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Immunohistochemical investigation of amyloid ß-protein (Aß) in the brain of aged cats

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Summary. To clarify the immunohistochemical features of amyloid deposits and cerebral amyloid angiopathy (CAA), the distribution of the amyloid ß-protein subtypes Aß40, Aß42, Aß43 and Aß precursor protein (APP) were examined in the brains of fourteen aged cats (7.5-21 year-old). Two types of plaques were detected. The first type was characterized by Aß positive antigenic material and detected in the cortical layers of the frontal and parietal lobes of all examined cats. The second type was characterized by diffuse positive immune staining representing diffuse plaques, which were detected only in the very aged cats (17-21 years old) and distributed throughout the cortical layers of the parietal lobes. Vascular amyloid and the amyloid deposits were strongly positive-stained with the antibody Aß42. APP was exhibited in neurons and axons while the staining was stronger in the very aged cats (17-21 years old). Our findings suggest that the feline forms a spontaneous model for understanding the early changes of normal brain aging and the early stage of amyloid ,-protein deposition.

Key words: Amyloid ß-protein, Cat, Senile plaque, Ageing

Introduction

In human Alzheimer's disease (AD) amyloid depositions, neurofibrillary tangles (NFTs) and loss of synaptic neuronal connections (Miller et al., 1993) are prominent histopathological brain lesions. Senile plaques (SP) and cerebral amyloid angiopathy (CAA) have been detected in the brain of animal species, such as non-human primates (Selkoe et al., 1987), dogs (Wisniewski et al., 1970; Selkoe et al., 1987; Giaconne et al., 1990; Ishihara et al., 1991; Uchida et al., 1990; 1991, 1993a,b; Shimada et al., 1992; Cummings et al., 1993,1996a; Uchida et al., 1993a; Gruys, 1995; Wegiel et al., 1995a; Russell et al., 1996; Kuroki et al., 1997; Borras et al., 1999; Papaioannou et al., 2001), bears (Selkoe et al., 1987; Uchida et al., 1993b; Tekirian et al., 1996), cats (Cummings et al., 1996b; Nakamura et al., 1996; Nakayama et al., 2001) and camels (Nakamura et al., 1997).

NFTs are not only found in the brains of aged dementing people but they are also prominent after repeated trauma and in aged persons with Down's syndrome (Uchida et al., 1991; Miyawaki et al., 2002). However, they are rare in dogs (Papaioannou et al., 2001).

The major component of the senile plaques and the amyloid deposits in the walls of larger arteries (CAA) is the amyloid ß-protein, a polypeptide of 39-44 amino acids. It has a high content of ß-pleated sheet as its secondary molecular structure, and it is produced by proteolysis of the Aß precursor protein (APP) (Roher et al., 1993; Selkoe, 1994; Wegiel et al., 1995b; Head and Torp, 2002).

Amyloid plaques are subtyped into three categories based on morphology, fibrils rich in ß-sheet conformation and their association with additional degenerative and reactive elements (i.e. dystrophic neurites, astrocytes and microglia) (Cotman et al., 1991). The subtypes are generally referred to as (i) diffuse without histological amyloid (non proven accumulation of ß-sheeted proteins), (ii) primitive (congophilic, ß-sheeted fibrillar protein accumulation) lacking a layered structure with a central core of amyloid and containing few dystrophic neurites or reactive glia), or (iii) neuritic plaques (with a central core of amyloid and containing extensive dystrophic neurites and reactive glia) (Wisniewski and Terry, 1973). Some scientists report that dogs have predominantly diffuse plaques (Giaconne et al., 1990; Cummings et al., 1993; Wegiel et al., 1995a) as well as primitive and neuritic plaques (Uchida et al., 1993a; Cummings et al., 1996b, Papaioannou et al., 2001; Miyawaki et al., 2002). Additionally, neurofibrillary tangles have also been reported in aged dogs (Papaioannou et al., 2001).

