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Invited Review

Recent advances in research on neuropathological aspects of familial amyotrophic lateral sclerosis with superoxide dismutase 1 gene mutations: Neuronal Lewy body-like hyaline inclusions and astrocytic hyaline inclusions

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Summary. Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease that primarily involves the motor neuron system. Of all patients with ALS, approximately 5%-10% of them are familial and most of the others are sporadic. Superoxide dismutase 1 (SOD1) gene mutations are shown to be associated with about 20% of familial ALS (FALS) patients. FALS is neuropathologically classified into two subtypes: classical FALS in which degeneration is restricted to only motor neurons and FALS which is characterized by the degeneration of the posterior column in addition to the lesion of the motor neuron system. The neuronal Lewy body-like hyaline inclusion (LBHI) is a characteristic neuropathological marker of mutant SOD1-linked FALS with posterior column involvement. Inclusions similar to the neuronal LBHIs have been discovered in astrocytes in certain patients with FALS exhibiting SOD1 gene mutations. The purpose of this review is to discuss the novel neuropathological significance of the astrocytic hyaline inclusions (Ast-HIs) and neuronal LBHIs in brain tissues from individuals with the posterior-column-involvement-type FALS with SOD1 gene mutations. In hematoxylin and eosin preparations, both Ast-HIs and neuronal LBHIs are eosinophilic inclusions and sometimes show eosinophilic cores with paler peripheral halos. Immunohistochemically, both inclusions are intensely positive for SOD1. At the ultrastructural level, both inclusions consist of approximately 15-25 nm-sized granule-coated fibrils and granular materials. Immunoelectron microscopically, these abnormal granule-coated fibrils and granular materials are positive for SOD1. Therefore, the FALS disease process originating from *SOD1* gene mutations occurs in astrocytes as well as neurons and is involved in the formation of both inclusions.

Key words: Familial amyotrophic lateral sclerosis (FALS), Neuronal Lewy-body like hyaline inclusions (LBHIs), Astrocytic hyaline inclusions (Ast-HIs), Superoxide dismutase 1 (SOD1), Granule-coated fibrils

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Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal and age-associated neurodegenerative disorder that primarily involves both upper and lower motor neurons (Hirano, 1995, 1996). Despite this disease having been studied for over 120 years, its etiology is still unknown. Of all patients with ALS, approximately 5%-10% of them are familial and most of the others are sporadic (Hudson, 1981; Juneja et al., 1997). The prevalence rate of ALS is approximately 5 to 10 cases per 100,000 population (de Belleroche et al., 1996). Neuropathologically, ALS is classified into three major types: 1) classical sporadic ALS (SALS), in which degeneration is restricted to only motor neurons; 2) familial ALS (FALS); and 3) Guamanian ALS, in which there is the widespread development of Alzheimer's neurofibrillary tangles (NFTs). The SALS patients are predominantly male (male:female = 2:1, Hudson, 1981) and the disease mostly begins in the fifth and sixth decade of life. This disease process is progressive, and most patients who are not placed on respirators die of respiratory muscle paralysis within 5 years of onset. The main clinical feature of the patients is progressive muscular atrophy generated from the loss of lower motor neurons and spasticity from the degeneration of upper motor neurons. The FALS patients demonstrate an autosomal-dominant

transmission (Mulder et al., 1986) and show a lesser male preponderance (male:female = 1.3:1, from the data based on the 210 patients of 48 families; Hudson, 1981). In comparison with the SALS patients, the clinical course of the FALS patients exhibits a younger mean age of onset and more rapid progression, and the average duration from the first symptom until death is shorter (Hudson, 1981; Mulder et al., 1986). The clinical symptom of the many FALS patients starts from the muscle weakness of the lower extremities (Hudson, 1981; Mulder et al., 1986). Morphologically, FALS is subclassified into two types (Engel et al., 1959). One type is the classical form similar to SALS. The other is the form with posterior column involvement, and it is characterized by the degeneration of middle root zones of the posterior column, Clarke nuclei and posterior spinocerebellar tracts, in addition to the lesion of the motor neuron system (Fig. 1). Therefore, FALS should be neurologically and neuropathologically distinguishable from SALS.

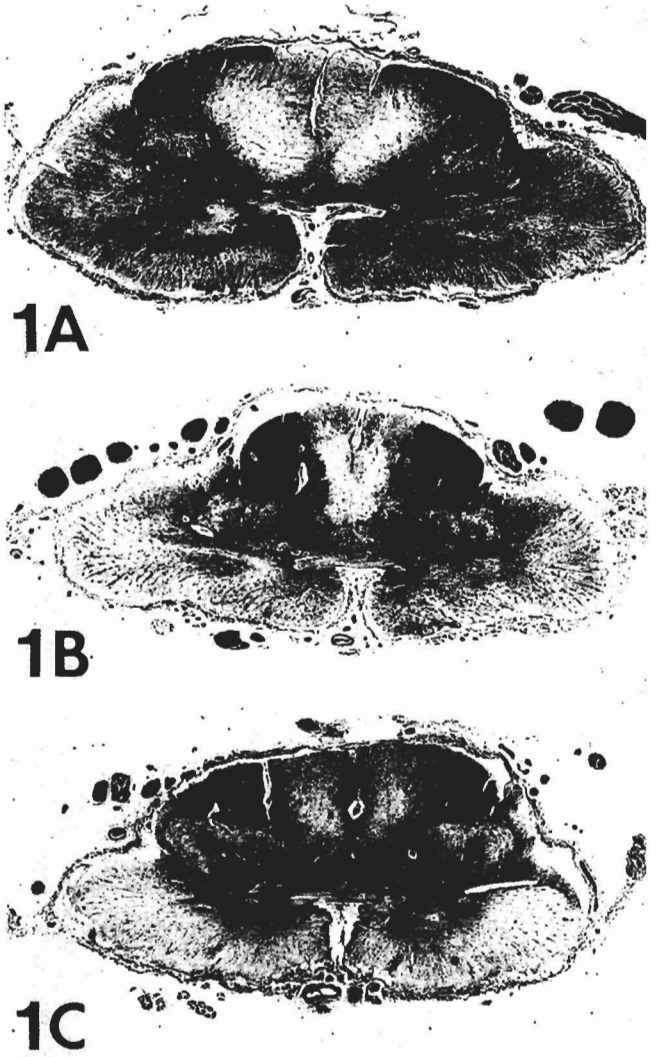

Fig. 1. The spinal cord from an individual with posterior-column-involvement-type familial amyotrophic lateral sclerosis (FALS) with a two base pair deletion in the codon 126 of superoxide dismutase 1 (SOD1) gene. **A:** C5, **B:** T2, and **C:** L1 showing severe degeneration of the corticospinal and spinocerebellar tracts and of the middle portions including middle root zones of the posterior column. Luxol fast blue (20 μm-thick sections). A, x 7; B, x 8; C, x 7.5. From Kato et al. (1996b). Reproduced with permission from the Journal of Neuropathology and Experimental Neurology.

