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Loss of Calbindin-D_{28K} is Associated with the Full Range of Tangle Pathology within Basal Forebrain Cholinergic Neurons in Alzheimer's Disease

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Abstract

Basal forebrain cholinergic neurons (BFCN) are selectively vulnerable in Alzheimer's disease (AD). We have shown that the majority of BFCN in the human brain contain the calcium binding protein calbindin-D_{28K} (CB), a large proportion lose their CB in the course of normal aging, and the BFCN which degenerate in AD lack CB. Here we investigated the relationship between CB in the BFCN and the process of tangle formation in AD using antibodies to Tau epitopes that appear early, intermediate or late in the process of tangle formation. Very small percentages (0-3.7%) of CB-positive BFCN contained pre-tangles/tangles and very small percentages (0-5%) of the total BFCN pre-tangles/tangles were in CB-immunoreactive neurons. The number of CB-positive BFCN which contained Tau immunoreactivity was highest for the early epitope and lower for intermediate epitopes. A late appearing epitope was absent from CB-positive BFCN. Age-related loss of CB appears to coincide with tangle formation in the BFCN and is associated with the full range of Tau pathology, including late appearing epitopes.

Keywords

Alzheimer's disease; Calbindin-D_{28k}; Basal Forebrain Cholinergic Neurons; Selective Neuronal Vulnerability; Tangles; Tau epitope

1. Introduction

The reasons for selective vulnerability of specific neuronal populations in neurodegenerative disorders remain poorly understood. The basal forebrain cholinergic neurons (BFCN) constitute one such population that is vulnerable to damage and loss in many

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This work is dedicated to the memory of our colleague Dr. Lester Binder.

neurodegenerative disorders that afflict the elderly, particularly Alzheimer's disease (AD) (Arendt, et al. 1985; Geula and Mesulam, 1999; Perry, et al. 1993; Schliebs and Arendt, 2011; Whitehouse, et al. 1985). The BFCN comprise a complex population of neurons, the most extensive component of which is located within the nucleus basalis of Meynert – cholinergic cell group 4 (nbM-Ch4) that is highly vulnerable to tangle formation (Geula et al., 2008; Mesulam, et al. 1983a; Mesulam, et al. 1983b). The BFCN have been implicated in the cognitive processing of selective attention and memory. Many lines of evidence converge to support cholinergic modulation of memory by these neurons (Berger-Sweeney, et al. 1994; Fine, et al. 1997; Sarter and Parikh, 2005; Stoehr, et al. 1997). In humans, the role of cortical cholinergic innervation, which originates exclusively from the BFCN, in memory derives from the observation in AD that loss of BFCN, cortical choline acetyltransferase (ChAT) activity and basal forebrain tangle density correlate with memory loss and severity of dementia (Geula and Mesulam, 1989, 1999).

We have demonstrated that BFCN and their cortically projecting axons are vulnerable to abnormalities early in the course of normal aging (Geula, et al. 2008). Alterations in cortical cholinergic axons such as thickened fibers and ballooned terminals were evident as early as midlife. Furthermore, neurofibrillary tangles (NFT) and pre-tangles which represent pathological hallmarks of AD, but are also present in the brains of normal elderly (Arriagada, et al. 1992; Price and Morris, 1999), were present in the BFCN as early as the third decade of life and showed a progressive increase in normal aging, pathologically mild AD and pathologically severe AD (Geula, et al. 2008; Mesulam, et al. 2004).

Age is the primary risk factor for neurodegenerative diseases of the elderly, indicating that normal age-related changes must figure prominently in selective neuronal vulnerability in these disorders. Our observations have revealed one such age-related alteration that is associated with the vulnerability of BFCN in AD. The majority of BFCN in the human and non-human primate brain contain the calcium (Ca^{2+}) binding protein calbindin-D_{28K} (CB) (Geula, et al. 1993), which is known to act as both a Ca^{2+} buffer and a Ca^{2+} sensor (Lutz, et al. 2003; Roberts, 1994). Of great interest, we have shown that a large proportion of BFCN lose their CB in the course of normal aging (Wu, et al. 1997), leading to the suggestion that age-related loss of the Ca^{2+}-buffering and thus protective role of this protein may contribute, at least in part, to the selective vulnerability of BFCN in neurodegenerative disorders of the elderly. Our work has further established that loss of BFCN in AD occurs primarily in the subpopulation that lack CB, and that virtually all of the remaining ChAT-positive BFCN are CB-positive (Geula, et al. 2003; Riascos, et al. 2011). Our preliminary observations indicate that CB-positivity in BFCN may be associated with protection from the process of tangle formation (Riascos, et al. 2011). Using Thioflavin-S positivity and phosphorylated tau (PHF-1) immunoreactivity, we found that, unlike CB deficient BFCN, a very small percentage of CB-positive BFCN contained tangles and pretangles in the normal elderly and AD brains.

As in other neurons, the process of tangle formation in the BFCN involves abnormalities in the microtubule associated protein Tau. During the aging process and in AD, post-translational modifications occur, which cause Tau to undergo conformational changes resulting in its dissociation from microtubules and self-association into aggregates (Chun

and Johnson, 2007). Aggregation of Tau into NFT in AD requires abnormal phosphorylation (PH) (Ballatore, et al. 2007). In addition, Tau undergoes multiple truncations as it transforms from an unfolded monomer to the structured polymer characteristic of NFT (Guillozet-Bongaarts, et al. 2005).