There is evidence in the literature that senile plaques

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were also present in the brain of aged cats. They were found in the cerebral cortex of three very aged cats (more than 18 years old), which consisted of a coarse assembly of silver staining-positive materials but were morphologically different from the well-known classical, primitive and diffuse plaques. They were located mainly in the temporal lobe and secondarily in the occipital lobe (Nakamura et al., 1996). Additionally, Aß depositions were described in the brain of old cats (16 and 20 year-old) (Cummings et al., 1996b). Nakayama et al., (2001) reported that he found diffuse plaques only in one examined cat, 20 years old, He used a computer-aided method in order to estimate the morphology of animal and human senile plaque. Congophilic amyloid angiopathy was observed in a few cortical arterioles of the old (6-20 year old) cat (Nakamura et al., 1996).

The Aß protein deposits in human brain include two major subtypes that have different carboxyl termini: Aß40 and Aß42/43 (Selkoe et al., 1987, Miller et al., 1993; Iwatsubo et al., 1994; Tamaoka et al., 1994; Mann et al., 1995). In aged dogs immuno-histological investigations have indicated that diffuse plaques and dense plaques consist mainly of Aß42/43 and Aß40, respectively (Wisniewski et al., 1996). The CAA in meningeal vessels appeared to be positive for both (Nakamura et al., 1997; Rofina et al., 2003). However, research regarding these proteins in senile plaques in the brain of aged cats is scarce and there are a limited number of publications.The case where they have been found, referred to cats aged 16 years and more (Cummings et al., 1996b). The diffuse plaques within the feline brain were immunoreactive for Aß42 but not for Aß40 (Cummings et al., 1996b) while others report that they were immunoreactive for Aß40 (Nakamura et al., 1996). Concerning the vascular deposits, they were immunoreactive for both Aß40 and Aß42 (Cummings et al., 1996b; Nakamura et al., 1996).

The objective of the present study was to investigate the SPs and CAA in cats. The brains of fourteen cats aged from 7.5 to 21 years, were examined immunohistochemically for Aß subtypes. For immunostaining of SPs and CAA various commercial antibodies were applied. The topographic distribution of the plaques in the cerebral hemispheres was also studied.

Materials and methods

The brains of 14 cats of different ages (varied from 7.5 to 21 years old) were studied. The cats were seven Siamese and seven DSH. They had been routinely necropsied at the Department of Animal Pathology, Aristotle University of Thessaloniki. The cats were euthanased or died spontaneously suffering from a variety of disorders (Table 1). Their brains were routinely fixed by immersion in 4% buffered formaldehyde. Subsequently they were embedded in paraffin wax and cut into 5 μm thick sections.

Coronal sections of different areas from the cerebral hemispheres (frontal, parietal, temporal and occipital lobes), cerebellum, midbrain and pons were stained with hematoxylin and eosin (H&E) as well as alkaline Congo red. In the latter staining, congophilic material was identified as amyloid when it exhibited apple-green birefringence in polarized light.

Single-labeling immunohistochemistry by avidin-biotin complex (ABC) with several primary antibodies was performed in serial coronal sections of different areas from the cerebral hemispheres. In order to enhance the immunoreactivity of the primary antibodies, pretreatments were performed for each antibody. All primary antibodies used in this study, including the source, dilution and pretreatment performed, are listed in Table 2. Endogenous peroxidase was inhibited using 0.3% hydrogen peroxide in methanol for 30 min at room temperature (RT). Normal horse/goat serum (1:10 and 1:25 respectively) was used at RT for 30 min to block non-specific reactions. The sections were incubated with the primary antisera at 4°C overnight in a humidity

Table 1. Age, gender, breed and reason of euthanasia or death of the aged cats used.

CASE No.	AGE (YEARS)	GENDER	BREED	REASON OF EUTHANASIA OR DEATH
1	7.5	Male	DSH	Chronic renal failure-euthanasia
2	10	Female	DSH	Hypertrophic myocardiopathy-sudden death
3	11	Female	DSH	Chronic pancreatitis-death
4	12	Male	Siamese	Pulmonary carcinoma-death
5	12	Male	Siamese	Inflammatory bowel disease-dilated cardiomyopathy-death
6	13	Male	DSH	Chronic renal failure-euthanasia
7	13	Female	Siamese	FIV, hyperthyroidism, hypertrophic myocardiopathy-death
8	14	Female	Siamese	Mammary carcinoma-metastatic pulmonary neoplasm-death
9	15	Male	Siamese	Mammary carcinoma, metastatic pulmonary neoplasm-euthanasia
10	16	Female	DSH	Chronic pancreatitis-pancreatic insular amyloidosis-diabetes mellitus-euthanasia
11	17	Female	DSH	Mammary carcinoma-euthanasia
12	17	Female	DSH	Hyperthyreoidism, hypertrophic cardiomyopathy, mammary carcinoma-metastatic pulmonary neoplasm - euthanasia
13	20	Female	Siamese	Hypertension-retinal detachment-euthanasia
14	21	Female	Siamese	Pulmonary carcinoma-euthanasia