Discovery of superoxide dismutase 1 (SOD1) gene mutations in familial amyotrophic lateral sclerosis (FALS)

Since Charcot and Joffroy (1869) reported the presence of ALS in 1869, over 120 years of research had not been able to determine its etiology. However, there have been epoch-making discoveries about FALS in recent years. Linkage analysis of FALS by Siddique et al. (1991) has showed that the genetic locus is strongly linked to chromosome 21q. Single strand conformation polymorphism analyses of polymerase chain reaction products by Deng et al. (1993) and Rosen et al. (1993) have indicated that this locus partially overlaps the genetic locus of superoxide dismutase 1 (SOD1): the SOD1 gene lies on chromosome 21 (21q22.1), spans 11 kb of chromosomal DNA, and consists of 5 exons interrupted by 4 introns, which encodes a protein of 153 amino acids (Levanon et al., 1985). The SOD1 exists as a homometric dimer (Weisiger and Fridovich, 1973). In general, superoxide dismutase is an antioxidant metalloenzyme that catalyzes the conversion of superoxide radical to hydrogen peroxide and molecular oxygen (Fridovich, 1986). Three isoforms of SOD have been identified: SOD1 (Cu/Zn-binding homodimeric cytosolic SOD; 32 kDa), SOD2 (Mn-dependent homotetrameric mitochondrial SOD; 88 kDa) and SOD3 (Cu/Zn-binding homotetrameric extracellular SOD; 135 kDa) (Fridovich, 1986). They are encoded by their own genes on different chromosomal loci: 21q22 (SOD1), 6q21 (SOD2) and 4pter-q21 (SOD3) (Weitkamp and Franke, 1978; Levanon et al., 1985; Hendrickson et al., 1990).

To date, more than 50 different mutations (Cudkowicz and Brown, 1996; Siddique et al., 1997) within all exons of the SOD1 gene and introns have been identified to be involved in the development of chromosome 21q-linked FALS (Fig. 2). However, published literature concerning FALS autopsy cases with characterized mutations of SOD1 is remarkably limited in number (Table 1). As for the relationship between this disease mechanism and SOD1 gene mutation, the dominant hypothesis at first was the loss-of-function theory. Because mutant SOD1 protein originating from SOD1 gene mutations is produced, wild-type (normal) SOD1 protein with normal activity is reduced in patients with FALS. Activity levels of SOD1 enzyme in FALS patients with SOD1 gene mutations are commonly reduced by 30% to 50% of the activity in normal individuals (Nakashima et al., 1995; de Belleroche et al., 1996). Superoxide radicals cannot completely be scavenged by SOD1 enzyme in FALS with SOD1 gene mutations, and the oxidative stress generated from the

Table 1. Autopsy cases of familial amyotrophic lateral sclerosis (FALS) with characterized mutations of superoxide dismutase 1 (SOD1).

SOD1 MUTATION	NEURONAL INCLUSIONS	CORTICOSPINAL TRACT INVOLVEMENT	POSTERIOR COLUMN INVOLVEMENT	REFERENCES
A4V	LBHI	+	+	Shibata et al., 1996b
	ICI	- or mild	+ (asymmetry)	Cudkowicz et al.,1998
A4T	LBHI	+	+	Takahashi et al.,1994
H46R	LBHI	+	+	Saida et al.,1999
H48Q	NF, HI	+	minimal	Shaw et al., 1997
E100G	SLI	+	+	Ince et al.,1996
I113T	NF	+	-	Orrell et al.,1995
	NF	-	ND	Rouleau et al.,1996
	NF	ND	ND	Kokubo et al.,1998
	NF	+	+	Ince et al.,1998
2-bp	LBHI	+	+	Kato et al.,1996a,b
deletion(126)	IEI	+	-	Kadekawa et al., 1997

+: present; -: absent; ND: not described; LBHI: Lewy body-like hyaline inclusion; ICI: intracytoplasmic inclusion; NF: neurofilamentous inclusion; HI: hyaline inclusion; SLI: skein-like inclusion; IEI: intracytoplasmic eosinophilic inclusion.

non-scavenged superoxide radicals plays a role in the toxic effect against the cells.

Some evidence contradictory to the loss-of-function theory have been reported. Support for the idea of gain of adverse function comes from the transgenic studies by Gurney et al. (1994): they have established a transgenic mouse model of mutant SOD1-related FALS that carries Gly93Ala for human mutant SOD1. Although the clinical symptoms and pathological findings of the mice are partially similar to those of human FALS, the SOD1 activity of the mice is about four times higher than that of normal mice. In addition, Andersen et al. (1995) have demonstrated that the SOD1 activity levels in homozygous FALS patients who have an Asp90Ala mutation

