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Alterations of Ca²⁺ responsive proteins within cholinergic neurons in aging and AD

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Abstract

The molecular basis of selective neuronal vulnerability in Alzheimer's disease (AD) remains poorly understood. Using basal forebrain cholinergic neurons (BFCN) as a model and immunohistochemistry, we have demonstrated significant age-related loss of the calcium binding protein calbindin-D_{28K} (CB) from BFCN, which was associated with tangle formation and degeneration in AD. Here we determined alterations in RNA and protein for CB and other Ca²⁺ responsive proteins Ca²⁺/calmodulin-dependent protein kinase I (CaMKI), growth-associated protein-43 (GAP43), calpain in the basal forebrain. We observed progressive downregulation of CB and CaMKI RNA in laser-captured BFCN in the normal-aged-AD continuum. We also detected progressive loss of CB, CaMKI-Delta, and GAP43 proteins in BF homogenates in aging and AD. Activated μ -calpain, a calcium-sensitive protease that degrades CaMKI and GAP-43, was significantly increased in the normal aged BF and was 10-times higher in AD BF. Overactivation of μ -calpain was confirmed using proteolytic fragments of its substrate spectrin. Substantial age and AD related alterations in Ca²⁺-sensing proteins most likely contribute to selective vulnerability of BFCN to degeneration in AD.

Keywords

Calbindin-D_{28K}; GAP43; CaMKI; calpain; basal forebrain; proteolysis; cholinergic system

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Conflict of Interest

The authors have no conflicts of interest to declare.

1. Introduction

The molecular basis of selective neuronal vulnerability in neurodegenerative disorders remains poorly understood. In a majority of neurodegenerative disorders, age is the major risk factor, suggesting that age-related changes figure prominently in selective neuronal vulnerability. One neuronal population which is selectively vulnerable to degeneration in most neurodegenerative disorders of the elderly, particularly in Alzheimer's disease (AD), is the cholinergic neurons of the basal forebrain (BFCN).

In AD, the BFCN and their cortically projecting axons are among the earliest degenerating neural elements (Geula and Mesulam, 1999). Recent reports have shown that the BFCN are vulnerable to phosphorylated tau accumulation and tangle formation early in the course of mild cognitive impairment (MCI) and AD (Mesulam, et al., 2004). Our observations indicate that phosphorylated tau accumulation, tangle formation and axonal abnormalities characterize the BFCN early in the course of aging, decades before the clinical manifestation of cognitive abnormalities (Geula, et al., 2008). The contribution of the BFCN to cognitive deficits in AD is underscored by a strong correlation between the loss of these neurons and the severity of dementia (Lehericy, et al., 1993) and the fact that the cholinergic system is among a handful of targets for available therapy in AD (Bullock, et al., 2006). Because of its contribution to cognitive deficits in neurodegenerative disorders, understanding the reasons for the selective vulnerability of the BFCN is of interest both scientifically and therapeutically.

Our work has uncovered an age-related change in the BFCN that is a likely contributor to the selective vulnerability of these neurons. Calbindin-D_{28K} (CB), a calcium-binding protein, is present in the majority of BFCN in the human and non-human primate brain but not in the rodent (Chang and Kuo, 1991, Geula, et al., 1993). We have observed a selective, significant and substantial loss of CB from the BFCN in the course of normal aging in both the human (Geula, et al., 2003) and the non-human primate (Wu, et al., 2003). This loss was selective since no change in CB-positive neurons was observed in 13 out of 17 cortical areas studied, and a small loss was observed in the remaining cortical area (ranging between 26–37%) (Bu, et al., 2003). Significantly we have shown that in AD loss of BFCN occurs nearly exclusively in neurons that have lost their CB and that CB-positive BFCN in aging and AD are protected against the process of tangle formation (Riascos, et al., 2011).

As a member of the family of calcium binding proteins, CB binds calcium (Ca²⁺) with high affinity and regulates its intracellular levels (Iacopino, et al., 1994). The presence of CB has been shown to protect neurons from modes of degeneration involving uncontrolled elevations of intracellular Ca²⁺ (D'Orlando, et al., 2001, Scharfman and Schwartzkroin, 1989). Based on these observations, we have hypothesized that the age-related loss of CB deprives the BFCN of one of the mechanisms to buffer intracellular Ca²⁺, thus leading to increased intracellular levels of this element following pathologic insults, resulting in loss of these neurons.

It is possible that the age-related loss of CB and the resultant increase in intracellular Ca²⁺ triggers activation of other Ca²⁺-related proteins and pathways. It is also possible that loss of CB in BFCN occurs in the context of a more general dysregulation of Ca²⁺. There is experimental evidence that BFCN may suffer Ca²⁺ dysregulation in normal aging in animal models (Murchison and Griffith, 2007). Indirect evidence of increased intracellular levels of Ca²⁺ in the human BFCN comes from the study of the AMPA glutamate receptors (AGR) in these cells. An age-related shift from the Ca²⁺ impermeable AGR subunit GluR2 to the Ca²⁺ permeable GluR1 and 4/5 subunits in BFCN has been reported and shown to be related to vulnerability to excitotoxicity (Ikonomovic, et al., 2000).

