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ORIGINAL PAPER

Age-related loss of calcium buffering and selective neuronal vulnerability in Alzheimer's disease

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Abstract The reasons for the selective vulnerability of distinct neuronal populations in neurodegenerative disorders are unknown. The cholinergic neurons of the basal forebrain are vulnerable to pathology and loss early in Alzheimer's disease and in a number of other neurodegenerative disorders of the elderly. In the primate, including man, these neurons are rich in the calcium buffer calbindin-D_{28K}. Here, we confirm that these neurons undergo a substantial loss of calbindin in the course of normal aging and report a further loss of calbindin in Alzheimer's disease both at the level of RNA and protein. Significantly, cholinergic neurons that had lost their calbindin in the course of normal aging were those that selectively degenerated in Alzheimer's disease. Furthermore, calbindin-containing neurons were virtually resistant to the process of tangle formation, a hallmark of the disease. We conclude that the loss of calcium buffering capacity in these neurons and the resultant pathological increase in intracellular calcium are permissive to tangle formation and degeneration.

Keywords Selective neuronal vulnerability · Aging · Alzheimer's disease · Calcium dysregulation · Cholinergic basal forebrain neurons · Tangle pathology

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Introduction

A major question remains unanswered in relation to neurodegenerative disorders: what are the causes of selective vulnerability of certain neuronal populations? Here we provide evidence that age-related loss of the calcium binding protein calbindin-D_{28K} (CB), which can lead to destabilization of calcium levels and abnormalities in calcium signaling, is a major factor allowing selective vulnerability of one such population, the basal forebrain cholinergic neurons (BFCN).

The human BFCN provide the entire cortical mantle with cholinergic innervation [30, 31] and participate in the cognitive processing of memory and attention [13, 41], which show deficits in Alzheimer's disease (AD). The BFCN are vulnerable in many neurodegenerative disorders of the elderly, including AD, Parkinson's disease and Lewy-body dementia [1, 2, 15, 36, 40]. In AD, the BFCN

and their axons are among the earliest degenerating neural elements [15]. The BFCN are vulnerable to phosphorylated tau accumulation and tangle formation early in mild cognitive impairment (MCI) and AD [29]. We found that phosphorylated tau accumulation in pre-tangles, tangle formation and axonal abnormalities characterize the BFCN early in the course of aging and AD [16]. Pre-tangles were detected in the BFCN as early as the third decade of life. A low density of pre-tangles and tangles was present in the BFCN of normal young individuals below 65 years of age. The density of tangles and pre-tangles in the BFCN displayed an increase in the brains of normal old individuals (above 65 years), and a progressive and significant increase in brains of pathologically mild and pathologically severe AD cases. In AD, there is a strong correlation between loss of BFCN and severity of dementia [25, 38] and the cholinergic system is among a handful of targets for available therapy [8]. Thus, understanding the causes of the vulnerability of BFCN is of interest both scientifically and therapeutically.

The BFCN display a number of changes, including loss of RNA and protein for high affinity nerve growth factor receptor (TrkA) [35], which may contribute to their degeneration. However, these changes occur concurrent with appearance of cognitive deficits in MCI and AD. Since age is the primary risk factor in AD and other neurodegenerative disorders of elderly, we speculated that age-related changes must make a significant contribution to vulnerability of the BFCN. Consistent with this expectation, we observed selective loss of the calcium binding protein calbindin-D_{28K} (CB) from BFCN in the course of normal aging [14, 45]. The number of CB-positive BFCN and the percentage of the ChAT-positive BFCN that contained calbindin immunoreactivity were significantly lower in brains from normal individuals above 65 years of age when compared with normal individuals below 65 years. Age-related loss of CB is a primate specific phenomenon; the rodent BFCN are devoid of CB [17] and age-related loss of CB from BFCN also occurs in non-human primates [43].

Calbindin binds calcium with high affinity, regulates its intracellular levels [20] and protects neurons from degeneration induced by elevations of intracellular calcium [10, 37, 39]. Therefore, we hypothesized that age-related loss of CB may lead to vulnerability of the BFCN to calcium insults in neurodegenerative disorders. Here, we confirm loss of CB protein from the BFCN in the course of normal aging and demonstrate that CB displays a further loss at the level of protein and RNA in AD, that loss of BFCN in AD occurs in neurons that have lost their CB in the course of normal aging and AD, and that presence of CB is associated with substantial protection against phosphorylated tau accumulation and tangle formation.

Materials and methods

Case information

De-identified brains from 12 normal young individuals (20–64 years, 7 males, 5 females), 25 normal, non-demented old individuals (68–99 years, 12 males, 13 females), without any signs of neurological or psychiatric disorders, and 28 clinically and pathologically confirmed AD patients (61–99 years, 16 males, 12 females) were obtained at autopsy and used in these experiments. Detailed clinical records were available for every subject. Clinical and neuropathological diagnoses were according to the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) [33, 34]. Postmortem intervals in the three groups of subjects (young group 14.75 ± 3.4, old group 14.32 ± 1.5 and AD group 17.1 ± 1.7) and age of the old and AD groups (old group 82.7 ± 1.6 and AD group 79.2 ± 2.1) were not significantly different ($p > 0.05$).

Immunohistochemistry

Series of sections from blocks containing the basal forebrain fixed in 4% paraformaldehyde for 36 h and cut at a thickness of 40 μm were processed immunohistochemically using the avidin–biotin-peroxidase (ABC) method as described elsewhere [14], utilizing the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA). The antibodies used were a specific polyclonal antibody against ChAT (gift of Dr. Louis Hersh, University of Kentucky Medical School, 1/300-1/500), mouse monoclonal and rabbit polyclonal antibodies against CB (Swant, Switzerland, 1/1000), a monoclonal antibody against $p75^{NTR}$ (Lab Vision Inc., Fremont, CA), and the PHF-1 monoclonal antibody that recognizes tau phosphorylated at Ser396/404 (gift of Dr. Peter Davies, Albert Einstein School of Medicine). To control for non-specific staining, sections stained using the above antibodies were compared with sections stained in the absence of primary antibodies or in the presence of non-specific IgG in place of primary antibodies.

