis), compared with the projected rate of placebo decline (calculated using a statistical model). The treatment difference at 52 weeks was greater than that observed at 26 weeks (5.7 *versus* 4.9 points, respectively)[13,35], indicating that cognitive decline was slower in patients who were on rivastigmine for 52 weeks. Furthermore, these patients who were on rivastigmine for the entire 52 weeks had a slightly better outcome than those who received placebo for the first 26 weeks and were then switched to active drug (1.4-point difference on the ADAS-cog).

Interestingly, a subgroup of 158 patients with moderately severe AD (baseline MMSE 16.3 points, Global Deterioration Scale [GDS] score $\geq$ 5) in this study showed an even more robust pattern of response[36]. Significant benefits on the ADAS-cog, both in terms of symptomatic benefits at the start of the study, and apparent slowing of disease progression (widening difference over time compared with placebo) were evident in patients receiving 1–4 or 6–12 mg/day over the first 26 weeks of the study. At 52 weeks, there was a striking improvement on the ADAS-cog of 8.1 points over the projected placebo decline in the patients who had received rivastigmine 6–12 mg/day during the first 26 weeks (Figure 5), and 4.6 points over the projected placebo decline in the lower dose group (OC analyses)[36]. Projected placebo declines at weeks 38, 44 and 52 were calculated using a modelling procedure based on

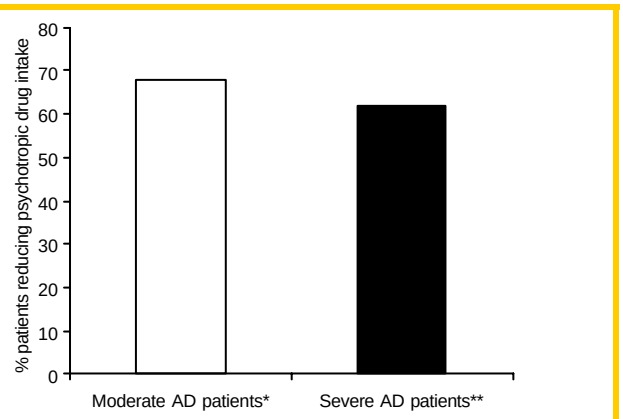
ADAS-cog scores in the original placebo group at baseline, weeks 12, 18 and 26. The increasing treatment effects over time may suggest that rivastigmine was slowing disease progression. Similarly, significant benefits on the ADAS-cog were seen in patients with milder disease (GDS score < 5). The OC analyses showed improvements over projected placebo decline of 3.7 points on the ADAS-cog in the higher dose rivastigmine group and 3.1 points in the lower dose group at 52 weeks[36].

The beneficial effects of rivastigmine on cognition persisted for at least 2 years, as shown by a meta-analysis of 2010 AD patients (mean baseline MMSE 19.4) taking part in four 26-week, placebo-controlled studies that were followed by open-label extension studies[55] (including the study reported by Farlow *et al*[13] above). Predictions of placebo decline beyond the first 26 weeks were based upon baseline-dependent mathematical models[15]. The validity of these modelled data were confirmed by a high concordance between actual ADAS-cog values at 26 weeks in patients receiving placebo in the placebo-controlled studies and the predicted values of these patients[55]. Over 2 years, rivastigmine-treated patients showed significantly less cognitive decline compared with projected placebo-treated patients[55]. OC analyses showed that mean ADAS-cog scores after 1 or 2 years declined by 4 to 5 points less than predicted, had patients been left untreated.

Further evidence of the effects of rivastigmine on the rate of cognitive decline in patients with mild to moderate AD (mean baseline MMSE 19.8) comes from a 142-week analysis, which demonstrated that the average annual rate of decline of 4.5 points on the ADAS-cog in patients receiving at least 6 mg/day of rivastigmine was about half that of annual decline of 8.2 points in patients receiving less than 6 mg/day[56].

Long-term benefits of rivastigmine in patients with mild-to-moderate or severe AD are also seen in behavioural domains over a 2-year treatment period. Behavioural symptoms of a group of mild-to-moderate AD patients (n = 98, mean baseline MMSE 19.0) were evaluated using the behavioural component of the Clinicians Interview Based Impression of Change – plus caregiver input (CIBIC-plus), which is adapted from the Behavioural Pathology in Alzheimer's Disease Rating Scale (BEHAVE-AD). After 26 weeks, CIBIC-plus behaviour scores in patients receiving rivastigmine 6–12 mg/day improved, while those in patients receiving placebo showed no improvement[57]. This difference was statistically significant (p = 0.02). At 52 weeks, additional improvements were observed in patients who had received rivastigmine since the start of the study, and at week 104 improvements in this group were still rated as mild to moderate[57]. In contrast, patients who had received placebo during the first 26 weeks (double-blind phase) of the study showed only mild improvements at week 52 and no benefits at week 104[57]. Throughout the 104 weeks, behavioural symptoms were significantly better in the patients who had received rivastigmine from the beginning of the study, compared with those who received placebo for the first 26 weeks ($p \leq 0.05$ at all time points). These data suggest that patients may benefit in the behavioural domain from early treatment, as this may have an impact on disease progression and lead to longer-term benefits. Rivastigmine appeared to provide particular improvements in mood disorders and hallucinations[57]. However, given the small number of patients in this study, replication will be needed in a larger cohort of patients.

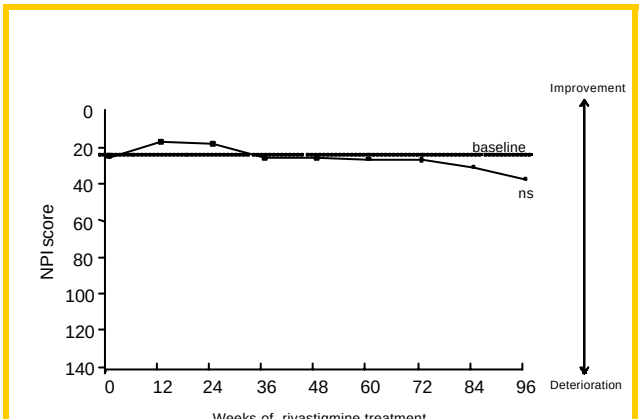
In an open study, 173 patients with moderate to severe AD (mean baseline MMSE 9.3) who were living in a nursing home received rivastigmine for 52 weeks[58].



*Moderate AD patients: MMSE > 10, n = 51
**Severe AD patients: MMSE ≤ 10, n = 54

(Data are based on a subset of patients who were taking psychotropic medications at baseline, from a total study population of 173 moderate to severe AD patients)

Figure 6. Percentages of nursing home patients reducing their psychotropic drug intake over a 52-week rivastigmine study[59].



ns: not statistically significantly different to baseline

On a scale of 0–144, higher scores indicate worse behavioural problems

Figure 7. Long-term efficacy of rivastigmine in patients with the Lewy body variant of AD (n = 120). NPI scores were sustained at baseline values for at least 96 weeks[61].

Overall, 57% of patients demonstrated improvements on the NPI–Nursing Home version (NPI-NH), and a mean improvement of 1.6 points was observed (OC analysis). The majority of patients in the study (n = 105) were receiving psychotropic medications at baseline and antipsychotic use decreased in both the severe and moderate groups by 62% and 68%, respectively, over the 52 weeks of rivastigmine treatment (Figure 6)[59]. More patients with moderate AD (47%) were able to discontinue psychotropic use completely, compared with patients with severe AD (28%), due to greater reductions in anxiolytics and hypnotic sedatives. NPI-NH mean changes from baseline over 12 months in the moderate and severe AD groups were –0.2 and –0.3, respectively.

In addition to providing meaningful long-term benefits in patients with mild, moderate or severe AD, rivastigmine has demonstrated significant long-term efficacy in patients with other forms of dementia. In a double-blind, placebo-controlled multicentre study, rivastigmine provided benefits in cognition, attention, apathy, indifference, anxiety, delusions, hallucinations and aberrant motor behaviour in 120 patients with the Lewy body variant of AD (mean baseline MMSE 18.6)[60] for 20 weeks. From this study, Grace *et al*[61] followed 29 patients receiving open-label rivastigmine for up to 96 weeks. By 96 weeks, neither MMSE nor NPI scores were significantly worse than at baseline – that is, there was no detectable deterioration in cognition or behaviour while patients were receiving rivastigmine (Figure 7). The authors explained that this finding was highly significant because the Lewy body variant of AD is a progressive illness and a substantial reduction in MMSE and NPI scores would have been expected in untreated patients[61].

Rivastigmine has also demonstrated significant benefits in cognitive outcomes over 1 year in a large cohort of patients with AD (mean baseline MMSE 19.6), with or without vascular risk factors, taking part in a double-blind, placebo-controlled study that was part of the drug development (ADENA) programme. At 26 weeks, benefits were even greater in those with vascular risk factors than in those with 'pure AD'[62], possibly indicating an effect on certain aspects of vascular dementia in addition to its known effects on Alzheimer's pathology. All patients were then given the opportunity to enter an open-label study during which they all received rivastigmine 2–12 mg/day, and 178 patients with vascular risk factors were followed for a further 26 weeks[63]. The OC analyses demonstrated that the cognitive benefits of rivastigmine were maintained in these patients for at least 52 weeks. Patients who were originally treated with placebo during the double-blind phase did not achieve the same ADAS-cog scores as those that had received rivastigmine for the full 52 weeks[63]. Since a substantial proportion of AD patients have underlying vascular pathology, a therapy that is effective in treating patients with AD and those with vascular risks would be of considerable benefit.

The suggestion that rivastigmine may affect vascular pathologies has been supported by a small 22-month study in which patients with subcortical VaD receiving rivastigmine 6 mg/day showed significant improvements compared with a control group receiving cardioaspirin 100 mg/day in executive function, behaviour (Figure 8) and depression – the core features of subcortical VaD that reflect frontal lobe dysfunction[64,65]. These improvements were reflected in significantly reduced caregiver stress over 22 months.

Galantamine

Like donepezil, galantamine is a rapidly reversible AChE-selective inhibitor. Galantamine has been shown to increase AChE levels by 209% in the CSF of AD patients after 6 months of therapy (Figure 2)[22]. Studies evaluating longer-term direct effects of galantamine on central enzyme activities do not appear to have been performed. It has been proposed that galantamine may have a unique pharmacological profile via the allosteric modulation at the presynaptic nAChR, and that this may result in an increased release of ACh, while at the postsynaptic nAChR allosteric binding may increase the frequency of opening of the receptor ion channel and potentiate the ACh-activated electrical currents[66,67]. In fact, clinically-relevant concentrations of tacrine, donepezil and galantamine have all been shown to interact *in vitro* with the allosteric activator site on the nAChR[68]. However, the modulatory effects of cholinesterase inhibitors on nicotinic receptors have, to date, been demonstrated mostly *in vitro* in a cellular system, and there is limited evidence that central activation of nAChR activity translates into increased cognitive performance *in vivo*. Therefore, the clinical relevance of these effects on nAChR is uncertain.

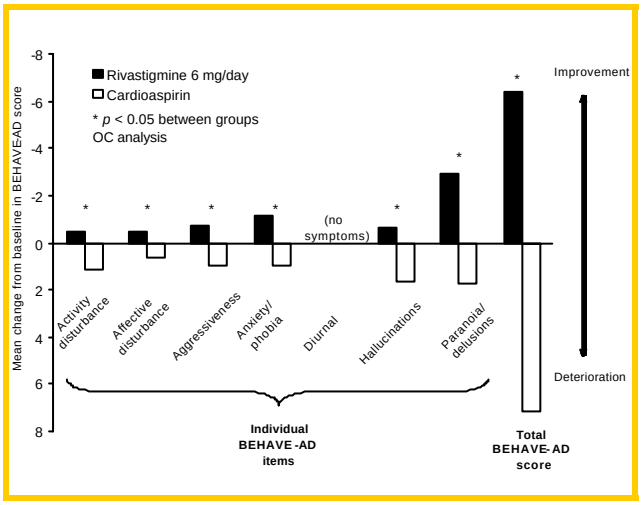


Figure 8. Benefits of rivastigmine on the behaviour of patients with subcortical VaD (n = 16). Changes from baseline in BEHAVE-AD scores (individual items and total score) in patients receiving rivastigmine or cardioaspirin for 22 months[64].

Raskind *et al*[14] reported the results of an open-label extension (to 52 weeks) of a 26-week double-blind, placebo-controlled study of galantamine. Of 636 patients with mild-to-moderate AD (mean baseline MMSE 19.3) entering the double-blind study, 353 entered the open-label phase. Patients received placebo or galantamine 24 or 32 mg/day during the double-blind phase, and all patients received galantamine 24 mg/day during the open-label phase. All treatment groups, including the placebo group, showed initial improvements on the ADAS-cog. By 26 weeks (the end of the double-blind phase), both galantamine-treated groups showed significant benefits over placebo, according to both the OC and ITT/LOCF analyses (all $p < 0.001$ *versus* placebo). However, by 52 weeks, ADAS-cog scores in the two groups of patients who had received placebo or galantamine 32 mg/day in the first 26 weeks (during the double-blind phase) had deteriorated by about 2 points below baseline (Figure 9). Only the group that had received galantamine 24 mg/day for the full study period maintained ADAS-cog scores near baseline[14]. A similar pattern of response was seen in ADL as assessed using the Disability Assessment for Dementia (DAD) scale[14].

Raskind & Truyen[69] presented a 36-month follow-up of these data. They showed that after this extended period, about 18% of patients remaining in the study maintained cognitive function near baseline levels. However, only about half of the patients entering this extension study completed the 36-month treatment period. Therefore, this responder analysis should be interpreted with caution since the fact that many patients not benefiting from treatment may have withdrawn before this time may have skewed the results in favour of a high responder rate in the remaining population. Mean ADAS-cog scores were not presented.

Morris *et al*[70] presented 18.5-month data from another open-label extension of a 5-month galantamine study[71] in which patients received placebo or galantamine 8, 16 or 24 mg/day for 5 months (n = 978, mean baseline MMSE 17.8). Morris *et al*[70] described the results of an OC analysis of 333 patients from the original placebo, 16 mg/day or 24 mg/day groups after they had been switched to galantamine 24 mg/day for the open-label extension (data from the original 8 mg/day group were not shown). All three groups achieved similar scores on the ADAS-cog within 4.5 months of switching (Figure 10). They then all declined below baseline, almost in parallel, until the end of the study.

Finally, Erkinjuntti *et al*[72] presented data on the long-term (1-year) efficacy of galantamine in patients with probable VaD (mean baseline MMSE 20.6). At the end of a 6-month double-blind study with patients diagnosed with probable VaD or AD combined with cerebrovascular disease, significant differences between galantamine 24 mg/day and placebo were reported on the ADAS-cog, DAD and NPI[73]. The probable VaD subgroup included 252 patients in both the placebo or galantamine 24 mg/day treatment arms, both demonstrating improvements over baseline on the ADAS-cog. Galantamine failed to provide significant benefits over placebo at 6 months in that subgroup. This study was followed by a 6-month open-label study involving 195 of the original 252 probable VaD patients, all of whom received galantamine 24 mg/day[72]. At the end of the study, ADAS-cog scores in both groups (ie, patients from the initial double-blind study who received galantamine and continued to do so, as well as patients who initially received placebo and then switched to galantamine) were still above baseline and there was still no statistically significant difference between them. Neither group declined below baseline at any stage of the study, probably because the entry criteria stated that patients had to have stable disease, and therefore worsening of symptoms caused by vascular pathologies was less likely.

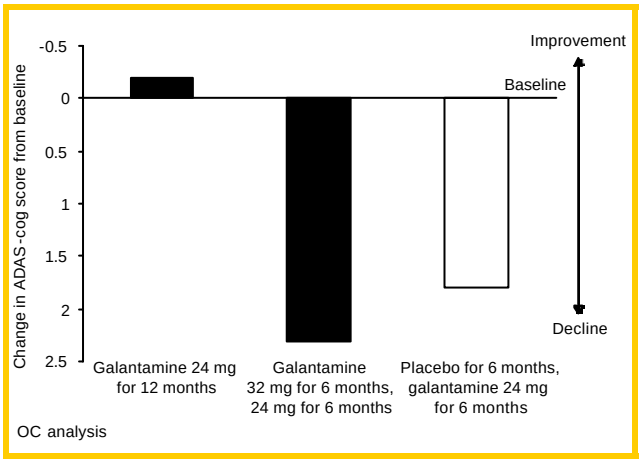


Figure 9. Changes from baseline on the ADAS-cog during a 52-week galantamine study. During the first 26 weeks, patients received placebo, galantamine 24 mg/day or galantamine 32 mg/day. This double-blind phase was followed by an open-label, 26-week phase during which all patients received galantamine 24 mg/day[14].

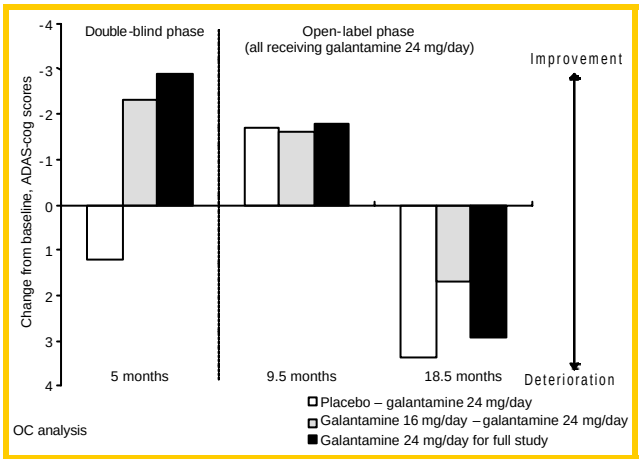


Figure 10. Long-term efficacy of galantamine in patients with mild to moderate AD (n = 333). ADAS-cog scores in placebo-treated patients caught up with those of actively treated patients during the open-label phase, following which all groups demonstrated similar rates of decline[70].

Long-term study withdrawals

In long-term studies, rates of withdrawal were fairly similar in studies evaluating donepezil, rivastigmine and galantamine. In patients with AD, 1-year completion rates for donepezil studies ranged from 33–92% of patients[49,50,52,53], those for rivastigmine were in the region of 70–80%[13,55,58], and that for galantamine was 42%[14]. Later, 2-year completion rates in AD studies appeared to be approximately 35–50% for donepezil[53] and 55% for rivastigmine[55]. In addition, 2-year data from studies in other dementia types indicated 70% completion rates for rivastigmine in patients with the Lewy body variant of AD[61] and 100% completion rates for rivastigmine in a small study of patients with VaD[64]. Finally, 3-year completion rates in AD studies were reported to be about 21% for donepezil[53] and 23% for galantamine[69].

However, these completion rates are considerably higher than those reported in audit studies of clinical practice in the USA and UK[41,42], and do not necessarily reflect the length of treatment duration in the real world. In clinical practice, physicians should be aiming for high rates of patient persistence. If this is not possible with the initial drug, due to a perceived lack of efficacy or problems with tolerability over the long term, pharmacological differences between available cholinesterase inhibitors provide a good rationale to switch to another drug in the same class[44].

Discussion

In the absence of direct comparative studies, it is difficult to compare data on the long-term efficacy of cholinesterase inhibitors. However, tentative comparisons may be made, which suggest that the pharmacological properties of these drugs may be driving important differences in clinical efficacy and safety.

Primarily, donepezil appears to exhibit modest, consistent efficacy on cognition over the long term. Long-term data for donepezil are only available in patients with mild-to-moderate AD. In studies performed to date, following the maintenance of cognition above baseline levels for about 6 months or more, patients apparently decline at the same rate as if they had been left untreated[49,50,52-54]. This is an example of 'delaying' rather than 'slowing' disease progression. Galantamine also clearly provides initial symptomatic benefits[14]. Published data suggest that once galantamine-treated patients begin to decline, they do so at a rate similar to that of untreated patients[4].

Studies of rivastigmine have shown evidence suggestive of slowing disease progression. In studies involving patients with mild, moderate or moderately severe AD[13,36,55], the projected placebo and rivastigmine lines of response diverged (increasing treatment differences) over time, suggesting that rivastigmine was slowing, rather than just delaying, clinical decline. Moreover, in a delayed-start design study, the group of patients who initially received placebo did not 'catch up' with the group that had received rivastigmine for the entire study[13,36,57], indicating that early rivastigmine treatment may have a disease modifying effect. Rivastigmine has shown both cognitive and behavioural long-term benefits in patients with mild, moderate or severe AD, the Lewy body variant of AD and subcortical VaD[36,57,58,61,64,64]. In patients with AD, rivastigmine was reported to provide particular benefits in mood disorders and hallucinations[57]. In the VaD study, statistically significant benefits were observed on all items of the BEHAVE-AD, demonstrating very broad effects[64]. The behavioural benefits of rivastigmine are associated with reduced antipsychotic and other psychotropic use[58] that should have favourable cost and safety implications.

These apparent benefits may reflect the pharmacological properties of the three agents. To begin with, since rivastigmine inhibits both AChE and BuChE, the agent is likely to remain pharmacologically effective over the long term, as changes in the brains of AD patients lead to rising levels of BuChE and declining AChE activities as the disease progresses[27,74]. AChE-selective agents may be rendered less effective when their target enzyme has a more limited role to play. Secondly, as a slowly reversible AChE inhibitor, rivastigmine is not associated with upregulation of AChE gene expression, which may lead to tolerance with rapidly reversible inhibitors such as donepezil and galantamine[21], and since AChE may accelerate the formation of β-amyloid, upregulation may also accelerate disease progression[28,29]. In addition, the G1 isoforms of AChE and BuChE are associated with compact neurotoxic plaques, and BuChE in particular may play an important role in the development of malignant plaques associated with degeneration and dementia. Therefore, as AD advances, the rationale for using rivastigmine becomes more compelling.

This is further endorsed by the effects of rivastigmine on behavioural symptoms, which tend to develop at more advanced stages of AD. Neither donepezil nor galantamine have yet demonstrated behavioural benefits over the long term. Again, this may reflect rivastigmine's dual inhibitory properties, since BuChE activities, which are inhibited only by rivastigmine, are particularly high in the hippocampus and amygdala[75], and the drug's preferential selectivity for the G1 form of AChE may lead to targeted actions in the frontal lobe[21]. Interestingly, the efficacy of rivastigmine on behavioural symptoms have been most markedly demonstrated in dementias with more of a subcortical frontal involvement, such as the Lewy body variant of AD, Parkinson's disease dementia and subcortical VaD[60,61,64,65,76]. However, more data are required to clarify the relative effectiveness of cholinesterase inhibitor treatments in the long-term management of behavioural symptoms in dementia.

Furthermore, as mentioned earlier, agents that inhibit both AChE and BuChE may have a greater potential than AChE-selective inhibitors for disease modification, through preventing inert plaques transforming into malignant ones associated with degeneration and dementia. It may not be surprising, therefore, that of the three available agents, only rivastigmine has shown evidence of possible slowing of disease progression, or disease modification. Therefore, in order to maximize clinical benefit with rivastigmine and possibly other cholinesterase inhibitors, it may be beneficial to start therapy early, or even at a point when the disease is not fully manifest – that is, in patients with mild cognitive impairment (MCI). In order to test this hypothesis, a prospective, multicentre, randomized study (InDDEx) is ongoing to examine the effect of rivastigmine therapy on prolonging the time to a clinical diagnosis of AD in individuals with MCI. Similar studies are ongoing with donepezil and galantamine.

It has been proposed that the interaction of certain cholinesterase inhibitors with a site on the nAChR that is different from the binding site of endogenous ACh (the allosteric site) may lead to modulatory effects. Tacrine, donepezil and galantamine have all been shown to interact *in vitro* with the allosteric activator site on the nAChR[68], but the clinical relevance of these effects are still under debate[21]. In fact, the putative clinical relevance of nAChR modulation may become even less likely as AD progresses and these receptors are generally lost early in the disease process[77].

Rivastigmine shows selectivity for central rather than peripheral enzymes that may be explained by its 4–5 times greater sensitivity for the G1 form of AChE[78]. The G1-selectivity of rivastigmine also leads to high brain region-selectivity for the hippocampus and cortex[78], especially areas of the cortex involved in attentional processes and behaviour[79-82]. In contrast, donepezil and galantamine show much less or no differential specificity for the different isoforms of AChE. This may result in less targeted efficacy[21].

In conclusion, the cholinesterase inhibitors have been shown to provide sustained benefits in patients with AD. Long-term treatment is likely to reduce the overall burden of the disease to patients, caregivers and society. Physicians should aim to keep their patients on active treatment over the long term, and should consider switching between the agents in cases of intolerability or failing efficacy. The data suggest that there may be important differences between the individual agents, although the results of long-term, direct comparative studies are required to clarify these. The results of one such study, the 2-year EXCEED trial comparing donepezil and rivastigmine in outpatients with a diagnosis of moderate to moderately-severe AD (MMSE 10–20), should be available within the next two years[83]. Meanwhile, it is interesting to consider the apparent differences between these agents based on the data available so far from long-term studies. It will be of even greater importance to follow ongoing randomized studies evaluating these drugs in order to ascertain potential differences between them over a long-term therapeutic perspective.

References

1. Rosenstein L.D., Differential diagnosis of the major progressive dementias and depression in middle and late adulthood: a summary of the literature of the early 1990s. *Neuropsychol Rev* 1998; 8:109-167.
2. Davies K.L., Maloney A.J., Selective loss of central cholinergic neurons in Alzheimer's disease. *Lancet* 1976; 2:1403.
3. Lopez O.L., Becker J.T., Wisniewski S., Saxton J., Kaufer D.I., DeKosky S.T., Cholinesterase inhibitor treatment alters the natural history of Alzheimer's disease. *J Neurol Neurosurg Psychiatry* 2002; 72: 310-314.
4. Rainer M., Mucke H.A.M., Long-term cognitive benefits from galanthamine in Alzheimer's disease. *Int J Geriatr Psychopharmacol* 1998; 1: 197-201.
5. Winblad B., Brodaty H., Gauthier S., Morris J.C., Orgogozo J-M, Rockwood K., Schneider L., Takeda M., Tariot P., Wilkinson D., Pharmacotherapy of Alzheimer's disease: is there a need to redefine treatment success. *Int J Geriatr Psychiatry* 2001; 16: 653-656.
6. Gauthier S., Gélinas I., Gauthier L., Functional disability in Alzheimer's disease. *Int Psychogeriatr* 1997; 9(Suppl 1):163-165.
7. Cummings J.L., Kaufer D., Neuropsychiatric aspects of Alzheimer's disease: the cholinergic hypothesis revisited. *Neurology* 1996; 47: 876-883.
8. Bryant J., Clegg A., Nicholson T., McIntyre L., De Broe S., Gerard K., Waugh N., Clinical and cost-effectiveness of donepezil, rivastigmine and galantamine for Alzheimer's disease: a rapid and systematic review. *Health Technol Assess* 2001; 5: 1-137.
9. Doody R.S., Stevens J.C., Beck C., Dubinsky R.M., Kaye J.A., Gwyther L., Mohs R.C., Thal L.J., Whitehouse P.J., DeKosky S.T., Cummings J.L., Practice parameter: Management of dementia (an evidence-based review). Report of the Quality Standards Subcommittee of the American Academy of Neurology. *Neurology* 2001; 56: 1154-1166.
10. Fox N.C., Scahill R.I., Crum W.R., Rossor M.N., Correlation between rates of brain atrophy and cognitive decline in AD. *Neurology* 1999; 52:1687-1689.
11. Farlow M.R., Do cholinesterase inhibitors slow progression of Alzheimer disease? *Int J Clin Pract* 2002; Suppl 127: 37-44.
12. Kawas C.H., Clark C.M., Farlow M.R., Knopman D.S., Marson D., Morris J.C., Thal L.J., Whitehouse P.J., Clinical trials in Alzheimer's disease: debate on the use of placebo controls. *Alzheimer Dis Assoc Disord* 1999;13: 124-129.
13. Farlow M., Anand R., Messina Jr J., Hartman R., Veach J., A 52-week study of the efficacy of rivastigmine in patients with mild to moderately severe Alzheimer's disease. *Eur Neurol* 2000; 44: 234-241.
14. Raskind M.A., Peskind E.R., Wessel T., Yuan W and the Galantamine USA-1 Study Group. Galantamine in AD, a 6-month randomized placebo-controlled trial with a 6-month extension. *Neurology* 2000; 54: 2261-2268.
15. Stern R.G., Mohs R.C., Davidson M., Schmeidler J., Silverman J., Kramer-Ginsberg E., Searcey T., Bierer L., Davis., K.L., A longitudinal study of Alzheimer's disease: measurement, rate, and predictors of cognitive deterioration. *Am J Psychiatr* 1994;151: 390-396.
16. Watkins P.B., Zimmerman H.J., Knapp M.J., Gracon S.I., Lewis K.W., Hepatotoxic effects of tacrine administration in patients with Alzheimer's disease. *JAMA* 1994; 271: 992-998.
17. Weinstock M., Selectivity of cholinesterase inhibition. *CNS Drugs* 1999;12: 307-323.
18. Mesulam M., Guillozet A., Shaw P., Quinn B., Widely spread butyrylcholinesterase can hydrolyze acetylcholine in the normal and Alzheimer brain. *Neurobiol Dis* 2002; 9: 88-93.

19. Greig N.H., Utsuki T., Yu Q., Zhu X., Holloway H.W., Perry T.A., Lee B., Ingram D.K., Lahiri D.K., A new therapeutic target in Alzheimer's disease treatment: attention to butyrylcholinesterase. *Curr Med Res Opin* 2001;17: 1-7.
20. Ballard C.G., Advances in the treatment of Alzheimer's disease: benefits of dual cholinesterase inhibition. *Eur Neurol* 2002; 47: 64-70.
21. Poirier J., Evidence that the clinical effects of cholinesterase inhibitors are related to potency and targeting of action. *Int J Clin Pract* 2002; Suppl 127: 6-19.
22. Davidsson P., Blennow K., Andreasen N., Eriksson B., Minthon L., Hesse C. Differential increase in cerebrospinal fluid-acetylcholinesterase after treatment with acetylcholinesterase inhibitors in patients with Alzheimer's disease. *Neuroscience Letters* 2001; 300:157-160.
23. Darreh-Shori T., Almkvist O., Guan Z.Z., Garlind A., Strandberg B., Svensson A-L., Soreq H., Hellström-Lindahl E., Nordberg A., Sustained cholinesterase inhibition in AD patients receiving rivastigmine for 12 months. *Neurology* in press.
24. Haroutunian V., Greig N., Pei X.F., Utsuki T., Gluck R., Acevedo L.D., Davis K.L., Wallace W.C., Pharmacological modulation of Alzheimer's beta-amyloid precursor protein levels in the CSF of rats with forebrain cholinergic system lesions. *Brain Res Mol Brain Res* 1997; 46:161-168.
25. Wolf B.A., Wertkin A.M., Jolly Y.C., Yasuda R.P., Wolfe B.B., Konrad R.J., Manning D., Ravi S., Williamson J.R., Lee V.M., Muscarinic regulation of Alzheimer's disease amyloid precursor protein secretion and amyloid beta-protein production in human neuronal NT2N cells. *J Biol Chem* 1995; 270: 4916-22.
26. Guillozet A.L., Smiley J.F., Mash D.C., Butyrylcholinesterase in the life cycle of amyloid plaques. *Ann Neurol* 1997; 42: 909-918.
27. Arendt T., Bruckner M.K., Lange M., Bigl V., Changes in acetylcholinesterase and butyrylcholinesterase in Alzheimer's disease resemble embryonic development – a study of molecular forms. *Neurochem Int* 1992; 21: 381-396.
28. Inestrosa N.C., Alvarez A., Garrido J., Calderon F., Bronfman F.C., Dajas F., Gentry M.K., Doctor B.P., Acetylcholinesterase accelerates assembly of amyloid-beta-peptides into Alzheimer's fibrils: possible role of the peripheral site of the enzyme. *Neuron* 1996; 16: 881-891.
29. Rees T., Brimoijoin S., Soreq H., Younkin S., Acetylcholinesterase promotes amyloid beta plaque deposition *in vivo*. *Neurobiol Aging* 2002; 23(Suppl 1): 243.
30. Nyquist-Battie C., Hagler K.E., Windberg L., Thottassery J.V., Acetylcholine levels, choline acetyltransferase and acetylcholinesterase molecular forms during thyroxine-induced cardiac hypertrophy. *Neurochem Int* 1993; 22: 143-151.
31. Siek G.C., Katz L.S., Fishman E.B., Korosi T.S., Marquis J.K., Molecular forms of acetylcholinesterase in subcortical areas of normal and Alzheimer disease brain. *Biol Psychiatry* 1990; 27: 573-580.
32. Inglis F., The tolerability and safety of cholinesterase inhibitors in the treatment of dementia. *Int J Clin Pract* 2002; Suppl 127: 45-63.
33. Rogers S.L., Farlow M.R., Doody R.S., Mohs R., Friedhoff L.T. and the Donepezil Study Group. A 24-week double-blind, placebo-controlled trial of donepezil in patients with Alzheimer's disease. *Neurology* 1998; 50: 136-145.
34. Bateson A.N., Basic pharmacologic mechanisms involved in benzodiazepine tolerance and withdrawal. *Curr Pharm Des* 2002; 8: 5-21.
35. Corey-Bloom J., Anand R., Veach J., for the ENA-713 B352 Study Group. A randomized trial evaluating the efficacy and safety of ENA-713 (rivastigmine tartrate), a new acetylcholinesterase inhibitor, in patients with mild to moderately severe Alzheimer's disease. *Int J Geriatr Psychopharmacol* 1998;1: 55-65.
36. Doraiswamy M., Krishnan K.R.R., Anand R., Sohn H., Danyluk J., Hartman R.D., Veach J., Long-term effects of rivastigmine in moderately severe Alzheimer's disease: does early initiation of therapy offer sustained benefits? *Prog Neuropsychopharmacol Biol Psychiatry* 2002; 26: 705-712.
37. Potkin S.G., Anand R., Hartman R., Veach J., Impact of Alzheimer's disease and rivastigmine treatment on activities of daily living over the course of mild to moderately severe disease. *Prog Neuropsychopharmacol Biol Psychiatry* 2002; 26: 713-720.
38. Feldman H., Gauthier S., Hecker J., Vellas B., Subbiah P., Whalen E., A 24-week, randomized, double-blind study of donepezil in moderate to severe Alzheimer's disease. *Neurology* 2001; 57: 613-620.
39. Gauthier S., Feldman H., Hecker J., Vellas B., Emir B., Subbiah P., Exploratory analysis of the effects of donepezil in moderate and severe Alzheimer's disease patients. *Neurobiol Aging* 2002; 23(Suppl 1): 72.
40. Tariot P.N., Cummings J.L., Katz I.R., Mintzer J., Perdomo C.A., Schwam E.M., Whalen E., A randomized double-blind, placebo-controlled study of the efficacy and safety of donepezil in patients with Alzheimer's disease in the nursing home setting. *J Am Geriatr Soc* 2001; 49: 1590-1599.
41. Tinklenberg J., Newkirk L., Thompson J. Donepezil treatment of Alzheimer's disease patients in California clinical practice: one-year follow-up. 14th Congress of the European College of Neuropsychopharmacology (ECNP), Istanbul, Turkey, 13-17 October 2001 (Abstract 444).
42. Cameron I., Curran S., Newton P., Petty D., Wattis J. Use of donepezil for the treatment of mild–moderate Alzheimer's disease: an audit of the assessment and treatment of patients in routine clinical practice. *Int J Geriatr Psychiatry* 2000; 15: 887-891.
43. Novartis Pharmaceuticals. NDC project number 88-2072-NCS01. Data on file, August 2002.
44. Emre M., Switching cholinesterase inhibitors in patients with Alzheimer's disease. *Int J Clin Pract* 2002; Suppl 127: 64-72.
45. Auriacombe S., Pere J-J., Loria-Kanza Y., Vellas B., Efficacy and safety of rivastigmine in patients with Alzheimer's disease who failed to benefit from treatment with donepezil. *Curr Med Res Opin* 2002; 18: 129-138.
46. Bullock R., Connolly C., Switching cholinesterase inhibitor therapy in Alzheimer's disease – donepezil to rivastigmine, is it worth it? *Int J Geriatr Psychiatry* 2002; 17: 288-289.
47. Farlow M., Brashear A., Rationale, benefits and guidelines for switching to rivastigmine. *Neurobiol Aging* 2002; 23(Suppl 1): S77.
48. Ferris S.H., Switching previous therapies for Alzheimer's disease to galantamine. *Clin Ther* 2001; 23(Suppl A): A3-7.
49. Winblad B., Engedal K., Soininen H., Verhey F., Waldemar G., Wimo A., Wetterholm A-L., Zhang R., Haglund A., Subbiah P. and the Donepezil Nordic Study Group. A 1-year, randomized, placebo-controlled, study of donepezil in patients with mild to moderate AD. *Neurology* 2001; 57: 489-495.
50. Mohs RC., Doody RS., Morris JC., Ieni JR., Rogers SL., Perdomo CA., Pratt RD for the "312" Study Group. A 1-year, placebo-controlled preservation of function survival study of donepezil in AD patients. *Neurology* 2001; 57: 481-488.
51. Weiner M.F., Martin-Cook K., Foster B.M., Saine K., Fontaine C.S., Svetlik D.A., Effects of donepezil on emotional/behavioural symptoms in Alzheimer's disease patients. *J Clin Psychiatry* 2000; 61: 487-492.
52. Doody RS., Geldmacher DS., Gordon B., Perdomo CA., Pratt RD for the Donepezil Study Group. Open-label, multicenter, phase 3 extension study of the safety and efficacy of donepezil in patients with Alzheimer disease. *Arch Neurol* 2001; 58: 427-433.
53. Rogers S.L., Doody R.S., Pratt R.D., Leni J.R., Long-term efficacy and safety of donepezil in the treatment of Alzheimer's disease: final analysis of a US multicentre open-label study. *Eur Neuropsychopharmacol* 2000; 10: 195-203.
54. Rogers S.L., Friedhoff L.T., Long-term efficacy and safety of donepezil in the treatment of Alzheimer's disease: an interim analysis of the results of a US multicentre open label extension study. *Eur Neuropsychopharmacol* 1998; 8: 67-75.
55. Grossberg G., Irwin P., Satlin A., Mesenbrink P., Spiegel R. ,Rivastigmine in Alzheimer's disease: efficacy over two years. *Am J Geriatr Psychiatry* in press.

