FULL PAPER *Pathology*

Age-Related Histological Changes in the Canine Substantia Nigra

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ABSTRACT. Age-related changes in the canine substantia nigra, were examined using immunostaining for tyrosine hydroxylase (TH), glial fibrillary acidic protein (GFAP), neurofilament (NF), ubiquitin, single stranded DNA (ssDNA), and alpha-synuclein (αSN). Brain sections from 34 necropsied dogs, ranging from 2 months to 18 years old, were used for this study. On general histological examinations, several age-related changes, including lipofuscin deposition, polyglucosan bodies, amorphous basophilic inclusions and eosinophilic crystal inclusions, were found in the aged dogs. Immunohistochemically, TH-positive neurons were located only in the substantia nigra. The number of TH-positive neurons was well preserved in all dogs examined, however, the ratio of TH-positive neurons to GFAP-positive glial cells tended to show slight decrease in aged dogs. By ssDNA immunostaining for apoptotic cells, there were no significant results in the number of ssDNA-positive neurons. The number of ubiquitin- and NF-positive swollen neurites was increased markedly in aged dogs. Ubiquitin immunostaining revealed a small number of basophilic and eosinophilic inclusions, although both types of inclusions were negative for NF. By αSN immunostaining, no neurons were immunoreactive and no basophilic or eosinophilic intracytoplasmic inclusions were revealed. These results indicate that in the substantia nigra of aged dogs the dopaminergic neurons are well preserved, but intracytoplasmic inclusions and ubiquitin-positive degenerative neurites are commonly found.

KEY WORDS: aging, canine, inclusion body, senile change, substantia nigra.

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Parkinson's disease (PD) is a systemic neurodegenerative disorder characterized by resting tremor, rigidity, and bradykinesia. Pathologically, PD is defined by a massive loss of dopaminergic neurons of the substantia nigra, and the appearance of intraneuronal inclusions called Lewy bodies [8]. Age-related neuronal cell loss and decline of neurotransmitter systems are sometimes implicated in the pathogenesis of these neurodegenerative disorders. A decrease in number of dopaminergic neuronal cells with aging may contribute to the development of PD, especially in sporadic PD [13, 24, 27, 32]. Recently, some genetic factors such as alpha-synuclein (αSN) and "parkin" have been identified in autosomally dominant inherited PD and in autosomally recessive juvenile parkinsonism [14, 16, 25]. Although these genetic factors may explain the pathogenesis of familial PD, the cause of selective neurodegeneration in sporadic PD in aged patients without genetic changes remains unknown.

On the other hand, various age-related changes have been reported in the central nervous system of the dogs [5]. Beta-amyloid deposition, forming senile plaques and cerebral amyloid angiopathy, has been well examined in the brain of aged dogs, and the usefulness of the dog as an animal model for beta-amyloidogenesis in Alzheimer's disease have been discussed [6, 23, 31]. Although a number of reports concerning these senile changes in the canine cerebral cortex have been published, there are a few data about age-related changes in the canine substantia nigra. In addition, spontaneous disease entry mimicking human PD has not been established in dogs. In the present study, morphological features of canine substantia nigra are examined in a number of dogs of various ages. To determinate the basic morphological changes that occur with aging, we performed immunostaining for glial fibrillary acidic protein (GFAP) to elucidate the proliferation of astrocytes [4], and for ubiquitin and neurofilament (NF) to visualize degenerative neurites and axonal spheroids [18].

Since tyrosine hydroxylase (TH) is a rate-limiting enzyme in the synthesis of catecholamines, including dopamine, the antibody for this enzyme is very useful for visualizing dopaminergic neurons [26]. We employed the antibody to confirm canine substantia nigra. Lewy bodies, representing as intracytoplasmic neuronal inclusions, are considered to be relevant diagnostic and pathogenetic features of PD, and are known to contain more than 20 different proteins, including structural and cytosolic proteins, enzymes, and proteins such as ubiquitin elicited as a cellular response [2, 17]. Recently, a presynaptic protein, αSN, has been identified as specific constituent of the Lewy body [1, 11, 19, 28, 29], and pathological aggregation of α N is thought to be responsible for neurodegeneration and apoptotic neuronal cell loss in the substantia nigra of PD patients [1, 19, 34]. In this study, we performed immunohistochemistry for αSN to detect Lewy-body-like pathology and for single-stranded DNA (ssDNA) to visualize apoptotic cell or fragmented DNA in the canine substantia nigra.

