Involvement of 4-hydroxy-2-nonenal Accumulation in Multiple System Atrophy

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Recent studies have suggested implications for α-synuclein cytotoxicity in the patho-mechanism of multiple system atrophy (MSA). Given *in vitro* evidence that α-synuclein generates oxidative stress, it is proposed that lipid peroxidation may be accelerated in MSA. To address this issue, we performed an immunohistochemical analysis of protein-bound 4-hydroxy-2-nonenal (P-HNE) in sections of archival, formalin-fixed, paraffin-embedded pontine materials of eight sporadic MSA patients and eight age-matched control subjects. In the MSA cases, P-HNE immunoreactivity was localized in all of the neuronal cytoplasmic inclusions and glial cytoplasmic inclusions, both of them identified with α-synuclein and ubiquitin. It was also detectable in reactive astrocytes and phagocytic microglia but undetectable in activated microglia. By contrast, P-HNE immunoreactivity in the control cases was only very weak or not at all in the parenchyma including neurons and glia. The present results provide *in vivo* evidence that HNE participates in α-synuclein-induced cytotoxicity and neuroinflammation in MSA.

Key words: alpha-synuclein, 4-hydeoxy-2-nonenal, immunohistochemistry, multiple system atrophy, oxidative stress

I. Introduction

Multiple system atrophy (MSA) is a neurodegenerative disease characterized by selective involvement of the pontocerebellar, striatonigral, and autonomic systems, corresponding to the classical clinical entities such as olivo-pontocerebellar atrophy (OPCA), striatonigral degeneration (SND) and Shy-Drager syndrome (SDS), respectively [41]. Compelling evidence identifies α-synuclein cytotoxicity in the pathological processes of α-synucleinopathies including neurodegenerative disorders such as MSA as well as Parkinson's disease [9, 10, 39, 42]. Several studies focused on implications for aggregation and ubiquitination of α-synuclein molecules [21, 26] in neuronal cytoplasmic inclusions (NCIs) and (oligodendro)glial cytoplasmic inclusions (GCIs), both of which are known to be pathognomonic hallmarks of MSA and appear in the affected systems [40, 41].

Recent investigations have documented the occurrence of oxidative stress in human MSA and its animal models [8, 18, 33] as well as in vitro evidence that α-synuclein molecules generate oxidative stress [34]. It is well known that oxidative stress gives rise to the formation of oxidative modification products derived from nucleic acids, proteins and lipids [11]. A previous study demonstrated oxidative injury evidenced by the presence of 4-hydroxy-2-nonenal (HNE) in Purkinje cells in the cerebellum of patients with OPCA [43]. HNE, a member of 4-hydroxyalkenals, is a well characterized lipid peroxidation product derived from n-6 polyunsaturated fatty acids (PUFAs), including linoleic and γ-linoleinic acids; it attacks and modifies protein amino acid residues to form its protein-bound forms such as "hemiacetal adduct" with histidine, cysteine or lysine, "pyrrole adduct" with lysine, and "fluorescent crosslinker" with lysine (Fig. 1) [38]. These processes give rise to stress response such as expression of proinflammatory gene pro-