For concurrent visualization of two antigens within the same section, the double-immunohistochemical method of Levy et al. [26] was used. Tissue sections were first processed for one antigen using diaminobenzidine as chromogen. After the development of the DAB brown reaction product, tissue sections were processed for the second antigen with the peroxidase labeling visualized using benzidine dihydrochloride, which results in a granular blue reaction product. Alternatively, fluorescence double labeling was performed using secondary antibodies conjugated to Texas Red and FITC. The two antibodies used in each double immunohistochemical experiment

were obtained from different species (i.e. mouse and rabbit).

Quantitative analysis of stained profiles

Matching adjacent sections single or double stained for the antigens under study were available in all immunostained cases. For this analysis, all magnocellular immunoreactive neurons in 1–2 sections spanning each of the anterior, intermediate and posterior sectors of the nucleus basalis of Meynert—cholinergic cell group 4 (nbM-Ch4), the primary component of BFCN, were counted. Counting was carried out at 10× magnification using a counting box placed in the eyepiece of a compound microscope. Data were compiled as the number of immunoreactive profiles per section of the entire Ch4-nbM.

In five-six cases per group, enough sections with systematic random sampling of basal forebrain stained for ChAT and CB were available satisfying the requirements for unbiased stereological estimation of immunoreactive profiles. Stereological analysis using these sections was carried out according to procedures previously described in detail [44], employing the fractionator method and the Stereologer (Systems Planning) software. Using this method, stereological estimates of the total number of immunoreactive profiles in each case were obtained.

Laser capture microdissection and qRT-PCR

Brain slabs containing the basal forebrain were prepared from fresh tissue immediately following autopsy, frozen in isopentane cooled on dry ice, and stored at −80°C until used. Ten μm thick sections through fresh frozen basal forebrains prepared on a cryostat were stained with histogene and magnocellular BFCN were collected on caps using a laser capture microdissector. Cells were extracted from caps and rapidly dissolved in Trizol. The concentration of total RNA was measured by UV spectrophotometry, and RNA quality was confirmed in an Agilent bioanalyzer. Quantitative RT-PCR was used to investigate differential expression of the CB gene in old and AD BFCN. Two μg of total RNA from each basal forebrain was reverse transcribed to synthesize cDNA using superscript reverse transcriptase (Invitrogen). The following primers were used: CB forward GACGGCAATGGATACATAGAT, CB reverse ACTGGCCTAAGCATAGACTTTC, GAPDH forward AGGTGAAGGTCGGAGTCAACGG, and GAPDH reverse CGGTGCCATGGAATTTGCC. Amplification of each gene was expressed as the amplification cycle at which its PCR product was first detected (threshold cycle, CT). All samples were processed in parallel. The relative quantification of target gene expression was performed according to the comparative CT method. For each run, the mean expression level of the CB gene was normalized to the mean expression levels of the GAPDH reference gene. Serial dilution of standard human cDNA was used to correct for differences in amplification efficiency.

Western blot analysis

The basal forebrain area, in which BFCN are located, was dissected from fresh tissue immediately after autopsy, frozen on dry ice and stored at −80°C until used. Analysis was carried out on whole cell protein extracts separated on 12% SDS-PAGE and transferred to PVDF membranes. Membranes were probed with antibodies to CB and GAPDH and immunoreactivity was visualized with peroxidase conjugated secondary antibody and electrochemiluminescent (ECL) detection kit (Amersham). Bands visualized on exposed film at the correct molecular weights were quantified for protein content using the Image J software.

Statistical analysis

Data for each portion of the study were found to be normally distributed; therefore, parametric statistical analyses were used. Analysis of variance followed by Bonferroni post hoc comparisons or *t* tests were used to detect significant group differences. Significant relationships were analyzed using Pearson correlations.

Results

Quantitative analysis confirms significant age-related loss of calbindin from human cholinergic neurons

Using immunohistochemistry for the specific cholinergic enzyme, choline acetyltransferase (ChAT; Fig. 1a, b), the low affinity neurotrophin receptor ($p75^{NTR}$) and CB (Fig. 1d, e), and counts in matching sections at three levels of nbM-Ch4, we investigated the expression of these proteins in aging and AD. Both ChAT (Fig. 1a, b) and $p75^{NTR}$ immunoreactivity visualized a large population of the BFCN. While $p75^{NTR}$ was present in a slightly lower number of BFCN, counts of the two markers did not differ significantly (Fig. 1g). Additionally, counts of ChAT and $p75^{NTR}$ were not different in young brains when compared with the old group (Fig. 1a, b, g). In contrast, the numbers of CB-positive BFCN displayed a significant decrease when old subjects were compared with the young (Figs. 1d, e, g, 2a–c). In young brains, nearly 70% of the ChAT-positive BFCN contained CB. In old brains, this percentage was reduced to 36, significantly lower than the young