Intracellular free Ca^{2+} is a critical modulator of signaling pathways that regulate neuronal development, plasticity, and pathologies. Thus, we further investigated alterations in proteins that demonstrate Ca^{2+} -dependent activity within the BFCN as a first step in determination of potential causes and / or consequences of the age-related loss of CB and dysregulation of Ca^{2+} . A preliminary microarray gene profiling analysis in BFCN from one AD and one non-demented case using laser capture microdissection indicated reduced expression of genes for Ca^{2+} / calmodulin dependent protein kinase I (CaMKI) and the growth-associated protein-43 (GAP43) (data not shown), in addition to the gene for CB (Riascos, et al., 2011).

CaMKI belongs to the family of multifunctional Ca^{2+} / calmodulin-dependent protein kinases, major transducers of Ca^{2+} , which are commonly involved in neuronal development and plasticity (Wayman, et al., 2008). CaMKI is a monomeric enzyme of 42 kDa with four isoforms (α , β , δ , and γ) which are encoded by separate genes and demonstrated to have a wide tissue distribution (Kamata, et al., 2007). CaMKI activity is governed by Ca^{2+} /CaM and its upstream activator calmodulin kinase kinase (CaMKK). The CaMKK/CaMKI cascade can phosphorylate and thus activate several substrates such as synapsins, cAMP response element-binding-protein (CREB) and extracellular signal-regulated kinases (ERKs). Through these interactions, CaMKI has been shown to play a contributing role in many neuronal functions including axon formation and elongation, dendritic arborization, spine formation and morphology, and synaptic plasticity (Wayman, et al., 2008). Furthermore, CaMKI has been shown to mediate the expression of the above genes via phosphorylating the initiation factor 4GII (Srivastava, et al., 2012). Robust expression of CaMKI isoform δ was reported in rodents in the extended amygdala, which includes the BFCN (Sakagami, et al., 2005). Furthermore, CaMKI δ expression may be regulated by synaptic inputs and is strong in neurons with high synaptic activity (Kamata, et al., 2007), features that are clearly established for the cholinergic system of the basal forebrain (Mesulam, 2004).

GAP43 is a neuronal specific 25 kDa protein selectively distributed to axons (Goslin, et al., 1988). In the presynaptic space, GAP43 is considered the main reservoir of CaM in the absence of Ca^{2+} (Gamby, et al., 1996). Elevation of intracellular levels of Ca^{2+} results in release of CaM after phosphorylation by activated-protein Kinase C (PKC) (Chapman, et al., 1991). Subsequently, activated-GAP43 promotes the polymerization of F-actin under the influence of several neurotrophic factors (He *et al.*, 1997), the recycling of synaptic vesicles, and regulation of AMPA receptors mediated by BDNF to facilitate genesis of neurites, synaptic plasticity, and nerve regeneration (Gupta, et al., 2009, Oestreicher, et al., 1997). Both *in vitro* and *in vivo* studies have demonstrated that GAP-43 expression and activation are regulated by glutamate and ionotropic glutamate receptors (McKinney, et al., 1999, Tsai, et al., 2007). Therefore, in addition to being a CaM-binding protein GAP-43 is considered a Ca^{2+} transducer that mediates neuronal plasticity in an activity-dependent manner.

Calpain is a cytoplasmic cysteine protease exclusively activated by Ca^{2+} , which consists of one heavy (80 kDa) catalytic subunit and one light (30 kDa) regulatory subunit. CaM-binding proteins are recognized substrates for activated calpain. (Wang, et al., 1989). Thus, as known CaM-binding proteins, CaMKI and GAP43 are both substrates for calpain (Naira, et al., 1985, Zakharov, et al., 2005). There are 15 calpain isoforms identified in humans; however, the extensively studied isoforms m- and μ -calpains, activated by millimolar and micromolar concentrations of Ca^{2+} respectively, are the most abundant in the brain (Liu, et al., 2008). In contrast to m-calpain, μ -calpain displays widespread activation in the AD brain and the relative enrichment of μ -calpain in neurons has been considered to explain this difference (Saito, et al., 1993). It is possible that increased intracellular Ca^{2+} levels in human

BFCN, precipitated by the age- and AD-related loss of CB, result in activation of calpain and greater degradation of CaMKI and GAP43 in BFCN.

