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## Original Article

# Caspase-cleaved glial fibrillary acidic protein within cerebellar white matter of the Alzheimer's disease brain

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**Abstract:** Although the cerebellum is generally thought of as an area spared of Alzheimer's disease (AD) pathology, recent evidence suggests that balance and mobility dysfunction may be magnified in affected individuals. In the present study, we sought to determine the degree of pathological changes within the cerebellum utilizing an antibody that specifically detects caspase-cleaved GFAP within degenerating astrocytes. Compared to control subjects, application of this antibody, termed the GFAP caspase-cleavage product (GFAPccp) antibody, revealed widespread labeling in cerebellar white matter with little staining observed in grey matter. Staining was observed within damaged astrocytes, was often localized near blood vessels and co-localized with other markers of apoptosis including TUNEL and caspase-cleaved tau. Of interest was the association of beta-amyloid deposition in white matter together with GFAPccp in cerebellar AD sections. In contrast, utilizing the tangle marker, PHF-1, neurotic pathology was completely absent in AD cerebellar sections. It is suggested that the observed pathological changes found in the white matter of the cerebellum may contribute to the declined motor performance in AD.

**Keywords:** GFAP, caspase, cerebellum, Alzheimer's disease, neurofibrillary tangles, TUNEL, beta amyloid, immunohistochemistry, PHF-1

## Introduction

The cerebellum is a subcortical brain structure that is essential for learning and controlling movement [1]. The cerebellum does not initiate movement, but it contributes to the proper timing, coordination, and fine-tuning precision of movement [2]. Consequently, lesions in the cerebellum may lead to postural instability, loss of balance and of normal gait [3]. While Alzheimer's disease (AD) is characterized by the extent of plaques and tangles predominantly within the hippocampus and cortex, the cerebellum is thought to be largely spared of pathology and is often used as an area of comparison (for example see, [4, 5]). In this regard, studies in AD have documented the relative lack of neuropathological changes in the cerebellum including the integrity of granule cell number and density [6]. However, several studies have indicated that despite the absence of tau pathology [7, 8], it is not uncommon to find diffuse amy-

loid deposits both in the granular cell layer as well as in white matter of the cerebellum of AD subjects [9-11].

In spite of the relative lack of pathology associated with the cerebellum, several studies have documented mobility dysfunction, loss of balance, and an increase risk for falls in Alzheimer's patients as compared to nondemented controls [12-16]. The motor impairments, including gait and balance dysfunctions in AD might suggest that pathological changes are evident in this structure that may occur independently of neurotic changes due to the lack of neuropathology found in the cerebellum. To examine this possibility, we tested for the presence of white matter changes utilizing an antibody (GFAPccp) that specifically detects caspase-cleaved GFAP within degenerating astrocytes of the AD brain [17-19]. Using this antibody, we now document the labeling of damaged astrocytes along blood vessels in cerebellar white matter of AD sub-

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jects. Moreover, there was a clear association in the pattern of labeling of the GFAPccp antibody with beta-amyloid deposition suggesting a potential interaction between astrocytes and deposited beta-amyloid. The presence of pathological white matter changes within the cerebellum may contribute to the gait and balance abnormalities associated with AD.

### Materials and methods

#### *Antibody dilutions*

The rabbit GFAPccp (in house, 1:100). The anti beta-amyloid mAb 1560 clone 6E10 (1:400), and PHF-1 (mouse monoclonal, 1:1000). The mAb TauC3 (caspase-cleaved tau) was utilized at 1:100. To visualize beta-amyloid staining, sections were pretreated for 5 minutes in 95% formic acid. To assess apoptosis, the Apoptag peroxidase kit was employed according to the manufacturer's instructions (Millipore).

#### *Immunohistochemistry*

Autopsy cerebellum brain tissue from five neuropathologically confirmed AD cases and five neuropathologically normal cases were studied. Human brain tissue sections used in this study was provided by the Institute for Memory Impairments and Neurological Disorders at the University of California, Irvine. Free-floating 50 µm-thick sections were used for immunohistochemical studies as previously described [20].