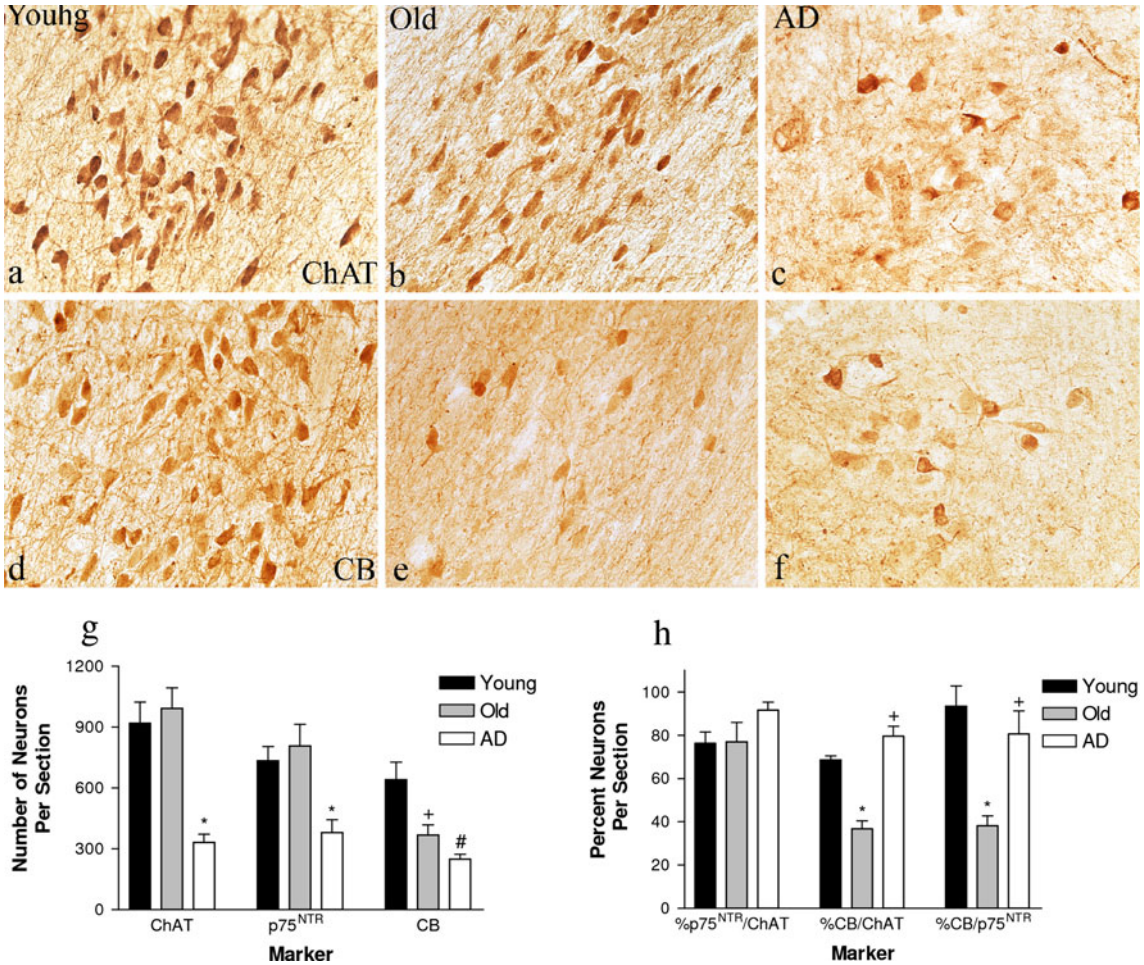

Fig. 1 Calbindin-containing basal forebrain cholinergic neurons (BFCN) survive in Alzheimer's Disease (AD). Choline acetyltransferase (ChAT) immunoreactivity identifies the BFCN in young (**a**), old (**b**) and AD (**c**) cases. In old brains, the number of BFCN remains the same while in AD, there is a significant reduction in the number of BFCN. Calbindin (*CB*) immunoreactivity in present in most of the BFCN in young brains (**d**), while in the old a majority of BFCN lose their CB (**e**). Calbindin immunoreactive neurons remain constant in AD (**f**) when compared with the old and appear similar in density to the ChAT-positive BFCN, indicating that the BFCN which survive in AD, are those that retain their CB in the course of normal aging. Quantitative analysis confirmed the preservation of CB-positive BFCN in AD. **g** counts obtained from young ($n = 8$, 26–61 years), old ($n = 15$, 68–99 years) and AD ($n = 14$, 65–89 years) brains at three levels of basal forebrain demonstrate that numbers of ChAT- and low affinity neurotrophin receptor ($p75^{NTR}$)-positive BFCN remain constant in aging ($p > 0.05$), but display a significant reduction in AD ($^{*}p < 0.001$ and $p < 0.05$ respectively). Counts of CB-positive BFCN, however, display a significant reduction in old brains and a further small reduction in AD when compared with the young ($^{+}p < 0.01$, $^{\#}p < 0.001$). The numbers of CB positive BFCN profiles in AD were not significantly different from that in the old nor were they significantly different from the number of ChAT-positive profiles in AD brains ($p > 0.05$), indicating the survival of CB-positive BFCN in AD. **h** The percentage of ChAT-positive BFCN that contained $p75^{NTR}$ was high and remained constant in young, old and AD brains ($p > 0.05$). However, the percentage of ChAT or $p75^{NTR}$ immunoreactive BFCN that contained CB displayed a significant decrease in the old when compared with the young brains ($^{*}p < 0.001$), indicating significant age-related loss of CB from these neurons. In AD, these percentages were significantly increased when compared with the old ($^{+}p<0.01$) and were not different from the young ($p > 0.05$), indicating that the loss of BFCN in AD occurs nearly exclusively in neurons that lose CB in the course of normal aging

(Fig. 1h). Thus, we confirmed the significant age-related loss of CB from BFCN in the human brain.