Recent advances in our understanding of tangle formation have revealed phosphorylated and cleaved Tau epitopes that appear early and thus make an initial contribution to tangle formation (Augustinack, et al. 2002; Guillozet-Bongaarts, et al. 2005; Lauckner, et al. 2003; Luna-Munoz, et al. 2005). Thus, early (Alz 50 – phosphorylated (PH)-Ser422, AT8 – PH-Ser202 / Thr205 and Tau oligomer complex 1 [TOC-1]) (Braak, et al. 1994; Patterson, et al. 2011), intermediate (PHF-1 – PH-Ser396/404, PH-Thr181 and caspase-3-cleaved Tau [Tau-C3]) (Augustinack, et al. 2002; Guillozet-Bongaarts, et al. 2005; Lauckner, et al. 2003) and late appearing Tau epitopes (truncated Tau [MN423], PH-Ser 262) (Guillozet-Bongaarts, et al. 2005; Lauckner, et al. 2003) have been identified in the course of tangle formation. In this study antibodies to Tau epitopes that appear at different stages of tangle formation were utilized to test the hypothesis that the presence of CB is associated with reduced phosphorylated and truncated Tau generation and pre-tangle / tangle formation in BFCN. We report that the presence of CB in BFCN is associated primarily with the epitopes of Tau seen in early stages of NFT formation, and epitopes that appear late in this process are completely absent from CB-positive BFCN.

2. Materials and Methods

2.1 Case Information and Tissue Processing

Basal forebrain tissue from 5 cases with clinical manifestation of Alzheimer type dementia and confirmed AD pathology were used in the present experiments (Table 1). Assuming a variation around the means of 20% or less, 5 brains allows detection of a difference of 22.5% or greater between the means with a type I error (probability) of 0.05 and 80% confidence level. Mean age of subjects at death was 76.6 ± 6.2 years and mean postmortem interval was 11.6 ± 3.4 hours. The Consortium to Establish a Registry for Alzheimer's Disease (CERAD) (Morris, et al. 1989) and the National Institute on Aging – Alzheimer's Association (McKhann et al., 2011) criteria were used for clinical diagnosis of AD. Neuropathological diagnoses were rendered according to the National Institute on Aging (NIA)-Reagan and National Institute on Aging-Alzheimer's Association (NIA-AA) criteria (Hyman, et al. 2012; Montine, et al. 2012; Newell, et al. 1999).

Immediately following autopsy, each brain was cut into 2–3 cm coronal slabs and placed in 4% paraformaldehyde (in 0.1 M phosphate buffer, pH 7.4) for 30–36 hours at 4°C and then taken through sucrose gradients for cryoprotection (10–40% in phosphate buffer). Slabs were sectioned at a thickness of 40 μm on a freezing microtome and stored in 0.1 M phosphate buffer containing 0.02% sodium azide at 4° C.

2.2 Immunohistochemistry and Thioflavin-S Stain

One in 24 series of sections spanning the entire nbM-Ch4, the major component of BFCN with projections to nearly all of the cortical mantle (Mesulam, et al. 1983a; Mesulam, et al.

1983b), were processed immunohistochemically using the avidin-biotin-peroxidase (ABC) method as described elsewhere (Geula, et al. 2003), utilizing the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA). The antibodies used (Table 2) were rabbit polyclonal antibody against CB (Swant, Switzerland, 1/2000); Tau oligomer complex-1 (TOC-1, gift of Dr. Lester Binder Northwestern University, 1/5000), a mouse monoclonal antibody that selectively recognizes Tau dimers and oligomers which appear early in the process of Tau aggregation, most likely in pre-tangles, but not fibrillar Tau that is a characteristic of NFT (Patterson, et al. 2011); AT8 (Innogenetics, Belgium, 1/500), mouse monoclonal antibody against early phosphorylated Tau at Ser202/Thr205; PHF-1 (generous gift of Dr. Peter Davies, Albert Einstein College of Medicine, NY, 1/1000), mouse monoclonal antibody that recognizes an intermediate epitope of Tau phosphorylated at Ser396/404; and MN423 (gift of Dr. Lester Binder, Northwestern University, 1/5000), mouse monoclonal antibody that recognizes a late stage truncated Tau epitope (Guillozet-Bongaarts, et al. 2005). For concurrent visualization of two antigens within the same section, the double immunohistochemical method of Levy et al. was used (Levey, et al. 1986). Tissue sections were first processed for one antigen using diaminobenzidine (DAB) 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. To control for non-specific staining, sections stained using the above antibodies were compared with sections stained in the absence of primary antibodies.

To visualize mature NFT, a series of sections was stained with Thioflavin-S, a fluorescent molecule that binds to the β-pleated sheet structure of tangles. Briefly, 40 μm thick sections were mounted on slides, dried at room temperature, defatted in chloroform and 100% ethanol, rehydrated in a decreasing gradient of ethanol to water, incubated in a 0.1% solution of Thioflavin S, differentiated in 80% ethanol and coverslipped with an aqueous medium.

2.3 Unbiased Stereological Analysis of Stained Profiles

Unbiased stereological analysis was carried out for quantitation of stained profiles using the StereoInvestigator software (MicroBrightField). The human nbM-Ch4 extends antero-posteriorly from the level just anterior to the crossing of the anterior commissure to the central nucleus of the amygdala (Mesulam and Geula, 1988). We analyzed 1 in 24 series of sections from each case spanning the entire nbM-Ch4 region for each stain. Counts were obtained of the magnocellular nbM-Ch4 containing tangles / pre-tangles immunoreactive for each Tau epitope, CB immunoreactivity, co-localization of CB with Tau pathology, or Thioflavin-s staining in each case. BFCN tangles were identified by their globose morphology and large size due to the magnocellular nature of nbM-Ch4 neurons. nbM-Ch4 tangles and pre-tangles were distinguished from neuropil threads, by the latter's thread like and thin appearance, and from plaque neurites, by the latter's small size and thin diameter and plaque-like, circular arrangement. The top and bottom 5 μm of each section were set as guard height. The region of interest was traced at 10X magnification, and counting was carried out at 40X. In all cases, enough sections with systematic random sampling of basal forebrain were available satisfying the requirement for unbiased stereological estimation of immunoreactive profiles. Stereological parameters such as dissector spacing, dissector

height and counting frame size were determined through trials to result in coefficients of error equal to or less than 0.1. Total estimated profiles were calculated based on the volume of the basal forebrain in sections used for analysis.

