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Synaptic pathology in Alzheimer's disease: relation to severity of dementia, but not to senile plaques, neurofibrillary tangles, or the ApoE4 allele

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Summary. Alzheimer's disease (AD) is characterised by an increased number of senile plaques (SP) and neurofibrillary tangles (NFT) as compared with that found in non-demented individuals of the same age, and a marked degeneration and loss of synapses. One of the main risk-factors for the disorder is inheritance of the apolipoprotein E4 (ApoE4) allele. To further study the relation between these pathogenetic substrates for AD, we quantified the synaptic vesicle membrane protein rab3a in brain tissue from 19 patients with AD and 9 age-matched control subjects. Rab3a levels were reduced in AD, both in the hippocampus (60% of control level, $p < 0.0001$), and in the frontal cortex (68% of control level, $p < 0.01$), but not in the cerebellum (92% of control level). Within the AD group, lower rab3a levels were found both with increasing duration and severity of dementia. These findings further support that synaptic pathology is closely correlated to the clinical dementia in AD. In contrast, no significant correlations were found between SP counts and duration or severity of dementia, while higher NFT counts in the frontal cortex were found with increasing severity of dementia ($r = 0.54$, $p < 0.05$). There were no significant correlations between the rab3a level and SP or NFT counts, and by immunohistochemistry, reduced rab3a immunostaining was found throughout the neuropil in AD brain, without relation to SP or NFT. These findings suggest that the synaptic pathology in AD is not closely related to the presence of SP and NFT. No significant differences in rab3a levels were found in any brain region between AD patients possessing different numbers of the ApoE4 allele, suggesting that, although ApoE4 is a risk factor for earlier development of AD, the degree of synaptic pathology does not differ between patients with or without the ApoE4 allele.

Keywords: Alzheimer's disease (AD), apolipoprotein E (ApoE), dementia, neurofibrillary tangles, rab3a, senile plaques, synapses.

Introduction

Alzheimer's disease (AD) is characterised by progressive dementia and brain changes of an increased number of senile plaques (SP) and neurofibrillary tangles (NFT), compared with brains from non-demented individuals of the same age (Tomlinson and Corsellis, 1984). Although rare genetic forms of AD exist, the majority of patients have no obvious family history and are classified as sporadic AD.

The aetiology and pathogenesis of sporadic AD is largely unknown. The leading hypothesis is that deposition of βA4 protein, the 39–43 amino acid break-down product of the amyloid precursor protein (Kang et al., 1987), in the microvessels (Glenner et al., 1984) and in the core of SP (Masters et al., 1985), is the "central event" (Hardy and Allsop, 1991), or plays the "seminal role" (Joachim and Selkoe, 1992) in the pathogenesis of AD (for review see Selkoe, 1994).

During the last years, several papers have revealed a marked synaptic pathology in AD. This has been established in several cortical regions using electron microscopy (DeKosky and Scheff, 1990), and immunohistochemistry using the synaptic vesicle proteins synapsin I (Hamos et al., 1989) and synaptophysin (Terry et al., 1991; Zhan et al., 1993; Heinonen et al., 1995) as markers. The major part of the synaptic pathology in AD is localised in the neuropil, without relation to SP and NFT (Masliah et al., 1991b), and the severity of the synaptic loss is also greater than the loss of large neurones in the same cortical region (Masliah et al., 1991a; Heinonen et al., 1995). The degree of synapse pathology correlates well with clinical measures of dementia, while SP and NFT show only a weak correlation (Terry et al., 1991; Dickson et al., 1995; Heinonen et al., 1995).

One of the proteins exclusively localised to synaptic vesicles is rab3a, a low molecular weight protein (Fischer von Mollard et al., 1990), that probably plays a central role in the regulation of exocytosis of small synaptic vesicles (Jahn and Südhof, 1993; Lledo et al., 1994). In the present study, we used rab3a as a marker for synaptic density to further study synaptic pathology in AD, and its relation clinical measures of dementia and the classical neuropathological changes (SP and NFT).

Recent data implicate that apolipoprotein E (ApoE) is involved in the pathogenesis of AD. Antibodies to ApoE label SP and NFT (Namba et al., 1991), and an increased frequency of the ApoE4 allele is found in both familial and sporadic AD (Corder et al., 1993; Poirier et al., 1993; Saunders et al., 1993; Strittmatter et al., 1993). The major hypotheses on the pathogenetic mechanism of ApoE in AD are: 1) that ApoE4 acts as a "pathological chaperone" that by binding to soluble βA4 protein makes it insoluble and sequestered in SP (Wisniewski and Frangione, 1992); and 2) that ApoE3, but not ApoE4, prevents the hyperphosphorylation of tau and its self-assembly into paired helical filaments and NFT (Strittmatter et al., 1994). However, Apo E

has also been suggested to be involved in neuronal repair and reactive synaptogenesis after injury (Snipes et al., 1986; Mahley, 1988; Poirier, 1994). It has also been suggested that ApoE3 increases and ApoE4 decreases neurite outgrowth (Nathan et al., 1994), and that ApoE4 carriers may have an impaired reactive synaptogenesis (Poirier, 1994), which may be of pathogenetic importance in AD. These findings induced us to study the possible relation between ApoE4 and synaptic pathology in AD.

Materials and methods

Patients

The present study included brains from 19 patients with AD, 7 men and 12 women, mean age ± SD 78 ± 9.3 years and 9 control subjects, 6 men and 3 women, mean age ± SD 71 ± 14 years. The mean age did not significantly differ between the AD and control groups.

The AD patients fulfilled the clinical criteria for probable AD (McKhann et al., 1984). The post-mortem examination revealed no infarcts or other changes that could account for the dementia, and the histopathological score (Alafuzoff et al., 1987) was five or above. The control group consisted of patients, who had died from cardiac disease or malignant disease. Their medical records revealed no history of dementia, or psychiatric or neurological diseases. The post-mortem examination revealed no macroscopic infarcts, and the histopathological score (Alafuzoff et al., 1987) was four or lower. The severity of dementia was estimated using the intellectual subscales in a geriatric rating scale (Adolfsson et al., 1981).

Histopathological examination

For assessment of the severity of the histopathological changes, the frontal lobe and the anterior part of the hippocampal formation from the right hemisphere were fixed in 10% buffered neutral formalin for 4–6 weeks, and thereafter embedded in paraffin blocks. Six μm sections were stained with Bodian-PAS stain. The absolute number of SP and NFT was counted in five randomly selected fields at magnification ×125, and a mean count of SP and NFT was obtained for each region, and rated on a four-step scale (0 = no, 1 = mild; 2 = moderate; and 3 = severe), to simplify the assessment of the severity of the histopathological changes (Alafuzoff et al., 1987).