There was no significant correlation between age and number/percentage of CB-positive BFCN in the young or the old group of subjects. However, when data for the two groups were combined, a significant correlation was detected between the age of the subjects and the number of CB-positive BFCN as well as the percentage of BFCN that contained CB immunoreactivity ($p < 0.0001$ for both). Thus, it appears that gradual loss of CB occurs throughout aging and that the rate of this loss is accelerated at approximately 65 years of age (the cutoff used to separate

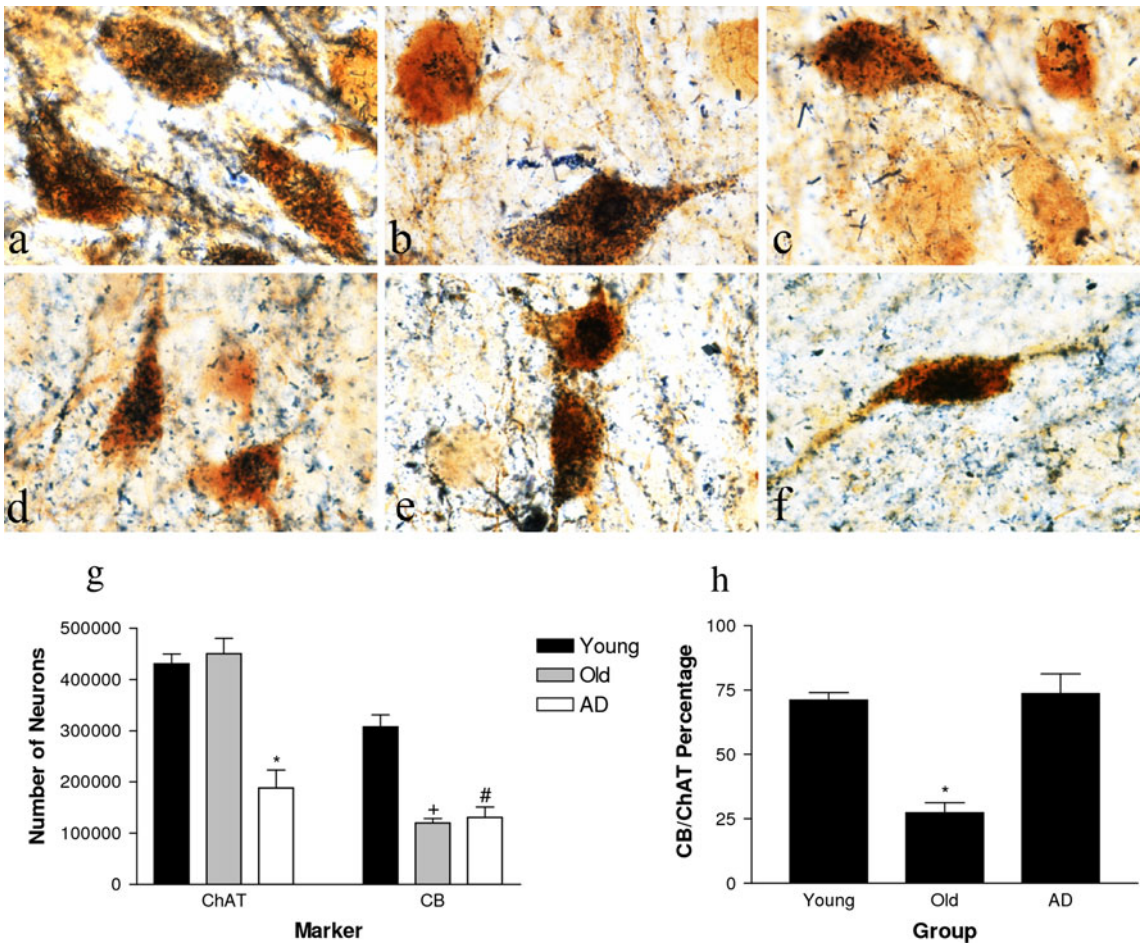

Fig. 2 Double immunostaining and unbiased stereology confirm preservation of CB-positive BFCN in AD. **a** Double staining for ChAT (*brown*) and CB (*granular blue–black*) in young brains indicated the presence of CB immunoreactivity in the majority of the BFCN. **b**, **c** In old brains, many of the BFCN that continued to exhibit ChAT immunoreactivity contained no CB, indicative of age-related loss of CB from these neurons. In AD (**d**–**f**), the number of ChAT-positive BFCN was significantly reduced. However, the majority of the remaining BFCN contained CB immunoreactivity. **g** Unbiased stereological counting methods in a subset of young ($n = 5$), old ($n = 5$) and AD ($n = 6$) cases, demonstrated the preservation of ChAT-positive BFCN in aging and a significant loss of these neurons in AD ($^{*}p < 0.001$). In contrast, CB-positive BFCN displayed a significant decrease in old brains when compared with the young ($^{+}p<0.001$) and were preserved in AD when compared with the old ($^{\#}p > 0.05$). **h** The stereologically determined percentage of the ChAT-positive BFCN that contained CB displayed a significant decrease in old brains when compared with the young ($^{*}p < 0.001$), but was significantly higher in AD when compared with the old ($^{*}p < 0.001$). There was no difference in this percentage between young and AD groups ($p > 0.05$), indicating that nearly all the surviving BFCN in AD contain CB and the degeneration of BFCN occurs nearly exclusively in neurons that have lost their CB in the course of normal aging

young from old in this study), resulting in significantly lower numbers of CB-positive BFCN in the old group when compared with the young.

Loss of cholinergic neurons in AD occurs primarily in neurons that lack calbindin

Consistent with the well-known loss of BFCN in AD, the numbers of ChAT and $p75^{NTR}$ immunoreactive BFCN displayed a significant decrease in AD brains when compared with old individuals (Fig. 1b, c, g). In contrast, the numbers of CB-positive BFCN displayed only a small and non-significant decrease in AD brains when compared with the old group (Figs. 1 e, f, g, 2b–f), indicating that the neurons with preserved CB immunoreactivity during aging survive in AD. The percentage of the ChAT- and $p75^{NTR}$-positive BFCN remaining in the AD brains that contained CB immunoreactivity displayed a significant increase when compared with the normal aged group and was the same as the percentage in young brains (Fig. 1h). Therefore, the loss of BFCN in AD occurs primarily in neurons that lose their CB in the course of normal aging. Importantly, the number of CB-positive BFCN did not display a correlation with PMI in the three groups of brains ($p > 0.05$).