2.4 Statistical Analysis

Statistical analysis was carried out using the Instat software (version 3.05). Data were found to be normally distributed, therefore, mixed model analysis of variance (ANOVA) followed by appropriate *post hoc* comparisons were used to detect significant group differences. Four separate analyses were carried out: A) comparison of total numbers of nbM-Ch4 tangles / pretangles stained for each Tau epitope; B) comparison of CB co-localization with each Tau epitope in nbM-Ch4; C) percentage of CB co-localization with each Tau epitope as a function of the total CB immunoreactive nbM-Ch4; and D) percentage of CB co-localization with each Tau epitope as a function of total Tau stained nbM-Ch4. The probability value for significant effects was set at $p<0.05$.

3. Results

3.1 Tangles and Pre-Tangles Containing Early, Intermediate and Late Appearing Tau Epitopes are Abundant in Basal Forebrain Cholinergic Neurons

The antibodies to the four Tau epitopes used in these experiments visualized pre-tangles, tangles and neuropil threads in the basal forebrain (Fig. 1). The TOC-1 antibody, which visualizes the early oligomeric states of PH-Tau, stained primarily pre-tangles in the BFCN, with cytoplasmic staining that also filled the dendrites, but was also present in tangles. The AT8 and PHF-1 antibodies, which recognize epitopes of Tau that are intermediate in the process of tangle formation, tended to stain both pre-tangles and tangles, and the MN423 epitope was present only in tangles. In addition to tangles and pre-tangles, phosphorylated and truncated Tau are also present in neuropil threads and plaque neurites, considered abnormal processes of neurons, particularly dendrites, that are found in the neuropil (Braak, et al. 1994; Markesbery, et al. 1993). All antibodies used also stained neuropil threads scattered among the BFCN. However, the basal forebrain contained fewer and thicker neuropil threads when compared with cortical regions in the same sections. The apparent density of basal forebrain neuropil threads stained with each antibody varied among different cases. Neuritic plaques were abundant in adjacent neocortical regions within the same sections, but were virtually absent from the basal forebrain region in which the BFCN are located (Fig. 1).

Quantitative analysis pointed to significant differences among the numbers of nbM-Ch4 tangles that were Thio-S-positive and the numbers of tangles / pre-tangles that contained each Tau epitope (Table 3 & 4, Fig. 2). The largest total numbers of nbM-Ch4 tangles / pre-tangles were Thio-S-positive, followed sequentially by PHF-1, MN423 and TOC-1 immunoreactive pretangles / tangles. The smallest numbers of nbM-Ch4 tangles / pre-tangles were AT8-positive. The total number of Thio-S-positive nbM-Ch4 tangles was significantly higher than the number of tangles / pre-tangles which displayed TOC-1 or AT-8 immunoreactivity ($p<0.05$; Table 4, Fig. 2).

3.2 Calbindin-Positive Cholinergic Neurons are Associated with Protection Against Tangle Formation

CB positive BFCN exhibited immunoreactivity that filled the neuronal cell body and the processes (Table 3, Fig. 1). Very small percentages of CB -positive BFCN were immunoreactive for each Tau epitope (0 to 3.7%, Table 4). Similarly, very small percentages of BFCN tangles and pre-tangles positive for each Tau epitope were found in CB-positive subpopulation (0-5.1%, Table 4). Thus, the overwhelming majority of tangles and pre-tangles (95-100%) were found in BFCN that lacked CB immunoreactivity, indicating an association between presence of calbindin and protection against the process of tangle formation.

3.3 Presence of Calbindin Retards the Process of Tangle Formation within Basal Forebrain Cholinergic Neurons

To determine the progression of the process of tangle formation within the CB-positive BFCN, neurons that showed co-localization of CB with the early, intermediate and late appearing Tau epitopes (Fig. 1 and 3) were counted. A substantially greater number of CB-positive BFCN displayed co-localization with the potentially earliest appearing TOC-1 epitope of Tau when compared with later appearing AT8 and PHF-1 epitopes (Fig. 4). The late appearing MN423 epitope was never co-localized with CB (Fig. 1, 3 and 4). This difference was statistically significant when CB-positive neurons that contained TOC-1 immunoreactivity where compared with those that contained the MN423 epitope ($p<0.05$) (Fig. 4).

We also compared the number of BFCN double-stained with CB and each Tau epitope as a percentage of total BFCN stained with CB or each Tau epitope (Table 4). The percentage of CB-positive BFCN which contained TOC-1 immunoreactivity, expected to be present at the earliest stages of Tau oligomer formation, was significantly larger than the percentage of CB-positive BFCN which contained the AT8 epitope which appears relatively early ($p<0.001$), PHF-1 epitope which appears intermediate ($p<0.01$), and the MN423 epitope which appears late in the process of tangle formation ($p<0.001$). The percentage of total TOC-1 immunoreactive BFCN which were CB-positive was also significantly higher when compared with the percentage of AT8, PHF-1 or MN423 immunoreactive BFCN which were CB-positive ($p<0.001$). Similarly, the percentage of AT8 immunoreactive BFCN which contained CB was significantly higher when compared with that of MN423 ($p<0.05$). However, this percentage displayed no significant differences when the AT8 epitope was compared with PHF-1 or when the PHF-1 epitope was compared with MN423 ($p>0.05$, Table).

4. Discussion

We had shown a selective loss of CB from the BFCN in the course of normal aging in the human and non-human primate brain (Wu, et al. 1997; Wu, et al. 2003). More recent observations from our laboratory indicate that the BFCN that degenerate in AD are those that lose their CB in the course of aging (Geula, et al. 2003; Riascos, et al. 2011), suggesting a potential protective role for CB in the BFCN against age-related neurodegenerative

processes. Consistent with our preliminary observations (Riascos, et al. 2011), the results of the present study, using immunoreactivity for a number of abnormal epitopes of Tau, demonstrate that the presence of CB in the BFCN is associated with protection of these neurons against the process of tangle formation. The overwhelming majority of pre-tangles and tangles within the BFCN (95-100%) were found in neurons that were devoid of CB or were extracellular tangles.

