DOI 10.7251/VETJSR018N **UDK 591.18.084:537.531**
Original Scientific Paper

CHARACTERISTIC MORPHOLOGICAL CHANGES IN THE BRAIN OF OLD DOGS

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Abstract: Aging of brain tissue is a natural universal process accompanied by numerous morphological changes. Some characteristic age-related changes are visible macroscopically, while most can only be detected by histological analysis.
The aim of this study was to identify and describe characteristic changes in the brain of elderly dogs.
Brain tissue from 36 dogs, divided into two groups, was used in the study. Following necropsy, the brain tissue was fixed in formalin and processed using standard histological protocols. Tissue sections were stained using the following methods: hematoxylin and eosin (HE), Congo red, PAS, and immunohistochemical staining using the streptavidin-biotin technique (LSAB2). Polyclonal and monoclonal antibodies against beta-amyloid (Aβ1-14/Aβ1-42), a monoclonal antibody against ubiquitin, and a monoclonal antibody against metallothionein I and II were used as primary antibodies.
In the experimental group, macroscopic examination revealed thickening of the leptomeninges, cortical atrophy with narrowing of the gyri and widening of the sulci, and enlargement of the lateral brain ventricles. Microscopic examination of the brain tissue showed fibrosis of the meninges and blood vessel walls, neuronophagia, satellitosis, astrocytosis, astrogliosis, amyloid deposits, lipofuscin accumulation, the presence of ubiquitin-positive granules, and polyglucosan bodies. The brain regions most commonly affected by age-related changes included the hippocampus, frontal cortex, and parietal cortex. Amyloid deposits, confirmed by Congo red staining and immunohistochemistry, were present both in the walls of cerebral blood vessels and in the brain parenchyma.
During the aging process, numerous changes occur in the nervous system of dogs, affecting all components of nervous tissue, including neurons, glial cells, blood vessels, and meninges. Due to the similarities between age-related changes in the brains of dogs and humans, the dog may serve as a valuable experimental model for better understanding and describing these alterations in further studies.
Keywords: age-related changes, amyloid, brain, dog, lipofuscin.

INTRODUCTION

The aging process is a universal biological process during which homeostasis in the body becomes disrupted. In the brain of old dogs, changes occur in the brain parenchyma, meninges, choroid plexuses and blood vessels (Nešić et al., 2017; Dimakopoulos and Mayer, 2002). Macroscopic changes include shrinkage of the cerebral hemispheres (narrowing of the gyri and widening of the sulci), thickening and calcification of the cerebellum, enlargement of the lateral ventricles, and other alterations (Dimakopoulos and Mayer, 2002). Histological changes in the brain parenchyma of old dogs include amyloid accumulation, neuronal and glial alterations, thickening of the blood vessel wall, lipofuscin deposition, among others (Nešić et al., 2017; Dimakopoulos and Mayer, 2002). The literature has established that age-related changes in the dog brain occur spontaneously and slowly, with certain similarities to changes observed in the human brain (Pirici et al., 2011). Amyloid also accumulates extracellularly in the brain parenchyma forming characteristic plaques. Literature data indicate the presence of senile plaques and amyloid deposits in the walls of cerebral blood vessels in a wide rangeof animal species, including monkeys, dogs, bears, cats and camels (Pirici et al., 2011). Some authors suggest that amyloid may also be localized within nerve cells (Head et al., 2010; Head et al., 2008). Numerous research findings indicate that amyloid is neurotoxic because it stimulates the development of oxidative stress in the cell. Oxidative stress accompanies age-related changes and contributes toneuronal degeneration (Vargas et al., 2010; Devi et al., 2006; Figueroa et al., 2006; Fukui et al., 2007). Proteins involved in cellular defense of cells against oxidative stress include metallothioneins. These are proteins that bind heavy metals such as copper and zinc in the cell, thereby neutralizing their toxic effects. There are three groups of metallothioneins (MT) with specific roles: MT I and II neutralize peroxynitrate, while MT III neutralizes hydroxide radicals and peroxynitrate (Uchida, 2010; Uchida et al., 2002). The degradation of worn-out proteins in the cell is carried out by the ubiquitin proteasome system. (Giannini et al., 2013; Bingol and Shuman, 2005). Disruption of the ubiquitin-proteasome system can lead to the development of neurodegenerative diseases in humans (Upadhya and Hegde, 2007). Impaired functioning of this system results in incomplete degradation of proteins, lipids and damaged organelles, leading to their aggregation into indigestible lipofuscin granules (Gray and Woulfe, 2005). Lipofuscin is a so-called “aging pigment”, produced during the aging process in postmitotic long-lived cells (Jung et al., 2010; Gray and Woulfe, 2005).

The aim of the study was to identify and characterizethe regions of the dog brain where age-related changes are most prominent, as well as to describe the associated histological alterations.

MATERIALS AND METHODS

The study included 36 autopsied dogs, divided in two groups. Six dogs up to five years old were used as the control group, while the remaining 30 dogs over ten years old were classified as the experimental group. After autopsy, the dog brains were fixed for 72 hours in 10% neutral formalin, then sectioned coronally, and fixed for an additional 24 hours. The frontal cortex, parietal cortex, hippocampus, cerebellum, and medulla oblongata of the left hemisphere were selected for analysis. After fixation, tissue modeling was performed, and processed using standard histological protocols. The prepared tissue sections were stained using the following staining methods: hematoxylin-eosin, Congo red, Periodic acid Schiff (PAS) and immunohistochemical. Histochemical and immunohistochemical stainings |of the examined brain tissue were performed at the Department of Pathology, Faculty of Veterinary Medicine, University of Belgrade. Stained sections were analyzed using a light microscope (Olympus, BX51). Preparations stained with Congo red were also analyzed under polarized light. Selected sections displaying characteristic age-related changes were digitally photographedusing a light microscope (Olympus, BX51) with a digital camera (Olympus ColorView III).