For single labeling, all sections were washed with 0.1 M Tris-buffered saline (TBS), pH 7.4, and then pretreated with 3% hydrogen peroxide in 10% methanol to block endogenous peroxidase activity. Sections were subsequently washed in TBS with 0.1% Triton X-100 (TBS-A) and then blocked for thirty minutes in TBS-A with 3% bovine serum albumin (TBS-B). Sections were further incubated overnight at room temperature in various primary antibodies as listed above. Following two washes with TBS-A and a wash in TBS-B, sections were incubated in anti-rabbit or mouse biotinylated anti-IgG (1 hour) and then in avidin biotin complex (1 hour) (ABC, Elite Immunoperoxidase, Vector Laboratories, Burlingame, CA, USA). Antibodies were visualized using Brown DAB substrate (Vector Laboratories). For bright-field immunohistochemical double labeling, primary antibody labeling was detected using the brown DAB substrate (Vector Labs), while the second

label was visualized using the Blue SG substrate (Vector Labs).

#### *Immunofluorescence microscopy*

Immunofluorescence studies were performed by incubating sections with primary antibody overnight at a room temperature, followed by secondary anti-rabbit or mouse biotinylated anti-IgG (1 hour) and then in ABC (1 hour). Visualization was accomplished by using a tyramide signal amplification kit (Molecular Probes, Eugene, OR) consisting of Alexa Fluor 488-labeled tyramide (green, Ex/Em = 495/519). For immunofluorescence co-localization studies, antigen visualization was accomplished using an Alexa fluor 488-labeled tyramide (green, Ex/Em = 495/519) for one label and streptavidin Alexa fluor 555 (red, Ex/Em = 555/565) for the second label, both from Invitrogen (Carlsbad, CA).

