

Changes in Glial Fibrillary Acidic Protein Immunoreactivity in the Dentate Gyrus and Hippocampus Proper of Adult and Aged Dogs

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ABSTRACT. Astrocytes perform neuron-supportive tasks, repair and scarring process in the central nervous system. In this study, we observed glial fibrillary acidic protein (GFAP), a marker for astrocytes, immunoreactivity in the dentate gyrus and hippocampus proper (CA1-3 region) of adult (2-3 years of age) and aged (10-12 years of age) dogs. In the adult group, GFAP immunoreactive astrocytes were distributed in all layers of the dentate gyrus and CA1-3 region, except in the stratum pyramidale of the CA1-3 region. In the aged group, GFAP immunoreactivity decreased markedly in the molecular layer of the dentate gyrus. However, GFAP immunoreactivity in the CA1-3 region increased in all layers, and the cytoplasm of GFAP immunoreactive astrocytes was hypertrophied. GFAP protein levels in the aged dentate gyrus decreased; however, GFAP levels in the CA1-3 region increased. These results suggest that the morphology of astrocytes and GFAP protein levels in the hippocampal dentate gyrus and CA1 region are changed, respectively, with age.

KEY WORDS: aging, astrocyte, canine, glial fibrillary acidic protein, hippocampus.

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Astrocytes, one of the neuroglia, perform neuron-supportive tasks, including the biochemical support of endothelial cells, which form the blood-brain barrier, the uptake of neurotransmitters from the synaptic cleft, the release of energetic substrates that are essential to sustain high metabolic activities at the synapse, and a principal role in repair and scarring process in the brain [8]. Astrocytes are characterized by the expression of the intermediate filament, glial fibrillary acidic protein (GFAP) within their cytoplasm [4]. A well-known feature of reactive astrocytes increases the production of intermediate filaments, and increases the expression of GFAP [21].

The hippocampus is a very important brain region to control cognitive functions [6], and is the brain region most vulnerable to aging processes [17, 18]. In addition, the hippocampus is one of the initial brain regions displaying pathology and neuronal loss in Alzheimer's disease [26]. The dog is widely accepted for aging study, because dogs have the same β -amyloid sequence to human [12] and the extent of β -amyloid has also been linked to cognitive dysfunction in dogs [3, 10].

Although many studies on age-related changes of astrocytes in the central nervous system in many species are conducted [1, 2, 7, 9, 23, 25, 27], few reports have demonstrated

the GFAP expression in the aged dog brain. In this study, therefore, we investigated age-related changes in GFAP immunoreactivity and its protein levels in the hippocampus to elucidate the correlation between age and change in astrocyte morphology.

MATERIALS AND METHODS

Experimental animals: The present study used the progeny of German shepherd obtained from the special weapons and tactics (SWAT), South Korea. Male dogs were used at 2-3 years of age for adult group and 10-12 years of age for aged group. These animals did not show any clinical and other signs in neural disorders. The procedures for handling and caring for the animals adhered to the guidelines, which are in compliance with the current international laws and policies (NIH Guide for the Care and Use of Laboratory Animals, NIH Publication No. 85-23, 1985, revised 1996), and they were approved by the Institutional Animal Care and Use Committee at Hallym's Medical Center. All of the experiments were conducted to minimize the number of animals used and the suffering caused by the procedures used in this study.

Immunohistochemistry: For immunohistochemical analysis, adult and aged dogs ($n=5$ at each age) were anesthetized with intravenous injection of 0.15 mg/kg ketamine (Yuhan, Seoul, South Korea) and 0.05 mg/kg xylazine (Bayer Animal Health, Suwon, South Korea) and perfused transcardially with 0.1 M phosphate-buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde in 0.1 M phosphate-buffer (PB, pH 7.4). The tissue processing and immunohistochemical staining was conducted by the methods of our

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previous study [11]. In brief, tissues were serially sectioned into 30- μ m coronal sections, and they were stained with diluted rabbit anti-GFAP antibody (1:1,000, Chemicon, Temecula, CA, U.S.A.) and subsequently exposed to biotinylated goat anti-rabbit IgG (1:200, Vector, Burlingame, CA, U.S.A.) and streptavidin peroxidase complex (1:200, Vector). They were then visualized by staining with 3,3'-diaminobenzidine in 0.1 M Tris-HCl buffer (pH 7.2).

