Elevation of cytoskeletal protein breakdown in aged Wistar rat brain

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1. Introduction

Dysfunction in the homeostatic regulation of calcium (Ca2+) has been one of the many notable theories proposed to account for age-related neuronal degradation [7,12,13,16,30,56,59] and (of more immediate therapeutic potential) neurodegenerative diseases such as stroke and Alzheimer’s disease [6,49]. Our study was designed to focus on experimental evidence supporting the calcium hypothesis for aging [29,54]. Concerning this theory, past investigators have focused on studying the regulation of Ca2+ in intracellular and extracellular areas and its dysfunctional link to degenerative alterations in the physiology of neuronal activities. Of specific interest are the effects of Ca2+ on synaptic transmissions [36,53] and hypoxic-ischemic cellular death [9,24]. Many laboratories, including ours, have focused on the secondary dysfunctional role of Ca2+ on cellular physiology and the potential role of calpain overactivation in causing neurodegenerative responses [53,60]. It has been shown in a number of studies that a sustained elevation in intracellular Ca2+ triggers calpain activation, which leads to cellular damage [5,21,24,51]. Activated calpain exerts its damage by degrading various cellular substrates such as αII-spectrin, calmodulin binding proteins, microtubule-associated proteins (MAPs) and neurofilaments. These processes are often associated with oncotic necrosis (oncosis). However, certain pathophysiological conditions (such as hypoxia or moderate excitotoxicity) can also trigger activation of the pro-apoptotic protease caspase-3. Like calpain, caspase-3 can degrade a large number of neuronal proteins, leading to apoptosis [57]. In fact, both caspase-3 and calpain-mediated proteolytic events have been implicated in neurodegenerative diseases such as Alzheimer’s disease [1,4,25,26,44,48,50].
Therefore, it is conceivable that calpain and caspase-3 proteases might also be activated in the normal aging process [10,61]. In fact, there have been some sporadic reports showing increased activities of calpains [3,5,21,52] and caspases [28,62] in aging brain and/or other organs. Hinman et al. [18] also recently showed that there is a significant increased of activated calpain-1 immunoreactivity in antigen-DR (HLA-DR) positive microglia in the white matter of the aged rhesus monkey. In the present study, we evaluated the hypothesis that proteolysis plays a role in cellular damage due to aging by examining calpain- and caspase-mediated proteolysis in neuronal tissue during the process of normal aging in the Wistar rat.

2. Materials and methods

2.1. Tissue preparation

All procedures were carried out in strict compliance with the Institutional Animal Care and Use Committee of Parke-Davis and Pfizer Global Research and Development and the Society of Neuroscience. Male Wistar rats (Charles River Laboratories), age’s 3, 12, 7, 21 and 23.5 months were anesthetized with 4% isoflurane before being sacrificed via cardiac puncture. Animals were first perfused with saline, then 10% glycerol, 10 mM DT, 0.5 mM EGTA and 40 mM acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (Ac-DEVD-MCA, Peptide International). After 2h, the assay was measured on a Millipore CytoFluor 2300 plate-reader [31] using a fluorescence of excitation 380 ± 15 nm and emission 460 ± 15 nm.

2.2. Protein extraction and sample analysis

Protein samples were extracted from neuronal tissue using a modified Triton extraction method. A proportionate volume of 20 mM Tris-HCl buffer (pH 7.4), 5 mM EDTA, 5 mM EGTA, 150 mM NaCl, 1 mM DTT, 1% Triton X-100 and protease inhibitor (Roche #11 836 153 001) was added to the frozen (−70 °C) powdered tissue and stored in wet ice for 12 h before processing for immunohistochemical staining. Immediately preceding protein extraction, frozen brains were crushed into powder using a pre-cooled mortar and pestle over dry ice.

2.3. Assay for caspase-3 activity in neuronal tissue

Paraformaldehyde-stored brains were transversely sectioned at 100 µm, using a chilled tissue slicer. The slices were placed in a sucrose solution (20%) before being placed on glass microscope slides and stored at 4 °C. Samples were washed in a series, which included phosphate buffered saline (PBS), 80% formic acid, 0.5% Triton buffer (to permeate the cell membrane), 1% hydrogen peroxide (to quench endogenous peroxidase activity) and blocked in 2% horse serum (PBS) overnight at 4 °C. Tissue sections were visualized with a diaminobenzidine tetrahydrochloride (DAB) solution and counter-stained with Hematoxylin and Eosin (H&E) Staining. All sections were examined with a phase contrast microscope at 400× resolution.