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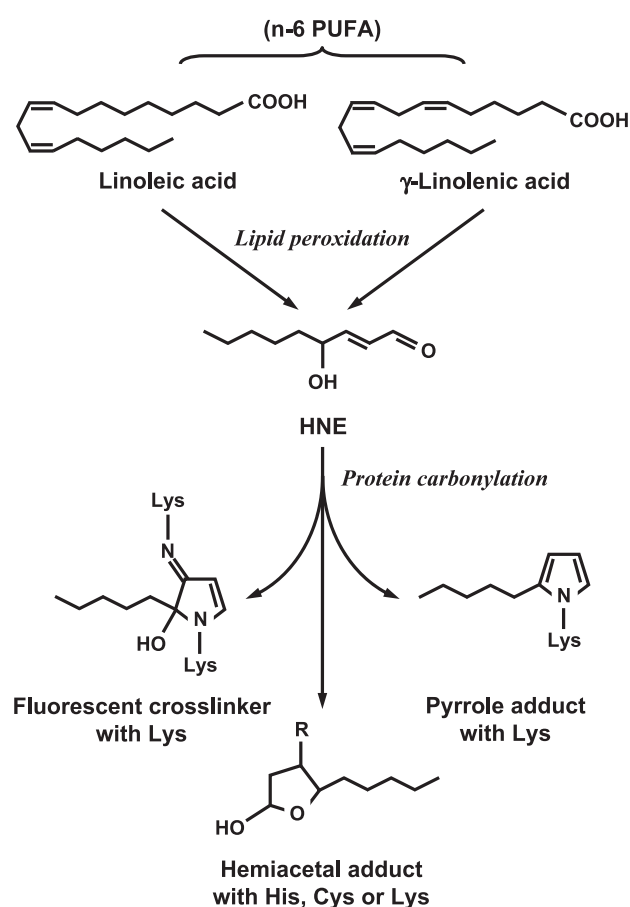
Fig. 1. Schematic diagram showing nonenzymatic processes of n-6 polyunsaturated fatty acid (PUFA) peroxidation and subsequent protein carbonylation to form several protein adducts of 4-hydroxy-2-nonenal (HNE). His, Cys, and Lys indicate protein amino acid residues of histidine, cysteine, and lysine, respectively.

Table 1. *Clinical features of examined cases*

Case No.	Age at death	Sex	Disease (phenotype)
1	55	M	MSA (SDS)
2	55	F	MSA (SDS)
3	57	F	MSA (SND)
4	59	F	MSA (OPCA)
5	61	M	MSA (OPCA)
6	62	M	MSA (SDS)
7	65	M	MSA (SDS)
8	68	F	MSA (SND)
9	46	F	Sepsis
10	53	F	Middle ear carcinoma
11	55	F	Systemic lupus erythematosus
12	66	M	Panhypopituitarism
13	68	F	Cardiac valvular disease
14	73	M	Pancreatic carcinoma
15	75	M	Sepsis
16	87	M	Cardiac valvular disease

Abbreviations: F, female; M, male; MSA, multiple system atrophy; OPCA, olivopontocerebellar atrophy; SDS, Shy-Drager syndrome; SND, striatonigral degeneration.

ducts including monocyte chemoattractant protein-1 (MCP-1) and cyclooxygenase-2 (COX-2) in macrophages [20, 25]. Given such conditions, it is plausible that HNE triggers neuroinflammation in MSA. However, the relationship between α-synuclein aggregation, lipid peroxidation, and glial inflammatory response in MSA has not been fully understood. To address this issue, we investigated and compared immunohistochemical localizations of protein-bound HNE (P-HNE), α-synuclein, ubiquitin and glial cell markers in MSA pons, which is essentially involved in this disease.

II. Materials and Methods

Subjects

This study was performed after obtaining written consent from the family member of each case, in accordance with the Declaration of Helsinki, and carried out on archival, formalin (10%)-fixed, paraffin-embedded brain stem pontine materials of eight sporadic MSA patients (age: 55–68 yr; sex: 4 males and 4 females) and eight age-matched

control subjects (age: 52–68; sex: 6 males and 2 females). There was no significant difference in age at death between the control and MSA groups (P>0.8 by unpaired Student's t-test). The clinical features of the cases examined are summarized in Table 1. Multiple 6-μm-thick sections of each pons were used for histopathological staining with hematoxylin-eosin (H&E) and Klüver-Barrera (K&B) and for immunohistochemical staining.

Antibodies

The primary antibodies employed in immunohistochemistry were mouse monoclonal IgG$_1$ against P-HNE (Clone HNEJ2; NOF, Tokyo, Japan) at a concentration of 0.1 μg/mL, rabbit polyclonal IgG against α-synuclein (Cat. No. 18671; IBL, Fujioka, Japan) at a concentration of 1 μg/mL, rabbit polyclonal IgG against ubiquitin (Cat. No. Z458; Dako, Glostrup, Denmark) at a dilution of 1:1,000, rabbit polyclonal IgG against glial fibrillary acidic protein (GFAP) (Cat. No. GTX16997; GeneTex; Irvine, CA, USA) at a dilution of 1:1,000, and rabbit polyclonal IgG against glucose transporter-5 (GLUT5) (Cat. No. 18905; IBL) at a concentration of 0.5 μg/mL. Of these antigens, GFAP and GLUT5 were used as histological markers of astrocytes and microglia, respectively. As described in the data sheet, the antibody HNEJ2 specifically recognizes hemiacetal adduct with histidine among the protein-bound forms of HNE [37].

