A subset of calretinin-positive neurons are abnormal in Alzheimer's disease

Abstract The distribution of the calcium-binding protein calretinin was investigated by immunohistochemistry in the hippocampus, the subicular areas, and the entorhinal cortex in patients with Alzheimer's disease and in control subjects. By double immunolabelling, the calretinin immunoreactivity was compared to the immunoreactivity for β/A4 amyloid or for tau proteins. Calretinin-positive neurons were mainly observed in the molecular layer of the gyrus dentatus, the stratum radiatum of the Ammon's horn, and in layers II and III of the entorhinal cortex. The general pattern of calretinin immunoreactivity was conserved in Alzheimer's disease. Calretinin-positive neurons appeared normal in the hippocampus but had a reduced dendritic tree in the entorhinal cortex. Dystrophic calretinin immunoreactive fibres were often observed in the outer molecular layer of the gyrus dentatus and in the CA4 sector in Alzheimer's disease. Most neurons containing neurofibrillary tangles were not calretinin immunoreactive and most senile plaques were not associated with calretinin positive fibres. These results show that entorhinal calretinin-positive neurons are affected in Alzheimer's disease in spite of an absence of systematic association with neurofibrillary tangles and senile plaques.

Key words Calretinin • Calcium-binding protein Alzheimer's disease • Hippocampus • Entorhinal cortex

Introduction

Neurofibrillary tangles and senile plaques are characteristic histological lesions abundant in the brain of people affected with Alzheimer's disease, the most common cause of dementia in elderly people. Neurofibrillary tangles are made of bundles of abnormal filaments (paired helical filaments, PHF) accumulating in neurons, in abnormal neurites in the neuropil (neuropil threads) and in some dystrophic neurites in the senile plaques. Immunochemical and biochemical studies have demonstrated that the main component of isolated PHF is the microtubule-associated protein tau [5, 26]. Tau proteins play an important role in the stabilisation of the microtubule network and are involved in the morphogenetic events leading to axonal differentiation. Tau proteins present in PHF (PHF-tau) differ from the normal tau isoforms, the best documented of these differences being a state of increased phosphorylation [7, 13, 16]. The phosphorylation of tau affects its ability to trigger the polymerisation of microtubules in vitro and an increased phosphorylation of tau in Alzheimer's disease might underly the loss of microtubules and disturbances of axoplasmic flows observed in neurons containing neurofibrillary tangles. The senile plaques are constituted of an extracellular deposit of amyloid fibres surrounded by dystrophic neurites. This amyloid deposit is composed of a 42-amino acid polypeptide, called β/A4 amyloid peptide [30]. This β/A4 amyloid peptide derives from a larger precursor (the amyloid peptide precursor) which is abundant in neurons.

A disturbance of calcium homeostasis leading to a calcium-mediated neuronal death has been proposed to play a role in Alzheimer's disease. For instance, the activation of calcium-dependent enzymatic pathways might be instrumental in an abnormal processing of tau proteins. An increase in intracellular calcium could lead to the activation of selected kinases or kinase cascades involved in the abnormal or excessive phosphorylation of PHF-tau. The calcium-calmodulin-dependent pro-
tein kinase II has been found to phosphorylate in vitro the serine 405 in bovine tau protein [44]. The same serine was reported to be phosphorylated in PHF-tau [19]; other phosphorylated serines have been identified in PHF-tau and several other kinases are also potential candidates involved in the phosphorylation of tau in vivo. The neuronal death in Alzheimer's disease has also been suggested to result from excessive activation of receptors for excitatory amino acids like glutamate, the neurotoxic effects of glutamate being mediated by an increase in intracellular calcium secondary to the stimulation of N-methyl D-aspartate (NMDA) receptors. Another rationale for the role of calcium-mediated neurotoxic effects of glutamate has been suggested in recent studies showing that in neuronal cultures treated with glutamate or calcium ionophores, neurons develop antigenic changes similar to those observed in Alzheimer's disease [31]. In addition beta-amyloid, which accumulates in the brain tissue in the disease, was also reported to destabilise calcium homeostasis and to render neurons more sensitive to excitotoxic damage [32].

