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UNIVERSITÀ DEGLI STUDI DI TORINO

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Parenchymal and Vascular Lesions in Ageing Equine Brains: Histological and Immunohistochemical Studies

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Summary

Many age-related changes are described in the nervous system of different species, but detailed studies of brain lesions in ageing horses are lacking. The aim of the present study was to systematically characterize lesions in the brains of 60 horses aged from 7 to 23 years. No gross changes were present in any brain. Microscopically, spongiform changes, lipofuscin storage, corpora amylacea, gliosis and satellitosis were common, together with axonal and neuronal swellings. The most important findings were the presence of pseudocalciumcalcium (pCaCa) deposits and arterial wall degeneration. Scanning electron microscopical examination of two cases with vascular mineralization revealed marked deposition of an amorphous substance in the vessel walls that was probably formed by a polyanionic protein matrix and a mineral component. Immunohistochemically, numerous axonal spheroids were positively labelled for ubiquitin. No PrPsc was detected in sections with neuronal vacuolation. Neuronal swelling, corpora amylacea, hippocampal Tau-positive neurons and methenamine-positive diffuse (preamyloid) plaques were also detected. Congo red staining failed to detect amyloid deposition. The characterization of age-related lesions in the brains of these horses will allow these changes to be discriminated from pathological processes in future studies. Some lesions described here, including some vascular changes, the presence of diffuse plaques and tau accumulation in hippocampal neurons, have not been described previously in the horse.

Keywords: ageing; brain; histopathology; horse

Introduction

A wide range of changes has been described in the ageing brain of many species (Cork *et al.*, 1988; Mirra *et al.*, 1993; Summers *et al.*, 1995; Borrás *et al.*, 1999; Schultz *et al.*, 1999; Ferrer *et al.*, 2005). The most detailed investigations have been of man, monkeys, mice and dogs, but some studies have investigated other

species including cows (Yanai *et al.*, 1994a; Gavier-Widen *et al.*, 2001), sheep and goats (Braak *et al.*, 1994; Nelson *et al.*, 1994; Hooper, 1999), pigs and horses (Furuoka *et al.*, 1996; Yanai *et al.*, 1996; Jahns *et al.*, 2006).

The most commonly reported age-related changes include neuroaxonal dystrophy, calcification and inflammation, but the pathogenesis and functional consequences of these changes are largely unknown. The aim of the present study was to characterize the histo-pathological changes present in the brains of 60 aged

horses and to compare these with changes previously described in the ageing equine brain (Hurst, 1934; Saunders, 1953; Yanai *et al.*, 1996; Jahns *et al.*, 2006).

Materials and Methods

Animals

The brains of 60 heavy farming horses (aged 7e23 years) and of five young control horses (aged 3 months to 5 years) were examined. The aged animals included 30 males and 30 females imported from Eastern Europe and slaughtered in an abattoir in Torino. The aged horses were grouped by age, so that group A comprised seven horses aged 7e9 years, group B comprised 17 horses aged 10e13 years and group C consisted of 36 horses aged 15e23 years. The control animals included three young males and two females, living in Italy, with no evident neurological signs and which had died from different causes unrelated to the nervous system. These horses underwent post-mortem examination at the Department of Animal Pathology, University of Torino.

Histopathology

Brains were collected immediately after death and fixed in 10% neutral buffered formalin. Coronal slices of the frontal and temporoparietal cerebral cortex, basal nuclei area, hippocampus, thalamus, midbrain, cerebellum, pons and medulla oblongata were embedded in paraffin wax. Sections (5 mm) were stained with haematoxylin and eosin (HE) in addition to Weigert's Van Gieson (for collagen), von Kossa (for calcium), Turnbull blue (for iron), methenamine silver (for plaques), Gallyas silver (for neurofibrillary tangles) and Congo red (for amyloid).

The severity of microscopical lesions was graded as: no lesions (—), a low number of focal to multifocal lesions (+), a moderate number of lesions disseminated through one or more areas (++) or diffuse and severe lesions (+++).

Immunohistochemistry

Eight of the most severely affected brains of horses aged 9e15 years, including those with vascular calcification, and the brains of 10 animals older than 15 years were selected for methenamine silver staining and immunohistochemistry (IHC). Primary antibodies employed in IHC included reagents specific for glial fibrillary acidic protein (rabbit anti-cow GFAP; 1 in 2,000; Dako, Glostrup, Denmark; code number Z0334), ubiquitin (rabbit anti-human ubiquitin; 1 in 100; Dako; Z0458), b-amyloid protein (mouse anti-human bA4; 1 in 50; Dako; M0872), total Tau protein (rabbit anti-Tau protein irrespective of the state of phosphorylation; 1 in 500;

Dako; A0024), phosphorylated Tau AT8 (mouse anti-human; 1 in 50; Innogenetics, Pierce Endogen, Rockford, Illinois; 90206), 3-repeat Tau isoform RD3 (mouse anti-human; 1 in 800; Upstate Biotechnology, Lake Placid, New York; 05-803) and 4-repeat Tau isoform RD4 (mouse anti-human; 1 in 50; Upstate Biotechnology; 05-804).

The sections were incubated with 2% hydrogen peroxide in 10% methanol for 15 min at room temperature, followed by incubation with 5% normal goat serum for 2 h at room temperature, then overnight at 4°C with primary antibodies. Secondary detection was by use of an avidinebiotin complex (ABC) kit (Pierce, 32020) or a labelled streptavidin-biotin (LSAB) system (Dako). Immunolabelling was 'visualized' with 0.005% 3, 3'-diaminobenzidine-tetrahydrochloride (DAB; SigmaAldrich, St. Louis, Missouri) and 0.025% hydrogen peroxide in 200 ml phosphate buffered saline (PBS; 137 mM NaCl, 12 mM PO₄, 2.7 mM KCl, pH 7.4), followed by light haematoxylin counterstaining.

In the seven sections in which neuronal vacuolation was identified, PrPsc expression was evaluated by application of bovine PrPsc-specific monoclonal antibody (clone 12F10; Commissariat a l'Energie Atomique, Paris) using the ABC method (Vectastain™; Vector Laboratories, Burlingame, California).