Immunohistochemistry

Immunohistochemistry was performed on 8 μm thick 4% formaldehyde-fixed sections from the frontal gyrus (Brodmann area 9) and anterior hippocampal formation from one AD patient (histopathological score 10), and one control case (histopathological score 0). The rab3a monoclonal antibody (MAb; clone 42.2) was generated using recombinant rab3a as antigen (Matteoli et al., 1991). This MAb recognises specifically rab3a on western blotting, and has by conventional and electron microscopic immunohistochemistry been found to specifically label synaptic vesicles in presynaptic nerve terminals similar to MAbs directed against other synaptic vesicle proteins such as synaptophysin and synaptotagmin (Matteoli et al., 1991). The sections were pre-treated with peroxidase and pre-incubated with normal serum for 60 min. Thereafter, the sections were incubated at +4°C overnight with the rab3A MAb diluted 1/50 in 0.05 M Tris-buffered saline containing 1% bovine serum albumin. The sections were then processed by the avidin-biotin method with the Vectastatin ABS kit (Vector Laboratories, Burlingame, CA), counterstained with hematoxylin, dehydrated and coverslipped with DPX. The specificity of the rab3a labelling procedure was determined by omitting the primary antibody in the control sections.

Quantitative Western blotting

For determination of the rab3a level in brain tissue, the hippocampal formation, frontal cortex (Brodmann area 9), and cerebellum were dissected from the left hemisphere, homogenised in liquid nitrogen, and stored at −80°C pending biochemical analyses. Samples of homogenised brain tissue (≈ 100 mg wet weight) were delipidised in chloroform/methanol/water (4/8/3 v/v), and centrifuged at 2.000 × g for 10 min. The pellet was resuspended in chloroform/methanol/water, centrifuged, and the supernatant containing the lipids was discarded. The pellet was dried under a stream of nitrogen, dissolved in SDS sample buffer (0.08 M Tris-HCl, pH 6.8, containing 0.17 M sodium-dodecyl sulphate, 6.5 mM dithioerithiol, 0.5 M urea, and 0.1% bromphenol blue), ultrasonicated in a water bath for 15 min, and boiled for 5 min. The total protein concentration was determined in each sample using the bicinchoninic acid method (Smith et al., 1985). Finally, the volume was adjusted to get a total protein concentration of 0.5 μg/μL.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was run in a Mini-PROTEAN II cell (BioRad Labs, Richmond, CA), on 12% gels using the buffer systems of Laemmli (1972). A volume of 10 μL (corresponding to 5.0 μg of total protein) of each sample was loaded to the gel. After electrophoresis, the proteins were blotted from the gel onto a polyvinyl difluoride (PVDF) membrane (Millipore, Bedford, MA, USA), by the semidry technique using the NovaBlot System (Pharmacia, Uppsala, Sweden). Non-specific binding sites were blocked with 5% milk powder in phosphate buffered saline (58 mM Na_2HPO_4, 17 mM NaH_2PO_4*H_2O, 68 mM NaCl, pH 7.4), containing 0.05% Tween 20. After that, the membrane was incubated overnight with a specific monoclonal antibody against rab3a (clone 42.2; Matteoli et al., 1991) diluted 1/500. After washing, the membrane was incubated with alkaline phosphatase conjugated goat anti-mouse Ig (Jackson, West Grove, PA) diluted 1/4.000 for 1 hour as detecting antibody, and the colour reaction was developed with 0.015% 5-bromo-4-chloro-3-indolyl phosphate and 0.030% nitro-blue tetrazolium in 0.1 M carbonate buffer containing 1.0 mM $MgCl_2$ (Leary et al., 1983).

Rab3a was quantified by densitometric scanning of the Western blots on a Camag Scanner II (Mattenz, Switzerland) at 620 nm. The standard curve consisted of hippocampal, frontal cortex and cerebellar brain homogenates (separate standards for each brain region) from one control case, prepared as described above. Three dilutions (10, 5.0, and 2.5 μg total protein) of the standard from the brain region to be analysed were run on each gel. The band intensity in the 5.0 μg standard was set as 1 arbitrary unit of rab3a, and after that, all values were corrected by dividing with the mean rab3a level in controls.

ApoE genotyping

ApoE genotyping was performed on brain tissue samples, by amplification of the forth exon of the ApoE gene by PCR with biotinylated primers, followed by reverse DNA hybridisation on nitrocellulose strips, using the INNO-LiPA ApoE kit (Innogenetics N.V., Gent, Belgium).

Statistical analysis

The Mann-Whitney U-test was used for comparisons between two groups, and the Kruskal-Wallis one-way analysis of variance test for comparisons between three or more groups. The Spearman correlation coefficient was used for correlations.

Results

Immunohistochemistry

Immunohistochemistry using the rab3a MAb showed a granular immunopositive reaction mostly localised in the neuropil of the cortex and the hippo-