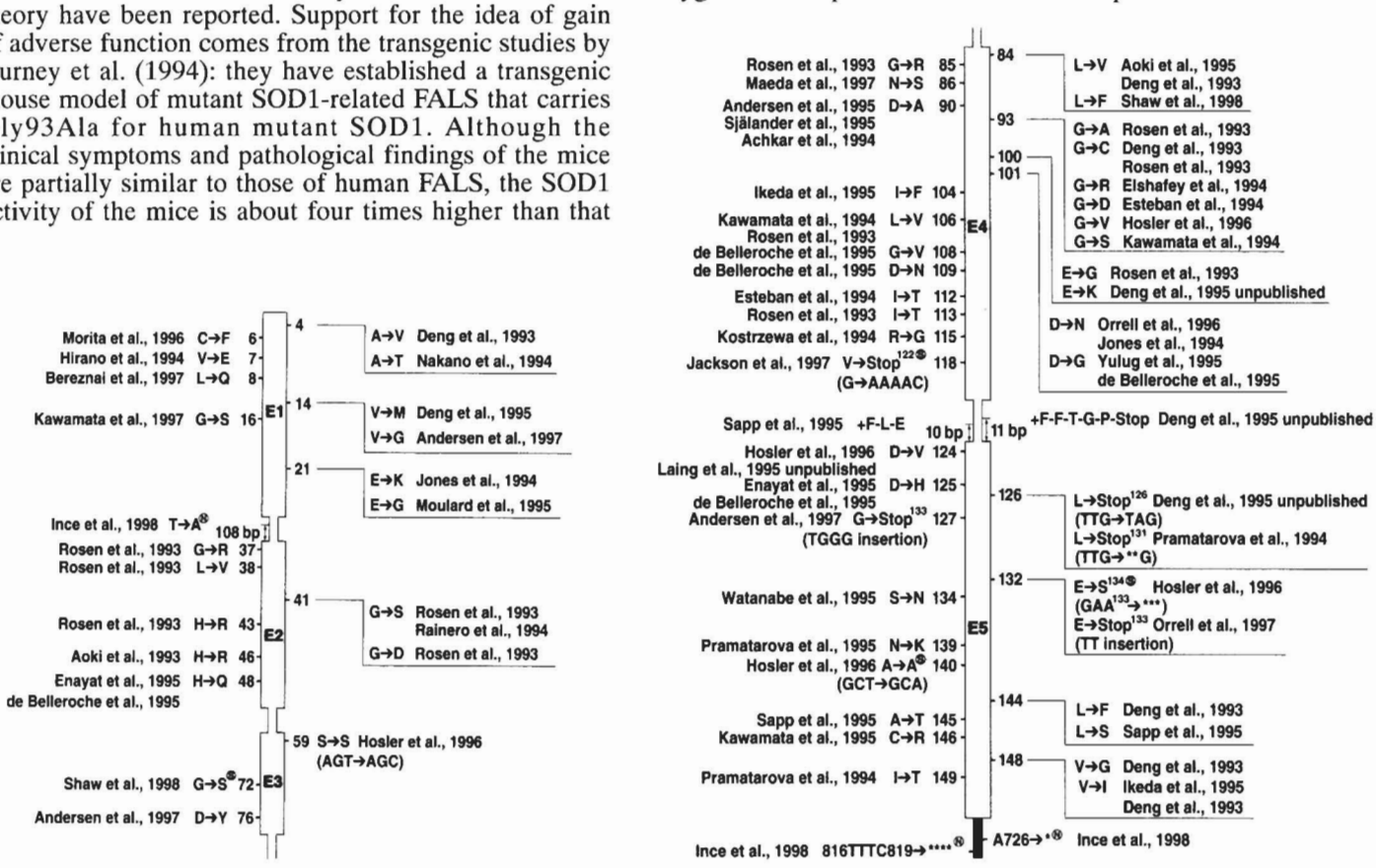

Fig. 2. Mutations, polymorphisms and variants in the SOD1 gene in amyotrophic lateral sclerosis (ALS). The structure of the gene is shown diagrammatically. Shaded areas (E1-E5): exon 1 to 5; open areas: intron; black area: 3' untranslated region. Amino acid or nucleotide substitutions are indicated at appropriate codons according to the accepted international nomenclature. Although the majority is FALS, even sporadic ALS (SALS) has an SOD1 abnormality; each encircled superscript S indicates the SALS. In addition, each encircled superscript N demonstrates ALS without familial history, in which familiality or sporadicity is not clear.

are equivalent to those in normal individuals. Further, no pathological evidences have been reported in SOD1 knockout mice at up to 4 months in age, although increased vulnerability to injury is indicated (Reaume et al., 1996). These observations support the hypothesis that mutant SOD1 itself acquires novel toxic effects (gain-of-function theory). At present, this gain-of-function theory is strongly supported by most of the ALS investigators, replacing the loss-of-function theory. There have been several reports that even SALS patients possess SOD1 gene mutations (Fig. 2: Jackson et al., 1997; Shaw et al., 1998). However, the gain-of-function theory alone cannot completely explain the overall disease mechanism of human FALS. As a matter of fact, SOD1 gene mutations are found in only 20% of all the FALS patients (Juneja et al., 1997).

The discovery of SOD1 gene mutations in FALS patients has a strong impact on morphological analysis in the neuropathological field. Especially, neuronal Lewy body-like hyaline inclusions (LBHIs) are thought to be a significant morphological hallmark of mutant SOD1-related FALS with posterior column involvement (Kato et al., 1996a,b, 1997; Shibata et al., 1996b). To explain the neuropathological characterization of neuronal LBHIs in association with the genetic alteration, this review focuses on the neuronal LBHIs.

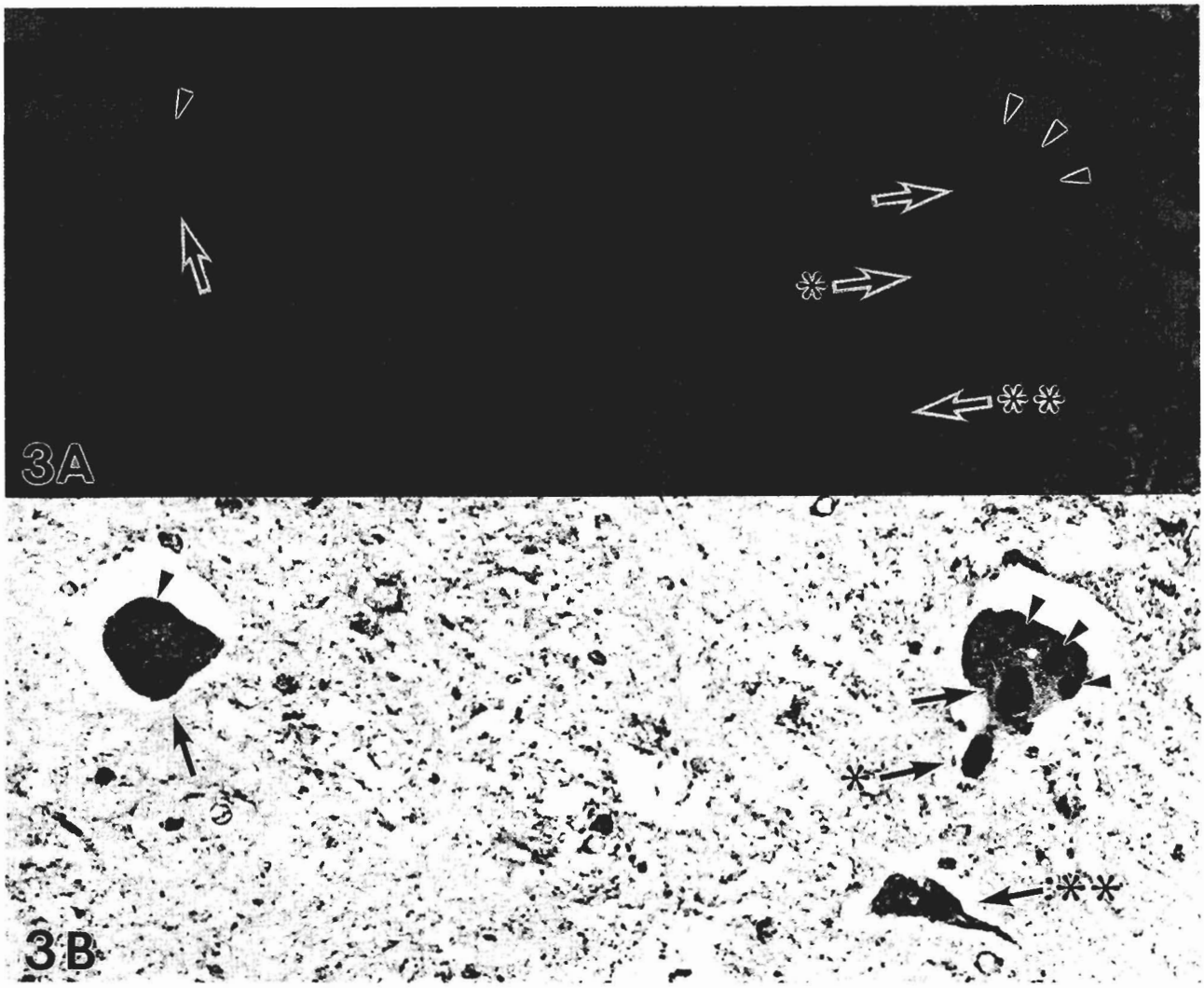

Fig. 3. Serial sections of neuronal Lewy body-like hyaline inclusions (LBHIs) in the spinal cord of an FALS patient. **A.** Round (arrows) and ill-defined (arrowheads) LBHIs are seen in the cytoplasm of the chromatolytic anterior horn cells. The round LBHIs are composed of eosinophilic cores with paler peripheral halos (arrows). Other LBHIs consist of obscure slight eosinophilic materials (arrowheads). The proximal cord-like swollen neurite has a sausage-like LBHI (arrow and asterisk). An atrophic anterior horn cell without inclusions is also observed (arrow and double asterisks). H&E, x 580. **B.** Immunostaining with the antibody against SOD1. Each inclusion including an intra-neuritic LBHI is intensely labeled by the anti-SOD1 antibody. Although the SOD1 immunoreactivity in the LBHIs with cores and halos (arrows) is almost restricted to the halos, the immunostaining in ill-defined LBHIs (arrowheads) and an intra-neuritic LBHI (arrow and asterisk) is distributed in the entire portion of each of the inclusions. x 580

Moreover, astrocytic hyaline inclusions (Ast-HIs), which are composed of the same components as neuronal LBHIs (Kato et al., 1996a,b, 1997), are also described in detail, together with the authors' contributions to the discovery of the Ast-HIs.