Scanning Electron Microscopy

Samples in which mineral deposits were identified were analyzed by scanning electron microscopy (SEM). These samples were dewaxed and dried by the critical point method using liquid CO₂ in a Critical Point Dryer CPD 030 (Balzers Union, Fiirstentum Liechtenstein). The samples were then mounted on 13 mm diameter stubs with colloidal graphite (EML Laboratories, Berkshire, UK) and carbon coated using a Polaron PS 100 sputter coater (Agar Scientific, Stansted, UK). Analysis was with a Stereoscan 120 scanning electron microscope (Cambridge Instruments, Cambridge, UK) with a Link ISIS 300 dispersive X-ray analyzer (EDX) equipped with the Cameo™ program for X-ray colour imaging (Oxford Instruments, High Wycombe, UK) (Torre and Mattutino, 2000). A working distance of 24 mm, an accelerating voltage of 20 kV and an X-ray detector take-off angle of 30° were used in all analyses. Details of the colour X-ray vision, electron microscopical and microanalysis techniques are given by Statham (1996).

Statistical Analysis

The chi squared test was used to assess differences between the three groups of horses. To investigate for

possible associations between the histological findings and age, parametric or non-parametric tests (AN-OVA or the KruskalWallis test) were also performed. A post-test was performed only when the *P* value was <0.05 (Bonferroni multiple comparison test for ANOVA and Dunn's multiple comparison test for the KruskalWallis test, respectively). The statistical analyses were carried out with GraphPad InStat™ (GraphPad Software, San Diego California).

Results

No gross changes were observed in the brain of any of the horses in this study. The main microscopical features in the brain of horses in groups A to C are summarized in Table 1.

No significant differences were found between these three age groups, except for the presence of lipofuscinosis ($P < 0.05$), satellitosis ($P < 0.05$) and vascular mineralization ($P < 0.01$).

Neuronal vacuolation was observed in seven horses (12%). Vacuoles were most frequently found in the nuclei of the pons and the medulla oblongata, especially in the raphe of the obex and in the vestibular nuclei. The vacuoles were single, of variable size, empty and only occasionally contained amorphous material (Fig. 1a). All vacuoles were observed in the brains of the oldest horses, except for one animal that was aged 8 years (group A). No labelling for PrPsc was detected in any brain.

Vacuolation of the neuropil was present in 20 animals (33%) and always occurred in the area of the brainstem in the nuclei of the pons, medulla and in the rubber nucleus of the mesencephalon (Fig. 1b). Vacuoles varied in size and were focally distributed; diffuse vacuolation was found in only two cases and involved most of the pons. White matter spongiosis was observed in 26 animals (43%) and primarily involved the capsula interna area (Fig. 1c) and the brainstem. The severity and distribution of neuropil vacuolation and white matter spongiosis is summarized in Table 1. The frequency of the different types of vacuolation did not significantly differ between the groups.

Lipofuscin storage chiefly affected the large cortical neurons and numerous neurons of the brainstem nuclei (nucleus vestibularis, formatio reticularis of the pons, nucleus tractus spinalis nervus trigemini, nucleus parasympathicus, nervus vagi and rubber nucleus) in 57 horses (95%). The three horses not affected were aged 8e10 years. No pigment was present in the brains of the young control animals. Lipofuscin was observed mostly in the perikaryon, between the nucleus and the axon hillock. No degenerative changes were noted in the affected neurons.

Lipofuscin storage increased significantly with age ($P < 0.05$).

Multifocal or disseminated astrogliosis was present in 18 cases (30%), occurring mainly in the brainstem (especially in the thalamus) and sometimes in the globus pallidus, where increased GFAP expression was observed. Gliosis was mainly observed in the white matter and accompanied by perivascular astrocytes. This change was moderate, with no significant difference in incidence related to age in the old horses. No alteration in microglia or oligodendroglia was seen.

Small foci of satellitosis were present in 55 animals (92%), particularly in the frontal cerebral cortex, thalamus and midbrain sections and this change was significantly more noticeable among the older animals (100% in group C; $P < 0.05$) (Fig. 1d). Five animals in group A were also affected. ANOVA and Bonferroni post-testing revealed a significant increase in severity of satellitosis ($P < 0.05$) between groups A and C. Oseydineuronophagia (i.e. intense satellitosis) was sporadically observed in the cerebral cortex of five older horses (group C).

Subependymal glial nodules were present in the brain of 14 horses (23%), mostly near the lateral ventricles or the Sylvian aqueduct. These nodules were composed of small, dark, round-oval cells, morphologically resembling glial cells and showing an altered level of expression of GFAP. The ependymal lining was discontinuous over the glial nodules. The number of these nodules did not increase with age (Table 1). Nodules and an irregular ependymal pattern were particularly evident in the brains of seven animals (12%).

Granulomas with lipid crystals were observed in the brains of three animals (5%) aged 10e23 years (groups B and C). These were noted in the subcortical white matter, the pons and the meninges of the cerebral hemispheres. The granulomas were characterized by an accumulation of fusiform empty structures similar to lipid crystals, surrounded by sparse macrophages and glial cells. An increase in collagen fibres was also detected in the meninges overlying these lesions.

Diffuse melanosis and fibrosis of the pineal gland were observed in the brains of five cases (8%). The pigment was present in the pinealocytes, but also in the stroma of the gland between the glial fibres. The presence of melanosis and fibrosis was unrelated to the age of the animals.

The brains of eight horses from group B and 10 from group C were selected for further examination by means of histochemical and immunohistochemical techniques for detection of additional lesions (Table 2).