While based on a relatively small sample, the present results suggest that the protection associated with the presence of CB, may be mediated by presence of only early, and absence of late epitopes of tau in the process of tangle formation. A higher number of the relatively low density of pre-tangles / tangles found in the CB-positive BFCN contained primarily the early appearing epitopes of Tau, particularly the TOC-1 epitope, and the epitopes that appear later in this process were found in substantially fewer CB-positive BFCN. Importantly, the MN423 epitope, known to appear very late in the process of tangle formation, was completely absent from CB-positive BFCN, suggesting that tangle formation is either halted or is present only in the earliest stages as evidenced by absence of late tau epitopes. Consistent with this suggestion, a recent report indicates that the progression of tangle formation within the BFCN population as a whole is slower than that in cortical areas. Using antibodies to PH-Ser422, which appears early in the process of tangle formation, and caspase-3 cleaved Tau (Tau-C3), which appears later, the authors found a greater proportion of PH-Ser422 to Tau-C3 immunoreactive tangles in the basal forebrain when compared with cerebral cortex, indicative of slower progression of tangle formation in BFCN (Vana, et al. 2011). This finding is consistent with the results of the present study, and raise the possibility that the slow progression of Tau pathology observed may be mediated, at least in part, by the presence of CB in a subpopulation of BFCN.

Of great interest, the presence of each tau epitope in CB-positive BFCN is independent of the presence of that epitope in the total BFCN population. Thus, the TOC-1 epitope, which was present in a relatively low number of BFCN, was present in a greater number of CB-positive BFCN, when compared with PHF-1, which was present in a substantially higher number of BFCN. The extent to which various tau epitopes and Thioflavin-S stain co-localize within the same neuron or tangle is not clear. Within the BFCN, it is very likely that early and intermediate epitopes might display a certain degree of overlap, as would intermediate and late epitopes. Thioflavin-S stains all mature tangles. Given that all tau epitopes were present in at least some tangles, overlap between Thioflavin-S and tau epitopes is very likely, particularly the MN-423 late appearing epitope of tau.

Calcium acts as an important signal for a variety of neuronal processes including neurotransmitter release (Katz, 1996), neuronal excitability (Marty, 1989), synaptic plasticity (Malenka, et al. 1989) and gene expression (Szekely, et al. 1990). Importantly, unregulated elevation of intracellular Ca^{+2} is toxic to cells and can cause programmed cell death (Chalfie and Wolinsky, 1990) or degeneration through other cellular pathologies (D. W. Choi, 1990; Meldrum and Garthwaite, 1990).

Calbindin binds Ca^{2+} with high affinity (Miller, 1991) and CB-positive neurons are able to regulate intracellular Ca^{2+} and to reduce excessive Ca^{2+} levels (Mattson, et al. 1991).

Presence of CB confers protection to neurons against modes of damage involving elevated intracellular Ca^{2+} (Mattson, et al. 1991; Rintoul, et al. 2001).

The calcium binding proteins CB, parvalbumin and calretinin are present in GABAergic cortical interneurons (Celio, 1990), and these inhibitory cortical interneurons are virtually tangle free in normal aged and AD brains (Hof and Morrison, 1991), reinforcing a potential neuroprotective role of CB and other calcium binging proteins. Some studies have reported presence of weak CB immunoreactivity in cortical pyramidal neurons (Hof and Morrison, 1991). Cortical pyramidal neurons are selectively vulnerable to tangle formation, and one study has shown that the loss of such neurons is correlated with the density of cortical tangles in AD (Hof and Morrison, 1991). However, it is important to note that other studies, including our own studies in the human brain, do not find appreciable immunoreactivity for CB or other calcium binding proteins in cortical pyramidal neurons (Bu, et al. 2003). Therefore, at this juncture, no definitive statements can be made regarding the relationship between CB and tangle formation in these neurons. If these contradictory results are resolved and cortical pyramidal neurons are found to be CB-positive, double immunohistochemical procedures will be needed to determine whether tangles are formed in CB-positive cortical pyramidal neurons.

The possibility must be considered that CB may be merely a protein sensitive to tau pathology, and is lost in the BFCN as tau pathology progresses. However, it should be noted that, at the time loss of CB is observed during the normal aging process, only a small subpopulation of cholinergic neurons contain tangles or pre-tangles, while nearly 70% of these neurons have lost their CB (Geula, et al. 2003). Thus, CB loss occurs prior to pre-tangle and tangle formation, and therefore it is unlikely to be caused by tau pathology. Furthermore, recent observations from our laboratory indicate that age-related loss of CB from the BFCN may take place in the greater context of dysregulation of other Ca^{2+}-responsive proteins within these neurons (Riascos, et al. 2014). For example, we have reported that RNA in laser captured BFCN and protein measured in basal forebrain homogenates display significant age- and AD-related reductions of not only CB, but also the Ca^{2+}-responsive proteins Ca^{2+}/calmodulin-dependent protein kinase I (CaMKI) and the growth-associated protein-43 (GAP43) (Riascos, et al. 2014). These findings strengthen the possibility that loss of CB and other Ca2+-responsive proteins are reflective of overall Ca2+ dysregulation in BFCN, which may contribute to tangle formation.