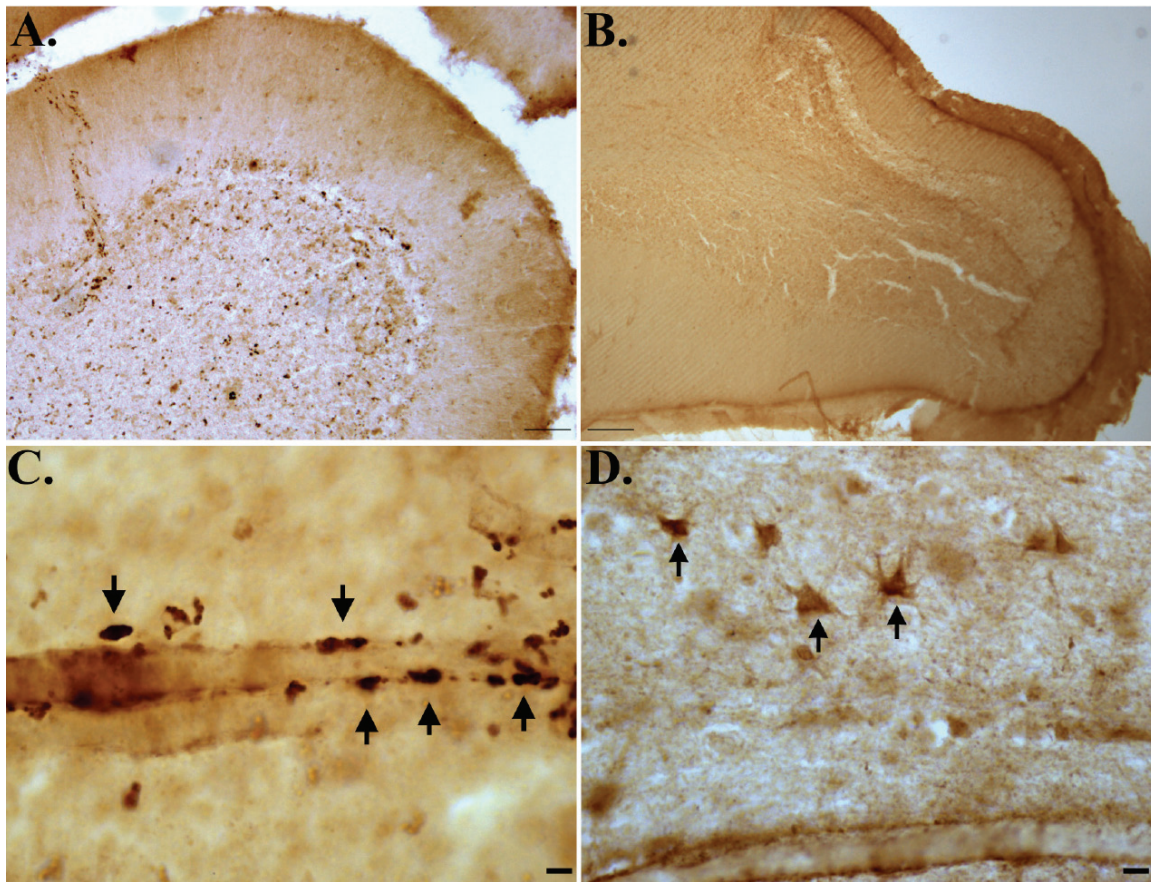
### Results

#### *Caspase-cleaved GFAP in cerebellar white matter of the AD brain*

As an initial approach, we screened cerebellum tissue sections from AD subjects or age-matched controls utilizing our in house GFAPccp antibody that detects caspase-cleaved GFAP within degenerating astrocytes [17]. Immunohistochemical analysis revealed widespread labeling of the GFAPccp antibody principally within white matter of AD cerebellum tissue sections (**Figure 1A**). In contrast, we observed very little staining of the GFAPccp antibody in age-matched control sections in either white or gray matter of the cerebellum (**Figure 1B**). Closer examination of the GFAPccp labeling in AD sections indicated strong staining of damaged astrocytes that were in close proximity to blood vessels. This type of staining was very similar to previous results that we found in hippocampal regions of the AD brain [17].