Table 1 Microscopical features of lesions of the brain in aged horses

<i>Neuropathological features</i>	<i>Areas affected</i>	<i>Group A, severity score and number of cases</i>	<i>Group B, severity score and number of cases</i>	<i>Group C, severity score and number of cases</i>	<i>Number and percentage of positive cases</i>
Neuronal vacuolation	Pons, medulla (raphe of the obex; vestibular nuclei)	-(6) + (1)	-(17)	-(30) + (6)	7 (12%)
Neuropil vacuolation	Medulla, pons, mesencephalon	-(2) + (2) ++ (3)	-(13) + (3) ++ (1)	-(25) + (5) ++ (5) +++ (1)	20 (33%)
White matter spongiosis	Capsula interna (globus pallidus), pons, medulla, mesencephalon	-(3) + (2) ++ (2)	-(8) + (4) ++ (4) +++ (1)	-(23) + (3) ++ (6) +++ (4)	26 (43%)
Neuronal lipofuscin storage	Cortical neurons, brainstem nuclei	-(1) + (6)	-(2) + (9) ++ (6)	-(0) + (20) ++ (15) +++ (1)	57 (95%)
Gliosis	Brainstem, midbrain, globus pallidum	-(4) + (3)	-(12) + (5)	-(26) + (4) ++ (6)	18 (30%)
Satellitosis	Frontal cerebral cortex, thalamus, midbrain	-(2) + (3) ++ (2)	-(3) + (5) ++ (4) +++ (5)	-(0) + (10) ++ (14) +++ (12)	55 (92%)
Subependymal nodules*	Lateral ventricles, Sylvian aqueduct	2	4	8	14 (23%)
Irregularities of the ependymal surface*	Sylvian aqueduct	—	2	5	7 (12%)
Lipid granulomas*	Subcortical white matter, pons, meninges of cerebral hemispheres	—	1	2	3 (5%)
Melanosis and fibrosis*	Pineal gland	1	1	3	5 (8%)
Vascular degeneration	Meningeal vessels	-(1) + (6)	-(6) + (6) ++ (5)	-(18) + (12) ++ (6)	35 (58%)
Neovascularization*	Capsula interna	—	1	1	2 (3%)
Vascular mineralization	Capsula interna (globus pallidum), thalamus, cerebellum	-(4) + (2) ++ (1)	-(8) + (1) ++ (4) +++ (4)	-(32) + (1) ++ (1) +++ (2)	16 (27%)
Choroid plexus degeneration	Lateral ventricles, fourth ventricle	-(2) + (2) ++ (3)	-(12) + (0) ++ (5)	-(20) + (3) ++ (13)	26 (43%)
Cholesteatosis*	Lateral ventricles	1	—	1	2 (3%)

*No grading assigned; group (A) 7–9 years old; group (B) 10–13 years old; group (C) 15–23 years old; (—) no lesions; (+) a low number of focal to multifocal lesions; (++) a moderate number of disseminated lesions; (+++) severe and diffuse lesions.