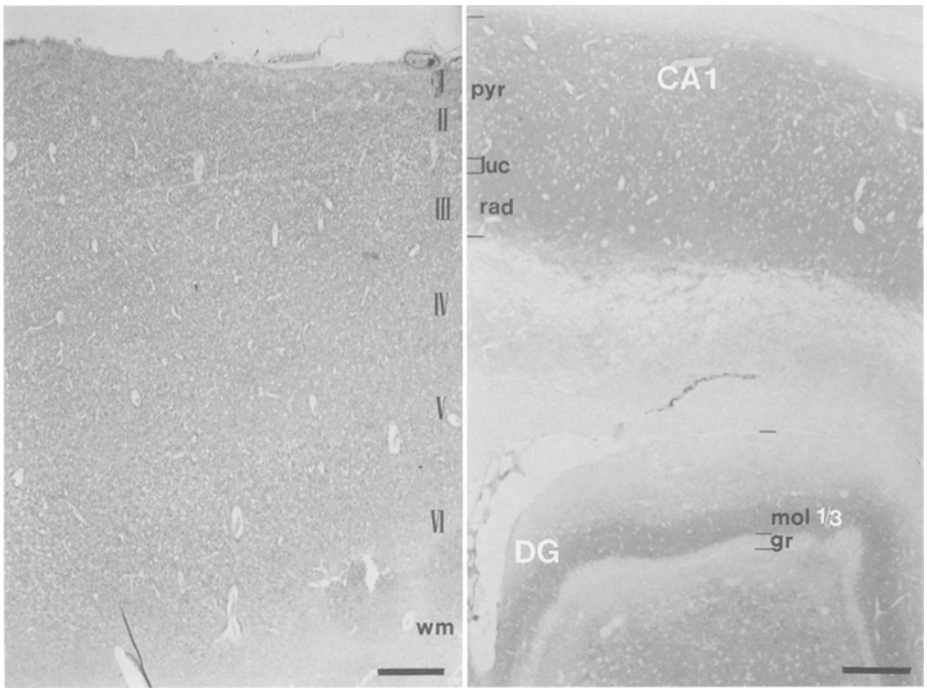

Fig. 1. Distribution of rab3a immunoreactivity in the frontal cortex (left) and the hippocampus (right) in control brain. Magnification ×25. Bar = 0.5 mm. HTX counterstaining. Abbreviations: *I–VI* cortical layers I–VI, *wm* white matter, *pyr* pyramidal layer, *luc* stratum lucidum, *rad* stratum radiatum, *mol* molecular layer 1/3, *gr* granular layer, *DG* dentate gyrus

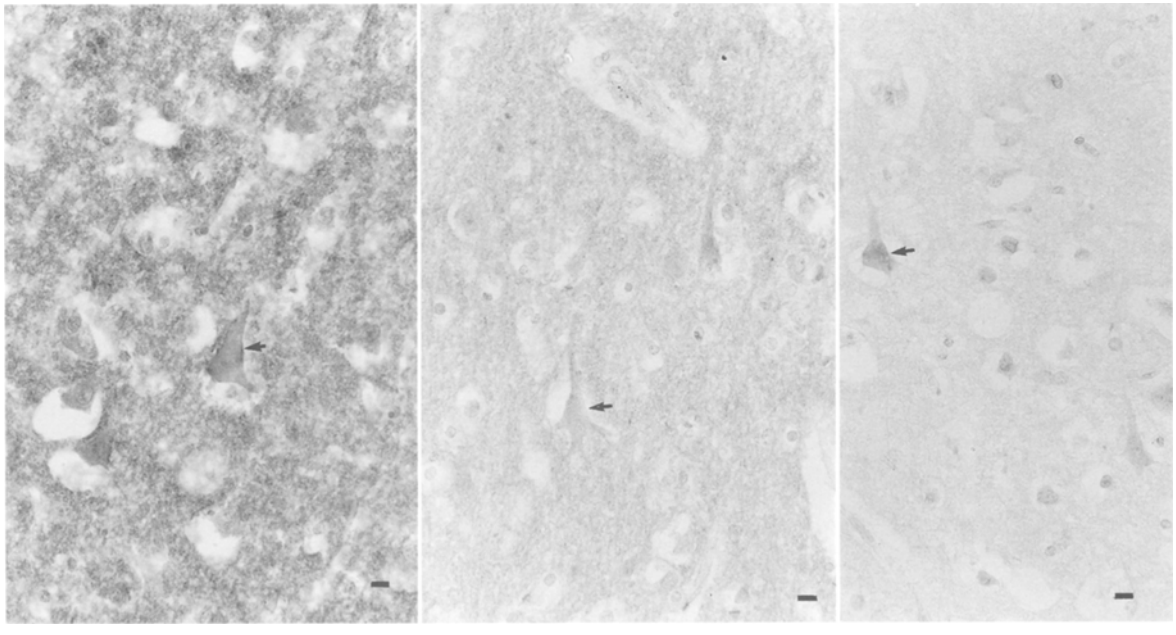

Fig. 2. Distribution of rab3a immunoreactivity in the neuropil of the frontal cortex layer IIIb in the control brain (left) and a marked reduction of rab3a immunoreactivity in AD brain (middle). In the right figure control staining where the rab3a MAb was omitted. HTX counterstaining. Magnification ×400. Bar = 10 μm. Arrows = pyramidal cells

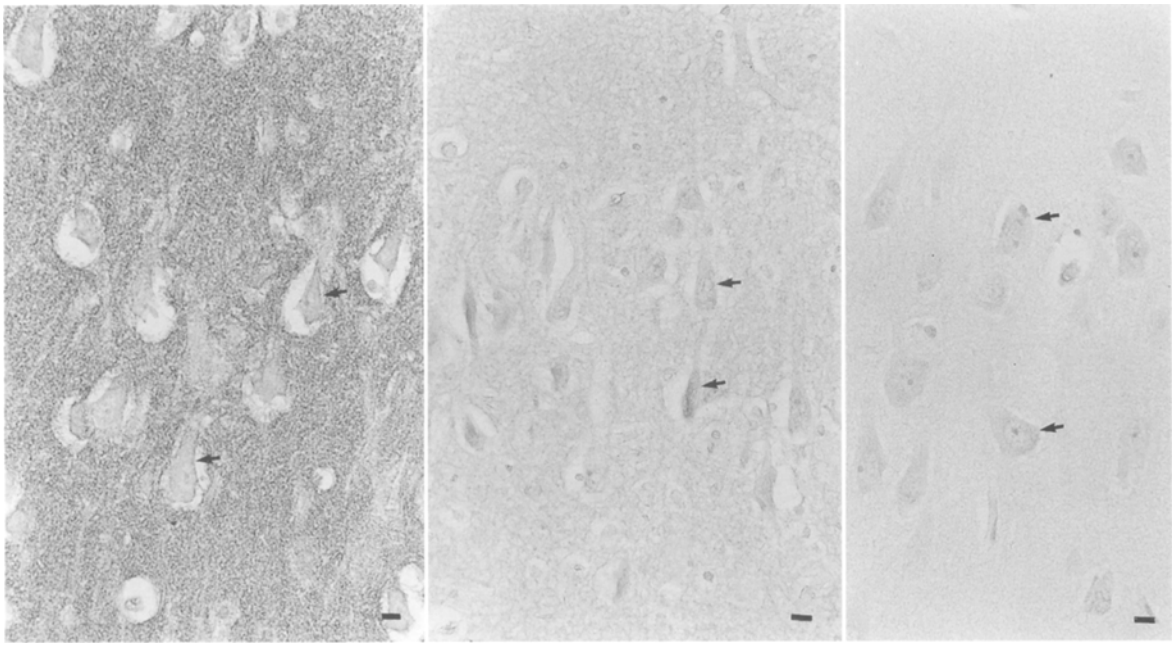

Fig. 3. Distribution of rab3a immunoreactivity in the neuropil of the CA1 hippocampal subfield in the control brain (left) and a marked reduction of rab3a immunoreactivity in AD brain (middle). In the right figure control staining where the rab3a MAb was omitted. HTX counterstaining. Magnification ×400. Bar = 10 μm. Arrows = pyramidal cells

campus (Fig. 1). Control staining did not reveal any positivity in the tissue (Figs. 2 and 3, right).