There are at least two potential mechanisms through which the age-related loss of CB and putative dysregulation of intracellular Ca^{2+} within the BFCN may influence the process of tangle formation. The first, involves cyclin-dependent kinase 5 (CDK5). Tau is abnormally phosphorylated by a number of protein kinases (Drewes, et al. 1992; Ekinci and Shea, 1999; Wang, et al. 1998), including CDK5 (Baumann, et al. 1993; Hosoi, et al. 1995; Kobayashi, et al. 1993). CDK5 is the catalytic kinase component, the other component being the regulatory subunit p25, derived from p35 through proteolytic activity of the Ca^{2+} activated protease calpain (Hisanaga, et al. 1993). *In vitro* and *in vivo* evidence indicates that calpain activation results in cleavage of p35 to p25 and subsequent prolonged activation of CDK5 and phosphorylation of Tau (Hashiguchi, et al. 2002; Hung, et al. 2005; Lee, et al. 2000). Activation of calpain-1 (μ calpain [activated by μM Ca^{2+}]) in AD animal models has been

implicated in direct cleavage of Tau into a 17KDa fragment with toxic effects on neurons (Hashiguchi, et al. 2002; Hung, et al. 2005; Lee, et al. 2000; Roberts, 1994). Thus, one neuronal alteration triggered by increased intracellular Ca^{2+} that is of immediate relevance to the process of tangle formation is increased activation of the protease calpain (Liu, et al. 2008). Some studies indicate that calpain levels and activity may be increased in AD brains (Saito, et al. 1993; Tsuji, et al. 1998). Our earlier findings revealed significantly elevated levels of activated calpain in the basal forebrain region within which BFCN are located, in the process of normal aging and a 10-fold increase in AD (Riascos, et al. 2014). Importantly, calpain degrades CaMKI and GAP43 (Nairn, et al. 1985; Zakharov and Mosevitsky, 2007), which as mentioned above, are decreased in homogenates of basal forebrain in AD (Riascos, et al. 2014).

A second potential mechanism set in motion by loss of CB that is relevant to the process of tangle formation pertains to caspase-3 (Cas-3). CB contains a structural motif for binding procaspase-3 (Kojetin, et al. 2006), and inhibits Cas-3 activation both through this binding activity and by buffering intracellular Ca^{2+} (Bellido, et al. 2000; W. S. Choi, et al. 2008). Caspases, particularly Cas-3, cleave Tau (Kang, et al. 2005). Cas-3 cleavage of Tau significantly increases its polymerization, a process that accelerates oligomerization and tangle formation (Rissman, et al. 2004). Loss of the inhibitory function of CB is likely to promote Cas-3 activation, Tau cleavage by Cas-3 and tangle formation within the BFCN.

In addition to loss of CB, one other age-related mechanism has been identified that is likely to contribute to further dysregulation of Ca^{2+} in the BFCN. An age-related loss of the Ca^{2+} impermeable AMPA glutamate receptor subunit GluR2 has been reported in human BFCN (Ikonomovic, et al. 2000). This loss is likely to result in increased intraneuronal Ca^{2+} through unopposed activation of Ca^{2+} permeable GluR1 and 4/5 AMPA receptors in BFCN. Similar loss of GluR2 AMPA receptors has been reported in the entorhinal cortex and hippocampus prior to NFT formation (Ikonomovic, et al. 1997). These findings reinforce the potential contribution of intracellular Ca^{2+} dysregulation to tangle formation not only within the BFCN, but also in other neuronal populations vulnerable to early Tau pathology and tangle formation.

Ubiquitous presence of CB within BFCN indicates high levels of intracellular Ca^{2+} trafficking in these neurons, perhaps because of their extensive synaptic connections throughout the cortex, necessitating the presence of a strong calcium buffer to sequester excess Ca^{2+} intracellularly. While our findings suggest the contribution of age-related loss of CB to tangle formation and degeneration in the BFCN, other tangle-prone neurons in AD, such as those in the entorhinal cortex and hippocampus, do not contain CB. The age-related loss of Ca^{2+} impermeable AMPA receptors from these neurons reviewed above suggests that the resultant increased Ca^{2+} concentrations may also contribute to tangle formation in the entorhinal cortex, hippocampus and perhaps other tangle-prone neurons. Thus it is possible that Ca^{2+} dysregulation, brought about by different age-related mechanisms depending on neuronal type and population, is a primary cause of tangle formation in AD.

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Highlights

- Human basal forebrain cholinergic neurons display age-related loss of calbindin.
- Calbindin-positive cholinergic neurons are resistant to tangle formation.
- Calbindin is associated only with early, but not late tau epitopes in cholinergic neurons.