In view of the potential role played by a disturbance of calcium regulation in the disease, the status of calcium-binding proteins in Alzheimer's disease is of particular interest, since reasonable evidence suggests that these proteins play a role in maintaining calcium homeostasis (for review see [2, 20]). Alternatively, since calcium-binding proteins appear during embryonic brain development in parallel with neuritic outgrowth, far before synaptogenesis, it has been postulated that they might be functionally related to the microtubules [10].

Calretinin is a recently discovered calcium-binding protein [36] which shares extensive homologies with calbindin-D28K [35] but is found in largely separated sets of neurons [33, 38]. Calretinin-positive non-granule and non-pyramidal neurons are present in rodent [1, 17, 33] and monkey [34, 42] hippocampus and entorhinal cortex [37, 42]. Recently the distribution of calretinin-immunoreactive (IR) neurons in the frontal and temporal cortex in Alzheimer's disease has been reported [21].

In this study, we describe the distribution of calretinin-IR neurons and fibres in the hippocampus and the entorhinal cortex of human control subjects and in patients with Alzheimer's disease, i.e. in areas known to be strongly lesioned in Alzheimer's disease. The calretinin immunoreactivity was detected simultaneously with the tau or the beta-amyloid immunoreactivity, to investigate to which extent there is an anatomical association between histological lesions and calretinin immunoreactivity.

### Materials and methods

#### Tissue preparation

Tissue blocks including the human hippocampus and the adjacent parahippocampal gyrus were taken at autopsy from two normal subjects (71 and 75 years, post-mortem delays of 12 and 24 h) and from three patients with Alzheimer's disease (57, 73 and 76 years, post-mortem delays of 7, 10 and 20 h). These tissue blocks were fixed for 72 h in 4% paraformaldehyde in phosphate-buffered saline (pH 7.2). Other brain slices were fixed in formalin and embedded in paraffin for neuropathological examination. The patients had a clinical diagnosis of probable Alzheimer's disease and this diagnosis was confirmed by the histological examination showing the presence of numerous neurofibrillary tangles and senile plaques in the hippocampus and in several neocortical areas. The tissue blocks fixed in paraformaldehyde were cut on a Lancer vibratome in tissue sections with a thickness of 30 μm and processed for immunohistochemistry.

#### Immunocytochemistry

Tissue sections were immunolabelled with a rabbit antiserum to calretinin using the peroxidase-antiperoxidase method as previously reported [6]. Some sections were double immunolabelled with the calretinin antiserum in association either with a mouse monoclonal antibody to beta-amyloid or with a mouse monoclonal antibody to bovine tau proteins. For the double immunolabelling, the antibody to calretinin was detected with a goat anti-rabbit antibody (Nordic) followed by a rabbit peroxidase-antiperoxidase complex (Nordic) and diaminobenzidine (DAB) as chromogen, whereas the beta-amyloid or tau antibody was detected using a goat anti-mouse antibody conjugated to fluorescein (Sigma). In some experiments, the calretinin and beta-amyloid immunoreactivities were demonstrated on the same section using a double-colour technique. The anti-calretinin antibody was detected as above with DAB as chromogen, giving a brown colour; the beta-amyloid antibody was detected with the same method (using a mouse peroxidase-antiperoxidase complex) and a mixture of DAB and cobalt chloride as chromogen, giving a blue colour.

The calretinin rabbit antiserum (a kind gift of Dr. J.H. Rogers) has been previously described [36]. It does not cross-react at all with calbindin-D28K [39]. The anti-beta-amyloid antibody is a mouse monoclonal antibody prepared using a synthetic peptide (residues 12–28 of the beta-amyloid peptide) and was purchased from Boehringer. For the labelling of beta-amyloid deposits with the anti-beta-amyloid antibody, sections were pretreated with concentrated formic acid [24]. The tau-1 antibody is a mouse monoclonal anti-tau antibody purchased from Boehringer. For optimal labelling of neurofibrillary tangles with the tau-1 antibody, sections were pretreated with alkaline phosphatase, as previously