56. Farlow M., Messina J., Anand R., Hartman R., Veach J., Attenuation in the progression of cognitive deterioration in Alzheimer's disease with rivastigmine: a dose-dependent effect. Poster presented at the American Psychogeriatric Association (APA), New Orleans, USA, 2001.
57. Rösler M., Retz W., Retz-Junginger P., Dennler H.J., Effects of two-year treatment with the cholinesterase inhibitor rivastigmine on behavioural symptoms in Alzheimer's disease. *Behav Neurol* 1998; 11: 211-216.
58. Cummings J., Anand R., Koumaras B., Hartman B., Behavioral benefits in Alzheimer's disease patients residing in a nursing home following 52 weeks of treatment with rivastigmine. Poster presented at the American Psychogeriatric Association (APA), New Orleans, USA, 2001.
59. Edwards K., Goodman W., Anand R., Koumaras B., Hartman R., Effect of Alzheimer's disease severity on psychotropic drug use and behavior in nursing home patients treated with rivastigmine. *Neurobiol Aging* 2002; 23(Suppl 1): S78.
60. McKeith I., Del Ser T., Spano P., Emre M., Wesnes K., Anand R., Cicin-Sain A., Ferrara R., Spiegel R., Efficacy of rivastigmine in dementia with Lewy bodies: a randomised, double blind, placebo-controlled international study. *Lancet* 2000; 356: 2031-2036.
61. Grace J., Daniel S., Stevens T., Shankar K.K., Walker Z., Byrne E.J., Butler S., Wilkinson D., Woolford J., Waite J., McKeith I.G., Long-term use of rivastigmine in patients with dementia with Lewy bodies: an open-label trial. *Int Psychogeriatr* 2001; 13: 198-205.
62. Kumar V., Anand R., Messina J., Hartman R., Veach J., An efficacy and safety analysis of Exelon in Alzheimer's disease with concurrent vascular risk factors. *Eur J Neurol* 2000; 7: 159-169.
63. Kumar V., Messina J., Hartman R., Anand R., Long-term cognitive benefits of rivastigmine in Alzheimer's disease patients with vascular risk. Poster presented at the 6th International Stockholm/Springfield Symposium on Advances in Alzheimer Therapy, Stockholm, Sweden, 5-8 April 2000 (Abstract 38A).
64. Moretti R., Torre P., Antonello R,M., Cazzato G., Bava A., An open-label pilot study comparing rivastigmine and low-dose aspirin for the treatment of symptoms specific to patients with subcortical vascular dementia. *Curr Ther Res* 2002; 63: 443-458.
65. Moretti R., Torre P., Antonello RM., Cazzato G., Bava A., Rivastigmine in subcortical vascular dementia: an open 22-month study. *J Neurol Sci* 2002; in press.
66. Maelicke A., Allosteric sensitization of nicotinic receptors by galantamine, a new treatment strategy for Alzheimer's disease. *Biol Psychiatry* 2001; 49: 279-288.
67. Samochocki M., Zerlin M., Jostock R., Groot Kormelink PJ., Luyten WH., Albuquerque EX., Maelicke A. Galantamine is an allosterically potentiating ligand of the human alpha4/beta2 nAChR. *Acta Neurol Scand Suppl* 2000; 176: 68-73.
68. Svensson A-L., Cholinesterase inhibitors in Alzheimer's disease: an experimental study on mechanisms for interaction with muscarinic and nicotinic receptors and neuroprotection. Doctoral Dissertation, Karolinska Institutet, Huddinge University Hospital, Huddinge, Sweden, 1997.
69. Raskind M., Truyen L., Galantamine has long-term cognitive benefits in Alzheimer's disease – an analysis of responder rates at 36 months. Poster presented at the 7th International Geneva/Springfield Symposium on Advances in Alzheimer Therapy, Geneva, Switzerland, 3-6 April 2002.
70. Morris JC., Kershaw P., Cognitive benefits of long-term, continuous galantamine treatment in patients with Alzheimer's disease. Poster presented at the 7th International Geneva/Springfield Symposium on Advances in Alzheimer Therapy, Geneva, Switzerland, 3-6 April 2002.
71. Tariot P.N., Solomon P.R., Morris J.C., Kershaw P., Lilienfeld S., Ding C. A 5-month, randomized, placebo-controlled trial of galantamine in AD. *Neurology* 2000; 54: 2269-2276.
72. Erkinjuntti T., Lilienfeld S., Damaraju C.V., Long-term treatment with galantamine is effective in slowing decline in patients with probable vascular dementia: preliminary results. Poster presented at the 7th International Geneva/Springfield Symposium on Advances in Alzheimer Therapy, Geneva, Switzerland, 3-6 April 2002.
73. Erkinjuntti T., Kurz A., Gauthier S., Bullock R., Lilienfeld S., Damaraju C.V. Efficacy of galantamine in probable vascular dementia and Alzheimer's disease combined with cerebrovascular disease: a randomised trial. *Lancet* 2002; 359: 1283-1290.
74. Perry EK., Perry RH., Blessed G., Tomlinson BE. Changes in brain cholinesterases in senile dementia of Alzheimer type. *Neuropathol Appl Neurobiol* 1978; 4: 273-277.
75. Darvesh S., Grantham DL., Hopkins DA. Distribution of butyrylcholinesterase in the human amygdala and hippocampal formation. *J Comp Neurol* 1998; 393: 374-390.
76. Reading PJ., Luce AK., McKeith IG. Rivastigmine in the treatment of parkinsonian psychosis and cognitive impairment: preliminary findings from an open trial. *Mov Disord* 2001; 16: 1171-1195.
77. Nordberg A., Alafuzoff I., Winblad B. Nicotinic and muscarinic subtypes in the human brain: changes with aging and dementia. *J Neurosci Res* 1992; 31: 103-111.
78. Enz A., Amstutz R., Boddeke H., Gmelin G., Malanowski J. *Brain selective inhibition of acetylcholinesterase: a novel approach to therapy for Alzheimer's disease*. In: Cuello AC (ed), Progress in Brain Research. Netherlands: Elsevier Sciences Publishers, 1993; 98: 431-438.
79. Adler G., Brassen S. Short-term rivastigmine treatment reduces EEG slow-wave power in Alzheimer patients. *Neuropsychobiology* 2001; 43: 273-276.
80. Sharma T., Kumari V., Mitterschiffthaler M Cognitive effects of rivastigmine in Alzheimer's disease: an fMRI study. Poster presented at the American College of Neuropsychopharmacology 40th Annual Meeting, Waikoloa, Hawaii, 9-13 December 2001.
81. Venneri A., Shanks M.F., Staff R.T., Pestell S.J., Forbes K.E., Gemmell H.G., Murray A.D., Cerebral blood flow and cognitive responses to rivastigmine treatment in Alzheimer's disease *NeuroReport* 2002; 13: 83-96.
82. Nordberg A., Stefanova E., Almkvist O., Nilsson A., Wall A., Långstrom B. Improved cortical glucose metabolism in Alzheimer's disease (AD) patients treated with rivastigmine for 1 year. *J Neurol Sci* 2001; 187(Suppl 1): P0142.

Culture and Dementia

Adesola Ogunniyi[1#], Kathleen S Hall[2], Olusegun Baiyewu[1], Oyewusi Gureje[1], Hugh C. Hendrie[2].

[1] Departments of Medicine and Psychiatry, University College Hospital, Ibadan, Nigeria;

[2] Department of Psychiatry, Indiana University School of Medicine, Indianapolis, IN, USA

Paper presented in part at the 8th International Conference on Alzheimer's Disease and Related Disorders, Stockholm, Sweden, July 21, 2002.

Abstract

Dementia is a rising global challenge. Appropriate selection and harmonization of screening instruments is essential for overcoming educational and cultural biases before transnational studies can be successfully and meaningfully undertaken. Recognition of impairment in the activities of daily living is influenced by the state of technological development and the culture. Cross-cultural studies have shown lower prevalence and incidence of dementia and Alzheimer's disease (AD) in developing countries. Apolipoprotein E studies have shown inconsistent association between possession of the e4 allele and AD in different ethnic groups, and appear to support a gene-environment interaction hypothesis. Cultural variations in behavioural and psychological symptoms of dementia (BPSD) as well as care arrangements are highlighted to advocate differences in approach to management in various ethnic groups.

Dementia in all its forms constitutes a major public health concern worldwide because of the unprecedented increase in the number of elderly individuals (aged 65 years and above) who are at risk of developing the condition. Dementia is characterized by considerable decline in cognitive ability to the extent that personal activities of daily living are interfered with. The manifestations and recognition of the disease depend on the social and cultural setting in which the afflicted individual lives. Culture, which refers to the customs, habits, and social norms of a particular group of people, determines the pattern of activities of daily living, roles, expectations, care arrangements for the elderly, as well as the recognition and acceptance of dementia and other disease states.

In many societies, the elderly is revered and regarded as a repository of wisdom; cultural attitudes may therefore influence what is perceived as senility. African culture guarantees social and psychological superiority to older people, thus conferring them with high status and prestige[1]. A high tolerance for what would be described as aberrant or deviant behaviour elsewhere appears to be condoned so as to preserve family integrity[2]. It therefore means that a condition like dementia may be concealed at home, and those afflicted may not be taken to hospital to avoid the stigma attached to mental illness. Amongst the Igbos, in the Eastern part of Nigeria, dementia is regarded as a form of demonic attack of the brain by religious ministers[3].

In a country like India with diverse cultures; there are differences in the conceptualization of dementia. In Goa for example, it is recognized and explained as brain weakness or deterioration but not an illness, whereas in Benares, personality changes appear to be more easily recognized as a feature of dementia than memory failure[4,5]. In the Far East, it is documented that medical attention is usually preferentially sought for stroke, but not for elderly relatives with failing memory that is usually ascribed to metaphysical factors[6]. This may partly explain the preponderance of vascular dementia documented in that region. These various peculiarities in different regions of the world highlight the complexity of understanding dementia in various cultures.

Culture and Diagnostic Instruments

The diagnosis of dementia requires objective documentation of cognitive decline that may involve multiple domains including: memory, orientation, judgement, abstraction, language, thinking and concentration. Comparison of results obtained from different studies is hampered when different diagnostic instruments are used and when the diagnostic criteria are not uniform. Such difficulties limit the utilization of data for generating hypotheses about the putative risk factors involved. The development of culture fair instruments for assessment in cross-cultural studies is therefore a major challenge. Virtually all the commonly used instruments like the Mini-Mental State Examination (MMSE), the Cognitive Abilities Screening Instrument (CASI), Elderly Cognitive Assessment Questionnaire (ECAQ), Cross Cultural Cognitive Examination (CCCE), Informant Questionnaire

#Correspondence: Adesola Ogunniyi, MBChB, Department of Medicine, University College Hospital, Ibadan, NIGERIA, Tel/Fax: +234 2 2410489, E-mail: dementia.uch@skannet.com.ng

on Cognitive Decline in the Elderly (IQCODE), Geriatric Mental State Examination (GMS), Cambridge Mental Disorders of the Elderly Questionnaire (CAMDEX) and the Community Screening Interview for Dementia (CSI'D) suffer from cultural and educational bias[7,8]. Adaptation of the test items to the cultural, linguistic and educational norms of the population to be studied, referred to as harmonization, constitutes an important first step in their application. The MMSE is available in many languages including Chinese and Hindi[9,10]. The Chinese version was shown to be poorly suited for use in elderly illiterate persons[9]. It was observed in a cross-cultural study comparing Chinese, American and Finish subjects that differences existed in their performances on MMSE items with the Chinese subjects being better on recall items, while the American and Finish subjects performed better on constructional praxis[9]. Therefore, poor recall by Chinese subjects would appear to be more predictive of cognitive decline than poor drawing skills.

The CSI'D, containing items selected from the CAMDEX, MMSE, Dementia Rating Scale, East Boston Memory Test, and the Comprehensive Assessment and Referral Evaluation, is available in English, Yoruba, Jamaican patois, Kenyan, Chinese and Cree versions[8,11]. It was successfully applied in five communities with very different socio-economic backgrounds (Cree Indians and Caucasians in Winnipeg, Canada; African Americans in Indianapolis, USA; Yoruba in Ibadan, Nigeria; and Jamaicans in Kingston, Jamaica) with consistent results[11]. That has made it a choice instrument for dementia screening in different cultures. The CSI'D had been adopted for the 10/66 studies in many developing countries. A combination of scores on the cognitive and informant items is computed to derive a discriminant function score, which produced better sensitivity and specificity for the diagnosis of dementia than cognitive scores alone. The predictive validity of the CSI'D for dementia diagnosis ranged from 82% in Ibadan to 97% in Cree Indians and Caucasians[11]. The informant score alone was shown to predict dementia independent of cognitive scores in all the sites.

One item included in the CSI'D is the Stick Design Test (SDT) which assesses visuo-constructional ability in illiterate individuals. The test involves arranging matchsticks to copy stimulus designs. Preliminary data on its application to 275 Yoruba subjects in Ibadan showed that it was better in discriminating between normal and demented subjects than the construction praxis item on MMSE. In addition, it was less influenced by education and gender[12]. Another innovative test for assessing recall and visual perception in illiterate cultures is the use of the Indiana University Tokens Test as part of the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) neuropsychological test battery. This involves recognition and recall of shapes, sizes and colours. Preliminary data from a Kenyan pilot study revealed significant difference in test scores on this item between normal and demented subjects (Ayuo P , personal communication).

Assessment of impairment in activities of daily living is the second component of dementia diagnosis. The expectations and roles of the elderly vary in different cultures, and according to the state of technological development. Erkinjuntti et al.[13] identified assessment of social activities as an important cause of disagreement in dementia diagnosis. In many developing countries like Nigeria, the demands are much limited, as is exposure to modern domestic appliances. Most of the elderly individuals in low technology societies had neither used nor seen microwave ovens, dishwasher, and hairdryer before. There are also gender differences in expected roles, and elderly males, even if widowed, are not involved in meal preparations. The wives, children and extended family members take up most of the chores. Therefore, asking an old man about food preparation using microwave oven may not be appropriate. He would however be expected, if cognitively intact, to remember the essential ingredients for the preparation of local meals, and retain decision-making role in the family. The situation is however changing with increasing westernization in the urban communities. Cultural adaptation of diagnostic instruments in that setting included asking about handicrafts, traditional games, and participation in community activities. Such cultural adaptation for the assessment of impairment in daily activities was similarly carried out in Ballabgarh, India as a prerequisite for accurate dementia diagnosis in that environment[14].

Culture and age determination:

Prevalence data on a disease that is age associated requires accurate age determination for meaningful comparison of rates. In illiterate societies where birth records are not available, age determination becomes an important issue. In one study in India, under-reporting by an average of three years was documented, while the Chinese appeared to overestimate their age by an extra year[6]. Age adjustment of the rates obtained is essential before data can be meaningfully compared between communities studied[7]. Uncorrected rate in a community with a large proportion of the "young old" individuals would result in spuriously low disease prevalence. The use of historical events for near accurate age determination has been validated in a Nigerian study with very high correlation[15].

Burden of dementia and Alzheimer's disease across cultures

The prevalence of dementia in subjects older than 65 years in western countries ranges between 4 and 11%,[16] however, there is paucity of information on dementia burden in developing countries. The few isolated studies

available have consistently reported lower rates except in Shanghai, China[17] where the figures obtained were similar to those in Caucasians. That study also reported a higher frequency of AD (65%), unlike previous studies in China, which reported a preponderance of vascular dementia[7]. Providing reliable and comparable information about true differences in disease rates, on which to base preventive strategies, have relied more on a cross-cultural and international approach[18]. The objective is to study populations in Diaspora that are living in different environments in terms of technological development using the same methodology. Finding significant differences in disease rates between any two of such migrant populations would facilitate delineation of risk factors like diet, vascular and socio-cultural factors including education, which may be environmentally determined. It would also facilitate the detection of possible interactions between genetic and environmental factors. Data from cross-cultural studies have been obtained from Yoruba Africans and African Americans (Indianapolis-Ibadan), Native Americans (Cree) and Caucasians in Canada, Hindi Indians compared with Americans (Indo-US), and Japanese Americans vs. Japanese (Ni-Hon-Sea)[14, 19-23].

In the Ni-Hon-Sea Project, the prevalence rates of dementia in aging Japanese populations were obtained at three sites namely: Hiroshima, Japan, Kings County, Washington (The KAME Project), and Oahu, Hawaii. The rates were similar to those reported in Caucasians, and ranged between 5.3 and 7.6%[19,20]. In all these sites, AD was the predominant subtype, especially in women. The prevalence of dementia in the Japanese-American cohort in the Washington project was reported to be higher than the rates reported from studies in Japan, which would suggest some environmental influence[19]. In the Cree study, although the overall prevalence of dementia was the same in the Cree and Canadians resident in Winnipeg, the rate for AD was significantly lower[21]. In the Indianapolis-Ibadan study, the prevalence of dementia and AD were found to be significantly lower in Yoruba subjects (2.29% and 1.41% respectively) than in African Americans (8.24% and 6.24% respectively)[22]. In the Indo-US Cross National Dementia Epidemiology Study involving elderly population in Ballabgarh, a rural district of Northern India, and elderly subjects living in the Monongahela Valley in Pennsylvania in the USA (MoVIES Project), the overall age-adjusted prevalence of dementia in the Indian population was 1.36%, and for Alzheimer's disease, the rate was 1.07%[14]. Table 1 shows a compilation of the prevalence rates reported from cross-cultural studies. The lower dementia rates in "low-tech cultures" have been ascribed to many possibilities including biological selection, low life expectancy, shorter disease duration, lower incidence and environmental protection.

Prevalence, unlike incidence, is affected by the survival of those afflicted, and one of the criticisms of the lower prevalence in developing countries is possible bias due to higher mortality. As a result of these, less reliance is placed on prevalence data. Incidence rates are therefore regarded as better measure of true disease burden. Much fewer incidence than prevalence studies of dementia have been carried out worldwide[7]. In developing countries, incidence rates from two cross-cultural studies are available. In Ibadan, the incidence rates of AD and

Table 1: Prevalence of dementia and Alzheimer's disease in selected cross-cultural studies*.

Site	Dementia	Alzheimer's disease
Western countries[16]	4-11%	50-75% of total
Cree-Manitoba Study[21]		
Cree	4.2%	0.5%
Winnipeg	4.2%	3.5%
Ni-Hon-Sea Study[19]		
Seattle	6.3%	Predominantly AD
Honolulu (70+; men)	7.6%	Predominantly AD
Hiroshima (60+)	5.3%	Predominantly AD
Indianapolis-Ibadan Study[22]		
Indianapolis	8.24%	6.24%
Ibadan	2.29%	1.41%
Indo-US Study[14]		
Ballabgarh, India	1.36%	1.07%

* References as superscripts

dementia were: 1.35% and 1.15% respectively, whilst among African Americans in Indianapolis, the respective rates were 3.24% and 2.52%[22]. In the Indo-US study, the incidence rates were roughly six times lower in Ballabgarh than in the comparative US group[24] (Table 2). Many studies like these are required.

It would thus appear from these cross-cultural, trans-national studies that the burden of dementia is lower in the developing countries or more traditional societies, which would suggest influence of environmental factors like industrial exposure or diet on genetic predisposition. These factors could either be protective or interact with genetic factors to lower the risk of dementia in individuals residing in developing countries.

Age has been universally shown to be a risk factor for dementia[7,16]. Rural living before the age of 19 years has been reported to increase the risk of AD in African Americans[25]. Low educational attainment is also with increased risk in many communities, but not amongst the Yoruba[14,17, 18,22,25]. Amongst Caucasians, possession of the ε4 allele of apolipoprotein E (APOE) has been consistently shown to increase the risk of AD in a dose-dependent fashion. However, this association appears not to be present in all ethnic groups[26]. In African Americans, the association between APOE e4 and AD is weaker than in Caucasians, and the effect appeared to be attenuated in Hispanics[26, 27]. In a Nigerian study, there was lack of association between AD and APOE[28]. In agreement with this observation, the frequencies of vascular risk factors were lower in the Nigerian cohort than in African Americans[29]. The *APO*E protein is involved in cholesterol transport, and is encoded on chromosome 19. In African Americans, there was demonstrable significant interaction among total serum cholesterol, APOE genotype and AD risk, [30] but not in the Yoruba cohort. One striking difference between the Indianapolis and Ibadan cohorts is in their diet, which is reflected in lower cholesterol levels found in the Yoruba when compared with the African-American population (mean cholesterol levels in the Yoruba, 166 mgs/ml; mean levels in African Americans, 220 mgs/ml)[29]. It has been speculated that there may be some dietary interaction, perhaps involving cholesterol or other lipids, which in association with *APO*E accounted for the differences in rates of illness between the two sites. It is also possible that the relative lack of vascular risk factors by itself in the Yoruba may play an important role in modifying disease onset in that population.

In the Indo-US study on the other hand, although the frequency of APOE e4 allele was lower in the Hindi subjects than in the American cohort (7% vs.11%), the association with AD was maintained in the two cohorts[31]. Differences in diet may possibly explain the differences in association of APOE and AD in Yoruba and Hindi subjects. It would be worthwhile carrying out further analysis on dietary constituents to tease out the particular components involved and/or the mode of interaction.

Culture and behavioural disturbances of dementia

Behavioural and psychological symptoms of dementia (BPSD) occur commonly in the various subtypes. BPSD may be the presenting symptoms or appear in its course, and constitute a major cause of caregiver burden[32, 33]. In western countries, BPSD are responsible for institutionalization of demented individuals. The distressing ones include: wandering, aggression, incontinence, depression, psychosis, sleep disturbance, sexual disinhibition and poor eating habits[32, 33]. Up to 90% of those afflicted manifest some of these symptoms depending on severity and duration of the disease, as well as prevailing co-morbid psychiatric conditions[32]. Their recognition, impact and management vary in different cultures, and according to literacy level. The pattern of BPSD in Taiwanese subjects is similar to the findings in Caucasians[34]. In Turkey, suspiciousness, visual hallucinations, sleep disturbances, wandering and hiding of objects appeared to be more frequently reported than in other European countries. There was no significant difference in aggressive or agitated behavior in African Americans as compared with Caucasians[35]. In Argentina,

Table 2: Incidence of Dementia and Alzheimer's disease in cross-cultural studies

Sites	Dementia	Alzheimer's disease
Indianapolis-Ibadan Study[23]		
Indianapolis	3.24%	2.52%
Ibadan	1.35%	1.13%
Indo-US Study*[24]		
Ballabgarh, India	n/a	3.24%
Monongahela, USA	n/a	19.32%

* Alzheimer's disease only; References as superscript

different BPSD appear problematic in the different ethnic groups. In those of European descent, aggressiveness, sleep disorders and delusions were prominent, while in the more illiterate and rural communities; more tolerance for these symptoms was evident. Fear of stigmatization was associated with either denial or concealment amongst the Volga German descendants[36]. In India, rural-urban differential in troublesome symptoms was observed. Pacing and wandering that were considered worrisome in the urban settings because of associated trauma were less so in the rural communities where family support is stronger[37]. Although BPSD are not currently a major cause of distress for caregivers, there is the impression that this trend may change with time because of economic pursuit and decreasing family ties.

In the Yoruba culture, hallucinations and delusions, which tend to connote mental illness, may be concealed by family members to avoid any stigma[1, 2,6]. A cross-cultural study of BPSD in migrant African populations involving African Americans, Jamaicans and Yoruba found more problems with personal care in Jamaicans and African Americans than in the Yoruba demented subjects. Getting lost was most frequent in the Jamaicans, while personality changes were most frequent in African Americans. The Nigerian caregivers were reported to be most concerned about their demented relative being involved in embarrassing situations[38, 39]. It was observed that treatment was less often sought for behavioral disturbances in Nigeria because of limited facilities and dearth of trained manpower[38].

The pattern of presentation, tolerance, management seeking behaviours of BPSD thus vary in different cultures. It is dependent on the prevailing economic activities, the literacy level, place of residence, and who the caregivers may be. In most developing countries, family members appear more tolerant, and concealment was not uncommon. Different approaches for recognition and management need to be devised for this important aspect of dementia in different cultures.

Culture and care of the demented patient:

Demented individuals are either cared for within the community or are institutionalized, and the cultural setting influences the type of care available. In western countries, families are relatively small, and economic pursuits demand a lot of mobility. The elderly people value their independence, and therefore most of them prefer to live alone. Between 30 and 50% of elderly people are reported to live alone in these countries, the highest being in Denmark[40]. In Indianapolis, more than 40% of elderly African Americans live alone[41]. Thus living alone is not peculiar to any particular ethnic group, but would seem to depend on the environment in which the older person lives. Most subjects having mild to moderate dementia are managed at home, and the spouses or the children generally provide care. In the advanced stage, when constant supervision is required, institutionalization becomes the rule rather than the exception[7]. In developed countries, as many as two-thirds of all the demented cases may be institutionalized[7, 40,42]. The cost of providing care for these patients is enormous and could be as high as $47,000 per person[42]. In 1992, the total direct and indirect costs were estimated at $113 billion in the US alone,[43] which is far beyond the total annual budget and allocation to the health sector in many developing countries.

In developing countries, community-based care is favoured because families are larger with many generations living together including extended family members. This pattern has been reported in studies from China, India and Nigeria[3,6,7 44]. In a community in Ibadan, about 15% of the elderly Yoruba subjects lived alone[41]. With an average family size of 5.5 persons, and about 62% of them containing more than one generation, family support is strong with increased chance of psychosocial stimulation. The Ibadan data on household characteristics were similar to the observations from many countries involved in the 10/66 studies. In India, household size averaged between 4 and 6; in China, Cuba and selected South and Central America countries, the number averaged between 2 and 6 (Prince M, personal communication). Therefore in these communities, the older persons receive constant stimulation from family members, and they, in return, serve as surrogate parents for the grandchildren when still cognitively intact. In Goa, India, 'old age homes' are only available for older persons in good health, and who can afford the cost of care[4]. Thus, demented subjects are not likely to be kept in such facilities.

In Nigeria, because there is no health insurance policy, and very few of the older persons receive pensions, the

burden of their care falls on family members. Nursing homes for the elderly are culturally unacceptable because of the notion that it is 'more respectable' to die at home than in hospital[44]. Individuals without strong family ties however utilize the few facilities that exist. This stigma of destitution has limited public acceptance of institutionalization. The use of private nurses and/or domestic helpers by some wealthy families is not sufficiently widespread. Exorbitant cost of care, poor response to therapy and bureaucratic delays were some of the reasons adduced for high default rate and poor compliance with hospital treatment[3, 45]. In a recent review of the financial implications of caring for demented individuals in Nigeria, Uwakwe reported that a lot of money is spent on sacrifices, alternative medical care, etc. with minimal benefit. Increasing use of church facilities to care for demented subjects in some communities in Eastern Nigeria appeared to be a novel approach to management. Uwakwe argued that the use of music and dancing that form the core of African tradition, constitute a form of psychotherapeutic intervention[45]. The impact of these and other innovative cultural methods need to be assessed.

Conclusion

Available data from epidemiological and genetic studies have combined to enhance our understanding of some aspects of dementia, and have shown that culture plays an important part in disease recognition, diagnosis, care and support system. Results from cross-cultural studies have strengthened the impression that the environment interacts in some way with genetic predisposition to determine disease risk. Analytic studies appear to implicate diet as being partly responsible. Similarities and differences in BPSD have revealed that different approaches are needed for managing troublesome symptoms in different cultures, bearing in mind tolerance for deviant behavior in some ethnic groups. Strong family ties, larger household sizes and multigenerational linkages, characteristic of developing countries, make community-based care of demented subjects feasible, unlike the situation in western cultures. However, with changing lifestyles and family dynamics, and some of the highlighted cultural differences will disappear. The developing countries need to be aware of this ominous trend and prepare for the challenges that may lie ahead since time is of essence.

References

1. Ihezue U.H., Okpara E., Psychiatric disorders of old age in Enugu, Nigeria. *Acta Psychr. Scand.* 1989; 79: 332-337
2. Iniechen B., The epidemiology of dementia in Africa: a review. *Soc Sci Med.* 2000; 50: 1673-1677.
3. Uwakwe R., Knowledge of religious organizations about dementia and their role in care *Int. J. Geriatr. Psychiat.* 2000; 15: 1152-1153.
4. Patel V., Prince M., Ageing and mental health in a developing country: who cares? Qualitative studies from Goa, India. *Psychol. Med.* 2001; 31: 29-38.
5. Cohen L., Towards an anthropology of senility: anger, weakness, and Alzheimer's in Banaras. *India Med. Anth. Quarterly* 1995; 9: 314-334.
6. Ineichen B., The geography of dementia: an approach through epidemiology. Health and Place 1998; 4: 383-394.
7. Henderson A.S., Dementia. In: *Epidemiology of Mental Disorders and Psychosocial problems.* World Health Organization, Geneva. 1994: 6-8.
8. Hall K.S., Ogunniyi A.O., Hendrie H.C., A cross-cultural community-based study of dementias: methods and performance of the survey instrument, Indianapolis, USA, and Ibadan, Nigeria. *Int. J. Methods Psychiatr.* Res. 1996; 6: 129-142
9. Salmon D.P., Riekkinen P.J., Katzman R., Zhang M., Jin H., Yu E., Cross-cultural studies of dementia: a comparison of Mini-Mental State Examination performance in Finland and China. *Arch. Neurol.* 1989; 46: 769-772.
10. Ganguli M., Ratcliff G., Chandra V., A Hindi version of the MMSE: the development of a cognitive screening instrument for a largely illiterate rural elderly population in India. *Int. J. Geriatr. Psychiatr.* 1995; 52: 224-227.
11. Hall K.S., Gao S., Emsley C.L., Ogunniyi A.O., Morgan O., Hendrie H.C. Community screening interview for dementia (CSI'D'): performance in five disparate study sites. *Int. J. Geriatr. Psychiatr.* 2000; 15: 521-531.
12. Baiyewu O., Unverzagt F.W., Gureje O., Ogunniyi A.O., Hall K.S., Hendrie H.C. The Stick Design Test: a new measure of visuoconstuctional ability in subjects with limited education. Abstract # 63. *NeuroBiol Aging* 2000; 21 (1S): S14.
13. Erkinjuntti T., Osibye T., Steenhuis R., Hachinski V. The effect of different diagnostic criteria on the prevalence of dementia. *N. Eng. J. Med.* 1997; 337: 1667-1674.