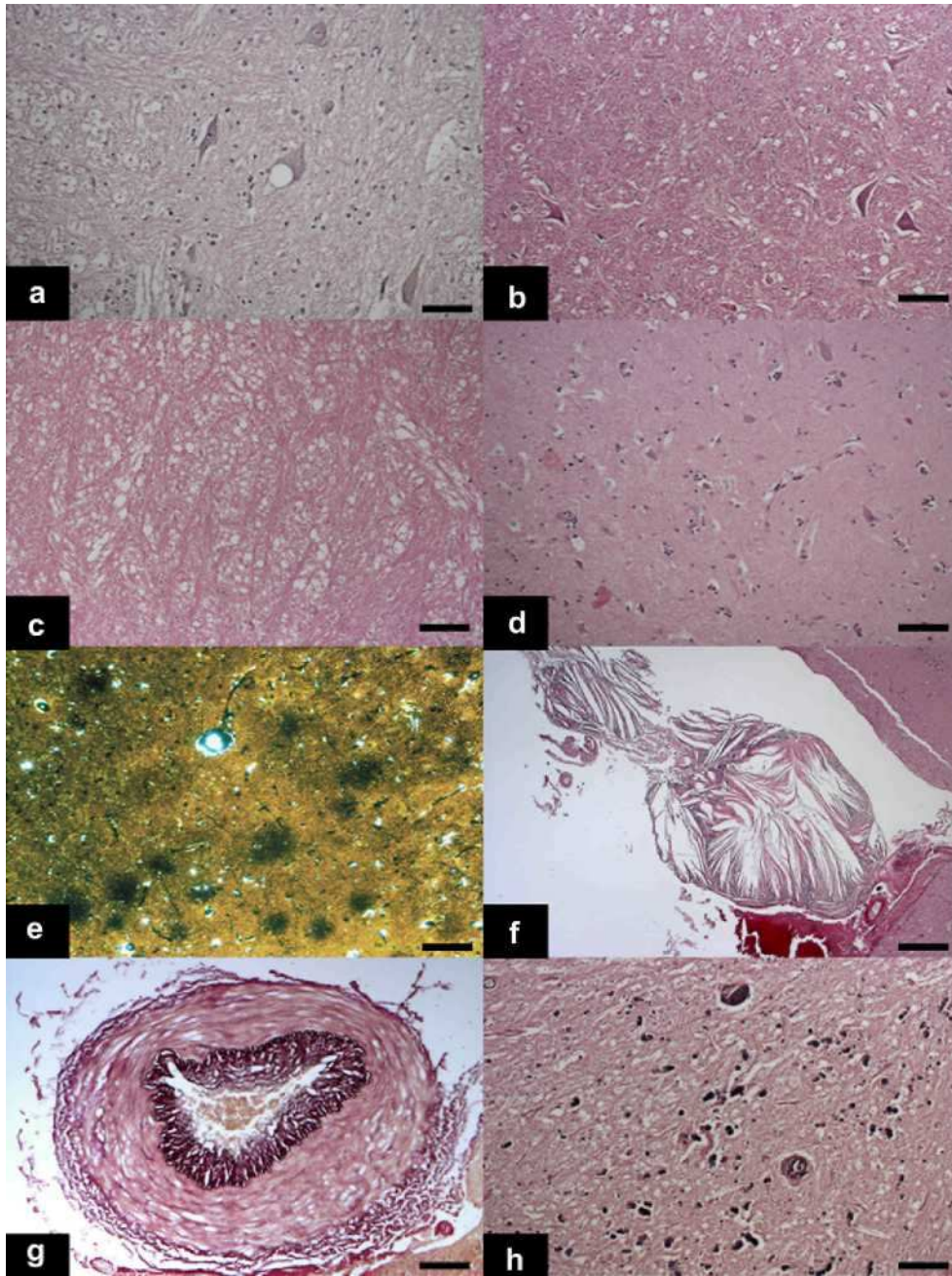


Fig. 1. Sections of equine brain. (a) Section from the pons showing a neuron with an intracytoplasmic vacuole. HE. Bar, 50 mm. (b) Section from mesencephalon showing vacuolation of the neuropil. HE. Bar, 100 mm. (c) Diffuse spongiosis of the white matter of the globus pallidum. HE. Bar, 100 mm. (d) Satellite glial cells surrounding cortical neurons. (e) Diffuse plaques in the neocortex. Methenamine silver. Bar, 50 mm. (f) A cholesterol granuloma of the choroid plexus of the lateral ventricle. HE. Bar, 400 mm. (g) Degeneration of a meningeal artery showing fibroelastic proliferation of the intima and hyperplasia and hypertrophy of the smooth muscle cells of the tunica media. Weigert's Van Gieson. Bar, 100 mm. (h) Capsula interna showing numerous small mineral deposits along vessels walls and in the neuropil. HE. Bar, 100 mm.

Ubiquitin-labelled axonal spheroids and neuronal swellings (Figs. 2a,c) were observed principally in the midbrain and striatum in almost all horses. Ubiquitin-positive corpora amylacea were noted throughout the brain in all animals (Fig. 2d). Most of the

horses had sporadic neurons labelled for Tau in the hippocampus (Figs. 2e, f). These Tau-positive neurons did not label for hyperphosphorylated Tau AT8. A similar proportion of neurons expressed Tau 3 (3R) and 4 (4R) repeat isoforms (Figs. 2g, h). No

Table 2 Histochemical and immunohistochemical studies of selected brains from horses in groups B and C

Horse number and group	Age (years)	Ubiquitin positive		Tau-positive neurons in the hippocampus	Methenamine silver-positive diffuse plaques
		Corpora amyloacea	Spheroids and neuronal swellings		
1 (B)	9	+ / +++	+	-	-
2 (B)	10	+	-	++ / ++++	-
3 (B)	10	+	- / +	-	++ / ++++
4 (B)	10	++	- / +	- / +	-
5 (B)	10	+ / +++	++ / ++++	- / +	+
6 (B)	10	++	+++	+ / ++	-
7 (B)	12	+ / +++	+ / +++	+	+
8 (B)	15	++	++ / ++++	+ / ++	-
9 (C)	>15	+ / +++	+	++ / ++++	NA
10 (C)	>15	+	- / +	-	-
11 (C)	>15	+	+	- / +	-
12 (C)	>15	+	+	+	-
13 (C)	>15	+	+	+	-
14 (C)	>15	++	- / +	++	-
15 (C)	>15	+ / +++	+	+	-
16 (C)	>15	++	+	++ / ++++	-
17 (C)	>15	++ / ++++	- / +	-	+
18 (C)	>15	++ / ++++	++	+++	-

The number of positive structures was scored as none (-), low (+), moderate (++) and numerous (+++); NA, not available.

neurofibrillary tangles were detected by the Gallyas silver stain. Methenamine silver staining revealed diffuse plaques of 50 µm mean diameter in the neocortex of 25% of the horses examined (Fig. 1e). b-amyloid (bA4) IHC and Congo red staining failed to detect amyloid deposition in the form of amyloid plaques or amyloidotic angiopathy in any of the horses. No significant differences were recorded in the severity of the lesions described in Table 2 when horses from groups B and C were compared.

Oedema, fibrosis and hyaline degeneration of the choroid plexus, especially in the lateral ventricles, were observed in 26 cases (43%). Against all expectations, choroid plexus degeneration was found across animals in all three age groups and was highest in group A, but the difference in incidence among the groups was not significant. The young controls showed no degenerative changes in the choroid plexus.

Cholesteatosis of the choroid plexus of the lateral ventricles, in the form of small nodules termed cholesteatomas or cholesterol granulomas, occurred in two horses, aged 8 and 16 years (Fig. 1f).

In 35 horses (58%), vascular degeneration was moderate to severe, with the development of intimal fibroelastic proliferation. Although there was no significant relationship with age, lesion severity was found to increase with age and was limited to scattered vessels in different areas of the brain. Intimal proliferation primarily affected the meningeal vessels and was usually associated with hyperplasia and hypertrophy of the smooth muscle cells of the tunica media. These

muscle cells sometimes seemed to be transformed into 'foamy cells', similar to those observed in human atherosclerosis. The internal elastic membrane of these vessels was often irregular and sometimes duplicated, fragmented or discontinuous (Fig. 1g), as seen in the initial stages of human arterial degeneration.

Vascular mineralization of varying degrees was found in 16 cases (27%) and significantly increased with age ($P < 0.01$), with a higher frequency in animals of group B. No vascular mineralization was detected in the control animals. Mineralized lesions were mainly observed near the capsula interna, especially in the globus pallidus and in the thalamus. The severity of these lesions is reported in Table 1. Mineral accumulation was significantly greatest in the brain of horses in group B. One animal, aged 12 years, had severe diffuse lesions, also involving the white matter of the cerebellum. Mineralization occurred in or around the vessel walls, but sometimes the deposits totally occluded the lumina of the affected blood vessels. The most frequent amorphous deposits were in the form of small globules along the capillaries or around the walls of the arterioles and veins. Small deposits were occasionally found free in the parenchyma, with no relationship to the blood vessels. The largest deposits were found in and around the arterioles, sometimes showing a concentric lamellar structure. The deposits were intensely PAS positive. No inflammatory or glial reactions were observed around the mineralized lesions, but in two brain samples there was vascular neoformation, probably secondary to vessel occlusion.