3. Results

3.1. Immunoblot analysis of αII-spectrin in young and aged rat brains

Western immunoblots were prepared to evaluate a time course of degradation of two protease-sensitive neuronal proteins (αII-spectrin and MAP-2/B) to gauge an overall picture of biochemical functions during the aging process. Gel loading controls with beta-actin were performed for all data.
subjected to staurosporin treatment) [33]. (D) For comparison, SBDP150/SBDP145 and SBDP150i/SBDP120, respectively. Analysis of variance (ANOVA) was also performed on SBDP levels with respect to age. (Cell Signaling Technology) was shown (samples as in top panel of A). The far right lane (+) is SBDP150i positive control (lysate from human SH-SY5Y cells).

203 When compared to young rats (3 months) (Fig. 2A and B). Further, ANOVA (analysis of variance) showed that SBDP145 significantly increases in both cortex and cerebellum with respect to animal age, while age-related SBDP150 increasing did not reach statistical significance. In addition, evenness of gel loading was confirmed with beta-actin as the standard (Figs. 1A and 2A).

204 To compare the SBDPs observed in the aged rat brains to those generated by these proteases (calpain and caspase-3), we subjected naive 3-month rat cortex lysate to in vitro calpain and caspase-3 digestion, respectively (Fig. 1C). Both SBDP150 and SBDP145 were prominently generated by calpain proteolysis [43,47,57]. In contrast, in vitro caspase-3 digestion generated a fragment just below 150 kDa band termed “SBDP150i” and a characteristic 120 kDa spectrin breakdown product (SBDP120) [33,58]. Both SBDP150 and SBDP145 were prominently generated by calpain proteolysis [47,57]. In comparison, while both SBDP150 and SBDP145 were prominently observed in aged rat cortex and cerebellum, the SBDP120 was noticeably absent (Figs. 1A and 2A).

205 We further examined, if there was also a lack of caspase-3-generated SBDP150i. Indeed, using an anti-SBDP150i-specific antibody (cell signaling), we confirmed that caspase-3-generated SBDP150i was also absent in cortex and cerebellum from all age groups (Fig. 1C).
Fig. 2. Age-related αII-spectrin breakdown in rat cerebellum. (A) Two sets of representative immunoblots of all-spectrin breakdown during time course are shown (total n = 5–8). Spectrin intact bands (280 kDa), SBDP150 and SBDP145 are indicated by arrows. (B) Time course of protein extracts of cerebral cortex tissue at 3, 17, 21 and 23.5 months were assayed in immunoblots against αII-spectrin antibody (Affiniti) at 1:1000 concentration. Dark bars indicated 150-kDa spectrin breakdown products (SBDP150) while shaded bars indicate SBDP145. Data are shown as SBDP/intact spectrin ratio. Data are means ± S.E.M. (n = 5 for control and 23.5 month, n = 6 for 21 month, n = 8 for 17 month). Values showing significance different from 3-month control animal are indicated by * p ≤ 0.02 or ** p < 0.05, with Student’s unpaired t-test. In the αII-spectrin intact band (280 kDa), arrows indicate SBDP150 and SBDP145. (C) One set of representative immunoblots of caspase-produced SBDP150i was shown (samples as in top panel of A). The far right lane (+) is SBDP150i positive control as in Fig. 1 [33]. ANOVA was also performed on SBDP levels with respect to age.

3.2. Immunoblot analysis of MAP-2A/B in young and aged rat brains

Young and aged rat cortical and cerebellar samples were also subjected to analysis with Western immunoblotting using a microtubule associated protein 2A and 2B (MAP-2A/B) antibody. In this case, the intact MAP-2A/B appeared as a doublet of about 300 kDa in all lanes (Fig. 3A (cortex) and 4A (cerebellum)). A dramatic and decreased intact MAP-2A/B density over the time course is seen in the cortex (Fig. 3B) and in the cerebellum (Fig. 4B) (based upon Student’s t-test analysis). The reduction of MAP-2A/B protein levels could not be due to uneven gel loading, since equal amounts (20 μg) were loaded to each lane. Furthermore, results with beta-actin showed constant levels between control and aged samples in both cortex and cerebellum, indicating sample loadings were even (see “actin” panels in Fig. 7). In parallel with the reduction of intact MAP-2A/B in aged rat brains, multiple breakdown products of MAP-2A/B were also apparent in cortex and cerebellum from aged rats (Figs. 3A and 4A, respectively). Similar to SBDP analysis, evenness of gel loading was confirmed with beta-actin as standard (Figs. 3A and 4A).