14. Chandra V., Ganguli M., Pandav R., Johnston J., belle S., DeKosky S.T. Prevalence of Alzheimer's disease and other dementias in rural India. The Indo-US Study. *Neurology* 1998; 51: 1000-1008.
15. Ogunniyi A., Osuntokun B.O. Determination of ages of elderly Nigerians through historic events: validation of the Ajayi-Igun 1963 listing. *W. Afr. J. Med*. 1993; 12: 189-190.
16. Hendrie H.C., Epidemiology of dementia and Alzheimer's disease. *Ame. J Geriatr. Psychiatr.* 1998; 6: 3-18.
17. Zhang M., Katzman R., Salmon D., The prevalence of dementia and Alzheimer's disease in Shanghai, China: impact of age, gender and education. *Ann Neurol.* 1990; 27: 429-437.
18. Osuntokun B.O., Hendrie H.C., Ogunniyi A.O., Cross-cultural studies in Alzheimer's disease. *Ethnicity Dis.* 1992; 2: 352-357.
19. Graves A.B., Larson E.B., Edland S.D., prevalence of dementia and its subtypes in the Japanese American population of King county, Washington State. The Kame Project. *Am. J. Epidemiol.* 1996; 144: 760-771.
20. Yamada M., Sasaki H., Mimori Y ., Prevalence and risk of dementia in the Japanese population. *J. Am. Geriatr Soc.* 1999; 47: 189-195.
21. Hendrie H.C., Hall K.S., Pillay N., Alzheimer's disease is rare in Cree. *Int. Psychogeriatr*. 1993; 5: 5-14.
22. Hendrie H.C., Osuntokun B.O., Hall K.S., Prevalence of Alzheimer's disease and dementia in two communities: Nigerian Africans and African Americans. *Am J. Psychiatr.* 1995; 152: 1485-1492.
23. Hendrie H.C., Ogunniyi A., Hall K.S., Incidence of dementia and Alzheimer's disease in 2 communities: Yoruba residing in Ibadan, Nigeria, and African Americans residing in Indianapolis, Indiana. *JAMA* 2001; 285: 739-747.
24. Chandra V., Pandav R., Dodge HH., Incidence of Alzheimer's disease in a rural community in India. The Indo-US Study. *Neurology* 2001; 57: 985-989.
25. Hall K.S., Gao S., Unverzagt F.W., Hendrie H.C. Low education and childhood rural residence. *Neurology* 2000; 54: 95-99.
26. Farrer L.A., Cupples A., Haines J.L., Effects of age, sex, and ethnicity on the association between apolipoprotein E genotype and Alzheimer's disease: a meta-analysis. *JAMA* 1997; 278: 1349-1356.
27. Hendrie H.C., Hall K.S., Hui S., Apolipoprotein E genotypes and Alzheimer's disease in a community study of elderly African Americans. *Ann Neurol.* 1995; 37: 118- 120.
28. Osuntokun BO., Sahota A., Ogunniyi A.O., Lack of association between apolipoprotein E e4 and Alzheimer's disease in elderly Nigerians. *Ann neurol.* 1995; 38: 463-465.
29. Ogunniyi A., Baiyewu O., Gureje O., Epidemiology of dementia in Nigeria: results from the ndianapolis-Ibadan study. *Euro J. Neurol.* 2000; 7: 485-490.
30. Evans R.M., Emsley CL., Gao S., Serum cholesterol, APOE genotype, and the risk of Alzheimer's disease: a population-based study of African Americans. *Neurology* 2000; 54: 240-242.
31. Ganguli M., Chandra V., Kamboh MI., Apolipoprotein E polymorphism and Alzheimer's disease: The Indo-US cross-national dementia study. *Arch. Neurol.* 2000; 57: 824-830.
32. Finkel S., Silva J., Cohen G., Miller S., Sartorius N. Behavioural and psychological signs and symptoms of dementia. *Int. J Geriatr. Psychiatr.* 1997; 12: 1060-1061.
33. Mega M.S., Cummings J.L., Fiorello T., Gornbein J. The spectrum of behavioral changes in Alzheimer's disease. *Neurology* 1996; 46: 130-135.
34. Hwang J-P., Yang C-H., Tsai S-J., Liu K-M. Behavioral disturbances in psychiatric inpatients with dementia of the Alzheimer type in Taiwan. *Int J. Geriatr. Psychiatr* 1997; 12: 902-906.
35. Mintzer J.E., Nietert P., Costa K., Waid L.R. Cross-cultural perspectives, agitation in demented patients in the United States. *Int. Psychogeriatr.* 1996; 8 (Suppl.3): 487-490.
36. Mangone C.A. Cross-cultural perspectives: Argentina. *Int Psychogeriatr* 1996; 8 (Suppl 3): 473-478.
37. Chandra V. Cross-cultural perspectives: India. Int. Psychogeriatr. 1996; 8 (Suppl.3): 479-481.
38. Hendrie H.C., Baiyewu O., Eldemire D., Prince C. Cross-cultural perspectives: Caribbean, Native American, and Yoruba. *Int. Psychogeriatr.* 1996; 8 (Suppl. 3): 483-486.
39. Hendrie H.C., Gao S., Baiyewu O. A comparison of symptoms of behavioral disturbances in Yoruba and African American individuals with dementia. *Int. Psychogeriatr.* 2000; 12 (Suppl. 1): 403-408.
40. Ineichen B. Caring for people with dementia around the world. Alzheimer Disease Int. Factsheet 4. March 1998.
41. Hall KS., Gureje O., Gao S., Risk factors and Alzheimer's disease: a comparative study of two communities. *Austr. NZ J. Psychiatr.* 1998; 32: 698-706.
42. Max. W. The economic impact of Alzheimer's disease. *Neurology* 1993; 43 (Suppl 4): S6-S10.
43. Cummings J.L. Dementia: the failing brain. *Lancet* 1995; 345: 1481-1484.
44. Akanji B.O., Ogunniyi A., Baiyewu O. Healthcare for older persons, a country profile: Nigeria. *J. Am. Geriatr. Soc.* 2002 (in press).

Characterizing Rat p18 Amyloid Beta (Aβ) Responsive Protein p18AβrP

Klaus Heese[#], Yasuo Nagai, Tohru Sawada
BF Research Institute, Inc., Osaka, Japan

Abstract

Alzheimer's disease (AD) is characterized by the presence of beta-amyloid (Aβ) peptide deposits in the brain and increased Aβ production is thought to be one of the early events in the neuropathogenesis of AD. Here we describe a new protein, p18AβrP, which is up-regulated at mRNA level in oligodendroglial cells by Aβ (1-42). Transfection experiments with p18AβrP as a GFP (green fluorescent protein)-fusion-protein show that this protein is mainly located in the cytoplasm of the cell. Two-hybrid-system-analysis revealed that p18AβrP interacts with rat 70 kd heat shock cognate protein hsc70 and with rat tumor suppressor protein Tid-1. Moreover, p18AβrP inhibits NGF-induced neurite outgrowth and its over-expression leads to neuronal cell death pointing to its pivotal role in the control of cellular survival.

Keywords: apoptosis; differentiation; Gemin6; neurodegeneration; ras activation

Introduction

Alzheimer's Disease (AD) is the most common neurodegenerative disorder of the elderly, and it is characterized clinically by progressive memory loss, as well as other cognitive impairments. The neuropathological hallmarks of AD include neuritic amyloid plaques, cerebrovascular amyloidosis and neurofibrillary tangles. Deposits of amyloid fibers consist mainly of an amyloid beta-peptide (Aβ) and increasing experimental and genetic evidences point to an essential role of Aβ, a 39-43 amino acid peptide derived from proteolytic processing of amyloid precursor protein (APP), in the pathogenesis of AD[1]. Mutations in the APP encoding gene and also in presenilin 1 and presenilin 2, which lead to early-onset AD, are associated with excess Aβ deposition in the brain of AD patients. Aβ itself can be cytotoxic and different lines of evidence support a causative role of Aβ in the pathogenesis of AD[2].

However, the mechanism for the degeneration of nerve cells and synaptic connections that underlies the emergence of dementia, in particular in sporadic AD, is still unknown. To further explore the cause of neuronal degeneration in AD, we started a fundamental gene expression analysis using the cDNA subtraction technology. We used this technology to elucidate the genetic mechanism involved in AD by investigating the toxic effect of Aβ (1-42) and its influence on neuronal and glial gene expression[3,4].

In the present study we describe a new protein, rat p18AβrP, which showed the most significant up-regulation in oligodendrocytes upon stimulation with Aβ (1-42) and we confirmed the result by reverse transcription-polymerase chain reaction (RT-PCR) analysis.

Moreover, we have subcloned the open reading frame of p18AβrP in-frame with the green fluorescent protein (GFP) to study the subcellular localization of p18AβrP by using fluorescent light microscopy as it has been described recently as a novel visual classification approach[5]. To characterize the functional role of p18AβrP we have analyzed the pathophysiological outcome of neuronal p18AβrP expression. Finally, we investigated the cellular function of p18AβrP by applying the two-hybrid system - which is an *in vivo* yeast-based system that identifies the interaction between two proteins (X and Y) by reconstituting an active transcription factor - and we detected that rat 70 kd heat shock cognate protein hsc70 and rat Tid-1 tumor suppressor protein act as new p18AβrP-interacting proteins.

Materials and methods

Reagents

Unless indicated, all reagents used for biochemical methods were purchased from Sigma-Aldrich (Tokyo, Japan).

Reverse transcription-polymerase chain reaction (RT-PCR)

The RT-PCR method was used for mRNA expression analyses as described previously[6]. Briefly, total cellular RNA

#**Correspondence:** Klaus Heese, PhD, BF Research Institute, Inc., c/o National Cardiovascular Center, 5-7-1 Fujishiro-dai, Suita, Osaka 565-0873, Japan 81-6-6834-7646 (Phone), 81-6-6872-8761 (Fax), e-mail: heesek@silver.ocn.ne.jp

was isolated according to the TRIzolÒ Reagent-protocol (Gibco BRL, Grand Island, NY, USA). After extraction with chloroform, RNA was precipitated by adding 1 volume isopropyl alcohol to the aqueous phase, washed with 75% ethanol, resolved in RNase-free water and quantified spectrophotometrically by absorbance at 260 nm. Total RNA (0.2 µg/µl) of each sample was first reverse-transcribed into cDNA (oligo (dT)-primed-SMART^{TM}-cDNA-synthesis (Clontech, Tokyo, Japan); Superscript II^{TM} (Gibco)) according to the manufacturer's protocol, which (0.5 µl) in turn was subjected to PCR amplification (25 µl reaction-volume) using *p18AbrP*-specific primers (sense: 5'-atgagtgaatggacgaagaaaagccccttagaatgggaggat-3'; anti: 5'-tctgggaagctgaaagatggccttgaataagatcctgaattcggg-3'). The numbers of cycles used to amplify each cDNA were chosen to allow the PCR to proceed in a linear range according to the Elongase^{TM} enzyme mix-protocol (Gibco). The amplification steps involved denaturation at 94°C for 1 min, annealing for 50 s at 65°C (AnnT) with specific primers and extension for 1 min at 68°C (AnnT: 65°C/24 cycles). PCR amplification of the constitutively expressed ribosomal protein S12 (AnnT: 60°C/16 cycles) cDNA was used as a measure of input RNA. Controls using RNA samples without RT or controls without RNA were used to demonstrate the absence of contaminating DNA. The PCR reactions were analyzed by electrophoresis in 1.5 % agarose gels followed by alkaline blotting of the fragments onto nylon membranes and subsequent hybridization with specific fluorescein-labelled DNA (S12- and p18AβrP-PCR-products; using PCR fluorescein labeling mix (Roche Diagnostics, Mannheim, Germany) probes. Detection and appropriate analysis of the membranes were done with the Fluor Imager 595 / Image Quant ver. 5.0 (Molecular Dynamics, Tokyo, Japan). In addition to non-parametric statistical testing (Kruskal-Wallis test), statistical evaluation of results was performed by analysis of variance (ANOVA) and the statistical error was indicated as the SEM (standard error of the mean).

cDNA cloning

After cDNA subtraction rat *p18AβrP* EST (expressed sequence tag) sequence was obtained. Full-length cDNA has been gained using oligonucleotides designed from partial cDNA/EST sequences in public databases (http://www.ncbi.nlm.nih.gov) to screen an appropriate cDNA library (ClonCapture-Ready^{TM} Super DNA (rat brain); Clontech) for 5'-RACE and RT-PCR experiments. A *p18AβrP* construct for sequence analysis was generated by inserting rat *p18AβrP* cDNA into the pCR®II-TOPO® T/A cloning vector (Invitrogen, Tokyo, Japan).

A p18AβrP expression construct was generated by inserting rat *p18AβrP* cDNA in-frame with the green fluorescent protein (GFP) of pcDNA3.1CT-GFP-TOPO® (Invitrogen) at the C-terminus of p18AβrP (p18AβrP-CT-GFP).

p18AβrP *cDNA and protein analyses*

p18AβrP cDNA and protein sequences were used as search tools in the National Center for Biotechnology information (NCBI) Blastp 2.0 program against non redundant GenBank CDS translations + PDB + SwissProt + PIR + PRF databases, in addition to the UniGene database (NCBI)[7]. Homology searching was performed using the Blast and FASTA (Wisconsin Package Version 10.0, Genetics Computer Group (GCG), Madison, WI) algorithms and hits were aligned using BestFit (Wisconsin Package Version 10.0, GCG). Protein sequence motif searching was performed with the PROSITE Profile-, BLOCKS-, ProDom-, PRINTS-, Pfam- and PSORTII-programs[8-10]. Phosphorylation sites were searched by using NetPhos 2.0 protein phosphorylation prediction server[11]. Additionally, protein sequence analysis was performed using the following programs at the ExPASy–www–server (http://www.expasy.ch): softberry: http://www/softberry.com/index.html; and Amino Acid Composition Search (AACompIdent): http://kr.expasy.org/tools/aacomp/.

Cell culture

B104 neuroblastoma and PC12 cells were propagated in Dulbecco's Modified Eagle Medium (D-MEM)/F12 (1:1) containing N2-supplement and 10 % fetal calf serum (FCS; Gibco) at 37 °C in humidified 5% CO_2/95% air. The CHO (Chinese hamster ovary) cell line was propagated in DMEM plus 10 % FCS. The CG-4-(oligodendrocyte progenitor)-cell-line-culture was performed according to Espinosa de los Monteros et al.[12]: Briefly, the CG-4 oligodendrocyte progenitor cell line (kindly provided by Dr. Kazuhiro Ikenaka; Okazaki National Research Center, Aichi prefecture, Japan) was propagated in DMEM/F-12 (1:1 v/v), N1-supplement (5 mg/l of insulin, 16.1 mg/l putrescine, 50 mg/l transferrin, 4.6 mg/l of D-galactose, 8 mg/l sodium selenite, 2.4 g/l HCO_3) + 30% (v/v) conditioned serum-free medium from B104 neuroblastoma cells. For maturation into oligodendrocytes, CG-4 cells were incubated without the B104 mitogenic source for 24 h. Thereafter, 2% FCS (Gibco) was added to the differentiation medium to enhance cell survival as described previously[12,13].

For induction of cell death, cells were incubated without FCS ± Aβ (1-42) (10 mg/ml diluted in serum-free medium, stock-solution: 1mg/ml in phosphate-buffered saline (PBS) pH7.4, 24 h pre-incubation at 37°C; Peptide Institute, Osaka, Japan) for 60 h - 72 h. Thereafter, cell survival was measured by CellTiter 96® AQ_{ueous} One Solution cell proliferation assay (according to the manufacturer's protocol (Promega))[3]. For induction of neurite outgrowth PC12 cells were stimulated with NGF

(50 ng/ml) 24 hs after transfection with p18AβrP-CT-GFP or control vectors (see below).

cDNA subtraction

The application of PCR-Select™-cDNA subtraction (Clontech), a technique based on selective amplification of differentially expressed sequences, enabled us to compare two populations of mRNA and to obtain clones of genes that were expressed in one population (Aβ (1-42)-activated cells) but not in the other (control sample).

For these studies CG4 cells were incubated without FCS ± (Aβ (1-42)) for 60 h and thereafter, cDNA-subtraction was performed as reported previously[3,4].

Both mRNA populations were converted into cDNA by the SMART™-PCR-cDNA-synthesis (Clontech). A modified oligo(dT) primer (CDS primer) primed the first strand synthesis reaction. The SMART™-oligonucleotide-anchor sequence and the polyA$^+$ sequence served as universal priming sites for end-to-end cDNA amplification (LD-PCR). Aβ (1-42)-activated- and control-cDNAs were hybridized, and the hybrid sequences were then removed. Consequently, the remaining unhybridized cDNAs represented genes that were expressed in the Aβ (1-42)-activated population, but were absent from the control mRNA. The subtracted cDNA was cloned into a TOPO®-T/A cloning vector (Invitrogen) and differentially expressed genes were confirmed by Southern-blots and sequencing (ABI PRISM™ BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer, Branchburg, NJ; Sequencer: ABI PRISM Model 310).

Cell transfection

PC12 or CHO cells were transiently transfected with p18AβrP-CT-GFP, GFP (Clontech) expression vector (Electroporation: 0.36 kV/750 µF, BioRad Gene Pulser II system (Bio-Rad, Hercules, CA, USA)) or empty plasmid (controls) and maintained in D-MEM)/F12(1:1)/N2 medium containing 10 % FCS (Gibco) at 37 °C in humidified 5% CO_2/95% air. Transfection efficiency and subcellular distribution of p18AβrP-CT-GFP were assessed by fluorescence microscopy after 72 hrs (Olympus IX70, Tokyo Japan).

Tissue p18AβrP *expression analysis*

For the tissue specific gene expression analysis of *p18AβrP* Rapid-Scan™-Gene-Expression panels (Origene Technologies, Rockville, MD, USA) were used as ready to use tissue cDNAs to perform a non-quantitative RT-PCR analysis. PCR products were analyzed by using a standard 2% DNA electrophoretic agarose E-gel™ (Invitrogen).

Yeast Two-Hybrid System

Rat *p18AβrP* was sub-cloned from pENTR/D-TOPO®- into the pDEST™32-vector (Invitrogen) containing the GAL4 DNA binding domain. pEXP-AD502 was used as an activation domain expression vector containing the ProQuest™ two-hybrid rat brain cDNA library (Invitrogen). The used yeast strain for ProQuest™ two-hybrid system-Gateway™-technology was MaV203.

For selection three reporter genes were used: A single copy of each of three reporter genes (*HIS3*, *URA3* and *lacZ*) are stably integrated at different loci in the yeast genome. The promoter regions of *URA3*, *HIS3*, and *lacZ* are unrelated (except for the presence of GAL4 binding sites). In the ProQuest™ two-hybrid system, in comparison to standard two-hybrid systems, false positives are reduced because three independent transcription events (from distinct promoters) must occur at independent chromosomal loci. Induction of the *HIS3* and *URA3* reporter genes allow two-hybrid-dependent transcription activation to be monitored by cell growth on plates lacking histidine or uracil, respectively. Induction of the *lacZ* gene results in a blue color when assayed with X-gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside). Moreover, two-hybrid-dependent induction of *URA3* results in conversion of the compound 5-fluoroorotic acid (5FOA) to 5-fluorouracil, which is toxic. Hence, cells containing interacting proteins grow when plated on medium lacking uracil, but growth is inhibited when plated on medium containing 5FOA.

This system therefore reduces false positives by:

a) providing four phenotypes [His$^+$ (3AT^R), b-gal, Ura$^+$ and 5FOA^S] for assessing true interactors and

b) using low-copy-number (ARS/CEN) vectors that reduce expression levels and toxicity.

Positive clones were confirmed by retransformation assay: Yeast cells containing potentially interacting proteins harbor both DB-rat *p18AβrP* and AD-Y (Y= e.g. rat *TID-1*). Plasmid DNA isolated from yeast cells containing DB-rat *p18AβrP* and AD-Y (Y= e.g. rat *TID-1*) was introduced into E. coli by electroporation and transformants containing AD-Y (Y= e.g. rat *TID-1*) were selected with ampicillin (or DB-rat *p18AβrP* with gentamicin). The plasmid DNA AD-Y (Y= e.g. *rat TID-1*) from these E. coli cells was transformed into MaV203 together with pDBLeu or DB-rat *p18AβrP* and tested for induction of the reporter genes. True positives induced the reporter genes with pDB-rat *p18AβrP* but not with the pDBLeu control vector alone.

Results

RT-PCR analysis of Aβ (1-42)-induced up-regulation of p18AβrP

At 60 h of treatment with Aβ (1-42) 50% of CG4 cells were dead. Control cultures with serum-free medium alone showed cell death of < 10% at the same time point due to serum deprivation (data not shown).

Applying the cDNA subtraction method we identified p18AβrP as a gene activated by Aβ (1-42) in rat CG4

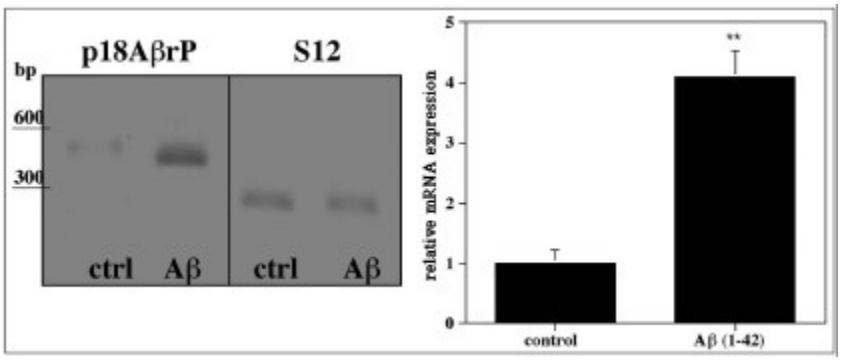


Figure 1. Southern blot analysis of rat CG4 oligodendrocytes stimulated with Aβ (1-42) (10 mg/ml) shows up-regulation of *p18AβrP* mRNA. Cells were stimulated (lane 1+2 serum-free conditions) for 60 h with the indicated stimuli (lane 1: control (ctrl); lane 2: Aβ (1-42)-stimulated cells (Aβ). Left: Southern blot analysis of PCR-products. Right: Quantification of *p18AβrP* mRNA transcripts. Values are the ratio of densitometric scores for *p18AβrP*- and S12- PCR-products ± SEM of six independent experiments. (** P< 0.01, compared to unstimulated controls).

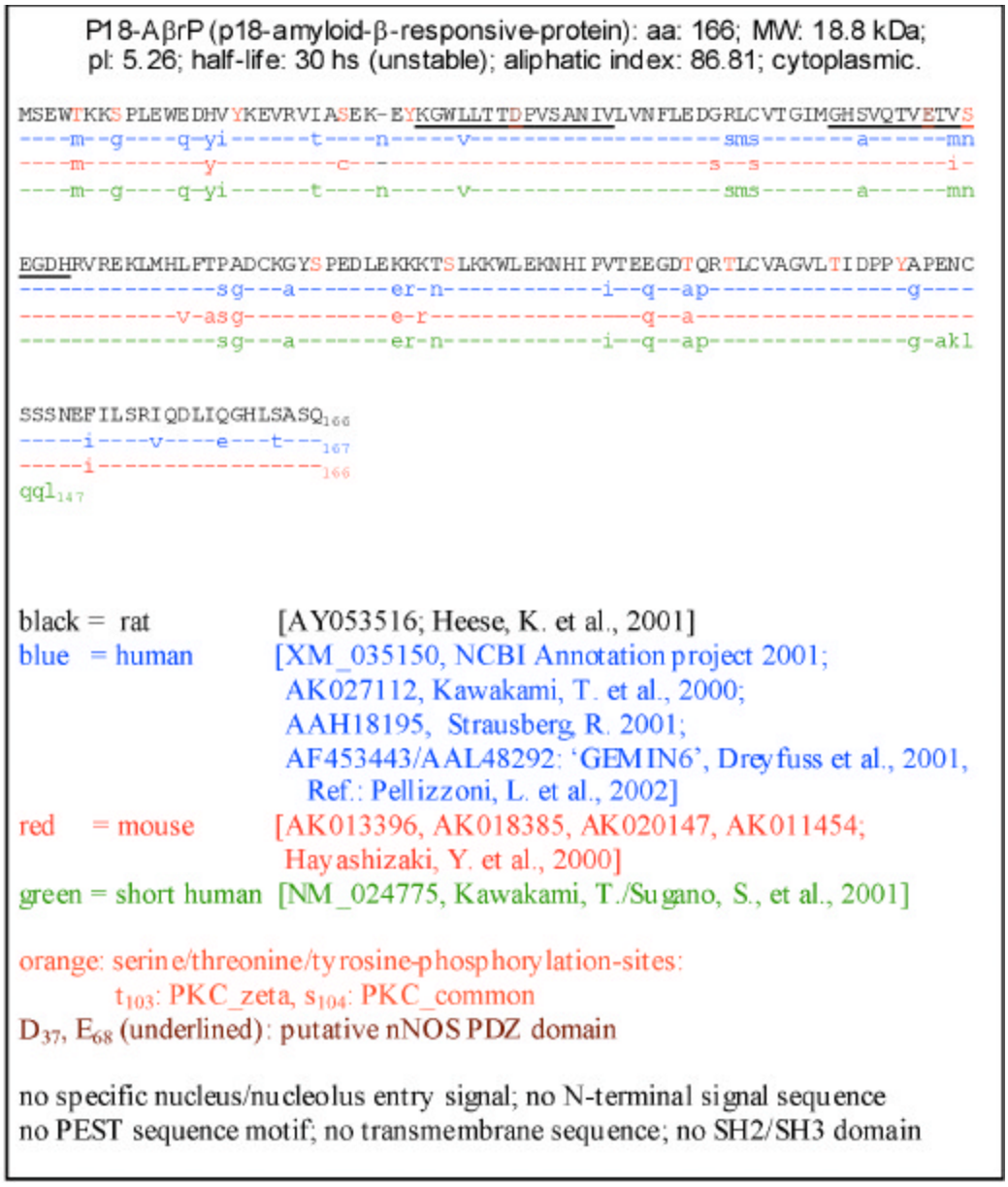


Figure 2.
Characteristic features of p18AβrP/Gemin6.

oligodendroglial cells. We confirmed the Aβ (1-42)-induced increase of p18AβrP mRNA expression by RT-PCR analysis (Figure 1).

The data presented clearly show that Aβ (1-42) up-regulates p18AβrP mRNA in rat CG4 oligodendroglial cells.

Our protein sequence examinations testify that this protein does not belong to any other protein family and no functional protein domain could be identified (Figure 2).

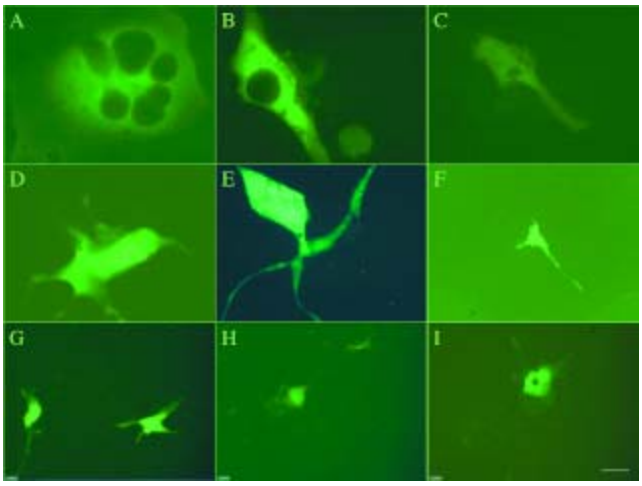


Figure 3.
Expression of p18AβrP as a GFP fusion protein in CHO cells. Scale bar represents 75 µm (a-e) and 25 µm (f-i), respectively.

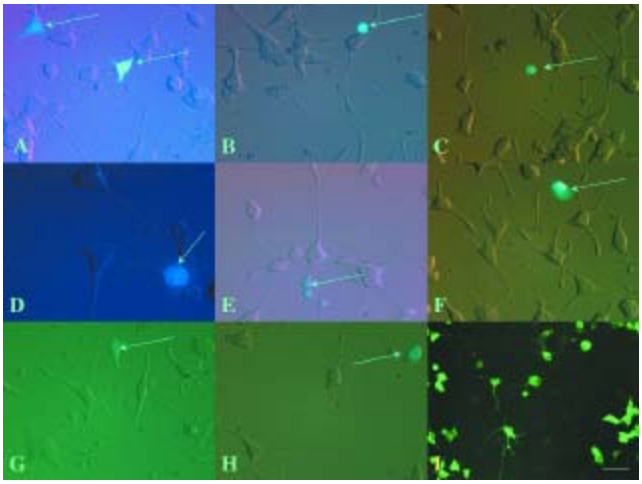


Figure 4. p18AβrP inhibits NGF-induced neurite outgrowth in neuronal PC12 cells.
Expression of p18AβrP as a GFP fusion protein in PC12 cells. 24 hrs post-transfection, cells were treated for 120 hrs with NGF (50 ng/ml); I: Control PC12 cells expressing only GFP. Scale bar represents 75 µm (a), 50 µm (b-h) and 25 µm (i), respectively.

Expression of p18AβrP *in CHO and neuronal PC12 cells*

To investigate the possible physiological function of p18AβrP we transfected CHO cells with a p18AβrP-GFP fusion protein. The characterization by fluorescence microscopy demonstrates that p18AβrP is particularly located in the cytoplasm (Figures 3 and 4). However, as shown in figures 3D and 3F, it could also be detected in the nucleus of the cell.

p18AβrP *inhibits NGF-induced neurite-outgrowth in PC12 cells*

To explore the cellular relevance of Aβ (1-42)-mediated up-regulation of p18AβrP, we expressed this protein in neuronal PC12 cells. Figure 4 indicates that p18AβrP-positive cells do not show neurites upon stimulation with NGF for 120 hs. In contrast, non-transfected cells and cells expressing only GFP clearly display neurite-outgrowth upon NGF stimulation under the same cell culture conditions. Moreover, oligodendrocytes

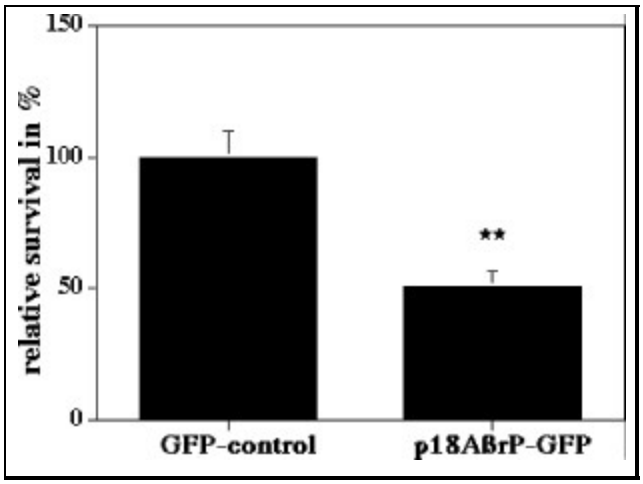


Figure 5.
Effect of p18AβrP expression on survival of PC12 cells. ELISA-CellTiter 96® AQ_{ueous} Assay (Promega). Cells were incubated as described in material and methods. Data are shown as mean ± SEM of eight independent experiments, each done in duplicate (**P < 0.01, compared to controls (only GFP-transfected cells), ANOVA).

Figure 6. Expression of *p18AβrP* mRNA in various tissues: non-quantitative mRNA expression analysis by RT-PCR.
1 = brain; 2 = heart; 3 = kidney; 4 = spleen; 5 = liver; 6 = colon; 7 = lung; 8 = small intestine; 9 = muscle; 10 = stomach; 11 = testis; 12 = salivary; 13 = thyroid; 14 = adrenal gland; 15 = pancreas; 16 = ovary; 17 = uterus; 18 = prostate; 19 = skin; 20 = plasma blood leucocytes; 21 = bone marrow; 22 = fetal brain.

(cell death occurred immediately post transfection, data not shown) and neurons cannot survive for longer than about two weeks after p18AβrP transfection (see for instance Figures 4C, 4F and 5).

p18AβrP *is an ubiquitous expressed protein*

By using the RT-PCR method we analyzed the expression pattern of p18AβrP and detected that its mRNA is expressed in all tissues investigated (Figure 6).

p18AβrP *interact with hsp70 and Tid-1*

Using the yeast ProQuest™ two-hybrid system we found that p18AβrP interacts with various proteins (Table 1), in particular a) with rat 70 kd heat shock cognate protein hsc70, a cytosolic molecular chaperon that is a constitutively expressed member of the HSP70 family and b) with rat Tid-1 (tumorous imaginal disc protein Tid56-like protein intermediate form), a member of the DnaJ family of proteins which serve as co-chaperons to/and interacts with Hsp70/Hsc70 proteins.

In addition, gephyrin and rat brain creatine kinase Ckb were considered to be new binding partners of p18AβrP.

Discussion

In the present study we describe for the first time p18AβrP as a new protein interacting with proteins of the Hsp70 and Tid-1 families and which is up-regulated upon cellular Aβ-peptide stimulation. Moreover, we present data clearly demonstrating the inhibitory effect of p18AβrP on neuronal differentiation and survival.

Interestingly, Tid-1 belongs to the ubiquitously expressed DnaJ family of proteins and serves as a regulatory factor to the conserved heat shock 70 (Hsp70) superfamily of molecular chaperones[14]. Tid-1 has been shown to be a mitochondrial modulator of apoptosis and associated with Hsp70 proteins[15]. The molecular chaperone complex is involved in cellular signaling pathways linked to apoptosis, protein folding, and membrane translocation and in modulation of the activities of tumor suppressor proteins, including retinoblastoma, p53, and WT1[16]. In addition, Tid-1 defines a ras-GTPase-activating protein (rasGAP)-binding protein[17]. Among the three identified isoforms Tid-1_I (the intermediate-isoform which we have found to interact with p18AβrP) binds preferentially to rasGAP, particularly upon growth factor activation. Although the outcome of this interaction is still unclear (activation or suppression of p21ras; Trentin et al., 2001)[17], neuronal differentiation/survival is modified as p21ras is a key regulator of this pathway[18].

As pointed out by Trentin et al., the cellular background in which Tid-1 is expressed can influence whether it resides in the cytosol, mitochondria, or is found in the nucleus[17]. Thus, the cellular context is crucial for the in vivo function of Tid-1 and therefore also for p18AβrP. This is of particular interest with respect to the recent observation by Pellizzoni et al. (2002) who identified p18AβrP by immunoprecipitation-experiments and called p18AβrP as Gemin6 due to its association with the survival of motor neuron (SMN) protein[19].

Spinal muscular atrophy (SMA) is an autosomal-recessive neurodegenerative disorder that is caused by homozygous mutations or deletion of the telomeric copy of the *SMN* gene on human chromosome 5q13. The SMN protein is part of multiprotein complexes in the cytoplasm and the nucleus that are involved in spliceosomal small-nuclear RNP assembly. Additionally, the SMN protein is involved in critical steps of ribosome production, messenger RNA transcription and pre-mRNA splicing[20]. Moreover, it is of interest to mention that the SMN protein activity is down-regulated during neuronal differentiation[21]. Thus, up-regulation of p18AβrP/Gemin6 may act contrary to this SMN-down-regulation during neuronal differentiation and may finally result in cell death as we could observe in PC12-transfected cells.