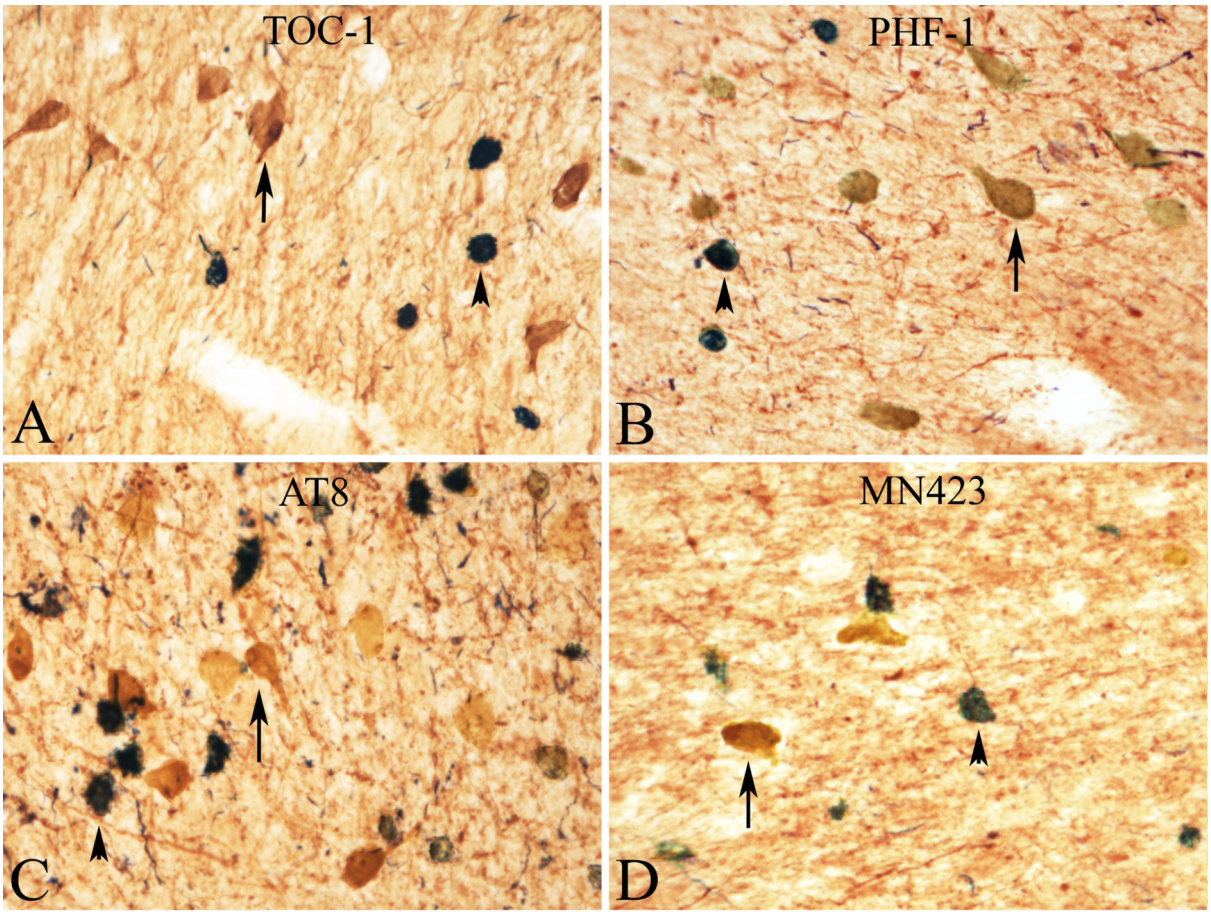

Figure 1. Calbindin staining and immunoreactivity for various Tau epitopes show little overlap in basal forebrain cholinergic neurons (BFCN)

Double immunohistochemistry demonstrates the presence of calbindin (brown, DAB) and Tau epitopes (granular blue, BDHC) (A) TOC-1, which appears early in Tau oligomers, (B) AT8 and (C) PHF-1, which are intermediate in the process of tangle formation, and (D) MN423, which appears late in this process, in BFCN. Immunoreactivity for all four Tau epitopes was observed within BFCN in all the AD cases investigated. Immunoreactivity for calbindin and Tau epitopes was virtually non-overlapping, with minor co-localization of calbindin with TOC-1, AT8 or PHF-1 (see Fig 3 and 4). CB was never co-localized with the late appearing Tau epitope MN423 in BFCN. Note that while neuropil threads stained for each tau epitope are present among the BFCN (elongated blue thread-like structures), no neuritic plaques are seen. Magnification 10X. Arrows point to single CB immunoreactive neurons and arrowheads to neurons singly stained for each Tau epitope.

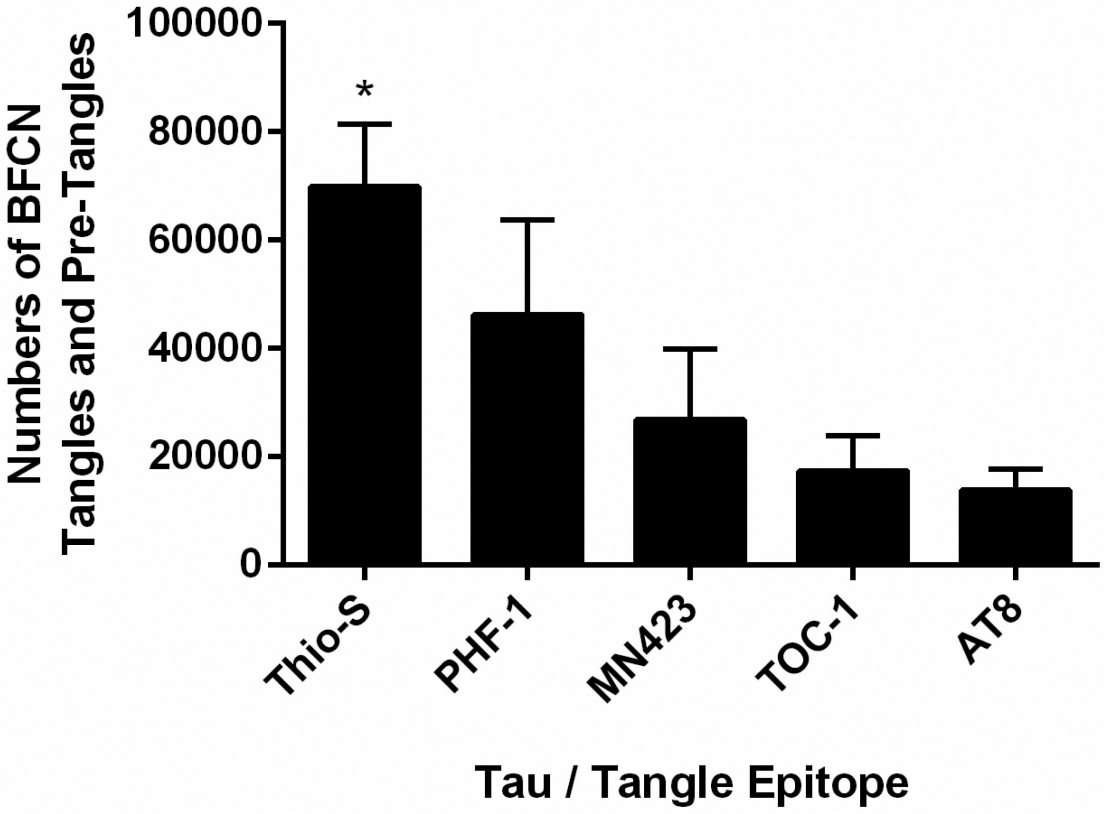

Figure 2. Stereological quantitation of the number of tangles / pre-tangles in the basal forebrain cholinergic neurons containing various Tau epitopes

Unbiased stereological counting methods demonstrated that Thioflavin-S (Thio-S), which binds to β-pleated sheet conformation in mature tangles, is present in the largest number of tangles / pre-tangles in BFCN, followed sequentially by Tau epitopes PHF-1, MN423, TOC-1 and AT8. The number of Thio-S-positive BFCN tangles was significantly larger than the numbers of BFCN containing TOC-1 or AT8 immunoreactive tangles / pre-tangles (*$p<0.05$). Bars represent means and standard errors.