Following up on our preliminary microarray results, here we report substantially reduced RNA for CB, CaMKI and GAP43 in human BFCN in AD, and in CB and GAP43 RNA in the course of normal aging. We also report substantially decreased CB, CaMKI and GAP43 protein in BF homogenates in the course of normal aging and in AD. Finally, we describe increased levels of the active form of the Ca^{2+} -dependent protease μ -calpain in aging and AD. Thus, while age-related loss of CB is likely to result in increased intracellular Ca^{2+} levels and toxicity in BFCN, alterations in levels of other Ca^{2+} related proteins also occur in these neurons through age- and AD-related changes in gene expression as well as activation of the protease μ -calpain. We propose that age-related dysregulation of Ca^{2+} levels and signaling not only contributes to degeneration of BFCN, but may also contribute to selective vulnerability of other neuronal populations to degeneration.

2. Materials and Methods

2.1. Samples and neuropathological diagnosis

Brains from normal young individuals (20–64 years), normal non-demented aged individuals (68–99 years) without any signs of neurologic or psychiatric disorders, and from clinically and pathologically confirmed AD patients (61–99 years) were obtained at autopsy and used in the present experiments. Only brains with sufficient clinical information to allow a clinical diagnosis of normal or AD and absence of other neurological signs or other systemic disorders were used. Clinical and neuropathological diagnoses were according to the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) (Morris, et al., 1989). Sex and as appropriate age were matched across subject groups. (PMI) in the three groups of subjects (young 15.8 ± 2.8 hours, old 13.6 ± 3.3 hours, AD 18.2 ± 4.2) and age of the old and AD groups (old 84 ± 2.9 years, AD 74.8 ± 4.4) were not significantly different ($p > 0.05$).

2.2. Laser Capture Microdissection (LCM), RNA Isolation, and Quantitative Real Time Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

Fresh frozen basal forebrain tissue from 4 normal old and 6 AD brains were cut at a thickness of 10 μm using a cryostat. Similar tissue with preserved RNA was available from 2 normal young cases (<65 years) and was processed in a similar manner and used for non-statistical comparison. Sections were immediately fixed in 70% ethanol for 1 minute and stained with histogene. Stained sections were dehydrated in graded ethanols and xylenes. BFCN were identified by their magnocellular and hyperchromic morphology within the basal forebrain and captured using CapSure caps and a laser capture microscope (Arcturus). At least 250 BFCN were harvested from sections spanning the nucleus basalis of Meynert (NbM)-Ch4 sector of BFCN in each case and rapidly dissolved in Trizol. The concentration of total RNA was measured by UV spectrophotometry, and RNA quality was confirmed in an Agilent bioanalyzer. qRT-PCR was used to investigate differential expression of the CB, CaMKI and GAP43 genes in old and AD BFCN. Briefly, 2 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, CaMKI forward GCTGGTCCATAGGTGTCATCGC, CaMKI reverse CACGCAGCAGTCTCGACAGCA, GAP43 forward AGAAGGCAAGGGACGAGACAAC, GAP43 reverse CAGCAGCAGGCACATCGG, 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 (cycle threshold, CT). All samples were processed in parallel. The relative quantification of the target gene expression was performed according to the comparative CT method. For each run, the mean expression levels of the CB, CaMKI, and GAP43 genes were normalized to the mean expression levels of the GAPDH reference gene. Serial dilution of standard cDNA was used to correct for differences in amplification efficiency.

2.3. Western Blot Analysis

Basal forebrain (BF) samples from subsets of 6 normal young, normal old and AD brains each were used for analysis of protein levels. Western blot procedures were carried out on whole cell protein homogenates extracted from dissected and frozen basal forebrains and analyzed on 12% SDS-PAGE and transferred to PVDF membrane. After blocking with 1% dry milk for 1 hour, the membranes were probed for 4 hours with a monoclonal antibody against CB (Swant, Belinzona, Switzerland), a monoclonal antibody against GAP43 (Clon B-5, Santa cruz biotechnology, Santa Cruz, CA), and overnight with a polyclonal antibody against CaMKI δ isoform (3365 Cell Signaling, Boston, MA), and a polyclonal antibody against μ -calpain (Clon H-65, Santa Cruz biotechnology, Santa Cruz, CA). After washes in 1% TBST, membranes were incubated for 1 hour in appropriate peroxidase conjugated secondary antibodies (1/5000 in all cases) and immunoreactivity was visualized with an electrochemiluminescent (ECL) detection kit (Amersham). GAPDH polyclonal antibody (14C10 Cell Signaling, Boston MA 1/3000) was used as internal control for all target antibodies after stripping procedures. Bands were visualized on exposed film at the correct molecular weights and quantified for protein content using densitometry on an image analyzer (ImageJ of NIH, USA).

Activation of calpain involves autolytic degradation of the heavy subunit (80 kDa) into multiple lighter fragments (Goll, et al., 1992). Therefore, as one measure of the activity of this Ca²⁺-dependent protease, we analyzed the optical density of multiple bands between the heavy and the light subunit (30 kDa) in the μ -calpain blots. As a second measure of calpain activity, we investigated degradation of the calpain substrate α II-spectrin (α -Spc) (Czogalla and Sikorski, 2005; Nicolas, et al., 2002) into a 150 kDa fragment. For this purpose, we probed membranes with a polyclonal antibody to α -Spc (1/5000, Cell Signaling).

