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Selective loss of synaptic proteins in Alzheimer's disease: Evidence for an increased severity with *APOE* ε4

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Abstract

A pathological feature of Alzheimer's disease (AD) is an area-specific neuronal loss that may be caused by excitotoxicity-related synaptic dysfunction. Relative expression levels of synaptophysin, dynamin I, complexins I and II, N-cadherin, and αCaMKII were analysed in human brain tissue from AD cases and controls in hippocampus, and inferior temporal and occipital cortices. Synaptophysin and dynamin I are presynaptic terminal proteins not specific to any neurotransmitter system whereas complexin II, N-cadherin, and αCaMKII are specific for excitatory synapses. Complexin I is a presynaptic protein localised to inhibitory synapses. There were no significant differences in synaptophysin, dynamin I, N-cadherin, or αCaMKII protein levels between AD cases and controls. The complexin proteins were both markedly lower in AD cases than in controls ($P < 0.01$). Cases were also categorised by *APOE* genotype. Averaged across areas there was a 36% lowering of presynaptic proteins in AD cases carrying at least one ε4 allele compared with in AD cases lacking the ε4 allele. We infer that synaptic protein level is not indicative of neuronal loss, but the synaptic dysfunction may result from the marked relative loss of the complexins in AD, and lower levels of presynaptic proteins in AD cases with the *APOE* ε4 allele.

Keywords: Excitotoxicity; Presynaptic; Postsynaptic; Excitatory; Inhibitory; Neurodegeneration

1. Introduction

AD is a devastating degenerative brain disease that causes progressive dementia. The brain tissue of AD patients shows many structural and biochemical changes (Bahr et al., 1994). These include a build-up of β-amyloid in neuronal cell bodies, high β-amyloid concentrations in plaques, high concentrations of ceroid-lipofuscin, neurofibrillary tangle formation within neurones, neuronal atrophy, a decrease in electron transport chain enzyme activity, and granulovacuolar degeneration (Parker et al., 1990; Braak and Braak, 1991; Mukaetova-Ladinska et al., 1993; Bahr et al., 1994; Simonian and Hyman, 1994; Parker and Parks, 1995; Mattson, 1997; Swerdlow et al., 1997).

Genetic risk factors play an integral rôle in the ætiology of AD. The best established risk factor is the presence of the ε4 allele of apolipoprotein E (Rubinsztein, 1997). The mechanism by which apoE influences AD is not known, but the protein is postulated to play a rôle in synaptogenesis (Pirttila et al., 1996; Mukaetova-Ladinska et al., 1997; Rubinsztein, 1997). The ε4 allele is associated with increased risk of AD in a dose-dependent manner. Individuals with one ε4 allele have a three-fold risk of developing AD, while ε4 homozygotes have an eight-fold risk of AD, compared with ε3 homozygotes (Rubinsztein, 1997). The *APOE* ε4 allele is associated with a greater accumulation of amyloid deposits (Mukaetova-Ladinska et al., 1996; Pirttila et al., 1996), accounts for about 50% of AD risk, and is the strongest risk factor for late-onset AD (Pirttila et al., 1996; Rubinsztein, 1997).

The excitotoxicity theory has been implicated in many neurodegenerative disorders including AD, and may be involved in the process by which neurones are destroyed. Excitotoxic insults may be elicited by a synaptic dysfunction. To investigate this possibility, synaptic proteins associated with excitatory and inhibitory neurotransmitter systems at the pre- and postsynaptic terminal were quantified. N-cadherin is localised to excitatory synapses (Benson and Tanaka, 1998), and the α-subunit of calcium/calmodulin-dependent protein

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kinase II (αCaMKII) is the major protein of the excitatory postsynaptic density (PSD; Fox, 2003). Complexins I and II are specific for inhibitory and excitatory presynaptic terminals, respectively (Harrison and Eastwood, 1998). Synaptophysin and dynamin I were chosen to represent all presynaptic terminals (Wiedenmann and Franke, 1985; Robinson et al., 1994). The functions of these proteins range from synaptic vesicle recycling and exocytosis to synaptic maintenance and signal transduction. By analysing the levels of these proteins in AD cases and controls in the context of *APOE* genotype, we aimed to investigate the nature of the synaptic dysfunction in AD and further understand the rôle excitotoxicity plays in neuronal cell death.

2. Materials and methods

The monoclonal mouse anti-synaptophysin antibody SY38 was obtained from Dako International (Sydney, NSW). Highly species-specific horseradish peroxidase-conjugated sheep anti-mouse IgG antibody was purchased from Amersham (Castle Hill, NSW). Maxisorp 96-well Immunoplates plates were used (Nalge Nunc International, Medos Company, Mt. Waverley, Vic.). Complexin antibodies were gifts from Dr. K. Sawada, and the dynamin I antibody and purified ovine dynamin I protein were gifts from Dr. P. Robinson. The N-cadherin antibody was a gift from Dr. D.R. Colman. The antibody to αCaMKII was obtained from Santa Cruz Biotechnology Inc. (Monarchmedical, Stafford, Qld).