Immunohistochemical staining

Sections were deparaffinized, rehydrated, quenched for 10 min at room temperature with 3% hydrogen peroxide, rinsed in phosphate-buffered saline, pH 7.6 (PBS), pre-treated for 20 min at room temperature with 3% bovine

serum albumin (BSA) in PBS, and then incubated overnight at 4°C with the primary antibodies. Antibody binding was visualized by the polymer-immunocomplex (PIC) method using the appropriate EnVision system (Dako). 3,3'-Diaminobenzidine tetrahydrochloride (DAB) was the chromogen, and hematoxylin, the counterstain. Sections processed with omission of the primary antibodies or incubated with nonimmune serum derived from the same animal species as those producing the antibodies gave negative reaction controls. The specificity of the anti-P-HNE antibody on sections was comfirmed by preimmunoabsorption test; sections were incubated with the antibody preincubated with 1 mg/mL BSA-HNE conjugates in PBS.

Immunohistochemical evaluation

Immunohistochemical localization of P-HNE was verified by comparison with consecutive sections stained with H&E and immunostained with the antibodies to α-synuclein, ubiquitin, GFAP, and GLUT5, and it was also identified by the double-immunolabeled staining method as described previously [32]. Sections were incubated with one antibody, and immunoreaction was detected by the PIC method using DAB as the chromogen. After taking microphotographs, sections were processed by eluting the immune complex by microwaving (400 W, 95°C, 10 min) in citrate buffer, pH 6.0, and these sections were subsequently incubated with another antibody. Immunoreaction was detected by the PIC method using $NiCl_2$/DAB as the chromogen. Immunohistochemical localizations of the examined substances on $NiCl_2$/DAB-colored sections were compared with the initially taken microphotographs at the corresponding regions of DAB-colored sections.

III. Results

Histopathological observations

Histopathological examination revealed that the MSA pons showed the characteristic features of this disease as described previously [41], whereas the control pons displayed no significant abnormality. In brief, at a low magnification of K&B-stained sections, the MSA patients showed prominent atrophy of pontine base and myelin pallor of transverse fibers (Fig. 2b), while the control subjects seemed to be morphologically intact (Fig. 2a). At a higher magnification of H&E-stained sections, the MSA cases exhibited atrophy and reduced number of pontine neurons, reactive gliosis, and appearance of NCIs and GCIs (Fig. 2d), but by contrast the control cases did not show any histological alteration (Fig. 2c).

Immunohistochemical observations

Immunoreaction products were invisible on sections processed for negative reaction control (Fig. 2g) and preimmunoabsorption test of the anti-P-HNE antibody (Fig. 2h). P-HNE immunoreactivity was only very weak or not at all in the control cases (Fig. 2e). In the MSA cases, the immunoreactivity was mainly distributed in the pontine

base and distinct in the abnormal structures NCIs and GCIs, as well as the cytoplasm of all of the normal-looking and degenerative pontine neurons and reactive glia, and it was more intense in these abnormal structures (Fig. 2f). As described in the literature [7], microglial morphology in pathological conditions was defined as the following: activated microglia were characterized by thin perikarya and double-branching, arborized, thick cell processes, and phagocytic microglia were characterized by hypertrophic perikarya. Moreover, activated microglia were scattered in the MSA lesions (Fig. 3h), while phagocytic microglia were aggregated to constitute so-called glial nodules (Fig. 3j). P-HNE immunoreactivity in the MSA pons was colocalized with all of the NCIs and GCIs immunoreactive for α-synuclein (Fig. 3a, b) and ubiquitin (Fig. 3c, d), and it was also detectable in almost all of the GFAP-immunoreactive reactive astrocytes in the lesions (Fig. 3e, f) and GLUT5-immunoreactive "aggregated" phagocytic microglia (Fig. 3i, j) but not in any of the GLUT5-immunoreactive "scattered" activated microglia (Fig. 3g, h). No distinct α-synuclein immunoreactivity was detectable in GFAP-immunoreactive astrocytes (Fig. 4a, b) or GLUT5-immunoreactive microglia (Fig. 4c, d) in the MSA cases. There was no significant difference in these immunohistochemical results among the MSA cases.