2.4. Statistical Analysis

Data from normalized gene expression and integrated OD of protein bands were not normally distributed. Therefore significant differences were determined using the non-parametric Kruskal-Wallis test, followed by Dunn's post hoc comparison tests. Relationships were determined using Pearson correlations. The probability value for accepting results as significant was set at 0.05.

3. Results

3.1. Basal Forebrain Cholinergic Neurons Display Substantial Age- and Alzheimer-Related Changes in Calbindin

We have shown substantial age- and AD-related loss of CB from the BFCN using immunohistochemical procedures (Geula, *et al.*, 2003; Riascos *et al.*, 2011). Here, we determined changes in CB mRNA and protein using qRT-PCR and Western blot analysis. Calbindin mRNA and protein were detected in BFCN isolated via laser capture microdissection and in BF homogenates in all cases studied. Calbindin mRNA was substantially down-regulated (73%) in the normal aged BFCN when compared with the two young cases, and significantly so in AD BFCN (95%, $P < 0.05$) when compared with the normal aged. In BF homogenates, we observed a 25% loss of CB in the normal aged

($P < 0.05$) when compared with the young and a 56% loss of CB ($P < 0.01$) in AD when compared with the normal aged. These observations confirm our immunohistochemical findings of age- and AD-related loss of calbindin from the BFCN. CB mRNA and protein comparisons are shown in Fig. 1.

3.2. Loss of CaMKI and GAP43 in Aged and Alzheimer Basal Forebrain

To determine changes in other Ca^{2+} related proteins and based on our preliminary microarray findings, we investigated the levels of CaMKI and GAP43 mRNA and protein in BFCN and BF homogenates. CaMKI mRNA was down-regulated 17% in the normal aged BFCN when compared with the two young controls and 75% in AD BFCN in comparison to the normal aged. The latter comparison only approached significance ($p < 0.075$). A significant decrease was observed when the AD group was compared with the combined young and old group ($p < 0.05$). At the protein level, the full-length specie of CaMKI isoform δ corresponding to the molecular weight of 43 kDa in BF homogenates displayed a 47% loss in the normal aged when compared with the young ($P < 0.001$). A further 63% loss of CaMKI was observed in AD when compared with the old ($P < 0.01$). Moreover, we observed several bands between 25–32 kDa in the 3 groups. These fragments presented with a decrease in the normal aged of 43% when compared to the young, but a 34% increase in AD BF compared to the normal aged; and none of these differences were significant ($p > 0.05$). Additionally, we identified a lower band at 15 kDa which was exclusively seen in some of the AD cases. We quantified only the full-length and the 25–32 fragments since these bands were common to all cases. CaMKI δ mRNA and protein comparisons are presented in Fig. 2.

GAP43 mRNA was upregulated by 153% in the normal aged when compared with the two young controls. In contrast, GAP43 mRNA was downregulated by 80% in AD when compared with the old ($p < 0.02$). The 43 kDa band, corresponding to the full-length specie of GAP43 displayed a loss of 47% within the BF in the normal aged in comparison with the young ($P < 0.05$) and a further non-significant loss of 28% in AD when compared to the normal aged. Similar to CaMKI δ , we were able to detect several lighter species of GAP43. These additional bands were broadly spread and poorly defined between 10–43 kDa. Furthermore, bands were not consistently observed in all groups. Only the lightest band at 10 kDa was obvious and present in all groups. Thus, numerous but indistinguishable fragments were found in young BF, fewer in the normal aged, and virtually none in AD. Due to the difficulty to distinguishing specific fragments, we analyzed the sum of intensities of all fragments seen beneath 43 kDa. This analysis revealed non-significant decrease of 22% in the normal aged BF when compared with the young, but a significant 27% decrease in AD when compared with the normal aged ($P < 0.05$). Alterations in GAP43 mRNA and protein are presented in Fig. 3.

Levels of CB displayed a significant correlation with measures of both CaMKI ($r = 0.63$, $p < 0.009$) and GAP43 ($r = 0.81$, $p < 0.0001$).

3.3. Levels of Active Ca^{2+} -Dependent Protease μ -Calpain are Increased in Aged and Alzheimer Basal Forebrain Homogenates

Our observed age- and AD-related changes in CaMKI and GAP43 mRNA were smaller than the observed changes in their protein products. This raised the possibility that one factor contributing to decreased levels of these proteins may be increased protein degradation. As discussed earlier, increased intracellular Ca^{2+} resulting from loss of CB in aged and AD BFCN is expected to result in increased activity of the Ca^{2+} activated protease calpain, which among other substrates, is known to degrade both CaMKI and GAP43 (Naira, et al., 1985; Zakharov, et al., 2005). Following Ca^{2+} stimulation, the 80-kDa heavy subunit of calpain, specific for each isoform, is autocatalytically processed (Goll, et al., 1992). Thus,

we measured activation of μ -calpain by means of identification of the bands below 80-kDa and above 30-kDa (light subunit). We did not observe fragments below 30-kDa. We detected increases in μ -calpain fragments of 239% ($p>0.05$) in the normal aged when compared with the young and of 355% ($P<0.05$) in AD when compared with the normal aged.