MATERIALS AND METHODS

Brain samples: A total of 34 brain samples were obtained from euthanized dogs, ranging from 2 months to 18 years old. The ages, sex, and major pathological findings of these

Table 1. Age, sex, and major pathological findings of 34 dogs examined

Case No	Age (years)	Sex*	Major pathological findings
1	0.2	M	Catarrhal enteritis
2	0.4	M	Acute pneumonia
3	1.7	F	Hemorrhagic enteritis
4	2	M	Cerebral cortical necrosis
5	3	F	Spinal malacia
6	4.5	F	Endocardiosis
7	6	N.D.**	Granulomatous meningoencephalomyelitis
8	6	M	Malignant histiocytosis
9	8	F	N.D.**
10	8	M	Meningioma
11	10	M	Cardiovascular disease
12	10	F	N.D.**
13	11	F	Mammary gland tumor
14	12	F	Dirofilariasis
15	12	M	Hemangiosarcoma
16	12	F	Meningioma
17	12	F	Dirofilariasis
18	13	F	Osteosarcoma
19	13	F	N.D.**
20	14	F	Mammary gland tumor
21	14	M	Cerminal adenocarcinoma
22	15	F	Cerebral malacia
23	15	F	Mammary adenocarcinoma
24	15.6	F	Pulmonary carcinoma
25	16	F	Cardiomyopathy
26	16	F	Insulinoma
27	16	M	Dysstasia
28	16.3	M	Nephritis
29	17	F	N.D.**
30	17	M	N.D.**
31	17	M	Hepatic nodular hyperplasia
32	18	F	Hepatocellular carcinoma
33	18	M	N.D.**
34	18	N.D.**	N.D.**

*Sex: M; Male and F; Female. **N.D.= No data.

dogs are summarized in Table 1.

Histopathology: For histopathological investigation, these brain samples were fixed in 10% formalin and embedded in paraffin. The blocks were taken from the cerebrum, hippocampus, and midbrain, including the substantia nigra. Paraffin sections 4 to 6 μm thick, were made and stained with hematoxylin and eosin (HE) for light microscopy. Some selected sections were also stained with periodic acidic Schiff (PAS) and phosphotungstic acid hematoxylin (PTAH).

Antibodies: As primary antibodies, rabbit sera against TH (1:100, Rotos Boptech Cooperation, New York, NY., U.S.A.), GFAP (Prediluted, DAKO-Japan, Kyoto, Japan.), and ssDNA (1:400, DAKO-Japan), and ubiquitin (1:100, DAKO-Japan) as well as a goat anti-αSN antiserum (1:5, Chemicon, International Inc., Temecula, CA., U.S.A.), and a mouse monoclonal antibody for low and high molecules of NF (Prediluted, DAKO-Japan) were used. As a secondary antibody for αSN immunostaining, biotinylated-rabbit serum against goat immunoglobulin was used.

Immunohistochemistry: Immunohistochemistry was performed using streptoavidin biotin peroxidase complex (SAB, Nichirei, Tokyo, Japan) for αSN and an Envision polymer (DAKO-Japan) for the others. For the immunostaining of TH, ubiquitin and NF, hydrated autoclave pretreatment was attempted to enhance immunoreactivity. For αSN immunostaining, the sections were immersed in 0.1% trypsin solution at room temperature for 20 min to enhance immunoreactivity. Endogenous peroxidase activity was quenched by adding 0.3% hydrogen peroxide in methanol for 10 min at room temperature. All of the sections were incubated with 3% (w/v) bovine serum albumin in PBS for 30 min at 37°C. The sections were then incubated with primary antibodies at 37°C for 30 min, and with an Envision polymer reagent at 37°C for 30 min except in the case of the αSN immunostaining. For this immunostaining, the sections were incubated with SAB at 37°C for 30 min. The chromogenic reaction was carried out with 3,3-diaminobenzidine. The sections were counterstained with Mayer's hematoxylin for the TH, ubiquitin, GFAP, NF, and αSN immunostaining, and with light green for the ssDNA immunostaining.

Quantitative analysis: Quantitative analysis for each immunostaining was performed on 5 randomly selected fields at a magnification of × 400, and the average number of positive cells or area per 1 mm^2 was calculated.