Western blot analysis: To confirm changes in GFAP levels in the hippocampus of adult and aged dogs, 2 animals in each group were sacrificed and used for Western blot analysis. After sacrificing them and removing the brain, the hippocampi were dissected with a surgical blade. The tissues were homogenized in 50 mM PBS (pH 7.4) containing 0.1 mM ethylene glycol bis (2-aminoethyl Ether)-N,N,N',N' tetraacetic acid (pH 8.0), 0.2% Nonidet P-40, 10 mM ethylenediamine tetraacetic acid (EDTA) (pH 8.0), 15 mM sodium pyrophosphate, 100 mM β -glycerophosphate, 50 mM NaF, 150 mM NaCl, 2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride and 1 mM dithiothreitol (DTT). After centrifugation, the protein level was determined in the supernatants using a Micro BCA protein assay kit with bovine serum albumin as the standard (Pierce Chemical, Rockford, IL, U.S.A.). Aliquots containing 50 μ g of total protein were boiled in loading buffer containing 150 mM Tris (pH 6.8), 3 mM DTT, 6% SDS, 0.3% bromophenol blue and 30% glycerol. The aliquots were then loaded onto a 10% polyacrylamide gel. After electrophoresis, the gels were transferred to nitrocellulose transfer membranes (Pall Crop, East Hills, NY, U.S.A.). To reduce background staining, the membranes were incubated with 5% non-fat dry milk in PBS containing 0.1% Tween 20 for 45 min, followed by incubation with rabbit anti-GFAP antiserum (1:1,000), peroxidase-conjugated goat anti-rabbit IgG (Sigma, St. Louis, MO, U.S.A.) and an ECL kit (Pierce Chemical).

Quantification of data: Thirty sections per animal were selected in order to quantitatively analyze the relative optical density (ROD) of GFAP immunoreactive structures in the hippocampus. The studied tissue sections were selected according to anatomical landmarks corresponding to the Figs. 54 and 55 of the Lim *et al.* dog brain atlas [14]. The corresponding areas of the hippocampus were measured on the monitor at a magnification of 25–50 \times . Images of all GFAP immunoreactive structures taken from 3 layers (strata oriens, pyramidale and radiatum in the hippocampus proper, and molecular, granule cell and polymorphic layers in the dentate gyrus) were obtained through an AxioM1 light microscope (Carl Zeiss, Göttingen, Germany) equipped with a digital camera (AxioCam, Carl Zeiss, Germany) connected to a PC monitor. Analysis of a region of interest in the hippocampus was performed using an image analysis system. Video images were digitized into an array of 512 \times 512 pixels corresponding to a tissue area of 140 \times 140 μ m (40 \times primary magnification). Each pixel resolution was 256 gray levels. The intensity of GFAP immunoreactivity was evaluated by means of an optical density (OD), which

was obtained after the transformation of the mean gray level using the formula: $OD = \log(256/\text{mean gray level})$. The OD of background was determined in unlabeled portions and the value subtracted for correction, yielding high OD values in the presence of preserved structures and low values after structural loss using NIH Image 1.59 software: A ratio of the relative optical density (ROD) was calibrated as %.

The result of the Western blot analysis was scanned, and the quantification of the Western blotting was done using Scion Image software (Scion Corp., Frederick, MD, U.S.A.), which was used to count ROD: A ratio of the ROD was calibrated as %.

Statistical analysis: The data shown here represent the means \pm SEM of experiments performed for each dentate area. Differences among the means were statistically analyzed by two-tailed Student *t*-test using SPSS program. Statistical significance was considered at $P < 0.05$.