For immunohistochemical examination, a three-stage indirect method was used with appropriate antigen retrieval.Immunohistochemical staining were used the streptavidin-biotin technique (commercial kit Labelled streptavidin-biotin LSAB 2 method, DAKO, Denmark) and the following primary antibodies : polyclonal antibody for beta amyloid (Aβ1-14, 1:400, manufacturer ABD serotec), antibody for beta amyloid (Aβ 1-42, 1:500, manufacturer Invitrogen), monoclonal antibody for ubiquitin (1:3000, manufacturer ABD serotec), monoclonal antibody for metallothionein (1:50, manufacturer DAKO). Antigen retrieval was performed thermally, proteolytically, or by incubation in formic acid. Inhibition of endogenous peroxidase activity was performed using a solution of 3% H2O2 in methanol for 15 minutes. During immunohistochemical staining, phosphate-buffered saline (PBS, pH 7.2-7.4) was used for all washing steps. 25% goat serum was used for the 20-minute preincubation phase. Primary antibodies were incubated in a humid chamber at room temperature for 60 minutes. Following washing, tissue sections were treated with detection kit components (Dako, Cytomation, LSAB 2). The chromogen diaminobenzidine (DAB+, DAKO) was used to visualize the antigen-antibody complex.

The following histological changes were evaluated in the brains of dogs: gliosis (astrogliosis and astrocytosis), satellitosis, neuronophagy, vascular alterations, amyloid and lipofuscin deposits, and the presence of metallothionein and ubiquitin. Amyloid deposits were demonstrated using both immunohistochemical staining and histochemical Congo red staining. Lipofuscin accumulation was visualized by staining tissue sections with the PAS method. The presence of ubiquitin and metallothionein was examined by immunohistochemical staining.

RESULTS

The following macroscopic changes were observed in the brains of dogs from the experimental group: atrophy (narrowing) of the gyri, widening of the sulci and lateral ventricles, and diffuse thickening of the leptomeninges. Narrowing of the gyri and widening of the sulci were observed exclusively in the oldest dogs, aged between 16.5 and 18 years. (Figure 1.).

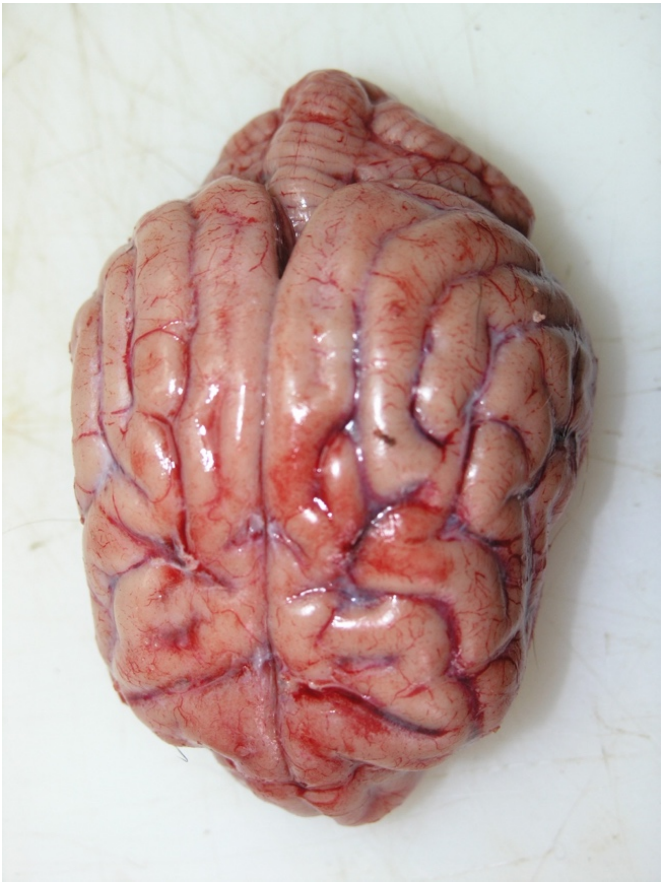

Figure 1. Dog, mixed breed, 17 years old, macroscopic appearance of the cerebellum (thickening whitish cloudy fields)

Atrophy of the cerebral gyri with consequent widening of the sulci was found in 5 (16.67%) dogs from the experimental group. Mild widening of the lateral ventricles was diagnosed in all dogs older than 15 years (19 dogs, 63% of the experimental group). Diffuse thickening of the meninges was observed in 23 older dogs (76%). The thickening of the leptomeninges was clearly visible at the level of the cerebral gyri as a whitish opacity of the meninges. Gliosis refers to glial hypercellularity in the central nervous system (CNS), resulting from the proliferation and/or hypertrophy of glial cells. Tissue sections were stained with hematoxylin and eosin and immunohistochemically labeled for metallothioneins to determine changes in astrocytes. In the CNS of the control group, gliosis was found in the frontal and parietal

cortex (50%) and the medulla oblongata (33.33%). In contrast to the control group, gliosis in the experimental group was found in all examined parts of the CNS, with the frontal cortex most frequently affected—present in 25 dogs (83.33%). Gliosis in the examined brain tissue was characterized by astrocytic changes, which can present in two forms: astrogliosis (hypertrophy of astrocytes) and astrocytosis (proliferation of astrocytes).

Immunohistochemical staining of dog brain tissue for metallothioneins I and II revealed that astrogliosis occurred more frequently than astrocytosis. In most dogs, metallothionein-immunopositive astrocytes appeared hypertrophied (Figure 2).