The fact that SMN can serve as an anti-apoptotic factor in neuronal cells is in agreement with other recent outcomes showing that disturbance of the functional interaction between SMN and p53 (p53 is a multifunctional factor

Table 1 The Yeast-Two Hybrid system analysis reveals four new p18AβrP-interacting proteins

Protein name	GenBank No.	Beta-gal-activity
Rat Tid-1, intermediate form (#)	AY077460	******
Rat Hsc70	NM_024351	*****
Rat gephyrin	X66366	***
Rat brain creatine kinase	M14400	*

* = indicates the strength of each interaction. # = rat sequence identified by Heese et al.

Ebixa® – the name

Product information (abbreviated from the SmPC) Name: Ebixa®. **Active substance:** Memantine. **Indication:** Treatment of patients with moderately severe to severe Alzheimer's disease. **Contraindications:** Hypersensitivity to active substance or any of the excipients. **Special warnings and precautions:** Not recommended for patients with severe renal impairment. Caution is recommended with patients suffering from epilepsy. Clinical data are limited on patients with myocardial infarction, congestive heart failure and uncontrolled hypertension and patients with these conditions should be closely supervised. **Interactions:**Concomitant use of amantadine, ketamine or dextromethorphan should be avoided. Effects of L-dopa, dopaminergic agonists and anticholinergics may be enhanced. Effects of barbiturates and neuroleptics may be reduced. Effect of dantrolene and baclofen may be modified. Plasma levels of cimetidine, ranitidine, procainamide, quinidine and nicotine may be increased. Urinary pH increase may elevate plasma levels of memantine. **Common adverse reactions:** Hallucinations, confusion, dizziness, headache and tiredness (none above 2%). Uncommon adverse reactions (0,1 – 1%): Anxiety, increased muscle tone, vomiting, cystitis and increased libido.

to remember

PostScript Picture
(Eba_4c.eps)

The first and only drug in a new class – effective even in severe stages of Alzheimer's disease[1,2]

PostScript Picture
(Lm_sp+4C.eps)

Posology: Maintenance dose is 20 mg, (10 mg twice daily) taken with or without food. Treatment starts with 5 mg in the morning for a week; the 2nd week 5 mg twice daily; the 3rd week 10 mg in the morning and 5 mg in the afternoon and from 4th week 10 mg twice daily. Reduce dose to 5 mg twice daily in patients with moderate renal impairment. **Overdose:** Symptomatic treatment. Elimination: Mainly in unchanged form via the kidneys. **Administration:** Orally as tablets (10 mg) or solution (10 mg/g). **Marketing authorisation:** H. Lundbeck A/S, 9 Ottiliavej, DK-2500 Valby, Denmark. **References: 1.** Winblad B, Poritis N. Memantine In Severe Dementia: Results Of The M-Best Study (Benefit And Efficacy In Severely Demented Patients During Treatment With Memantine). Int J Geriat Psychiatry 1999;14:135-146 **2.** Reisberg B, Windscheif U, Ferris SH, Stoeffler A, Moebius H-J, and the Memantine Study Group. Memantine in moderately severe to severe Alzheimer's disease: results of a 6-month multicenter randomized controlled trial. Neurobiology of Aging 2000; 21 (1S): S1275.

involved in cell cycle control, DNA repair, transcription activation and apoptosis) leads to apoptosis [22,23].

Taking these findings into account, our presented data indicate that Aβ may induce cell death via up-regulation of p18AβrP and thereby: a) impairing the anti-apoptotic functionality of SMN and/or b) modifying the p21ras-/Tid-1-signaling pathways. However, further analyses are necessary to clarify the physiological and pathophysiological role of p18AβrP/Gemin6.

In conclusion, our findings point to the pivotal role of p18AβrP as a new key component in the Tid-1/Hsp70-protein complex modulating neuronal cell growth, differentiation and survival - probably via controlling the p21ras-signaling pathway. In addition, the fact that p18AβrP is up-regulated by Aβ peptide and associated with the SMN-complex points to its involvement in processes of neuronal degeneration and makes it to a possible new therapeutic target for the treatment of neurodegenerative diseases.

References

1. Bayer T.A., Wirths O., Majtenyi K., Hartmann T., Multhaup G., Beyreuther K., Czech C., Key factors in Alzheimer's disease: beta-amyloid precursor protein processing, metabolism and intraneuronal transport. *Brain Pathol* 2001; 11**:** 1-11.
2. Selkoe D.J., Alzheimer's disease: genes, proteins, and therapy. *Physiol Rev* 2001; 81: 741-766.
3. Heese K., Nagai Y., Sawada T., Induction of rat L-phosphoserine phosphatase by amyloid-b (1-42) is inhibited by interleukin-11. *Neurosci Lett* 2000; 288: 37-40.
4. Heese K., Nagai Y., Sawada T., Identification of a new synaptic vesicle protein 2B mRNA transcript which is up-regulated in neurons by amyloid beta peptide (Aβ) fragment (1-42). *Biochem Biophys Res Commun* 2001; 289: 924-928.
5. Hoja M.R., Wahlestedt C., Höög C., A visual intracellular classification strategy for uncharacterised human proteins. *Exp Cell Res* 2000; 259: 239-246.
6. Heese K., Nakayama T., Hata R., Masumura M., Akatsu H., Li F., Nagai Y., Yamamoto T., Kosaka K., Suemoto T., Sawada T., Characterizing CGI-94 (comparative gene identification-94) which is down-regulated in the hippocampus of early stage Alzheimer's disease brain. *Eur J Neurosci* 2002; 15: 79-86.
7. Altschul S.F., Madden T.L., Schäffer A.A., Zhang J., Zhang Z., Miller W., Lipman D.J., Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Res* 1997; 25: 3389-3402.
8. Bateman A., Birney E., Durbin R., Eddy S.R., Finn R.D., Sonnhammer E.L.L., Pfam 3.1: 1313 multiple alignments match the majority of proteins. *Nucleic Acids Res* 1999; 27: 260-262.
9. Horton P., Nakai K., A probabilistic classification system for predicting the cellular localization sites of proteins. *Intellig Syst Mol Biol* 1996; 4: 109-115.
10. Horton P., Nakai K., Better prediction of protein cellular localization sites with the k nearest neighbor classifier. *Intellig Syst Mol Biol* 1997; 5: 147-152.
11. Blom N., Gammeltoft S., Brunak S., Sequence- and structure-based prediction of eukaryotic protein phosphorylation sites. *J Mol Biol* 1999; 294: 1351-1362.
12. Espinosa de los Monteros A., Yuan J., McCartney D., Madrid R., Cole R., Kanfer J.N., de Vellis J., Acceleration of the maturation of oligodendroblasts into oligodendrocytes and enhancement of their myelinogenic properties by a chemically defined medium. *Dev Neurosci* 1997; 19: 297-311.
13. Yonemasu T., Nakahira K., Okumura S., Kagawa T., Espinosa de los Monteros A., de Vellis J., Ikenaka K., Proximal promoter region is sufficient to regulate tissue-specific expression of UDP-galactose: ceramide galactosyltransferase gene. *J Neurosci Res* 1998; 52: 757-765.
14. Kelley W.L., Molecular chaperons: How J domains turn on Hsp70s. *Curr Biol* 1999; 9: R305-R308.
15. Syken J., De-Medina T., Münger K., TID1, a human homolog of the Drosophila tumor suppressor l(2)tid, encodes two mitochondrial modulators of apoptosis with opposing functions. *Proc Natl Acad Sci U S A* 1999; 96: 8499-8504.
16. Cheng H., Cenciarelli C., Shao Z., Vidal M., Parks W.P., Pagano M., Cheng-Mayer C., Human T cell leukemia virus type 1 Tax associates with a molecular chaperone complex containing hTid-1 and Hsp70. *Curr Biol* 2001; 11: 771-775.
17. Trentin G.A., Yin X., Tahir S., Lhotak S., Farhang-Fallah J., Li Y.,Rozakis-Adcock M., A mouse homologue of the Drosophila tumor suppressor l(2)tid gene defines a novel Ras GTPase-activating protein (RasGAP)-binding protein. *J Biol Chem* 2001; 276: 13087-13095.

Plasma Lipids in Patients Newly Diagnosed With Probable Alzheimer's Disease

Annica Algotsson[#], Bengt Winblad

Department of Neurotec, Division of Geriatric Medicine, Karolinska Institutet, Sweden

Abstract

Plasma lipids and lipoproteins have been assessed in 109 Swedish patients (38 men and 71 women, mean age 70.2±9.8 years, range 49 - 90) newly diagnosed with probable Alzheimer's disease (AD). The apolipoprotein E ε4 allele (apoE4) had an impact on total cholesterol (cholesterol) values. Lipid values of female and male patients did not exceed those of female and male subjects in a large Swedish population study. However, the cholesterol values of the majority of the patients were not optimal according to European guidelines, and half of the patients in the present study had other vascular risk factors. Results from several clinical studies as well as studies on transgenic mouse models and cultured neural cell lines indicate that cholesterol might contribute to the pathogenesis of AD. Treatment with 3-hydroxy-3-metylglutaryl coenzyme A reductase inhibitors (statins) early in the disease process might be indicated in the near future in AD and also in patients with mild cognitive impairment (MCI). However, randomized, placebo-controlled trials ought to precede such therapeutic strategies.

There were gender differences with higher values in female patients and in female subjects ≥60 years of age in the large study concerning cholesterol and high-density lipoprotein cholesterol (HDL-C). It has previously been suggested that the incidence and prevalence of AD is greater in women. Differences in plasma lipids between the genders might offer an explanation to these findings.

Keywords: Alzheimer's disease; Swedish population; total cholesterol; high-density lipoprotein cholesterol; apolipoprotein E alleles; statins; gender.

Introduction

Results from clinical studies as well as studies on transgenic mouse models and cultured neural cell lines indicate that cholesterol might contribute to the pathogenesis of AD. Notkola et al.,1998[1] and Kivipelto et al., 2001[2] found that a previous high serum cholesterol level was a significant predictor of AD in population-based studies. In two epidemiological, cross-sectional studies, treatment with statins decreased the prevalence of AD[3] and of all types of dementia[4]. However, in a randomized, placebo-controlled trial in 20 536 patients,15454 men and 5082 women (with 5806 subjects aged at least 70 years at study entry), with various forms of occlusive arterial disease and/or diabetes mellitus and/or hypertension, treatment with simvastatin (a hydrophobic statin) 40 mg/d had no effect on the percentage of participants classified as cognitively impaired in the two treatment groups at follow up after 5 years of treatment. The diagnostic instrument used in this classification was a modified telephone interview for cognitive status. Similar numbers of participants (n = 31) were reported to have developed dementia[5].

Statins do more than just lower cholesterol: they also improve endothelial function[6], decrease platelet activity[7] and reduce inflammation[8]. There is evidence that inflammation in the central nervous system (CNS) is an early event in the pathogenesis of AD[9] and there is evidence of oxidative damage in Alzheimer's disease brain[10]. In combination with cholesterol, a number of other vascular risk factors are now considered risk factors for AD as well[11]. The pathologic event common to all forms of AD is the abnormal accumulation of the amyloid beta-peptide (Abeta). Abeta is derived by proteolytic cleavage from the amyloid precursor protein (APP), a type I integral membrane protein that is ubiquitously expressed. Recent studies suggest a link between the processing of APP and cholesterol. In neural cells overexpressing APP, reduction of cholesterol levels stimulated the non-amyloidogenic pathway by means of

#**Correspondence:** Annica Algotsson, Geriatric Clinic, B56, Huddinge University Hospital, SE-141 86 Huddinge, Sweden. Telephone +46 8 585 854 39; fax +46 8 585 854 82. Email address: Annica.Algotsson@neurotec.ki.se

increasing the activity of the α-secretase ADAM 10. The increase in α-secretase activity resulted in decreased secretion of Abeta[12]. Eckert et al., 2000[13], found that the cholesterol content in hippocampal membranes of AD brains was correlated with the membrane-disordering effects of Abeta. Cholesterol-reducing drugs decreased serum concentrations of Abeta in humans with elevated low density lipoprotein cholesterol[14] and reduced the accumulation of Abeta in the brain in a transgenic mouse model of AD[15].

The apoE4, being an important lipid regulatory protein and at the same time the major susceptibility gene for AD, represents a link between cholesterol and AD. Alzheimer patients who are apoE4 homozygotes have earlier ages of onset, increased amyloid burden, decreased number of nicotinic receptor binding sites and faster rates of cognitive decline[16]. The apoE frequency and the association between apoE genotype and AD are reported to vary in different populations[17]. In a healthy Swedish population, the relative frequency of apoE4 amounted to 0.20 (0.15 in individuals >60 years of age)[18]. Lipid profiles have also been reported to differ between different populations [19,20]. Studies on plasma cholesterol and HDL-C in Swedish Alzheimer patients are sparse. The aim of the study was to assess plasma lipids in patients with probable Alzheimer's disease early in the disease process after exclusion of cases with conditions predisposing to secondarily deranged lipid homeostasis.

Methods

Study population

Records were examined of patients diagnosed with probable Alzheimer's disease according to the ICD-10, codes F000 and F001[21] and the NINCDS-ADRDA-criteria[22] during the period of January 1998 - November 2001 at the Geriatric clinic of Huddinge University Hospital. The clinic bears resposibility for investigation of all patients with early onset dementia in Greater Stockholm and for investigation of patients with late-onset dementia in the south-western part of Stockholm (the latter catchment area included approximately 35 000 inhabitants > 65 years of age). Patients who were hospitalized during the diagnostic procedures were not considered for inclusion - only cholesterol values but not HDL-C and triglyceride values were available for these patients. Additional exclusion criteria (to exclude patients with conditions predisposing to secondarily deranged lipid homeostasis): Treatment with lipid-lowering drugs or acetylcholine esterase inhibitors before the diagnosis, alcohol overconsumption, acute infection or other significant acute or chronic diseases (for instance gastrointestinal bleeding, cancer disease, liver or kidney disease or untreated thyroid disease) before and during the diagnostic procedures. Patients with insulin-dependent diabetes mellitus were excluded, but patients with non insulin-dependent diabetes (n = 8) were accepted in the study. Patients with symptoms and sign of cerebrovascular disease were also excluded. Mild to moderate non-specific white matter lesions were not reasons for exclusion. 109 patients, 71 women and 38 men (mean age 70.2±9.8 years, range 49 - 90), fullfilled the inclusion/exclusion criteria. 50 patients, 18 men and 32 women, had early onset of the disease. 20 very healthy, age-matched, non-obese, non-smoking individuals without medication, (10 men and 10 women) from earlier studies[23,24] served as controls. The controls < 65 years of age belonged to the staff of Huddinge University Hospital and the rest of the controls were volunteers from pensioners' associations in the south-western part of Stockholm. For inclusion in the study, the mini-mental state examination (MMSE) values[25] of the controls had to be ≥ 28/30.

Procedures

The patients were examined according to the clinical routine of the Geriatric clinic. At the first visit to the geriatric clinic non-fasting blood samples were drawn when the patient had been in the sitting position for at least 10 minutes and the blood samples were directly analysed by the chemical laboratory of Huddinge University Hospital. Plasma concentration of cholesterol[26], and triglycerides[27] were measured by enzymatic techniques. HDL-C was also measured directly by an enzymatic technique[28]. On the same day the patients underwent a physical examination including one measure of blood pressure in the recumbent position. Body mass index (BMI) was calulated as weight (kg)/height $(m)^2$. Their medical history was obtained both from the patient himself and from a near relation. The diagnostic investigations performed on the patients included neuropsychological testing, EEG, magnetic resonance imaging (MRI) or computed tomography (CT) and single proton emission computed tomography of the brain (SPECT). Values of tau protein in the cerebrospinal fluid (CSF) were measured in 53 patients (21 men and 32 women). The controls underwent a physical examination and had their fasting blood samples examined at the laboratory of Huddinge University Hospital. Because of methodological changes in the assessement of HDL-C in 1996 and 1998, HDL-C values of the controls have been slightly upregulated (3-10%, according to recommendations from the laboratory) in order to allow comparisons with the values of the patients.

The identities of the patients were carefully protected, the controls gave their informed consent and the study was accepted by the Ethics Committe of Huddinge University Hospital.

Statistics

Descriptive data are presented as the mean±standard deviation (SD). Triglyceride values and values of the cholesterol/HDL cholesterol quotient were not normally

distributed and were because of that transformed into natural logarithms before being used in the analyses. Associations between plasma lipids and the ApoE4 genotype, gender and presence of vascular risk factors were assessed by analyses of covariance (ANCOVA). Group differences were evaluated by an unpaired two-tailed Student's t-test. The significance level was adjusted according to Bonferroni/Dunn.

Results

The relative frequency of the apolipoprotein E alleles of all patients was 0.04 for the E2 allele, 0.53 for the E3 allele and 0.43 for apoE4. The corresponding values for the patients with early onset of disease were 0.02 for the E2 allele, 0.48 for the E3 allele and 0.50 for apoE4. There were no gender differences with respect to apoE4. The mean value of CSF tau protein for 53 patients (see above) exceeded 700 ng/L. In ANCOVA tests cholesterol was independently associated with gender ($p < 0.001$) and the apoE4 genotype ($p = 0.001$) and there were independent positive associations between cholesterol on one side and triglycerides ($p = 0.001$) and BMI (< 0.001) on the other. There was also a trend ($p = 0.059$) towards an independent positive association between cholesterol and HDL-C. HDL-C was independently associated with gender ($p < 0.001$) and negatively associated with triglycerides ($p < 0.001$) and there was a trend towards a negative association between HDL-C and BMI ($p = 0.066$). Triglyceride levels were independently and positively associated with BMI ($p = 0.002$). The cholesterol/HDL-C quotient was independenly associated with apoE4 ($p = 0.039$) and independently and positively associated with triglycerides ($p < 0.001$) and BMI ($p < 0.001$). Selected characteristics of the Alzheimer patients are presented in Table 1. The patients have been grouped according to presence of apoE4 and gender. Table 2 shows the presence of vascular risk factors in the patient groups divided according to gender and presence of apoE4. Half of the patients had vascular risk factors and 14 patients had more than one vascular risk factor. There were no significant differences between these groups and the values were therefore pooled. The presence of one to three vascular risk factors had no

Table 1. Selected characteristics of patients with probable Alzheimer's disease. MEAN±SD.

	MEN		WOMEN	
	without apoE4	with apoE4	without apoE4	with apoE4
number	12	26	21	50
age(years)	72.9±11.2	70.5±8.6	70.1±11.0	69.5±9.6
cholesterol (mmol/L)	4.8±0.7*	5.8±0.7†	6.2±0.9‡	6.7±1.1
HDL-C (mmol/L)	1.5±0.4?	1.5±0.3#	1.9±0.5	1.8±0.5
chol/HDL	3.52±0.93	4.18±1.09**	3.50±1.00	4.00±1.23
triglyceride (mmol/L)	1.2±0.4	1.7±1.1	1.3±0.6	1.4±0.9
BMI	23.3±3.5	23.9±3.2	25.0±3.0	24.0±4.8
MMSE	22.0±5.7	22.3±4.7	21.3±3.9	22.0±0.5
duration (years)	4.0±3.2††	3.7±2.0††	2.3±1.5	3.2±1.8

SD = standard deviation; apoE4 = apolipoprotein E ε4 allelle; cholesterol = total cholesterol; HDL-C = high-density lipoprotein cholesterol; chol/HDL = the quotient of total cholesterol/high-density lipoprotein cholesterol; BMI = body mass index; MMSE = mini-mental state examination; duration = duration of the disease.
p-values < 0,0083 are considered significant (according to Bonferroni/Dunn).
*men without E4 differed from men with E4 and from women without and with E4 ($p < 0.001$).
†men with E4 differed from women with E4 ($p < 0.001$).
‡there was a trend towards a difference between women without E4 and women with E4 ($p = 0.048$).
?there were trends towards differences between men without E4 and women without E4 ($p = 0.017$) and women with E4 ($p = 0.026$).
#men with E4 differed from women without E4 ($p < 0.001$) and women with E4($p = 0,002$).
**there was a trend towards a difference between men with E4 and women without E4 ($p = 0.028$).
††there was a trend towards a difference between men without E4 ($p = 0.046$) and with E4 ($p = 0.016$) and women without E4.

independent impact of the plasma lipids of the patients when controlling for gender and presence of apoE4. 13 female patients were on oral medication with oestrogens. Oestrogen therapy had no impact of plasma lipids when controlling for presence of apoE4 (values not shown). Table 3: The cholesterol and HDL-C values and the cholesterol/HDL-C quotient of men without apoE4 and with early onset of the disease did not differ from the values of men without apoE4 and late onset of the disease. The same was also true for the men with apoE4 and the women with and without this allele - the age of onset of the disease did not matter. Table 4: The cholesterol and HDL-C values and the cholesterol/HDL-C quotient of male patients did not differ from the values of

Table 2. Probable Alzheimer's disease. Number of patients (precent within parentheses) with different vascular risk factors.

	MEN		WOMEN	
	without apoE4	with apoE4	without apoE4	with apoE4
smokers	1/12 (8%)	4/26 (15%)	6/21 (29%)	6/50 (12%)
NIDD	0	1/26 (4%)	4/21 (19%)	3/50 (6%)
cardiac disease	1/12 (8%)	4/26 (15%)	3/21 (14%)	5/50 (10%)
BP≥160/90 (or medication for high BP)	5/11* (45%)	7/23† (30%)	10/20‡ (50%)	13/46? (28%)
HDL-C≤0,9	1/12 (8%)	1/26 (4%)	0	0
BMI>30	0	0	0	1/50 (2%)

apoE4 = the apolipoprotein E ε4 allelle; NIDD = non insulin-dependent diabetes; cardiac disease = ischemic heart disease and /or cardiac insuffiency and/or atrial fibrillation and/or valvular disease; BP = blood pressure (mm Hg). HDL-C = high-density lipoprotein cholesterol; BMI = body mass index. *BP missing for 1 male· †BP missing for 3 males. ‡BP missing for 1 woman. ?BP missing for 4 women.
The presence of one to three vascular risk factors had no independent impact of the plasma lipids of the patients when controlling for gender and presence of apoE4.

Table 3. ProbableAlzheimer's disease - cholesterol, HDL-C and cholesterol/HDL-C values in early and late onset of the disease (Mean±SD).

	MEN							
	EARLY ONSET				LATE ONSET			
	nr	chol	HDL-C	chol/HDL-C	nr	chol	HDL-C	chol/HDL-C
without apoE4	4	4.7±0.8	1.3±0.5	3.97±1.12	8	4.9±0.6	1.5±0.3	3.29±0.81
with apoE4	14	5.9±0.7	1.5±0.3	4.16±1.10	12	5.8±0.7	1.5±0.4	4.21±1.13

	WOMEN							
	EARLY ONSET				LATE ONSET			
	nr	chol	HDL-C	chol/HDL-C	nr	chol	HDL-C	chol/HDL-C
without apoE4	7	5.8±0.8	1.9±0.4	3.05±0.53	14	6.4±1.0	1.8±0.5	3.72±1.12
with apoE4	25	6.6±0.9	1.9±0.5	3.81±1.13	25	6.8±1.3	1.8±0.5	4.18±1.33

SD = standard deviation; nr = number; chol = total cholesterol (mmol/L); HDL-C = high-density lipoprotein cholesterol (mmol/L); chol/HDL-C = the quotient of total cholesterol/ high-density lipoprotein cholesterol; apoE4 = the apolipoprotein E ε4 allelle.
There were no significant differences between early and late onset of disease within gender and apoE4 groups with respect to cholesterol, HDL-C and the cholesterol/HDL-C quotient.

Table 4. Cholesterol, HDL-C and cholesterol/HDL-C values in patients with probable Alzheimer's disease versus healthy controls. Mean±SD.

	male patients	male controls	female patients	female controls
number	38	10	71	10
age(years)	71.3±9.4	70.9±6.1	69.7±10.0	69.4±6.6
cholesterol (mmol/L)	5.5±0.8*	6.0±1.1	6.6±1.1	6.6±1.1
HDL-C (mmol/L)	1.5±0.3†	1.5±0.2‡	1.8±0.5	1.9±0.4
chol/HDL	3.97±1.08	4.01±0.77	3.85±1.19	3.64±0.85

SD = standard deviation; cholesterol = total cholesterol; HDL-C = high-density lipoprotein cholesterol; chol/HDL = the quotient of total cholesterol and high-density lipoprotein cholesterol.
p-values < 0,0083 are considered significant (according to Bonferroni/Dunn).
*male patients differed from female patients ($p < 0.001$) and female controls ($p = 0.001$).
†male patients differed from female patients ($p < 0.001$) and female controls ($p = 0.002$).
‡there was a trend towards a difference between male controls and female patients ($p = 0.031$).

male controls. Likewise, the corresponding values for female patients did not differ from those of female controls.

Discussion

Plasma lipids and lipoproteins have been assessed in 109 Swedish patients newly diagnosed with probable AD after exclusion of cases predisposed to secondarily deranged lipid homeostasis. The patients were in relatively early stages of the disease, mean value of the MMSE being 21.9±4.8. The apoE4 genotype was more frequent among patients with early onset of disease, maybe because of selective mortality among older patients with this allele. The relationship between apoE4 and cholesterol, with higher values of cholesterol in the patients with ApoE4 (Table1), are consistent with findings from population studies[29]. The relationship is also consistent with findings of some authors [1,30,31] but at variance with those of other authors[32] studying patients with AD. The differences in results might be due to effects of ethnicity[17] and differences concerning selection of patients.

The values of cholesterol, HDL-C and the cholesterol/HDL-C quotient of the patients did not differ within the gender groups from those of 20 very healthy controls (Table 4). The patients were non-fasting while the controls were in the fasting state. It has been shown that this factor is of minor importance concerning values of total cholesterol and HDL cholesterol; the nutritional status is, however, of more importance for triglyceride values [33,34].

A population study which provides lipid values from 175 000 Swedish subjects is available[20]. The subjects were mainly recruited from screening programmes. 2/3 had fasted overnight. Little was known about clinical characteristics, the presence of risk factors or confounding effects of possible ongoing treatment, but no one was hospitalized. For the whole material the mean cholesterol value (mmol/L) was approximately 5.9 and did not differ between men and women. In the subjects ≥60 years of age (approximately 16 700 men and 19 900 women), however, the mean cholesterol value was slightly higher among women (approximately 6,6 mmol/L versus 6,1 mmol/L among men). The cholesterol values of male patients seem to be at a slightly lower level (especially when male patients without apoE4 are considered) compared with male subjects in the Swedish population study, while the values of female patients seem to be at approximately the same level as the values of female subjects. However, the cholesterol values of the patients were not optimal (with the exception of some of the men without apoE4) according to European guidelines (total cholesterol should be ≤5.0 mmol/l irrespective of concomitant vascular risk factors)[35]. The HDL-C values of male and female patients seem to be at approximately the same or a somewhat higher level compared with values of male and female subjects. However, methodological differences make the comparison somewhat difficult. The triglyceride values of the patients seem to be at the same or a somewhat lower level than those of the subjects in the population study, in spite of the fact that all of the patients were in the non-fasting state.

The BMI values of the patients were within normal ranges. However, the mean BMI value of the 50 patients with early onset of disease in the present study were lower than the BMI value of 653 men and women 55-64 years of age in a population sample from Göteborg, Sweden[36].

Due to the relatively high HDL-C values of the patients, their lipid profiles seem favourable with cholesterol/HDL-C quotients ≤4.0 and the triglyceride values of the patients were also at a low level (Table 4). Nevertheless, half of the patients had other vascular risk factors (Table 2). High HDL-C values might be dysfunctional and actually represent a risk factor for ischemic heart disease in white women when caused by a

common mutation in the cholesteryl ester transfer protein, CETP[37]. Furthermore, there is evidence of oxidative damage in AD brain, leading to neuronal lipid peroxidation, which may be linked to Abeta[9]. Not only low density lipoprotein cholesterol but also HDL-C particles might be susceptible to oxidative modification. Kiviatinitz et al.,1997[38], found that HDL-C particles, aggregated by oxidation, produced neurodegeneration in cultured cerebral cells. The process was accompanied by disorganization of the cellular microtubular cytoskeleton and hyperphosphorylation of the microtubule-associated protein tau. In a population-based autopsy study, Launer et al., 2001[39], found a linear association for increasing late-life blood HDL-C levels and an increasing number of hippocampal and neocortical neurofibrillary tangles and neocortical neuritic plaques. Thus, the constituents of HDL-C might play a role in the formation of AD pathology.

Changes in plasma cholesterol might alter the input of sterol into the CNS. Virtually all statins cross the blood-brain barrier and might inhibit the rate of cholesterol synthesis in the brain[40]. Statins might also have beneficial effect on markers of inflammation in the CNS[8,9] and on concomitant vascular risk factors. Concerning the putative effect on developement of dementia, however, the effects of various types of statins might differ41. In the epidemiological, cross-sectional study of Wolozin et al., 2000, treatment with lovastatin and pravastatin, but not simvastatin, was associated with a decreased prevalence of AD. However, bias concerning selection of patients might have influenced the results[3].

Conclusions

The cholesterol values of the majority of the patients in the present study were not optimal, and half of the patients had other vascular risk factors. Results from many studies indicate that cholesterol might contribute to the pathogenesis of AD[1,2,11,12,13,14], Furthermore, in some clinical studies treatment with statins was found to reduce the prevalens of AD [3,4] but the findings of a large, randomized, placebo-controlled study, focused on simvastatin treatment (40 mg/d) in cardiovascular disease, are at variance[5]. Treatment with statins early in the disease process in AD and also in MCI (a condition believed to represent a high risk for the development of clinically probable AD)[42] might be justified in the near future. However, randomized, placebo-controlled trials, focused on cognitive disturbances, ought to precede such treatment strategies

In individuals ≥60 years of age in the large Swedish population study[20] and in the present study of Alzheimer patients, cholesterol and HDL-C values of females are higher than in males. Gender differences are also evident in elderly subjects in several ethnically different populations with divergent dietary habits[19]. It has previously been suggested that the incidence and prevalence of AD is greater in women than in men[43,44]. The gender differences concerning plasma lipids might offer an explanation to these findings.

References

1. Notkola I.L., Sulkava R., Pekkanen J., Erkinjuntti T., Ehnholm C., Kivinen P., Tuomiletho J., Nissinen A., Serum total cholesterol, Apolipoprotein E ε4 allele and Alzheimer's disease. *Neuroepidemiology* 1998; 17: 14-20.
2. Kivipelto M., Helkala E.L., Laakso M.P., Hanninen T., Hallikainen M., Alhainen K., Soininen H., Midlife vascular risk factors and Alzheimer's disease in later life: longitudinal, population based study. *BMJ* 2001; 322: 1447-1451.
3. Wolozin B., Kellman W., Rousseau P., Celesia G.G., Siegel G. Decreased prevalence of Alzheimer disease associated with 3-hydroxy-3-methyglutaryl coenzyme A reductase inhibitors. *Arch Neurol* 2000; 57: 1439-1443.
4. Jick H., Zornberg G.L., Jick S.S., Seshadri S., Drachman D.A., Statins and the risk of dementia. *Lancet* 2000; 356: 1627-1631.
5. Heart Protection Study Collaborative Group., MRC/BHF Heart Protection Study of cholesterol lowering with simvastatin in 20 536 high-risk individuals: a randomized placebo-controlled trial. *Lancet* 2002; 360: 7-22.
6. Stroes E.S., Koomans H.A., de Briun T.W., Rabelink T.J., Vascular function in the forearm in hypercholesterolaemic patients off and on lipid-lowering medication. *Lancet* 1995; 346: 467-471.
7. Lacoste L., Lam J.Y., Hung J., Letchacovsky G., Solymoss C.B., Waters D., Hyperlipidemia and coronary disease: correction of the increased thrombogenic potential with cholesterol reduction. *Circulation* 1995; 92: 3172-3177.
8. Strandberg T.E., Vanhanen H., Tikkanen M.J., Effect of statins on C-reactive protein inpatients with coronary artery disease. *Lancet* 1999; 353: 1274.
9. Cagnin A., Brooks D.J., Kennedy A.M., Gunn R.N., Myers R., Turkheimer F.E., Jones T., Banati R.B., In-vivo measurement of activated microglia in dementia. *Lancet* 2001; 358: 461-467.
10. Butterfield D.A., Drake J., Pocernich C., Castegna A., Evidence of oxidative damage in Alzheimer's disease brain: central role for amyloid beta-peptide., *Trends Mol Med* 2001; 7: 548-554.
11. Skoog I., Kalaria R.N., Breteler M.M., Vascular factors and Alzheimer disease. Alzheimer *Dis Assoc Disord* 1999; 13 Suppl 3: S106-114.
12. Korjo E., Gimpl G., Lammich S., Mätrz., Fahrenholz F., Low cholesterol stimulates the nonamyloidogenic pathway by its effect on the α-secretase ADAM 10. *Proc. Natl. Acad. Sci USA* 2001; 98: 5815-5820.
13. Eckert G.P., Cairns N.J., Maras A., Gattaz W.F., Müller W.E., Cholesterol modulates the membrane-disordering effects of beta-amyloid peptides in the hippocampus: specific changes in Alzheimer's disease. *Dement Geriatr Cogn Disord* 2000; 11: 181-186.
14. Friedhoff L.T., Cullen E.I., Geoghagen N.S., Buxbaum J.D., Treatment with controlled-release lovastatin decreases serum concentrations of human beta-amyloid (Abeta) peptide. *Int J Neuropsychopharmacol* 2001; 4: 127-130.