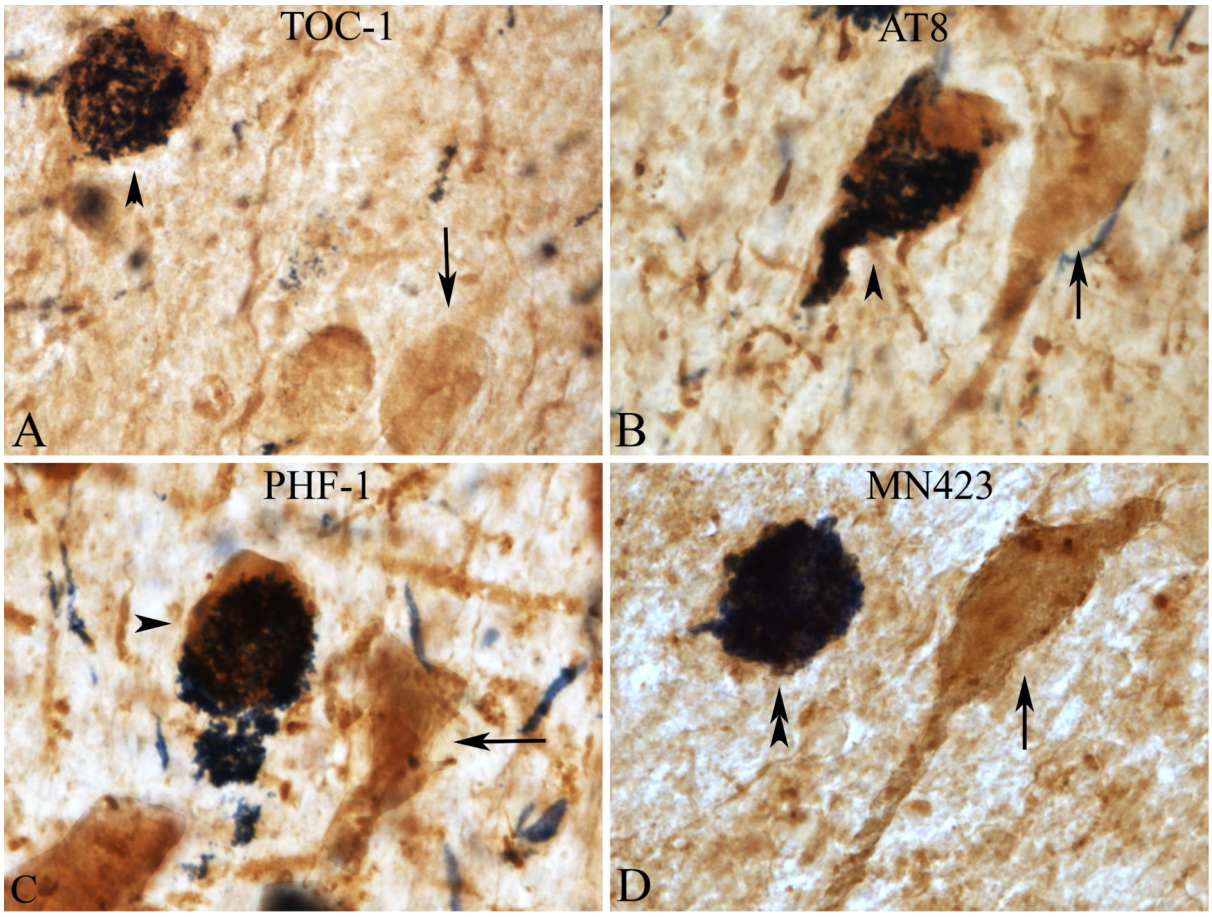

Figure 3. Abnormal Tau epitopes are rarely co-localized with calbindin immunoreactivity in basal forebrain cholinergic neurons (BFCN)

High power photomicrographs, showing examples of BFCN containing calbindin immunoreactivity only (brown, arrows), calbindin immunoreactivity and each Tau epitope (Tau epitopes in granular blue, arrowheads) or Tau epitope immunoreactivity only (granular blue, double arrowhead). BFCN were infrequently double-stained for calbindin and the early Tau epitope TOC-1 (A), or the intermediate Tau epitopes AT8 (B) and PHF-1 (C). Calbindin immunoreactivity was never co-localized with the late appearing Tau epitope MN423 in BFCN (D). Magnification 40X.