In the frontal cortex of control brain, rab3a positive staining was present almost evenly throughout all cortical layers from the pial surface to the white matter border (Fig. 1), while neurones showed a slightly positive reaction localised on the membrane of the neuronal cell body (Fig. 4, left). In the hippocampus and the dentate gyrus, layer specific distribution of immunostaining was revealed: the pyramidal cell layer, stratum lucidum and stratum radiatum of the CA hippocampal subfield as well as the proximal 1/3 of the molecular layer in the gyrus dentatus were immunostained (Fig. 1). Pyramidal CA1 neurones also showed positive immunostaining on the neuronal body membrane (Fig. 4, right), a pattern similar to the cortical neurones.

In AD brain, a marked reduction of rab3a positive staining was found as compared with control brain, both in the frontal cortex (Fig. 2) and in the hippocampus (Fig. 3). In addition, weak staining was found in some SP-like structures in the cortical neuropil in AD brain (Fig. 5).

Quantitative Western blotting

An example of a typical standard curve is given in Fig. 6. As can be seen, the standard curve is virtually linear ($r = 0.996$) from 0–2 arbitrary units of rab3a. There were no significant correlations between post-mortem delay time and

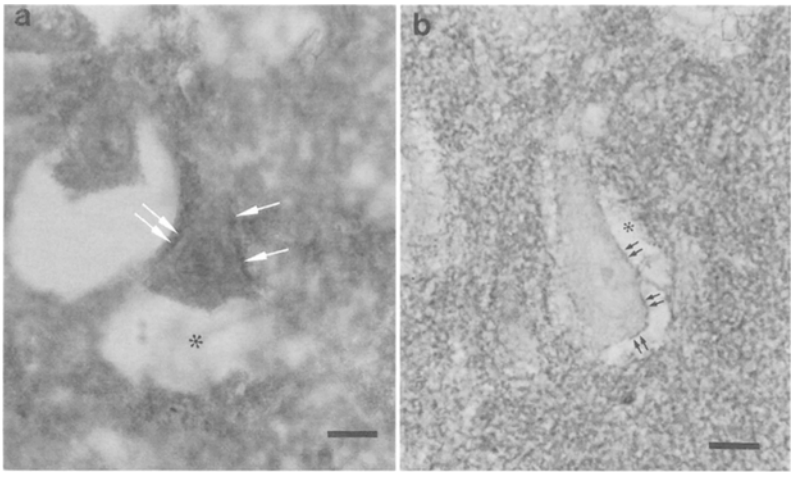

Fig. 4. Rab3a immunoreactivity on the cell membranes (arrows) of pyramidal cells in layer III of the frontal cortex (a) and CA1 pyramidal layer (b) of the hippocampus from a control case. Perineuronal artefact due to tissue preparation (asterix). HTX counterstaining. Magnification × 1.000. Bar = 10μm

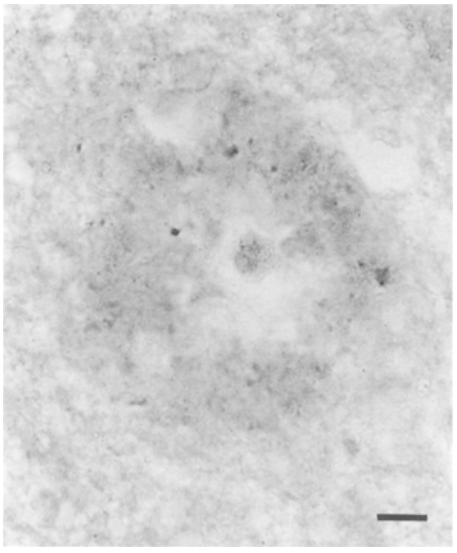

Fig. 5. Senile plaque-like positive rab3a immunoreactivity in the superficial layer III of the frontal cortex from a Alzheimer's disease case. Magnification ×1.000. Bar = 10μm

rab3a in any brain region, neither in the AD group ($r = 0.09$ in hippocampus, $r = 0.20$ in frontal cortex, and $r = 0.12$ in cerebellum), nor in the control group ($r = 0.00$ in hippocampus, $r = -0.37$ in frontal cortex, and $r = -0.21$ in cerebellum).

In the hippocampus (Fig. 7, left), rab3a was significantly reduced in the AD group (0.60 ± 0.23) as compared with the control group group (1.00 ± 0.18; $p < 0.0001$). Also in the frontal cortex (Fig. 7, middle), rab3a was significantly reduced in the AD group (0.68 ± 0.28) as compared with the

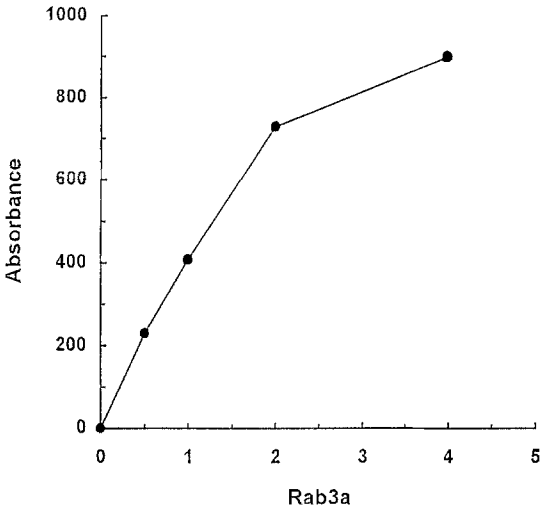

Fig. 6. Standard curve of rab3a quantified by densitometric scanning of Western blots. Brain homogenate from one control case diluted to 0, 0.25, 0.50, 1.0, and 2.0 μg/μL of total protein (corresponding to 0, 0.5, 1, 2, and 4 arbitrary units) was used as a standard curve (the rab3a level in the standard containing 0.50 μg/μL total protein was set as 1 arbitrary unit). The standard curve was virtually linear (r = 0.996) up to 2 arbitrary units