15. Refolo L.M., Pappola M.A., LaFrancois J., Malester B., Schmidt S.D., Thomas-Bryant T., Tint G.S., Wang R., Mercken M., Petanceska S.S., Duff K.E., A cholesterol-lowering drug reduces beta-amyloid pathology in a transgenic mouse model of Alzheimer's disease. *Neurobiol Dis* 2001; 8: 890-899.
16. Craft S., Teri L., Edland S.D., Kukull W.A., Schellenberg G., McCornick W.L., Bowen J.D., Larson E.B., Accelerated decline in apolipoprotein E ε4 homozygotes with Alzheimer's disease. *Neurology* 1998; 51: 149-153.
17. Farrer L.A., Cupples L.A., Haines J.L., Hyman B., Kukull W.A., Mayeux R., Myers R.H., Pericak-Vance M.A., Risch N., VanDuin C.M., Effects of age, sex and ethnicity on the association between apolipoprotein E genotype and Alzheimer's disease. *JAMA* 1997; 278: 1349-1356.
18. Eggertsen G., Tegelman R., Ericsson S., Angelin B., Berglund L., Apolipoprotein E polymorphism in a healthy Swedish population: variation of allele frequency with age and relation to serum lipid concentrations. *Clin Chem* 1993; 39: 2125-2129.
19. Contois J.H., Wu A.H.B., Li Z., Feroce A..H., Grunenberger F., Haller J., deGroot L., Lammi-Keefe L.J., Distribution of serum apolipoproteins A-1 and B and lipoprotein(a) in European elderly. The SENECA study. *Clinica Chimica Acta* 2000; 295: 1-12.
20. Walldius G., Jungner I., Holme I., Aastveit A.H., Kolar W., Steiner E., High apolipoprotein B, low apolipoprotein A-1, and improvement in the prediction of fatal myocardial infarction (AMORIS study): a prospective study. *Lancet* 2001; 358: 2026-2033.
21. World Health Organization., *The The ICD-10 classification of mental and behavioural disorders: clinical description and diagnostic guidelines.* WHO, editor, Geneva, Switzerland,1992.
22. McKann G., Drachman D., Folstein M.F., Katzman R., Price D., Stadlan E.M. Report of the NINCDS-ADRDA Work group under the auspices of the Department of health and human services task force on Alzheimer's disease. *Neurology* 1984; 34: 939-944.
23. Algotsson A., Nordberg A., Winblad B., Influence of age and gender on skin vessel reactivity to endothelium-dependent and endothelium-independent vasodilators tested with iontophoresis and a laser Doppler perfusion imager. *J Gerontol Med Sci* 1995; 50A: M121-M127.
24. Algotsson A., Nordberg A., Winblad B., Apolipoprotein E ε4 allele has an impact on vascular reactivity in Alzheimer's disease. *Arch Gerontol Geriatr* 2000; 221-231.
25. Folstein M.F., Folstein S.E., McHugh P.R., Mini-mental state: a practical method for grading the cognitive state of patients for the clinician. *J Psychiatr Res* 1975; 12: 189-198.
26. Siedel J., Hägele E.O., Ziegenhorn J., Wahlefeld A.W. Reagent for the enymatic determination of serum total cholesterol with improved lipolytic efficency. *Clin Chem* 1983; 29: 1075-1080.
27. Siedel J., Schuck R., Staepels J., Town M-H., Long term stable, liquid ready-to-use monoreagent for the enzymatic assay of serum or plasma triglycerides (GPO-PAP method). ACC Meeting abstract 34. *Clin chem* 1993; 39: 1127.
28. Sugiuchi H., Uji U., Okabe H., Irie T., Uekama K., Kayahara N., Miyauchi K., Direct measurement of high-density lipoprotein cholesterol in serum with polyethylene glycol-modified enzymes and sulfated alpha-cyclodextrin. *Clin Chem* 1995; 41: 717-723.
29. Lahoz C., Schaefer E.J., Cupples L.A., Wilson P.W., Levy D., Osgood D., Parpos S., Pedro-Botet J., Daly J.A., Ordovas J.M., Apolipoprotein E genotype and cardiovascular disease in the Framingham heart study. *Atherosclerosis* 2001; 154: 529-537.
30. Wehr H., Parnowski T., Puzyn'ski S., Bednarska-Makaruk M., Bisko M., Kotapka-Minc S., Rodo M., Wolkowska M., Apolipoprotein E genotype and lipid and lipoprotein levels in dementia. *Dement Geriatr Cogn Disord* 2000; 11: 70-73.
31. Isbir T., Agachan B., Yilmas H., Aydin M., Kara I., Eker E., Eker D., Apolipoprotein-E polymorphism and lipid profiles in Alzheimer's disease. *Am J Alzheimers Dis Other Demen* 2001; 16: 77-81.
32. Dupuy A.M., Mas E., Ritchie K., Descomps B., Badion S., Cristol J.P., Touchon J., The relationship between Apolipoprotein E4 and lipid metabolism is impaired in Alzheimer's disease. *Gerontology* 2001; 47: 213-218.
33. Rifai N., Merril J.R., Holly R.G., Postprandial effect of a high fat meal on plasma lipid, lipoprotein cholesterol and apolipoprotein measurements. *Ann Clin Biochem* 1990; 27: 489-493.
34. Craig S.R., Amin R.V., Russel D.W., Paradise N.F., Blood cholesterol screening. Influence of fasting state on cholesterol results and management decisions. *J Gen Intern Med* 2000; 15: 395-399.
35. Wood D., de Backer G., Faergeman O., Graham I., Mancia G., Pyörälä K., Prevention of coronary heart disease in clinical practice. Recommendations of the second joint task force of European and other societies on coronary prevention. *Eur Heart J* 1998; 19: 1434-1503
36. Wilhelmsen L., Johansson S., Rosengren A., Wallin I., Dotevall A., Risk factors for cardiovascular diease during the the period 1985-1995 in Göteborg, Sweden. The GOT-MONICA project. *J Int Med* 1997; 242: 199211.
37. Agerholm-Larsen., Nordestgaard B.G., Steffensen R., Jensen G., Tybjaerg-Hansen A., Elevated HDL cholesterol is a risk factor for ischemic heart disease in white women when caused by a common mutation in the cholesteryl ester transfer protein gene. *Circulation* 2000; 101: 1907-1912.
38. Kivatinitz S.C., Pelsman M.A., del C. Alonso A., Bagatolli L., Quiroga S., High-density lipoprotein aggregated by oxidation induces degeneration of neuronal cells. *J Neurochem* 1997; 69: 2102-2114.
39. Launer L.J., White L.R., Petrovitch H., Ross G.W., Curb J.D., Cholesterol and neuropathologic markers of AD: a population-based autopsy study. *Neurology* 2001; 57: 1447-1452.
40. Hayley R.W., Is there a connection between the concentration of cholesterol circulating in plasma and the rate of neuritic plaque formation in Azheimer's disease? (Editorial comment) *Arch Neurol* 2000; 57: 1410-1412.
41. Ichihara K., Satoh K., Disparity between angiographic regression and clinical event rates with hydrophobic statins. *Lancet* 2002; 359: 2195-2198.
42. Petersen R.C., Doody R., Kurz A., Mohs R.C., Morris J.C., Rabins P.V., Ritchie K., Rossor M., Thal L., Winblad B., Current concepts in mild cognitive impairment. *Arch Neurol* 2001; 58: 1985-1992.
43. Fratiglioni L., Viitanen M., von Strauss E., Tontodonati V., Herlitz A., Winblad B., Very old women at highest risk of dementia and Alzheimer's disease. Incidence data from the Kungsholmen Project, Stockholm. *Neurology* 1997; 48: 132-139.

Calcium/Calmodulin Dependent Protein Kinase II in Alzheimer's Disease

Maria Nordlinder[1], Wen-Lin An[1], Irina Alafuzoff[2], Nenad Bogdanovic[1], Jin-Jing Pei[1#]

[1]Karolinska Institutet, NEUROTEC, Division of Experimental Geriatrics, Novum, Huddinge, Sweden

[2]Kuopio University, Department of Neuroscience and Neurology, Section of Neuropathology, Kuopio University Hospital, Kuopio, Finland

Abstract

Neuropathologically, Alzheimer's disease (AD) is characterized by synapse loss, the presence of large numbers of senile plaques and neurofibrillary tangles (NFT). NFT constitutes primarily of paired helical filaments (PHF), which are the aggregates of the abnormally hyperphosphorylated microtubule binding protein tau (PHF-tau). It is believed that PHF-tau is caused by an imbalance of protein phosphatase and kinase activities. Ca^{2+}/calmodulin dependent protein kinase II (CamKII) has previously presented itself as a possible kinase candidate for tau phosphorylation. In this study the level and distribution of CamKII in relationship to PHF-tau were investigated. CamKII immunoreactivity was compared with that obtained using antibodies to PHF-tau, normal tau and a synaptic marker, respectively, in homogenates from AD and control brains. Although there is no apparent increase of total or active CamKII level, a significant positive correlation between total CamKII, and PHF-tau was detected in homogenates of AD brain as compared with control. Active CamKII is significantly correlated with AT8 labeled PHF-tau in AD homogenates. The significant correlation between total CamKII and activated CamKII in the homogenates of AD cases suggested that accumulation of total CamKII in NFT-bearing neurons and pretangle-like neurons might be in part contributed by the increased activated CamKII. The data support the concept that CamKII is involved in formation of PHF-tau in AD.

Keywords: CamKII, Neurofibrillary tangle, Tau phosphorylation, Alzheimer's disease

Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder. The Diagnosis of AD is confirmed by postmortem detection of severe neuronal loss, the abundant number of intraneuronal neurofibrillary tangles (NFTs) and extracellular senile plaques[1,2]. Intraneuronal NFTs are composed of bundles of filaments, called paired helical filaments (PHF), which are the aggregates of abnormally hyperphosphorylated microtubule-associated protein tau (PHF-tau)[2]. PHF-tau shows decreased affinity for microtubules and high affinity for normal tau and other microtubule-associated proteins that lead to microtubule destabilization and disassembly[3-5].

Intensive studies have shown that tau is phosphorylated by a number of kinases in vitro. Ca^{2+}/calmodulin-dependent protein kinase II (CamKII), which is a highly enriched serine/threonin kinase in nervous system, has been shown to phosphorylate sites within tau that are important for microtubule interaction (Ser262 and Ser356)[6-11]. However, it has not been established if CamKII is responsible for tau hyperphosphorylation in vivo. CamKII is regulated by the level of intracellular Ca^{2+} and activated by an interaction with the Ca^{2+}/calmodulin complex. Binding of Ca^{2+}/calmodulin alters the conformation of the kinase, disrupting an intramolecular inhibiting interaction, which leads to a rapid autophosphorylation of the Thr-286 (α-isoform). The autophosphorylation prolongs the activity of the enzyme due to an entrapment of the bound Ca^{2+}/calmodulin and a conversion of the kinase to a Ca^{2+}-independent form[7]. The kinase activity continues until the phosphate is removed from the inhibitory domain by a CamKII phosphatase.

CamKII is required for synaptic plasticity such as long-term potential, which is a cellular model of learning and memory[12]. It is believed that the autophosphorylation of CamKII and the following Ca^{2+}/calmodulin-independent activity works as a molecular switch leading to a long-term storage of graded information in response to calcium influx[7].

#**Correspondence:** Jin-Jing-Pei MD, PhD, Karolinska Insitutet, NEUROTEC, Division of Experimental Geriatrics, Novum, KFC, plan 4, S-141 86 Huddinge, Sweden, Phone: +46 8 585 837 87 Fax: + 46 8 585 838 80, E-mail: Jin-Jing.Pei@neurotec.ki.se

Because CamKII is involved in memory and learning it is considered to play a role in the AD development.

To be a candidate kinase for tau hyperphosphorylation, CamKII should be overactive in neurons containing NFT and be present in the pathology wherever the neurofibrillary changes occur. CamKII has been previously found in NFT-bearing neurons[13] and to be associated with PHF[14]. However, whether or not CamKII activity is changed in correspondence to tau pathology is not well understood. In this study, the immunoreactivity of CamKII was observed in NFT-bearing neurons using antibodies against total and active form of CamKII. Using ELISA method, the levels of both total and active CamKII were measured and analyzed in AD and control brains.

Materials and methods

Homogenate preparation

Brain tissues from medial temporal cortex of 22 AD and 10 control cases (Table 1) were used in this study. The gray matter was separated from the white matter and homogenized in 1:10 w/v protease inhibitor cocktail buffer (PICB) (Sigma, Saint Louis, Missouri). Protein concentration was measured by Bradford method[15].

Antibodies

Information about the antibodies used in the study is described in detailed in Table 2.

Western blotting

The specificity of the antibodies against synaptophysin (SP), total- and active CamKII was analyzed in homogenates from 5 controls and 5 ADs using 10% sodium dodecly sulfate polyacrylamide gel electrophoresis (SDS-PAGE). For each case 10 mg sample were loaded per lane. The gels were run at 200V for 30-40 min. After separation, proteins were transferred to a nitrocellulose membrane for 90 min at 100V using a multiblot electrophoresis unit from BioRad. After transferring, the membranes were blocked for unspecific antibody binding sites using 5% non-fat milk in 0.1% TBS-Tween (TBS-T). The membranes were treated with primary antibodies against total CamKII, active CamKII, and synaptophysin (SP), respectively. After incubation, the membranes were washed with TBS-T and treated with secondary horseradish peroxidase linked anti-rabbit or anti-mouse IgGs at 1:2000 (Amersam, Pharmacia Biotech, England). Both anti-rabbit and anti-mouse IgGs were diluted in 5% milk powder in TBS-T. ECL reagent kit (Amersham Pharmacia Biotech, England) was used for developing membrane signals to films. After incubation, the films were exposed for about 2 min and then developed using a Curix 60 machine, type 9462 (Agfa-Gevaert AB, Kista, Sweden).

Table 1. Detailed information for the cases used in the Western blot and ELISA studies

Case number	Gender	Age	Post-mortem delay (hrs)	Diagnosis
1	M	67	2	Control
2	M	76	3	Control
3	F	96	10	Control
4	M	75	10	Control
5	M	79	5	Control
6	F	84	7	Control
7	M	84	7	Control
8	M	83	8	Control
9	F	98	9	Control
10	F	82	4	Control
11	M	86	7	AD
12	M	71	7	AD
13	F	54	3	AD
14	F	76	6	AD
15	M	88	7	AD
16	F	73	7	AD
17	M	82	6	AD
18	F	100	6	AD
19	F	90	10	AD
20	F	82	7	AD
21	F	74	6	AD
22	F	84	7	AD
23	F	92	6	AD
24	F	84	2	AD
25	F	78	6	AD
26	F	97	9	AD
27	F	68	4	AD
28	F	91	4	AD
29	F	84	4	AD
30	F	74	4	AD
31	F	87	5	AD
32	F	68	3	AD

Table 2. Primary antibodies used in the study.

Antibody	Specificity	Phosphorylation site*	Dilution	Reference
Tau-1	Normal tau	depSer199/202	1:20 000	Ref 27
AT8	PHF-tau	pSer202/Thr205	1:200-1:500	Ref 28
PHF-1	PHF-tau	pSer395/404	1:200	Ref 29
Anti-CamKIIα	CamKIIα		1:200-1:30 000	Sigma Saint Louis, Missouri
Anti-Active CamKII	Active CamKII	pThr286	1:1000-1:10 000	Promega Madison WI
Anti-Synaptophysin clone SVP-38	Synaptophysin		1:4000-1:6000	Sigma, Saint Louis, Missouri

dep, dephosphorylated; p, phosphosylated

Table 3. Simple correlation analyses of normal tau, PHF tau, synaptophysin (SP), total CamKII, and active CamKII

Antibody 1	Antibody 2	r-value	p-value
Tau-1	total CamKII	-0.24	>0.05
AT8	total CamKII	0.45	<0.01
PHF-1	total CamKII	0.43	<0.05
Tau-1	active CamKII	0.06	>0.05
AT8	active CamKII	0.37	<0.05
PHF-1	active CamKII	0.24	>0.05
Total CamKII	active CamKII	0.60	<0.01
Tau-1	SP	0.28	>0.05
AT8	SP	-0.26	>0.05
PHF-1	SP	-0.28	>0.05
Total CamKII	SP	0.31	>0.05
Active CamKII	SP	0.39	<0.05

Indirect enzyme-linked immunosorbent assay (ELISA)

The levels of total-, active CamKII, normal- and PHF-tau and SP in AD and control groups was analyzed using ELISA. Briefly, 96-well microtiter plates were coated with 2 mg/100ml/well sample, diluted in 20 mM Tris-HCl, pH7.4 containing 2.5mM EDTA, 2.5 mM EGTA, 0.02 g/ml protease inhibitor cocktail (Sigma, St. Louis, MO), and 0.1% NaN3. The samples were incubated in room temperature for 2 hr. Non-specific sites were blocked with blocking buffer (0.85% NaCl, 10mM Tris-HCl pH7.4, 0.3g/ml BSA, 2% Tween-20) for 1 hr. Primary antibody was added to each well and incubated with coated samples overnight at 4°C. Secondary anti-mouse (1:1000) or anti-rabbit (1:2000) antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) linked to horseradish peroxidase was added and incubated for 2 hr at room temperature. For enzyme reaction, KPL enzyme substrate tetramethylbenzidine (TMB) (SMS gruppen, Helsingborg, Sweden) was added. Reaction time was set between 10 to 30 min and stopped by adding 100 ml/well 1M phosphoric acid. The absorbances were read with an ELISA reader (Septra MAX 250 machine from Molecular devices, Sunnyvale, CA) at 450 nm.

Immunocytochemistry and confocal microscopy

To visualize the localization of CamKII in relation to NFT, sections from human hippocampus from 2 AD cases (post-mortem delay: 24 and 48h, age: 72 and 74 yr) and 2 healthy control cases (post-mortem delay: 24 and 48h, age:

76 and 78 yr) were stained and studied with confocal microscopy. Formalin-fixed frozen tissue blocks were sliced to 30 mm thin sections, which were mounted on glass slides. The sections were rinsed in 0.1% TBS-T and blocked in 5% BSA and 3% normal goat serum in TBS-T for 30 min. The sections were incubated with a mixture of one rabbit polyclonal antibody (total or active CamKII) and mouse monoclonal antibody (AT8) over night in a humid chamber. After washing with TBS-T, the sections were incubated with a mixture of CY^{TM}3-labeld anti-rabbit IgG (red) (1:500) and CY^{TM}2 labeled anti-mouse IgG (green) (1:250) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 4 hr in room temperature. A BioRad Laser Scanning Confocal Imaging System (Radiance Plus) was used to determine co-localization of the CY^{TM}3 (red) labeled anti-rabbit IgG to CY^{TM}2 (green) labeled anti-mouse IgG. The system was equipped with a Nikon Eclipse inverted microscope (TE300). An argon ion laser that excites at 488 nm with a dichroic beamsplitter 560DCLP and a bandpass filter HQ515/30 was used to detect CY^{TM}2 (green) labeled PHF-tau (AT8). A HeNe laser that excites at 543 nm with E570LP emission filter was used to measure total or active CamKII labeled by CY^{TM}3 (red). Laser light illuminates a Nikon 60´ /1.4 NA oil immersion objective. Images scanned on the two channels (red and green) were merged to produce a single profile.

Statistics

Microsoft Excel Student's t-test was used to analyze the significance of the change between AD and controls. The OD values from each of the examined antibodies used in the ELISA-experiments were analyzed against each other by Pearson simple correlation analysis using the SPSS 9.0 software program.

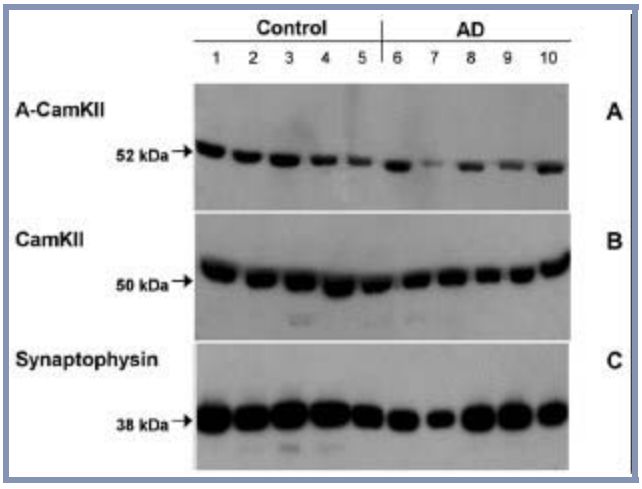


Figure 1: Immunoblots of 5 controls and 5 AD cases, labeled with antibodies against active-CamKII (A), total CamKII (B) and synaptophysin (C) at a dilution of 1:30 000, 1:10 000 and 1:4000, respectively. The antibodies used were specific, labeling only one band. The bands correlate with the expected molecular weight for each of the three proteins.

Results

Analysis of antibody specificity using Western blots

Homogenates from the medial temporal cortex of 5 AD and 5 control cases were used to analyze the specificity of the antibodies on Western blots. As figure 1 illustrates, the antibodies used against active CamKII, total CamKII and SP were specific. The antibodies labeled one single band located around the expected molecular weight for each antibody, 52, 50 and 38 kDa, respectively (Figure 1 A, B, C).

Levels and correlation of total CamKII, active CamKII and synaptophysin in AD and control

The mean OD value and SD of antibodies to total CamKII, active CamKII, synaptophysin, and to tau (Tau-1, AT8, PHF-1) are shown from 10 control and 22 AD cases in figure 2. The results showed a significant decrease in normal-tau (Tau-1) and SP and a significant increase in PHF-tau (AT8, PHF-1) in AD as compared with controls. The immunoreactivity of active and total CamKII did not change significantly between two groups.

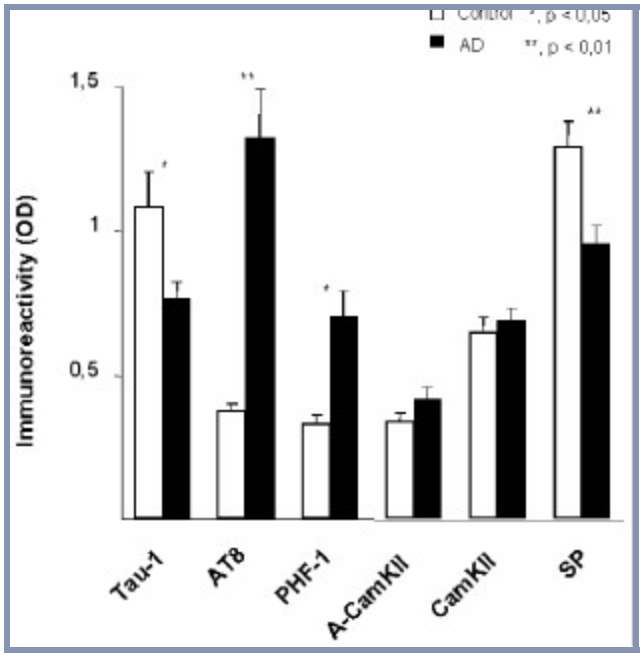


Figure 2: Histographical illustration of the result from the ELISA experiments, showing the difference in mean OD value ± SD between 10 control cases and 22 AD cases. Non-paired Student t-test showed a significant decrease in normal tau (p-value < 0.05) and synaptophysin (p-value < 0.01), and a significant increase in PHF-tau (AT8 and PHF-1) (p-value < 0.01 and 0.05, respectively) Levels of active and total CamKII remains unchanged between AD

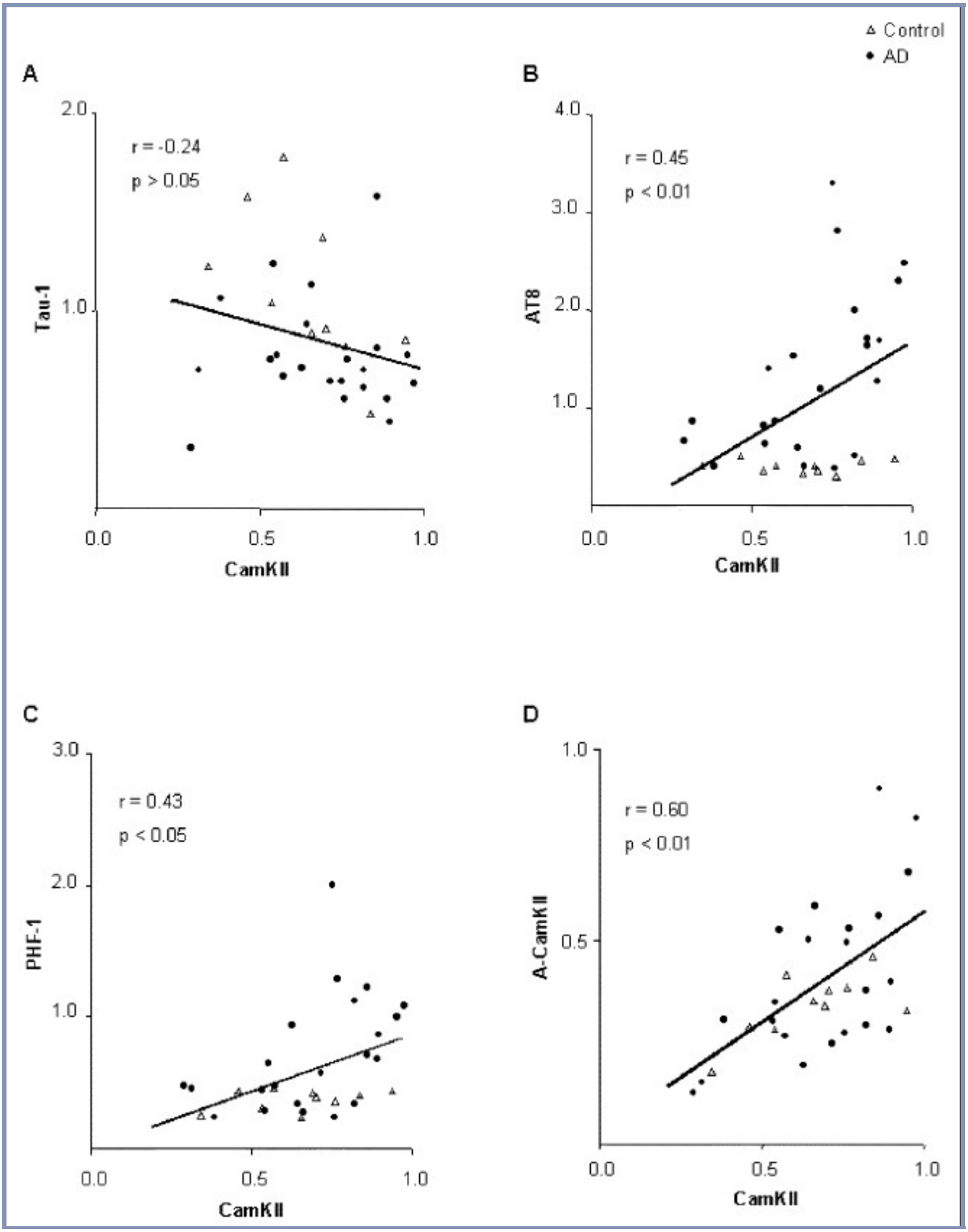


Figure 3: Representative data from simple correlation analysis. There is a significant positive correlation between total CamKII and PHF-tau labeled by AT8 and PHF-1 (B, C) and between total and active CamKII (D), no significant correlation between normal tau and total CamKII (A).

The data from the ELISA experiment was further analyzed by a simple Pearson correlation analysis (shown in Figure 3 and summarized in Table 3). The results showed no significant correlation between the immunoreactivity of normal tau (Tau-1) and the immunoreactivities of total CamKII, active CamKII, synaptophysin. Total and active CamKII did correlate significantly with AT8 but only total CamKII correlated significantly with PHF-1. However, there was a positive significant correlation between active CamKII and total CamKII with the highest r-value at 0.60. Synaptophysin did not correlate significantly to any of the markers of tau. Synaptophysin does, however, correlate to active CamKII. Age and postmortem delay were not significantly correlated with any of the antibodies used in the study.

Co-localization of CamKII and PHF-tau

The immunocytrochemical results revealed that the brain sections (Figure 4) contained many classical tangles positive for both PHF-tau (A1) and total CamKII (A2) in the entorhinal area of an AD case. The tangles were surrounded by several unaffected neurons, with CamKII spreading throughout the entire cytoplasm (A1, A2, A3). It is possible that these surrounding healthy looking neurons are at an early stage of neurofibrillary degeneration. A figure at a higher magnification (B3) on a couple of tangle-containing neurons showed clearer overlap between PHF-tau and CamKII (B, B2, B3). In addition, more fibers positive for AT8 than total CamKII were observed. However, we failed to locate any significant signal of active CamKII by immunocytochemistry.

Discussion

Neurofibrillary tangles are one of the major lesions in AD brain. They constitutes of the aggregates of abnormally hyperphosphorylated tau. It is of great importance to identify the kinases responsible for tau hyperphosphorylation in order to understand the mechanisms behind the pathogenesis of this AD lesion. It has previously been shown that CamKII is able to phosphorylate tau in vitro[6-11] and associates with PHF[14].

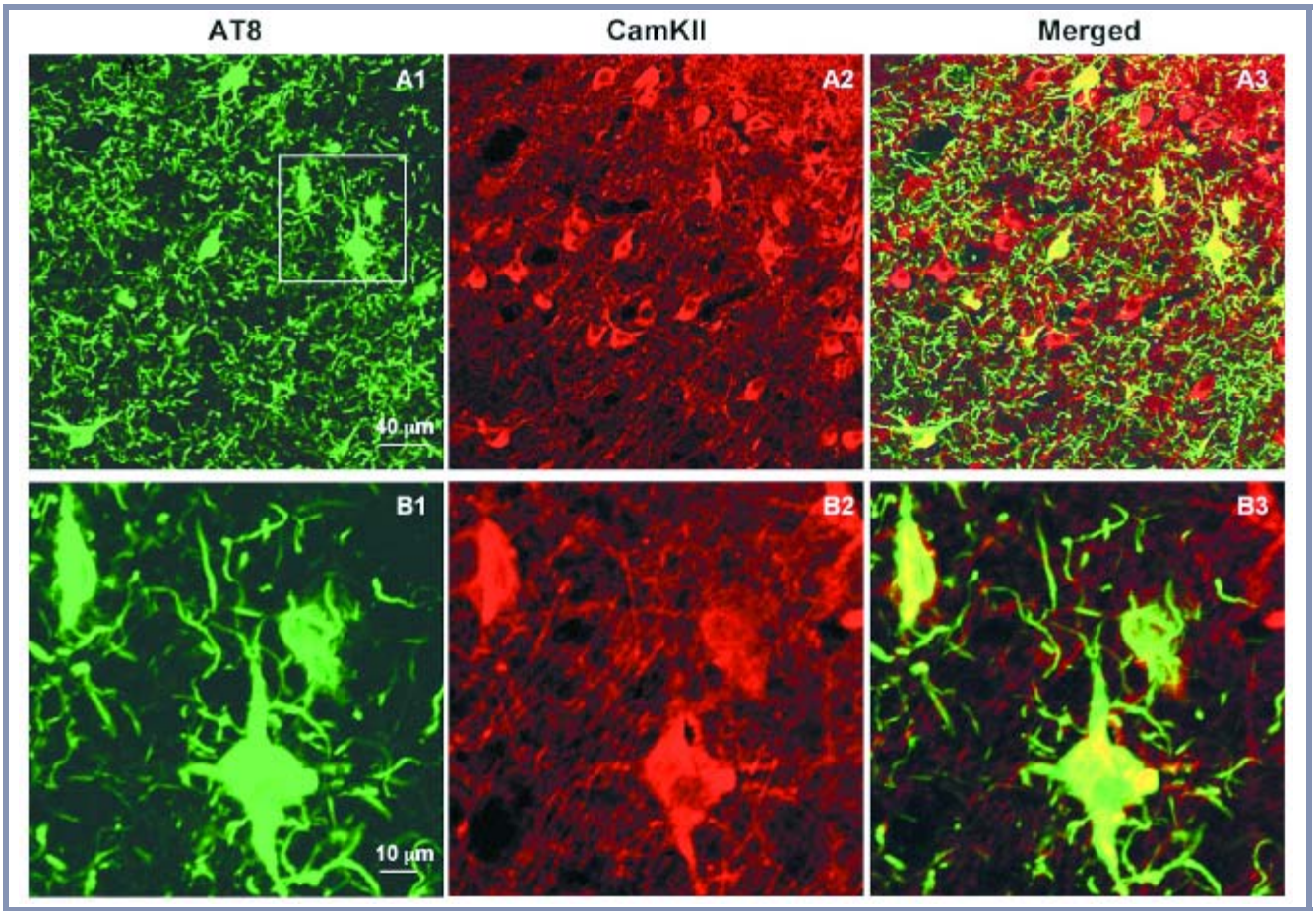


Figure 4: Immunocytochemistry of sections from AD medial temporal cortex with antibodies directed at PHF-tau (A1) and total CamKII (A2). The section contains several tangle-bearing neurons positive for AT8 that are overlapped with total CamKII (A1, A2, A3). Surrounding the tangle-bearing neurons, some healthy looking neurons are only positive for CamKII (A2, A3). B1, B2, and B3 are higher magnification of A1, A2, and A3, respectively.

The aim of this study was to investigate the molecular relationship between CamKII and tau phosphorylation in situ. We found: 1) no increase in either total-, or active CamKII in AD homogenates as compared with controls, but a significant positive correlation between total CamKII and PHF-tau labeled by both AT8 and PHF-1; 2) a significant positive correlation between active CamKII and AT8; and 3) that total CamKII and PHF-tau are co-localized in NFT affected neurons.

In consistent with previous in situ hybridization study showing a co-expression of CamKII and tau mRNA[16], CamKII immunoreactivity was found to be co-existed with PHF-tau in neurons containing NFT and those that are likely to form NFT in the current study. It seems that there is no increase in total or activated CamKII in AD brain. The positive correlations between total CamKII and PHF-tau labeled by AT8 and PHF-1, between active CamKII and the PHF-tau labeled by AT8, and between total CamKII and active CamKII suggest that the immunoreactivity of total CamKII in NFT-bearing neurons are at least partially contributed by active CamKII, and the activated CamKII is more favorable to the PHF-tau phosphorylation sites recognized by AT8, which is considered as an early marker of tau pathology[17]. This sustained or likely increased CamKII activity relative to PHF-tau phosphorylation sites recognized by AT8 and PHF-1 is likely due to the reduced activity of a CamKII phosphatase that could prolong CamKII activity and reduce its sensitivity to intracellular Ca^{2+} levels. Zinc has a complete inhibitory effect on a CamKII specific phosphatase[18], its level is increased in AD pathology[19]. Zinc has also been shown to induce autophosphorylation and activation of CamKII in a non-Ca^{2+}/calmodulin dependent-manner[20].

PP2A could serve as a candidate CamKII phosphatase since it has been previously shown to be an efficient phosphatase for both CamKII[21] and tau[22,23]. PP2A inhibition results in an up-regulation of CamKII and induces tau hyperphosphorylation[24]. It is likely that the reduced activity of PP2A in AD brain[22,24] might lead to a reduced dephosphorylation of CamKII, which prolongs CamKII activity that could phosphorylate tau, as well as a compromised ability of PP2A to dephosphorylate PHF-tau, resulting in formation of PHF-tau in AD.

The reduced synaptophysin level in AD could contribute to the impaired cognitive function at a relatively early state of AD[26]. Upon activation, CamKII is known to localize to the synapse that is severely lost in AD. Our results showed a significant positive correlation between active CamKII and synaptophysin, which indicates that CamKII could be degraded together with synaptophysin during the development of AD. The remained level of CamKII in AD is likely mobilized and concentrated more to the sites where tau hyperphosphorylation is taken place[14] (and current data). This might also explain why we could not detect the increased level of total or active CamKII in AD as compared with control, but we did observe a complete co-existence between PHF-tau and total CamKII in neurons affected by NFTs, as well as in the surrounding healthy looking neurons (pretangle neurons).

In conclusion we found that total CamKII significantly correlates with PHF-tau labeled by AT8 and PHF-1 and present in neurons affected by NFTs and pretangle neurons. Active CamKII significantly correlates AT8 labeled PHF-tau and total CamKII. This suggests that CamKII activation might be involved in formation of PHF-tau at an early stage in AD.