RESULTS

GFAP immunoreactivity in the dentate gyrus: In the adult group, GFAP immunoreactive astrocytes (GFAP⁺ As) were detected in all layers of the dentate gyrus (Fig. 1A). Many cell bodies of GFAP⁺ As were distributed in the polymorphic layer. GFAP⁺ As in the dentate gyrus had thin processes and thread-like morphology: Especially, the processes of GFAP⁺ As in the molecular layer were well ramified.

In the aged group, although GFAP⁺ As were detected in all layers of the dentate gyrus, the morphology of astrocytes was different from that in the adult group (Fig. 1B), and the density of GFAP⁺ As in all layers was significantly lower than that in the adult group (Fig. 2A). Many GFAP⁺ As were detected in the subgranular zone in the polymorphic layer and showed hypertrophied cytoplasm. Especially, GFAP⁺ processes in the molecular layer were very low in density compared to those in the adult group (Fig. 2A).

GFAP immunoreactivity in the CA1–3 region: In the CA1–3 region of the adult group, GFAP⁺ As were detected mainly in the strata oriens and radiatum; however, GFAP⁺ As were rarely detected in and near the stratum pyramidale (Fig. 1C and 1E).

In the aged group, GFAP⁺ As were detected in all layer of the CA1–3 region (Fig. 1D and 1F): The density of GFAP⁺ structures in all layers was significantly increased compared to the adult group (Fig. 2B). Especially, GFAP immunoreactivity in the CA1–3 regions was significantly increased in the stratum pyramidale compared to that in the adult group (Fig. 2B). In addition, the cell bodies and processes in the aged group were hypertrophied compared to those in the adult group (Fig. 1D and 1F).

GFAP levels in the hippocampus: In this study, we found that changes in GFAP levels were somewhat different in the dentate gyrus and CA1 region in adult and aged dogs. GFAP protein levels in the dentate gyrus of aged dogs were lower than that in the adult group. However, GFAP protein levels in the CA1 region of aged dogs were higher than that