Additional ANOVA post hoc analysis also showed that intact MAP-2A/B levels significantly decreased with respect to animal age (p < 0.05) in both cortex and cerebellum. Since MAP-2A/B was reported to be extremely sensitive to calpain-mediated proteolysis [15,19,20,51] and no reports have been published on its sensitivity to caspase-3, we subjected naive cortex (3 month) lysate to calpain digestion and found that the in vitro calpain-proteolysed MAP-2A/B also shows multiple lower molecular weight fragments (Fig. 3C), as was the case observed in aged rat cortex and cerebellum (Figs. 3A and 4A).

We also performed immunohistochemical analysis with MAP-A/B antibody with cortex and cerebellum samples from young (3 month) and aged (23.5 month) rats. Paraformaldehyde-fixed brains were transversely sectioned at 100 μm and slices were incubated overnight at 4 °C with MAP-2A/B (1:500, Sigma Chemical) and visualized with a diaminobenzidine tetrahydrochloride (DAB) (brown) (Fig. 5). Slices were also counter-stained with Hematoxylin and Eosin Staining (blue/red). Fig. 5 showed representative slices from young cortex have more MAP-2 reactivity when compared to aged counterpart (compare panel A to B, Fig. 5). Consistent with the Western blot results, the decline of MAP-2A/B staining in aged cerebellum appeared more severe (compare panel D to C, Fig. 5).
Fig. 3. Alteration of MAP-2A/B levels in rat cortex during aging. (A) Two representative immunoblots (total n = 4) of MAP-2A/B breakdown during time course are shown. Indicated is the intact MAP-2A/B (280 kDa). Arrows indicate multiple breakdown products (BDPs). (B) Time course of intact MAP-2A/B levels of cerebellum tissue at 3, 17, 21 and 23.5 months were assayed in immunoblots. Data are ratios of intact MAP-2A/B at time X/MAP-2A/B of 3-month control animals. Data are means ± S.E.M. (n = 4). Values showing significance different from 3-month control animal are indicated by * p ≤ 0.05 or ** p < 0.001 with Student’s unpaired t-test. ANOVA was also performed on MAP-2A/B levels with respect to age.

Fig. 4. Alteration of MAP-2 levels in rat cerebellum during aging. (A) Two representative immunoblots (total n = 4) of MAP-2 breakdown during time course are shown. Indicated is the intact MAP-2 (300 kDa). Arrows indicate multiple breakdown products (BDPs). (B) Time course of intact MAP-2A/B levels of cerebellum tissue at 3, 17, 21 and 23.5 months were assayed in immunoblots. Data are ratios of intact MAP-2A/B at time X/MAP-2A/B of 3-month control animals. Data are means ± S.E.M. (n = 4). Values showing significance different from 3-month control animal are indicated by * p ≤ 0.05 with Student’s unpaired t-test. ANOVA was also performed on MAP-2A/B levels with respect to age.
Fig. 5. Immunohistochemical staining of MAP2A/B of young and aged rat brains. Paraformaldehyde-stored brains were transversely sectioned at 100μm. Cortex (A and B) and cerebellum (C and D) sections from young (3 months, panels A and C) or old (23.5 months, panels B and D) rats were treated and incubated with anti-MAP-2A/B (1:500, Sigma Chemical), washed, and visualized with a diaminobenzidine tetrahydrochloride (DAB) solution (brown). The immunoreactive product appears brown in color over a background of Hematoxylin and Eosin (H&E) staining (blue/red). All sections were examined with a phase contrast microscope at 400x resolution. Shown here are representative sections. Scale bar represents 100μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

3.3. Analysis of caspase-3 processing and activity in aged rat brains

The lack of increase of SBDP120 in aged rat brains prompted us to further investigate the status of caspase-3 processing and activity during aging in neuronal tissue. Immediately following protein extraction, a time course of fresh cortical and cerebellar samples were assayed against a fluorogenic caspase-3 substrate acetyl-DEVD-AMC to examine if aging produced an elevated caspase-3 hydrolytic activity. Fig. 5 shows that the opposite trend was seen: a significant decrease in caspase-3 activity (rather than increase) in both cortex (28% decrease) and cerebellum (25% decrease) was seen with aged (23.5 month) rats compared to the 3-month control animals (Fig. 6). ANOVA also showed that caspase activity declines significantly with age.

Fig. 6. Time course of caspase-3 activity in rat cortex and cerebellum. Triton X-100 extracts were incubated in reaction mixtures the caspase-3 preferred Ac-DEVD-AMC peptide substrate. Solid bars indicate cortex levels and shaded bars indicate cerebellum levels. All data are measured as fluorescence units divided by sample protein level (ng). Data are means ± S.E.M. (n = 5 for control and 23.5 month, n = 6 for 21 month, n = 12 for 17 month). Values showing significance different from 3-month control animal are indicated by *p < 0.05 or **p < 0.005, with Student’s unpaired t-test.