chamber. This was followed by reaction with the horse anti-mouse biotinylated IgG (1:125, Vector laboratories, Burlingame, Ca, USA) or goat anti-rabbit biotinylated IgG (1:250, Vector) at RT for 60 min. Between treatments, rinsing steps were performed using PBS. Finally, all sections were incubated with avidin-biotin peroxidase complex (ABC, Vector laboratories, Burlingame, Ca, USA) and rinsed with PBS. Visualization was performed with 3,3'-diaminobenzidine solution (DAB, Sigma, St. Louis, MO, USA) counter-stained for 10 min with Mayer's hematoxylin (Merck, Darmstadt, Germany) for 30 sec. The slides were dehydrated and mounted with Eukitt (Kindler GmBH and Co., Freiburg, Germany).

Positive controls were brain sections of dogs that were found positive for SPs, CAA and NFTs (Papaioannou et al., 2001). For negative control, brain sections of dogs were incubated while omitting the first antibody.

Results

The immunoreactivity of SPs and CAA with the used antibodies in the brains of the cats is summarized in Table 3.

Aß-protein: SPs, although some variability in the results among individuals was observed, the samples of each cat examined in this study demonstrated measurable areas of Aß distribution that were restricted to the gray matter. The Aß-immunostaining labeled two types of Aß plaques in the neuropil, which were detectable only after formic acid pretreatment. The first type was characterized as non-well circumscribed Aß positive antigenic material and was detected in all examined brains. Especially in the younger cats this type of Aß deposits presented primarily in the deeper layers of the cortical gray matter, while the older ones presented this material all over the cortical gray matter layers (Fig. 1). The Aß positive antigenic material was distributed in the cellular layers of the frontal and parietal lobes around neurons and capillaries and was evident in all samples of the examined cats. The second type was characterized by the diffuse accumulation of beta amyloid antigenic material without compact congophilic amyloid deposition. These plaques did not have obvious degenerative neurites and were not detectable either by HE or alkaline Congo-red staining. Morphologically, they were characterized by an immunoreactive core and less well outlined crown. They can be considered as diffuse plaques in comparison to the human senile plaque types (Fig. 2). Diffuse plaques were evident only in the brain of the very aged cats (17-21 years old) and they were distributed throughout the cortical layers, especially the cellular layers of the parietal lobes. It is interesting to note that a limited number of diffuse plaques were detected within the brain sections of a 12 year old Siamese cat. In two cases these plaques were detected mainly in the hippocampus.

Some positive immune staining, representing both types of deposits, was seen dispersed in the cortical white matter of the very aged cats (17-21 years old) (Fig.

Table 2. Primary antibodies used in this study, including the source, the dilution and the pretreatment performed.

ANTIBODY	DILUTION	SOURCE	PRETREATMENT
Aß(8-17)	1:50	DAKO	Formic acid(85%),10min RT
Panß(15-30)	1:400	Biosource	Formic acid(85%),10min RT
AßPP	1:450	Biosource	Formic acid(85%),10min RT
Aß40	1:50	Biosource	Formic acid(85%),10min RT
Aß42	1:50	Biosource	Formic acid(85%),10min RT
Aß43	1:50	Biosource	Formic acid(85%),10min RT

Table 3. Semiquantitive evaluation of cerebral amyloid deposits and vascular amyloid.