#### *The GFAPccp antibody colocalizes with other markers of apoptosis*

To confirm that labeling in cerebellar white matter of the GFAPccp antibody was occurring in astrocytes undergoing apoptosis, double-label experiments were performed using two additional markers for apoptosis including TUNEL



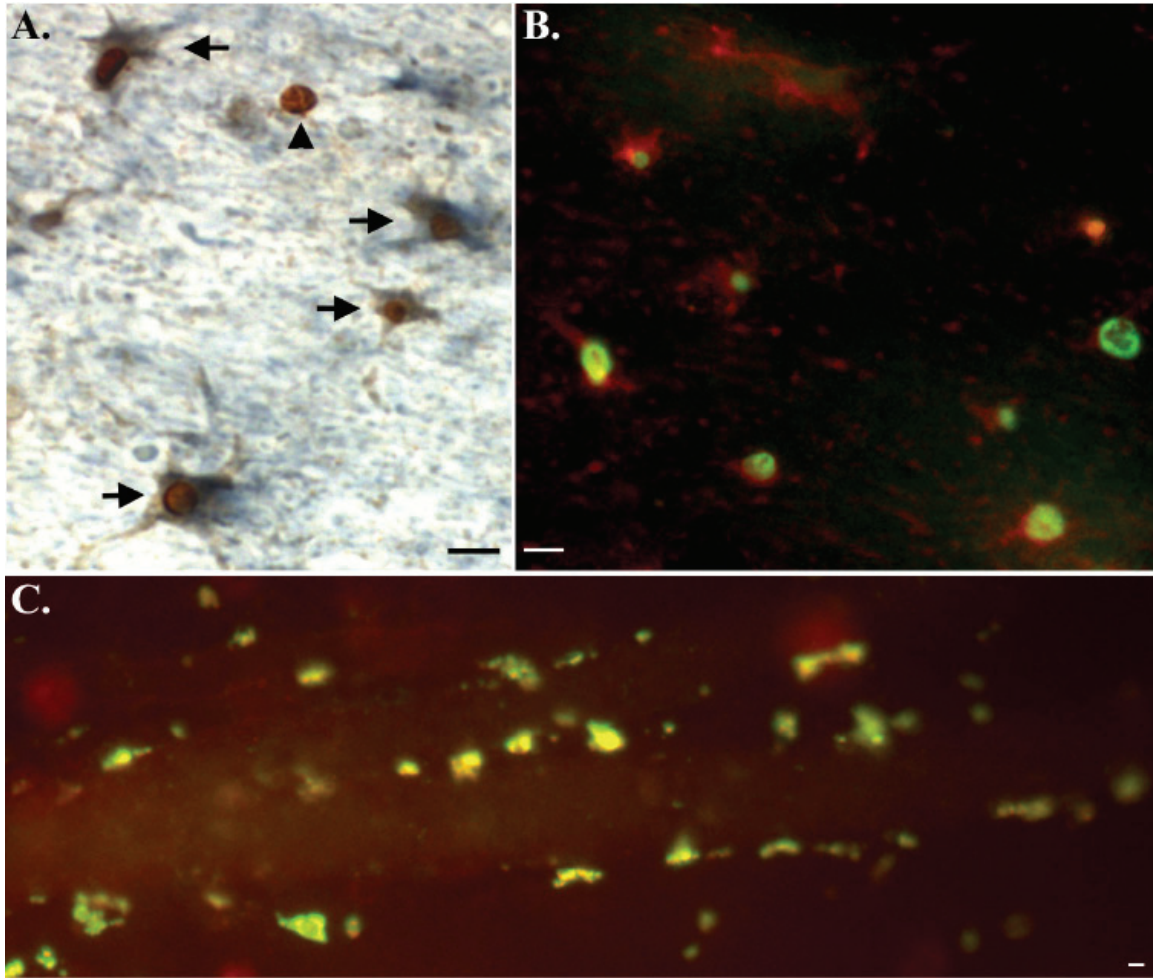
**Figure 1.** Caspase-cleaved GFAP in cerebellar white matter of the AD brain. A: Representative labeling in the cerebellum of an AD case at low magnification utilizing the GFAPccp antibody indicated widespread labeling primarily in white but not gray matter. B: Representative labeling in a control case with the GFAPccp antibody revealed a relative absence of labeling in both white and gray areas of the cerebellum. C and D: High magnification of staining with the GFAPccp antibody in representative AD cases indicating labeling of degenerating astrocytes along blood vessels in the white matter of the cerebellum (arrows, C and D). Scale bars are: A, 100  $\mu$ m; B, 300 $\mu$ m; C and D, 10  $\mu$ m.