RESULTS

General histopathology: The results of general histological examinations in the substantia nigra were summarized in Table 2. In aged dogs, intracytoplasmic lipofuscin deposits in the neurons and irregularly extended myelin sheaths, sometimes accompanied by spheroids, were frequently observed. Intracytoplasmic neuromelanin pigments were also prominent in aged dogs. In 9 aged dogs, amorphous basophilic inclusions were occasionally found in the neuronal cells (Fig. 1). These inclusions were intensely positive for PAS stain, and appeared as aggregates of PAS-positive granules larger than lipofuscin. Moreover, in two aged dogs, inclusion bodies that were crystalline acidophilic, and intracytoplasmic, and characterized as single, round granules mimicking Negri bodies were detected (Fig. 2). All of these inclusions were negative for PAS and some were stained with PTAH. Immunohistochemically, both types of inclusions were negative for αSN and NF, and a few were feebly positive for ubiquitin.

Tyrosine hydroxylase: TH-positive neurons were localized in the substantia nigra, but not in the other regions including the cerebral cortex and hippocampus. The cytoplasm of the large neuronal cell and their processes in the substantia nigra showed very intense and selective immunoreactivity (Fig. 3). In addition, almost all melanin-bearing neurons were positive for TH. The number of TH-positive neurons was well preserved in all dogs examined, and tended to increase slightly in aged dogs (Fig. 4).

Table 2. Summary of histological lesions in the substantia nigra of dogs examined

Case No.	Age (years)	General histological lesions in the substantia nigra
1	0.2	N.S.*
2	0.4	N.S.
3	1.7	N.S.
4	2.0	Ischemic neurons
5	3.0	N.S.
6	4.5	N.S.
7	6.0	Perivascular cuffing
8	6.0	Infiltration of histiocytes
9	8.0	Severe congestion
10	8.0	N.S.
11	10.0	N.S.
12	10.0	N.S.
13	11.0	N.S.
14	12.0	Alzheimer's type II glia, Perivescular cuffing
15	12.0	Basophilic inclusions
16	12.0	Basophilic inclusions
17	12.0	Basophilic inclusions, Lipofuscin deposits, Ischemic neurons, Alzheimer's type II glia,
18	13.0	Lipofuscin deposits, Polyglucosan bodies
19	13.0	Basophilic inclusions, Lipofuscin deposits, Polyglucosan bodies
20	14.0	Lipofuscin deposits
21	14.0	Lipofuscin deposits
22	15.0	Lipofuscin deposits, Ischemic neurons, Perivascular cuffing
23	15.0	Basophilic inclusions, Lipofuscin deposits
24	15.6	Basophilic inclusions, Lipofuscin deposits
25	16.0	Lipofuscin deposits
26	16.0	Lipofuscin deposits, Alzheimer's type II glia
27	16.0	Basophilic inclusions, Lipofuscin deposits
28	16.3	Eosinophilic crystal inclusions, Basophilic inclusions, Lipofuscin deposits, Polyglucosan bodies
29	17.0	Basophilic inclusions, Lipofuscin deposits
30	17.0	Basophilic inclusions, Lipofuscin deposits, Polyglucosan bodies
31	17.0	Basophilic inclusions, Lipofuscin deposits
32	18.0	Lipofuscin deposits
33	18.0	Lipofuscin deposits
34	18.0	Eosinophilic crystal inclusions, Basophilic inclusions, Lipofuscin deposits

*N.S.: No significant lesions.

GFAP: GFAP-positive cells, suggesting astrocytes, were found relatively less frequently in the substantia nigra than in the other regions examined. The number of GFAP-positive cells per 1 mm^2 tended to increase slightly in aged dogs (Fig. 5). In addition, the ratio of TH-positive neurons to GFAP-positive glial cells tended to decline in aged dogs (Fig. 6).

ssDNA: Immunostaining for ssDNA revealed the nuclei of the neuronal, glial, and vascular smooth muscle cells. The number of ssDNA-positive neuronal cells in the substantia nigra was very small. There was no significant relationship between the number of ssDNA-positive neurons and individual age.

Ubiquitin: Ubiquitin-positive materials varied in size and shape, and most appeared as granular structures within the neuropile. There were small amounts of ubiquitin-positive materials within the cytoplasm of neurons. Immunoreactivity for ubiquitin of the neuronal nucleus was detected in a few cases, but there was no significant trend. The average index of positive ubiquitin reactivity for the neuropile increased markedly in aged dogs (Fig. 7).

NF: Immunostaining for NF revealed some large neuronal cells, their processes, and axonal spheroids within the extended myelin sheaths of the substantia nigra as well as of the other regions examined. The distribution of NF-positive spheroids closely matched that of ubiquitin-positive large global materials in the neuropile. The average index of NF-positive spheroids increased markedly in aged dogs like as ubiquitin-positive neuropile.