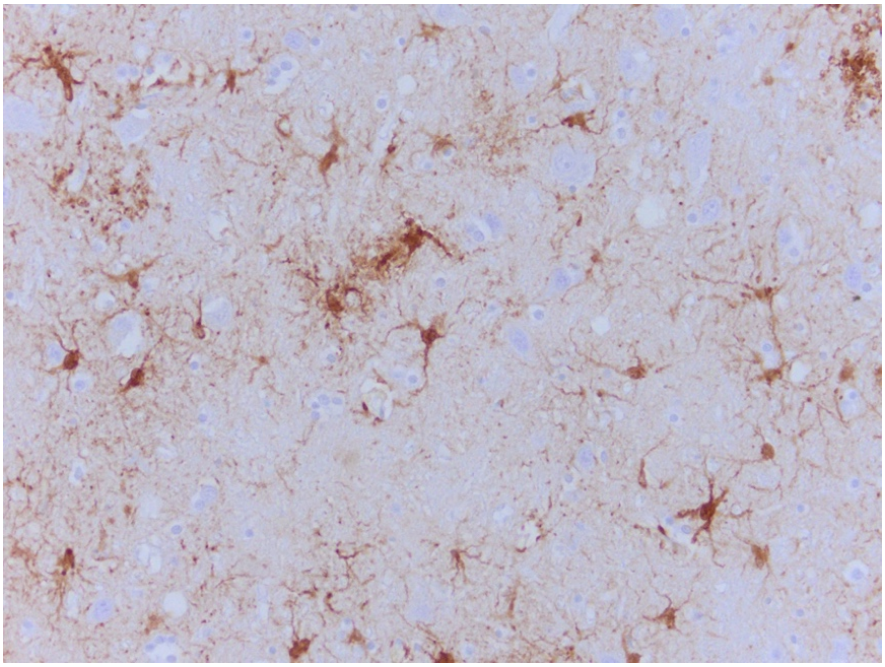

Figure 2. Dog, Labrador, 13 years old, medulla oblongata, immunohistochemical staining of metallothionein I and II antibodies in astrocytes (brown color), magnification 400X.

In dogs from the experimental group, astrogliosis was detected in 80 - 87.5% of cases in different regions of the CNS, while astrocytosis was present in 30-37.5% of cases. Both changes were more commonly found in the white matter than in the gray matter. The intensity of the immunohistochemical staining for metallothioneins was greater in older animals, indicating that glial changes ware more intense in older dogs.

Satellitosis and neuronophagy (Figure 3) are two processes in the CNS that accompany each other and serve as indicators of neuronal damage caused by harmful agents or alterations in the perineural microenvironment. Disruption of neuronal homeostasis activates perineural satellite oligodendroglia, leading to their hypertrophy and proliferation, while microglial cells phagocytose the damaged neurons.

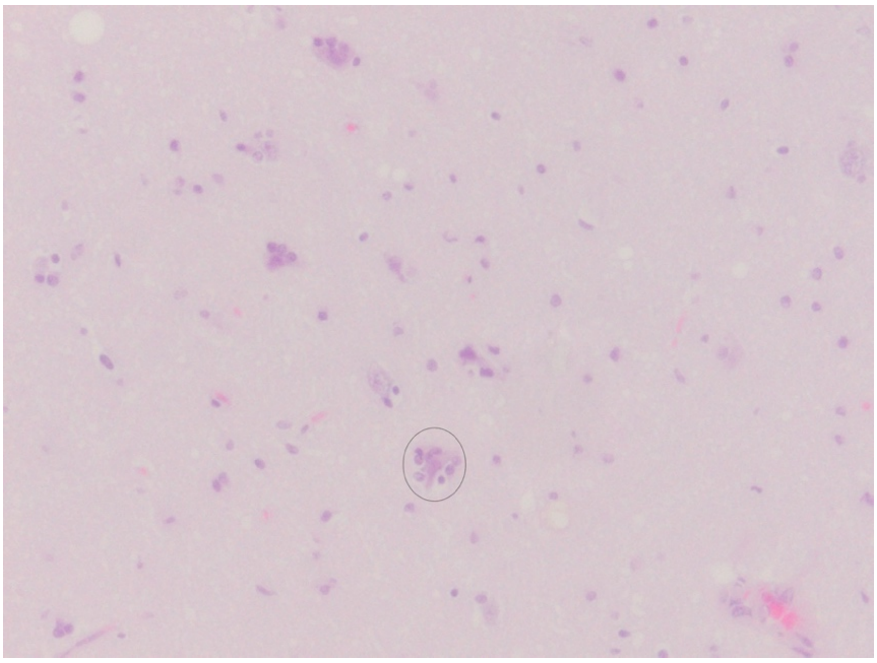

Figure 3. Dog, Irish Setter, 14 years old, hippocampus, hematoxylin eosin method, satellitosis and neuronophagia (circle), magnification 400X.

In dogs from the control group, satellitosis and neuronophagy were observed in the frontal cortex and medulla oblongata in three dogs, in the parietal cortex in two dogs, and in the hippocampus in one dog. Unlike the control group, satellitosis and neuronophagia were more frequently observed in the brains of dogs from the experimental group. Both changes were most commonly detected in the frontal cortex of dogs in the experimental group (76.67%). The cerebellum was the only part of the CNS in which satellitosis and neuronophagy were not observed, whereas in other examined regions (parietal cortex, hippocampus, and medulla oblongata), these changes were present in 30-70% of cases.

Pathohistological examination of the blood vessels revealed fibrosis of the vessel walls and the presence of amyloid deposits in the vessel walls. Fibrosis was observed in only one dog from the control group, affecting the hippocampus, medulla oblongata and cerebellum. In the brains of dogs from the experimental group, fibrosis was a very common finding in the frontal (70%) and parietal cortex (63.33%) and hippocampus (66.67%), while it occurred in a smaller percentage in the cerebellum (40%) and medulla oblongata (36.67%). It was noted that all dogs with vascular fibrosis in the brain tissue were older than13 years.

The presence of amyloid deposits in the brains of dogs was proven by histochemical staining by Congo red (Figure 4) and immunohistochemical staining (Figure 5). The presence of amyloid was not proven in the brains of dogs from the control group. However, in the brains of dogs from the experimental group, amyloid deposits were identified in both the blood vessel walls and the parenchyma using Congo red staining and immunohistochemical technique. The vast majority of dogs, in whose brain parenchyma amyloid deposits were detected, were older than 15 years.