We also directly measured calpain activity in BF homogenates using measures of cleavage of the major calpain substrate, α -Spc into a 150 kD fragment. Levels of calpain cleaved α -Spc displayed a 66% increase in the normal aged BF when compared with the normal young ($p<0.05$), and a further 23% increase in AD when compared with the normal aged, which approached significance ($p<0.08$). Analysis of calpain activity is shown in Fig. 4.

3.4. Levels of Proteins in Very Young and Middle-Aged Basal Forebrains

In aging studies, it would be desirable to divide the population in several groups according to age and determine whether the changes observed take place across the age span or are restricted to a particular age. We were unable to obtain adequate numbers of specimens from individuals below age 65 to allow a thorough analysis across various age groups. Hence, we divided the 6 cases in our young group into a group of 3 very young individuals (20, 26 and 36 years) and 3 middle-aged individuals (49, 56 and 61 years) for a preliminary analysis of the levels of proteins in these two groups. We detected reductions of 8.9% in CaMKI 25–32 KD fragment, 12% in total GAP43, 52% in GAP43 10–43 KD fragment and 7.6% in CB and an increase of 52% in μ -Calp in the middle aged group when compared with the very young group. However, none of these changes were statistically significant ($p>0.05$).

4. Discussion

A large body of evidence indicates that disturbances in Ca^{2+} homeostasis contribute to the neurodegenerative changes in the continuum of normal aging and AD. The normal age-related aspect of these changes is a likely factor in increased vulnerability of neurons to the additional stress imposed by pathological processes. In AD, the amyloid- β ($\text{A}\beta$) peptide is considered one of the most important stressors (Harkany, et al., 2000). Of particular interest, $\text{A}\beta$ has been shown to disturb Ca^{2+} homeostasis and to result in increased intracellular Ca^{2+} levels. It has been suggested that these effects are mediated by inhibition of glutamate transporters, enhancing NMDA receptor activation, or directly by causing membrane oxidative stress (Harkany, et al., 2000, Mattson, 2004, Molnár, et al., 2004). The age-related shift of AMPA subunits observed in the human BFCN, resulting in greater numbers of the Ca^{2+} permeable receptor subtype, might explain, in part, the vulnerability of the BFCN to degeneration (Ikonomic, et al., 2000). The main goal of the present study was to elucidate changes in Ca^{2+} -responsive proteins that may be involved in the selective vulnerability of BFCN and their projecting fibers in neurodegenerative disorders.

4.1. Loss of Calbindin is an Age-Related Change which is Exacerbated in Alzheimer's Disease

We have previously reported substantial loss of CB from the human and non-human primate BFCN in the course of normal aging (Wu, et al., 1997). We also previously found other brain areas with age-related loss of neurons immunopositive for CB such as the primary visual and visual association cortices, posterior cingulate gyrus, and parahippocampal gyri. However, these losses were mild in comparison to the loss in BFCN (Bu, et al., 2003). We found that the number of BFCN remains stable in the normal aged when compared with young controls (Geula, et al., 2003). Thus, the biochemically determined age-related loss of CB in the present study reflects a selective downregulation of CB protein, consequent to a general shift in the CB mRNA profile in BFCN during aging. We also found a significant

loss in both gene expression and protein levels of CB in AD. One other group has reported a similar decrease in CB mRNA in the human aged and AD BF homogenates (Iacopino and Christakos, 1990). With few exceptions (Tortosa and Ferrer, 1994), the presence of CB has been found to confer protection to a variety of neurons against several modes of damage such as excitotoxicity, hypoxia/ischemia, and prolonged stimulation (D'Orlando, et al., 2001, Scharfman and Schwartzkroin, 1989). Thus the age-related loss of CB in BFCN is likely to explain, in part, the vulnerability of these neurons in AD.

Of great interest, loss of CB mRNA and protein within the BF in aging and AD was greater and more consistent than loss of CaMKI and GAP43. This fact, along with the strong correlation we found in the levels of CB protein and those of CaMKI and GAP43 suggest that loss of CB may precede and cause, at least in part, loss of the other two proteins from the BFCN.

4.2. Loss of CaMKI isoform δ in the normal aged and AD BFCN

In order to appreciate the potential effects of progressive loss of CaMKI δ in normal aging and in AD, it is important to review the crucial neuronal-survival pathways that may be subsequently affected. CaMKI δ is exclusively localized in the cytosol in a non-membrane-bound manner. Similar to CaMKI α , which is the other exclusively cytoplasmic CaMKI isoform, CaMKI δ carries a nuclear export signal (Senga, et al., 2012). Indeed, activation of CaMKI by Ca^{2+} /CaM binding, induced by glutamate, and subsequent phosphorylation by CaMKK, results in nuclear translocation of CaMKI δ (Sakagami, et al., 2005).