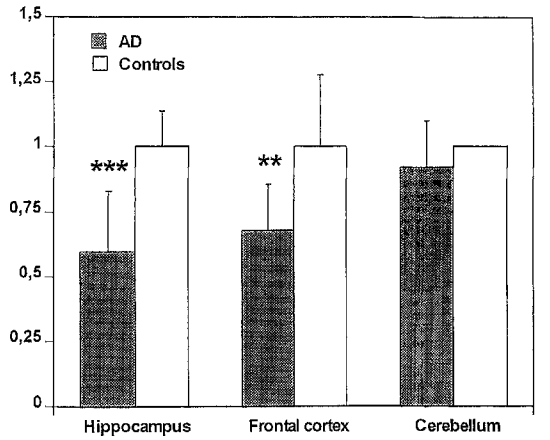

Fig. 7. Rab3a levels in the hippocampus (left), frontal cortex (middle), and cerebellum (right). Values are given as arbitrary units of rab3a per 5.0 μg of total protein (one arbitrary unit defined as the mean band intensity in controls). Abbreviations: *AD* Alzheimer's disease. Significances (Mann-Whitney U-test): Hippocampus: $p < 0.0001$; frontal cortex: $p < 0.01$

control group (1.00 ± 0.18; $p < 0.01$). However, rab3a did not significantly differ between the AD group (0.92 ± 0.19) and the control group (1.00 ± 0.21) in the cerebellum (Fig. 7, right).

Within the AD group, there was a significant correlation between duration of dementia and rab3a in hippocampus ($r = -0.55$; $p < 0.01$), while the correlation coefficient in the frontal cortex ($r = -0.39$) was not statistically significant. There was also a significant correlation between severity of

dementia and rab3a in hippocampus ($r = -0.53$; $p < 0.05$), while also for this parameter, the correlation coefficient in the frontal cortex ($r = -0.47$) was not statistically significant.

Within the AD group, there were no significant correlations between duration of dementia and hippocampal SP ($r = -0.02$) or NFT ($r = 0.13$) counts, or between duration of dementia and frontal cortex SP ($r = -0.29$) or NFT ($r = 0.11$) counts. Neither were there any significant correlations between severity of dementia and hippocampal SP ($r = 0.21$) or NFT ($r = -0.05$) counts, or between severity of dementia and frontal SP counts ($r = -0.15$), while NFT counts in the frontal cortex showed a positive correlation ($r = 0.54$; $p < 0.05$) with severity of dementia.

Within the AD group, there were no significant correlations between the rab3a level and SP or NFT counts, neither in the hippocampus ($r = -10$ and $r = -0.21$ respectively), nor in the frontal cortex ($r = 0.00$ and $r = 0.03$ respectively).

The ApoE4 allele frequency was 45% in the AD group (7 with none, 7 with one, and 5 with two ApoE4 alleles) and 28% in the control group (4 with none, 4 with one, and 1 with two ApoE4 alleles). The relatively high ApoE4 allele frequency in the control group may simply reflect the small sample size. None of the controls had evidence of clinical dementia, nor other than negligible evidence of Alzheimer-type pathology at histochemistry (no control subject had NFT in the hippocampus or frontal cortex, and of the four control cases without any ApoE4 allele, one had some hippocampal SP; of the four control cases with one ApoE4 allele one had some SP in the frontal cortex, and one had some SP both in the hippocampus and in the frontal cortex; and the control case with two ApoE4 alleles had no SP either in the hippocampus or in the frontal cortex).

Table 1. Comparison between Alzheimer patients with different numbers of ApoE4 alleles

ApoE4 alleles	none (n = 7)	one (n = 7)	two (n = 5)
Age at death (years)	77.6 ± 5.6	78 ± 13	79 ± 9.6
Duration of disease (years)	8.9 ± 4.4	8.9 ± 4.4	7.0 ± 4.8
Severity of dementia	54 ± 6.4	52 ± 6.9	42 ± 17
SP in hippocampus	2.3 ± 1.0	2.3 ± 0.8	1.8 ± 1.3
NFT in hippocampus	2.1 ± 0.9	2.7 ± 0.8	2.0 ± 1.0
SP in frontal cortex	2.5 ± 0.6	2.0 ± 1.0	1.8 ± 1.0
NFT in frontal cortex	1.8 ± 1.0	1.0 ± 0.7	1.2 ± 1.3
Rab3a in hippocampus	0.63 ± 0.25	0.52 ± 0.18	0.66 ± 0.28
Rab3a in frontal cortex	0.63 ± 0.34	0.70 ± 0.25	0.72 ± 0.27
Rab3a in cerebellum	0.94 ± 0.10	0.92 ± 0.20	0.91 ± 0.06

Values are given as mean ± SD. Abbreviations: *SP* senile plaques, *NFT* neurofibrillary tangles. Severity of dementia evaluated according to Adolfsson et al. (1981). The number of SP and NFT was rated using a four-step scale according to Alafuzoff et al. (1987). No significant differences were found between Alzheimer patients with different numbers of ApoE4 alleles

Within the AD group, there were no significant differences in age at death, age at onset, or severity of dementia between patients with different numbers of ApoE4 alleles (Table 1). Neither were there any significant differences in SP of NFT counts in the hippocampus and frontal cortex between patients with different numbers of ApoE4 alleles (Table 1). There were no significant differences in rab3a levels between patients with different numbers of ApoE4 alleles, either in the hippocampus or in the frontal cortex (Table 1).

Discussion

We found a decrease in rab3a in the hippocampus and in the frontal cortex in AD, while no change was found in the cerebellum. Rab3a is specifically located in the membrane of small synaptic vesicles (Fischer von Mollard et al., 1990). A decrease in rab3a may have several explanations, including a decreased gene expression of rab3a, a disturbed axonal transport of synaptic vesicles, a reduced number of synaptic vesicles within synapses, or a reduced number of synaptic terminals with normal number of vesicles. Since no correlation between post-mortem delay time and rab3a was found, the reduction is probably not due to differences between the diagnostic groups in the agonal state, handling of the brain tissue material or other artefacts. A synaptic loss in AD has been established in several cortical regions using electron microscopy (Davies et al., 1987; DeKosky and Scheff, 1990). Therefore, the most plausible explanation for the decrease in rab3a in AD is a synaptic loss.

By quantitative immunoblotting, we found a decrease in rab3a in AD in the hippocampus and in the frontal cortex, while no changes were found in the cerebellum. These changes matches the regional distribution of the disease, with marked pathology in the hippocampus and in the association cortices, while no changes are found in the cerebellum (Tomlinson and Corsellis, 1984). Also by immunohistochemistry, there was a marked difference in rab3a staining, both in the hippocampus and in the frontal cortex, between AD and control brain. These findings are in agreement with immunoblotting and immunohistochemical studies using anti-synaptophysin antibodies (Terry et al., 1991; Lassman et al., 1992; Dickson et al., 1995; Heinonen et al., 1995).

