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SHORT REPORTS

REDUCTION OF CALBINDIN mRNA EXPRESSION IN THE TEMPORAL CORTEX IN ALZHEIMER'S DISEASE

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ABSTRACT

An alkaline phosphatase-labelled antisense oligonucleotide probe specific for the mRNA of the important calcium binding protein (calbindin D_{28K}) was used in non-radioactive hybridisation histochemical studies of normal post-mortem human brain and the brains from patients with Alzheimer-type dementia (ATD). The results revealed a significant decrease in calbindin mRNA signal from hippocampal neurones especially in the dentate gyrus (granule cells) and Ammon's horn (pyramidal cells). The loss of calbindin mRNA signal indicates that calbindin containing neurones are affected in ATD and suggests that a reduction in calcium binding or buffering capacity predisposes these key neurones to damage in ATD.

INTRODUCTION

A number of studies suggest that there is a disturbance in calcium homeostasis in Alzheimer's disease. These have included studies of intracellular calcium[1, 2] and demonstraties that the number of neurones containing calcium binding or buffering proteins are selectively reduced in Alzheimer's disease (ATD)[3, 4, 5] with for example calbindin D_{28k} containing neurones being specifically lost from the nucleus basalis[5] and frontal and temporal cortices[3, 4] in ATD.

Although much research attention has concentrated on the proteins of the plaques and tangles[6, 7, 8, 9, 10, 11] no evidence has been produced to show that the accumulation of these proteins such as B-amyloid[9], or the microtubule protein (tau)[7] in plaques and tangles are the primary cause of cell death in Alzheimer's disease, and indeed an elevation in cell calcium content due to excitoxin damage[12] or local ischaemia[13] could result in abnormal proteolysis

and the accumulation of proteins into tangles or plaques. Given this possibility of disturbed calcium homeostasis in ATD we have investigated the expression of the calcium binding protein (calbindin D_{28k}) in the temporal cortex and hippocampus of patients dying with a diagnosis of Alzheimer's disease. Calbindin D_{28k} is a member of a family of high affinity calcium binding proteins which may act to bind or buffer intracellular Ca^{++} and protect neurones against damage due to elevated intracellular calcium[14, 15] Our results show a dramatic reduction in the expression of calbindin D_{28k} mRNA in the hippocampus/temporal cortex of ATD case's including a reduction of calbindin expression in the pyramidal cells and dentate granule cells of the temporal cortex, consistent with a reduction in synthesis of this calcium buffering protein which may predisope these cells to damage due to elevated intracellular Ca^{++}.

MATERIALS AND METHODS

Human Brain Specimens

Brains removed post-mortem from 3 clinically and neuropathologically diagnosed (plaques and tangles) ATD patients (age at death 61-73 years) and 3 neurologically normal control patients (age at death 46-75 years) were supplied by the MRC Brain Bank (Addenbrooke's Hospital, Cambridge, UK) and the Department of Anatomy, University of Auckland, New Zealand. The collection, storage and dissection of the tissues has been described previously (Bird and Iversen, 1974)[16]. The controls had no history of neurological or psychiatric illness with 3-4 h post-mortem delay. In the ATD cases the clinical diagnosis was confirmed by light microscopic observations after silver staining for plaques and tangles[3, 4].

Case Histories

Case 1. - Male; age at death 61 years; onset at age 54; duration of the disease was 7 years; 3 h post-mortem delay.

Case 2. - Female, age at death 73 years; onset at age 65; duration 8 years; 5 h post-mortem delay.

Case 3. - Male, age at death 70 years; onset at age 64; duration 6 years; 6 h post-mortem delay.

Probe preparation

For detection of calbindin mRNA an antisense alkaline phosphatase-labelled oligodeoxynucleotide (30 mer) was used. Its sequence corresponds to bases 143-173 of the human calbindin cDNA sequence (Parmentier et al, 1987)[17]. The method of labelling and purification of the probe were described previously by Jablonski et al (1986)[18] and Kiyama et al (1990)[19].