and caspase-cleaved tau. Using TUNEL labeling, we confirmed both by bright field and immunofluorescence the colocalization with GFAPccp within astrocytes in cerebellar white matter of the AD brain (**Figure 2A** and **2B**). Additional experiments were carried out using an antibody that selectively detects caspase-cleaved tau. This antibody, termed TauC3 recognizes the C-terminal cleavage fragment of tau following cleavage at D421 and has been used as a marker for the activation of apoptosis in the AD brain [21]. Double-label immunofluorescence experiments utilizing GFAPccp together with the TauC3 antibody indicated strong colocalization between the two markers (**Figure 2C**). Double labeling appeared to be confined to a large extent within damaged astrocytes along blood vessels in cerebellar white matter (**Figure 2C**).

#### *Association between beta-amyloid deposition and GFAPccp in cerebellar white matter of the AD brain*

Experiments were performed to determine any possible relationship between the presence of caspase-cleaved GFAP with pathological features of AD including beta-amyloid deposition and neuritic pathology. Staining of cerebellum sections with anti beta-amyloid mAb 1560 clone 6E10 revealed diffuse, punctate beta-amyloid deposition predominantly within white but not gray matter of the AD brain (**Figure 3A** and **3B**). The diffuse pattern of labeling was in contrast to staining of beta-amyloid in AD frontal cortex sections that led to the characteristic labeling of senile plaques containing a well-defined core (**Figure 3C**). To identify a possible association between beta-amyloid deposition





**Figure 2.** Colocalization of the GFAPpccp antibody with markers of apoptosis in the cerebellum of the AD brain. A: Representative bright field microscopy image from the cerebellum of AD brain sections utilizing the GFAPpccp antibody (blue) together with TUNEL labeling (brown) revealed labeling of apoptotic nuclei within degenerating astrocytes (arrows, A). The arrowhead in Panel A represents TUNEL labeling in the absence of GFAPpccp staining. B: Confirmation of colocalization in Panel A was confirmed following double-label immunofluorescence imaging, with GFAPpccp fluorescence (red), TUNEL fluorescence (green), and where the two markers colocalized (yellow). C: Double-label immunofluorescence merged image utilizing an antibody to caspase-cleaved tau (red) and GFAPpccp (green). The data indicated colocalization between the two markers along a blood vessel in white matter of the cerebellum (yellow). All scale bars represent 10µm.