On immunohistochemistry, it is clear that rab3a positive staining was present in the cortical and hippocampal neuropil, where presynaptic axonal branches as well as postsynaptic dendritic elements are situated. In the cortex, the distribution was even, but in the hippocampal structure and the gyrus dentatus rab3a immunostaining followed certain anatomical stratification, that to some extent differs from synaptophysin immunostaining (Heinonen et al., 1995). The stratum radiatum and stratum lacunare of the CA hippocampal subfield as well as the proximal 1/3 of the molecular layer in the gyrus dentatus were immunostained by the rab3a MAb. These layers receive axonal input from the septal nucleus and commisural axons from opposite site (Andersen et al., 1971). In addition, mossy fibers from the dentate granular layer end up within stratum lucidum of CA3. In comparison, the entorhinal inputs in gyrus

dentatus (distal 2/3 of the molecular layer) and CA1 (stratum moleculare) were only weakly immunostained by the rab3a MAb, in contrast to anti-synaptophysin immunostaining (Heinonen et al., 1995; Blennow et al., unpublished). Thus, these neurones in perforant pathways from the entorhinal area may express less rab3a than synaptophysin. Interestingly, no synaptic pathology was found in the entorhinal area in AD in a study by Scheff and co-workers (1993). However, it is too early to speculate whether neurotransmitter-related or projection-related specific pattern exists in the expression of rab3a protein, but the reduction in the cortex and layer-specific reduction in the hippocampus correspond with the distribution of at least the cholinergic axons, which are one of the most affected neurotransmitter systems in AD (Roth, 1986; Perry, 1994). Moreover, the staining of SP with the rab3a antibody is less intense than with anti-synaptophysin antibodies (Heinonen et al., 1995). Similarly, we have also found that MAbs against the synaptic vesicle specific protein synaptotagmin give a less marked immunostaining than using a MAb against synaptophysin (unpublished data). These differences found in rab3a and synaptophysin immunostaining may suggest a different role for these proteins in different neuronal systems in the brain.

We found negative correlations between duration of dementia and the level of rab3a in the hippocampus and the same trend in the frontal cortex, i.e., with increasing duration, the number of synapses decline. These findings are in agreement with the study by DeKosky and Scheff (1990), in which a more pronounced synapse loss was found in end-stage AD patients at autopsy, than in the earlier phases of the disorder in biopsies. There were also similar correlations between severity of dementia and the level of rab3a, with increasing severity of dementia with lower rab3a levels. Also other studies have found similar correlations between severity of dementia and synaptic loss (DeKosky and Scheff, 1990; Terry et al., 1991; Lassman et al., 1992; Dickson et al., 1995; Heinonen et al., 1995). The correlations between duration and severity of dementia and rab3a levels suggest a close relations between the synaptic pathology and the cognitive decline in AD.

In contrast, we found no significant correlations between the rab3a level and SP or NFT counts. Likewise, Honer et al. (1992) found no significant correlations between the synaptophysin level and SP counts in AD, and only a weak correlation to NFT counts. Heinonen et al. (1995) found no correlation between synaptophysin immunoreactivity and β/A4 protein positive plaques or paired helical filaments-positive neurons, also in agreement with our findings. In contrast, Lassman et al. (1992) found significant correlation between SP/NFT counts and the synaptic vesicle proteins synaptophysin, synaptotagmin, and SV2. Using the rab3a MAb, we found a marked reduction of the characteristic granular immunostaining throughout the neuropil in AD, without relation to SP or NFT. Using an antibody against synaptophysin, Masliah et al. (1991a) also found that the major part of the synaptic pathology in AD is in the neuropil, without relation to SP and NFT. These pattern of immunostaining and the absence of a correlation between rab3a and SP and NFT counts in most studies support the hypothesis that synaptic pathology in AD is not closely related to SP and NFT.

We did not find any significant correlations between SP counts and duration or severity of dementia, only NFT counts in the frontal cortex showed a significant correlation, with higher NFT counts with increasing severity of dementia. Several other studies have also found that NFT correlates better with disease severity than SP (Wilcock and Esiri, 1982; Morris et al., 1991; Arriagada et al., 1992). In contrast, studies on the relation between severity of dementia and SP counts have found no (Morimatsu et al., 1975; DeKosky and Scheff, 1990) or only weak (Terry et al., 1991) correlations. Moreover, studies in which significant correlations between SP counts and severity of dementia have been found (Blessed et al., 1968; Duyckaerts et al., 1986) have been criticised (Terry et al., 1991; Arriagada et al., 1992) for including two different populations (both non-demented controls and AD patients) in the statistical calculations. However, a recent study found that markers indicative of cytoskeletal changes, e.g. SP, NFT and PHF protein accumulation might correlate better to dementia than markers of synapse loss (Dickson et al., 1995).

Many authors argue that amyloid deposition, and SP, is the "central event in the aetiology of AD" (Hardy and Allsop, 1991) or play a "seminal role in the pathogenesis of AD" (Joachim and Selkoe, 1992). Other authors have questioned the pathogenetic role of SP, and suggested that deposition of βA4 protein may occur without relation to the synaptic loss, and thus, that amyloid deposition may be a secondary response to the synaptic and neuronal degeneration (Wilcock and Esiri, 1982; Masliah et al., 1991b; Terry et al., 1991; Regland and Gottfries, 1992; Hoyer, 1993; Masliah et al., 1993; Zhan et al., 1993; Heinonen et al., 1995). Using immunohistochemistry, it has also been shown that deposition of βA4 protein in the form of diffuse plaques does not accentuate the synaptic loss in AD (Masliah et al., 1991b). The results of the present study give further support to the hypothesis that synaptic pathology may be an important pathogenetic event in AD.

In a preliminary report, it was found that AD patients possessing the ApoE4 allele displayed a more severe reduction in synaptophysin immunoreactivity, than AD patients without the ApoE4 allele (Miller et al., 1994). In the present study, we found no significant differences in rab3a levels, either in the hippocampus or in the frontal cortex, between AD patients possessing different numbers of the ApoE4 allele. The reason for this discrepancy is unclear, but may be caused by methodological differences (immunohistochemistry versus quantitative immunoblotting), or differences in patient materials. Alternatively, although ApoE4 is a risk factors for earlier development of AD, the degree of synaptic pathology does not differ between patients with and without the ApoE4 allele.