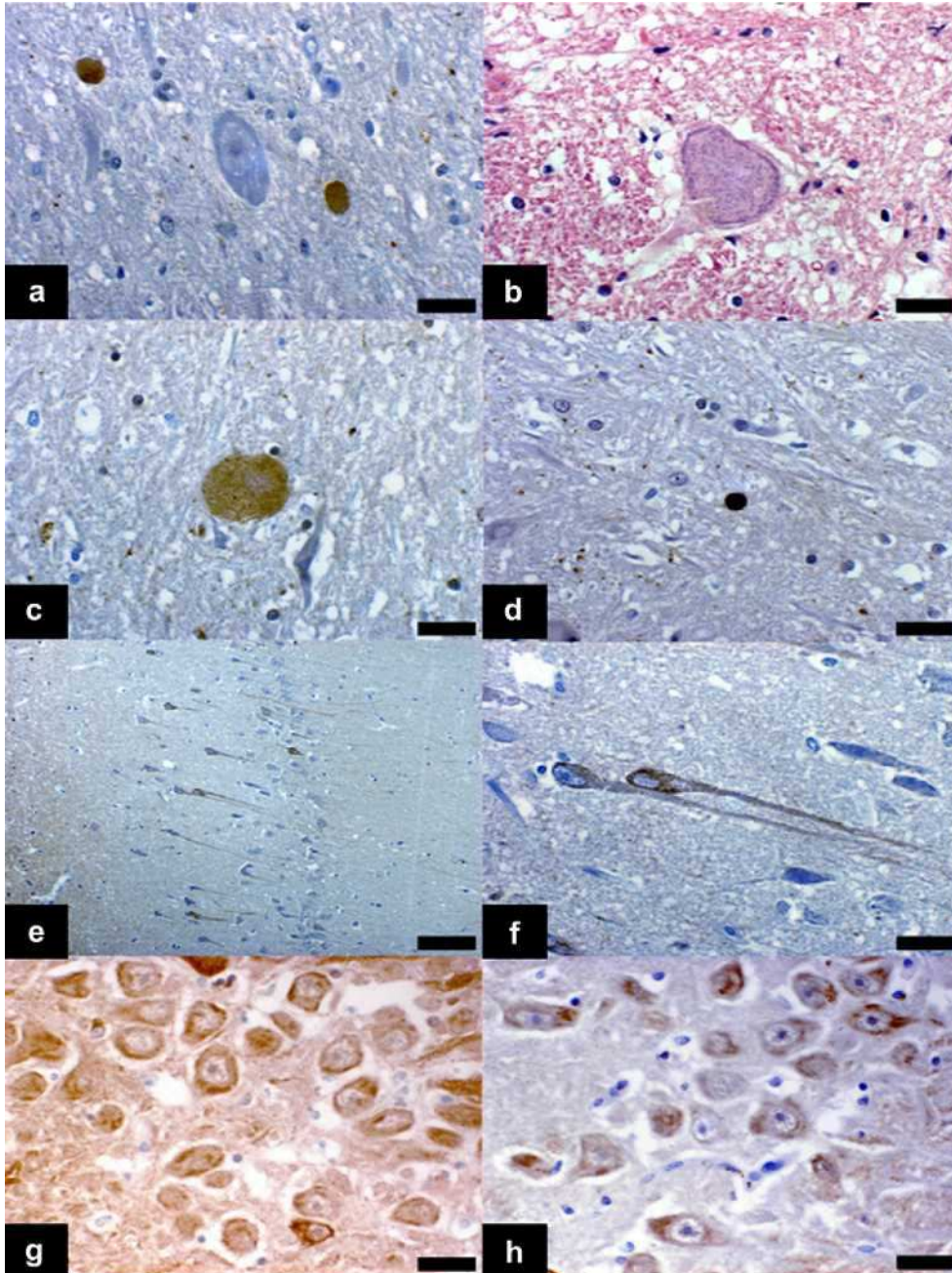


Fig. 2. Sections of equine brain. (a) Axonal spheroids labelled for ubiquitin. IHC. Bar, 25 mm. (b) A swollen neuron. HE. Bar, 25 mm. (c) A swollen neuron labelled for ubiquitin. IHC. Bar, 25 mm. (d) Corpora amylacea labelled for ubiquitin. IHC. Bar, 25 mm. (e) Hip-pocampal neurons labelled for expression of Tau. IHC. Bar, 100 mm. (f) Higher magnification of (e) showing two labelled neurons. IHC. Bar, 25 mm. (g) Hippocampal neurons labelled for Tau 3R expression. IHC. Bar, 25 mm. (h) Hippocampal neurons labelled for Tau 4R expression. IHC, Bar, 25 mm.

The calcified material stained intensely by HE (Fig. 1h), von Kossa and Turnbull blue.

In two cases of severe and diffuse vascular mineralization, SEM revealed marked deposition of amorphous substance in the wall of small vessels, sometimes almost totally occluding the lumina or forming concentric structures. The Cameo™ program for X-ray colour allowed characterization of these mineral deposits.

Calcium, phosphorus and magnesium were observed most frequently, with small quantities of other minerals not sufficiently consistent for analysis (Fig. 3).

Discussion

The microscopical changes observed in the brain of aged horses appear similar to those described in old