IV. Discussion

In the present study, noteworthy was the coexistence of P-HNE accumulates with α-synuclein- and ubiquitin-containing NCIs and GCIs in the MSA pons. These observations suggest a close link between α-synuclein, ubiquitin, and HNE in the pathomechanism of MSA. A number of investigations have indicated that oligomers of α-synuclein molecules generate reactive oxygen species (ROS) such as hydrogen peroxide and hydroxyl radical [35], which are responsible for lipid peroxidation [11, 38]. The representative lipid peroxidation product HNE modifies and crosslinks amino acid residues of α-synuclein to promote molecular aggregation of this protein [28]. HNE also inhibits normal proteasomal and lysosomal functions but not ubiquitination in neurons [4, 5, 15, 17], impairs glutamate and glucose transport in neurons [16, 27], and induces programmed cell death of neurons and oligodendrocytic progenitors [6, 19, 22]; these mechanisms are mediated by HNE-driven oxidative stress. Thus, our results suggest a critical role for HNE, as an α-synuclein-induced lipid peroxidation product, in the processes of neuronal and oligodendroglial degeneration in MSA, including ROS-induced cell damage, inhibition of the proteolytic mechanisms, and formation of the NCIs and GCIs.

Another interesting finding in this study was the disclosure of P-HNE accumulates in pontine base astrocytes and microglia in the MSA cases. Given that α-synuclein immunoreactivity was localized in NCIs and GCIs, it is likely that HNE primarily occurs in lesional neurons and oligodendroglia in the processes of α-synuclein aggregation
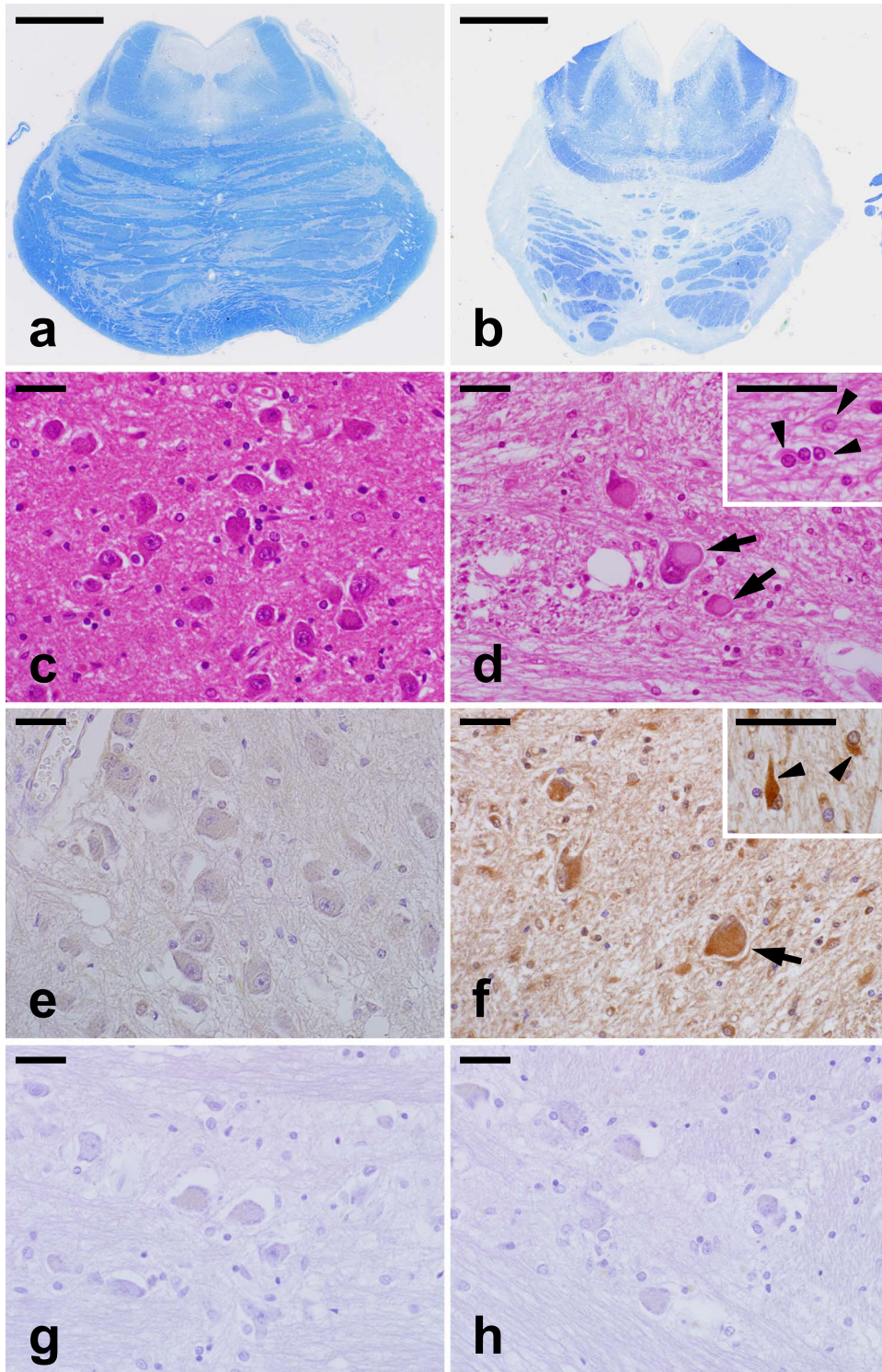
Fig. 2. Semimacroscopic findings of Klüver-Barrera-stained pontine sections (**a, b**) and microscopic findings of pontine nucleus sections stained with hematoxylin-eosin (**c, d**) and immunostained for protein-bound 4-hydroxy-2-nonenal (**e, f**) in control subjects (**a, c, e**) and multiple system atrophy (MSA) patients (**b, d, f**). Panels (**g, h**) indicate negative reaction control and preimmunoabsorption test sections, respectively, in MSA pontine nuclei. Polymer-immunocomplex method using 3,3'-diaminobenzidine tetrahydrochloride (brown in **e–h**) as the chromogen. Arrows and arrowheads indicate neuronal cytoplasmic inclusions and glial cytoplasmic inclusions, respectively. Bars=5 mm (**a, b**) and 100 μm (**c–h**).

and diffuses into the surrounding parenchyma. The finding that α-synuclein immunoreactivity was undetectable in astrocytes and microglia suggests that these cells digested this protein after uptake. The lack of P-HNE immuno-