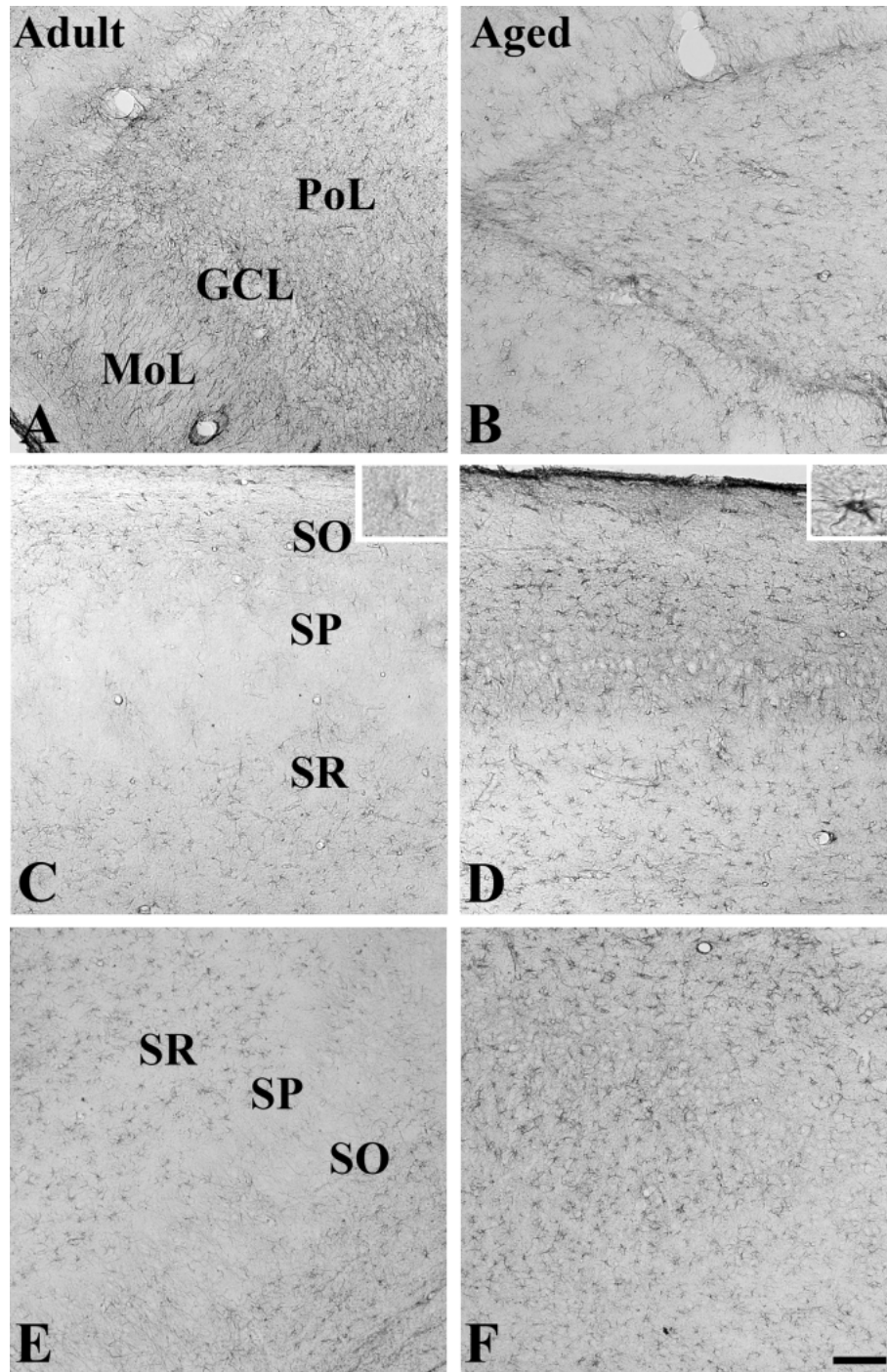


Fig. 1. GFAP immunohistochemistry in the dentate gyrus (A and B), CA1 (C and D) and CA3 region (E and F) of adult (A, C and E) and aged (B, D and F) dogs. GFAP⁺ structures in the adult dentate gyrus are abundant in all layers; however, GFAP⁺ structures in the aged dentate gyrus decrease. In the CA1–3 region of the adult group, GFAP⁺ structures are mainly detected in strata oriens and radiatum, however, GFAP immunoreactivity in the aged CA1–3 region is markedly increased in all layers. Note that GFAP⁺ astrocytes in the adult group have fine processes (inset in C), however, in the aged group, GFAP⁺ astrocytes show the hypertrophy of cytoplasm (inset in D). GCL, granule cell layer; MoL, molecular layer, PoL, polymorphic layer; SO, stratum oriens; SP, stratum pyramidale; SR, stratum radiatum. Bar = 100 μ m (A-F), 25 μ m (insets in C and D).

