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ORIGINAL PAPER

Diffuse beta-amyloid plaques and hyperphosphorylated tau are unrelated processes in aged dogs with behavioral deficits

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Abstract Single and double-labeling immunocytochemistry has been used to learn about the localization, distribution, and possible relationship between beta-amyloid protein (Aβ) deposition and tau hyperphosphorylation in the canine cerebral cortex with age. Behavioral impairment, as reported by the owners and tested in all dogs, correlated with increased Aβ burden in old dogs. Aβ plaques were diffuse and they were not accompanied by modifications in synaptic protein expression. Plaques were not associated with increased active mitogen activated protein kinase (MAPK/ERK-P) and p38 kinase (p38-P) expression, and tau hyperphosphorylation in neighboring cell processes. Yet tau hyperphosphorylation, as revealed with phospho-specific antibodies to tauThr181 and tauSer396, increased with age in individual neurons. Moreover, the subcellular pattern shifted from perinuclear localization to granular cytoplasmic and nuclear distribution with age. Our results in dog suggest that Aβ diffuse plaque formation and tau hyperphosphorylation are independent events, both occurring during the process of aging. Although increased cognitive dysfunction is associated with increased tau hyperphosphorylation, further investigation is needed to understand whether tau hyperphosphorylation is causative of cognitive impairment or an independent process related to aging.

Keywords Diffuse beta-amyloid plaques · Dog · Tau phosphorylation · Aging · Cognitive deficits

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Introduction

The principal hallmarks of Alzheimer's disease (AD) are the formation and deposition of beta-amyloid protein (Aβ) in the form of senile plaques (diffuse and neuritic plaques) and cerebral amyloid angiopathy, and the hyperphosphorylation and fibrillization of tau, which comprises neurofibrillary tangles (NFT_s), neuropil threads, and dystrophic neurites of neuritic plaques [15, 65]. Combined clinical and neuropathological studies in AD have shown a strong relationship between cognitive impairment and load of neuropathological hallmarks [12]. In the same line, behavioral deficits correlate with age-related changes described in the brain of old dogs [3, 8, 10, 11, 14, 48]. Aged dogs spontaneously developed Aβ diffuse plaques that are neuropathologically and sequentially identical to those observed in humans [30]. The deposition of the Aβ protein is widely distributed in cortex and hippocampus, but the brain stem and cerebellum are usually spared [29]. Yet amyloid plaques in dogs are only diffuse plaques that do not fulfill the β-pleated-sheet

conformation and are Congo red and thioflavine negative, with no apparent neuritic component [8, 14, 48, 57]. In humans, diffuse plaques are also encountered in Down's syndrome [22, 27], in normal aging [31, 38], and in response to trauma [42, 47, 53], in addition to AD.

Despite the similarity of Aβ deposition and cognitive decline, development of NFT_s is markedly different between canine and human brain. In human tissue, NFT_s are mainly composed of hyperphosphorylated tau that aggregates as paired helical filaments (PHF_s). In addition to NFT_s, hyperphosphorylated tau is found in neuropil threads and in dystrophic neurites surrounding senile plaques [1, 32]. Increased expression of several kinases including p38 kinase (p38), cyclin-dependent kinase-5 (CDK-5), glycogen synthase kinase-3β (GSK-3β), mitogen-activated protein kinase, and extracellular signal-regulated kinase (MAPK/ERK1-2) has been implicated in tau hyperphosphorylation with different efficiencies for particular sites, and formation of PHF_s [18, 19, 25, 45, 46]. Thus, phosphorylation of tau at Thr181 and at Ser396 can be conveyed by MAPK/ERK, stress-activated protein kinases c-Jun N-terminal kinase (SAPK/JNK), p38 or GSK-3β, whereas at Tau Ser202 phosphorylation may be due to MAPK/ERK, SAPK/JNK, p38 or GSK-3α/β [2]. Neurofibrillary changes have been described, in the absence of Aβ deposition, in nonmammalian models [61], and in aged nonhuman primates [59], ruminants [5], polar bears and cats [9, 24, 52].

Phosphorylation at the tau-1 site—amino acid residues 189 and 207 of the human tau sequence [21]—takes place in several neuronal populations and oligodendrocytes in dogs [64]. However, no transition from normal specific tau phosphorylation to excessive phosphorylation leading to cell degeneration and NFT formation has been detected in dogs [10, 44].