Initial studies suggested that AD patients homozygous for ApoE4 have higher average SP density than patients without or with only one ApoE4 allele (Rebeck et al., 1993; Schmechel et al., 1993), and that ApoE4 homozygotes have more intense βA4 immunoreactivity in SP and microvessels (Schmechel et al., 1993). Later studies have not been able to replicate these findings (Soininen et al., 1995). We were also not able to find any difference in SP counts between AD patients with different numbers of ApoE4 alleles.

Similarly, Gearing et al. (1995) found no significant differences in β/A4 protein-positive SP between AD cases possessing different numbers of the ApoE4 allele, and Harrington et al. (1994) found that the level of βA4 protein do not significantly differ between AD patients with and without the ApoE4 allele. These findings do not support a close relation between the ApoE4 allele and amyloid deposition. In contrast, ApoE-positive SP are more frequent in AD patients possessing the ApoE allele than in those who do not (Gearing et al., 1995). The number of β/A4 protein positive SP are many times more abundant than ApoE positive SP (Gearing et al., 1995). ApoE immunoreactivity is preferentially found in the core of classical senile plaques (consisting of fibrillar amyloid), while it is not found, or weak, in diffuse plaques (consisting of non-fibrillar "preamyloid" βA4 protein) (Kida et al., 1994; Gearing et al., 1995). These findings do not support that ApoE is involved in the early stages of fibrillar amyloid formation, which may explain our failure to find any relation between SP counts and the number of ApoE4 alleles.

In summary, the findings in the present study show by quantitative means, a marked synaptic loss in AD. This synaptic loss correlates to the clinical dementia symptoms, further supporting the central role of synaptic pathology in the pathogenesis of AD. In contrast, no relation was found between synaptic pathology and SP, NFT, or the ApoE4 allele.

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References

Adolfsson R, Gottfries CG, Nyström L, Winblad B (1981) Prevalence of dementia in institutionalised Swedish old people: the work load imposed by caring for these patients. Acta Psychiatr Scand 63: 225–244

Alafuzoff I, Iqbal K, Fridén H, Adolfsson R, Winblad B (1987) Histopathological criteria for progressive dementia disorders: clinical-pathological correlation and classification by multivariate data analysis. Acta Neuropathol (Berl) 74: 209–225

Andersen P, Bliss TVP, Skrede KK (1971) Lamellar organization of hippocampus excitatory pathways. Exp Brain Res 13: 222–238

Arriagada PV, Growdon JH, Hedley-Whyte ET, Hyman BT (1992) Neurofibrillary tangles but not senile plaques parallel duration and severity of Alzheimer's disease. Neurology 4: 631–639

Blessed G, Tomlinson BE, Roth M (1968) The association between quantitative measures of dementia and of senile change in the cerebral grey matter of elderly subjects. Br J Psychiatry 114: 797–811

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

Davies CA, Mann DMA, Sumpter PQ, Yates PO (1987) A quantitative morphometric analysis of the synaptic content of the frontal and temporal cortex in patients with Alzheimer's disease. J Neurol Sci 78: 151–164

DeKosky ST, Scheff SW (1990 Synapse loss in frontal cortex biopsies in Alzheimer's disease: correlation with cognitive severity. Ann Neurol 27: 457–464

Dickson DW, Crystal HA, Benova C, Honer W, Vincent I, Davies P (1995) Correlations of synaptic and pathological markers with cognition of the elderly. Neurobiol Aging 16: 285–304

Duyckaerts C, Hauw JJ, Bastenaire F, Piette F, Poulain C, Rainsard V, Javoy-Agid F, Berthaux P (1986) Laminar distribution of neocortical senile plaques in senile dementia of the Alzheimer type. Acta Neuropathol (Berl) 70: 249–256

Fischer von Mollard G, Mignery GA, Baumert M, Burger PR, Perin M, Jahn R, Südhof TC (1990) Rab3 is a small GTP-binding protein exclusively localised to synaptic vesicles. Proc Natl Acad Sci USA 87: 1988–1992

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

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

Hamos JE, DeGennaro LJ, Drachman DA (1989) Synaptic loss in Alzheimer's disease and other dementias. Neurology 39: 355–361

Hardy J, Allsop D (1991) Amyloid deposition as the central event in the aetiology of Alzheimer's disease. Trends Pharmacol Sci 12: 383–388

Harrington CR, Louwagie J, Rossau R, Vanmechelen E, Perry RH, Perry EK, Xuereb JH, Roth M, Wischik CM (1994) Influence of apolipoprotein E genotype on senile dementia of the Alzheimer and Lewy body types. Am J Pathol 145: 1472–1484

Heinonen O, Soininen H, Sorvari H, Kosunen O, Paljärvi L, Koivisto E, Riekkinen PJ (1995) Loss of synaptophysin-like immunoreactivity in the hippocampal formation is an early phenomenon in Alzheimer's disease. Neuroscience 64: 375–384

Honer WG, Dickson DW, Gleeson J, Davies P (1992) Regional synaptic pathology in Alzheimer's disease. Neurobiol Aging 13: 375–382

Hoyer S (1993) Sporadic dementia of Alzheimer's disease: role of amyloid in the etiology is challenged. J Neural Transm [P-D Sect] 6: 159–165

Jahn R, Südhof TC (1993) Synaptic vesicle traffic: rush hour in the nerve terminal. J Neurochem 61: 12–21

Joachim CL, Selkoe DJ (1992) The seminal role of β-amyloid in the pathogenesis of Alzheimer's disease. Alzheimer Dis Assoc Disord 6: 7–34

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

Kida E, Golabek AA, Wisniewski T, Wisniewski KE (1994) Regional differences in apolipoprotein E immunoreactivity in diffuse plaques in Alzheimer's disease. Neurosci Lett 167: 73–76

Laemmli UK (1972) Cleavage of structural proteins during the assembly of the head of bacteriophage. Nature 227: 680–685