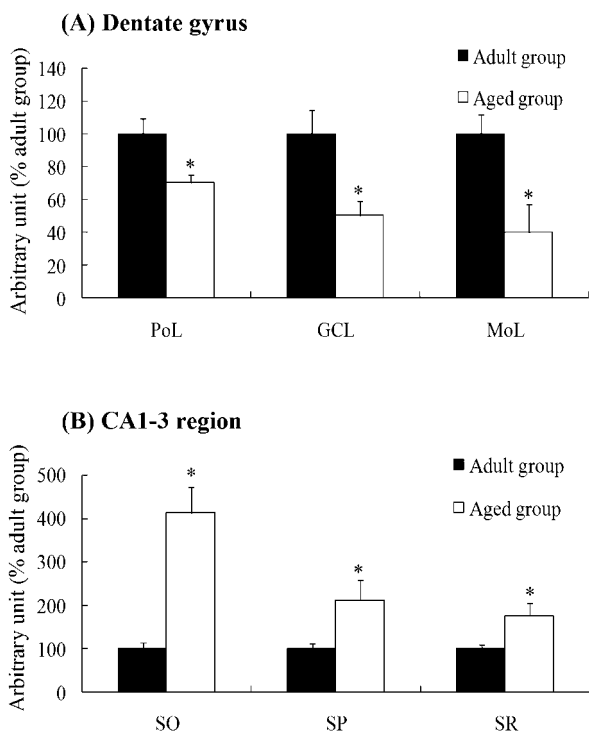


Fig. 2. Relative optical density (ROD) as % GFAP immunoreactivity in the dentate gyrus (A) and CA1–3 region (B) of adult and aged dogs (n=5 per group; * $P < 0.05$, significantly different from the adult group). Data are expressed as the means \pm SEM.

in the adult group (Fig. 3). GFAP proteins in the whole hippocampal homogenates were high in the aged group compared to those in the adult group (Fig. 3).

DISCUSSION

The increase of GFAP immunoreactivity may be associated with the morphological activation of astrocytes as astrogliosis under pathological condition. The astrogliosis is characterized by the hypertrophy of cellular processes of astrocytes and the up-regulation of GFAP in the CNS ischemia, trauma or neurodegeneration [22]. In addition, GFAP auto-antibodies are prevalent in the cerebrospinal fluids in dogs with various diseases [16, 24].

In this study, we used normal aged dogs and examined that there were age-related differences in GFAP immunoreactivity and its protein levels in the dentate gyrus and CA1–3 region between adult and aged dogs. The GFAP immunoreactivity and protein levels were decreased in the dentate gyrus and markedly increased in the CA1–3 region in aged dogs, however, GFAP⁺ As in both dentate gyrus and CA1–3 region were hypertrophied.

There are some contradictory reports on the age-related changes of GFAP⁺ As in rodent brains. A few studies reported that the significant age-related changes of astrocytes were not examined in the mouse hippocampus [15, 19]; however, many studies showed the age-related elevations of GFAP mRNA in the rat hippocampus and striatum [20], protein levels in the mouse striatum [13, 21] and the number of GFAP⁺ As in various brain regions (*i.e.*, hippocampus, cerebral cortex and striatum) [1, 2, 7, 9, 23, 25, 27]. However, these studies did not demonstrate the changes of

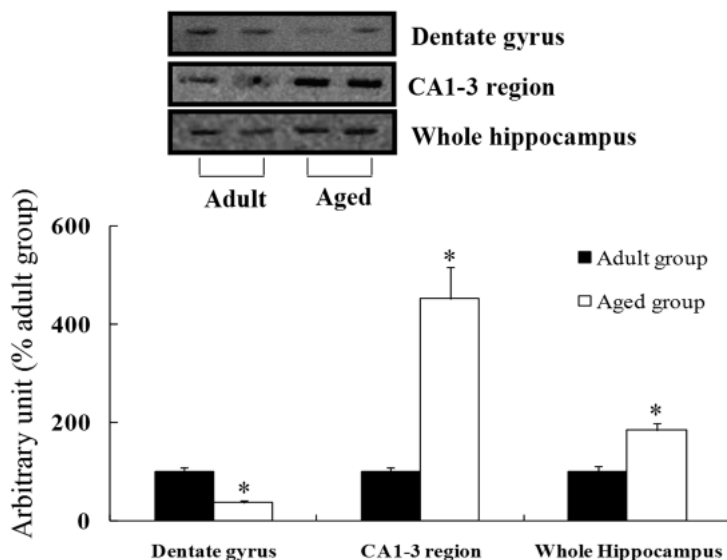


Fig. 3. The top panel shows Western blot analyses for GFAP in the dentate gyrus, CA1–3 region and whole hippocampus of the adult and aged group. The bottom panel shows relative optical density (ROD) as % of immunoblot bands (* $P < 0.05$, significantly different from the adult group). Data are expressed as the means \pm SEM.