The present study examines the relationship among Aβ deposition, tau phosphorylation, and synaptic protein expression in the vicinity of cerebral Aβ plaques in aged dogs with clinically verified progressive cognitive deficits. This study is geared to increasing our understanding of the neuropathological bases of cognitive deficits in old dogs.

Materials and methods

Dog samples

The study was performed on the brains of ten dogs, seven males and three females, of various breeds and from 1 to 20 years of age, all certified by their medical records from the veterinary hospital *Ars Veterinaria* of Barcelona, Spain (Table 1). In addition to the clinical history, the cognitive status of every dog was evaluated using a cognitive test with the aid of the pet's owner [49]. The final total score reflected the cognitive status of the animal. In all cases, brain donation was formally approved by the owner and euthanasia justified for medical reasons; the animals were euthanized with an intravenous overdose of sodium thiopental (75 mg/kg) (Thiobarbital, Braun Medical S.A., Spain). All animals were treated according to European legislation on animal handling and experiments (86/609/EU) and procedures were approved by the Ethical Committee of the University of Barcelona, Barcelona, Spain. All efforts were made to minimize animal suffering and to use no

Table 1 Characteristics of dogs used in this study, cognitive evaluations and neuropathological findings

Dog no.	Age (years)	Sex	Weight (kg)	Breed	Cognitive evaluation	Main pathology	Stage of Aβ deposition	Tau Thr181	Tau Ser396	Tau Ser202, SNAP-25, Synaptophysin, MAPK/ERK-P, p38-P
1	1	F	15	Mixed	YC (9)	Hip trauma	–	+	+	–
2	3	M	13.5	Beagle	YC (10)	Urinary incontinence	–	+	+	–
3	6	M	6.3	Mixed	YC (10)	Arthritis	–	++	++	–
4	8	M	32	Giant Schnauzer	LCD (13)	Chronic renal disease	I	++	++	–
5	10	M	33	German Shepherd	LCD (21)	Spleen neoplasm	II	++	++	–
6	14	M	5.5	Poodle	SCD (30)	Cognitive deficits	II	++++	++++	–
7	15	F	6	Pekingese	SCD (27)	Cognitive deficits	II	+++	+++	–
8	16	M	12	Fox Terrier	SCD (27)	Cognitive deficits	III	++++	++++	–
9	16	F	12	Mixed	SCD (29)	Cognitive deficits	IV	++++	++++	–
10	20	M	7.1	Mixed	SCD (40)	Cognitive deficits	IV	+++++	+++++	–

Stage of Aβ deposition was evaluated based upon [48, 57]

F female, *M* male, *YC* young control, *LCD* light cognitive deficits, *SCD* severe cognitive deficits. + – +++++ indicates from moderate to strong total neuronal staining intensity

more than the number of animals needed for reliable scientific data.

Tissue preparation

The tissue preparation was adapted from human brain bank methods [34]. Immediately after death, the brains were rapidly removed from the skull and 1-cm-thick coronal sections of cerebral cortex were immersion-fixed in 10% neutral buffered formalin for 2 weeks. After a 3-day cryoprotection, they were frozen on powdered dry ice and stored at −40°C until use.