reactivity in activated microglia may result from evidence that these cells promptly scavenge HNE via their strong antioxidative defense system using a large amount of intra-cellular glutathione produced by cystine that is taken up
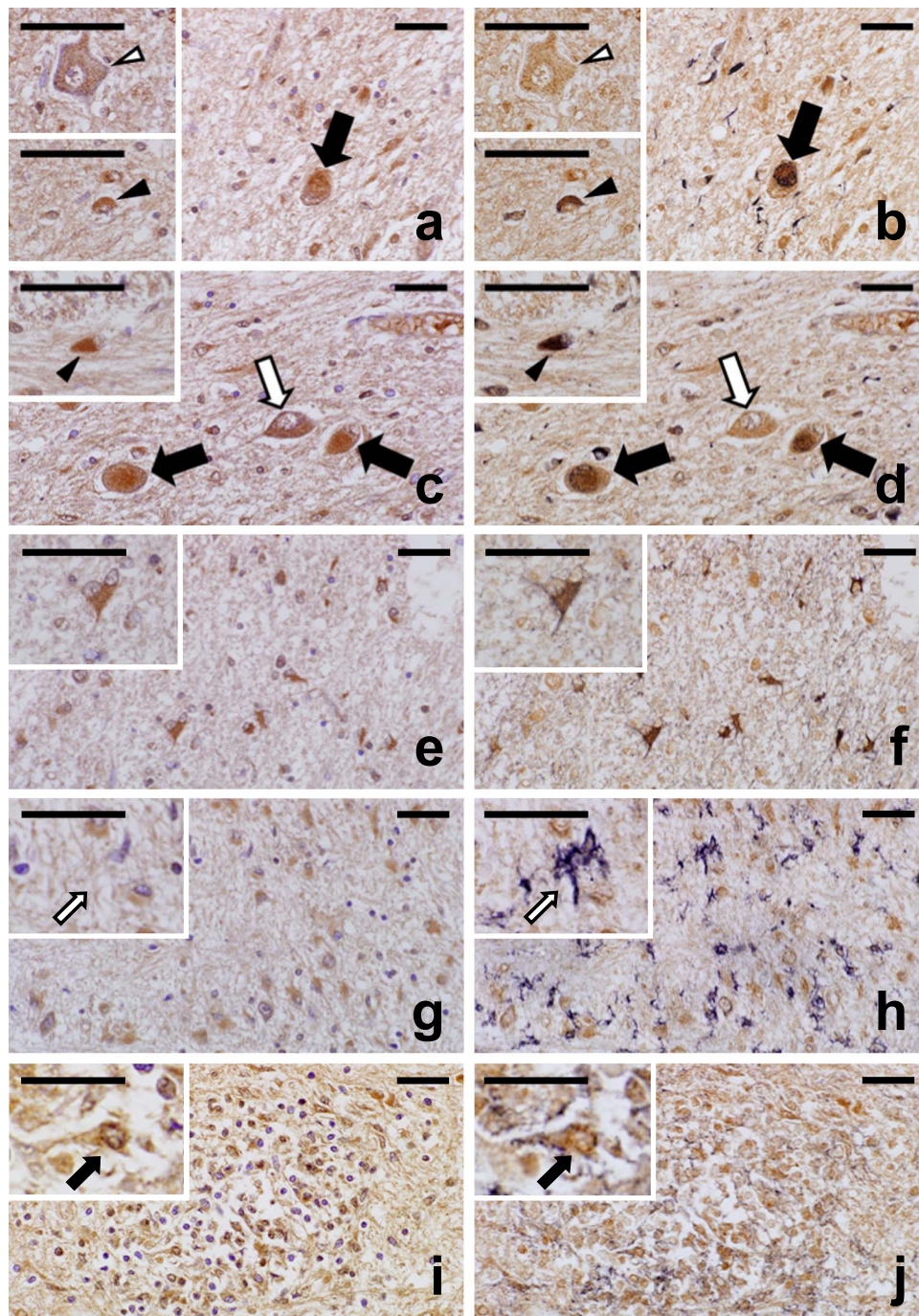
Fig. 3. Immunohistochemical comparison of protein-bound 4-hydroxy-2-nonenal (**a, c, e, g, i**) with α-synuclein (**b**), ubiquitin (**d**), glial fibrillary acidic protein (**f**) and glucose transporter-5 (**h, j**) in pontine sections from multiple system atrophy patients. Panels (**b, d, f, h, j**) including their insets indicate double staining at the identical regions on the same sections as panels (**a, c, e, g, i**), respectively. Large blank and solid arrows indicate atrophic pontine neurons and neuronal cytoplasmic inclusions, respectively. Blank and solid arrowheads indicate normal-appearing pontine neurons and glial cytoplasmic inclusions, respectively. Small blank and solid arrows indicate activated microglia scattered in the neuropil and phagocytic