In situ Hybridisation

The in situ hybridisation was carried out according to the method of Kiyama et al (1990)[19]. In brief, frozen sections (15 μm) were fixed with 4% paraformaldehyde, hybridised with hybridisation buffer (4×SSC, 50% formamide, 500 μg/ml sheared salmon sperm DNA, 10% dextran sulphate, 1×Denhardt's solution) containing alkaline phosphatase-labelled probe

at a concentration of 12.5 fmol/μl. The hybridisation was carried out at 37°C overnight. The sections were then washed four times in preheated 1×SSC at 55°C for 20 min and washed in 1×SSC at room temperature for 30 min. After preincubation in 0.1 M Tris HCl (pH 9.5), 0.1 m NaCl and 0.05 M $MgCl_2$ for 15-30 min, the sections were incubated in the same buffer containing alkaline phosphatase substrates, nitroblue tetrazolium (340 μg/ml) and 5-bromo-4-chloro-3-indolyl phosphate (170 μg/ml). The reaction was terminated after 24 h, the sections rinsed in 10 mM Tris-HCl (pH 7.5), 10mM EDTA and 0.9% NaCl. Finally the sections were mounted in the Tris-EDTA buffer containing 50% glycerol.

The optical density (525 nm) of the cellular mRNA signal (alkaline phosphatase product) was measured on a 2 μm^2 area of the cell cytoplasm using a Vickers Micro Densitometer M85 (Vickers Instrument, UK). Counts of the numbers of labelled cells were carried out as described previously[3, 4] and numbers of cells expressed as mean+S.E.M. Statistical analysis was carried out using Student's t-test.

RESULTS

The non-radioactive in situ hybridisation technique demonstrated the preserve of calbindin mRNA in the dentate gyrus, granule cells (Figure 1A) and in a number of pyramidal cells of the hippocampus/temporal cortex (Figure 1C). In the ATD cases there was a dramatic reduction in the amount of calbindin mRNA signal in the dentate gyrus granule cells (although the cells are still present) (Figure 1B). The decrease in mRNA signal (Figure 2A) being over 50% of the calbindin mRNA content found in neurologically normal controls (57.2%, Table 1). The calbindin D_{28k} containing interneurones of the hilus (HI, in Figure 1B) were relatively little affected with no obvious change in their signal content. In the temporal cortex the amount of calbindin mRNA signal in those pyramidal cells remaining was also significantly reduced (36.7%) relative to the same cells in neurologically normal controls (Figure 2B and Table 1) note that there was also a definite loss of large calbindin mRNA containing pyramidal cells with a loss of some 42% of pyramidal cells in the ATD cases (control cell count 86.7+2.7 mean+S.E.M., ATD cell count 48.7+2.4 mean+S.E.M., n=3 for each group).

DISCUSSION

The results presented here show unequivocally that the expression of the intracellular calcium binding protein calbindin D_{28k} is affected in the temporal cortices of patients dying with Alzheimer's disease. The cells affected included those of the dentate gyrus which normally survive the developing pathology of ATD (although the dentate gyrus develops plaques), and the pyramidal cell of the cortex (CA1) which are selectively lost in ATD. These results suggest strongly that at the very least the affected cells have a reduced capacity to synthesize the calcium buffer, calbindin D_{28k} which may render these cells more vulnerable to calcium damage[13, 15] The finding of others that cultered fibroblasts from patients with a diagnosis of Alzheimer's disease have disturbed calcium regulation or elevated intracellular calcium[1, 2] points to a wide spread of disturbance of calcium regulation in ATD. This disturbance may as in ischaemia, affect those cells of the temporal and frontal cortices which are most vulnerable to Ca^{++}

TABLE 1 Effects of Alzheimer's disease in calbindin D_{28k} mRNA. Value are means+S.E.M. of optical density measurements carried out at 525 nm on 2 μm^2 area. n is the number of cells sampled from three normal cases and three ATD cases.