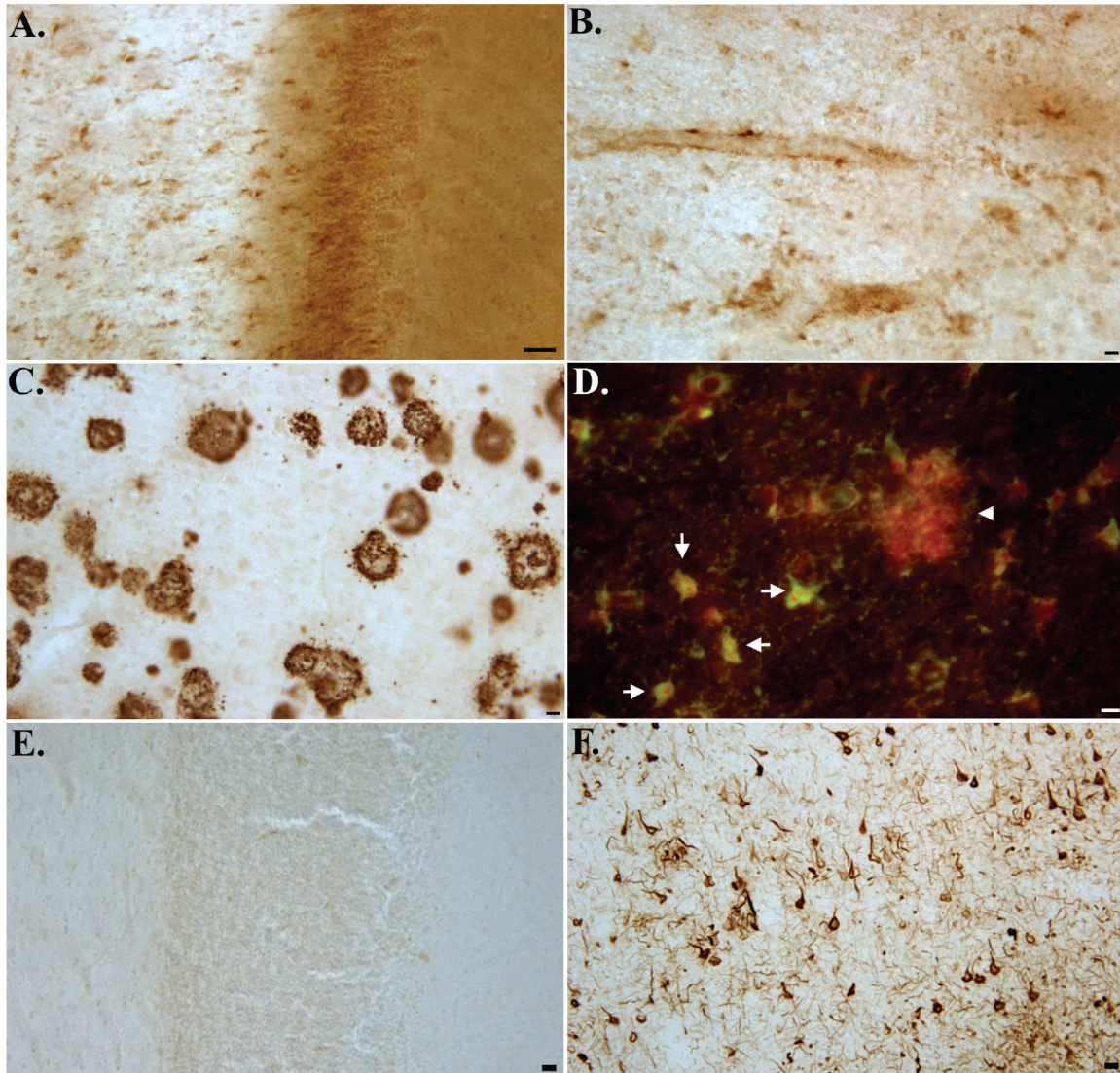
and GFAPpccp, double-labeling experiments were performed. In this case, the two markers often appeared localized within the same areas of white matter in the cerebellum (**Figure 3D**). In this regard, we often found damaged astrocytes labeled with GFAPpccp that were in close proximity to diffuse plaques (arrowhead, **Figure 3D**). In addition, GFAPpccp-labeled astrocytes appeared to exhibit punctate beta-amyloid labeling upon their surface (arrows, **Figure 3D**). In contrast to the diffuse beta-amyloid deposition, we could find no evidence of any neuritic changes as indicated by the complete absence of PHF-1 labeling in the cerebellum of AD sub-

jects (**Figure 3E**). As a positive control, we performed side-by-side staining of AD frontal cortex sections with PHF-1, leading to the extensive staining of NFTs and neuropil threads as expected (**Figure 3F**).