Lassman H, Weiler R, Fischer P, Bancher C, Jellinger K, Floor E, Danielczyk W, Seitelberger F, Winkler H (1992) Synaptic pathology in Alzheimer's disease: immu-

nological data for markers of synaptic and large dense-core vesicles. Neuroscience 46: 1–8
Leary JJ, Brigati DJ, Ward DC (1983) Rapid and sensitive colorimetric method for visualizing biotin-labelled DNA probes hybridized to DNA or RNA immobilized in nitrocellulose Bio-Blots. Proc Natl Acad Sci USA 80: 4045–4049
Lledo PM, Johannes L, Vernier P, Zorec R, Darchen F, Vincent JD, Henry JP, Mason WT (1994) Rab3a proteins: key players in the control of exocytosis. TINS 17: 426–432
Mahley RW (1988) Apolipoprotein E: cholesterol transport protein with expanding role in cell biology. Science 240: 622–630
Masliah E, Terry RD, Alford M, DeTeresa R, Hansen LA (1991a) Cortical and subcortical patterns of synaptophysinlike immunoreactivity in Alzheimer's disease. Am J Pathol 138: 235–246
Masliah E, Hansen L, Albright T, Mallory M, Terry RD (1991b) Immunoelectron microscopic study of synaptic pathology in Alzheimer's disease. Acta Neuropathol 81: 428–433
Masliah E, Mallory M, Hansen L, DeTeresa R, Terry RD (1993) Quantitative synaptic alterations in the human neocortex during normal aging. Neurology 43: 192–197
Masters CL, Simms G, Weinman NA, Multhaup G, McDonald BL, Beyreuther K (1985) Amyloid plaque core protein in Alzheimer's disease and Down syndrome. Proc Natl Acad Sci 82: 4245–4249
Matteoli M, Takei K, Cameron R, Hurlkbut P, Johnston PA, Sudhof TC, Jahn R, De Camilli P (1991) Association of rab3a with synaptic vesciles at late stages of the secretory pathway. J Cell Biol 115: 625–633
McKhann G, Drachman D, Folstein M, Katzman R, Price D, Stadlan EM (1984) Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA Work Group under the auspices of department of health and human services task force on Alzheimer's disease. Neurology 34: 939–944
Miller A, Alford M, Katzman R, Thal L, Saitoh T, Masliah E (1994) The expression of the Apo-ε4 allele in Alzheimer's disease accentuates the synaptic loss and the severity of the dementia (Abstract). Ann Neurol 36: 268–269
Morimatsu M, Hirai S, Muramatsu A, Yoshikawa M (1975) Senile degenerative brain lesions and dementia. J Am Geriatr Soc 23: 390–406
Morris JC, McKeel DW, Strorandt M (1991) Very mild Alzheimer's disease: informant-based clinical, psychometric, and pathologic distinction from normal aging. Neurology 41: 469–478
Namba Y, Tomonaga M, Kawasaki H, Otomo E, Ikeda K (1991) Apolipoprotein E immunoreactivity in cerebral amyloid deposits and neurofibrillary tangles in Alzheimer's disease and kuru plaque amyloid in Creutzfeldt-Jakob disease. Brain Res 541: 163–166
Nathan BP, Bellosta S, Sanan DA, Weisgraber KH, Mahley RW, Pitas RE (1994) Differential effects of apolipoproteins E3 and E4 on neuronal growth in vitro. Science 264: 850–852
Perry EK (1994) Cholinergic component of cognitive impairment in dementia. In: Burns A, Levy R (eds) Dementia. Chapman & Hall, London, pp 143–157
Poirier J, Davignon J, Bouthillier D, Kogan S, Bertrand P, Gauthier S (1993) Apolipoprotein E polymorphism and Alzheimer's disease. Lancet 342: 697–699
Poirier J (1994) Apolipoprotein E in animal models of CNS injury and in Alzheimer's disease. TINS 17: 525–530
Rebeck GW, Reiter JS, Strickland DK, Hyman BT (1993) Apolipoprotein E in sporadic Alzheimer's disease: allelic variation and receptor interactions. Neuron 11: 575–580
Regland B, Gottfries CG (1992) The role of amyloid β-protein in Alzheimer's disease. Lancet 340: 467–469
Roth M (1986) The association of clinical and neurological findings and its bearing on the classification and aetiology of Alzheimer's disease. Br Med Bull 42: 42–50

Saunders AM, Strittmatter WJ, Schmechel D, St George-Hyslop PH, Pericak-Vance MA, Joo SH, Rosi BL, Gusella JF, Crapper-MacLachlan DR, Alberts MJ, Hulette C, Crain B, Goldgaber D, Roses AD (1993) Association of apolipoprotein E allele ε4 with late-onset familial and sporadic Alzheimer's disease. Neurology 43: 1467–1472

Scheff SW, Sparks DL, Price DA (1993) Quantitative assessment of synaptic density in the entorhinal cortex in Alzheimer's disease. Ann Neurol 34: 356–361

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

Selkoe DJ (1994) Alzhemier's disease: a central role for amyloid. J Neuropathol Exp Neurol 53: 438–447

Smith PK, Krohn RI, Hermansson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC (1985) Measurement of protein using bicinchoninic acid. Anal Biochem 150: 76–85

Snipes GJ, McGuire CB, Norden JJ, Freeman JA (1986) Nerve injury stimulates the secretion of apolipoprotein E by nonneuronal cells. Proc Natl Acad Sci USA 83: 1130–1134

Soininen H, Kosunen O, Helisalmi S, Mannermaa A, Paljärvi L, Talasniemi S, Ryynänen M, Riekkinen Sr P (1995) A severe loss of choline acetyltransferase in the frontal cortex of Alzheimer patients carrying apolipoprotein ε4 allele. Neurosci Lett 187: 79–82

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

Strittmatter WJ, Weisgraber KH, Goedert M, Saunders AM, Huang D, Corder EH, Dong LM, Jakes R, Alberts MJ, Gilbert JR, Han SH, Hulette C, Einstein G, Schmechel D, Pericak-Vance MA, Roses AD (1994) Hypothesis: microtubule instability and paired helical filaments formation in the Alzheimers disease brain are related to apolipoprotein E genotype. Exp Neurol 125: 163–171

Terry RD, Masliah E, Salmon DP, Butters N, DeTeresa R, Hill R, Hansen LA, Katzman R (1991) Physical basis of cognitive alterations in Alzheimer's disease: synapse loss is the major correlate of cognitive impairment. Ann Neurol 30: 572–580

Tomlinson BE, Corsellis JAN (1984) Ageing in the dementias. In: Hume Adams J, Corsellis JAN, Duchen LW (eds) Greenfield's neuropathology. Edward Arnold, London, pp 951–1025

Wilcock GK, Esiri MM (1982) Plaques, tangles and dementia: a quantitative study. J Neurol Sci 56: 343–356

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

Zhan SS, Beyreuther K, Schmitt HP (1993) Quantitative assessment of the synaptophysin immuno-reactivity of the cortical neuropil in various neurodegenerative disorders with dementia. Dementia 4: 66–74

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