Pathological characterization of neuronal Lewy body-like hyaline inclusions (LBHIs)

In 1967, neuronal LBHIs were described as a characteristic feature of FALS with posterior column involvement by Hirano et al. (1967). As seen in hematoxylin and eosin (H&E) preparations, neuronal LBHIs show eosinophilic cores with paler peripheral halos (Figs. 3A, 4A), and the name is derived from the H&E staining feature that they look like Lewy bodies seen in the brain stem of patients with Parkinson's disease. Since neuronal LBHIs light microscopically display an amorphous core and a less dense amorphous halo, the term "hyaline" is also included in this name, i.e. Lewy body-like hyaline inclusion. The major light-microscopical differences between neuronal LBHIs and Lewy bodies are as follows: the neuronal LBHIs are blue to violet after Masson trichrome staining (Kato et al., 1996b, 1997), while the Lewy bodies show bright red cores with pale blue halos (Hirano, 1981). Although

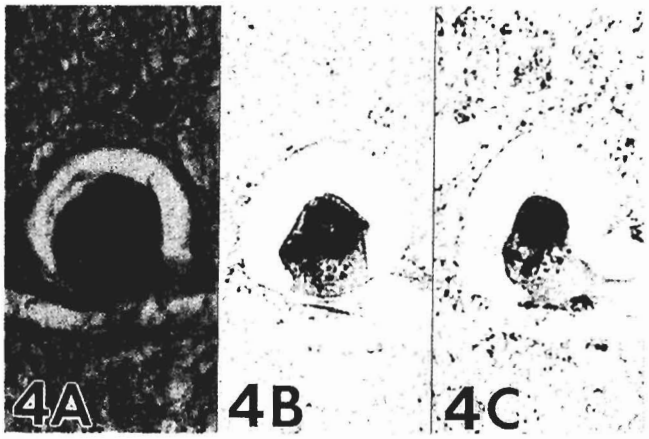

Fig. 4. Serial sections of a neuronal LBHI in the cingulate gyrus of an FALS patient. **A.** The neuronal LBHI consists of an eosinophilic core and a distinct halo. H&E, x 600. **B.** Immunostaining with the antibody to SOD1 is restricted to the periphery of the neuronal LBHI. x 600. **C.** Immunostaining with the antibody against ubiquitin. The ubiquitin-positive peripheral structure coincides with the SOD1-positive structure. x 600. From Kato et al. (1996b). Reproduced with permission from the Journal of Neuropathology and Experimental Neurology.

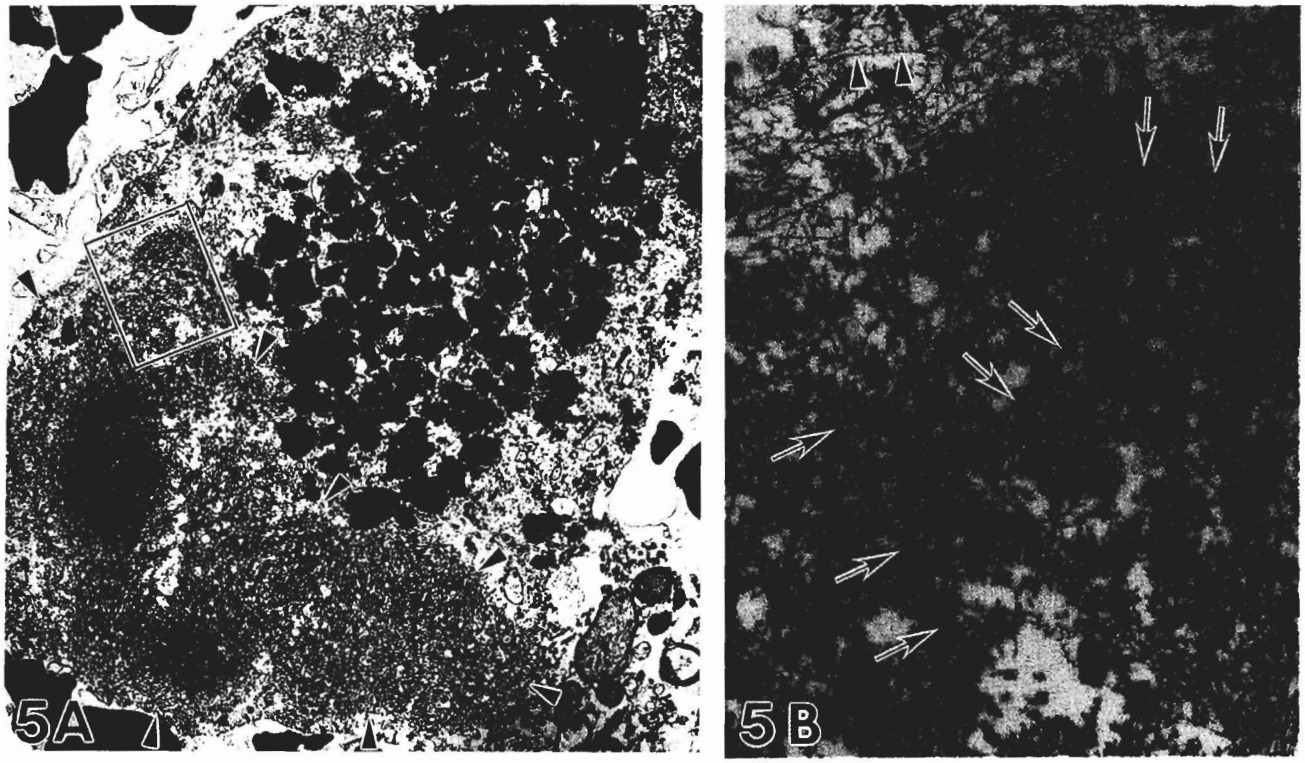

Fig. 5. Electron micrographs of a neuronal LBHI (arrowheads in A) in an anterior horn cell of an FALS patient. **A.** The major components of the neuronal LBHI are fibrils and granular materials. The fibrils and granular materials in the central portion of the neuronal LBHI corresponding to the core is more densely aggregated than those in the periphery. x 2,700. **B.** At higher magnification (inset in A), the neuronal LBHI is composed of approximately 15-25 nm-sized granule-coated fibrils (arrows) and granular materials, intermixed with 10-nm neurofilaments (arrowheads). x 18,000. From Kato et al. (1996b). Reproduced with permission from the Journal of Neuropathology and Experimental Neurology.