#### Discussion

Although the major symptoms associated with AD are cognitive in nature, emerging data suggests that motor dysfunction is an important component to the disease process. Thus, recent studies have documented gait and balance impairments as well as an increase risk

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**Figure 3.** Association of GFAPccp with beta-amyloid deposition within white matter of the cerebellum in the AD brain. A-B: Representative labeling with anti-Ab antibody, clone 6E10 at low (A) and high (B) magnification in representative cerebellum AD brain sections indicated diffuse staining predominately within white matter (left half of Panel A). Scale bars represent 20  $\mu$ m in Panel A and 10  $\mu$ m in Panel B. C: Illustrates comparison staining with the same antibody utilizing frontal cortex AD sections revealing labeling of amyloid plaques with a central core. D: Double-immunofluorescence labeling in the cerebellum of a representative AD case utilizing anti-Ab antibody, clone 6E10 (red) and GFAPccp antibody (green) indicated a close association between the two markers within white matter. Arrows in Panel D represent putative degenerated astrocytes while arrowhead in same panel designates a diffuse plaque. Scale bars in Panels C and D are equivalent to 10  $\mu$ m. E and F: Representative staining of the AD brain using the tangle marker, PHF-1 in cerebellum (E) and frontal cortex (F) indicated a complete lack of neuritic pathology in the cerebellum as compared to frontal cortex. Scale bars in Panels E and F represent 20 $\mu$ m.

for falls [12-16]. Because of the predominant role that the cerebellum plays in providing for precise timing for coordinated movement, a likely hypothesis for explaining motor dysfunction in AD is some underlying dysfunction in this brain structure. However, the cerebellum has the distinction of being spared from the classic pathology of AD, namely plaques and tangles

and because of this is often used as control region for comparison purposes (for example see, [4, 5]). Several studies have documented the presence of beta-amyloid deposition in the cerebellum [9-11] but there is very little evidence for any neuropathological changes including the presence of NFTs [7, 8]. The purpose of the present study was to examine the



cerebellum in AD for other pathological features in addition to beta-amyloid and NFTs. Numerous studies have suggested the activation of apoptotic pathways in the AD brain. In this regard, the activation of caspases and cleavage of critical proteins including beta-amyloid, tau, fodrin, and GFAP may promote the underlying pathology associated with AD (for review see [22]). To date, whether the activation of apoptotic pathways occurs in the cerebellum has not been investigated. Therefore, we examined cerebellar AD brain sections employing a custom-made, in house antibody (GFAPccp) that specifically detects caspase-cleaved GFAP within degenerating astrocytes [17-19]. Application of GFAPccp revealed widespread labeling in white matter of cerebellar AD sections, while little staining was noted in age-matched control sections. Specifically, GFAPccp labeled damaged astrocytes in white matter that were often localized near blood vessels. The morphological appearance of GFAPccp-labeled astrocytes suggested that these cells were severely damaged and exhibited characteristics of apoptosis. These results mirrored our previous findings with the GFAPccp antibody in AD hippocampal sections [17]. One important role of astrocytes is in the formation of the blood-brain barrier and in this role, astrocytes confer a protective role against hypoxia and aglycemia by extending end feet that encapsulate brain capillaries [23]. Our current results suggest that activation of apoptotic pathways and cleavage of cytoskeletal proteins, such as GFAP and tau, may be one of many factors that contribute to the compromised blood-brain barrier observed in AD [24-27].

Another finding of the present study was the apparent association between beta-amyloid deposition and caspase-cleaved GFAP in cerebellar white matter. Diffuse beta-amyloid staining was observed in white matter but was rarely found in gray matter of AD cerebellum sections. Moreover, double-label immunofluorescence experiments revealed punctate beta-amyloid staining on GFAPccp-labeled astrocytes (arrows, **Figure 3D**). We observed a similar relationship in AD hippocampal brain sections [17] and taken together the results suggest that astrocytes are activated and recruited to sites of beta-amyloid deposition. Several studies have now documented the important role that astrocytes may play in clearing beta-amyloid

via the ability to internalize deposited beta-amyloid peptides [28, 29]. In the process of clearing beta-amyloid, astrocytes themselves may be subjected to beta-amyloid-induced toxicity leading to the activation of apoptosis and eventual degeneration. Indeed, in a previous report we documented the ability of beta-amyloid to induce apoptosis and caspase-cleavage of GFAP in cultured astrocytes [17]. White matter labeling of degenerating astrocytes were independent of any neuropathological changes as evidenced by the complete lack of PHF-1 labeling in all regions of AD cerebellar tissue sections (**Figure 3E**).