Acknowledgments

Financial support was provided by Alzheimerfonden, Fredrik och Ingrid Thurings Stiftelse, Gamla Tjänarinnor Foundation, Gun och Bertil Stohnes Stiftelse, Karolinska Institutets Stiftelser, Loo and Hans Ostermans Foundation and Åke Wibergs Stiftelse.

References

1. Glenner G.G., Wong C.W., Alzheimer's disease: initial report of the purification and characterization of a novel cerebrovascular amyloid protein. *Biochem Biophys Res Commun* 1984; 120: 885-90.
2. Grundke-Iqbal I., Iqbal K., Quinlan M., Tung Y.C., Zaidi M.S., Wisniewski H.M., Microtubule-associated protein tau. A component of Alzheimer paired helical filaments. *J Biol Chem* 1986; 261: 6084-9.
3. Alonso A.C., Zaidi T., Grundke-Iqbal I., Iqbal K., Role of abnormally phosphorylated tau in the breakdown of microtubules in Alzheimer disease. *Proc Natl Acad Sci USA* 1994; 91: 5562-6.
4. Alonso A.D., Zaidi T., Novak M., Barra H.S., Grundke-Iqbal I., Iqbal K., Interaction of tau isoforms with Alzheimer's disease abnormally hyperphosphorylated tau and in vitro phosphorylation into the disease-like protein. *J Biol Chem* 2001a; 276:37967-73.
5. Alonso A., Zaidi T., Novak M., Grundke-Iqbal I., Iqbal K., Hyperphosphorylation induces self-assembly of tau into tangles of paired helical filaments/straight filaments. *Proc Natl Acad Sci U S A.* 2001b; 98: 6923-8.
6. Baudier J., Cole R.D., Phosphorylation of tau proteins to a state like that in Alzheimer's brain is catalyzed by a calcium/calmodulin-dependent kinase and modulated by phospholipids. *J Biol Chem* 1987; 262: 17577-83.
7. Colbran R.J., Schworer C.M., Hashimoto Y., Fong Y.L., Rich D.P., Smith M.K., Soderling T.R., Calcium/calmodulin-dependent protein kinase II. *Biochem J* 1989; 258: 313-25.
8. Litersky J.M., Johnson G.V., Jakes R., Goedert M., Lee M., Seubert P., Tau protein is phosphorylated by cyclic AMP-dependent protein kinase and calcium/calmodulin-dependent protein kinase II within its microtubule-binding domains at Ser-262 and Ser-356. *Biochem J* 1996; 316: 655-60.
9. Singh T.J., Wang J.Z., Novak M., Kontzekova E., Grundke-Iqbal I., Iqbal K., Calcium/calmodulin-dependent protein kinase II phosphorylates tau at Ser-262 but only partially inhibits its binding to microtubules. *FEBS Lett* 1996; 387: 145-8.
10. Sironi J.J., Yen S.H., Gondal J.A., Wu Q., Grundke-Iqbal I., Iqbal K., Ser-262 in human recombinant tau protein is a markedly more favorable site for phosphorylation by CaMKII than PKA or PhK. *FEBS Lett* 1998; 436: 471-5.
11. Steiner B., Mandelkow E.M., Biernat J., Gustke N., Meyer H.E., Schmidt B., Mieskes G., Soling H.D., Drechsel D., Kirschner M.W., Phosphorylation of microtubule-associated protein tau: identification of the site for Ca2(+)-calmodulin dependent kinase and relationship with tau phosphorylation in Alzheimer tangles. *EMBO J* 1990; 9: 3539-44.

12. Giese K.P., Fedorov N.B., Filipkowski R.K., Silva A.J., Autophosphorylation at Thr286 of the alpha calcium-calmodulin kinase II in LTP and learning. *Science* 1998; 279: 870-3.
13. Ferrer I., Blanco R., Carmona M., Puig B., Phosphorylated mitogen-activated protein kinase (MAPK/ERK-P), protein kinase of 38 kDa (p38-P), stress-activated protein kinase (SAPK/JNK-P), and calcium/calmodulin-dependent kinase II (CaM kinase II) are differentially expressed in tau deposits in neurons and glial cells in tauopathies. *J Neural Transm* 2001; 108: 1397-415.
14. Xiao J., Perry G., Troncoso J., Monteiro M.J., alpha-calcium-calmodulin-dependent kinase II is associated with paired helical filaments of Alzheimer's disease. *J Neuropathol Exp Neurol* 1996; 55: 954-63.
15. Bradford M.M., A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principal of protein-dye binding. *Anal Biochem* 1976; 72: 249-54.
16. Mah V.H., Eskin T.A., Kazee A.M., Lapham L., Higgins G.A., In situ hybridization of calcium/calmodulin dependent protein kinase II and tau mRNAs; species differences and relative preservation in Alzheimer's disease. *Brain Res Mol Brain Res* 1992; 12: 85-94.
17. Braak E., Braak H., Mandelkow E-M., A sequence of cytoskeleton changes related to the formation of neurofibrillary tangles and neuropil threads. *Acta Neuropatho (Berl)* 1994; 87: 554-67.
18. Ishida A., Kameshita I., Fujisawa H., A novel protein phosphatase that dephosphorylates and regulates Ca2+/calmodulin-dependent protein kinase II. *J Biol Chem* 1998; 273: 1904-10.
19. Cherny R.A., Atwood C.S., Xilinas M.E., Gray D.N., Jones W.D., McLean C.A., Barnham K.J., Volitakis I., Fraser F.W., Kim Y., Huang X., Goldstein L.E., Moir R.D., Lim J.T., Beyreuther K., Zheng H., Tanzi R.E., Masters C.L., Bush A.I., Treatment with a copper-zinc chelator markedly and rapidly inhibits beta-amyloid accumulation in Alzheimer's disease transgenic mice. *Neuron* 2001; 30: 665-76.
20. Lengyel I., Fieuw-Makaroff S., Hall A.L., Sim A.T., Rostas J.A., Dunkley P.R., Modulation of the phosphorylation and activity of calcium/calmodulin-dependent protein kinase II by zinc. *J Neurochem* 2000; 75: 594-605.
21. Strack S., Barban M.A., Wadzinski B.E., Colbran R.J., Differential inactivation of postsynaptic density-associated and soluble Ca2+/calmodulin-dependent protein kinase II by protein phosphatases 1 and 2A. *J Neurochem* 1997; 68: 2119-28.
22. Gong C.X., Shaikh S., Wang J.Z., Zaidi T., Grundke-Iqbal I., Iqbal K., Phosphatase activity toward abnormally phosphorylated tau: decrease in Alzheimer disease brain. *J Neurochem* 1995; 65:732-8.
23. Kins S., Crameri A., Evans D.R., Hemmings B.A., Nitsch R.M., Gotz J., Reduced protein phosphatase 2A activity induces hyperphosphorylation and altered compartmentalization of tau in transgenic mice. *J Biol Chem* 2001 276:38193-200.
24. Bennecib M., Gong C.X., Grundke-Iqbal I., Iqbal K., Inhibition of PP-2A upregulates CaMKII in rat forebrain and induces hyperphosphorylation of tau at Ser 262/356. *FEBS Lett* 2001; 490: 15-22.
25. Gong C.X., Singh T.J., Grundke-Iqbal I., Iqbal K., Phosphoprotein phosphatase activities in Alzheimer disease brain. *J Neurochem* 1993; 61: 921-7.
26. Sze C.I., Troncoso J.C., Kawas C., Mouton P., Price D.L., Martin L.J., Loss of the presynaptic vesicle protein synaptophysin in hippocampus correlates with cognitive decline in Alzheimer disease. *J Neuropathol Exp Neurol* 1997 5:933-44.
27. Liu W.K., Moore W.T., Williams R.T., Hall F.L., Yen S.H., Application of synthetic phospho- and unphospho- peptides to identify phosphorylation sites in a subregion of the tau molecule, which is modified in Alzheimer's disease. *J Neurosci Res* 1993; 34: 371-6.
28. Su J.H., Cummings B.J., Cotman C.W., Early phosphorylation of tau in Alzheimer's disease occurs at Ser-202 and is preferentially located within neurites. *Neuroreport* 1994; 5: 2358-62.
29. Otvos L. Jr., Feiner L., Lang E., Szendrei G.I., Goedert M., Lee V.M., Monoclonal antibody PHF-1 recognizes tau protein phosphorylated at serine residues 396 and 404. *J Neurosci Res* 1994; 39: 669-73.

Pupil Dilation Test for Alzheimer's Disease

Zhiqing Xiang, Yuemin Chen, Wenwei Yan#
Department of Psychiatry, Shanghai Medical Center, Shanghai, China

Abstract:

Objective: To determine whether pupil response to dilute tropicamide could be used as a diagnostic test for Alzheimer's disease (AD). **Method:** Pupil diameters of both eyes were measured simultaneously by an infra-red automatic pupil-diameter recorder and analyzer every 0.1 second for 30 minutes after instillation of 0.01% tropicamide to one eye and normal saline to the other. Three groups were studied: 52 patients with AD, 33 with vascular dementia (VD), and 58 elderly controls. After finding the cut-off point for differential diagnosis by receiver operator characteristic curve (ROC), its sensitivity, specificity and Kappa coefficient (degree of agreement) were calculated. **Results:** Mean percent change in diameters of the treated eye showed a trend of fastest maximum dilation in AD group, and was significantly different from other groups at all the measurement time points after 10th minute instillation. The difference was most significant at the 18th minute after instillation, and 15% was used as a cut-off point, the sensitivity was 0.81, specificity 0.79-0.82, and Kappa coefficient 0.62-0.67. **Conclusions:** Pupil dilation test could be used as a screening method in the diagnosis of Alzheimer's disease, or as a tool for differential diagnosis between AD and VD.

Keywords: pupil dilatation test, screening, Alzheimer's disease

There is a great need for an early, non-invasive, sensitive, and easily administered diagnostic test for Alzheimer's disease, which could only be definitely diagnosed by histological examination of brain tissue in nowadays. Nov. 1994, Scinto et al[1] has reported that the pupil dilation test might be able to identify Alzheimer's disease. The paper gave an impact to the medical professionals in the field of neurological science. Many replications were performed in 6 years, and some gave positive conclusions, while some found negative results[2-10]. The authors have replicated it again and again since 1995, and found there were some factors, which could influence the test results. The first one was the 'contamination' of control group, because some very early cases of Alzheimer's disease could behave normally and have normal memory and cognition. So, it is most important to ensure they would never contaminate that normal control group. The second was that the pupil size would be changed from time to time due to some factors, including the changing of aim of sight. We have to develop a new device that could automatically determine both eye pupils simultaneously, and correct the test eye by the other eye as a control. Besides, the sample, which size should be large enough, should be randomly selected and the clinical diagnostic criteria used should have good specificity. The following is the last study performed after further improvement of the device and the research methods mentioned above.

Subjects and methods

Subjects

All the inpatients with dementia in the Main and Branch Hospital of Shanghai Mental Health Center were recruited in the study except those who could not sit still. In total, 52 patients with Alzheimer's disease (AD) and 33 patients with vascular dementia (VD) were determined. Besides, **all** the healthy elderly from two communities were recruited in the study. After neuro-psychological measurement, only 58 persons met the inclusion criteria of normal controls (NC) and were determined. The inclusion and exclusion criteria were listed respectively in table 1, 2, and 3, and the demographic data were listed in table 4.

Methods

1. Pupil dilation test:

The test was performed in a semi-darkened, quiet room. Patient was instructed to sit comfortably in a chair, with nothing attached on his face or eyes. He only needed to be mentioned having a look at a small screen before him, on which some pictures were shown (Figure 1). After dark adaptation, one drop of 0.01% tropicamide was instilled into one eye (usually the left eye), while one drop of normal saline

#**Correspondence:** Wenwei Yan, MD, Department of Psychiatry, Shanghai Mental Health Center, 600 Wan ping Nan Rd, Shanghai 200031, China, Tel. 0086 21 64387250, Fax.0086 21 64387986, E-mail. yanww_2000@yahoo.com

Table 1. Inclusion criteria of AD:

1, met the diagnostic criteria of probable AD in NINCDS-ADRDA;
2, Hachinski score < 4;
3, no history of any other mental disorders;
4, no any indications of vascular dementia on CT or MRI
5, no glaucoma or any contraindication of pupil dilating drugs.
6, no medications which could influence pupil size.

Table 2. Inclusion criteria of VD:

1, met the diagnostic criteria of probable vascular dementia in NINCDS-ADREN;
2, Hachinski score > 7;
3, no history of any other mental disorders;
4, no any indications of severe extensive brain atrophy on CT or MRI.
5, no glaucoma or any contraindication of pupil dilating drugs.
6, no medications which could influence pupil size.

Table 3. Inclusion criteria of normal controls:

1, no memory deficit recently;
2, no manifestations or history of mental disorders;
3, no prominent physical diseases;
4, normal neuropsychological assessments including:
   1) MMSE (according to the educational level: illiteracy > 17, primary school > 20, middle school above > 24);
   2) Fuld object memory test (FOM) (total scale > 11, both the first and the last memory > 6);
   3) RVR (according to the educational level: illiteracy > 17, primary school > 20, middle school > 25);
   4) BD in WAIS (illiteracy > 9, primary school > 14, middle school above > 19),
   5) DS in WAIS (illiteracy > 5, primary school > 6, middle school above > 7)
5, no prominent brain atrophy or infarction on CT/MRI.
6, no glaucoma or any contraindication to pupil dilating drugs.
7, no medications that could influence pupil size.

Table 4. Demographic data of 3 groups

	No.			Age	MMSE	ADL	Duration of dementia
	M	F	total				
AD	23	29	52	76.8 ± 7.0	12.05 ± 7.55	38.58 ± 9.75	3.83 ± 2.87
VD	22	11	33	76.1 ± 8.3	13.29 ± 6.74	44.57 ± 5.54	2.85 ± 2.75
NC	24	34	58	71.0 ± 6.4	normal	/	/

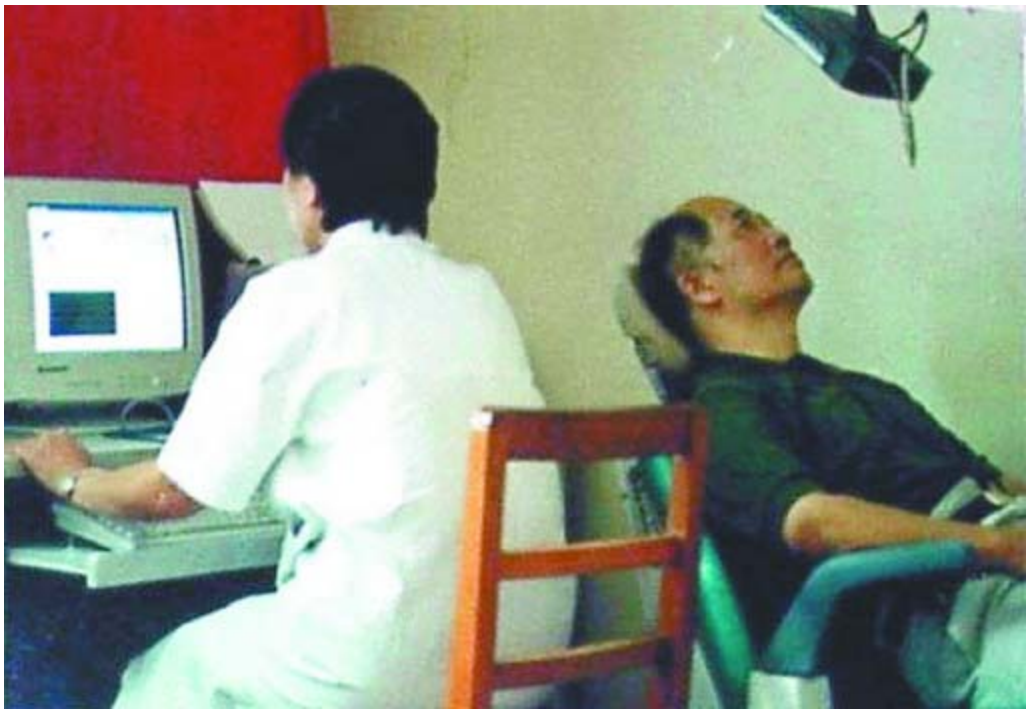


Figure 1. Patient sitting on a chair was instructed to have a look at a small video screen, while the recorder was measuring and recording the changes of two pupils simultaneously.

into the other as control. The both pupils were followed automatically and their diameters were recorded 10 times per second simultaneously by an infra-red automatic pupil-diameter recorder and analyzer (ZhongXin, Shanghai, China). We used the 5th minute after the instillation as baseline value. The mean value of total data recorded for each pupil in each minute (600 samples of pupil diameter per minute) would be shown on the screen of recorder as a point, and all the points in the period would be connected into a line showing the change in pupil size, each for an eye (Figure 2, blue color line for treated eye, and green color line for control eye). The real percent changes of the pupil diameter of treated eye, corrected by the other eye as control, were calculated and analyzed by the pupil recorder automatically and shown as the third line (red color) on the screen. The measuring and recording would be lasted for 30 minutes since 3 or 4 minutes after the instillation.

Real percent change of treated eye pupil diameter = $(Tn/Cn) / (T_5/C_5)$

Tn—treated eye pupil diameter at minute n

Cn—control eye pupil diameter at minute n

T_5—baseline of treated eye pupil diameter at minute 5

C_5—baseline of control eye pupil diameter at minute 5

2. Statistical Analyses

With the help of SPSS 10.0, we used t-test to compare the mean percentage change in pupil diameter of each group at every minute. From the data, we would find the time point, which could most significantly differentiate AD from NC, by the help of Receiver Operator Characteristic (ROC). We calculated the sensitivity and specificity of each point of percent change at each minute after instillation, and plotted the probability of having a true positive result against that of a false positive one for these points as a series of Receiver Operator Characteristic (ROC) curves. Then the point most close to the left-upper corner was chosen as the most ideal cut-off point that could obviously distinguish the AD case from normal controls. Each participant whose pupil percent change exceeded the cut-off point would be coded as (+), and vise versa. At last, we compared the test results with the clinical diagnoses by means of the sensitivity, specificity and Kappa coefficient (degree of agreement), just as follows:

	AD	NC	
+	a	b	a + b
–	c	d	c + d
	a + c	b +d	a + b + c + d

sensitivity = a/(a + c), specificity = d/(b + d),
Kappa coef. = 2(ad - bc)/[(a + b)(b + d) + (a + c)(c + d)]

Besides, we used correlation analysis to analyze the relationship between the percent change in pupil diameter of the treated eye and the severity of dementia, duration of dementia, age and MMSE in each group.

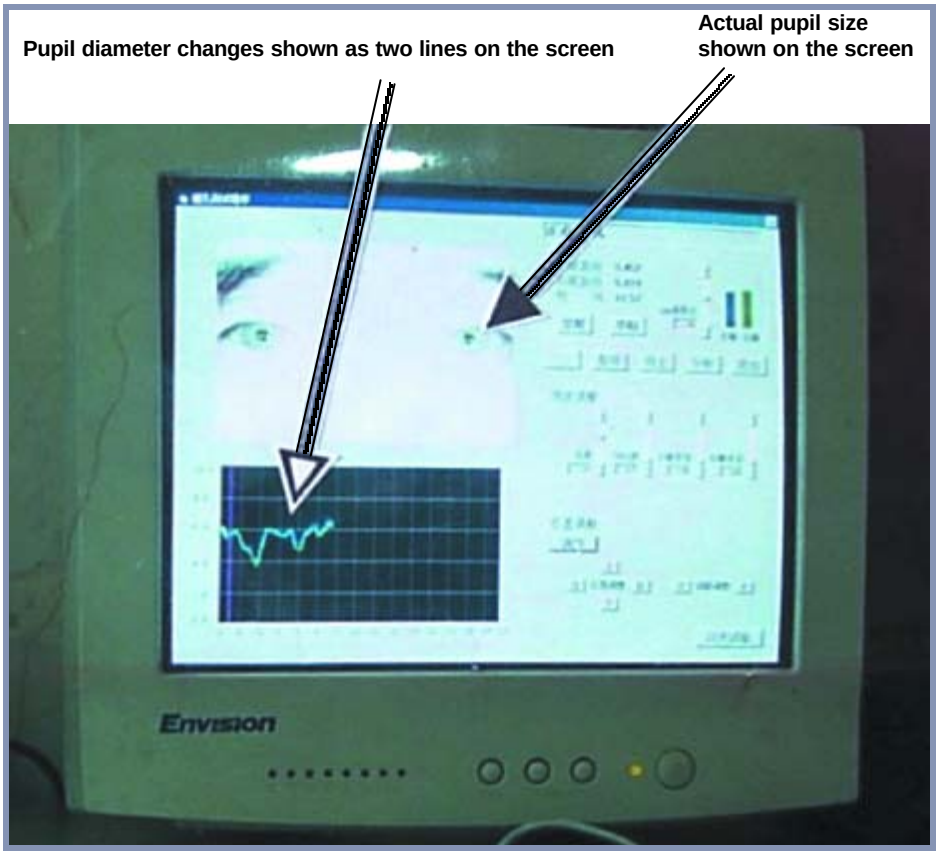


Figure 2. Screen of the automatic recorder showing the actual pupil size and the pupil diameter changes.

Results

After the instillation of 0.01% tropicamide, the pupil diameter largely increased in AD patients early since 7-10 minutes (Figure. 3), while remained almost unchanged in the other two groups (Figure. 4, 5). The results revealed that there were significant differences in the percent changes of treated eye pupil diameter between AD and VD or NC, especially at 18th minute after instillation ($p < 0.01$, Figure.6, 7, Table. 5). When the 'pupil diameter percent change?15% at 18 minutes after instillation' was chosen as the cut-off point, there were 42 cases with positive results in AD group (n = 52), while only 5 cases in VD group (n = 33), , and 13 cases in NC group (n = 58). As a diagnostic test for distinguishing AD from VD or NC, the sensitivity was 0.81, and the specificity was 0.82, 0.79 respectively, and the Kappa coefficient was 0.62, and 0.60.

There were no relationships between the percent change in pupil diameter of the treated eye and the severity of dementia, duration of dementia, age and MMSE in each group ($p > 0.05$).

Discussion

Alzheimer's disease (AD) exists on two planes: the clinical and pathological. The pathology of AD may be an insidious process developing over many years before the onset of even subtle clinical symptoms of dementia, while there is no problem that overt clinical manifestations of the disease occur after the presence of significant neuropathological abnormality[11]. So, there is great need for a diagnostic test that can detect the presence of the disease well before clinical symptoms become evident. In late 1994, Scinto et al published a finding that patients with probable AD could be distinguished from age-matched, healthy controls on the basis of an exaggerated pupil dilation response to a very dilute solution of tropicamide (a cholinergic antagonist). They reported that AD patients had a percentage increase in pupil diameter over baseline of ?13% by minute 29, while both non-Alzheimer dementia and normal controls were below 8%[1]. After Scinto's original report, some 29 or more publications have appeared evaluating the use of the pupil test. Despite a bewildering array of measurement techniques, drugs, and experimental conditions, 16 of 24 reports comparing AD patients with normal controls found that, as a group, AD patient pupils dilated more rapidly and/or to a greater extent than normal controls[11]. It's worth paying attention to the fact that it was consensus in all the studies (only except one[10]) that the AD patients exhibited a marked hypersensitivity to the diluted tropicamide. The problems did lay in the normal control (NC) group. If the NC group in a study contained more patients with earliest AD who had pathological changes in brain but without any clinical manifestation, the test results of this so-called 'normal' control group would have no significant difference with that of AD group.

We replicated the pupil dilation test early since 1995. In the first study, only one pupil was examined[2]. Although we got a positive result, there were a lot of problems. In 1997, we examined both two pupils with a delay of only 0.1 second between them, but we found even such a delay still could introduce factors that render the measurements nonequivalent in terms of other influences acting on pupil diameter. In 1998, we measured both eyes simultaneously with a helmet-like instrument[12]. The result was not ideal enough, because the demented patients could not bear the helmet on their head. At last, we developed the untouched device, which could follow both eyes automatically and measure both pupils simultaneously[13]. Besides, we found that the result could be influenced by the strictness of diagnostic criteria of the disease and definition of the normal control, and could be influenced by the size of sample as well. So, we chose strict diagnostic criteria of NINCDS-ADRDA for AD, and NINCDS-ADREN for VD, and we stipulated strict inclusion criteria for the normal control group, which included the neuropsychological test batteries and normal CT or MRI.

This test showed that the patients with probable AD displayed a pronounced response to the pupil dilating effects of tropicamide, which was significantly different from other groups ($p < 0.01$). It is suspected that patients with AD, which is characterized by the lack of endogenous acetylcholine, are hypersensitive to the acetylcholine receptor antagonist (e.g. tropicamide), and are easy to exhibit the pupil dilation. Furthermore, Scinto et al have found that in all 8 AD cases studied, many Aβ-positive plaques were distributed throughout the EW (Edinger-Westphal) nucleus after they died, while only 3 of 10 normal aged persons had only little neuropathological changes in their brains after death[11,14]. This might be the other reason why AD patients exhibited marked hypersensitivity to dilute tropicamide.

We found that AD group was most significantly different from other dementia and normal controls not at 29 minutes, which has reported by Scinto et al, but at 13-22 minutes. Pupil dilated earlier in AD than in patients with other dementia and normal controls. The pupils of patients with AD obviously dilated 13-22 minutes after instillation, while the latter still remained unchanged or dilated only a little. By using ROC curve, we chose 'percent change in pupil diameter over baseline of ?15% at minute 18' as the cut-off point to distinguish AD patients with normal controls and VD patients. In addition, it was founded that the severity of dementia and the course of dementia were not significantly related to the percentage of pupil dilation. In fact, in our AD and VD groups, there were many patients who had less than one-year duration (27% and 41% respectively). Some AD patients only suffered from dementia several months exhibited typical positive results. It is suggested that pupil dilation test not only could be used as a diagnostic tool for

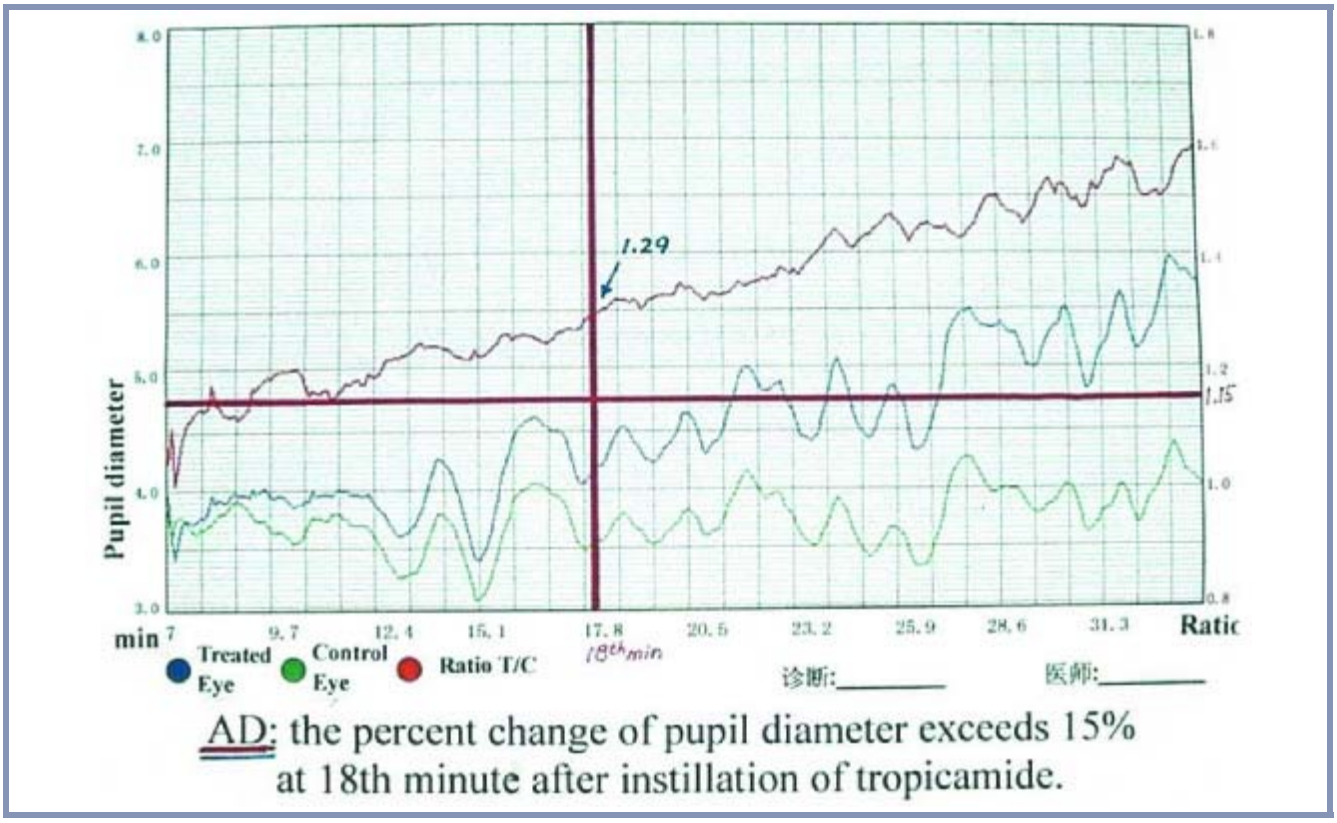


Figure 3. Pupil diameter percent change of a patient with Alzheimer's disease.

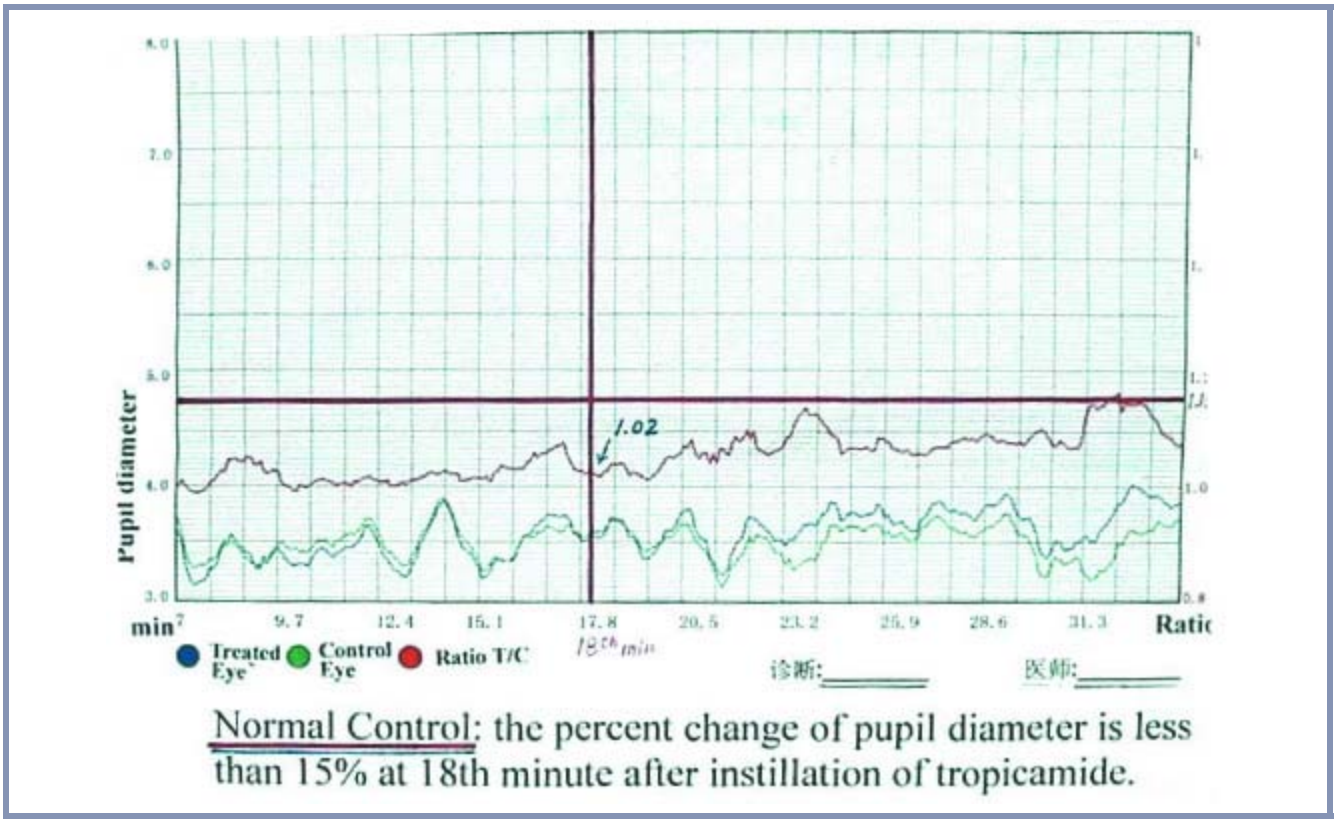


Figure 4. Pupil diameter percent change of an elder normal control.