Lewy bodies are immunohistochemically positive for α-synuclein (Baba et al., 1998), the neuronal LBHIs are negative for α-synuclein (Dr. Iwatsubo: personal communication). At the ultrastructural level the neuronal LBHIs are completely different from Lewy bodies, although both inclusions which consist of filamentous materials exhibit dense cores with rough peripheral halos and have no limiting membranes. In the neuronal LBHIs, the filaments of the peripheral portion corresponding to the halo are randomly oriented (Fig. 5) (Kato et al., 1996a,b, 1997), but the filamentous materials of the Lewy bodies in the peripheral portion are regularly oriented with a radial alignment (Duffy and Tennyson, 1965). At higher magnification, the neuronal LBHIs are comprised of approximately 15-25 nm-sized granule-coated fibrils and granular materials (Fig. 5) (Kato et al., 1996a,b, 1997), while the Lewy bodies are composed of about 7- 8 nm-sized filaments (Duffy and Tennyson, 1965). Both inclusions have, of course, 10 nm neurofilaments as normal intermediate filaments (Duffy and Tennyson, 1965; Kato et al., 1996a,b, 1997). The neurofilaments of the neuronal LBHIs are mainly

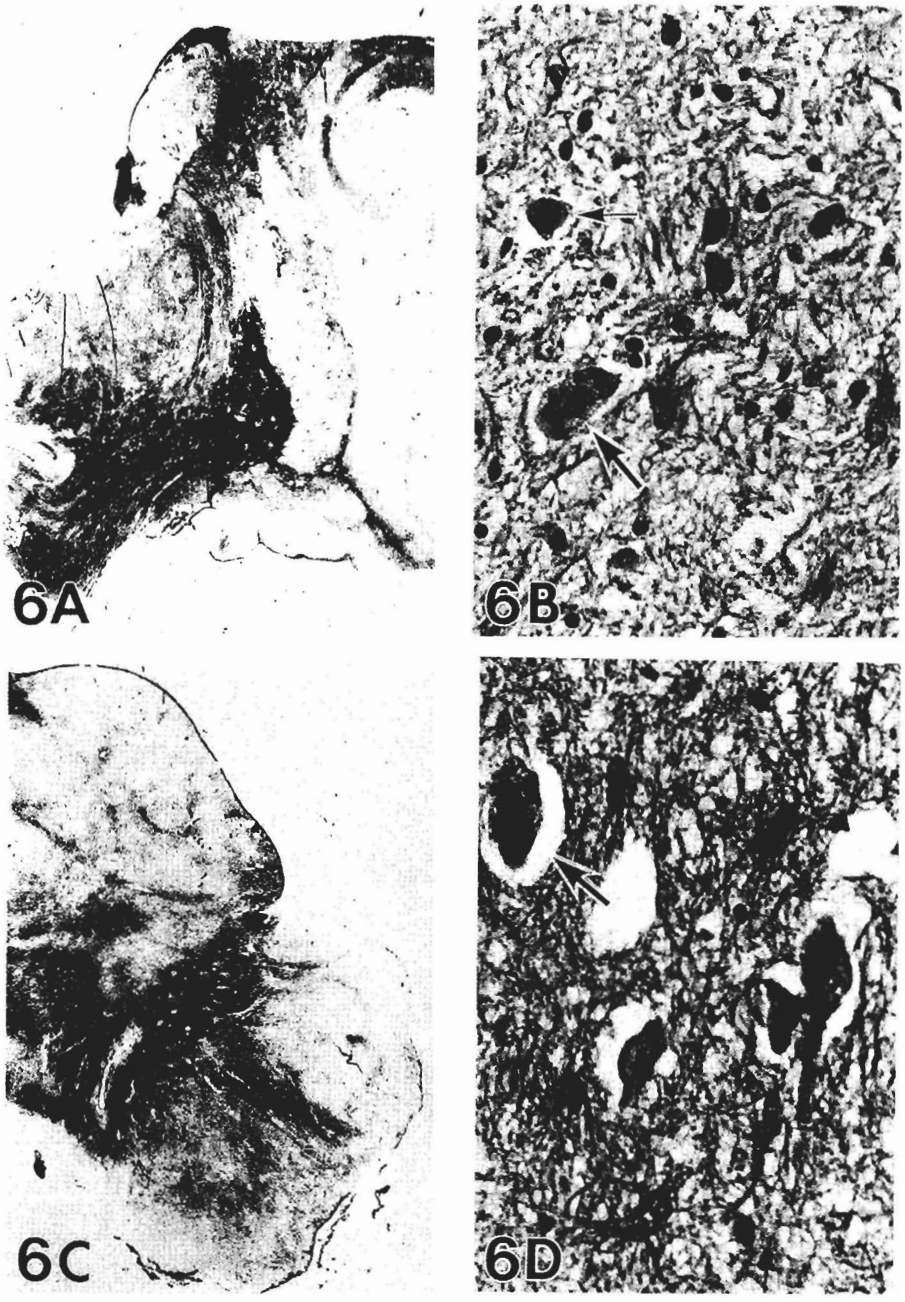

Fig. 6. A. Fibrous gliosis in the globus pallidus and subthalamic nucleus of the long-term surviving FALS patient of the Oki family. Holzer, x 2.4. **B.** Subthalamic nucleus. Marked neuronal depletion and infiltration of reactive astrocytes are evident. A remaining neuron bears a neuronal LBHI (large arrow) and one astrocyte has an astrocytic hyaline inclusion (Ast-HI) (small arrow). The Ast-HI resembles a neuronal LBHI. H&E, x 520. **C.** Severely degenerated substantia nigra, red nucleus and corticospinal tract. Holzer, x 5.0. **D.** Oculomotor nucleus. Showing neuronal loss and gliosis. One neuron contains a neuronal LBHI (arrow). H&E, x 520. From Kato et al. (1996b). Reproduced with permission from the Journal of Neuropathology and Experimental Neurology.

located in the halo and are sometimes also seen in the central portion corresponding to the core (Fig. 5) (Kato et al., 1996a,b, 1997).

The neuronal LBHIs are mainly distributed in motor neurons of mutant SOD1-linked FALS patients with posterior column involvement (Takahashi et al., 1994; Kato et al., 1996a,b, 1997; Shibata et al., 1996b; Saida et al., 1999). Together with the fact that FALS is a motor neuron disease, this distribution is readily understandable for the pathologists. In fact, autopsy cases of FALS with posterior column involvement revealed that the distribution of the neuronal LBHIs was generally limited to the motor neuron system (Takahashi et al., 1972; Nakano et al., 1984; Kato et al., 1987; Takahashi et al., 1994, 1995; Enayat et al., 1995; Chou et al., 1996; Kato et al., 1996a,b, 1997; Shibata et al., 1996a,b; Kadekawa et al., 1997; Ince et al., 1998; Saida et al., 1999). However, the above-mentioned concept had to be abandoned when the authors performed an autopsy on a male FALS patient, a member of a Japanese family of the island of Oki (Oki family), who died at age 65 with an 11-year clinical course. This FALS patient showed multisystem degeneration in addition to the motor neuron disturbance (Fig. 6) (Kato et al., 1996a,b). It is noteworthy that many neuronal LBHIs in this patient were observed not only in the motor neuron system (Fig. 3) but also in the affected non-motor neuron system (Figs. 4, 6B,D) (Kato et al., 1996a,b). We also had the opportunity to examine the pathological materials of his younger sister, who died at age 46 with a clinical course of only 18 months. Her pathological findings were compatible with FALS with posterior column involvement: the neuronal LBHIs were limited to the anterior horn cells of the spinal cord (Takahashi et al., 1972; Kato et al., 1996a,b). Despite the dissimilar neuropathological features, the two siblings had the same two base pair deletion (TTG to **G) in codon 126 of exon 5 of the SOD1 gene (Pramatarova et al., 1994; Nakashima et al., 1995; Kato et al., 1996a,b). An essential difference between the two siblings is the clinical duration of FALS: the long-term duration of the illness in FALS patients causes the degeneration to extend beyond the motor neuron system to the non-motor neuron system. Many neuronal LBHIs are also present in the affected neurons of the multisystem-degenerative lesions (Figs. 4, 6B,D) (Kato et al., 1996a,b, 1997). Similar considerations also pertain to certain respirator-assisted, long-surviving SALS patients who show extensive involvement extending beyond the motor neuron system: widespread degenerative changes have been described in these patients (Mizutani et al., 1992; Sasaki et al., 1992; Kato et al., 1993b).