Immunohistochemistry

Serial consecutive 40-μm-thick sections of the dorsal anterior prefrontal cortex corresponding to area 8a on the proreal gyrus [28] were obtained with a cryostat and processed free-floating with the streptavidin–biotin (LSAB) method (Dako LSAB + kit, Dako, Barcelona, Spain) following the instructions of the supplier. Briefly, after blocking endogenous peroxidases, the sections were incubated with normal serum for 2 h and then incubated overnight at 4°C with one of the primary antibodies. The monoclonal antibody to synaptic-associated 25,000 molecular weight protein (SNAP-25; raised against recombinant SNAP-25) and the monoclonal antibody against synaptophysin (Dako) were used at dilutions of 1:500. The phospho-specific tau Thr181, tau Ser202, and tau Ser396 polyclonal rabbit antibodies (Calbiochem, Madrid, Spain) were used at dilutions of 1:500, 1:500, and 1:100, respectively. The phospho-specificity of these antibodies was previously tested in human brain samples with AD by pre-incubation of the antibodies with alkaline phosphatase (Sigma, Madrid, Spain). Immunostaining of neurofibrillary tangles largely faded following this treatment. Similar results were obtained in the aged canine brain, after pre-incubation of the antibody with phosphatase. The immunoreaction was abolished thus indicating specificity of the antibody for phosphorylated species. The anti-MAP kinase phospho-specific (Tyr204) rabbit polyclonal antibody (MAPK/ERK-P) was used at a dilution of 1:200. The phosphorylation-dependent rabbit polyclonal antibody to p38(Thr180/Tr182) (p38-P) (Calbiochem) was used at a dilution of 1:100. For Aβ immunohistochemistry, a slight variation of the protocol was introduced. Before blocking endogenous peroxidases, the sections were incubated with 98% formic acid for 3 min to enhance antigenicity. The $A\beta_{8-17}$ mouse monoclonal antibody (Dako) was used at a dilution of 1:50. After washing, the sections were incubated with LSAB for 15 min each at room temperature. The peroxidase reaction was visualized, as a dark blue precipitate, with NH_4NiSO_4 (0.05 M) in phosphate buffer (0.1 M), 0.05% diaminobenzidine, NH_4Cl and 0.01% hydrogen peroxide, or as a brown precipitate with 3,3′-diaminobenzidine and hydrogen peroxide. Finally, the sections were dehydrated and mounted with DPX (Scharlau Chemie, Barcelona, Spain). Blank sections stained only with the secondary antibodies were used as negative controls.

Double-labeling immunofluorescence and confocal microscopy

Cryostat sections, 40 μm thick, were rinsed in PBS and incubated with 98% formic acid for 3 min. After that, they were mounted on glass slices and stained with a saturated solution of Sudan black B (Merck, Madrid, Spain) for 10 min to block the autofluorescence of lipofucsin granules present in nerve cell bodies, rinsed in 70% ethanol, and washed in distilled water. The sections were incubated in a blocking solution containing 0.2% gelatine, 0.2% azide, 0.2% Triton X-100, and 20% fetal bovine serum in PBS, pH 7.5 for 1 h at room temperature. Immediately afterwards, the sections were incubated at 4°C overnight with the $A\beta_{1-42}$ rabbit polyclonal antibody (kindly provided by Dr. M. Sarasa, Zaragoza, Spain) [50] at a dilution of 1:500 and with monoclonal antibody against synaptophysin (Dako) (1:100) in a vehicle solution composed of 0.2% gelatine, 0.2% azide, 0.2% Triton X-100, and 1% fetal bovine serum in PBS, pH 7.5. Other sections were incubated with the $A\beta_{8-17}$ monoclonal antibody at a dilution of 1:50 and with one of the following rabbit polyclonal antibodies against SNAP-25 (1:500), phospho-specific tau Thr181 (1:500), tau Ser202 (1:100), or Ser396 (1:100) (Calbiochem). Finally, other sections were double-stained for amyloid and anti-MAPK/ERK-P (1:200), or anti-p38-P (1:100) (Calbiochem), in the same vehicle solution. After washing in PBS, the sections were incubated in the dark with a cocktail of secondary antibodies and diluted in the same vehicle solution as the primary antibodies for 45 min at room temperature. Secondary antibodies were Alexa488 antirabbit and Alexa546 antimouse (both from Molecular Probes, Madrid, Spain); these were used at a dilution of 1:400. Sections only with the cocktail of secondary antibodies were used as controls. Finally, TO-PRO-3 (Invitrogen Life Technologies, Barcelona, Spain) was used to detect the cell nuclei. After that, sections were mounted in Immuno-Fluore Mounting medium (ICN Biomedicals, Barcelona, Spain), sealed and dried overnight. Sections were examined with a Leica TCS-SL confocal microscope. For each marker,

two sections per animal were evaluated for total neuronal staining intensity, and rankings were done by one investigator (MP) blind with respect to the age of the animal.