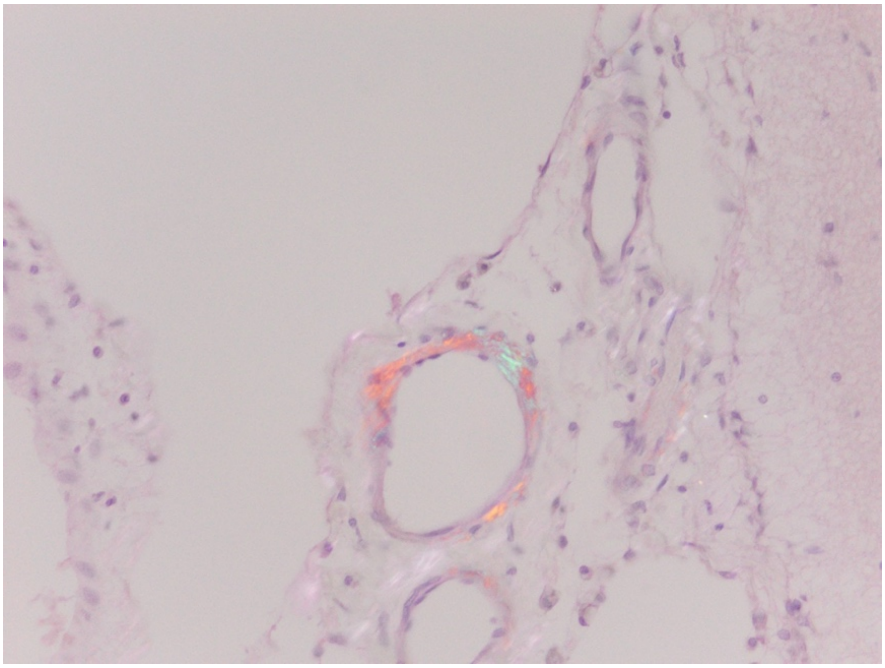

Figure 4. Dog, mixed breed, 17 years old, frontal cortex, Congo red staining, amyloid in the meningeal blood vessel wall (polarized light, fluorescent yellow-green color), magnification 200X.

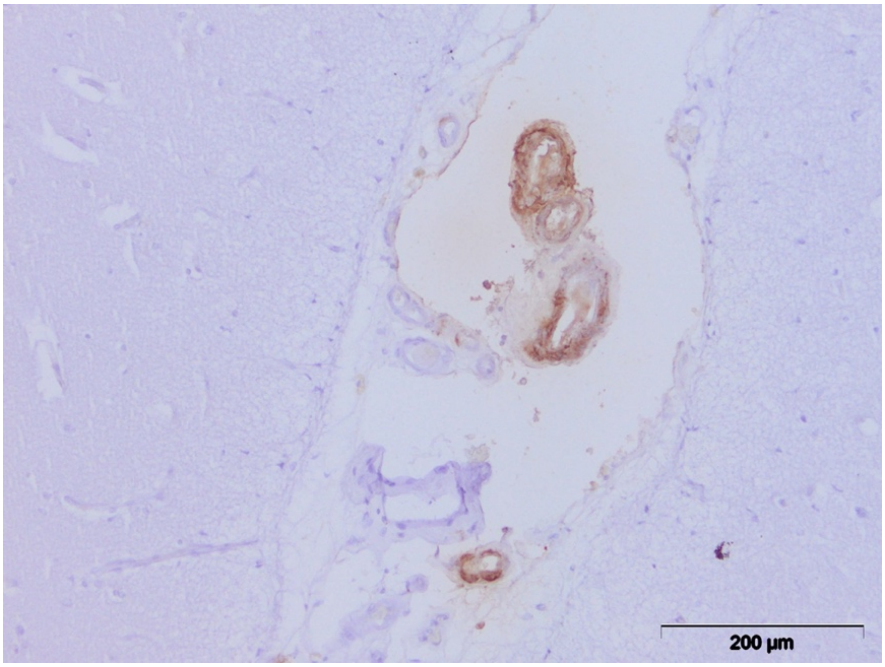

Figure 5. Dog, mixed breed, 17 years old, frontal cortex, immunohistochemical staining with Aβ 1-42 antibody, amyloid in meningeal blood vessels (brown), magnification 600X.

Histochemical staining with Congo red revealed amyloid deposits in the walls of meningeal and parenchymal blood vessels in all examined brain regions, except the

medulla oblongata, in dogs from the experimental group. The amyloid deposits in the form of diffuse plaques in the brain parenchyma did not stain positively with Congo red. The blood vessel walls of the frontal cortex were most frequently affected by amyloid deposition, with Congo red positivity observed in 63% of cases, while the cerebellum was the least affected (16.67%). In the parietal cortex and hippocampus, amyloid was proven by Congo red in about 40% of cases. In all dogs in which the amyloid was detected by Congo red, the deposits were localized within the walls of meningeal blood vessels. Amyloid deposits in the walls of parenchymal blood vessels were identified in 13 dogs in the frontal cortex, 9 dogs in the parietal cortex, 7 dogs in the hippocampus, and 4 dogs in the cerebellum.

Immunohistochemical amyloid was proven using two antibodies: anti-beta amyloid (Aβ 1-14) and anti-beta amyloid (Aβ 1-42). In the brain tissue of dogs from the experimental group, amyloid was detected in three forms of accumulation: within the walls of blood vessels, as diffuse plaques in the parenchyma, and intracellularly within neurons. All three forms of amyloid deposits were detected using the Aβ 1-14 antibody, while the Aβ 1-42 antibody demonstrated amyloid deposits in cerebral blood vessels and in the parenchyma in the form of senile plaques. In neurons, amyloid deposits were detected with the Aβ1-14 antibody in the hippocampus, frontal and parietal cortex in seven dogs (Figure 6).