The neuronal LBHIs seen in motor or non-motor neurons of the two siblings of the Oki family are essentially identical light microscopically and electron microscopically (Kato et al., 1996a,b). Noticeably, all of the neuronal LBHIs have strong SOD1 immunoreactivity (Figs. 3B, 4B) (Kato et al., 1996a,b). To investigate whether this SOD1 immunoreactivity to neuronal LBHIs is also characteristic of other families with several members suffering from mutant SOD1-linked FALS, the authors examined three FALS patients among the members of an American C family (C family) with the Ala4Val substitution in exon 1 of the SOD1 gene, which were obtained from the Montefiore Medical Center file. These three FALS patients of the C family (with FALS duration of 7 months, 8 months or 1 year, respectively) all had neuronal LBHIs that were generally limited to the motor neuron system (Nakano et al., 1984; Shibata et al., 1996b). As expected, all neuronal LBHIs observed in the three patients were intensely positive for SOD1 (Shibata et al., 1996a,b; Kato et al., 1997). Considered in connection with the facts that Bunina bodies and skein-like inclusions observed in SALS patients (Shibata et al., 1994) as well as Lewy bodies seen in Parkinson's disease (Shibata et al., 1996a) have no distinct immunoreactivity for SOD1, the appearance of SOD1-positive neuronal LBHIs in FALS patients with posterior column involvement can be thought to be

Fig. 7. Ast-HIs of the long-term surviving FALS patient. **A.** The Ast-HI is round and eosinophilic (arrow). The nucleus of the Ast-HI resembles that of a reactive astrocyte (double arrows) and not the nucleus of an oligodendrocyte (arrowhead). H&E, x 1,300. **B.** The Ast-HI has an eosinophilic core with a paler peripheral halo. H&E, x 1,300. From Kato et al. (1996b). Reproduced with permission from the Journal of Neuropathology and Experimental Neurology.

correlated with the SOD1 gene mutations.

Pathological characterization of astrocytic hyaline inclusions (Ast-HIs)

When the multisystem-degeneration-type FALS of the Oki family was examined, the notion that the FALS disease process due to the SOD1 gene mutation might lie in glial cells besides neurons sprang afresh in our minds. To make this clear, the authors observed the glial cells in the autopsied brain tissues of five FALS patients, members of the two different families, Japanese Oki and American C families, described above. As a consequence of the detailed pathological analysis of H&E preparations, glial hyaline inclusions resembling neuronal LBHIs were discovered in only the long-term surviving FALS patients with an 11-year clinical course among the members of the Oki family. The excitement of this discovery at that time remains even today a clear memory in the authors' minds. What we learned from the pathological analysis of the long-term surviving patient of the Oki family was the concept that the long clinical course caused the FALS degeneration process in the glial cells. Being open to this concept, we had to obtain further supportive data. So, we investigated a 54-year-old black female patient listed in the Montefiore Medical Center file: she was a 23-year surviving FALS patient, a member of the American family reported by Metcalf and Hirano (M-H family) (Metcalf and Hirano, 1971; Kato et al., 1997). Her histopathological findings were consistent with features of FALS with posterior column involvement. Like in the long-term surviving patient of the Oki family, glial inclusions in addition to neuronal LBHIs were observed in this FALS patient of the M-H family. Both patients are long-term surviving cases. Therefore, the degeneration process of FALS extends to glial cells beyond the neurons in two unrelated long-surviving FALS patients who come from different families and races. A similar situation also occurs in Alzheimer's disease. Thus, paired helical filaments, major components of NFTs that develop exclusively in neurons, are seen in astrocytes of a long-surviving Alzheimer disease patient with a clinical course of 25 years (Nakano et al., 1992). Although FALS cannot be readily compared with Alzheimer's disease, the concept that the FALS degeneration process which attacks neurons also affects glial cells is thought to be acceptable.

In H&E preparations, the nuclei of the inclusion-bearing glial cells resemble those of reactive astrocytes and not those of oligodendrocytes (Fig. 7). Immuno-histochemically, the glial inclusions themselves are not