microglia composing a glial nodule, respectively. Polymer-immunocomplex method using 3,3'-diaminobenzidine tetrahydrochloride (DAB) (brown in **a, c, e, g, i**) and nickel dichloride (NiCl$_2$)/DAB (blue in **b, d, f, h, j**) as the chromogen. Bar=100 μm (**a–j**).
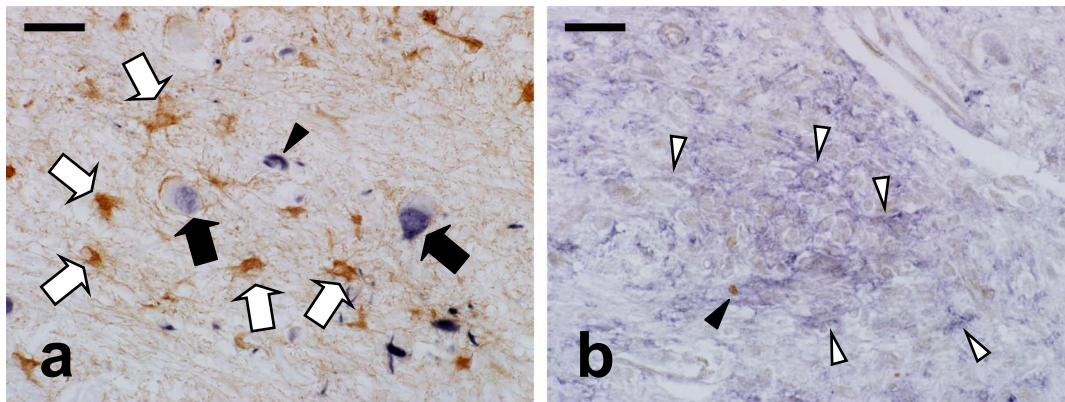
Fig. 4. Double immunohistochemical staining of α-synuclein (blue in **a**; brown in **b**) with glial fibrillary acidic protein-immunoreactive reactive astrocytes (brown in **a**) and glucose transporter-5-immunoreactive phagocytic microglia (blue in **b**) in pontine base obtained from multiple system atrophy cases. Solid arrows and arrowheads indicate neuronal and glial cytoplasmic inclusions, respectively. Blank arrows and arrowheads indicate reactive astrocytes in the lesion and phagocytic microglia to form a glial nodule, respectively. Polymer-immunocomplex method using 3,3'-diaminobenzidine tetrahydrochloride (DAB) (brown) and nickel dichloride $(NiCl_2)/\Delta AB$ (blue) as the chromogen. Bar=100 μm (**a, b**).