Stereological analysis confirms preservation of calbindin-positive cholinergic neurons in AD

We next used unbiased stereological analysis in five young, five old and six AD brains that had sufficient tissue to satisfy the requirements of stereology, to confirm the pattern of CB expression using counts of the entire BFCN population (Table 1). Stereological counts confirmed the preservation of ChAT-positive BFCN in aged brains when compared with the young (Fig. 2g). In contrast, there was a significant decrease in the number of BFCN that contained CB immunoreactivity in the old brains when compared with the young group (Fig. 2a–c, g). In AD, the numbers of CB-positive BFCN remained stable despite a significant loss of ChAT-positive BFCN (Fig. 2b–f, g). The percentage of the ChAT-positive BFCN that displayed CB was significantly lower in the old group (27%) when compared with the young (71%). In contrast, this percentage was significantly higher in the AD brains (74%) and was the same as that in the young (Fig. 2h). These results confirm that the BFCN which contain CB immunoreactivity are preserved in AD.

Presence of calbindin in AD cholinergic neurons is not due to damage-induced upregulation

Some modes of neuronal damage may be associated with upregulation of CB [9, 27]. To determine whether the presence of CB in nearly all AD BFCN is due to upregulation of this protein rather than preservation of CB containing neurons, we collected BFCN from fresh frozen tissue sections using laser capture microdissection and determined the levels of CB RNA by quantitative real-time PCR. We also determined the levels of CB protein in dissected basal forebrains from six young, old and AD brains each, using Western blot analysis. CB RNA levels were significantly lower in AD BFCN when compared with the old group (Fig. 3a). The levels of CB protein were significantly lower in the aged basal forebrain when compared with the young and showed a further significant decrease in AD (Fig. 3b, c). Thus, both CB RNA and protein display significant loss in AD BFCN, indicating no damage-induced upregulation. Despite this decrease, immunohistochemically demonstrable presence of CB identifies BFCN that survive the neurodegenerative insult.

Calbindin-positive cholinergic neurons are protected from the process of tangle formation

We and others have shown that accumulation of phosphorylated tau and formation of pre-tangles and tangles in the BFCN commence very early in the course of aging, show a progressive increase in the young-old-AD

Table 1 Unbiased stereological counts of ChAT and CB immunoreactive basal forebrain neurons

Young			Old			AD		
ChAT	CB	CB/ChAT%	ChAT	CB	CB/ChAT%	ChAT	CB	CB/ChAT%
463,473	326,127	70	492,855	110,567	22	296,125	195,641	66
478,558	392,621	82	490,216	134,002	27	114,921	85,189	74
418,022	267,166	64	349,841	145,808	42	113,909	85,632	75
371,964	267,518	72	505,889	97,154	19	227,159	191,911	84
424,018	284,289	67	415,265	113,553	27	269,774	115,874	43
						109,225	109,225	100
431,207 ± 18,733	307,544 ± 23,829	71 ± 3.1	450,813 ± 29,843	120,217 ± **9,701**	27 ± 3.9	188,518 ± 35,093	130,578 ± 20,613	74 ± 7.7

Cholinergic neurons in young, old and AD brains. The last row displays mean ± standard errors. For statistical differences see Fig. 2g, h

ChAT Choline acetyltransferase, *CB* calbindin

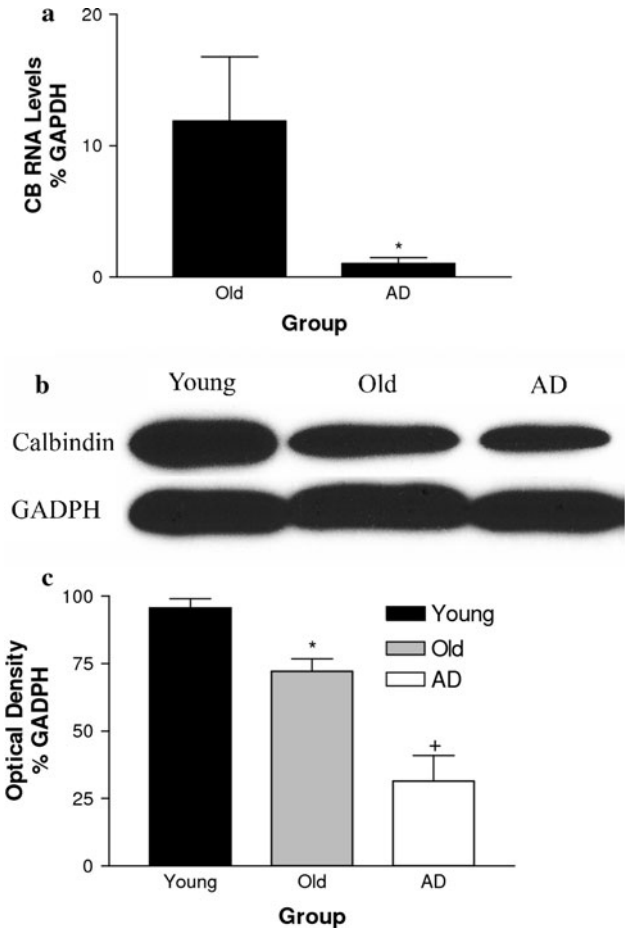

Fig. 3 Preservation of CB-positive BFCN in AD is not due to upregulation of CB. **a** Laser capture of BFCN from normal old ($n = 4$) and AD ($n = 6$) brains and qRT-PCR analysis revealed a significant reduction in CB RNA within these neurons in AD when compared with the normal old ($^{*}p < 0.02$). **b**, **c** Western blot analysis of the basal forebrain region containing the BFCN in young, old and AD brains ($n = 6$ each) confirmed a significant reduction in the levels of CB protein in the old when compared with the young ($^{*}p < 0.05$) and a further significant decrease in AD when compared with the old ($^{+}p<0.01$). However, despite reduced protein levels, the presence of CB identifies BFCN that survive the degenerative process in AD