Fig. 7. Lack of caspase-3 processing in aged rat cortex and cerebellum. Immunoblot analysis of pro-caspase-3 levels in 3-month control (n = 4) and aged (23.5 month; n = 5) rat cortex (A) and cerebellum (B) using anti-caspase-3 (Santa Cruz, H-277, 1:1000), which detects pro- and activated forms of caspase. No apparent processing of the 32kDa pro-form of caspase-3 (indicated by arrows) to the 17kDa activated form (indicated by open triangle) during aging was detected. As controls, Immunoblot analysis of beta-actin was also performed using anti-beta-actin antibody (Sigma). (C) Quantification of pro-caspase-3 levels (as pro-caspase-3/beta-actin ratio) is calculated for the young and aged rats’ cortex and cerebellum, respectively. In the cortex, 23.5 month (dark bars) values showing significance different from 3 month (white bars) samples are indicated by *p < 0.05 with Student’s unpaired t-test.
Parallel immunoblotting analysis of the same samples were then performed and probed with an anti-caspase-3 antibody, which detects both pro- and activated forms of caspase-3 (Fig. 7A). The 32 kDa pro-caspase-3 protein was observed in the cortex and cerebellum of both young and aged rats, while the fragment for the 17 kDa activated form was not observed, either in young or aged rat brains. Western immunoblots were also performed with anti-beta-actin antibody, which showed relatively constant levels between young and aged cortex and cerebellum sample group (Fig. 7A and B, respectively). In fact, densitometric analysis showed that, with respect to beta-actin levels (as controls), the pro-caspase-3 level was actually lower in cortex but remained constant in the cerebellum in aged rats with comparison to young rats (Fig. 7C). Taken together, these data would suggest that there might be an age-related reduction of pro-caspase-3 expression and no evidence of increased activation of caspase-3.

4. Discussion

The results of this study present evidence of elevated proteolysis of αII-spectrin (Figs. 1 and 2) and MAP-2A/B (Figs. 3 and 4) in a time course of aging in Wistar rats. Our results provide evidence that cytoskeletal protein degradation is a common occurrence in aged rat brains. Both of these proteins play important roles in the stability of the cytoskeletal system. αII-spectrin is the major structural component of the cortical membrane cytoskeleton and is abundant in axons and presynaptic terminals [16,42]. MAP-2A/B is a dendritic protein that interacts with and stabilizes microtubules, which provide both structural elements and tracks for organelle traffic [11,27]. The degradation of αII-spectrin and MAP-2A/B would seriously compromise the normal functions of neuronal tissue. Our results also indicate that age-related degradation of both αII-spectrin and MAP-2A/B appears less severe in the cortex than in the cerebellum.

Prior studies have looked at various aspects of cell death and calcium-related changes in the brain. Experiments have shown region-specific changes in calcium [17] and that homeostasis in the hippocampus and cortex are especially prone to dysfunction in aged rats, while the Ca$^{2+}$ changes in the cerebellum and striatum are less severe. Interestingly, in the present study, aging-induced cytoskeleton protein breakdown appears to affect both the cerebral cortex and the cerebellum (Figs. 1–4). Studies of the human brain have implicated that calpain levels are among the highest in the cerebellar region [3]. Also, evaluation of the rat brain has led researchers to conclude that aging produces a more drastic loss of neurons in the cerebellum than in the cortex [37]. When examining the effects of calpain-mediated spectrin breakdown, many studies have highlighted the role that proteases play on the integrity of the neuronal structure [24] and the increase in neuronal death [46]. Early studies focused on the relation of calpain-mediated proteolysis had on the young developing brain [14] and highlighted the susceptibility these proteins had on degeneration. They showed conclusively that MAP-2 was specifically at risk for being degraded due to the sensitivity a calpain substrate. Others highlighted the changes in phosphorylation that occurs with the MAP-2 protein [20], which, due to age-related changes, will increase its susceptibility with time. This happens when the regulation of binding changes in the microtubule network, making it more open to calpain hydrolysis. It is possible that changes due to spectrin breakdown could contribute to this. With the loss of MAP-2A/B, dendrites appear to collapse. One of the first studies to examine the effects of MAPs in an aged brain [8] saw almost total MAP-2A/B loss in the cortex and hippocampus with associated loss in synaptic plasticity.