CAT	AGE	CR				Aß40				Aß42			
	(years)	m	ba	c	p	m	ba	c	p	ma	ba	c	p
1	7,5	-	-	-	-	++	-	-	$+^{am}$	++	-	-	$+++^{am}$
2	10	-	-	-	-	+	-	-	-	++	-	-	$++^{am}$
3	11	-	-	-	-	+	-	-	-	++	-	-	$+^{am}$
4	12	-	-	-	-	+	+	+	$+^{am}$	++	+	++	$+^{am}$
5	12	-	-	-	-	+	+	+	$++^{am,d}$	++	++	++	$+++^{am,d}$
6	13	-	-	-	-	+	+	+	$+^{am}$	++	++	++	$+++^{am}$
7	13	-	-	-	-	+	+	+	$+^{am}$	++	++	++	$++^{am}$
8	14	-	-	-	-	±	+	+	$+^{am}$	++	++	++	$++^{am}$
9	15	-	-	-	-	+	+	+	$+^{am}$	++	++	++	$+++^{am}$
10	16	-	-	-	-	+	+	+	$+^{am}$	++	++	++	$+++^{am}$
11	17	+	+	+	-	++	+	+	$+++^{am}$	++	++	++	$++^{am,d}$
12	17	+	+	+	-	++	++	++	$++^{am}$	+++	+++	+++	$+++^{am,d}$
13	20	+	+	+	-	++	++	++	$++^{am}$	+++	+++	+++	$+++^{am,d}$
14	21	+	+	+	-	++	++	++	$++^{am}$	+++	+++	+++	$+++^{am,d}$

CR: Congo-red staining; ma: meningeal arteries; ba: brain arteries; c: capillaries; p: plaques; am: positive antigenic material; d: diffuse; +++: severe: ++: moderate; +: few; -: undetected.