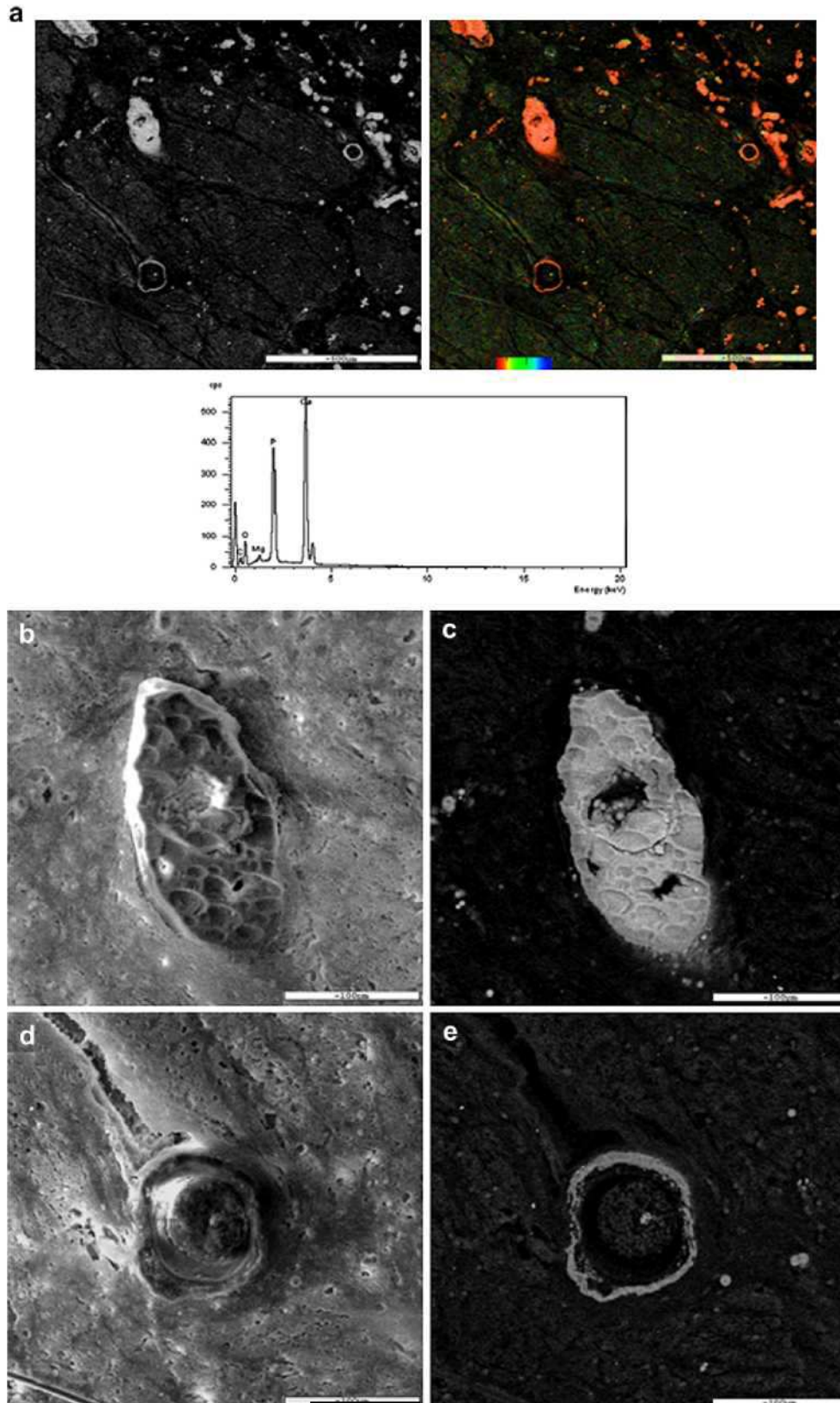


Fig. 3. Sections of equine brain. (a) Backscattered electron image. A visual response offset from 3.50 keV to 6.20 keV X-ray region showing the distribution of large amounts of mineral material where calcium and phosphorus have white colouration. (bee) Further details of (a) are shown. (b) and (d) Classical secondary electron images using scanning electron microscopy. (c) and (e) The same areas of (b) and (d) with retrodiffuse electrons showing marked deposition of amorphous substance in the wall of small vessels, sometimes almost totally occluding the lumina (b, c) or forming concentric structures (d, e). SEM. Bars, (a) 500 μ m; (bee) 100 μ m.

animals of other species and in elderly people (Cork *et al.*, 1988; Mirra *et al.*, 1993; Summers *et al.*, 1995; Borrás *et al.*, 1999; Schultz *et al.*, 1999; Jahns *et al.*, 2006). Neuronal and neuropil vacuolation and white matter spongiosis are degenerative lesions rarely reported in horses, and the mechanisms underlying development of these changes are poorly defined. According to Jahns *et al.* (2006) such vacuolation is not age related. The areas affected by neuronal vacuolation in the present study were similar to those described by Jahns *et al.* (2006) and included the nuclei of the brainstem, especially those of the pons and medulla oblongata. In contrast to the study of Jahns *et al.* (2006), vacuolation of Purkinje cells was not observed. The incidence of neuronal vacuolation in the brains of horses of the present study was low (12%), with no significant differences between the three age groups.

Neuropil vacuolation did not appear to be age related, although lesion severity was found to increase slightly with age. The aetiopathogenesis and clinical significance of this change are unknown. Spongy degeneration, brain oedema or autolysis (Summers *et al.*, 1995) might be responsible for this event. In the present study, autolysis may be excluded because the brains were adequately fixed in formalin immediately after slaughter.

White matter spongiosis occurred in the globus pallidus area and in the brainstem, but the reason for this localization is unknown. Similar lesions have been previously documented in horses (Jahns *et al.*, 2006) and cattle (Guarda, 1978) but remain unexplained. Numerous metabolic disorders, intoxication or infectious diseases with toxæmia may cause spongiosis, and cavitation generally occurs throughout the whole neuraxis, sometimes with bilateral and symmetrical distribution (Summers *et al.*, 1995).

Intraneuronal lipofuscin storage was observed in nearly all animals, as previously described (Whiteford and Getty, 1966; Summers *et al.*, 1995; Esiri *et al.*, 1997; Borrás *et al.*, 1999; Jahns *et al.*, 2006). The quantity of pigment appeared to increase in individual neurons and more neurons were affected in the same area with advancing age. No evidence of pigment accumulation was observed in the neurons of young control horses.

A quantitative analysis of neuronal loss was not performed, but satellitosis is generally associated with neuronal damage and depletion (Summers *et al.*, 1995; Kiatipattanasakul *et al.*, 1996), and satellitosis increases in aged animals (Fankhauser, 1972; Summers *et al.*, 1995).

Gliosis involving the grey and white matter has been described in aged mice and dogs (Summers *et al.*, 1995). The pathological significance of this change is poorly understood, but some authors consider it to

be an expression of senescence, secondary to neuronal damage (Shimada *et al.*, 1992). However, gliosis is also regarded as a common non-specific response of glial cells to many forms of injury. The observed subependymal glial nodules resemble those described in human neuropathology as a reaction to irritant non-specific insults such as trauma, inflammatory processes or imbalance in cerebrospinal fluid pressure (Sarnat, 1995).

Lipid granulomas in the parenchyma appear to be related to chronic inflammation and infiltration of the nervous tissue by lipid-containing macrophages. The lipid crystals that deposit in the tissue spaces stimulate productive inflammation, as occurs in a foreign body reaction (Maxie and Youssef, 2007).