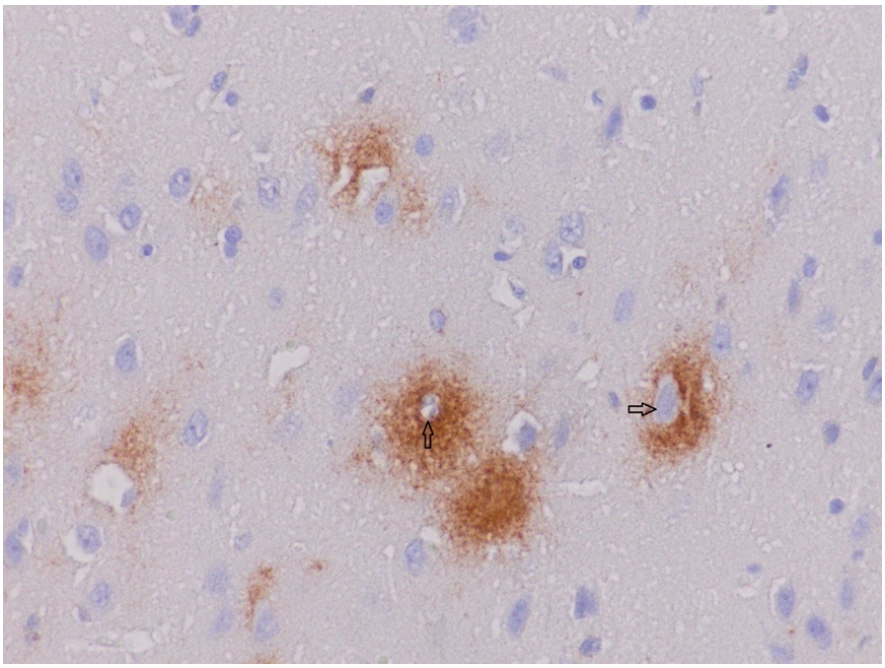

Figure 6. Dog, mixed breed, 15 years old, hippocampus, immunohistochemical staining with Aβ 1-42 antibody, amyloid in plaques (brown), preserved neurons (arrows), magnification 600X.

However, the neurons positive for amyloid were diffusely distributed around the senile plaques, and not in their center. Among the oldest dogs in the experimental group, intraneuronal amyloid deposits were observed in five dogs older than 16 years and in

two dogs aged 13 years. Amyloid deposits were also detected in the walls of both meningeal and parenchymal blood vessels. In some blood vessels, the deposits surrounded the entire blood vessel in the form of a ring, and in some, the deposits were intermittent. The amyloid was not detected in the blood vessels of the medulla oblongata, while it was present in all other examined brain regions The regions most frequently affected by amyloid deposition were the frontal cortex, followed by the parietal cortex and hippocampus, and finally the cerebellum. In meningeal blood vessels, amyloid was detected in the frontal region of 21 dogs (70%), the parietal region in 16 dogs (53.33%), the hippocampus in 15 dogs (50%), and the cerebellum in 5 dogs (16.67%). Amyloid deposits in parenchymal blood vessels were found in the parenchymal blood vessels of the brain in the frontal cortex in 70% of dogs, in the parietal cortex and hippocampus in approximately 50% of dogs, and in the cerebellum in 13.33% of dogs from the experimental group.

Amyloid deposits in diffuse plaques in the brains of dogs were demonstrated using both antibodies, in 19 dogs (63.33%) with Aβ1-14, and in 18 dogs (60%) with Aβ1-42. In the center of some plaques, there were “trapped” preserved neurons. The neurons within the diffuse plaques were negative for beta amyloid (Figure 7). The frontal cortex was the brain region most commonly affected by this change. In other brain regions where amyloid was detected, the shape and size of the plaques varied.

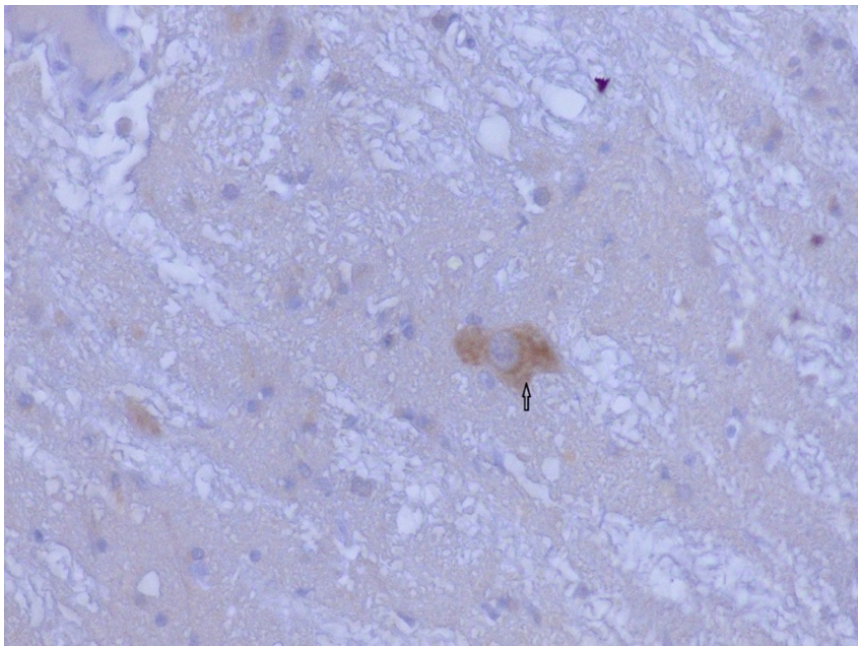

Figure 7. Dog, American Staffordshire Terrier, 14 years old, hippocampus, immunohistochemical staining with Aβ 1-14 antibody, amyloid in neurons (brown, arrow), magnification 600X.

A positive ubiquitin immunoreaction was not diagnosed in the brains of dogs from the control group. In the brains of dogs from the experimental group, an immunopositive signal for ubiquitin was detected in varying percentages. Ubiquitin-positive

immunoreactions were found in the hippocampus, frontal and parietal regions in a large number of dogs from the experimental group (83-87%), while in the cerebellum and medulla oblongata positivity was observed in approximately50% of dogs (Figure 8).