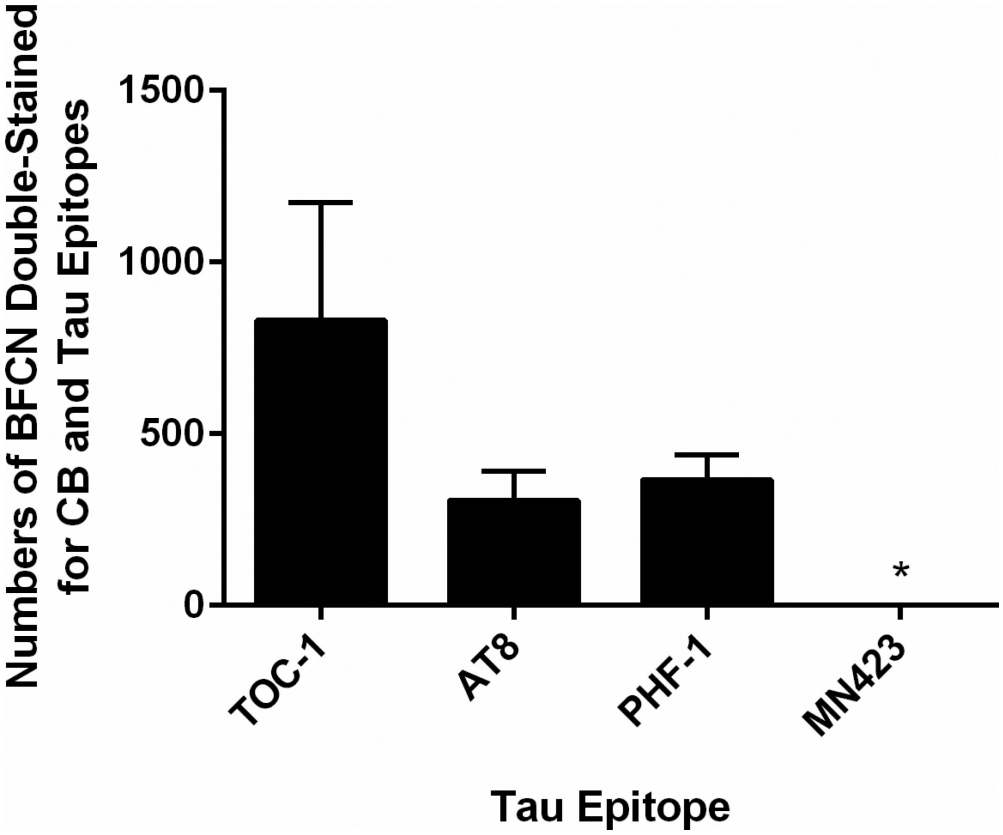

Figure 4. Presence of calbindin is associated primarily with early epitopes of tau in basal forebrain cholinergic neurons (BFCN)

While co-localization of calbindin with various Tau epitopes was infrequent when compared with total number of BFCN that contained calbindin or each Tau epitope, unbiased stereological counting methods demonstrated that the largest numbers of double stained calbindin immunoreactive BFCN contained the early appearing Tau epitope TOC-1, followed by the intermediate appearing epitopes AT8 and PHF-1. The late appearing Tau epitope MN423 was never co-localized with calbindin in BFCN. Thus calbindin-positive BFCN contain only tangles / pre-tangles that are in the initial stages of formation, and late stage tangles / pre-tangles are completely absent from these neurons. The number of BFCN double stained for calbindin and TOC-1 was significantly larger than the number containing calbindin and MN423 (* $p < 0.05$). Bars represent means with standard errors.

Table 1

Characteristics of Cases

Case #	Age (years)	Gender	PMI (Hours)	Braak Stage	MMSE	CDR
1	93	M	10	V	24 (7 Y BD)	1 (7 Y BD)
2	83	F	24	VI	11 (6 M BD)	2 (6 M BD)
3	80	F	4	V	11 (6 Y BD)	3 (6 M BD)
4	71	M	12	VI	1 (7 Y BD)	3 (1 Y BD)
5	56	M	8	VI	21 (6 Y BD)	3 (1 Y BD)

PMI – Postmortem Interval; Braak Stage – Stages of Neurofibrillary Tangle Formation; MMSE – Mini-Mental State Examination (highest possible score = 30); CDR – Clinical Dementia Scale (highest possible score = 3); Y – Year(s); M – Months; BD – before death

Table 2

Antibodies Used

Antibody	Epitope Recognized	Dilution
Calbindin, Rabbit Polyclonal	Calbindin-D_{28K}	1/2000
Tau Oligomer Complex-1 (TOC1), Mouse Monoclonal	Non-Fibrillar Tau Dimers and Oligomers	1/5000
AT8, Mouse Monoclonal	Tau Phosphorylated at Ser202/Thr205	1/500
PHF-1, Mouse Monoclonal	Tau Phosphorylated at Ser396/404	1/1000
MN423, Mouse Monoclonal	Truncated Tau	1/5000

Table 3

Total Counts of Basal Forebrain Cholinergic Neurons (nbM-Ch4) Immunoreactive for Calbindin and Tau Epitopes in Each Case

Case	Calbindin†	TOC1	AT8	PHF1	MN423
1	25,060	14,644	25,148	116,126	9,258
2	23,304	23,740	18,297	29,210	256
3	33,145	37,325	5,179	27,506	8,579
4	6,631	4,563	5,401	28,240	53,635
5	9,626	2,093	15,375	30,392	62,715

†Calbindin counts for each case represents the average of counts obtained from sections double-stained for calbindin and each tau epitope.

Table 4

Numbers and Percentages of BFCN Single or Double Stained for Calbindin and Each Tau Epitope

Tau Epitope	Total CB count	Total Tau Epitope count	Total double count	percentage of double to total CB	percentage of double to total
TOC-1	22554	1643	831*	3.68+	5.04^
AT8	17541	13880	307	1.75	2.21†
PHF-1	20048	46295	367	1.82	0.79
MN423	18069	26889	0	0	0

*Number of CB/TOC-1 double stained BFCN is significantly higher than CB/MN423 double stained BFCN ($p<0.05$).

+Percentage of CB-positive neurons which contain TOC-1 immunoreactivity is higher than the percentage that contain AT8 ($p<0.001$), PHF-1 ($p<0.01$) or MN423 ($p<0.001$) immunoreactivity.

^The percentage of total TOC-1 immunoreactive BFCN that contain CB is higher than the percentage of AT8, PHF-1 or MN423 immunoreactive neurons that contain CB ($p<0.001$).

†Percentage of AT8 immunoreactive BFCN that contain CB is significantly higher than the percentage of MN423 immunoreactive BFCN containing CB ($p<0.05$).