One of the major hypotheses that promoted this study was that caspase- and calpain-mediated proteolysis could also be identified as a contributor to the protein degeneration seen in the aging process. Many studies have identified its increase in ischemic events [31,33,35,40] and in head trauma [23,41,46]. The caspase family of proteases has been shown to mediate cell suicide [34] in cells either damaged by trauma or having undergone irreversible degeneration to the point where programmed death is required. Our experiments focused on the evaluation of caspase- and calpain-mediated proteolysis in aged rat brains. These studies, utilizing anti-caspase-3 Western immunoblots and an active caspase-3 assay (ac-DEVD-AMC) indicated no increase in activated forms of the 32 kDa pro-caspase fragment, or any increase in active caspase-3 (Figs. 5 and 6). Furthermore, anti-αII-spectrin immunoblots showed that there is significant age-related proteolysis to SBDP150 and SBDP145, while the previously described calpain-3 generated 120-kDa spectrin breakdown product (SBDP120) [57] was not observed (Figs. 1 and 2). It is worth noting that caspase-3 can also produce a fragment of about 150 kDa (termed SBDP150i, see [33,58]). It is, thus, possible that SBDP150i can in part contribute to the SBDP150 we observed in aged rat brain tissues. To test this possibility, we used a SBDP150i-specific antibody and found that there were no detectable signals of SBDP150i in either young or aged cortical or cerebellar samples (Figs. 1C and 2C). Thus, we conclude that caspase-mediated proteolysis is not significantly elevated in aged rat brains. Calpain-mediated αII-spectrin proteolysis in aging has in fact been documented. Bahr et al. [2] showed a rather selective increase of a calpain-mediated SBDP in both the cortex and telencephalon, but none in the cerebellum of aged mouse brains. Vicente and colleagues [54] also showed that a calpain-mediated SBDP was significantly elevated in the neocortex and hippocampus but not in thalamus or cerebellum in mid-aged mice (13 months). These data contrast our observations that calpain-mediated SBDP150 and SBDP145 are elevated with aging in both cortex and cerebellum (Figs. 1 and 2, respectively). One of the contributing factors could be species variation (mouse versus rat).

Our analysis of the extensive proteolysis of the cytoskeletal protein MAP-2A/B in the cortex and cerebellum of aged rats supports the importance of calpain activation in age-
related degeneration, since MAP-2A/B is known to be more sensitive to calpain- and not caspase-3 activation. MAP-2A/B (Figs. 3 and 4). It is worth noting that MAP-2A/B breakdown products in the cortex are in fact more intense than their counterparts in the cerebellum (Fig. 3A versus Fig. 4A). Yet, the levels of remaining intact MAP-2A/B in aged cerebel-

lum (23.5 months) were decreased to about 55% of young cortex controls, while the MAP-2A/B levels in aged cerebel-

lum (23.5 months) were further decreased to 25% of young cerebellum controls (Fig. 3B versus Fig. 4B). We hypothe-
sized that proteolysis in the cerebellum was even more ac-
tive, thus, not only reducing the level of intact MAP-1A/B, but more efficient in further degrading the fragments into

smaller peptides, as is the case with digestion with calpain in vitro (Fig. 3C). In addition, immunohistochemical analy-

sis, while not quantitative, showed a more dramatic reduction of MAP-2A/B levels in the cerebellum than in cerebral cor-
tex in the aged (23.5 month) rats, when compared to their younger counterpart (3 month) (Fig. 5). Hinnan et al. [18] recently showed that there is also a significant increase in ac-
tivated calpain-1 immunoreactivity in microglia and possibly myelin-bearing oligodendrocytes in the white matter of the aged rhesus monkey. It is, therefore, possible that there is a concerted increase of calpain activity in multiple cell-types within the aged brain.

Chronic neurological diseases, such as Parkinson’s and Alzheimer’s disease have both been found to be affected by

altered calcium homeostasis resulting in increased calpain ac-
tivity [48], oil-spectrin breakdown [18,43] and degradation of MAP-2A/B in the cytoskeleton [20]. It has been shown

that neurofibrillary tangles contain broken MAP-2 segments [22]. Although these results associate the affects of calpain

as having key roles in the aging process, studies of further detail are necessary to pinpoint all related breakdown prod-

ucts, their roles in the changing physiology of the cell, and the cascade effects on the system as a whole. It is understood that

many other effects of aging, from DNA damage to plaques and toxins, play fundamental parts in neural degeneration. With increased examination of these factors, working in con-
cert with calpain-mediated proteolysis, it is hoped that there can be further understanding of the aging of the human brain

and its relationship to the pathology of neurodegenerative diseases [7].

Uncited references

[32,39,55].

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