CREB is a nuclear basic / leucine zipper-containing transcriptional factor located in the nucleus, which is known to be critical for a variety of neuronal functions such as survival, differentiation, synaptic plasticity and learning and memory by regulating gene transcription through binding to cAMP responsive element regulatory sites (Shaywitz and Greenberg, 1999). The nuclear CaMKIV, activated by CaMKK, has proven to be the main kinase phosphorylating CREB. Indeed, the CaMKIV-dependent CREB activation is required for neuroprotection against excitotoxicity via blockage of the NMDA receptor (Bell, et al., 2013). However, some features of CaMKI δ favor the role of this isoform in inducing early CREB-related neuronal survival. First, CaMKI δ is localized primarily in the cytoplasm where CaMKK also is dominantly found. Likewise, the pair CaMKK/CaMKI is mutually induced by cytoplasmic Ca^{2+} influx. Additionally, CaMKI δ is involved in potentiating the MAPK pathway (Verploegen, et al., 2000). The MAPK pathway is thought to be critical for sustained CREB phosphorylation and induction of gene transcription. Thus, it is possible that CaMKI δ regulates CREB-dependent transcription not only via direct phosphorylation of CREB, but also via its positive regulation of the MAPK pathway (Sakagami, et al., 2005). Indeed, the selective inhibition of the network CaMKIV-CaMKK results in apoptosis of human neuroblastoma cells (McGinnis, et al., 2001), raising the possibility of similar toxic effects of reduced CaMKI. Therefore, loss of CaMKI δ is likely to contribute to loss of BFCN through loss of CREB-mediated neuronal survival against excitotoxicity.

It is also possible that loss of CaMKI δ in the BFCN contributes to degeneration of cortical cholinergic axons, which is a prominent feature of AD. CaMKI isoforms differentially influence axonal or dendrite elongation in a neuronal subpopulation specific manner. CaMKI α expression specifically impairs axonal but not dendritic growth after intracellular Ca^{2+} elevation (Ageta-Ishihara, et al., 2009). The structural and functional similarities between CaMKI α and CaMKI δ discussed earlier suggest possible distribution of CaMKI δ in the axons of BFCN. Further investigation is needed to determine whether loss of CaMKI δ contributes to the vulnerability of cholinergic axons against excitotoxicity, either alone or in combination with loss of GAP43.

4.3. Loss of GAP43 in Normal Aging and AD

We found loss of GAP43 in human BF in normal aging, which was exacerbated in AD. GAP43 is enriched in the BFCN (Augood, et al., 1995) and intense GAP43 expression is associated with aberrant cholinergic sprouting in AD (Masliah, et al., 1991). The well-known role of GAP43 in neuronal plasticity suggests that neuritic sprouting may represent an aberrant regenerative attempt of the cholinergic axons. The expression of GAP-43 mRNA is higher after peripheral nerve lesions and is associated with enhanced growth of axonal cones, which is observed only after injury (Cafferty, et al., 2004). Downregulation of GAP-43 correlates with neuritic degeneration (Keswani, et al., 2004). In particular, overexpression of GAP43 mRNA, identified by in situ hybridization, correlated with BFCN survival following cortical infarction (ischemic axotomy) (Figueiredo, et al., 1995).

GAP43-associated neuritic sprouting is seen in normal aging and AD and occurs in response to A β (Masliah, et al., 1991). Increased intracellular Ca²⁺ triggers normal GAP43-mediated genesis of neurites and synaptic plasticity (Oestreicher, et al., 1997). Thus, disturbance in Ca²⁺ homeostasis is a likely cause of GAP43-associated aberrant sprouting. Additional evidence in support of the relationship between Ca²⁺ levels and GAP43 induced aberrant sprouting is derived from the study of glutamate receptors. Overactivation of AMPA receptors is related to increased expression and activation of GAP43 resulting in neurite outgrowth after axotomy (Tsai, et al., 2007). GAP43 is believed to be an important modulator of the effects of brain-derived neurotrophic factor (BFNF) on AMPA receptor mediated neurite outgrowth and in BDNF-mediated neuroprotection against excitotoxicity (Gupta, et al., 2009). In fact, glutamate treatment decreases GAP43 mRNA levels and neurite regeneration following treatment with AMPA receptor antagonists (McKinney, et al., 1999).

There are contradictory results in the literature regarding a potential role for GAP43 in neuronal death. GAP-43 gene disruption has been shown to result in apoptotic neuronal loss *in vitro* and *in vivo* (Gupta, et al., 2009). Others have reported that GAP-43 gene knock-out leads to an increase in the total number of neurons (Gagliardini, et al., 2000) and that increased levels of neuronal GAP-43 can lead to apoptosis (Aigner, et al., 1995).