Alpha synuclein: By immunostaining for αSN, there were no immunoreactive neurons in the substantia nigra, and no inclusion-like bodies were revealed.

DISCUSSION

Immunostaining for TH was very useful for visualizing dopamine-synthesizing neuronal cells and their processes in the substantia nigra, as described in humans and other ani-

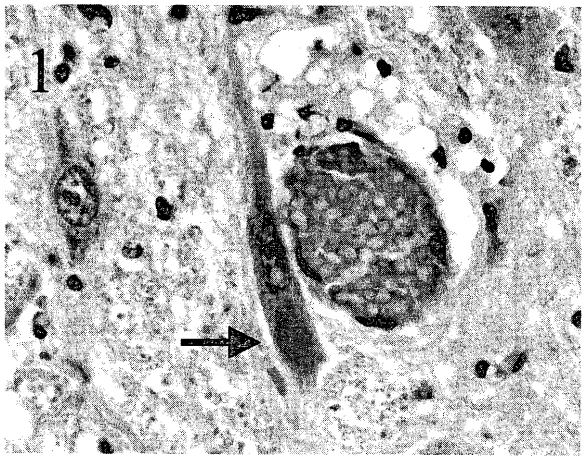

Fig. 1. Basophilic intracytoplasmic inclusion bodies (arrow) in neurons of the substantia nigra. HE. Stain. × 400.

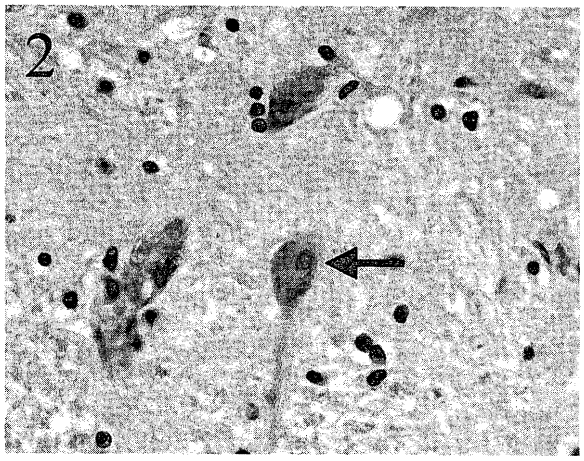

Fig. 2. Eosinophilic crystal inclusions mimicking Negri body (arrow) in the neurons of the substantia nigra. HE stain. × 400.

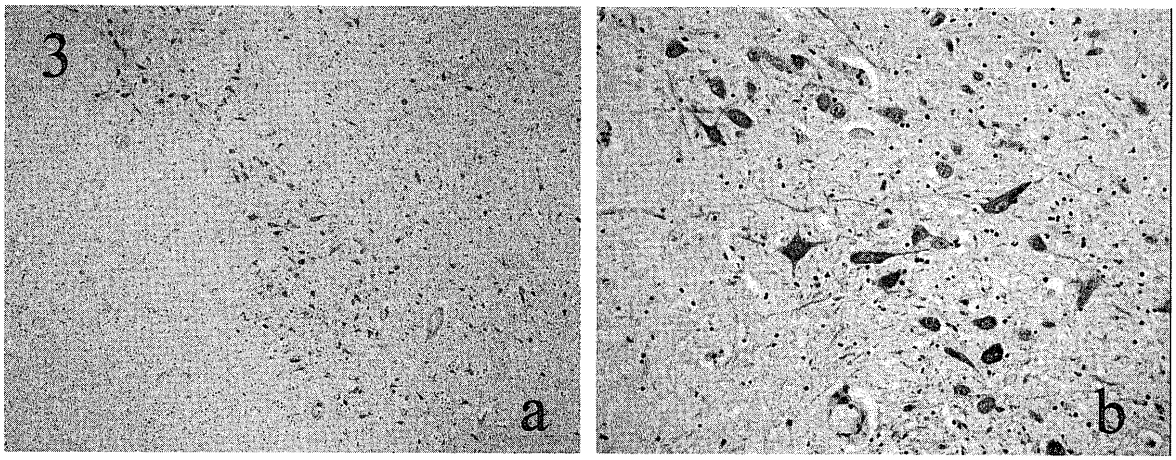

Fig. 3. Immunoreactivity for TH of the neurons limited in the substantia nigra. Note the intense positive reaction of the neurons and their processes. Immunostaining for TH. × 50 (a) × 200 (b).