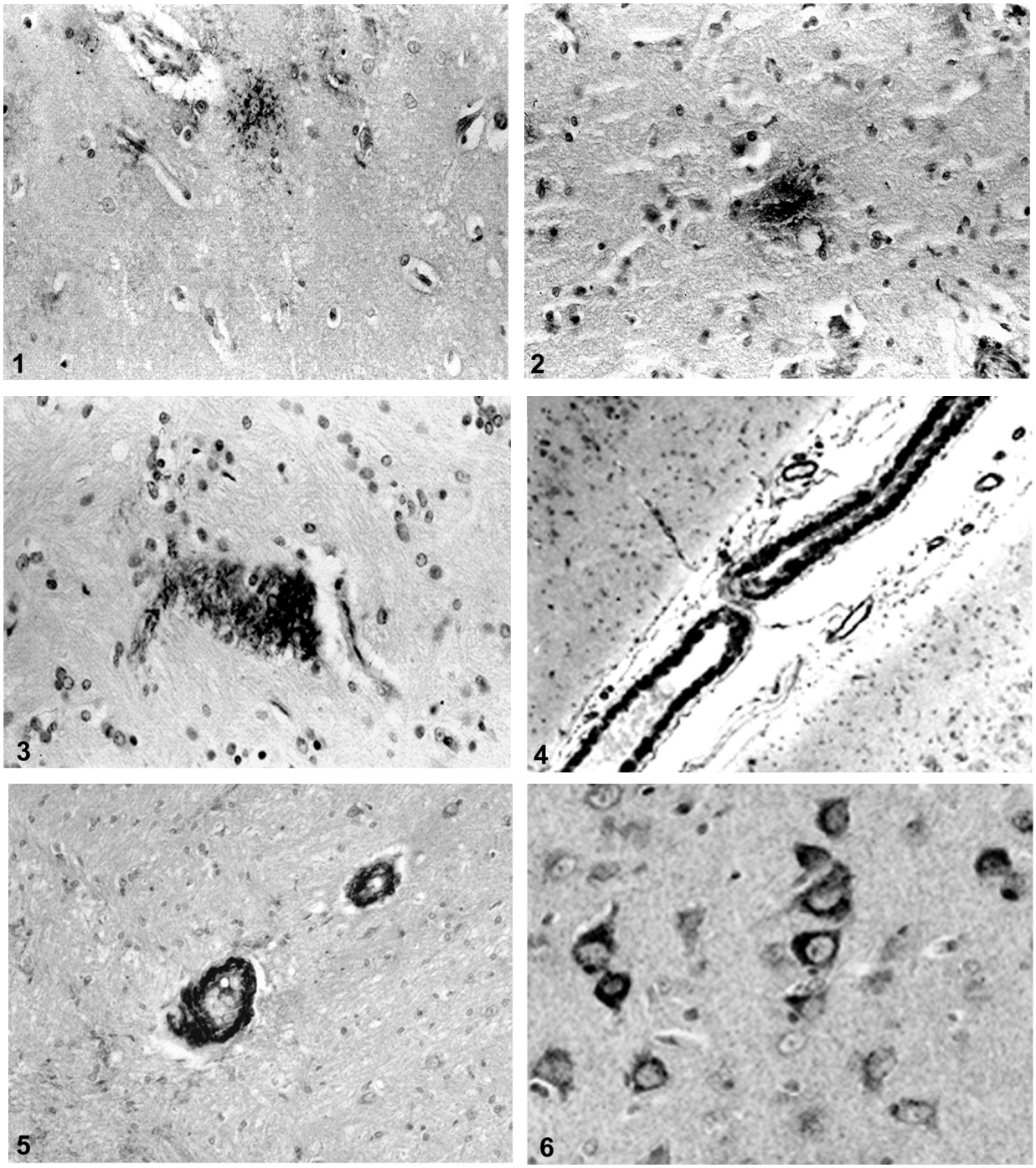

Fig. 1. Aß-positive antigenic material in the cortical gray matter layers, frontal lobe. Case 11, 17 years, ABC method, anti-Aß42 protein, x140

Fig. 2. Diffuse plaque in the neuropil of cerebral cortex, parietal lobe. Case 14, 21 years, ABC method, anti-Aß42 protein, x 320

Fig. 3. Aß-positive antigenic material in the cortical white matter, frontal lobe. Case 12, 17 years, anti-Aß42 protein, x 320

Fig. 4. Staining of amyloid deposit in the wall of meningeal vessels. Case 13, 20 years, ABC method, anti-Aß42 protein, x 140

Fig. 5. Staining of amyloid deposit in the wall of parenchymal vessels. Case 12, 17 years, ABC method, anti-Aß42 protein, x 320

Fig. 6. Neuronal cells show cytoplasmic immunoreaction for ß42. Case 11, 17 years, ABC method, anti-Aß42 protein, x 420

3).

No reaction was detected in the sections of cerebellum, midbrain and pons.

Amyloid was deposited in the wall of cerebromeningeal (Fig. 4) and cerebral vessels (Fig.5) and around the walls of cerebral capillaries. Associated with longevity, a gradual increase in the number of vascular deposits was detected. The Congo-red stained CAA only in the very old cats (Table 3). Moreover, numerous neurons within the cortex were found occupied by Aß deposition, especially in the old cats. The neuronal staining was prominent after using the antibody Aß42 (Fig. 6), whereas the antibodies Aß40, Aß (8-17) and Panß (15-30) revealed less clear positive results. Both monoclonal antibodies Aß40 and Aß42 stained plaques and vascular deposits, however the immunoreactivity of each was different (Table 4). Both the vascular amyloid and the two types of plaques were strongly positive-stained with the Aß42. The immunoreactivity of the amyloid deposits and the vascular amyloid for Aß (8-17) and Panß (15-30) was weaker than the staining with the Aß42. No staining was detected with the Aß43.

The Siamese cats appeared to react more strongly with the Aß antisera than the Domestic Shorthair (DSH) cats.

APP: Immunoreactivity was obtained with the polyclonal antibody used. Neuronal cells and axons were stained. The staining was stronger in the very aged cats. Immunoreactivity of the plaques and cerebrovascular amyloid in both capillaries and arterioles was not apparent.

Discussion

There are few studies describing senile plaque formation in the aged feline brain (Cummings et al., 1996b; Nakamura et al., 1996). To our knowledge the present study is the first attempt for a comparative immunohistochemical investigation for different types of Aß and APP in aged cat brains. Moreover the present study investigated the topographic distribution of positive-reacting deposits in cortical layers.