These observations suggest that GAP43 has multiple functions in relation to axonal sprouting and neuronal apoptosis, but that the precise effects of GAP43 may depend on other as yet unidentified factors. Thus, the loss we have observed in GAP43 in aging and AD is potentially involved in the degeneration of these neurons and / or aberrant sprouting of their axons in response to A β .

Given that GAP43 is a presynaptic protein, it is also possible that at least some of the reduction in GAP43 protein in BF homogenates is caused by loss of projections to the BFCN from other brain regions. Such synaptic loss is then likely to result in dysregulation of cortical cholinergic input.

4.4. Calcium-Dependent Proteolysis of CaMKI and GAP43 by Calpain in BFCN

We found substantial loss of the Ca²⁺ transducers and calmodulin-binding proteins, CaMKI and GAP43, within the BFCN in aging and AD at the protein level and smaller and less consistent reductions at the level of mRNA. This finding suggests that loss of these proteins in BFCN is likely influenced by increased degradation to a greater extent when compared with decreased synthesis.

We found an increase in the activity of the Ca²⁺/dependent protease calpain, and specifically of the isoform μ -calpain; directly by quantifying the autolytic fragments derived from heavy and specific subunit of the protease, and indirectly via the degradation of its recognized

substrate spectrin. We believe that our results suggest that the age- and AD related loss of CaMKI and GAP43 are due, at least in part, to the increased activation of μ -calpain, resulting from high Ca^{2+} levels most likely caused by the loss of CB.

In consequence, in protein blots, we observed bands below the respective full-length GAP43 and CaMKI δ . The pattern and abundance of these fragments did not display an inverse relationship with the progressive increase of μ -calpain activation. A progressive increase of fragments is expected if these fragments were derived specifically by μ -calpain overactivation. Nonetheless, we believe our findings are not necessarily inconsistent when considered in the context of the known profiles of fragments of each protein.

Given that m-calpain mediated proteolysis is negatively regulated by PKC, CaM and degraded GAP-43 fragments (Zakharov and Mosevitsky, 2007), our findings are consistent with a primary role of μ -calpain in degradation of this protein. We also observed multiple fragments of GAP43. The fact that cleavage of GAP43 by μ -calpain *in vitro* is known to result in formation of several large proteolytic fragments, in contrast to only one seen with m-calpain treatment (Zakharov, et al., 2005), lends additional support to the predominant role of μ -calpain in degradation of GAP43.

The abundance of GAP43 fragments displayed an inverse pattern to the progressive activation of μ -calpain. Specifically, these fragments displayed clearly higher levels in young BFCN but were nearly undetectable in AD. Some reports have demonstrated a role for GAP43 in modulation of growth cone resistance to inhibitory and repulsive action of neuritic-toxic factors (Aigner and Caroni, 1993). In this context, fragments of GAP43 derived from calpain proteolysis are involved in retraction of neurites against repulsive signals (Zakharov, et al., 2005, Zakharov and Mosevitsky, 2007). Thus, we believe that the enrichment of GAP43 fragments is indicative of healthy repulsive response capacity of neurites in synaptic rearrangements or against toxins. If so, this capacity is present in young BFCN and reduced in AD.

Calpain is not only expected to degrade all CaMKI isoforms, but also other CaMKs which strongly regulate CaMKI function, such as CaMKK and CaMKIV (Tremper-Wells and Vallano, 2005). The main CaMKI substrates such as synapsin and CREB are also cleaved by calpain (Liang, et al., 2007, Murrey, et al., 2006). In order to understand the reasons for lack of relationship, neither positive nor inverse, between CaMKI fragments and the progressive increase in μ -calpain activity, it is important to consider the role of other proteases in CaMKI degradation. As discussed previously, CaMKI is strongly related to CaMKIV structurally and through the mutual phosphorylation of CREB. CaMKIV is cleaved *in vitro* and *in vivo* by calpain and caspase-3. In fact, the presence of multiple fragments created through cleavage by the two proteases has made it impossible to distinguish protease-specificity for most of the observed fragments (McGinnis, et al., 2001). Therefore, we cannot exclude caspase-mediated degradation of CaMKI δ as a cause of the observed fragments. In fact, the online resource CASVM identifies several sites in the CaMKI δ molecule that could serve as targets for caspase cleavage (Wee, et al., 2006). Therefore, it is very difficult to establish the specific involvement of caspase or calpain in generation of the 25–30 kDa CaMKI δ fragments we observed in the blots. However, the 15 kDa fragment of CaMKI δ , which we observed exclusively in some cases of AD (Fig 2), may be generated from the cleavage of the protein at its arginine-74. This site receives the highest score when predicting cleavage by μ -calpain according to the online resource CaMPDB (duVerle, et al., 2010 Jan.). Although indirect, this evidence also suggests dominant degradation of CaMKI δ by over-activated μ -calpain in AD BF.

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Figure 1.