In conclusion, results from the present study support the caspase cleavage of cytoskeletal proteins including tau and GFAP within astrocytes in cerebellar white matter of the AD brain. The cleavage of critical cytoskeletal proteins may lead to the breakdown of the framework of the astrocyte and contribute to astrocytic degeneration along blood vessels. It is suggested that these events may be initiated in astrocytes following their interaction with deposited beta-amyloid in white matter of the cerebellum. Although the cerebellum has historically been defined as a brain structure largely spared from AD pathology, recent clinical evidence indicating an increase risk for falls and impairment of gait and balance suggest otherwise. Our results provide a potential molecular mechanism involving astrocyte degeneration and disruption of the blood brain barrier in cerebellar white matter as a putative contributing factor to mobility impairments associated with AD.

### Abbreviations

AD, Alzheimer's disease; A $\beta$ , beta amyloid; CCP, caspase-cleavage product; GFAP, glial acidic fibrillary protein; NFTs, neurofibrillary tangles; PHF, paired helical filaments; TBS, tris-buffered saline.

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### References

- [1] Bastian AJ. Moving, sensing and learning with cerebellar damage. *Curr Opin Neurobiol* 2011; 21: 596-601.
- [2] Marr D. A theory of cerebellar cortex. *J Physiol* 1969; 202: 437-470.
- [3] Fine EJ, Ionita CC and Lohr L. The history of the development of the cerebellar examination. *Semin Neurol* 2002; 22: 375-384.
- [4] Yakushev I, Landvogt C, Buchholz HG, Fellgiebel A, Hammers A, Scheurich A, Schmidtman I, Gerhard A, Schreckenberger M and Bartenstein P. Choice of reference area in studies of Alzheimer's disease using positron emission tomography with fluorodeoxyglucose-F18. *Psychiatry Res* 2008; 164: 143-153.
- [5] Soonawala D, Amin T, Ebmeier KP, Steele JD, Dougall NJ, Best J, Migneco O, Nobili F and Scheidhauer K. Statistical parametric mapping of (99m)Tc-HMPAO-SPECT images for the diagnosis of Alzheimer's disease: normalizing to cerebellar tracer uptake. *Neuroimage* 2002; 17: 1193-1202.
- [6] Andersen K, Andersen BB and Pakkenberg B. Stereological quantification of the cerebellum in patients with Alzheimer's disease. *Neurobiol Aging* 2012; 33: 197 e111-120.
- [7] Azzarelli B, Muller J, Ghetti B, Dyken M and Conneally PM. Cerebellar plaques in familial Alzheimer's disease (Gerstmann-Straussler-Scheinker variant?). *Acta Neuropathol* 1985; 65: 235-246.
- [8] Aikawa H, Suzuki K, Iwasaki Y and Iizuka R. Atypical Alzheimer's disease with spastic paresis and ataxia. *Ann Neurol* 1985; 17: 297-300.
- [9] Fukutani Y, Cairns NJ, Rossor MN and Lantos PL. Cerebellar pathology in sporadic and familial Alzheimer's disease including APP 717 (Val->Ile) mutation cases: a morphometric investigation. *J Neurol Sci* 1997; 149: 177-184.
- [10] Joachim CL, Morris JH and Selkoe DJ. Diffuse senile plaques occur commonly in the cerebellum in Alzheimer's disease. *Am J Pathol* 1989; 135: 309-319.
- [11] Li YT, Woodruff-Pak DS and Trojanowski JQ. Amyloid plaques in cerebellar cortex and the integrity of Purkinje cell dendrites. *Neurobiol Aging* 1994; 15: 1-9.
- [12] Suttanon P, Hill KD, Said CM, Logiudice D, Lautenschlager NT and Dodd KJ. Balance and mobility dysfunction and falls risk in older people with mild to moderate Alzheimer disease. *Am J Phys Med Rehabil* 2012; 91: 12-23.
- [13] Pedroso RV, Coelho FG, Santos-Galduroz RF, Costa JL, Gobbi S and Stella F. Balance, executive functions and falls in elderly with Alzheimer's disease (AD): a longitudinal study. *Arch Gerontol Geriatr* 2012; 54: 348-351.
- [14] Mazteras Munoz V, Abellan van Kan G, Cantet C, Cortes F, Ousset PJ, Rolland Y and Vellas B. Gait and balance impairments in Alzheimer disease patients. *Alzheimer Dis Assoc Disord* 2010; 24: 79-84.
- [15] Mirolsky-Scala G and Kraemer T. Fall management in Alzheimer-related dementia: a case study. *J Geriatr Phys Ther* 2009; 32: 181-189.
- [16] Alexander NB, Mollo JM, Giordani B, Ashton-Miller JA, Schultz AB, Grunawalt JA and Foster NL. Maintenance of balance, gait patterns, and obstacle clearance in Alzheimer's disease. *Neurology* 1995; 45: 908-914.
- [17] Mouser PE, Head E, Ha KH and Rohn TT. Caspase-mediated cleavage of glial fibrillary acidic protein within degenerating astrocytes of the Alzheimer's disease brain. *Am J Pathol* 2006; 168: 936-946.
- [18] Rohn TT, Wirawan E, Brown RJ, Harris JR, Maslah E and Vandenabeele P. Depletion of Beclin-1 due to proteolytic cleavage by caspases in the Alzheimer's disease brain. *Neurobiol Dis* 2011; 43: 68-78.
- [19] Acarin L, Villapol S, Faiz M, Rohn TT, Castellano B and Gonzalez B. Caspase-3 activation in astrocytes following postnatal excitotoxic damage correlates with cytoskeletal remodeling but not with cell death or proliferation. *Glia* 2007; 55: 954-965.
- [20] Rohn TT and Catlin LW. Immunolocalization of influenza A virus and markers of inflammation in the human Parkinson's disease brain. *PLoS One* 2011; 6: e20495.
- [21] Gamblin TC, Chen F, Zambrano A, Abrahama A, Lagalwar S, Guillozet AL, Lu M, Fu Y, Garcia-Sierra F, LaPointe N, Miller R, Berry RW, Binder LI and Cryns VL. Caspase cleavage of tau: linking amyloid and neurofibrillary tangles in Alzheimer's disease. *Proc Natl Acad Sci U S A* 2003; 100: 10032-10037.
- [22] Rohn TT. The role of caspases in Alzheimer's disease; potential novel therapeutic opportunities. *Apoptosis* 2010; 15: 1403-1409.
- [23] Nagele RG, Wegiel J, Venkataraman V, Imaki H, Wang KC and Wegiel J. Contribution of glial cells to the development of amyloid plaques in Alzheimer's disease. *Neurobiol Aging* 2004; 25: 663-674.
- [24] Viggars AP, Wharton SB, Simpson JE, Matthews FE, Brayne C, Savva GM, Garwood C, Drew D, Shaw PJ and Ince PG. Alterations in the blood brain barrier in ageing cerebral cortex in relationship to Alzheimer-type pathology: a study in the MRC-CFAS population neuropathology cohort. *Neurosci Lett* 2011; 505: 25-30.
- [25] Popescu BO, Toescu EC, Popescu LM, Bajenaru O, Muresanu DF, Schultzberg M and Bogdanovic N. Blood-brain barrier alterations in age-



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- ing and dementia. *J Neurol Sci* 2009; 283: 99-106.
- [26] Berzin TM, Zipser BD, Rafii MS, Kuo-Leblanc V, Yancopoulos GD, Glass DJ, Fallon JR and Stopa EG. Agrin and microvascular damage in Alzheimer's disease. *Neurobiol Aging* 2000; 21: 349-355.
- [27] Zipser BD, Johanson CE, Gonzalez L, Berzin TM, Tavares R, Hulette CM, Vitek MP, Hovanesian V and Stopa EG. Microvascular injury and blood-brain barrier leakage in Alzheimer's disease. *Neurobiol Aging* 2007; 28: 977-986.
- [28] Pihlaja R, Koistinaho J, Malm T, Sikkila H, Vainio S and Koistinaho M. Transplanted Astrocytes internalize deposited beta-amyloid peptides in a transgenic mouse model of Alzheimer's disease. *Glia* 2008; 56: 154-163.
- [29] Nielsen HM, Mulder SD, Belien JA, Musters RJ, Eikelenboom P and Veerhuis R. Astrocytic A beta 1-42 uptake is determined by A beta aggregation state and the presence of amyloid-associated proteins. *Glia* 2010; 58: 1235-1246.