GFAP immunoreactivity in the subregions of the dog hippocampus.

In this study, we found that GFAP⁺ structures were markedly decreased in the dentate gyrus in aged dogs. There is no study on decrease in GFAP⁺ structures in the dentate gyrus in aged animals. It has been reported that bouton density was significantly decreased within 200 μ m of compact β -amyloid plaques in the outer molecular layer of the dentate gyrus at 15–18 months of age in Tg 2576 mice [5]. Therefore, we suggest that the decrease of GFAP⁺ structures in the aged dentate gyrus may be related to the decrease of synapses.

On the other hand, it has been reported that moderate astrocytic gliosis was observed in the aged dog hippocampus [2, 25]. In addition, electron microscopy demonstrated the increased number of profiles of degenerative neural components in the vicinity of hypertrophic astrocytes in the cerebral cortex of the aged dogs [25]. However, these studies did not demonstrate the changes of GFAP immunoreactivity in the subregions of the dog hippocampus: They studied on the overall changes of GFAP⁺ As in the whole dog hippocampus. In conclusion, our present study shows that, in the aged dog, GFAP immunoreactivity is markedly increased in the CA1–3 regions, but slightly decreased in the dentate gyrus. The regional differences in GFAP⁺ As may be associated with neuronal vulnerability in these subregions.