mal species [10, 12, 20, 33]. The presence of neuromelanin is the one of the morphologic hallmarks for identification of the substantia nigra, and almost all melanin-bearing neurons were intensely positive for TH. Gaspar *et al.* [10] using by immunohistochemistry with 4% paraformaldehyde fixed cryostat sections, reported that TH-positive neurons were distributed not only in the substantia nigra, but also in human neocortex. We examined only limited regions of the brains including the midbrain, hippocampus, and some parts of the cerebral cortex, and TH-positive neurons was limited only in the substantia nigra. It seems that dopamine synthesizing neuronal cells are restrictively distributed within the substantia nigra of the canine brains, and that in the cerebral cortex may be extremely rare. By immunostaining for TH, we could not recognize apparent age-dependent decrease in the number of TH-positive neurons in canine substantia nigra, while the ratio of TH-positive neurons to GFAP-positive glial cells showed mild decrease in aged dogs. This fact indicates that dopamine-synthesizing neuronal cells may be well preserved during the aging process, and may be more resistant than those in the cerebral cortex or hippocampus to several negative influences, such as hypoxia or oxidative stress, which can induce neuronal cell death [3, 21]. Kiatipattanasakul *et al.* [15] reported that apoptotic neuronal cell death, as visualized by the TUNEL method, significantly increased during the aging process in canine brains, especially in the cerebral cortex. Their reports also described the incidence of apoptotic neurons in other regions including the hippocampus and thalamus, but they did not refer to the incidence in the substantia nigra. We employed ssDNA-immunostaining to detect the apoptotic process in brain cells, and in our dogs there was no signifi-

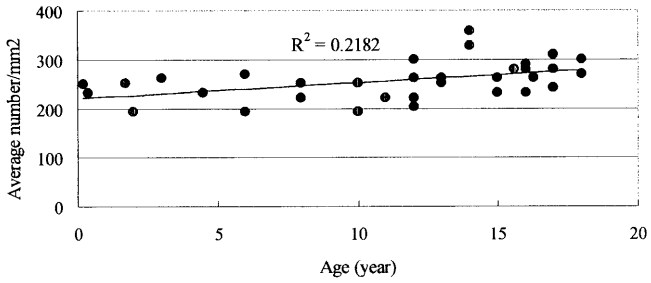

Fig. 4. The number of TH-positive neurons in the substantia nigra.

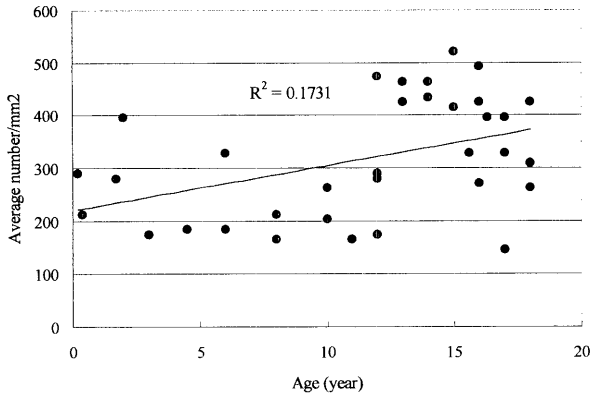

Fig. 5. The number of GFAP-positive glial cells in the substantia nigra.

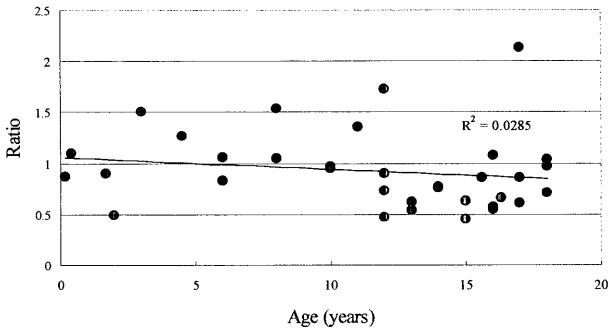

Fig. 6. The ratio of TH-positive neurons versus GFAP-positive glial cells in the substantia nigra.