	Control Neurologically Normal Cases	**Alzheimer's Disease Cases**
Dentate Gyrus	8.81+0.25	3.77+0.96*
(Granule Cells)	(n=50)	(n=50)
Temporal Cortex	8.83+0.19	5.59+0.23*
(Pyramidal Cells)	(n=50)	(n=50)

*P $\langle$ 0.0001, Student "t" test unpaired.

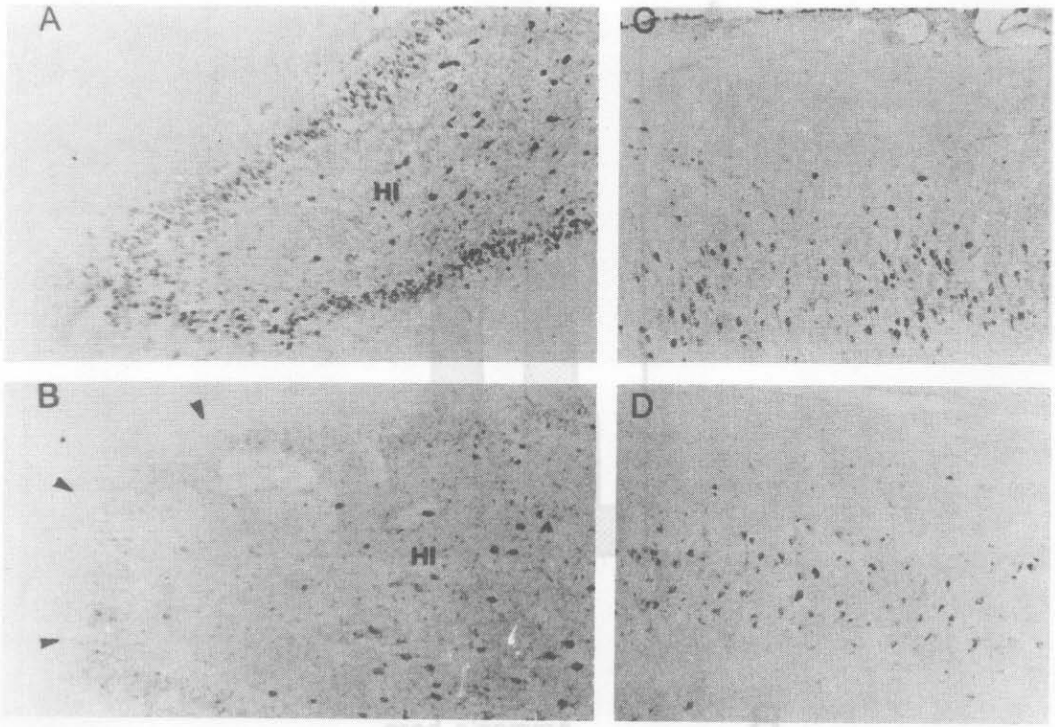

Fig. 1. Detection of calbindin mRNA in the dentategyrus of a neurologically normal control (A) and ATD case 1 (B), and Ammon's horn of the control (C) and ATD case 1 (D). Bar = 100 μm. Note the strong non-radioactive in situ/ mRNA signal in the dentate gyrus (A) and in the pyramidal cells (C) of the normal case. In ATD case there is a substantial reduction in calbindin mRNA content of the dentate gyrus cells (arrows, B) and a definite loss of pyramidal cells.