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REFERENCES

- Bjorklund, H., Eriksdotter-Nilsson, M., Dahl, D., Rose, G., Hoffer, B. and Olsen, L. 1985. Image analysis of GFA-positive astrocytes from adolescence to senescence. *Exp. Brain Res.* **58**: 163–170.
- Borrás, D., Ferrer, I. and Pumarola, M. 1999. Age-related changes in the brain of the dog. *Vet. Pathol.* **36**: 202–211.
- Cummings, B.J., Head, E., Ruehl, W.W., Milgram, N.W. and Cotman, C.W. 1996. The canine as an animal model of human aging and dementia. *Neurobiol. Aging* **17**: 259–268.
- Dahl, D. and Bignami, A. 1985. Intermediate filaments in nervous tissue. *Cell Muscle Motil.* **6**: 75–96.
- Dong, H., Martin, M.V., Chambers, S. and Csernansky, J.G. 2007. Spatial relationship between synapse loss and β -amyloid deposition in Tg2576 mice. *J. Comp. Neurol.* **500**: 311–321.
- Eichenbaum, H. 2001. The hippocampus and declarative memory: cognitive mechanisms and neural codes. *Behav. Brain Res.* **127**: 199–207.
- Hansen, L.A., Armstrong, D.M. and Terry, R.D. 1987. An immunohistochemical quantification on fibrous astrocytes in the aging human cerebral cortex. *Neurobiol. Aging* **8**: 1–6.
- Hatten, M.E., Liem, R.K., Shelanski, M.L. and Mason, C.A. 1991. *Astroglia in CNS injury.* *Glia* **4**: 233–243.
- Hayakawa, N., Kato, H. and Araki, T. 2007. Age-related changes of astrocytes, oligodendrocytes and microglia in the mouse hippocampal CA1 sector. *Mech. Ageing Dev.* **128**: 311–316.
- Head, E., Callahan, H., Muggenburg, B.A., Cotman, C.W. and Milgram, N.W. 1998. Visual-discrimination learning ability and beta-amyloid accumulation in the dog. *Neurobiol. Aging* **19**: 415–425.
- Hwang, I.K., Yoon, Y.S., Yoo, K.Y., Li, H., Sun, Y., Choi, J.H., Lee, C.H., Huh, S.O., Lee, Y.L. and Won, M.H. 2008. Sustained expression of parvalbumin immunoreactivity in the hippocampal CA1 region and dentate gyrus during aging in dogs. *Neurosci. Lett.* **434**: 99–103.
- Johnstone, E.M., Chaney, M.O., Norris, F.H., Pascual, R. and Little, S.P. 1991. Conservation of the sequence of the Alzheimer's disease amyloid peptide in dog, polar bear and five other mammals by cross-species polymerase chain reaction analysis. *Brain Res. Mol. Brain Res.* **10**: 299–305.
- Kohama, S.G., Gross, J.R., Finch, C.E. and McNeil, T.H. 1995. Increases of glial fibrillary acidic protein in the aging female mouse brain. *Neurobiol. Aging* **16**: 59–67.
- Lim, R.K.S., Liu, C.N. and Moffitt, R.L. 1960. A Stereotaxic Atlas of the Dog's Brain, Charles C, Thomas, Springfield.
- Long, J.M., Kalehua, A.N., Muth, N.J., Calhoun, M.E., Jucker, M., Hengemihle, J.M., Ingram, D.K. and Mouton, P.R. 1998. Stereological analysis of astrocytes and microglia in aging mouse hippocampus. *Neurobiol. Aging* **19**: 497–503.
- Matsuki, N., Fujiwara, K., Tamahara, S., Uchida, K., Matsunaga, S., Nakayama, H., Doi, K., Ogawa, H. and Ono, K. 2004. Prevalence of autoantibody in cerebrospinal fluids from dogs with various CNS diseases. *J. Vet. Med. Sci.* **66**: 295–297.
- Mesulam, M.M. 1999. Neuroplasticity failure in Alzheimer's disease: bridging the gap between plaques and tangles. *Neuron* **24**: 521–529.
- Miller, D.B. and O'Callahan, J.P. 2005. Aging, stress and the hippocampus. *Ageing Res. Rev.* **4**: 123–140.
- Mouton, P.R., Long, J.M., Lei, D.L., Howard, V., Jucker, M., Calhoun, M.E. and Ingram, D.K. 2002. Age and gender effects on microglia and astrocyte numbers in brains of mice. *Brain Res.* **956**: 30–35.
- Nichols, N.R., Day, J.R., Laping, N.J., Johnson, S.A. and Finch, C.E. 1993. GFAP mRNA increases with age in rat and human brain. *Neurobiol. Aging* **14**: 421–429.
- O'Callaghan, J.P. and Miller, D.B. 1991. The concentration of glial fibrillary acidic protein increases with age in the mouse and rat brain. *Neurobiol. Aging* **12**: 171–174.
- Pekny, M. and Pekna, M. 2004. Astrocyte intermediate filaments in CNS pathologies and regeneration. *J. Pathol.* **204**: 428–437.
- Pilegaard, K. and Ladefoged, O. 1996. Total number of astrocytes in the molecular layer of the dentate gyrus of rats at different ages. *Anal. Quant. Cytol. Histol.* **18**: 279–285.
- Shibuya, M., Matsuki, N., Fujiwara, K., Imajoh-Ohmi, S., Fukuda, H., Pham, N.T., Tamahara, S. and Ono, K. 2007. Autoantibodies against glial fibrillary acidic protein (GFAP) in cerebrospinal fluids from Pug dogs with necrotizing meningoencephalitis. *J. Vet. Med. Sci.* **69**: 241–245.
- Shimada, A., Kuwamura, M., Awakura, T., Umemura, T. and Itakura, C. 1992. An immunohistochemical and ultrastructural study on age-related astrocytic gliosis in the central nervous system of dogs. *J. Vet. Med. Sci.* **54**: 29–36.
- West, M.J., Kawas, C.H., Stewart, W.F., Rudow, G.L. and Troncoso, J.C. 2004. Hippocampal neurons in pre-clinical Alzheimer's disease. *Neurobiol. Aging* **25**: 1205–1212.
- Wu, Y., Zhang, A.Q. and Yew, D.T. 2005. Age related changes of various markers of astrocytes in senescence-accelerated mice hippocampus. *Neurochem. Int.* **46**: 565–574.