The observed fibrosis of the pineal gland was probably an age-related phenomenon, similar to the systemic fibrosis observed in aged animals of all species (Nieberle and Cohrs, 1962). Melanin may normally accumulate within human and animal pinealocytes and is not associated with clinical dysfunction (Koshy and Vettivel, 2001). Melanin is found in photoreceptors and photoreceptor cells derived from the pinealocytes and melanin accumulation increases in older people (Koshy and Vettivel, 2001). No specific data are reported in horses concerning accumulation of melanin within the brain with age, and no significant relationship was defined in the present study.

Neuroaxonal dystrophy (NAD; swollen neurons and spheroids) is commonly observed with increasing age in man and in other species such as the dog and primates (Bronson and Schoene, 1980; Arai *et al.*, 1993; Borrás *et al.*, 1999). NAD is considered to reflect neuroaxonal degeneration with age. As previously reported in NAD, spheroids observed in the brains of the horses in the present study were positively labelled for ubiquitin expression, indicating proteolytic activity occurring within these structures (Bronson and Schoene, 1980; Arai *et al.*, 1993; Borrás *et al.*, 1999). Ubiquitin is a highly conserved protein that targets proteins for non-lysosomal ATP-dependent proteolysis and is also known to bind to degenerate proteins and to have a role in the repair of degenerate axons (Migheli *et al.*, 1992; Lowe *et al.*, 1993; Gai *et al.*, 1995).

The corpora amylacea are polyglucosan bodies found throughout the brain, apparently free in the neuropil and/or associated with glial processes. These structures accumulate in the course of normal ageing in man and in a wide range of animal species (Cava-nagh, 1999). In the present study there was no significant difference in the number of corpora amylacea and in the labelling of these structures for ubiquitin in the brains of the horses examined.

Whereas the findings related to NAD and corpora amylacea are in agreement with those reported in other species and aged people, an unusual observation made in the present study was the expression of Tau protein by some neurons in the hippocampus. Tau protein is a highly soluble microtubule-associated protein (MAP) found in neurons. One of its main functions is to stabilize the axonal microtubules that are essential for fast axonal transport. Hyper-phosphorylation of this protein results in the self-assembly of fibres called neurofibrillary tangles (NFTs), or paired helical filaments and straight filaments, and in the accumulation of Tau in neurons (and glial cells). Accumulation of NFTs is a common feature of many neurodegenerative diseases such as Alzheimer's disease (AD) and other 'tauopathies' including Pick's disease (PiD), progressive supranuclear palsy, corticobasal degeneration, argyrophilic grain disease and familial frontotemporal dementia and Parkinson's disease (Ferrer *et al.*, 2005). An imbalance in the 4R/3R Tau isoforms has been observed in several tauopathies (Yoshida, 2006). Tau-positive hippocampal neurons observed in the present study did not express hyperphosphorylated Tau (AT8), indicating that the accumulated Tau was not hyperphosphorylated at position S202. However, phosphorylation at other amino acid residues cannot be ruled out. The similar immunolabelling observed for 3R and 4R Tau isoforms excludes the possibility of a proportional imbalance of the isoforms. Because Gallyas silver staining did not detect neurofibrillary tangles inside these neurons, we can only conjecture that non-phosphorylated-S202 Tau accumulates in some hippocampal neurons in a non-neurofibrillary tangle manner, perhaps due to axonal transport deficiencies occurring in ageing.

Brain ageing is frequently associated with deposition of β -amyloid in the form of senile and diffuse plaques or amyloid angiopathy in man and other species (Cork *et al.*, 1988; Mrak *et al.*, 1997; Walker, 1997; Borrás *et al.*, 1999; Kimura *et al.*, 2001; Nakayama *et al.*, 2001). In aged people, monkeys, dogs and bears, diffuse and mature plaques can be observed, whereas in cats and camels the diffuse type has been the only plaque type observed (Nakayama *et al.*, 2001). Diffuse plaques were rarely found in the brains of horses in the present study in comparison with the occurrence of spheroids, Tau accumulation or corpora amylacea. In the present study, diffuse plaques were the only plaque type detected by methenamine silver staining in the neocortex of some horses. These plaques did not stain by Congo red and were not labelled by IHC for bA4 expression. These results indicate the presence of pre-amyloid/non-congophilic diffuse plaques accumulating N-truncated Ab₄₂ with no Ab₄₀ as reported

in man and other species (Cummings *et al.*, 1996b). Ab₄₀ is known to accumulate in mature plaques and the lesions of amyloid angiopathy (Cummings *et al.*, 1996b), but neither of these changes was present in the horses of the present series. This suggests that forms of non-congophilic amyloid, other than Ab₄₀, may accumulate in the brain of aged horses.

Fibrosis and hyaline degenerative changes of the choroid plexus are frequently found in aged animals, but carry no specific significance (Summers *et al.*, 1995; Borrás *et al.*, 1999). In mature and aged horses, cholesterol granulomas (cholesteatomas) more commonly arise in the plexus of the fourth ventricles than in the lateral ventricles, where they may cause hydrocephalus (Summers *et al.*, 1995). The development of cholesteatomas appears to be related to chronic or intermittent congestion and oedema, with haemorrhages in the plexus. During these processes, the plexuses are infiltrated by lipid-containing macrophages, leading to the deposition of cholesterol crystals in the tissue spaces and stimulation of an inflammatory response (Maxie and Youssef, 2007).