continuum and are strongly related to the loss of BFCN [16, 29, 38]. We have demonstrated phosphorylated tau-positive pre-tangles within the BFCN as early as the third decade of life. The numbers of pre-tangles and tangles within the BFCN increased in old individuals (older than 65 years) and showed further progressive increase in pathologically mild and severe AD [16]. Therefore, we sought to determine whether CB-positive BFCN in aging and AD are protected from the process of tangle formation. Counts of BFCN positive for the PHF-1 epitope of abnormally phosphorylated tau and CB in double-stained sections revealed the formation of tangles and pre-tangles in old brains ($n = 4$, Table 2; Figs. 4, 5). While the CB-positive BFCN comprised about 27% of the total BFCN population in old brains (Fig. 2), on average less than 2% of the CB-positive BFCN contained PHF-1-positive tangles and pre-tangles (Table 2; Fig. 4a–d, g–h) and only 7.5% of the total population of PHF-1-positive BFCN pre-tangles and tangles were found in CB-positive neurons. Thioflavin S, which binds the abnormal β-pleated sheet protein conformations and is indicative of the formation of mature tangles, displayed a similar pattern. Less than 1% of the CB-positive BFCN contained thioflavin S-positive tangles in old brains and only 3.1% of thioflavin S-positive tangles within the aged BFCN were in CB-positive neurons (Table 2; Fig. 5a). While slightly higher, counts of PHF-1 immunoreactive pre-tangles and tangles and thioflavin S-positive tangles in AD BFCN were similar to that in the aged group. Even though CB-positive neurons comprise the overwhelming majority of the BFCN in AD (Fig. 2), on average only 5.2% of CB-positive BFCN contained PHF-1-positive tangles or pre-tangles in AD brains and only 1.9% of the PHF-1-positive BFCN tangles and pre tangles were in CB-positive neurons (Table 2; Figs. 4c, e, i–k, 5c, d). Similarly, on average 2.4% of CB-positive BFCN contained thioflavin-S-positive tangles and only 2.8% of thioflavin-S-positive tangles in BFCN were in CB-positive neurons. Therefore, CB-positive BFCN appear to be relatively protected from the process of tangle formation in aging and AD.

Discussion

The results of the present set of experiments clearly demonstrate that the presence of CB within the human BFCN confers protection against degeneration and tangle formation in aging and AD, and that age-related loss of calbindin from the BFCN identifies neurons destined to degenerate in AD. While the number of BFCN displayed a significant decrease in AD brains, the numbers of CB-positive BFCN remained the same when compared with the normal aged group, resulting in increased percentage of the remaining ChAT-positive BFCN that contained CB. Furthermore, a very small percentage of the total numbers of BFCN tangles and pre-tangles were detected in CB-positive BFCN in aging and AD. CB RNA and protein showed a significant decrease not only in the aged but also in AD BFCN, indicating that the presence of CB within the AD BFCN is not due to upregulation of this protein induced by neurodegenerative damage, but rather due to preservation of CB containing neurons.

In addition to binding calcium with high affinity, CB serves as a calcium sensor and upon binding calcium, interacts with a number of other proteins [23, 32]. The presence of intense CB immunoreactivity in the primate BFCN indicates the dependence of these neurons on the

Table 2 Counts of calbindin, PHF-1 and thioflavin-S stained basal forebrain cholinergic neurons in normal old and AD brains

Case	CB	PHF-1	CB + PHF-1	CB + PHF-1/CB%	CB + PHF-1/PHF1%
Old	283	166	3	1%	1.8%
Old	81	17	0	0%	0%
Old	54	15	2	3.7%	13%
Old	407	33	5.0	1.2%	15%
				1.5 ± 0.78%	**7.5 ± 3.8%**
AD	235	58	2	0.9%	3.4%
AD	70	398	5	8%	1.4%
AD	104	388	4	3.8%	1%
AD	38	186	3	7.9%	1.6%
				5.2 ± 1.7%	**1.9 ± 0.53**

Case	CB	TS	CB + TS	CB + TS/CB%	CB + TS/TS%
Old	280	60	5	1.8%	8.3%
Old	88	3	0	0	0
Old	45	12	0.5	1%	4.1%
Old	468	4	0	0%	0%
				0.7 ± 0.43%	**3.1 ± 1.9%**
AD	193	26	2	1%	7.8%
AD	61	104	0.8	1.3%	0.07%
AD	72	193	1	1.4%	0.5%
AD	34	74	2	5.8	2.7%
				2.4 ± 1.1%	**2.8 ± 1.8%**

calcium buffering and calcium sensing functions of this protein. Of great interest, while the age-related loss of CB from the BFCN may potentially interfere with the normal functions of these neurons, it does not automatically result in their degeneration since we found the number of BFCN to remain stable in the aging process. Neurodegenerative insults seem to be required to cause degeneration of the BFCN that have lost their CB in the course of aging. In particular, it is noteworthy that the amyloid-β peptide (Aβ), which accumulates in aged and AD brains, results in significant dysregulation of intracellular calcium levels [12, 28]. Thus, the age-related loss of CB from the BFCN appears to deprive these neurons of a major mechanism of intracellular calcium buffering and signaling. This leaves the BFCN vulnerable to the calcium dysregulation that follows neurodegenerative insults, such as that caused by Aβ accumulation, and results in the selective vulnerability of these neurons to degeneration.

The human BFCN are exquisitely vulnerable to formation of pre-tangles and tangles in aging and AD [16, 29]. In this process, abnormal phosphorylation of tau leads to its aggregation and its dissociation from microtubules [5, 42]. Aggregation of abnormally phosphorylated tau in pre-tangles and tangles, microtubule disintegration and potential direct toxic effects of tau oligomers [24] result in neuronal dysfunction and degeneration. Our findings and those of others [4, 19] indicate that calcium dysregulation is permissive of this process. They also suggest that restoration of calcium homeostasis is likely to protect the BFCN from loss in neurodegenerative disorders. CB interacts with the plasma membrane calcium pump [11]. Thus, therapeutic strategies that target calcium pumps may potentially protect neurons from calcium dysregulation and degeneration.