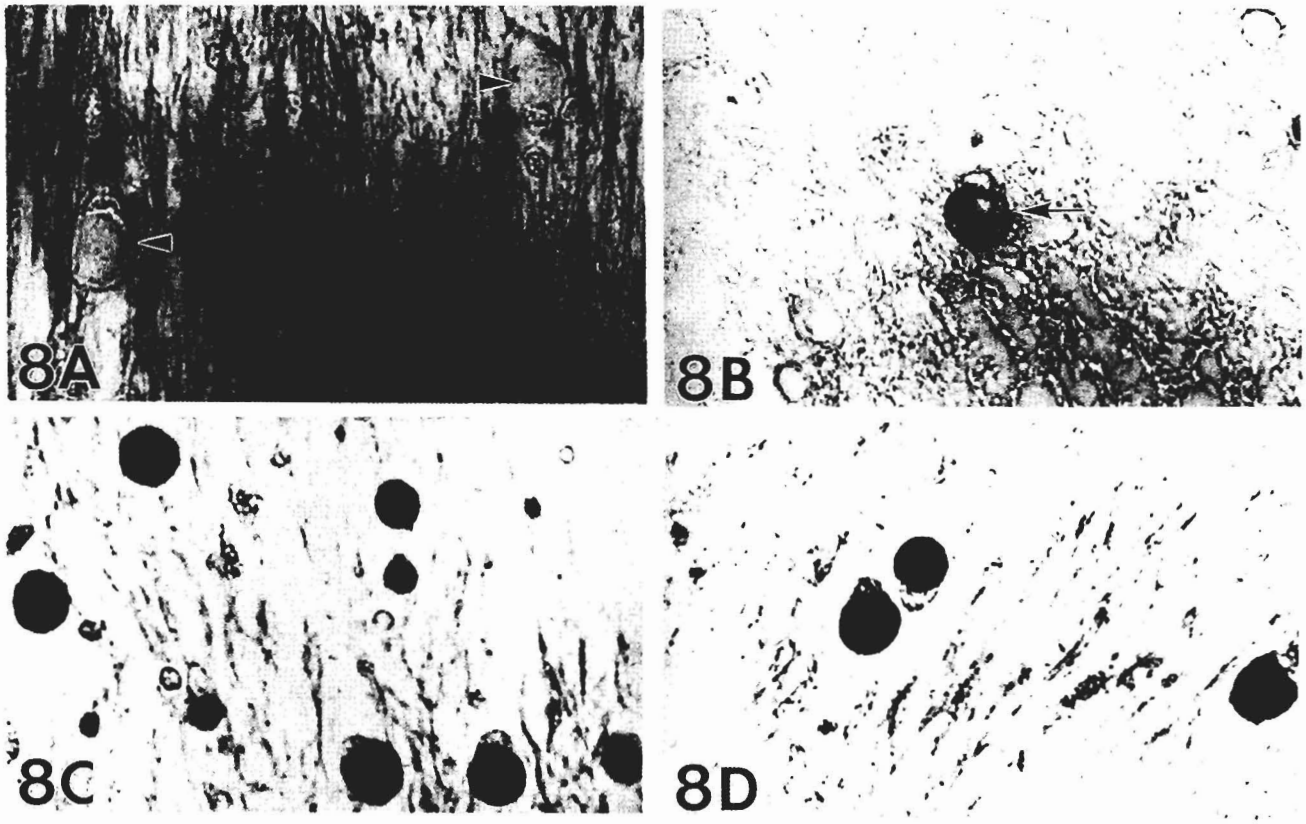

Fig. 8. Light microscopic characteristics of Ast-HIs. **A and B.** Immunostaining with the antibody to glial fibrillary acidic protein (GFAP). The Ast-HIs themselves are not stained (arrowheads in A), but the periphery is stained (arrow in B). **C.** Immunostaining with the antibody against SOD1. The Ast-HIs are intensely stained by the antibody. **D.** Immunostaining with the antibody to ubiquitin. The Ast-HIs are intensely positive. x 700. From Kato et al. (1997). Reproduced with permission from American Journal of Pathology.

stained by the antibody to glial fibrillary acidic protein (GFAP), a marker for astrocytes (Fig. 8A). However, in a few inclusion-bearing cells, only the periphery of the inclusions reacts with the anti-GFAP antibody (Fig. 8B). The inclusion-containing cells neither express the epitopes of oligodendroglial markers such as myelin basic protein (MBP), Leu 7 and 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNP) nor the epitopes of neuronal markers including phosphorylated neurofilament protein (pNFP), non-phosphorylated NFP (npNFP), synaptophysin and neuron-specific enolase (NSE). From these findings, the authors considered the inclusion-bearing cells to be astrocytes and named the inclusion astrocytic hyaline inclusion (Ast-HI) in 1994.

The Ast-HIs seen in each long-term surviving FALS patient of the Oki family or M-H family, are identical histochemically. They are eosinophilic inclusions when stained with H&E and sometimes show eosinophilic cores with paler peripheral halos (Fig. 7). The inclusions are generally round to oval and sometimes sausage-like. Like in the neuronal LBHIs, most Ast-HIs are generally blue to violet after Mallory azan or Masson trichrome staining, and are argyrophilic in Bielschowsky and Gallyas-Braak stainings. However, they are not stained by the following routine staining techniques: luxol fast blue, Holzer, phosphotungstic acid-hematoxylin, periodic acid-Schiff, alcian blue, Congo red, thioflavin S, Berlin blue stains, as well as oil red O, Sudan III and Sudan black B stains using cryostat sections.

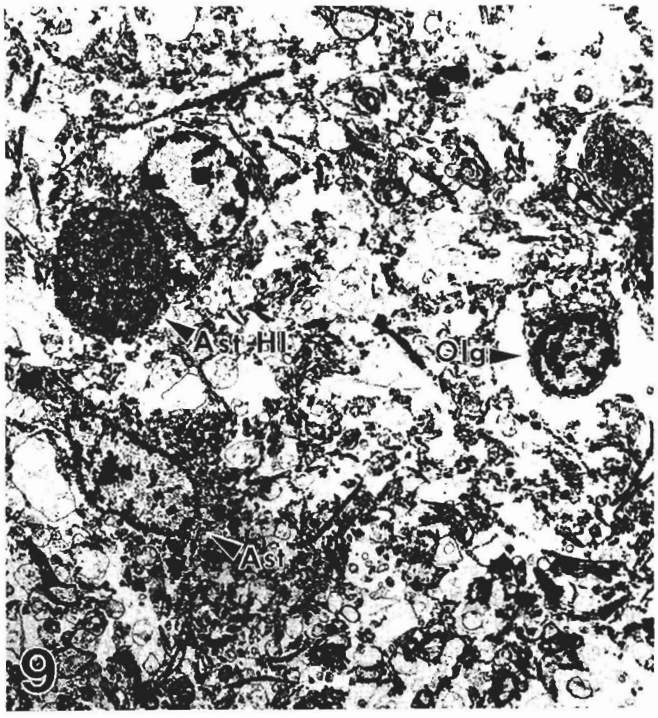

Fig. 9. An electron micrograph of an Ast-HI. The nucleus of the Ast-HI (Ast-HI) is similar to that of a reactive astrocyte (Ast), but differs from that of an oligodendrocyte (Olg). The Ast-HI is recognized as a globular structure that is well demarcated, but has no limiting membrane. x 2,000. From Kato et al. (1996b). Reproduced with permission from the Journal of Neuropathology and Experimental Neurology.

The Ast-HIs observed in the two long-surviving patients have the same ultrastructure. At low-power magnification, the nuclei of the inclusion-bearing astrocytes are the same as those of the reactive astrocytes, but not like the nuclei of oligodendrocytes (Fig. 9). The inclusions appear as globular structures that are well demarcated from other cytoplasmic structures, but have no limiting membranes (Figs. 9, 10A,C). The major components of the Ast-HIs are fibrils and granular materials (Figs. 9, 10A,C). The randomly-oriented fibrils coated with granular materials have a width that ranges from 15 to 25 nm (Fig. 10B). The granule-coated fibrils and granular materials resemble those of neuronal LBHIs (Figs. 5B, 10B). However, the Ast-HIs do not have neurofilaments. In most Ast-HIs, the cytoplasm is almost entirely replaced by components of the inclusion (Fig. 10A). Occasionally, small bundles of glial fibrils surround the inclusions or exist within the inclusion-bearing astrocytic cell bodies (Fig. 10C,D). These ultrastructural observations reflect the immunohistochemical finding that Ast-HIs themselves are not stained for GFAP but only the periphery is stained (Figs. 8B, 10C). Based on the view that the appearance of neuronal LBHIs is a morphological hallmark of mutant SOD1-linked FALS with posterior column involvement, the formation of the granule-coated fibrils and granular materials of the Ast-HIs may also be linked to the SOD1 gene mutations.

Analysis of granule-coated fibrils as essential common components between neuronal LBHIs and Ast-HIs

Ultrastructurally, the essential common constituents between Ast-HIs and neuronal LBHIs are granule-coated fibrils. Immunohistochemically, both neuronal LBHIs and Ast-HIs are intensely positive for SOD1 (Figs. 3B, 4B, 8C). These findings may lead the readers to think that the formation of the granule-coated fibrils may be directly related to the SOD1 gene mutations. As a matter of fact, the indirect immunogold technique reveals that colloidal gold particles labeled by the antibody to SOD1 are only found on the surface of the granule-coated fibrils (Fig. 11). This immunoelectron microscopical finding means that SOD1 is a common component of the granule-coated fibrils of neuronal LBHIs as well as those of Ast-HIs and is integrated into the granule-coated fibrils as a core protein (Kato et al., 1996b, 1997).