We found that the feline plaques were morphologically different from those of humans and other species (Wisniewski and Terry, 1973; Bergeron et al., 1987; Selkoe et al., 1987). Moreover, the morphological pattern of feline plaques in our study is not in agreement with data in literature concerning feline plaques. So, in the present study two types of A, deposits, were observed. The first type was the major one, and characterized by Aß positive antigenic material distributed throughout the cortical neuropil of the frontal and parietal lobes. This type was detected immunohistochemically in all examined cats, while others detected Aß positive material only in very old (20 years old) cats (Cummings et al., 1996b; Nakamura et al., 1996). The second type, the diffuse plaques, was distributed throughout the cortical layers of the parietal lobes and they were detected only in very old cats (17-21 years old). This finding is not in agreement with the findings of others (Nakayama et al., 2001), who found them only in one 20 year old cat. Typical primitive and neuritic plaques were not encountered in our study. This type of morphological pattern seen in the aged feline brain may indicate an early stage of plaque formation. Since human diffuse plaques are known via primitive plaques finally to progress to neuritic plaques, the current findings in cat brains suggest that the presence of longer Aß peptides is not the only prerequisite for seeding of amyloid *in vivo*. Therefore the lack of feline primitive plaques must be related to other factors (i.e. apolopoprotein E, α1-antichymotrypsin, metal ions etc).

In the aged canine brain the diffuse and primitive plaques contained a mixture of Aß17-42 and Aß42, and minimal Aß40 (Cummings et al., 1996b, Wisniewski et al., 1996). Some investigators have reported that the A, deposits in the aged feline brain were immunoreactive for Aß40 (Nakamura et al., 1996) and Aß42 (Cummings et al., 1996b) while the cortical capillaries and arterioles were immunoreactive for both (Cummings et al., 1996b; Nakamura et al., 1996). On the other hand, our results showed that the plaques and the vascular amyloid in the brain of the examined aged cats were strongly positive-stained with Aß42.The above finding shows that the feline diffuse plaques contain a significant proportion of Aß42.

Neuronal strongly positive- staining with Aß42 and weaker staining with the other used antibodies was also detected. However, the latter finding is contradictory to the results of other studies (Cummings et al., 1996b) where the neurons were weakly positive either with Aß40 or Aß42. The neuronal staining with anti Aß is in agreement with other studies, indicating that this staining, compared with the presence of plaques in the brains of aged cats, is strong evidence for the neuronal origin of plaques and the deposition of Aß (Cummings et al., 1996b; Wisniewski et al., 1996; Fernandez-Vizarra et al., 2004). Moreover, our finding concerning the neuronal staining is also supported by the findings of

Table 4. Immunoreactivity of senile plaques and vascular amyloid and brain tissues with antibodies examined

ANTIBODIES	Aß DEPOSITS				
	Aß positive antigenic material	Diffuse plaques	Arteries	Capillaries	Neurons
Aß8-17	+	+	+	+	+
Aß40	++	++	++	++	++
Aß42	+++	+++	+++	+++	+++
Aß43	-	-	-	-	-
Panß (15-30)	+	+	+	+	+
APP	-	-	-	-	+++

+++: severe; ++: moderate; +; few; -: undetected.

other researchers who identified the endoplasmic reticulum (ER), the trans-Golgi network (TGN) and the mitochondria as the sites for generation of Aß42 and Aß40 (Cook et al., 1997; Hartmann et al., 1997, Wilson et al., 1999, Fernandez-Vizarra et al., 2004, Rodrigo et al., 2004). Concerning the neuronal staining with APP, we detected stronger immune staining in the aged cats in comparison with the younger ones. This finding is in agreement only with the canine findings (Gruys personal communication). All the above lead us to suggest that further biochemical measurements and immunohistopathological findings are needed to investigate in detail the origin of plaques in the brain of aged cats.

It has been suggested that Siamese cats are prone to develop AA amyloidosis (Hol and Gruys, 1984; van der Linde-Sipman et al., 1997). From the present results it appeared that the Siamese cats were also more sensitive for developing Ab-positive deposits than the Domestic Shorthair (DSH) cats. It is interesting to note that we found diffuse plaques in one 12-year old Siamese cat while the majority of the diffuse plaques were detected in 17-year old cats and more. The latter finding compared with the sensitivity to the susceptibility for AA amyloidosis supports the view that some general factors, especially genetic, concerning amyloid other than SAA or Ab might occur in this breed of the cat.

In conclusion, the present immunohistochemical results indicate that as well as canines, felines appear to be a useful spontaneous model for understanding the changes of normal brain ageing or neurodegenerative diseases.

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