The age-related loss of CB from the BFCN is a neurochemically specific phenomenon. The BFCN in old brains displayed preserved ChAT and $p75^{NTR}$ immunoreactivity when compared with the young. This loss also appears to be regionally specific. Substantial age-related loss of neuronal CB has not been reported elsewhere in the nervous system. Our own relatively exhaustive survey of CB-positive neurons of the human cerebral cortex revealed preservation of these neurons in 13 of 17 cortical areas investigated, and statistically significant loss of CB-positive neurons ranging between 20 and 46% in only 4 cortical areas (visual association cortex, posterior cingulate cortex, primary visual cortex and parahippocampal gyrus) [7]. Similar age-related loss of CB from the BFCN in non-human primates [43] indicates that this phenomenon is related to the normal aging process and not a consequence of pathological processes or injury.

The BFCN are not the only neuronal population vulnerable to neurodegenerative insults. While the vulnerability of the BFCN in several neurodegenerative disorders

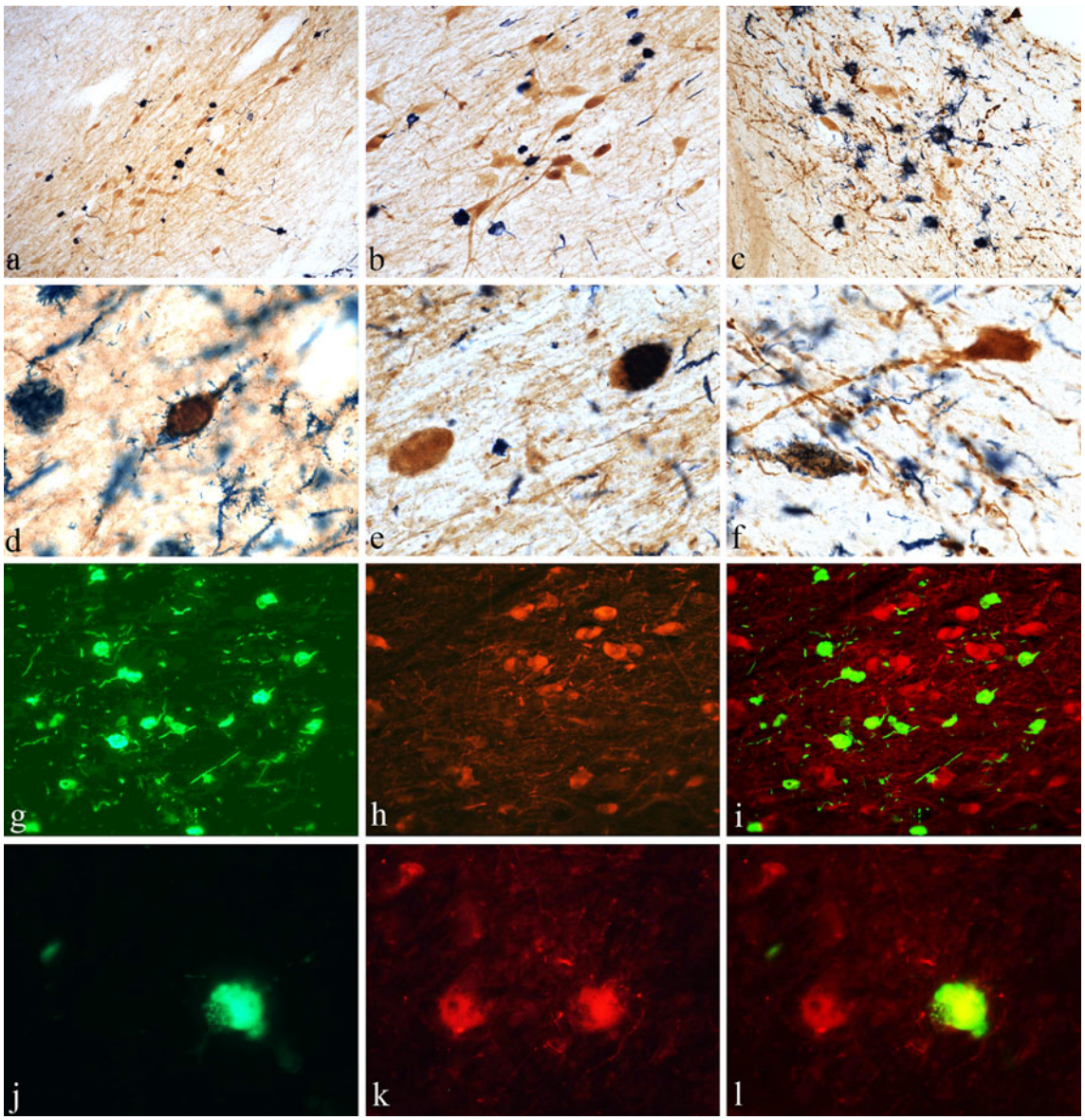

Fig. 4 Presence of CB confers protection to the BFCN against formation of pre-tangles and tangles. Low (**a**, 10×) and high power micrographs (**b**, **c**; 20×) of sections double-chromogen stained for CB (*brown*) and the PHF-1 epitope (*granular blue–black*) that identifies tau phosphorylated at Ser396/404 and is found in pre-tangles and tangles in the AD brain, clearly demonstrates nearly complete separation of BFCN that contain CB when compared with those that contain pre tangles or tangles in the normal aged (**a**, **b**) and AD brains (**c**). Only rare ChAT-positive BFCN were seen in the old (**d**, **e**) and AD (**f**) brains that also contained PHF-1 immunoreactivity. Fluorescent double staining confirmed the virtual lack of overlap between PHF-1-positive (**g**, *green*) and CB immunoreactive (**h**, *red*) BFCN in merged images (i). Again, only rarely were double stained BFCN seen containing PHF-1 (**j**, *green*) and CB (**k**, *red*) in merged images (**l**, *yellow–green*). Thus, CB-positive BFCN are virtually protected from the formation of phosphorylated tau-positive pre-tangles and tangles