The significance of the vascular changes observed in the brains of these horses is unclear. Cognitive dysfunction and behavioural changes without histological correlations have recently been recognized in aged dogs (Ruehl *et al.*, 1995; Cummings *et al.*, 1996a; Borrás *et al.*, 1999), but a similar syndrome is not yet reported in horses. Vascular degenerative changes such as atherosclerosis, so prominent in human pathology, are practically unknown in animals, with the exception of pigs and birds. Chronic arterial changes (arteriosclerosis) consisting of luminal narrowing, resulting from proliferative and degenerative changes in the media (increase in smooth muscle cells) and the intima (accumulation of mucopolysaccharides and fibrous tissue), are more common in domestic animals. Normally, there is no significant disturbance of blood flow associated with these changes. The arterial degenerative processes observed in the present study were characterized by changes in the internal elastic membrane (duplication and fragmentation) and hypertrophy/hyperplasia of the smooth muscle cells of the tunica media, with accumulation of lipid globules in the cytoplasm, similar to the foamy cells described in man. However, the lesions always involved the entire arterial wall and there were no protruding plaques locally compromising normal blood flow. Moreover, atheromas, fibro-fatty plaque or classical advanced lesions (e.g. necrosis, calcification) observed in human atherosclerosis were not seen in these horses and congophilic amyloid accumulation was not detected.

Vascular calcification is well documented in man (Hurst, 1934; Giordana, 1980; Adams and Graham,

1988; Lowe *et al.*, 1997), monkeys (Yanai *et al.* 1994b), cows (Hurst, 1934; Guarda, 1978; Yanai *et al.*, 1994a; Gavier-Widen *et al.*, 2001), mice (Fraser, 1968; Yanai *et al.*, 1984; Yanai *et al.*, 1987), rats (Yanai *et al.*, 1993), cats (Mandara, 2003), dogs (Fankhauser *et al.*, 1965) and horses (Hurst, 1934; Saunders, 1953; Fankhauser *et al.*, 1965; Yanai *et al.* 1996; Jahns *et al.*, 2006). In horses and mice, vascular calcification appears to be influenced by ageing, but the real significance of the process is not well understood. The location (globus pallidus), morphology and histochemical properties of these calcified deposits in the brains of horses of the present series resemble those reported in man and, previously, in horses (Hurst, 1926; Hurst, 1934; Saunders, 1953; Giordana, 1980; Adams and Graham, 1988; Yanai *et al.*, 1996; Lowe *et al.*, 1997; Jahns *et al.*, 2006).

Analytical studies performed on two cases with severe and diffuse vascular mineralization demonstrated that calcium and phosphorus, with smaller amounts of other minerals (e.g. magnesium), were the most important mineral constituents, and that these were associated with an organic matrix that was PAS positive and hematoxylinophilic. The deposits were also positively stained by Turnbull blue, but analytical studies did not confirm the presence of iron, suggesting that this staining pattern was non-specific and related to the presence of other minerals. The deposits in these two animals differed from those previously described in the horse (Yanai *et al.* 1996), as there was higher calcium content but an absence of aluminium, zinc, potassium and sodium. These differences may be related to the different geographical locations of the animals and their breed and breeding conditions. The high level of these minerals found in the basal ganglia may be a consequence of anoxia or a microvasculopathy, but the causes of mineralization remain unclear (Hurst, 1926; Slager and Wagner, 1956; Gomez *et al.*, 1989). It has been proposed that minerals such as calcium phosphate may precipitate on a polyanionic matrix formed by protein complexes with aminopolysaccharides (Schiffer, 1971).

Globoid mineralization of the vascular intima has been previously reported in equine small arterial vessels of numerous organs, but particularly those of the intestinal submucosa (Bollinger, 1869; De Oliveira *et al.*, 1985; Marcato, 2002). The mineralized deposits noted in the brain of horses of the present study were similar in size and composition to those described previously, but all blood vessels, not just arteries, were involved. Asteroid bodies are small structures in the subendothelial arterial wall that may cause hyperplasia of the tunica media, splitting of the internal elastic lamina, endothelial vacuolation and oedema

(De Oliveira *et al.*, 1985). The pathogenesis of formation of asteroid bodies is unknown, but there may be an association with calcium metabolism or with degeneration of smooth muscle cells of the tunica media, perhaps following mechanical injury (De Oliveira *et al.*, 1985). Asteroid bodies do not seem to be related to senescence because they are observed in young foals.

In the animals of the present study, arterial degenerative changes and mineralization were not observed in the same vessel. Although vascular degenerative changes were described, it is not clear whether these are age related or associated with a specific dysfunction in calcium metabolism. Calcium and phosphorus metabolism should be further investigated in order to determine whether there is any link to the formation of vascular mineral deposits.

Ageing in man is associated with a progressive decline in cognitive abilities. Age-related neuropathology is reported, but the significance of many changes remains undetermined. Common gross changes include thickening of the arachnoid, cortical atrophy, white matter loss, an increase in the volume of the ventricles and atherosclerosis of vessels (Esiri *et al.*, 1997). The most common microscopical changes are moderate neuronal loss, lipofuscin storage, glial cell changes and an increase in corpora amylacea and structures that are labelled for ubiquitin on IHC. More specific alterations such as neurofibrillary tangles, neuropil threads, senile plaques, cerebral amyloid angiopathy, Hirano bodies and granulovacuolar degeneration are considered to be related to cognitive dysfunction and dementia (Esiri *et al.*, 1997).

In the aged horses considered in the present study, no gross alterations were identified, whereas numerous microscopical changes were detected in the brain. Some of these were age related and similar to those described previously (Hurst, 1934; Saunders, 1953; Yanai *et al.*, 1996; Jahns *et al.*, 2006). The lesions identified within the vascular system of these aged horses have not been reported previously. Arterial degenerative changes were reported in 58% of the animals, but these were not associated with alteration of the parenchyma. The description of diffuse plaques and Tau accumulation in hippocampal neurons are also novel findings of the present study.

To our knowledge, a cognitive age-related syndrome has not yet been described in horses. However the pathological findings reported in this study suggest that such a syndrome may occur, as it does in aged dogs (Ruehl *et al.*, 1995; Cummings *et al.*, 1996a; Borras *et al.*, 1999). The characterization of the cerebral lesions of these aged horses will permit age-related change to be distinguished from pathological change in future studies. However, further research on aged

horse neuropathology is needed to better understand the pathogenesis of these changes and their possible link to the ageing process. Large-scale studies of horses of both sexes, differing in age, from different geographical areas and clinically assessed with blood biochemical data, could elucidate the incidence and prevalence of different lesions and their possible relationship to age and gender. Molecular studies of old horses may be required for understanding the pathogenesis of neuronal lesions, senile plaques and changes in synapses and blood vessels.

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