Age-related loss of calbindin-D28K (CB) from basal forebrain cholinergic neurons at the protein and gene expression levels. A. Laser capture of basal forebrain cholinergic neurons (BFCN) from normal young ($n = 2$), normal aged ($n = 4$), and AD ($n = 6$) brains and subsequent qRT-PCR analysis. Loss of mRNA expression of CB is observed from normal aging (73%) compared to young and significantly downregulated (95%, $P < 0.05$) in Alzheimer's disease (AD) when compared to normal aging. B&C. Representative blot and analysis of the basal forebrain region containing BFCN in young, normal aged, and AD brains ($n = 6$ per group). Levels of CB were normalized with GAPDH. Progressive loss of CB in the Aging and AD is observed; 25% loss of CB in the normal aged when compared with the young BF ($P < 0.05$) and 56% loss of CB from AD BF when compared with the normal aged ($P < 0.01$). Mean and standard errors are represented in the bars. † Indicates statistic difference when compared with the young and ‡ statistical difference when compared with normal aged.

Figure 2.

Changes in expression and degradation of CaMKI δ . **A.** Laser capture of basal forebrain cholinergic neurons from normal young ($n = 2$), normal aged ($n = 4$), and Alzheimer's disease (AD) ($n = 6$) brains and qRT-PCR analysis. CaMKI mRNA was down-regulated 17% in the normal aged BFCN when compared to young controls and 75% in AD BFCN in comparison to the normal aged ($p < 0.075$). **B.** Representative blot of human basal forebrain (BF) homogenates from young, normal aged, and AD brains ($n = 6$ per group). In addition to the full-length specie of CaMKI δ at 43 kDa, several indistinguishable bands were identified between 25–35 kDa. A light band at 15 kDa was occasionally detected only in AD cases. **C.** Analysis after normalization with GAPDH of the full-length 43kDa CaMKI δ revealed a 47% age-related loss of the protein ($P < 0.001$), and a further 63% reduction in AD vs the normal aged ($P < 0.001$). **D.** Analysis of the bands between 25–35 kDa did not display any particular pattern or statistical difference between groups. Mean and SE are represented in the bars. † Indicates statistic difference when compared with young and ‡ represents statistical difference when compared with the normal aged.

Figure 3.

Changes of the expression of full-length and proteolytic fragments of GAP43. A. Laser capture of basal forebrain cholinergic neurons (BFCN) from normal young ($n = 2$), normal aged ($n = 4$), and Alzheimer's disease (AD) ($n = 6$) brains and subsequent qRT-PCR analysis. mRNA expression of GAP43 increases in normal aging and subsequently decreases significantly 80% ($p < 0.02$) in AD vs normal aged BFCNs. B. Representative blot of human basal forebrain (BF) homogenates from young, normal aged, and AD brains ($n = 6$ per group). In addition to the full-length specie of GAP43 at 43 kDa, several and indistinguishable bands were identified beneath 43 kDa particularly in young BF, few bands in aged BF, and almost none in AD. C. Analysis after normalization with GAPDH of the full-length 43kDa GAP43 revealed loss of the protein in the normal aged vs young cases of 47% ($P < 0.05$); and a further non-significant loss of 28% in AD when compared to the normal aged. D. Analysis of the bands beneath 43 kDa full-length GAP43 demonstrated a progressive decrease resembling the changes for GAP43-43kDa. The decrease of 22% in the normal aged BF when compared with the young was non-significant in contrast to the loss of 27% in AD vs normal aged ($P < 0.05$). Mean and SE are represented in the bars. † Indicates statistic difference when compared with young and ‡ represents statistical difference when compared with the normal aged.

Figure 4.

Progressive increase in the activity of μ -calpain in normal aged and AD. A. Representative blot in human basal forebrain (BF) homogenates from young, normal aged, and AD brains ($n = 6$ per group). * Heavy and specific subunit of μ -calpain: no changes between groups are obvious. **Fragments corresponding to the autolytic-proteolysis of the heavy subunit which is Ca^{2+} -dependent. ***Light and non-specific subunit of μ -calpain, no changes are noted between groups. B. Analysis of the autolytic-fragments of μ -calpain shows progressive increase in the activity of the protease, of 239% ($p > 0.05$) in the normal aged when compared with the young and of 355% ($P < 0.05$) in AD when compared with the normal aged. C. Representative blot in human basal forebrain (BF) homogenates for the analysis of the cleavage of the major substrate of calpain, α -Spectrin (α -Spc). * Full-length protein of 240 kDa: no major differences between groups are seen. **150 kDa fragment, calpain cleavage product. D. Levels of calpain cleaved α -Spc displayed a 66% increase in the normal aged BF when compared with the normal young ($p < 0.05$), and a further 23% increase in AD when compared with the normal aged ($p < 0.08$). Mean and SE are represented in the bars. † Indicates statistic difference when compared with the young while ‡ represents statistical difference compared to the normal aged