from the extracellular space in return for HNE-induced glutamate release [1]. The fact that P-HNE accumulates were detectable in reactive astrocytes and phagocytic microglia points to the possibility that these glial cells may phagocytose extracellular P-HNE or undergo intracellular protein adduct formation of HNE diffused from the extracellular space. This discrepancy of P-HNE immunostaining between phagocytic and activated forms of microglia may reflect a difference in property of these cells [7].

Glial activation has been observed in the brain lesions of MSA patients and α-synuclein-overexpressing mice as an animal model of this disease [13, 34]. A number of studies have demonstrated that HNE impairs glutamate uptake by astrocytes and microglia and promotes glutamate release from these glial cells [1–3, 36], leading to exposure of neurons and oligodendrocytes to an excess of extracellular glutamate. It has been shown that an imbalance of glial glutamate release and uptake interacts with neuroinflammation [36]. Activated microglia modulate expression of astroglial enzymes responsible for oxidative and inflammatory stimuli [30]. Furthermore, several studies have focused on the role of α-synuclein in lipid peroxidation metabolism and downstream inflammatory response in the brain [10]. In fact, upregulation of proinflammatory gene products has been shown in reactive astrocytes and activated microglia in neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis [12, 23, 29], all of which are associated with increased tissue HNE levels [14, 24, 31]. These observations could indicate that glial cells in MSA lesions participate in local inflammation in response to oxidative stress.

Taken together, we demonstrated the accumulation of P-HNE in NCIs, GCIs, reactive astrocytes, and phagocytic microglia, but not activated microglia in MSA pontine lesions. Our results suggest the involvement of HNE-mediated cytotoxic effects on pontine neurons and oligodendroglia in association with the formation of NCIs and

GCIs, as well as inflammatory response and glutamate toxicity by astrocytes and microglia. Elucidating the mechanism by which α-synuclein-induced HNE production is regulated in the central nervous system will contribute to a better understanding of MSA and establishment of therapeutic strategies for this disease.

V. Acknowledgements

VI. References

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