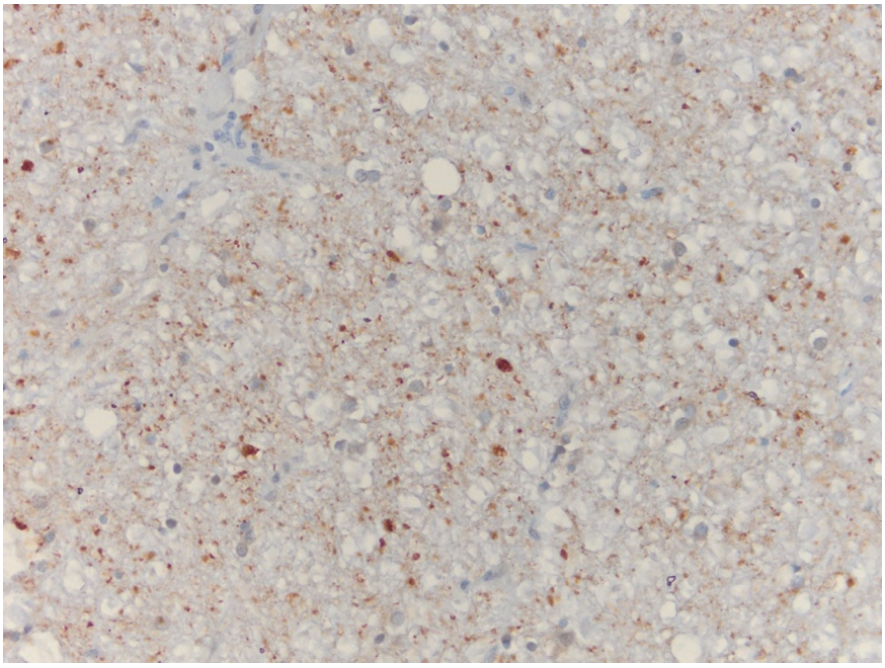

Figure 8. Dog, Irish Setter, 14 years old, hippocampus, immunohistochemical staining with ubiquitin antibody, positive reaction in dark brown neurons, magnification 400X.

The senile pigment lipofuscin was detected in neurons by hematoxylin-eosin and PAS methods. Lipofuscin had a granular structure that was slightly lighter in color than the pink cytoplasm. In tissue samples stained by the PAS technique, lipofuscin was also demonstrated in neurons of older dogs, i.e. dogs from the experimental group (Figure 9). Lipofuscin accumulations in neurons were observed in varying percentages across all examined brain regions. Lipofuscin granules were localized perinuclearly, as well as within the dendritic “stem” and the axonal “hill”.

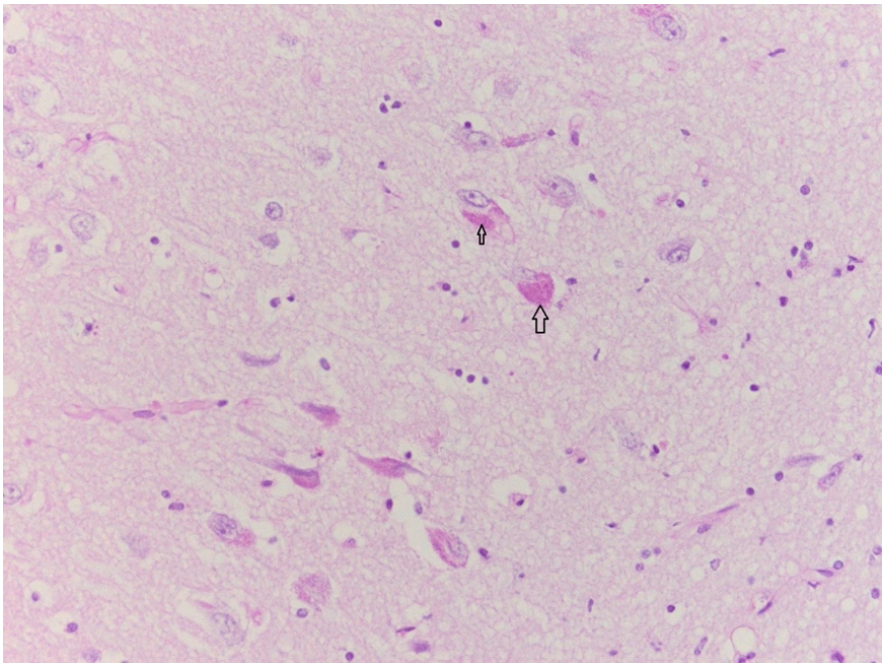

Figure 9. Dog, mixed breed, 18 years old, hippocampus, PAS method, lipofuscin granules in red neurons, magnification 400X.

In the large neurons of individual nuclei of the medulla oblongata, lipofuscin was present in 93.33% of cases, while in the other examined segments, it was detected in 56% and 74% of dogs from the experimental group.

DISCUSSION

In humans, the most commonly diagnosed macroscopic age-related changes in brain tissue are brain shrinkage and cerebral ventricle enlargement. These changes are thought to result from the loss of cortical neurons during human aging. In animals, unlike in humans, macroscopic age-related changes in the brain have been described less frequently in the literature. In our study we found that dogs older than ten years had atrophy of the cerebral gyri and widening of the cerebral sulci in 16.67% of cases, mild dilation of the cerebral ventricles in 63% of dogs and thickening of the leptomeninges in 76% of cases. The obtained results indicate a correlation between the aging process and the development of macroscopic changes in the brain. Histologically, astrogliosis (approximately 80%), astrocytosis (approximately 35%), satellitosis, and neurophagia (approximately 75%) were found. These findings of histological changes, together with macroscopic changes, support the conclusion that the brain tissue of older dogs shows a significant neuronal loss, which may affect the loss of function of the affected brain regions. Our study found that the hippocampus, frontal and parietal cortex were the brain regions most commonly affected by age-related changes. Based on these findings, a decline in cognitive abilities can be expected in older dogs, similar to the cognitive decline observed in aging humans due to comparable

neuropathological changes. All of these observations are consistent with previously published data regarding the aging process.

Vascular changes in the brain during aging significantly affect the vitality of neurons and glial cells, thereby affecting the preservation of central nervous system (CNS) structure and function. The changes in blood vessels during aging can occur in the perivascular space, within the vessel wall itself, or in the lumen of the blood vessel. The most significant and frequent changes associated with aging process occur within the walls of cerebral blood vessels, leading to changes in vascular permeability. Altered vascular permeability disrupts the normal flow of glucose and oxygen to neurons and glial cells, resulting in hypoxia and nutrient deprivation, which subsequently damage CNS cells (Dimakopoulos and Mayer, 2002).

In our study, fibrosis of the cerebral blood vessel walls was a common finding in older dogs (up to 70% of dogs in the experimental group). The brain regions most frequently affected by fibrosis were the hippocampus (66.67%), frontal (70%) and parietal (63.33%) cortex. Fibrosis of the blood vessel walls in these regions likely alters vascular permeability, leading to disruption of nutrient supply to all cells of the nervous tissue. It can be concluded that fibrosis is an age-dependent change, as in our study, it was observed in all dogs older than 13 years. Only a small number of dogs from the experimental group exhibited narrowing of the lumens of individual cerebral blood vessels, and this was noted exclusively in the oldest dogs (older than 16 years). The obliteration of a small number of blood vessels only in very old dogs indicates that the volume of cerebral blood vessels remains largely preserved during the aging process until the final stages of life. This suggests that the main cause of hypoxia and reduced nutrient supply to neurons and glial cells in certain regions of the central nervous system is increased vascular permeability, due to impaired architecture of the blood vessel wall. Our results are in accordance with the literature, as some authors have shown that the volume of cerebral blood vessels is largely preserved (Dimakopoulos and Mayer, 2002).