Bielschowsky staining

The Bielschowsky staining method was performed according to the Yamamoto and Hirano modification using 20% silver nitrate [68]. Briefly, 40-μm-thick sections were washed in distilled water and placed in 20% silver nitrate in the dark at 37°C for 30 min. After that, strong ammonia was added to the silver nitrate solution and the sections were left in this solution for 10 min at 37°C. Then the sections were washed in 0.1% ammonia and 6–10 drops of developer solution (10% formalin, distilled water, concentrated nitric acid and citric acid) were added to silver hydroxide solution and the sections were stained for 3–5 min. After that, the sections were toned in 0.2% gold chloride for 1–2 min and fixed in 5% sodium thiosulphate for 1 min. Finally, the sections were washed, dehydrated, and mounted in DPX (Scharlau Chemie).

Results

Based on the presence of established housetraining habits, disorientation in familiar surroundings, decreased activity, playfulness, vitality, decreased interaction with the owner, and modifications of the sleep/awake cycle, dogs were categorized as young control (YC), i.e. with no signs of behavioral disorder, light cognitive deficits (LCD), and severe cognitive deficits (SCD) (Table 1).

Starting at the age of 8 years, and increasing with age and with cognitive deficit severity, Aβ immunohistochemistry revealed the presence of delicate (Fig. 1a, c) and more compact (Fig. 1b, d) diffuse deposits throughout all cortical layers of the cerebral cortex in a characteristic four-stage distribution (I–IV) (Table 1) [48, 57]. No plaques were observed in the subcortical white matter. The Aβ plaques were not stained with antibodies to the synaptic proteins synaptophysin and SNAP-25 (data not shown). Increasing with age from moderate (+) to strong (+++++), immunoreactivity with phosphorylation-dependent tau antibodies Thr181 and Ser396 was found in all cases (Table 1). In young animals, the tau Thr181 and tau Ser396 immunoreactivity was observed in sparse neurons characterized by a widespread distribution within all the different cortical layers. Labeling was localized in the perinuclear cytoplasm and the nuclear membrane of some neurons (Figs. 2a, b, 3a). Yet increased tau Thr181 and tau Ser396 immunoreactivity in all the cortical layers was localized in the cytoplasm and, rarely, in the nuclear membrane in dogs aged between 8 and 20 years (Figs. 2c, 3b). No positivity was noticed with antitau antibodies Ser202.

In all situations, the distribution of phospho-tau immunoreactivity lacked any spatial relation with amyloid deposition. More precisely, neuronal Thr181 and Ser396 tau phosphorylation did not localize in the surroundings of diffuse plaques, thus indicating no

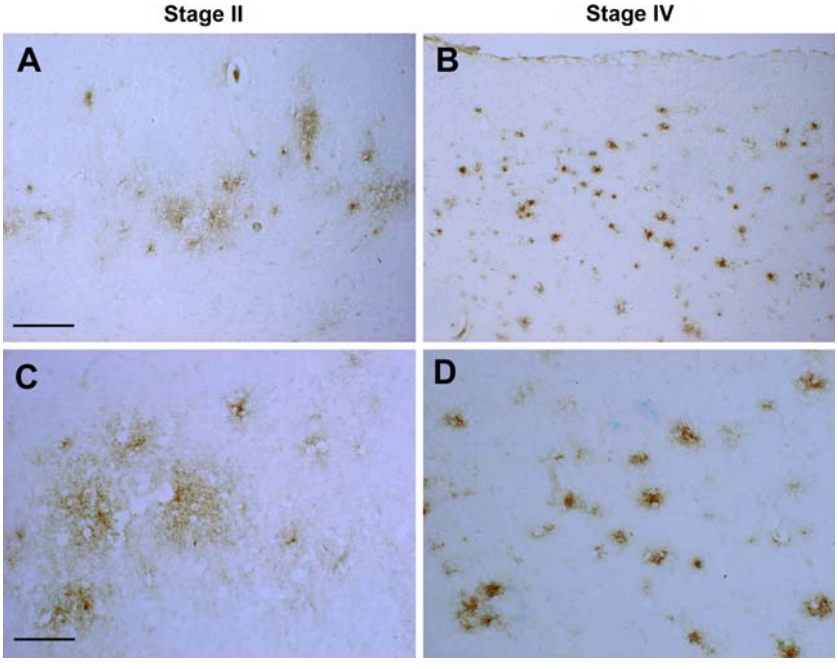

Fig. 1 Representative microphotographs of Aβ immunoreactivity in the prefrontal cortex of two dogs visualized with anti-$A\beta_{8-17}$ antibody (**a**–**d**). **a**, **c**: In stage II, Aβ deposition was localized in the deep layers of the cortex (V–VI) and showed a diffuse and cloud-like aspect (Animal no. 5, 10 year old). **b**, **d**: In stage IV, progressively more compact Aβ deposits extended throughout all cortical layers (Animal no. 9, 16 year old). **a**, **b** bar = 50 μm; **c**, **d** bar = 200 μm