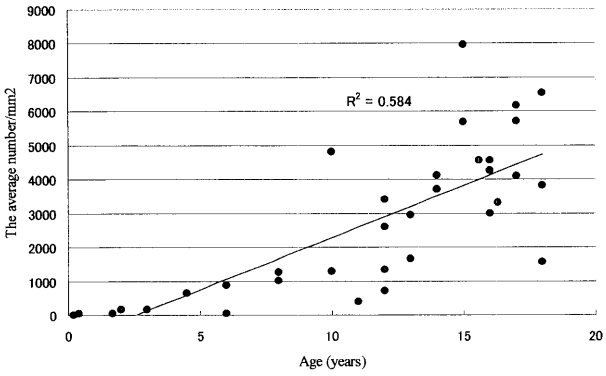

Fig. 7. The number of Ubiquitin-positive neurites in the substantia nigra.

cant increase in the number of ssDNA-positive neurons in the substantia nigra of aged dogs.

General histopathological examinations of the substantia nigra revealed intracytoplasmic lipofuscin deposition and the presence of axonal spheroids within the extended myelin sheaths; we considered that these changes were not pathologically significant. In the substantia nigra, those changes seemed to appear in older dogs. In two dogs from the very aged group, acidophilic inclusions mimicking Negri bodies were observed. Suzuki *et al.* [30] suggested that these inclusions might be formed by the aggregation of microtubules within the neurons predominantly in the substantia nigra of aged dogs. Moreover, similar inclusions have also been recognized in other aged animals [6]. These inclusions are supposed not to be associated with degeneration of the affected neurons [6, 30]. In our dogs, some eosinophilic inclusions were positive for PTAH, suggesting that those consisted of microtubules as described previously. In two aged dogs, these eosinophilic inclusions appeared not only in the substantia nigra, but also in other nuclei of the brain stem. Thus, we can assume that the inclusions are not specific features of the substantia nigra. On the other hand, amorphous basophilic inclusions were found more frequently in the substantia nigra, and morphologically these inclusions were quite different from the crystalline acidophilic inclusions. The basophilic inclusions varied in size, and were intensely positive for PAS, and negative for PTAH. Thus, we considered that the basophilic inclusions appeared because of the aggregation of some kinds of glycoprotein. Unlikely the eosinophilic inclusions, these basophilic inclusions were dominant in the substantia nigra. The inclusions might be more specific aged-related changes in the substantia nigra.

In humans, Lewy bodies and neurites are hallmarks of degenerating neurons in the brain stems of patients with PD and dementia with Lewy bodies. Classical Lewy bodies are found in the brain stem, especially in the substantia nigra, where these inclusions are associated with neuronal loss and clinical signs of idiopathic PD [34]. Lewy bodies are occasionally observed in other neurodegenerative diseases and in neurologically normal aged peoples [24, 34]. Their main constituents are both non-phosphorylated and phospholylated NF proteins, ubiquitin, and αSN [2, 19, 28, 29]. Recently, αSN accumulation in the Lewy body has been considered to be an important event in neurodegeneration in PD and dementia with Lewy bodies [1]. We did not find inclusion bodies containing similar constituents of Lewy

body in canine brains. In addition, αSN aggregation was not detected in TH-positive neurons. These results might indicate that αSN related pathology resulting in Lewy body formation and neuronal-cell-loss may not be evident in the dopaminergic neurons of the canine brains.

In the CNS, proteasome-mediated protein degeneration plays a major role in the break down of cellular proteins damaged by many kinds of stress or other insults causing glucose and oxygen storage [7]. Ubiquitin is the heat shock protein that is known to increase within neuropile with age and bind to degenerative proteins, and repair axonal degeneration [9, 18, 22]. The ubiquitin-positive materials in the neuropile increased obviously with age, as has previously been shown in the canine cerebrum [22]. Moreover, axonal spheroids are encountered in the CNS with increasing frequency with age. Spheroids are focal distensions of the axon with organelles that are normally in transit toward the axon termination or returning to the soma of the neuron. These abnormal axonal changes were well visualized by both ubiquitin-and NF-immunostaining. The reasons why there were abundant axonal and neuritic changes in the aged dogs, despite the rarity of pathologic lesions in the dopaminergic neuronal cells, are quite difficult. Although we considered that several aging changes of neuronal cells, including intracytoplasmic inclusions, did not cause neuronal cell loss or apoptosis, these deposits might inhibit axoplasmic transport, resulting degenerative changes of neurites and axons.

In conclusion, our findings suggest that dopamine-synthesizing neurons in the canine substantia nigra may be preserved during the aging process. The pathological implications of the intracytoplasmic inclusions and neuritic or axonal changes visualized by ubiquitin and NF immunostaining remain unknown. To elucidate the pathologic value of these aging changes, we need to perform further studies, using larger number of dogs, and including neurological examinations to evaluate the function of the substantia nigra.

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