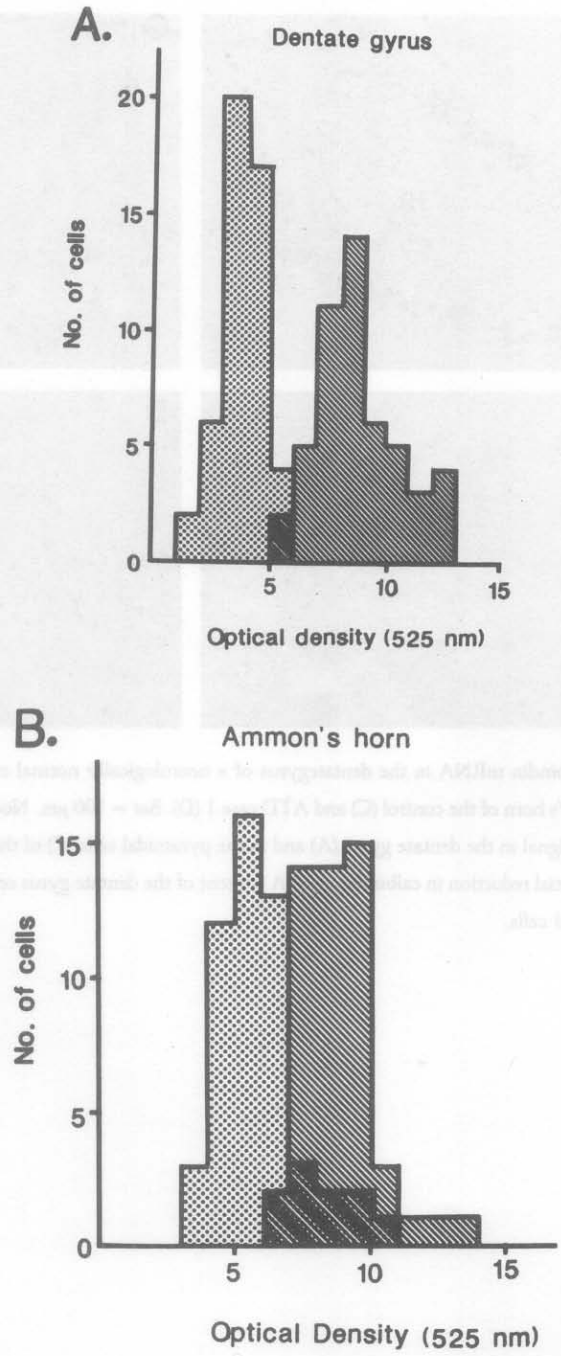

Fig. 2 Histogramsshowing the distribution of hybridisation signal from individual cells of dentate gyrus (A) and Ammon's horn (B) in controls (cross hatched histogram) and ATD cases (dotted histogram). The mean optical density values + S.E.M. for the control and ATD cases are given in **Table 1**.

damage[13, 20] including those cells of CA1 which we have shown here show a reduction in calcium binding protein expression in ATD. If our suggestion that disturbed calcium regulation/ buffering is critical to the developing neuronal pathology of ATD then this would have important consequences for the rationale therapy of Alzheimer's disease.

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บทคัดย่อ

การศึกษาการแสดงออกของ mRNA ของ calbindin D28K ในสมองของคนไข้โรค Alzheimer-type dementia (ATD) โดยวิธี non-radioactive *in situ* hybridization อาศัย alkaline phosphatase-labelled oligonucleotide probe ผลปรากฏว่าระดับของ calbindin mRNA ลดลงอย่างมีนัยสำคัญใน hippocampus โดยเฉพาะบริเวณที่เรียกว่า dentate gyrus (granule cells) และใน temporal cortex (pyramaidal cells) สรุปได้ว่าพยาธิสภาพของคนเป็นโรค ATD นี้มีผลกระทบต่อเซลล์สมองที่มี calbindin บรรจุอยู่ และชวนให้คิดว่าการลดลงของ calbindin มีผลต่อประสิทธิภาพการทำงานของ calcium binding หรือ buffering ภายในเซลล์สมองเหล่านั้น ดังนั้น calbindin อาจจะเป็นตัวแปรที่สำคัญในการตรวจวินิจฉัยโรค ATD