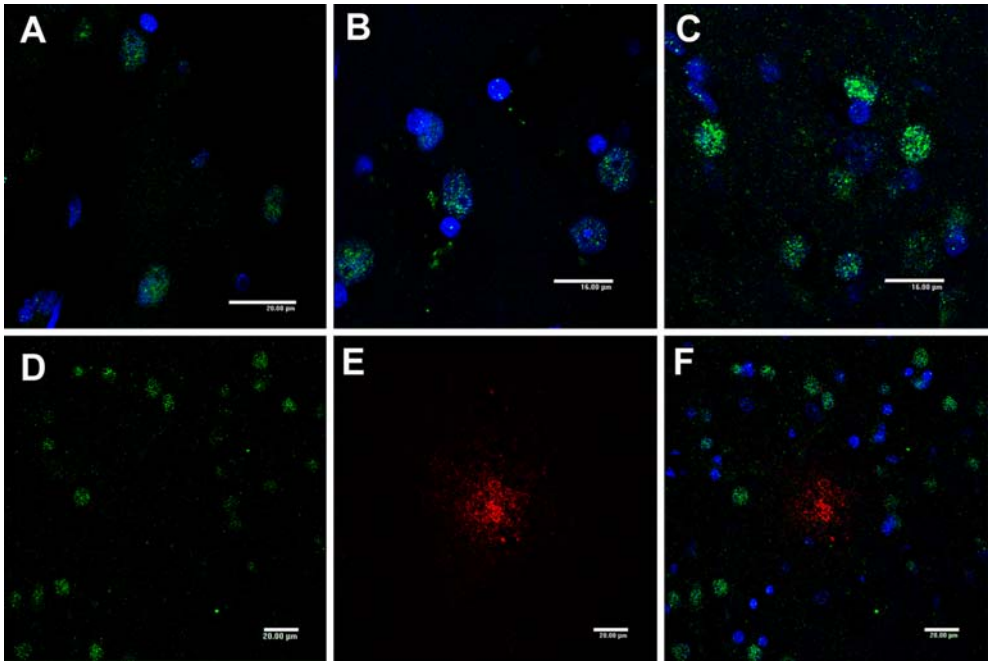

Fig. 2 Confocal images of progressive increased tau Thr181 immunoreactivity with age, without (**a**–**c**) and with Aβ deposition (**d**–**f**) in prefrontal cortex of dogs. **a** Dog no. 1 (1 year old) presented light tau Thr181 immunofluorescence (*green*) showing a granular pattern in the perinuclear cytoplasm and in the nuclear membrane. **b** In dog no. 4 (8 year old), the number of tau Thr181-immunoreactive neurons increased and the granular aspect became more evident. **c** In old dogs (in this example, dog no. 10, 20 year old), *tau* Thr181 immunofluorescence was very strong and the granular precipitate was localized mainly in the nucleus in cortical regions without Aβ deposition. **d**–**f** The same dog with strong tau Thr181 immunoreactivity (*green*) and Aβ deposition ($A\beta_{8-17}$, *red*). In merge (**f**), no relationship between tau Thr181 and Aβ deposition was found. No positive neurites surrounding plaque were detected. The sections were counterstained with TO-PRO-3 (*blue*). **a** bar = 20 μm, **b**–**f** bar = 16 μm

hyperphosphorylation of tau in neurites surrounding amyloid plaques (Figs. 2d–f, 3b–d). Conversely, amyloid deposition was absent in the vicinity of neurons bearing hyperphosphorylated tau (Fig. 3e, f). Moreover, diffuse plaque maturation between stage I and IV observed in aged dogs did not parallel increased tau Thr181 and Ser396 phosphorylation in neurons (Table 1). Bielschowsky silver staining confirmed the absence of dystrophic neurites and neurofibrillary tangles in all dogs. Finally, immunoreactivity to MAPK/ERK-P and p38-P in relation with amyloid plaques was negative in every case (Table 1).