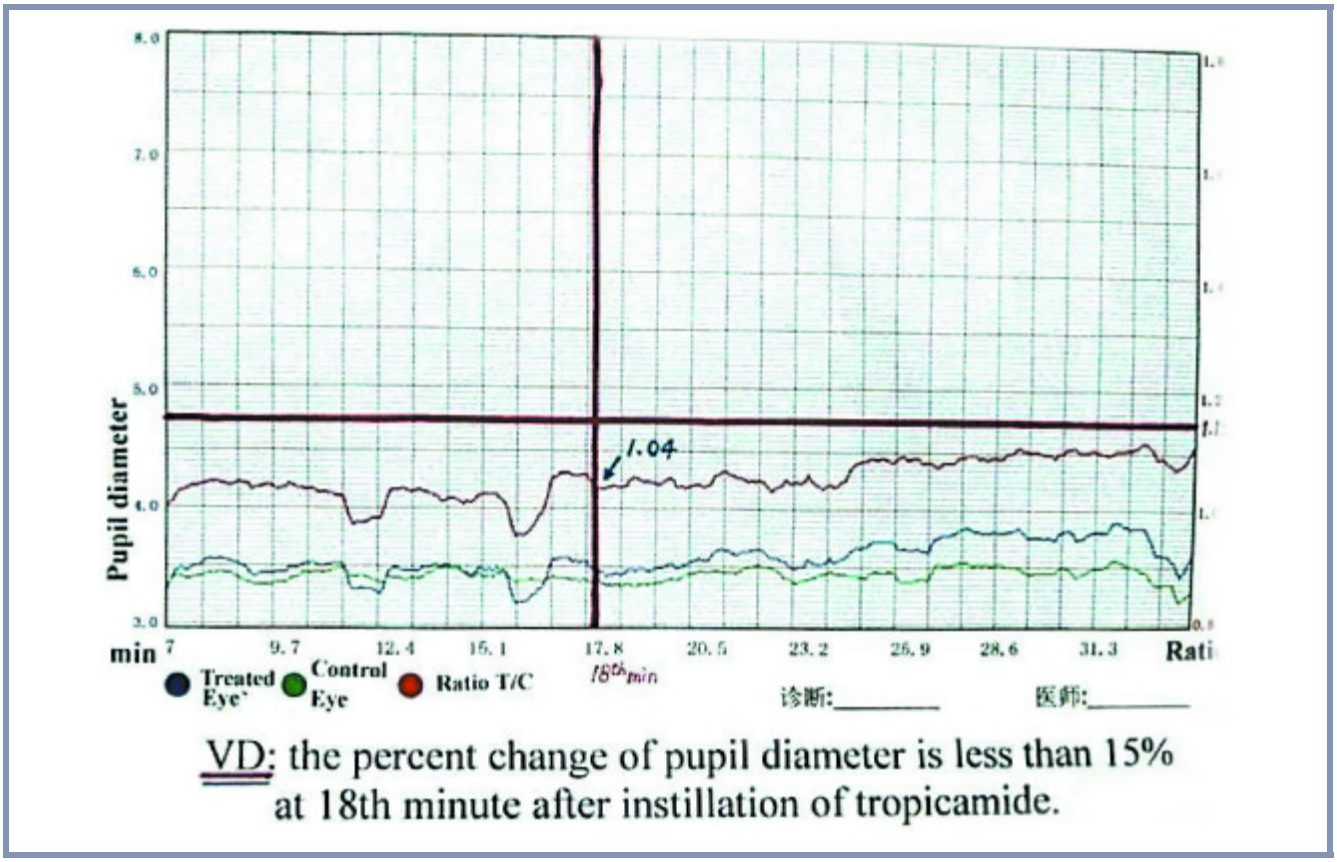


Figure 5. Pupil diameter percent change of a patient with vascular dementia.

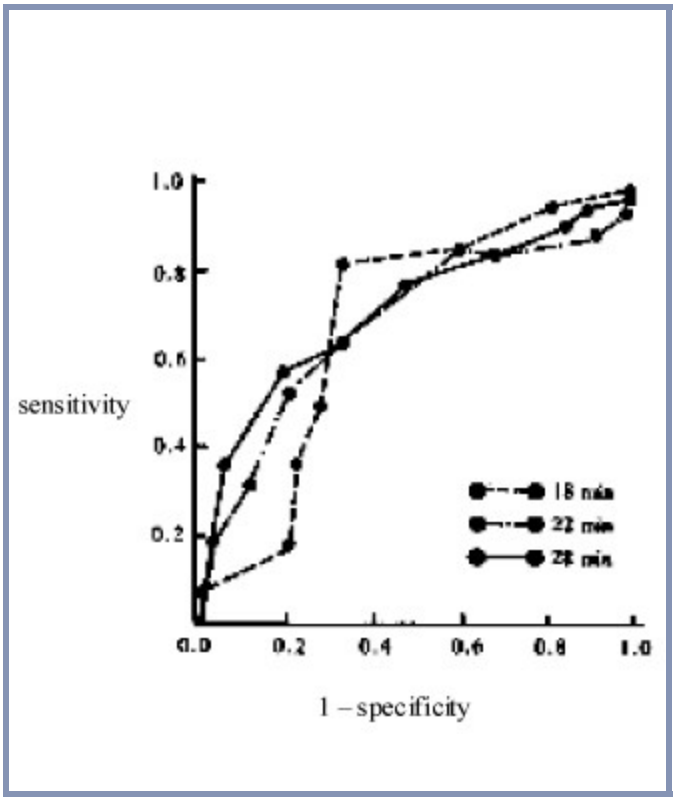


Figure 6. ROC curve of the pupil dilation test

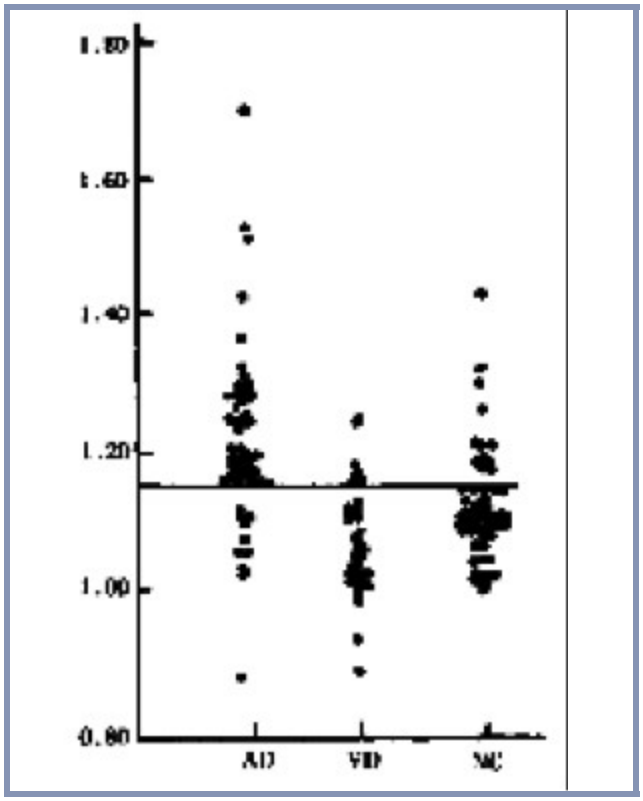


Figure 7. Pupil diameter percent changes of AD, VD, NC at min

Table 5.
Percent changes of treated eye pupil diameter at time points after instillation.

group	no.	min 10		min13		min16		min18	
		X±s	95%CI	X±s	95%CI	X±s	95%CI	X±s	95%CI
AD	52	1.045±0.100	(1.017, 1.073)	1.110±0.123	(1.075, 1.144)	1.163±0.143	(1.123, 1.203)	1.212±0.136	(1.174, 1.250)
VD	33	0.996±0.053	(0.978, 1.015)	1.004±0.087	(0.973, 1.035)	1.045±0.082	(1.016, 1.074)	1.071±0.085	(1.041, 1.101)
NC	58	1.007±0.042	(0.955, 1.018)	1.036±0.054	(1.022, 1.050)	1.086±0.078	(1.065, 1.106)	1.116±0.084	(1.094, 1.138)
group	no.	min20		min22		min25		min28	
		X±s	95%CI	X±s	95%CI	X±s	95%CI	X±s	95%CI
AD	52	1.238±0.142	(1.198, 1.277)	1.270±0.172	(1.222, 1.318)	1.316±0.188	(1.264, 1.369)	1.336±0.193	(1.281, 1.390)
VD	33	1.087±0.101	(1.051, 1.123)	1.098±0.121	(1.055, 1.141)	1.136±0.132	(1.089, 1.182)	1.166±0.148	(1.106, 1.226)
NC	58	1.153±0.107	(1.125, 1.181)	1.185±0.097	(1.160, 1.211)	1.219±0.116	(1.189, 1.250)	1.245±0.141	(1.208, 1.282)

detecting mild and severe AD cases but also for the earlier stage of the disease.

The sensitivity of pupil dilation test was 81%, not 100%, due to the reason that some atypical cases of AD, with an unusual distribution of pathology, would likely yield false negative results. Besides, AD group might be mixed up with the patients with other type dementia such as temporal lobe dementia and Lewy body dementia, which were similar in clinical manifestation with AD but have different pathological changes. As to VD group and normal control group, some cases exhibited positive response as well, because clinical diagnosed VD might be mixed up with some AD cases, and only could be proved by autopsy. And as well, other diseases that may affect similar areas of the brain could generate similar patterns as AD and thus 'false positive' results[14].

By the way, Scinto has said that he chose not to use the other eye as control for determining response to the drug in the treated eye because it was not possible to record the diameter of both pupils simultaneously with the equipment in their laboratory. He argued as well that 'as the treated eye responds to the influence of the drug, the untreated eye, as part of the consensual response, tends to constrict thus magnifying the response in the treated eye if the untreated pupil diameter was used as a control'[14]. According to our observation, the constriction of the untreated pupil, if any, after the instillation into treated eye was very transient. So, we chose the 5th minute as baseline, just after the influences of dark adaptation and the transient 'constriction', if any, and before the dilation in all three groups.

In conclusion, we considered that both the specificity and the sensitivity of pupil dilation test were good enough for clinical use. Not only it could be used as a diagnostic tool for screening early stage of AD, but also be used in distinguishing AD with VD. Of course, it needs further follow-up study to reveal if some elderly who behave normal now but show abnormal results in pupil dilation test would develop Alzheimer's disease later or not.

References

1. Scinto J.F., Daffner K.R., Dressler D., A potential noninvasive neurobiological test for Alzheimer's disease. *Science* 1994; 266: 1051-1054.
2. Cheng WH., Yan WW. Preliminary study of pupil dilation test for Alzheimer's disease. *Shanghai Archives of Psychiatry* 1997; 9: 11-15.
3. Arai H., Terajima M., Nakagawa T., Pupil dilation assay by tropicamide is modulated by apolipoprotein E4 allele dosage in Alzheimer's disease. *Neuroreport* 1996; 7: 918-920.
4. Kalman J., Kanka A., Magloczky E., Increased mydriatric response to tropicamide is a sign of cholinergic hypersensitiviy but not specific to late onset sporadic type of Alzheimer's disease. *Biol Psychiatry* 1997; 42: 909-911.
5. Robles A., Tourino R., Sesar A., Experience with pupil tropicamide tedt in Alzheimer's disease. *Rev Neurol* 1996; 24: 65-68.
6. Schweitzer I., Chang V., Kabov J., Anticholinergic eye drops in differentiating Alzheimer's disease and depression. *Biol Psychiatry* 1996; 39: 662.
7. Woodruff-Pak D.S., Romano R.M., Hinchliffe R.M., Detection of Alzheimer's disease with eyeblimk classical conditioning and the pupil dilation response. *Alzheimer's Res* 1996; 2: 178-180.
8. Gomez-Tortosa E. del Barrio A., Jimenez-Alfaro I., Pupil response to tropicamide in Alzheimer's disease and other neurodegenerative disorders. *Acta Neurol Scand* 1996; 94: 1104-1109.
9. Loupe D.N., Newman N.J., Green R.C., Pupillary response to tropicamide in patients with Alzheimer's disease. *Ophthamology* 1996; 103: 495-503.
10. Graff-Radford N., Lin SC., Brazis P., Tropicamide eye drops in Alzheimer's disease patients and controls. *Neurobiol Aging* 1996; 17(suppl 4): S164.
11. Scinto L.F., Frosch M., Daffner K.R., Specificity of neuronal loss in the Edinger-Westphal nucleus and its clinical implications. *Neurobiol Aging* 2000; 21(suppl 1): S164.
12. Cheng W.H., Chen Y.M., Yan W.W. Pupil dilation test for Alzheimer's disease. *Chinese Journal of Nervous Mental Disease* 2000; 26: 165-167.
13. Yan W.W., Chen Y.M., Xiang Z.Q. Pupil dilation test in the diagnosis of Alzheimer's disease. *Chinese J Gerontology* 2000; 19(6):

Carboxyl-Terminal Fragments of the Amyloid Precursor Protein in Alzheimer's Disease Brain: Detection of γ-Secretase Products

Geneviève Evin[1#], R. M. Damian Holsinger[1], Simone Eggert[2], Andreas Weidemann[2], Louise Canterford[1], David Hoke[1], Sam Gandy[3], Catriona McLean[1], Konrad Beyreuther[2], Colin L. Masters[1]

[1] Dept of Pathology, the University of Melbourne, and Mental Health Research Institute, Parkville, Australia;

[2] ZMBH, University of Heidelberg, Heidelberg,Germany;

[3] New York University School of Medicine, Nathan Kline Institute, Orangeburg, NY, USA

Abstract

To improve understanding of the amyloid precursor protein (APP) proteolytic processing that underlies AD pathogenesis, and to investigate the fate of APP cytoplasmic domain in a non-artificial system, we have analyzed total homogenates and subcellular fractions from human cortex samples for APP CTF. Antibodies to APP cytoplasmic domain detected βCTF as a major membrane-associated fragment of 14 kDa in Trizol extracts from frontal cortex. βCTF was significantly elevated by two-fold in AD as compared to controls. Another predominant species was a ~22 kDa fragment resulting from cleavage of APP upstream from the β-secretase site. αCTF was detected as a minor 10 kDa species. A fragment of 5.5 kDa that comigrated with εCTF recombinant standard was immunoprecipitated from cortex homogenates and found to be enriched in nuclear fractions.

Introduction

Aβ peptide, the major component of Alzheimer's disease amyloid plaques, is proteolytically derived from the type I, integral membrane protein, amyloid precursor protein (APP). APP can be shed from the membrane by two alternative cleavages due to α- and β-secretases (reviewed in 1,2). Besides releasing soluble APP ectodomain, α- and β-secretases produce membrane-associated fragments, termed αCTF and βCTF that consist of, respectively, 83 and 99 amino acids. βCTF contains the entire Aβ sequence with a free N-terminus and is the direct precursor of the amyloid peptides. γ-Secretase processing of βCTF within its transmembrane domain generates two major Aβ peptides, Aβ40 and Aβ42. Although Aβ42 is a quantitatively minor species, it is more aggregating than Aβ40 and is responsible for seeding amyloid deposition. γ-Secretase cleavage is expected to generate C-terminal fragments of 59 and 57 amino acids corresponding, respectively, to cleavage at position 40 and 42, but these have never been characterized, and the mechanism of γ-secretase cleavage remains poorly understood. Using transfected cells we, and others have previously identified a novel CTF of APP that results from cleavage at ε-site (atVal49), distal from the γ-secretase site and near the membrane/cytosol interface[3-5]. Products of a similar cleavage were also characterized and extracted from rat brain[6]. ε-Cleavage allows release in the cytosol of a fragment that contains the intact cytoplasmic domain of APP, a domain known to regulate Fe65 nuclear translocation[7-9] and thought to be involved in signal transduction[10] and regulation of phosphoinositide-mediated calcium signaling[11-12]. By its membrane topology and its requirement for an active presenilin complex, ε-cleavage closely resembles cleavage of Notch1 transmembrane domain at site 3. It remains unclear at present whether the same γ-secretase protease cleaves APP transmembrane domain at alternative sites (i.e. 40, 42 and 49). To clarify the proteolytic processing of APP within its transmembrane domain and to investigate the fate of APP cytoplasmic domain in a non-artificial system, we have analyzed total homogenates and subcellular fractions from human cortex samples for APP CTF.

#**Correspondence:** Dr Geneviève Evin, Dept of Pathology, University of Melbourne, Parkville 3010, Australia, tel + 613-8344-5878, + 613-8344-4004, email: gmevin @unimelb.edu.au

Materials and Methods

Brain samples and Extraction methods:

Human brain samples were obtained from the NH&MRC Tissue Resource Centre (Melbourne, Australia) and from the Institute for Brain Aging and Dementia Tissue Repository (Irvine, CA). Approximately 50 mg of brain tissue (frontal cortex) was homogenized in 1.0 mL of Trizol reagent (Gibco, Life Science Technology), using a pre-chilled mortar and pestle. After 15 min at 20^{o} C, 0.2 mL of chloroform was added and the samples were mixed by vortexing for 20 s. After 15 min at 20^{o} C, the samples were transferred to microtubes and centrifuged at 12,000 g for 15 min, at 4^{o} C. The upper aqueous phase containing RNA was precipitated with 0.5 mL of isopropanol. The protein was precipitated from the lower aqueous phase using 1.5 mL of isopropanol, sedimented at 12,000 g for 15 min, and the precipitate was washed three times with 0.3 M guanidine-hydrochloride and once with ethanol, and air-dried. The protein pellet was re-dissolved in 1% SDS, with heating at 50^{0} C and ultrasonic disruption. Preparation of membrane protein from brain cortex homogenates and Triton-X 100 extraction were carried out as described previously[13]. Preparation of subcellular fractions was as reported before[13]. Protein concentration was determined using the bicinchoninic assay (Pierce).

Immunoblotting

20 µg aliquots of protein were denatured by boiling for 5 min in Laemmli sample buffer containing 25 mM DTT. The proteins were separated on 4-20% Tris-Tricine polyacrylamide gels (Novex, Invitrogen, Australia) and transferred to nitrocellulose membrane (BioRad). After blocking with 0.5 % hydrolyzed casein, the blots were probed with primary antibody, followed by horseradish peroxidase secondary antibody conjugate (Dako, Botany, NSW, Australia) and developed with the enhanced chemiluminescence system (ECL, Amersham). Rabbit polyclonal anti-APP C-terminal antibodies, 369[14] and 93/2[15], and the anti-Aβ mouse monoclonal WO2[16] have been described previously.

Immunoprecipitation ofr γ/eCTF

Subcellular fractionswere diluted with 50 mM Tris-HCl, pH 7.4 containing 2% Triton X-100 and a cocktail pf protease inhibitors including 5 mM phenanthroline,to a final protein concwntration of 1 mg/mL. One mL aliquots (1 mg of protein) were mixed with Q-Sepharose slurry (1:0.5; v/v) (Pharmacia Biotech) to absorb APP. The Q-Sepharose gel was removed by centrifugation and the samples added to 5 mg of Protein A-Sepharose coated with affinity purified 369 (3 µL) or 93/2 antiserum (5 µL). Trizol extracts were diluted in Tris buffer saline, pH 7.5 (1:5, v/v) and 0.2 mg of protein were used for immunoprecipitation. The immunoprecipitates were washed three times with Tris saline buffer containing 2 mM EDTA and 0.2% Tween-20, and they were boiled in denaturing SDS sample buffer prior to separation by SDS-PAGE and Western blotting. *In vitro* translation of C50 and C59 proteins has been reported previously[3].

Results

1. βCTF is the major APP C-terminal fragment detected in human brain frontal cortex.

Analysis of APP species in 2% Triton X-100 extracts from human brain cortex by western blotting with polyclonal antibody 369 (directed to the APP cytoplasmic domain) showed presence of variable amounts of APP full-length, detected as a set of bands in the 110-120 kDa molecular range, and small CTF fragments in the 10-25 kDa range. The predominant CTF detected in both AD and CT brain samples had an apparent molecular weight of 14 kDa (Figure 1A) and) and an electrophoretic mobility similar to that of C100 overexpressed in transgenic mouse (not shown). It was immunoreactive with WO2, and with other antibodies directed to Aβ N-terminus, and was thus identified as βCTF. A second band of approximately 10 kDa was also detected in all samples. This corresponds to αCTF as it co-migrates with the major CTF species produced by COS7 cells stably overexpressing APP or by untransfected SH-SY5Y, and it cannot be immunoprecipitated with anti-Aβ N-terminal antibody WO2. Minor signals of 22 and 25 kDa were also detectable in some of the samples and would correspond to cleavages occurring upstream from the β-secretase site. The blot shown in Figure 1 suggests that βCTF is more elevated in AD samples than in control samples.

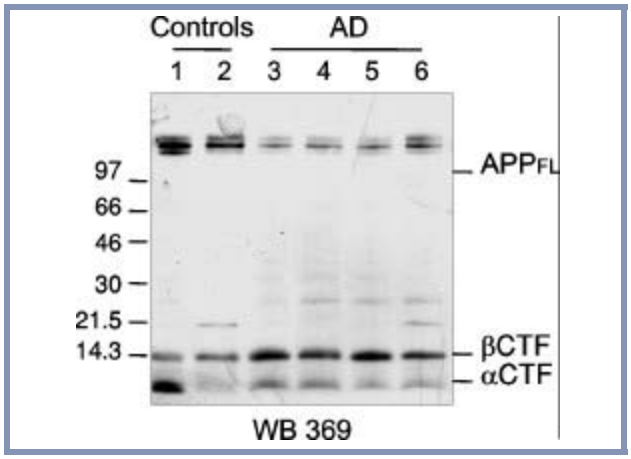


Figure 1 Detection of APP CTF in Triton X-100 extracts from human brain cortex.
20,000 x g pellets from total cortex homogenates were extracted with 2% Triton X-100. 20 µg of protein were resolved on Tris-Tricine 4-20% gels, transferred to PVDF and the blots probed with anti-APP cytoplasmic domain Ab 369. APP was detected as a 110-120 kDa doublet. The predominant C-terminal fragment detected was a 14 kDa product corresponding to βCTF. αCTF was detected as a 10 kDa signal.

2. βCTF is more elevated in frontal cortex from AD than from control subjects

To consolidate the previous finding, densitometric analysis of the western blot signals was performed on a larger series of samples. For the quantitative analysis, cortex samples were extracted with Trizol, the protein was precipitated with isopropanol, and was re-dissolved using 1% SDS. This procedure allowed optimal extraction of membrane proteins and, in particular, of APP CTF species A representative blot is shown in Figure 2A. In these conditions, βCTF and the 22 kDa fragment were enriched relatively to αCTF and APP, suggesting they required more stringent extraction conditions and possibly, were aggregated. Image densitometry was performed on the scans of the ECL films. Calculation of the band density of the βCTF signal relative to β-tubulin showed among seven controls and eight AD sample tested a statistically significant two-fold increase in AD. This finding is in agreement with our recent report of a 2.7-fold elevation of BACE immunoreactive species in AD[17].

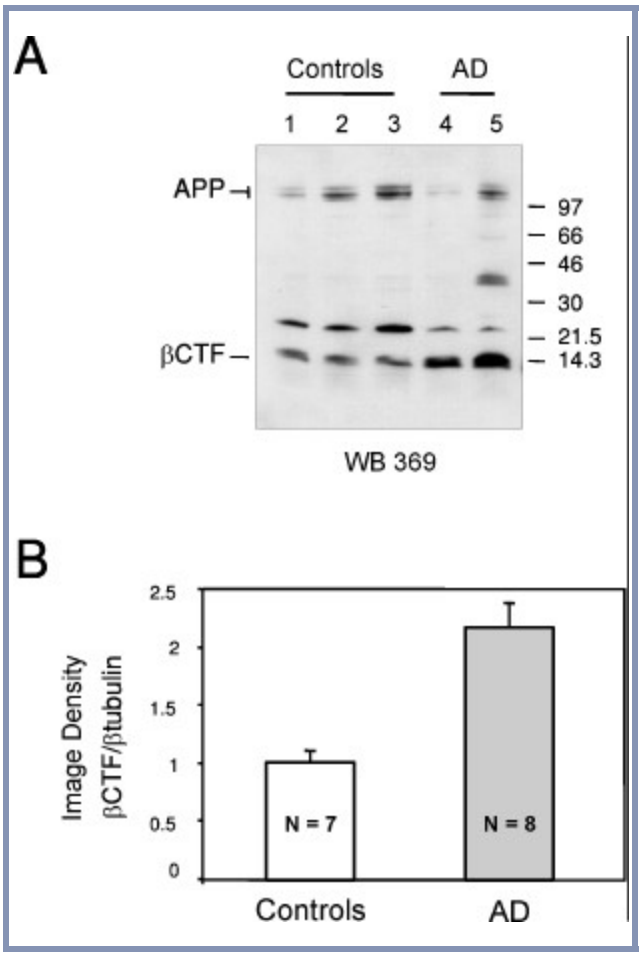


Figure 2 Extraction of APP CTF with Trizol demonstrates that βCTF is predominant and accumulates in AD.

Brain cortex tissue was homogenized in Trizol reagent, and total protein was precipitated and re-solubilized in 1% SDS. 20 µg of protein were analyzed by western blot as described for Figure 1. Panel A shows analysis of four control and two AD samples. Panel B shows quantitative analysis of the 14 kDa band (βCTF) in comparison to β-tubulin western blot signal (not shown) that was used as a neuronal marker (ref. 17). ECL films were scanned and the bands quantitated by image densitometry (NIH Image software). Statistical analysis indicates a significant two-fold increase in the βCTF/β-tubulin ratio in AD as compared to controls.

3. Detection of εCTF in lysates of SH-SY5Y cells and in human brain cortex fractions

To search for the presence of APP C-terminal fragments resulting from γ-secretase cleavage, lysates of neuroblastoma cells SH-SY5Y and various fractions from human brain cortex were immunoprecipitated with two alternative APP C-terminal antibodies: Ab 369 that was raised to the entire cytosolic region of APP, and 93/2 that was produced by immunization with a peptide corresponding to the last 15 carboxyl-terminal residues of APP. Figure 3A shows that both antibodies precipitated from the lysates of SH-SY5Y a similar C-terminal product of 5.5 kDa (lanes 2 and 4). From its apparent molecular weight and its immunoreactivity this fragment is likely to correspond to a γ-secretase CTF. Traces of a similar fragment were also detected in a 3000 g pellet from post nuclear fractionation of a control human brain (lane 3). This fraction corresponds to heavy membrane vesicles containing mostly lysosomes and some nuclei material[8].

To investigate further the presence and identity of the 5.5 kDa fragment in human brain, we analysed alternative fractions (Figure 3B). These included a nuclei-rich fraction (570 g pellet, that may also contain heavy lysosomes and membrane debris, and that we labeled N/L), and a cytosolic fraction (100,000 g supernatant obtained from centrifugation of a 10,000 g supernatant; labeled C). All samples were depleted from APP by absorption on Q-Sepharose[18] and similar amounts of protein were immunoprecipitated with Ab369. Western blot detection showed presence in the nuclei-rich fraction (lane 1) of a 5.5 kDa C-terminal fragment with an electrophoretic mobility similar to that of a C59 recombinant protein (lane 7) but faster than that of C50 standard (lane 6). Traces of the same 5.5 kDa species were also present in the precipitate of the cytosolic fraction (lane 5). Longer exposure of the blot (not shown) also revealed the fragment in the lysosomal fraction (lane 2). Together, these data indicate that the 5.5 kDa is relatively enriched in the nuclei fraction and also present to a lesser extent in the cytosol. By comparison with recombinant standard proteins it appears to correspond to C59, the εCTF product of Ps/γ-secretase complex.

To facilitate comparative analysis of various AD and control cortex samples we attempted to detect εCTF in total protein extracts from frontal cortex. Protein was extracted with Trizol, followed by precipitation with isopropanol and re-dissolution in 1% SDS. The samples were diluted ten-fold

to reduce SDS concentration and immunoprecioitated with Ab 369. Figure 3B, lane 2 shows that a 5.5 kDa fragment, similar to that produced by SH-SY5Y cells (lane 1), was detected in cortex homogenate from an AD patient. The band is fuzzy and possibly represents several isoforms. Next, we analyzed the APP small CTF products in a sample from a patient characterized previously as a carrier of a V→I APP mutation at codon 717[19]. Figure 3C shows presence of an ~6 kDa band that was more retained than the fragment detected in a sporadic AD and in a control sample. Traces of this new 6 kDa fragment were also detectable in the sporadic AD sample. Although it will require further characterization, we presume than this 6 kDa band represents the CTF produced by γ-secretase cleavage at position 40 or 42.

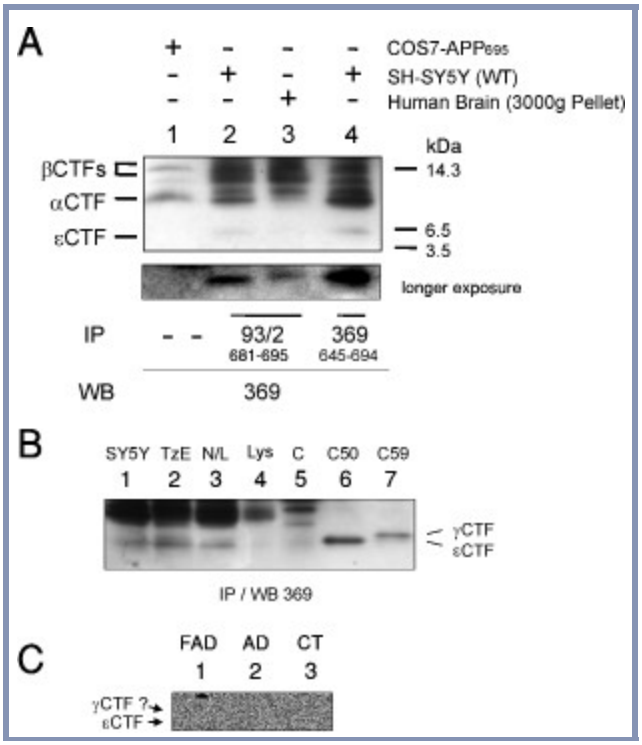


Figure 3 Detection of εCTF in SH-SY5Y lysates and in cortical nuclear fractions.

Panel A shows immunoprecipitation of SH-SY5Y lysate with two alternative APP C-terminal antibodies (lanes 2 and 4). Lane 2 corresponds to immunoprecipitation of a 3000 x g fraction from a human cortex post-nuclear supernatant. Lane 1 shows direct western blot of lysate of COS7 cells overexpressing APP695 that was used as a reference for α and βCTF. A 5.5 kDa band was detected in the precipitates from SH-SY5Y cells. A similar, faint signal was also identified in the brain fraction precipitate. Panel B shows immunoprecipitation of APP 5.5 kDa CTF from various human brain fractions. TzE corresponds to Trizol extract , N/L to a nuclei-enriched fraction (570 x g pellet), and Lys to a lysosomal preparation (3000 x g pellet from post-nuclear supernatant). SH-SY5Y lysate and recombinant C50 (γCTF) and C59 (εCTF) were used as reference standards. The 5.5 kDa band identified in human brain migrates with an electrophoretic mobility similar to that of a fragment produced in SH-SY5Y cells and to that of εCTF standard. Panel C shows analysis of immunoprecipitates of Trizol cortical extracts from an FAD (APP London mutation; lane 1), a sporadic AD (lane 2) and a control subject (lane 3). A signal at 6 kDa was observed in the FAD sample that may correspond to γCTF. Further characterization of these products is in progress.

Discussion

Accumulating evidence points to Aβ amyloid as a key component of AD pathogenesis. The processing of APP within its transmembrane region remains poorly understood although recent progress has allowed identification of the proteolytic activities that excise Aβ from its precursor APP. The membrane-anchored aspartyl protease BACE carries out the β-secretase cut to produce βCTF, and subsequent proteolysis of this fragment by a γ-secretase complex that includes PS1 completes liberation of the Aβ peptide (reviewed in 2). The precise mechanism of γ-secretase cleavage remains to be elucidated although a recent study demonstrates that it shares a striking resemblance with the cleavage and processing of signal peptides by signal peptide peptidase[20].

We have examined the APP C-terminal fragments present in human brain cortex homogenates. Triton X-100 extracted βCTF as the predominant CTF species together with substantial amounts of αCTF. Extraction in more stringent conditions, using Trizol, demonstrated that βCTF and a 22 kDa fragment were the two predominant species whereas αCTF bands were relatively minor. Quantitative analysis suggested that βCTF is increased by two-fold in the cortex of AD subjects in comparison to normal controls. This result is consistent with our recent report that BACE 70 kDa protein is elevated by more than two-fold in AD. Since βCTF represents the direct precursor of Aβ one may propose that the metabolic machinery processing this fragment, either the presenilin-dependent γ-secretase or a degradation pathway such as Detection of the proteasome[15] would be limiting. Other studies have examined APP CTF in human brain cortex[13,14, 21-23]. These identified similar 22, 14 (βCTF) and 10 kDa (αCTF) products. They also showed that these products can be resolved as multiple isoforms reflecting heterogeneity principally due to phosphorylation[22, 23]. Although a quantitative study by Russo et al suggested that βCTF might be more elevated in AD than in controls, the result was not statistically significant[22]. Sergeant et al reported that APP β and αCTF were actually decreased in AD[23]. These results were based on quantitation of Triton X-100 soluble species and the samples studied corresponded to temporal and occipital regions whereas we carried out our studies on frontal lobe samples only. Consistent with the report by Russo et al, we were unable to show a statistically significant difference in βCTF levels between AD and control samples using Triton X-100 extraction procedure. In contrast, we were able to identify a significant two-fold increase in βCTF in AD when using more stringent extraction conditions. This suggests that βCTF present in AD cortex exists as species of low solubility, possibly aggregates. This is in agreement with the report by Sergeant et al of the presence of a pool of βCTF in a detergent insoluble fraction in AD cortex. It is too early at present to speculate as to whether βCTF accumulation in AD brain may contribute to the disease pathogenesis.

The second part of our study examined APP γ-secretase CTFs. We showed that a 5.5 kDa fragment that co-migrates with recombinant εCTF standard was detectable in non-transfected human neuroblastoma SH-SY5Y cells and in AD cortex. This was immunoprecipitated by two alternative APP C-terminal antibodies. Precipitation with 93/2 indicated that this fragment contained the C-terminal portion of the APP cytosolic domain, beyond the caspase cleavage site we have previously identified[24]. Comparison of its electrophoretic mobility with *in vitro* translated C50 and C59 standards identified the 5.5 kDa fragment as εCTF (C50), the product of cleavage at position 49. A previous study has documented the presence of a 5.8 kDa fragment in lysosomal fractions from human cortex homogenates[25] and it probably represents the same εCTF species as we report here although we found that the levels of εCTF were low in lysosomal fractions. We have shown that the 5.5 kDa fragment was relatively enriched in nuclear fractions, as expected for the C-terminal domain of APP released from the membrane. Indeed, studies with transfected cell systems have shown that the APP C-terminal region interacts with the protein adaptor Fe65 and regulates its trafficking to the nucleus[7-9]. A recent study with neuronal cultures has also demonstrated that APP cytosolic fragment partly localized to the nucleus[26]. When we analyzed a sample from a subject carrying the APP London mutation (codon 717) we did not detect εCTF but we observed another fragment of ~6 kDa. This was also detectable, besides εCTF, in some AD samples and we presume it corresponds to a cleavage occurring upstream from ε-cleavage site. We are currently investigating further the nature of this 6 kDa species that has the expected electrophoretic mobility of the C-terminal product of γ-secretase cleavage at site 40 or 42. Our data may suggest that γ-secretase preferred cleavage site is at 49_50 (ε-site) but that in AD, and particularly in the presence of the London mutation, this cleavage may be impaired and may favor alternative cleavages at positions 40 and 42. Further analysis of these fragments in samples from AD and FAD subjects is in progress.

Acknowledgements

This work was supported by the NH-MRC (Program Grant 425618). D.E.H. is supported by a post-doctoral fellowship from NIH. K.T.B. is supported by the Deutsche Forchungsgemeinschaft and the Bundenministerium für Forschung und Technologie.