The most significant change in the cerebral blood vessel wall during aging is the accumulation of amyloid (Aβ deposits), a process known as cerebral amyloid angiopathy (CAA). In humans, Aβ deposits are commonly found during aging, in patients with familial CAA, and in nearly all patients with Alzheimer's disease (Kumar-Singh, 2008). In our study, amyloid accumulation was found in the cerebral blood vessel walls in 70% of dogs over 10 years of age. Our findings are in accordance with the literature data, and based on this observations, it can be assumed that this change is age-dependent (Nešić et al., 2017).

Plaques, or extracellular amyloid deposits, in the dog brain were not stained by the Congo red method, but were detected immunohistochemically. These results indicate that the plaques present in the brain tissue of dogs from the experimental group are composed of Aβ42, which is consistent with the literature data describing diffuse-type plaques (Kumar-Singh, 2008; Dimakopoulos and Mayer, 2002).

Amyloid deposits in the brain of dogs from the experimental group dogs were immunopositive for both anti-Aβ1-14 and anti-Aβ1-42 antibodies. Extracellular

amyloid deposits were immunopositive in approximately 60% of dogs from the experimental group. In some cases, preserved neurons were observed within the plaques.

The experimental group consisted of dogs of different breed status, which is probably the reason why plaques were demonstrated in only 60% of the dogs. It is well known that certain dog breeds of dogs have a shorter lifespan, making them more prone to developing age-related changes earlier than breeds with longer lifespans. These findings are consistent with the literature and with the findings of other researchers and indicate that clinical signs of cognitive decline can be expected in older animals (Nešić et al., 2017; Dimakopoulos and Mayer, 2002).

The literature data indicate that amyloid, in addition to affecting blood vessels permeability and indirectly damaging nervous tissue, can have a direct toxic effect on central nervous system cells. It exerts its direct effect by binding to specific receptors on cell membranes or by altering the architecture of cell membranes. Amyloid is also thought to generate reactive oxygen species by reacting with redox-active metals. The generation of free radicals in the cell initiates a series of chain reactions that ultimately lead to cell death (Vargas et al., 2010). Elevated levels of free radicals in the cell trigger the activation of defense mechanisms to eliminate them. In our study, the obtained results indicate an increased expression of metallothionein I and II in astrocytes, which participate in the defensive response against free radicals in the central nervous system. Our study also observed the reactivity of astrocytes around the plaques, but the reactivity of other glial cells was not observed. This may indicate that astrocyte reactivity is a consequence of neuronal damage or neuronal changes occurring during the aging process (. In older animals, a decrease in cognitive abilities should be expected due to the development of age-related changes in the hippocampus, frontal and parietal cortex, as indicated by astrocyte reactivity. Also, the results of our study indicate more intense changes in the white matter, because the intensity of the immunopositive signal for metallothioneins I and II was stronger in the white matter compared tothe gray matter.

It is known that protein degradation by the proteasome begins with the binding of ubiquitin. Ubiquitin is a highly conserved protein that, in addition to marking proteins that will be proteolytically degraded, has numerous other physiological roles in the cell. In the human population, ubiquitin immunopositive granules occur during aging, but also in some neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, motor neuron disease, etc. (Upadhya and Hegde, 2007).

Proteolytic processes during aging are intense, contributing to cellular damage within the nervous system. Our results from immunohistochemical staining for ubiquitin are in accordance with the literature data. In the study, a positive immunoreaction for ubiquitin was observed in 83-87% of the dogs from the experimental group in the hippocampus, frontal and parietal regions, and in 50% of the dogs in the medulla oblongata and cerebellum, while no positive immunoreaction was observed in the control group. These findings suggest that the amount of ubiquitin was significantly increased in nervous system cells of older individuals. The immunopositive signal was

present both in the cytoplasm of the cells and in the nuclei of the central nervous system cells in dogs from the experimental group. This suggests that nuclear proteins were bound to ubiquitin or that DNA repair mechanisms were activated. All this indicates that the aging process causes damage to DNA and nuclear proteins, impairing nuclear functionality and, consequently, overall cell function. Disruption of cellular functionality results in the formation of lipofuscin, representing the incomplete degradation of worn-out cellular organelles, primarily mitochondria. It is considered that PAS positivity of lipofuscin originates from insoluble lipid components, since PAS-positive substances are generally considered to be glycolipids or glycoproteins (Benavides et al., 2002). Our results are in accordance with the literature, showing that the amount of lipofuscin accumulated in dogs from the experimental group varied, with the most intense accumulation observed in the oldest dogs. Additionally, the amount of deposited lipofuscin differed between segments, but was highest in the medulla oblongata.

CONCLUSION

The nervous system of dogs undergoes numerous changes during the aging process, affecting all components of nervous tissue, including neurons, glial cells, blood vessels and meninges. One of the important factors is oxidative stress, which accelerates the aging process, with the brain being the most vulnerable organ compared to other tissues and organs. The structure and composition of nervous tissue largely explain this vulnerability, as the brain contains a high level of lipids—particularly unsaturated fatty acids, along with a high metabolic rate and elevated levels of reactive microelements. These characteristics promote the generation of free radicals, leading to lipid peroxidation and protein oxidation. Protein oxidation reduces the ability of neurons to maintain normal impulse transmission due to disruption of synapses and compromising the integrity of the cytoskeleton, which is essential for intracellular transport. Disruption of central nervous system homeostasis also leads to the accumulation of extracellular amyloid deposits in the neuropil and blood vessel walls, intracellular accumulation of lipofuscin, and the formation of ubiquitin granules and globules. Glial cells react to disturbances in the neuronal microenvironment, leading primarily to astrogliosis and, to a lesser extent, astrocytosis. All of the above changes observed in the brain of old dogs are the cause of cognitive dysfunction, but they are not fully understood and should be investigated in more detail in the future. It should also be emphasized that the results obtained in this study indicate that there is a similarity to age-related changes in humans, but also in patients with neurodegenerative diseases such as Alzheimer's disease. This leads to the conclusion that old dogs represent a valuable animal model for studying both the normal aging process and neurodegenerative disorders. Future research could address the extent to which relative age (the age of an individual in relation to the species' average lifespan) influences the development of brain changes, as well as the impact of these changes on cognitive abilities.