Discussion

The results of this study support previous observations regarding amyloid plaque formation and tau phosphorylation in aging canine brain. As previously reported [49], a variety of behavioral changes can be evidenced in aged dogs in the present series despite the limited number of cases studied. These alterations include disorientation in familiar surroundings, decreased activity and playfulness, altered social relationship and interaction with the owner and with other animals, decreased self-hygiene, alteration of adaptative capabilities, and modifications of the sleep/awake cycle [49]. As in humans, the severity of the cognitive deficit in dogs has been correlated with the density of the Aβ deposits in hippocampus and frontal cortex [11, 12]. In agreement with other reports, our results have shown that the aged canine brain contains predominantly diffuse plaques classified into four stages [8, 48, 57]. In this paper, we found that the first steps of Aβ deposition take place around 8 years, an age associated with high levels of oxidative stress and a dramatic drop in antioxidant defenses [23, 44] that clinically responds to behavioral enrichment and dietary fortification [36, 55]. In AD, Aβ deposition, a source of oxidative damage and oxidative stress, is considered to be a proximal event in the pathogenesis of AD [60]. A direct toxic effect of the Aβ deposition related with cognitive deficits has been demonstrated in humans and canines [11, 12]. The reduced dog life span may explain the absence of neuritic plaques, as it does not allow sufficient time for the 1–40 Aβ-fragment responsible for the cored plaque to precipitate [8, 13]. Other authors have shown that the presence of longer Aβ peptides is not central to the