References

1. Selkoe D. J., Alzheimer's disease: genes, proteins, and therapy. *Physiol. Rev.* 2001; 81:741-766
2. Evin G., Weidemann. A., Biogenesis and metabolism of Alzheimer's disease Aβ amyloid peptides. *Peptides* 2002; 23: 1285-129.
3. Weidemann A., Eggert S., Reinhard F. B. M., Vogel M., Paliga K., Baier G., Masters C. L., Beyreuther K., Evin G.,' A novel ε-Cleavage within the Transmembrane Domain of the Alzheimer Amyloid Precursor Protein demonstrates Homology with Notch Processing. *Biochemistry* 2002; 41: 2825-2835.
4. Sastre M., Steiner H., Fuchs K., Capell A., Multhaup G., Condron M. M., Teplow D. B., Haass C., Presenilin-dependent gamma-secretase processing of beta-amyloid precursor protein at a site corresponding to the S3 cleavage of Notch. *EMBO Rep* 2001; 2, 835-841.
5. Yu C., Kim S. H., Ikeuchi T., Xu H., Gasparini. L, Wang R., Sisodia S. S., Characterization of a presenilin-mediated amyloid precursor protein carboxyl-terminal fragment gamma. Evidence for distinct mechanisms involved in gamma -secretase processing of the APP and Notch1 transmembrane domains. *J. Biol. Chem.* 2001 276: 43756-43760.
6. Gu Y., Misonou H., Sato T., Dohmae N., Takio K., Iha ra Y. Distinct intramembrane cleavage of the beta-amyloid precursor protein family resembling gamma-secretase-like cleavage of Notch. *J. Biol. Chem.* 2001; 276: 35235-35238.
7. Minopoli G., de Candia P., Bonetti A., Faraonio R., Zambrano N., and Russo T., The β-amyloid precursor protein functions as a cytosolic anchoring site that prevents Fe65 nuclear translocation. *J. Biol. Chem.* 2001; 276: 6546-6550.
8. Cao X., Sudhof T. C. A transcriptionally active complex of APP with Fe65 and histone acetyltransferase Tip60. *Science* 2001; 293: 115-120.
9. Kimberly W. T., Zheng J. B., Guenette S. Y., Selkoe D. J. The intracellular domain of the beta-amyloid precursor protein is stabilized by Fe65 and translocates to the nucleus in a notch-like manner. *J. Biol. Chem.* 2001; 276: 40288-40292.
10. Gao Y, Pimplikar SW. The gamma-secretase-cleaved C-terminal fragment of amyloid precursor protein mediates signaling to the nucleus. *Proc. Natl. Acad. Sci. U.S.A.* 2001; 98: 14979-14984.
11. Leissring M. A., Murphy M. P., Mead T. R., Akbari Y., Sugarman M. C., Jannatipour M., Anliker B., Müller U., Saftig P., De Strooper B., Wolfe M. S., Golde T. E., LaFerla F., A physiologic signaling role for the γ-secretase-derived intracellular fragment of APP. *Proc. Natl. Acad. Sci. U.S.A.* 2002; 99: 4697-4702.
12. Kim J. H., Rah J. C., Fraser S. P., Chang K. A., Djamgoz M. B., Suh Y.H., Carboxyl-terminal peptide of beta-amyloid precursor protein blocks inositol 1,4,5-trisphosphate-sensitive Ca2+ release in Xenopus laevis oocytes. *J. Biol. Chem.* 2002; 277: 20256-20363.
13. Evin G., Cappai R., Li Q-X, Culvenor J. G., Small D. H., Beyreuther K., Masters C. L., Candidate γ-secretases in the generation of the carboxyl-terminus of the Alzheimer's disease βA4 amyloid: possible involvement of cathepsin D. *Biochemistry* 1995; 34: 14185-14192.
14. Nordstedt C., Gandy S. E., Alafuzoff I., Caporaso G. L., Iverfeldt K., Grebb J. A., Winblad B., Greengard P. Alzheimer beta/A4 amyloid precursor protein in human brain: aging-associated increases in holoprotein and in a proteolytic fragment. *Proc. Natl. Acad. Sci. U.S.A.* 1991; 88: 8910-8914.
15. Nunan J., Shearman M. S., Checler F., Cappai R., Evin G., Beyreuther K., Masters C. L., Small D. H.. The C-terminal fragment of the Alzheimer's disease amyloid precursor protein is degraded by a proteasome-dependent mechanism distinct from gamma-secretase. *Eur. J. Biochem.* 2001; 268: 5329-5336.
16. Ida N, Hartmann T, Pantel J, Schroder J, Zerfass R, Forstl H, Sandbrink R, Masters CL, Beyreuther K. Analysis of heterogeneous A4 peptides in human cerebrospinal fluid and blood by a newly developed sensitive Western blot assay. *J. Biol. Chem.* 1996; 271: 22908-22914.
17. Holsinger R. M. D., McLean C. A., Beyreuther K., Masters C. L., and Evin G. Increased expression of the amyloid precursor β-secretase in Alzheimer's disease. *Ann. Neurol.* 2002; 51: 783-786.

18. Moir R. D., Martins R. N., Bush A. I., Small D. H., Milward E. A., Rumble B. A., Multhaup G., Beyreuther K. , Masters C. L. Human brain beta A4 amyloid protein precursor of Alzheimer's disease: purification and partial characterization. *J. Neurochem.* 1992; 59: 1490-1498.
19. Evin, G. Smith M. J., Tziotis A., McLean C., Canterford L., Sharples R. A, Cappai R., Weidemann A., Beyreuther, R. G. H. Cotton, C. L. Masters, J. G. Culvenor R. G. Alternative transcripts of presenilin-1 associated with frontotemporal dementia. *Neuroreport* 2002; 13: 917-921.
20. Weihofen A., Binns K., Lemberg M. K., Ashman K., Martoglio B., Identification of signal peptide peptidase, a presenilin-type aspartic protease. *Science* 2002; 296: 2215-2218.
21. Estus S., Golde T. E., Kunishita T., Blades D., Lowery D., Eisen M., Usiak M. Qu X., Tabira T., Greenberg B. D., Younkin S. G. Potentially amyloidogenic, carboxyl-terminal derivatives of the amyloid protein precursor. *Science* 1992; 255: 726-728.
22. Russo C., Salis S., Dolcini V., Venezia V., Song X.-H., Teller J. K., and Schettini G. Identification of amino-terminally and phosphotyrosine-modified carboxy-terminal fragments of the amyloid precursor protein in Alzheimer's disease and Down's syndrome brain. *Neurobiol. Dis.* 2001; 8, 173-180.
23. Sergeant N., David J. P., Champain D., Ghestem A., Wattez A., Delacourte A. Progressive decrease of amyloid precursor protein carboxy terminal fragments (APP-CTFs), associated with tau pathology stages, in Alzheimer's disease. *J. Neurochem.* 2002; 81: 663-672.
24. Weidemann A., Paliga K., Dürrwang U., Reinhard F., Evin G., and Masters C. L. Proteolytic Processing of the Alzheimer's Disease Precursor Protein Within its Cytoplasmic Domain by Caspase-like Proteases. *J. Biol. Chem.* 1999; 274: 5823-5829.
25. Sun X., Tashiro T., Hirai S., Yamaguchi H. Identification of a 5.8 kDa C-terminal fragment of Alzheimer amyloid β protein precursor generated in the lysosomal system. Amyloid. *Int. J. Exp. Clin. Invest.* 1994; 1: 100-106.
26. Cupers P., Orlans I., Craessaerts K., Annaert W. and De Strooper B. The amyloid precursor protein (APP)-cytoplasmic fragment generated by γ-secretase is rapidly degraded but distributes partially in a nuclear fraction of neurons in culture. *J. Neurochem.* 2001; 78: 1168-1178.

Galantamine Significantly Improves All Aspects of Cognition in Patients With Advanced-Moderate Alzheimer's Disease

R. Blesa[1#], S. Schwalen[2]

[1] Neurology Department, Hospital Clinic, University of Barcelona, Spain

[2] Janssen-Cilag GmbH, Neuss, Germany

Abstract

Galantamine, an acetylcholinesterase inhibitor (AChEI) with an additional mechanism of action, nicotinic receptor modulation, is indicated for the treatment of patients with mild-to-moderate AD. A post hoc analysis was performed on pooled data from 4 pivotal studies to determine whether patients with advanced moderate AD benefit from galantamine treatment. Patients with a baseline ADAS-cog score of >30 or MMSE score of £12 were included in the analysis. Over 5 to 6 months, AD patients with advanced-moderate disease who were treated with galantamine demonstrated significant cognitive benefits (ADAS-cog scale) compared with placebo (p < 0.001). Global functioning also improved with galantamine treatment. Results from this analysis indicate that galantamine may be beneficial in patients with more advanced stages of AD.

Keywords: Acetylcholinesterase inhibitor; Advanced disease; Alzheimer's disease; Galantamine

Introduction

Alzheimer's disease (AD) is a progressive, neurodegenerative disease affecting millions of people worldwide. It is estimated that 5.7 % to 10 % of the US population aged 65 to 85 years and 25 % to 45 % of those aged 85 years or older have AD[1,2]. The most common presenting symptoms are memory loss and loss of other cognitive functions. This is typically followed by a progressive decline in the ability to perform activities of daily living (ADL); behavorial disturbances; increased caregiver time; increased utilization of resources and medical care; and eventual need for full-time, assisted-living or nursing home care before death. Although all manifestations of AD must be managed adequately, preservation of cognitive function is of central importance to the patient and is critical in assessing the response to therapy in clinical trials. Cognitive function typically declines rapidly, averaging 9 to 11 points on the cognitive subscale of the Alzheimer's Disease Assessment Scale (ADAS-cog) in untreated patients[3]. Although this decline is progressive, it is not linear. Patients in the milder stages of AD tend to deteriorate less rapidly than those patients in the moderate stages. Thus, patients with more advanced AD may benefit more measurably from treatment because untreated patients in this stage deteriorate much faster. If left untreated, patients with mild-to-moderate AD will exhibit severe decline within 2 years.

Currently, the acetylcholinesterase inhibitors (AChEIs) are the treatment of choice for AD, as these drugs have maintained or improved the cognitive decline seen in patients with AD[4-13]. However, AChEIs are indicated only for the treatment of mild-to-moderate AD. Because AD is a persistent disease that may affect patients for up to 10 years[14], patients may remain in its advanced stages for several years. Management of such patients with severe dementia poses a challenge to physicians, as studies evaluating cholinergic treatments for AD have traditionally focused on efficacy in patients with mild-to-moderate disease. Clinicians lack clear guidelines for treatment of the more advanced stages of AD[15], and therefore little is known about the efficacy of cholinergic drugs across different cognitive symptom domains. Galantamine, the most recent addition to the AChEI class that also modulates nicotinic receptors[16], has delayed the cognitive decline in patients with mild-to-moderate AD for up to 1 year and has also demonstrated significant benefits in behavior, ADL, and caregiver burden[4-7].

Here, the change in cognition over 5 to 6 months is evaluated in patients with advanced- moderate AD in 4 large-scale phase III studies of galantamine[4-7]. Because the methodology in these trials is consistent, it is possible to

Correspondence: Rafael Blesa, MD, Neurology Department, Hospital Clinic, University of Barcelona,Villarope 1 170, Barcelona, Spain 08036, Telephone: 011 34 93 227 5400, Fax: 011 34 93 227 5414, E-mail: rblesa@clinic.ub.es

pool the cognitive function data and perform a post hoc analysis of the effects of galantamine in both total ADAS-cog scores and individual item scores in patients with advanced moderate AD.

Methods

Design of Phase III Studies

All phase III studies were randomized, double-blind, placebo-controlled, multicenter trials conducted in Australia, Canada, Europe, South Africa, and the United States to assess the safety and efficacy of galantamine in patients with mild-to-moderate AD:

- 3-month international study comparing placebo (n = 125) and galantamine 24 to 32 mg/day (n = 261; 126 patients received 24 mg/day)[7]
- 5-month US study comparing placebo (n = 286), galantamine 8 mg/day (n = 140), galantamine 16 mg/day (n = 279), and galantamine 24 mg/day (n = 273)[5]
- 6-month US study comparing placebo (n = 213), galantamine 24 mg/day (n = 212) and galantamine 32 mg/day (n = 211)[4]
- 6-month international study comparing placebo (n = 215) and galantamine 24 mg/day (n = 220) or 32 mg/day (n = 218)[6]

In these studies, patients had mild-to-moderate AD, defined as a baseline Mini-Mental State Examination (MMSE) score of 10 to 22 and a score of ≥18 on the ADAS-cog in the 5-month US study[5], and a baseline MMSE score of 11 to 24 and a score of ≥12 on the ADAS-cog in the other 3 studies[4,6,7]. The primary outcome measure in all studies was change in cognition assessed with the ADAS-cog. Overall response to treatment was measured with the Clinician's Interview-based Impression of Change plus caregiver input (CIBIC-plus)[4-7]. Other outcome measures included changes in ADL[4-7] and behavioral symptoms[5].

In all trials, the patient and a responsible caregiver provided written informed consent to participate. These studies were conducted according to the Declaration of Helsinki and subsequent revision, and were approved by the institutional review boards of each center.

Patients With Advanced-Moderate AD

Data were pooled and a post hoc analysis was performed on the subgroup of advanced moderate AD patients. Galantamine 24 mg/day was selected for the post hoc analysis because it was a common dosethe recommended daily dose in the 4 studies. Two subgroups of patients with advanced-moderate disease were identified by ADAS-cog and MMSE scores as follows:

- Baseline ADAS-cog scores >30 (Group 1), or
- Baseline MMSE scores ≤12 (Group 2)

In this analysis, patients who were assessed and found to be toward or at the end of the moderate AD range of the continuum are classified as having advanced-moderate AD.

Changes in both total ADAS-cog scores and individual item scores over 5 to 6 months were used to evaluate the effect of galantamine on cognitive function. Global outcome was measured using the CIBIC-plus. The safety profile of galantamine was also analyzed.

Statistical Analyses

For the purposes of this pooled analysis, the patient numbers are higher at the baseline and 3-month assessments owing to the inclusion of the 3-month study, whereas the 5- to 6-month endpoint is a combination of data from the one 5-month and two 6-month studies.

All randomized patients who received at least one dose of trial medication were included in the analyses of baseline characteristics and safety data. For the post hoc analysis of patients with advanced-moderate AD, the primary statistical analysis of efficacy was the same as in the individual trials—the change in ADAS-cog score. In the individual trials, an intention-to-treat (ITT) analysis was performed as a sensitivity analysis, using the last observation carried forward (LOCF) method, in which the last observation available for each patient who received treatment is used in the analysis. Changes in ADAS-cog scores at 3 and 5 to 6 months were evaluated and compared with the treatment groups by means of an analysis of covariance with trial and baseline values as covariates. CIBIC-plus scores were evaluated using the Van Elteren test, controlling for trial.

Results

Patient Demographics

The database from the 4 phase III studies comprised 831 patients who received galantamine 24 mg/day and 839 patients who received placebo. All patients were included in the 3-month analyses. The 5- to 6-month analyses were based on the 705 and 714 patients who received galantamine and placebo, respectively, in the 5-month study and the two 6-month studies.

Overall, 245 and 257 patients receiving galantamine 24 mg/day and placebo, respectively, had baseline ADAS-cog scores >30. Of these, 215 and 221 patients respectively, entered the 5- and 6-month studies (the remaining patients were involved in the 3-month study). Sixty-three and 61 patients receiving galantamine 24 mg/day and placebo, respectively, had baseline MMSE scores of £12. Of these, 58 and 56, respectively, entered the 5- to 6-month studies. The baseline characteristics of these patients are described in Table 1. Other than AD severity, baseline characteristics

were similar to those seen in the overall populations of the 4 phase III studies.

Cognition

Patients with advanced-moderate AD who were treated with galantamine demonstrated significant improvement in ADAS-cog scores compared with placebo in the group with baseline ADAS-cog scores >30 (Group 1) as well as the group with MMSE scores of £12 (Group 2). Treatment differences were observed as early as 3 to 4 weeks into treatment in Group 1 ($p < 0.0001$) and Group 2 ($p = 0.072$) and cognitive abilities were maintained above baseline levels at 3 and 5 to 6 months.

At 6 months, mean changes from baseline in total ADAS-cog/11 scores were substantially greater with galantamine than with placebo (Figure 1). In the subgroup of patients with baseline ADAS-cog scores >30, patients receiving galantamine 24 mg/day improved by 2.8 points on the ADAS-cog after 5 to 6 months (n = 158), whereas those receiving placebo deteriorated by 3.6 points (n = 160). Treatment difference was 6.4 ADAS-cog points ($p < 0.0001$). In the subgroup of patients with baseline MMSE £12, ADAS-cog scores improved by 0.9 points (n = 41) in patients who received galantamine 24 mg/day and deteriorated by 5.8 points (n = 36) in patients who received placebo. Treatment difference at 5 to 6 months was 6.7 ADAS-cog points ($p = 0.001$).

Findings with total ADAS-cog scores were reflected by significant treatment differences across all individual ADAS-cog items in Group 1 and the majority of items (8/11) in Group 2 (Figure 2). Individual ADAS-cog items evaluated in this analysis included changes in commands, comprehension, constructional praxis, ideational praxis, naming of objects, orientation, remembering instructions, spoken language, word finding, word recall, and word recognition. The greatest changes in both groups were in comprehension, ideation, remembering instructions and word recognition items ($p < 0.0001$ for Group 1 and $p < 0.01$ for Group 2).

Global Impression

In addition to benefits in cognitive function, galantamine demonstrated beneficial effects in global functioning. Global responses to galantamine showed improvement or stabilization in significantly more patients compared with placebo at 5 to 6 months ($p < 0.001$). In Group 1, 55 % (n = 96) of those patients receiving galantamine 24 mg/day had improved or unchanged CIBIC-plus scores at 5 to 6 months, compared with 32 % (n = 57) of those receiving placebo. Improved or unchanged CIBIC-plus scores were seen in 49 % (n = 23) of galantamine-treated patients, compared with 23 % (n = 11) of those who were treated with placebo in Group 2 ($p = 0.008$) at 5 to 6 months.

Safety

Galantamine was well tolerated in patients with advanced-moderate AD in this post hoc analysis. Table 2 lists the most common adverse events (AEs) that were reported in at least 5 % of galantamine-treated patients compared with those receiving placebo. Nausea and vomiting were the most common adverse events in the galantamine-treated group; this was consistent with the original pivotal studies[4-7]. Gastrointestinal AEs are an AChEI class effect and may be managed by dosing with meals. Weight loss was also commonly reported in the galantamine-treated group. Galantamine treatment did not cause any sleep disturbances. Agitation occurred more frequently in the placebo-treated group.

Discussion

This post hoc analysis of data pooled from large-scale phase III studies shows that significant and clinically

Table 1. Baseline patient characteristics

	Baseline ADAS-cog >30		Baseline MMSE £12	
	Galantamine 24 mg/day	Placebo	Galantamine 24 mg/day	Placebo
Number of patients	245	257	63	61
Gender (male:female)	79:166	81:176	22:41	16:45
Mean age (SE)	76.2 (0.51)	75.9 (0.50)	75.8 (0.80)	73 (0.96)
Number (%) receiving concomitant medications	217 (88.6)	223 (86.8)	57 (90.5)	56 (91.8)
Mean ADAS-cog (SE)	38.7 (0.41)	39 (0.41)	42.3 (0.96)	43.1 (1.09)
Mean MMSE (SE)	15.4 (0.21)	15.4 (0.20)	11.1 (0.20)	11.2 (0.10)

Table 2. Adverse events reported ³5 % more frequently than with placebo

Adverse events	Baseline ADAS-cog >30		Baseline MMSE £12	
	Galantamine 24 mg (n = 245)	Placebo (n = 257)	Galantamine 24 mg (n = 63)	Placebo (n = 61)
Nausea	22.0 %	3.9 %	14.3 %	6.6 %
Vomiting	12.7 %	1.9 %	12.7 %	4.9 %
Weight decrease	8.6 %	1.2 %	11.1 %	0 %
Anorexia	12.7 %	3.9 %	12.7 %	1.6 %

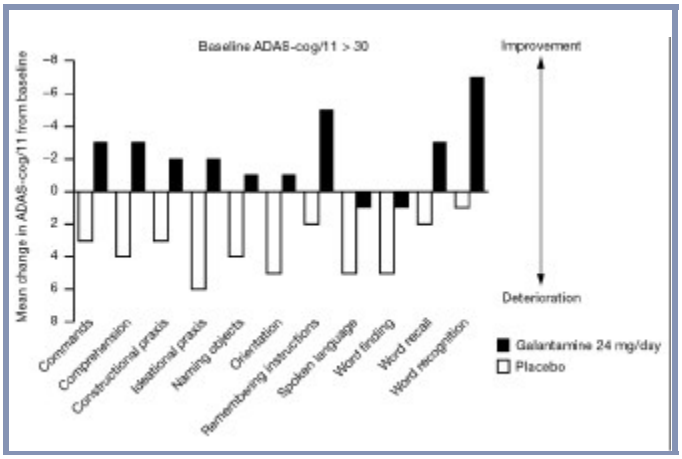


Figure 1. Mean changes from baseline in total ADAS-cog/11 scores over 6 months in galantamine- and placebo-treated patients.

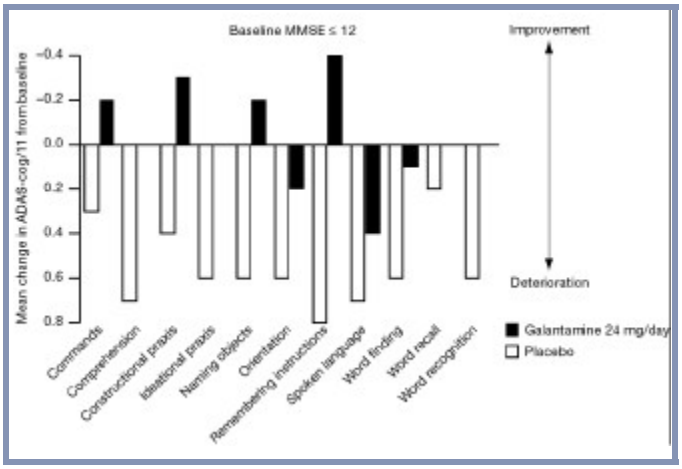


Figure 2. Mean changes from baseline in individual ADAS-cog item scores over 6 months in galantamine- and placebo-treated patients. Note that 8 of the factors have a possible score of 0 to 5 points. The exceptions are Orientation, 0 to 8 points; Word recall, 0 to 10 points; and Word recognition, 0 to 12 points.

important cognitive benefits can be achieved with galantamine in patients with advanced-moderate AD recruited into pivotal clinical studies.

The average treatment difference between galantamine- and placebo-treated patients in both subgroups was approximately 6.5 points on the ADAS-cog scale. It is believed that, to date, the magnitude of this treatment difference is the largest mean effect size reported with a cholinergic treatment for AD at 5 to 6 months. Furthermore, galantamine demonstrated beneficial effects in global functioning in patients with ADAS-cog scores >30 and MMSE scores £12.

Treatment guidance from evidence-based medical reviews points to insufficient evidence for the effectiveness of AChEIs in patients with more advanced AD, as there may be too few functioning cholinergic neurons on which cholinergic drugs can act[15]. However, according to recent studies, although neocortical cholinergic function characteristically declines in patients with severe AD, overt cholinergic deficits generally do not appear until late in the course of the disease[17]. Furthermore, the recent National Institute for Clinical Excellence (NICE) guidelines recommend that AChEIs be made available to patients with mild-to-moderate AD[18]. These guidelines also indicate that AChEIs exhibit no evidence of meaningful benefits or cost-effectiveness in patients with an MMSE score of £12[18]. However, the results of this post hoc analysis demonstrate that galantamine may provide cognitive as well as global functioning benefits in patients with more advanced stages of AD.

These benefits clearly have value for patients and may well decrease the burden placed on caregivers of patients with AD as well as associated societal costs. An enormous responsibility is placed on the caregivers (eg, spouse, child, sibling, friend) of patients with AD, as many of them spend significant time and money caring for a patient. As the role of the caregiver increases, he or she may begin to develop feelings of anger, grief, loneliness, hopelessness, and resentment, which can then affect personal health and overall well-being. Thus, any treatment that improves the patient's condition will invariably benefit the caregiver, making tasks less arduous and possibly more rewarding. Furthermore, this improvement will

most likely decrease the economic and societal costs of AD, as caregiver burden is an important determinant of when institutionalization is initiated[19].

Similar to the results seen in patients with mild-to-moderate AD who were studied in the pivotal clinical trials[4–7], galantamine was well tolerated in patients with more severe illness. As expected, gastrointestinal AEs were observed more frequently in the galantamine-treated group. Of note, agitation occurred even less frequently in the galantamine-treated group than in the placebo-treated group.

The results of this study, coupled with previous clinical experience[4-7], indicate that galantamine may be effective in a broad spectrum of AD, including more severe disease. Although several clinical studies of AChEIs have demonstrated the importance of early diagnosis and treatment of patients with mild-to-moderate AD[4,11,20], galantamine has also demonstrated beneficial effects in patients with advanced disease. In a progressive, degenerative condition such as AD, patients will eventually decline, but discontinuing treatment when an arbitrary level of cognitive function is reached or not initiating treatment for more severe impairment may deny patients cognitive benefits for the longest possible time period. Patients with AD should be maintained on treatment for as long as benefits persist. Further clinical investigation is needed for patients with advanced stages of AD to gain a better understanding of the clinical benefits of AChEIs used to treat these patients.

References

1. Evans D.A., Smith L.A., Scherr P.A., Albert M.S., Funkenstein H.H., Hebert L.E., Risk of death from Alzheimer's disease in a community population of older persons. *Am J Epidemiol* 1991; 134: 403-12.
2. U.S. General Accounting Office. Alzheimer's disease. Estimates of prevalence in the United States. GAO/HEHS-98-16, 1-45. Washington, DC: U.S. General Accounting Office; 1998.
3. Stern R.G., Mohs R.C., Davidson M., Schmeidler J., Silverman J., Kramer-Ginsberg E., Searcey T., Bierer L., Davis K.L., A longitudinal study of Alzheimer's disease: measurement, rate, and predictors of cognitive deterioration. *Am J Psychiatry* 1994; 151: 390-6.
4. Raskind M.A., Peskind E.R., Wessel T., Yuan W., Galantamine in AD: a 6-month randomized, placebo-controlled trial with a 6-month extension. *Neurology* 2000; 54: 2261-8.
5. Tariot P.N., Solomon P.R., Morris J.C., Kershaw P., Lilienfeld S., Ding C., A 5-month, randomized, placebo-controlled trial of galantamine in AD. *Neurology* 2000; 54: 2269-76.
6. Wilcock G.K., Lilienfeld S., Gaens E., Efficacy and safety of galantamine in patients with mild to moderate Alzheimer's disease: multicentre randomised controlled trial. *BMJ* 2000; 321: 1445-9.
7. Rockwood K., Mintzer J., Truyen L., Wessel T., Wilkinson D., Effects of a flexible galantamine dose in Alzheimer's disease: a randomised, controlled trial. *J Neurol Neurosurg Psychiatry* 2001; 71: 589-95.
8. Mohs R.C., Doody R.S., Morris J.C., Ieni J.R., Rogers S.L., Perdomo C.A., Pratt R.D., A 1-year, placebo-controlled preservation of function survival study of donepezil in AD patients. *Neurology* 2001; 57: 481-8.
9. Rogers S.L., Farlow M.R., Doody R.S., Mohs R., Friedhoff L.T., A 24-week, double-blind, placebo-controlled trial of donepezil in patients with Alzheimer's disease. Donepezil Study Group. *Neurology* 1998; 50: 136-45.
10. Rogers S.L., Doody R.S., Pratt R.D., Ieni J.R., Long-term efficacy and safety of donepezil in the treatment of Alzheimer's disease: final analysis of a US multicentre open-label study. *Eur Neuropsychopharmacol* 2000; 10: 195-203.
11. Doody R.S., Geldmacher D.S., Gordon B., Perdomo C.A., Pratt R.D., Open-label, multicenter, phase 3 extension study of the safety and efficacy of donepezil in patients with Alzheimer disease. *Arch Neurol* 2001; 58: 427-33.
12. Rosler M., Anand R., Cicin-Sain A., Gauthier S., Agid Y., Dal-Bianco P., Stahelin H.B., Hartman R., Gharabawi M., Efficacy and safety of rivastigmine in patients with Alzheimer's disease: international randomised controlled trial. *BMJ* 1999; 318: 633-8.
13. Corey-Bloom J., Anand R., Veach J. A randomized trial evaluating the efficacy and safety of ENA 713 (rivastigmine tartrate), a new acetylcholinesterase inhibitor, in patients with mild to moderately severe Alzheimer's disease for the ENA 713 B352 Study Group. *Int J Geriatr Psychopharmacol* 1998; 1: 55-65.
14. Walsh J.S., Welch H.G., Larson E.B. Survival of outpatients with Alzheimer-type dementia. *Ann Intern Med* 1990; 113: 429-34.
15. Clegg A., Bryant J., Nicholson T., et al. National Institute of Clinical Excellence, 2000.
16. Maelicke A., Samochocki M., Jostock R., Fehrenbacher A., Ludwig J., Albuquerque E.X., Zerlin M., Allosteric sensitization of nicotinic receptors by galantamine, a new treatment strategy for Alzheimer's disease. *Biol Psychiatry* 2001; 49: 279-88.
17. Davis K.L., Mohs R.C., Marin D., Purohit D.P., Perl D.P., Lantz M., Austin G., Haroutunian V., Cholinergic markers in elderly patients with early signs of Alzheimer disease. *JAMA* 1999; 281: 1401-6.
18. National Institute for Clinical Excellence. *Guidance on the Use of Donepezil, Rivastigmine, and Galantamine for the Treatment of Alzheimer's Disease.* Technology Appraisal Guidance No. 19, January 2001.
19. Annerstedt L., Elmstahl S., Ingvad B., Samuelsson S.M. Family caregiving in dementia—an analysis of the caregiver's burden and the „breaking-point" when home care becomes inadequate. *Scand J Public Health* 2000; 28: 23-31.

Guidelines for Authors

Manuscript format

Manuscripts can be sent by e-mail or mail. We accept 3.5-inch IBM-PC format floppy or CD. The manuscripts should be either in pure ASCII text or MS Word 6.0 or greater. The cover page should indicate the article's title, the full name, highest pertinent academic degrees, institutional affiliations, and current address of each author, the author handling all correspondence, e-mail address, telephone and fax numbers, and, if the manuscript was orally presented at a meeting, the name of the organization, place, and date when it was read.

The first use of an uncommon abbreviation should be preceded by the full name. Generic names of drugs are preferable. If a brand name is used, it should be in parentheses following the generic name, footnoted with the name and address of the supplier. Subheads should be inserted at suitable intervals.

MS Word files should *not* contain photographs, drawings or charts; these should be sent as separate files, indicating in the MS Word file the place where these should be placed. Drawings or charts are not accepted in MS Word format.

Do not use an extra hard return between paragraphs. Do not use tabs or extra space at the start of a paragraph or for list entries. Do not indent runover lines in references. Turn off line spacing. Turn off hyphenation. Do not specify page breaks, page numbers, or headers. Do not specify typeface. Care should be taken to correctly enter „one" (1) and lower case „el" (l), as well as „zero" (0) and capital „oh" (O).

Non-standard characters (Greek letters, mathematical symbols, etc.) should be coded consistently throughout the text. Please make a list of such characters and provide a listing of the codes used.

Figures & Tables

The Journal welcomes figures to accompany articles. Color figures are encouraged. Charts should be titled and numbered consecutively according to the citation in the text. Tables should be explained in the text. Short descriptive legends should be provided on a separate page. Legends for figures previously published should include a complete reference to the original publication, with the copyright designation. Copies of the publisher'' and author'' permission to use the figure must be provided. Tables, photos, and figures must be submitted in the following formats: TIF, EPS, or AI. JPEG and GIF files are not accepted. Tables can be submitted also as MS Word or MS Excel files. We accept also Corel Draw files.

The resolution of illustrations, charts, and photographs should be at least 300 dpi.

Manuscript review

All papers are subjected to editorial review. The editors will decide if a paper is suitable for the editorial profile of the Journal.

Articles containing original material are accepted for consideration with the understanding that they are contributed solely to the Journal. Authors should secure all necessary clearances and approvals prior to submission.

The Journal reserves the right to make editorial revisions prior to publication.

All manuscripts are acknowledged immediately, and every effort will be made to advise contributors of the status of their submission within 60 days.

Keywords

For indexing purposes, a list of 3-10 keywords is essential.

Abstract

Authors should provide a one paragraph (100-150 words) abstract summarizing the main points of the article. The abstract must clearly state the objectives, the methods used, the main results and the conclusions of the reported study.

References

References should be cited in the text in numerically consecutive order. References should be cited in the text by Arabic numerals superscript. Each reference should contain names of all authors. Journal titles should be abbreviated according to Index Medicus.

No., authors' names, title of the article. *Journal* Year; issue: pages. [for journal articles]

No., authors' names, title of the article. In: editors' names, *title of the book*. Place where it was published: Publishing House, Year: pages. [for edited books]

No., authors' names, *title of the book*. Place where it was published: Publishing House, Year. [for monographs]

Exemples:

5. Bierer L.M., Hof P.R., Purohit D.P., Carlin L., Schmeidler J., Davis K.L., Perl D.P., Neocortical neurofibrillary tangles correlate with dementia severity in Alzheimer's disease. *Arch Neurol* 1995; 52: 81-8. [for journal articles]
6. Benton A., Tranel D., Visuoperceptual, Visuospatial, and Visuoconstructive Disorders. In: Heilman K.M. and Valenstein E., eds., *Clinical Neuropsychology*. Oxford University Press, 1993: 195-212 [for edited books]
7. Luria A., *The Working Brain*. New York: Basic Books, 1973.[for monographs]

Reprints:

Twenty reprints of each article (free of charge) will be sent to the submitting author. For supplementary reprints, please contact the editorial office: office@brainaging.org

Copyright:

Authors submitting a paper do so on the understanding that if the manuscript is accepted for publication, copyright in the article, including the right to reproduce the article in all forms and media, shall be assigned exclusively to the Publisher. The submission of the manuscript by the authors means that the authors automatically agree to assign exclusive copyright to Eonia Publishers if and when the manuscript is accepted for publication.

The work shall not be published elsewhere in any language without the written consent of the Publisher. No material published in the Journal may be stored on microfilm or videocassettes or in electronic databases without prior written permission of the Publisher.

Miscellaneous:

It is necessary for the Journal to receive manuscripts as far ahead of deadline dates if possible. Time is needed for the manuscripts to be reviewed, returned to the editor, edited, composed, and printed. All manuscripts should be sent as soon as possible. We will inform the author when the article will be published.

The Journal expects authors to disclose any commercial or financial associations that might pose a conflict of interest in connection with the submitted article. All funding sources supporting the work should be acknowledged on the title page.