Acknowledgements: The work was supported by funds from the Ministry of Science, Technological Development and Innovation of the Republic of Serbia (Agreement No. 451-03-66/2024-03/200143).

Conflict of interest statement: The authors declare that there is no conflict of interest.

REFERENCES

Benavides S.H., Monserrat A.J., Fariña S. and Porta E.A. (2002): Sequential histochemical studies of neuronal lipofuscin in human cerebral cortex from the first to the ninth decade of life. *Archives of Gerontology and Geriatrics*, 34(3):219-231. DOI: 10.1016/S0167-4943(01)00223-0

Bingol B. and Schuman E.M. (2005): Synaptic protein degradation by the ubiquitin proteasome system. *Current Opinion in Neurobiology*, 15(5):536-541. DOI: 10.1016/j.conb.2005.08.016

Devi L., Prabhu B.M., Galati D.F., Avadhani N.G., Anandatheerthavarada H.K. (2006): Accumulation of amyloid precursor protein in the mitochondrial import channels of human Alzheimer's disease brain is associated with mitochondrial dysfunction. *Journal of Neuroscience*, 26(35):9057-9068. DOI: 10.1523/JNEUROSCI.2463-06.2006

Dimakopoulos A.C. and Mayer R.J. (2002): Aspects of neurodegeneration in the canine brain. *Journal of Nutrition*, 132(6):1579S-1582S.

Figueroa S., Oset-Gasque M.J., Arce C., Martínez-Honduvilla C.J., González M.P. (2006): Mitochondrial involvement in nitric oxide-induced cellular death in cortical neurons in culture. *Journal of Neuroscience Research*, 83(3):441-449. DOI: 10.1002/jnr.20756

Fukui H., Diaz F., Garcia S., Moraes C.T. (2007): Cytochrome c oxidase deficiency in neurons decreases both oxidative stress and amyloid formation in a mouse model of Alzheimer's disease. *Proceedings of the National Academy of Sciences of the USA*, 104(35):14163-14168. DOI: 10.1073/pnas.0701118104

Giannini C., Kloß A., Gohlke S., Mishto M., Nicholson T.P., Sheppard P.W., Kloetzel P.M., Dahlmann B. (2013): Polyubiquitin-substrate-degradative activity of the 26S proteasome is not impaired in the aging rat brain. *PLoS ONE*, 8(5):e64042. DOI: 10.1371/journal.pone.0064042

Gray D.A. and Woulfe J. (2005): Lipofuscin and aging: a matter of toxic waste. *Science of Aging Knowledge Environment*, 2005(5):re1. DOI: 10.1126/sageke.2005.5.re1

Head E., Pop V., Sarsoza F., Kayed R., Beckett T.L., Studzinski C.M., Tomic J.L., Glabe C.G., Murphy M.P. (2010): Amyloid β-peptide and oligomers in the brain and cerebrospinal fluid of aged canines. *Journal of Alzheimer's Disease*, 20(2):637-646. DOI: 10.3233/JAD-2010-1397

Head E., Pop V., Vasilevko V., Hill M., Saing T., Sarsoza F., Nistor M., Christie L.A., Milton S., Glabe C., Barrett E., Cribbs D. (2008): A two-year study with fibrillar beta-amyloid immunization in aged canines: effects on cognitive function and brain Abeta. *Journal of Neuroscience*, 28(14):3555-3566. DOI: 10.1523/JNEUROSCI.1787-08.2008

Jung T., Höhn A., Grune T. (2010): Lipofuscin: detection and quantification by microscopic techniques. *Methods in Molecular Biology*, 594:173-193. DOI: 10.1007/978-1-60761-411-1_12

Kumar-Singh S. (2008): Cerebral amyloid angiopathy: pathogenetic mechanisms and link to dense amyloid plaques. *Genes, Brain and Behavior*, 7(Suppl. 1):67-82. DOI: 10.1111/j.1601-183X.2007.00340.x

Nešić S., Kukolj V., Marinković D., Vučićević I., Jovanović M. (2017): Histological and immunohistochemical characteristics of cerebral amyloid angiopathy in elderly dogs, Veterinary Quarterly, 37(1):1-7.

Pirici D., Van Cauwenberghe C., Van Broeckhoven C., Kumar-Singh S. (2011): Fractal analysis of amyloid plaques in Alzheimer's disease patients and mouse models. *Neurobiology of Aging*, 32(9):1579-1587. DOI: 10.1016/j.neurobiolaging.2010.02.004

Uchida Y. (2010): Molecular mechanisms of regeneration in Alzheimer's disease brain. *Geriatrics and Gerontology International*, 10(Suppl. 1):S158-S168. DOI: 10.1111/j.1447-0594.2010.00612.x

Uchida Y., Gomi F., Masumizu T., Miura Y. (2002): Growth inhibitory factor prevents neurite extension and death of cortical neurons caused by high oxygen exposure through hydroxyl radical scavenging. *Journal of Biological Chemistry*, 277:32353-32359. DOI: 10.1074/jbc.M205340200

Upadhya S.C. and Hegde A.N. (2007): Role of the ubiquitin proteasome system in Alzheimer's disease. *BMC Biochemistry*, 8(Suppl. 1):S12. DOI: 10.1186/1471-2091-8-S1-S12

Vargas T., Ugalde C., Spuch C., Antequera D., Morán M.J., Martín M.A., Ferrer I., Bermejo-Pareja F., Carro E. (2010): Abeta accumulation in choroid plexus is associated with mitochondrial-induced apoptosis. *Neurobiology of Aging*, 31(9):1569-1581. DOI: 10.1016/j.neurobiolaging.2008.09.010