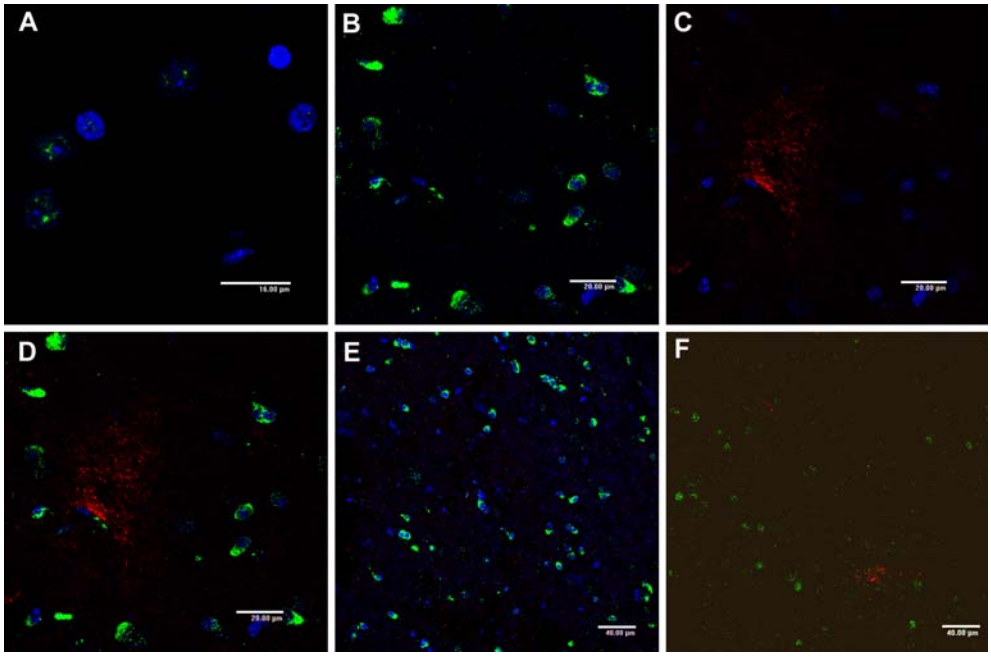

Fig. 3 Confocal images of progressive increased tau Ser396 immunoreactivity in aged dogs. **a** Dog no. 3 (6 year old) presented a light pattern of tau Ser396 (*green*) immunoreactivity mainly localized in the perinuclear region. The nuclei are visualized with TO-PRO-3 (*blue*). **b–d** Strong tau Ser396 immunofluorescence found in dog no. 8 (16 year old). Panel **b** corresponds to phospho-tau Ser396 only (*green*), **c** represents only $A\beta_{8-17}$ deposition (*red*), and **d** is merged. All sections were counterstained with TO-PRO-3 (*blue*). **e**, **f** Regional distribution of tau Ser396 staining in dog no. 8 (16 year old). Tau Ser396 immunoreactivity (*green*) was observed throughout all the cortical layers in region without amyloid deposition. The nuclei are visualized with TO-PRO-3 (*blue*) (**e**). When amyloid deposition was present, no apparent association between tau Ser396 phosphorylation (*green*) and amyloid plaque (*red*) was observed (**f**). **a** bar = 16 μm, **b–d** bar = 20 μm, **e**, **f** bar = 40 μm

seeding of amyloid in vitro [67], and, therefore, the absence of neuritic plaques in dogs may be related rather to other factors such as apolipoprotein E, metal ions or specific astroglial protein expression and microglial reaction [33, 39, 54, 63, 66]. Because the cognitive impairments seen in AD seem largely related with the concomitant regional and laminar tau pathology [4, 37, 40, 41], several authors correlate the functional deterioration of aged dogs with excessive tau phosphorylation and resulting abnormalities [64]. The present study has shown that tau phosphorylation is a common process in the dog brain during aging, and that this process occurs in the absence of intraneuronal neurofibrillary changes, neuropil threads, and dystrophic neurites surrounding amyloid deposits. Some studies have reported cytoskeletal abnormalities in dogs [44, 56], but our present results and others [16] indicate that the process of neurodegeneration does not involve NFT_s formation. Furthermore, in AD, abnormal synaptic protein expression occurs in human senile plaques [7, 17], and a similar situation is found in transgenic mice [26, 43, 62]. Yet no abnormal synaptic protein expression has been noticed in canine amyloid plaques.

In our study, no-housed aging dogs have been characterized under several paradigms that include in every animal, study of cognitive function, amyloid plaque stage deposition, and sites of Tau phosphorylation. As shown previously, despite the relatively small number of animals included in this study, our cognitive test is able to differentiate between three groups of canine cognitive status, that includes the LCD group proposed as the normal aging one [49]. Our data give evidences for the first time that increasing tau Thr181 and tau Ser396 immunoreactivity in canine cortical neurons may be associated, apart from aging, with the degree of cognitive dysfunction, but not with the stage of amyloid plaque deposition. Whether tau hyperphosphorylation is a cause of cognitive impairment or an independent phenomenon both related with aging is an open question. Discrete tau hyperphosphorylation in LCD animals, in absence of clinical signs of cognitive dysfunction, may reflect normal aging in dogs [49]. Because the presence of cognitive dysfunction, either light or severe, is also age-dependent, further experiments done with a large number of animals are needed to properly interpret the relationship between aging, cognitive dysfunction, and tau phosphorylation. Phosphorylation of tau at Ser202 observed by other authors in housed beagles [24, 35] has not been detected in the present series. Other studies have shown dog-specific

tau deposition, using Tau-1 monoclonal antibodies that recognize the 189 and 207 residues of the human tau sequence [21, 64]. The accumulation of phospho-tau-positive material in the nucleoplasm of neurons observed here is in agreement with the nuclear localization of tau reported in human frontal cortex [6].

Activation of stress kinases c-Jun N-terminal kinase (SAPK/JNK) and p-38 in association with tau phosphorylation has been reported in neurites surrounding Aβ plaques in double mutants for APP (Tg2576) and PS-1 (P264L) mice [58], APP transgenic mice [20, 50] and double APP/tau transgenic mice, which show Aβ plaques, neurofibrillary tangles, and dystrophic neurites in senile plaques [51]. However, no increased p38-P immunoreactivity has been found in the vicinity of amyloid plaques in aged dogs. Thus, the lack of reciprocal interactions between Aβ deposition and tau alterations challenges the idea that tau pathology is merely a downstream effect of Aβ production and deposition. In summary, our results in dogs suggest that Aβ diffuse plaque formation and tau hyperphosphorylation are independent processes, both occurring during the process of aging. Whether tau hyperphosphorylation is part of the process leading to severe cognitive dysfunction would have to be confirmed in future experiments.

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