of the aged [1, 2, 15, 36, 40] indicates that the pathological insults causing these disorders may all be conducive to calcium dysregulation, it remains to be seen whether this mechanism is operative in all vulnerable neurons. In AD, neurons in the entorhinal cortex are similar to the BFCN in that they are vulnerable to pathology and loss early in the course of the disease. They appear to be the first neurons within which pre-tangles and tangles are formed [6] and they are lost in very early AD [18], before signs of dementia become severe. There is evidence that the entorhinal neurons may also suffer from calcium dysregulation in AD. Loss of GluR2 subunit of AMPA receptors has been observed in neurons of layer 2 of entorhinal cortex in AD [3], prior to formation of tangles or neuronal loss. GluR2 is a calcium impermeable AMPA receptor subunit and its loss will result in preferential activation of the calcium permeable GluR1 and 4/5 subunits, thus potentially leading to increased intracellular calcium prior to tangle formation and

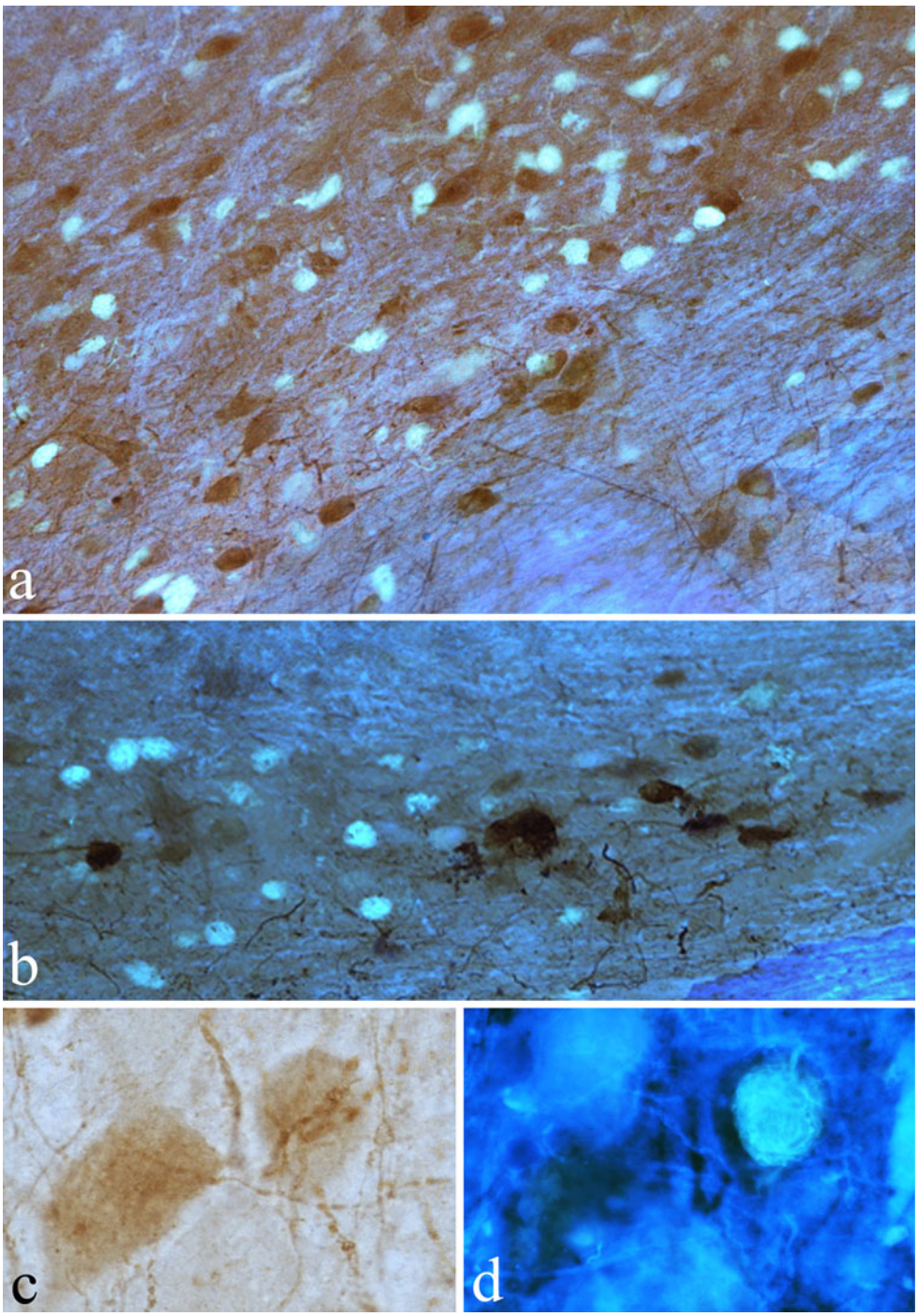

Fig. 5 Thioflavin S staining of mature tangles confirms resistance of CB immunoreactive BFCN to tangle formation. **a**, **b** Double staining for CB (*dark brown–black*) and thioflavin-S (light fluorescence) in old (**a**) and AD (**b**) brains demonstrates lack of overlap between tangle containing and CB immunoreactive BFCN. Rarely, a CB-positive BFCN (**c**, cell on the right) contained a thioflavin S-positive tangle (**d**) in double stained sections

cell death. Of interest, age-related loss of GluR2 from the BFCN has also been observed in the human brain [21]. Elucidation of further potentially shared calcium dysregulation mechanisms among various vulnerable neuronal populations in neurodegenerative disorders must await further investigations.

While age-related calcium dysregulation was hypothesized as the basis of neurodegenerative disorders over a decade ago [22], our observation of the loss of CB is the first clear calcium-related pathway identified that is associated with selective vulnerability of one neuronal population in these disorders.

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Conflict of interest The authors declare that they have no conflict of interest.

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