2.1. Synaptophysin antibody VL1SYN production and purification

The peptide sequence of the first vesicular domain from human synaptophysin, NKTESDLSIE-C, was manufactured by Mimotopes Pty. Ltd. (Clayton, Vic.) and conjugated to keyhole limpet hæmacyanin (KLH, Sigma, Castle Hill, NSW). The peptide-conjugated-carrier protein dissolved in PBS was emulsified with Titremax Gold adjuvant (Sigma) and injected into a New Zealand white rabbit and subsequently boosted three times at 2-week intervals. The titres were tested between boosts until the levels were deemed satisfactory. The serum was separated, and the antibody was purified using a double-affinity purification column system. In one column, KLH was coupled to 5 mL CNBr (Cyanogen Bromide)-activated Sepharose (Sigma), while the second column contained the peptide that the antibody was raised against coupled to 5 mL CNBr-activated Sepharose. The antiserum was first run through the CNBr-Sepharose–KLH column, then through the CNBr-Sepharose–peptide column. The antibody was eluted from the peptide column with 0.1 M glycine, pH 2.5. Antibody concentration was determined by absorbance at 280 nm. The antibody was tested for specificity on homogenised brain tissue by Western blot (Fig. 1).

2.2. Brain tissue preparation

Diagnosis of AD cases and controls was achieved according to the Australian guidelines for the CERAD criteria by a qualified neuropathologist (Halliday et al., 2002). Brain tissue from pathologically confirmed AD patients and controls was obtained at autopsy with informed written consent and frozen at −80 °C in 0.32 M sucrose (Dodd et al., 1986). The case-set for the sandwich ELISA included 15 AD cases and 15 controls. The case-set for the indirect ELISAs of the complexins and the Western blots of N-cadherin, dynamin 1, and αCaMKII consisted of 12 AD cases and 12 controls. There was an overlap of 10 AD cases and 8 controls between these groups. All these above cases were genotyped within a larger pool of 40 AD cases and 43 controls. Areas sampled were hippocampus, and cortex from inferior temporal gyrus and occipital pole. These areas were neuropathologically scored according to the severity of combined AD pathology, namely neuronal loss, neurofibrillary tangles, amyloid plaques, and gliosis. A score of 3 indicated the most severe combined AD pathology whereas a score of 0 indicated the absence of pathology. A small piece of brain tissue was excised from the three areas and the white matter removed. For the sandwich ELISA, the grey matter was homogenised in 10× volumes of 50 mM Tris, pH 7.5, containing 0.1% Triton X-100, in a motor-driven Teflon-glass homogeniser at 500 rpm until the suspension was uniform in consistency. The homogenate was centrifuged at 39,200 × *g* at 4 °C for 10 min and the supernatant collected. For use in Western blotting, grey matter was homogenised as described above in 50 mM Tris, pH 7.5. For the indirect ELISA, the Tris homogenates from the Western blot case-set were centrifuged as described above and the supernatant collected. The protein concentration of all samples was estimated by Lowry et al. (1951).

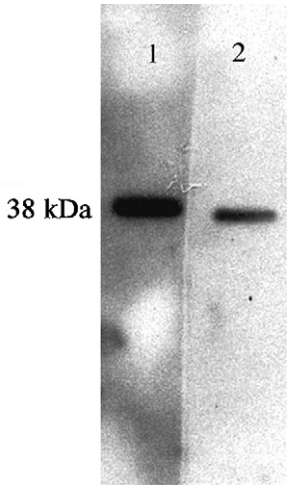

Fig. 1. Western blot comparison of the synaptophysin antibodies. SY38 (lane 1) and VL1SYN (lane 2) of homogenised human brain. The bands migrated at the approximate size of 38 kDa, the molecular weight of synaptophysin. See Section 2 for details.

2.3. Western blot

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (1970) using the Bio-Rad Mini-Protean III gel electrophoresis system (Bio-Rad Laboratories, Regents Park, NSW). An independent area from a non-sampled control case formed the protein standard, except for dynamin I where the standard consisted of purified ovine dynamin I. A range of standards (25, 50, and 100 ng for dynamin I; 3, 6, and 12 μg for N-cadherin; 4, 8, and 16 μg for αCaMKII) was diluted 1:1 (v/v) with sample buffer (2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 62.5 mM Tris–HCl, pH 6.8) and boiled for 5 min prior to loading. Samples were similarly treated. A prestained All Blue protein marker (Bio-Rad) was loaded onto every gel. N-cadherin and dynamin I were electrophoresed on a 7.5% separating gel, and αCaMKII on a 10% separating gel, and 3% stacking gel, at 200 V in running buffer (150 mM glycine, 20 mM Tris, 0.1% SDS) for ∼40 min. After transfer, the membranes were blocked in 5% Blotto (5% non-fat dried skim milk powder), Tris-buffered saline Tween (TBST; 137 mM NaCl, 3 mM KCl, 25 mM Tris, 0.1% Tween 20) for dynamin I, 0.5% Blotto for αCaMKII, and 10% Blotto for N-cadherin for 4 h with agitation, and incubated in the appropriate primary antibody at 4 °C overnight. Primary antibodies were diluted in 1% Blotto for dynamin I (1:10,000), 0.5% Blotto for αCaMKII (1:1000), and 5% Blotto for N-cadherin (1:30,000). The appropriate horseradish peroxidase-conjugated secondary antibody (Sigma) was added at a dilution of 1:10,000 in 1% Blotto for N-cadherin and dynamin I and 0.5% Blotto for αCaMKII, for 2 h with agitation. The immunoreactive bands were visualised on X-ray film (Super RX FujiFilm, Hanimex, Stafford, Qld) using ECL reagent (Amersham, see Fig. 2).

2.4. Synaptophysin sandwich ELISA

This method was performed as per Schlaf et al. (1996) with the following modifications. The coating antibody, VL1SYN, was applied at 10 μg/mL to 96-well plates. Capture antibody, SY38, was added at a dilution of 1:200. Highly species-specific anti-mouse IgG horseradish peroxidase-conjugated secondary antibody was added at a dilution of 1:4000. The diluent and wash buffer