There are transgenic mice that carry a transgene for human mutant SOD1: the Gly93Ala (G93A) mutation (Dal Canto and Gurney, 1994, 1995, 1997; Gurney et al., 1994), the Gly37Arg (G37R) mutation (Wong et al., 1995), the Gly85Arg (G85R) mutation (Bruijn et al., 1997) and the Asp90Ala (D90A) mutation (Brännström et al., 1997). These transgenic mice display a progressive hind leg paralysis with muscle atrophy that

resembles a clinical feature of mutant SOD1-linked FALS in humans (Dal Canto and Gurney, 1994, 1995). As in mutant SOD1-linked FALS, the disease of the transgenic mice is dominantly inherited. The international ALS symposium was held in October 1995 in Tokyo, Japan, and was organized by Drs. I. Nakano

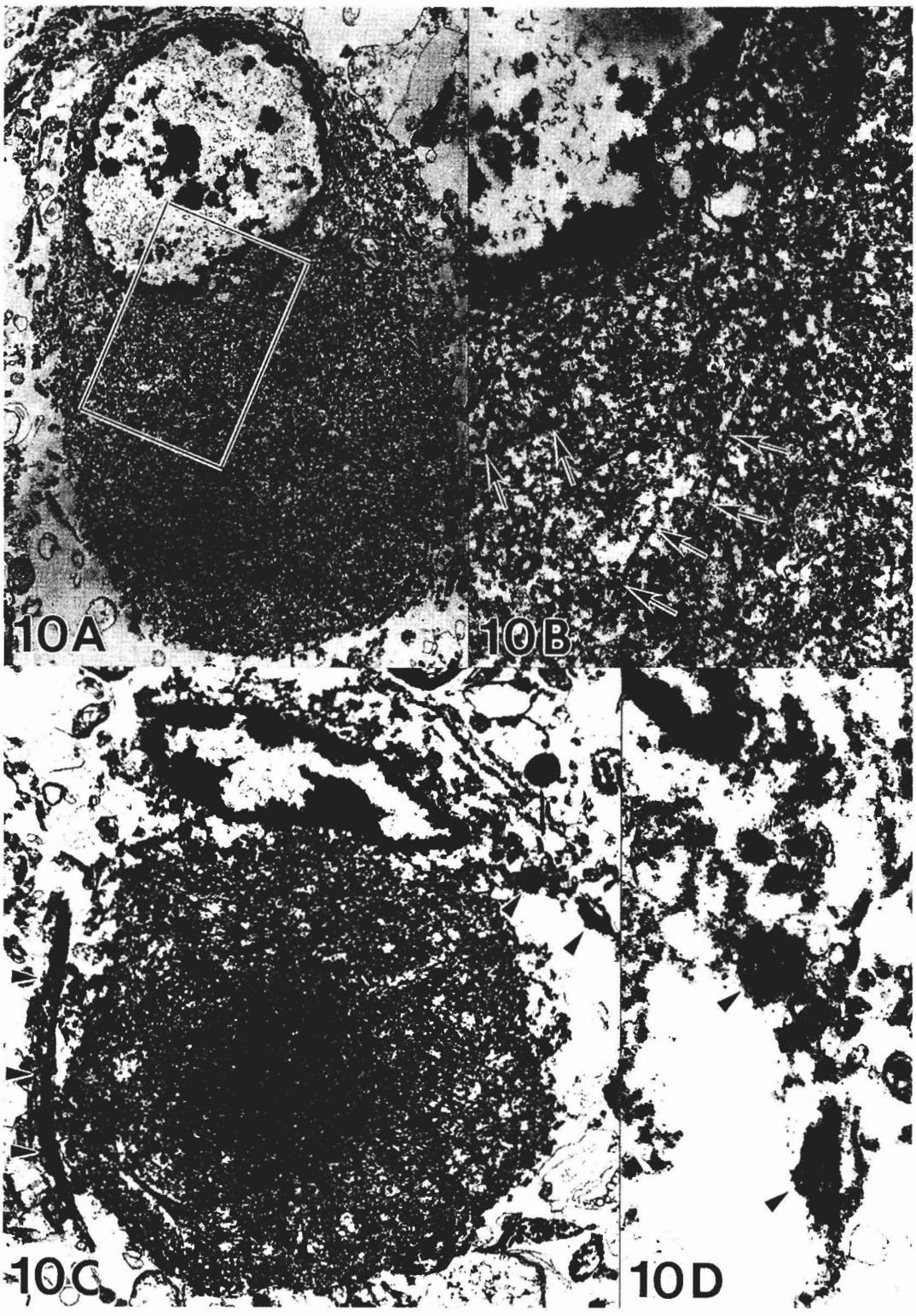

Fig. 10. Electron micrographs of Ast-HIs. **A.** The major components of the Ast-HI are fibrils and granular materials. The cytoplasm has been almost totally replaced by the components of the inclusion. x 6,400. **B.** At higher magnification (inset in A), fibrils appear composed of approximately 15- to 25-nm granule-coated fibrils (arrows). x 18,000. **C.** An electron micrograph of a core and halo type of Ast-HI. The Ast-HI components are densely aggregated in the central portion probably corresponding to the core. Bundles of glial fibrils are seen in the periphery of the Ast-HI within the cell body (arrowheads). This ultrastructural observation represents the immunohistochemical result that only the peripheral structures of the Ast-HI are positive for GFAP in Fig. 8B. x 10,000. **D.** At high-power magnification of C, some small bundles of glial fibrils are present within the cell body (arrowheads). x 28,000. From Kato et al. (1996b). Reproduced with permission from the Journal of Neuropathology and Experimental Neurology.

and A. Hirano. Just before the authors' presentation of the paper on Ast-HIs in human FALS patients at this symposium, Drs. L.I. Bruijn and D.W. Cleveland of the University of California presented their paper about the G85R-transgenic mice, which carried a small number of copies of a transgene for the G85R mutant human SOD1: both neuronal LBHIs and Ast-HIs observed in the G85R-transgenic mice (initial inclusions are in astrocytes, not in neurons), appear prior to clinical signs and have the epitope of SOD1 immunohistochemically (Bruijn et al., 1997). Mice overexpressing the wild-type SOD1 do not develop any clinical symptoms and pathological changes (Dal Canto and Gurney, 1994, 1995, 1997; Gurney et al., 1994; Wong et al., 1995; Bruijn et al., 1997). After listening to this paper, we discussed with them whether a mechanism similar to that of mutant SOD1-linked human FALS was applicable to the G85R-transgenic mice. The findings on mutant SOD1-linked human FALS, G85R-transgenic mice and wild-type SOD1 overexpressing mice suggest that the disease process based on SOD1 gene mutations extends to astrocytes beyond neurons and is related to the formation of inclusions in astrocytes and neurons; i.e., the formation of SOD1-positive granule-coated fibrils and granular materials. In fact, the granule-coated fibrils and granular materials as the essential common constituents between Ast-HIs and neuronal LBHIs are positive for SOD1 immunoelectron microscopically

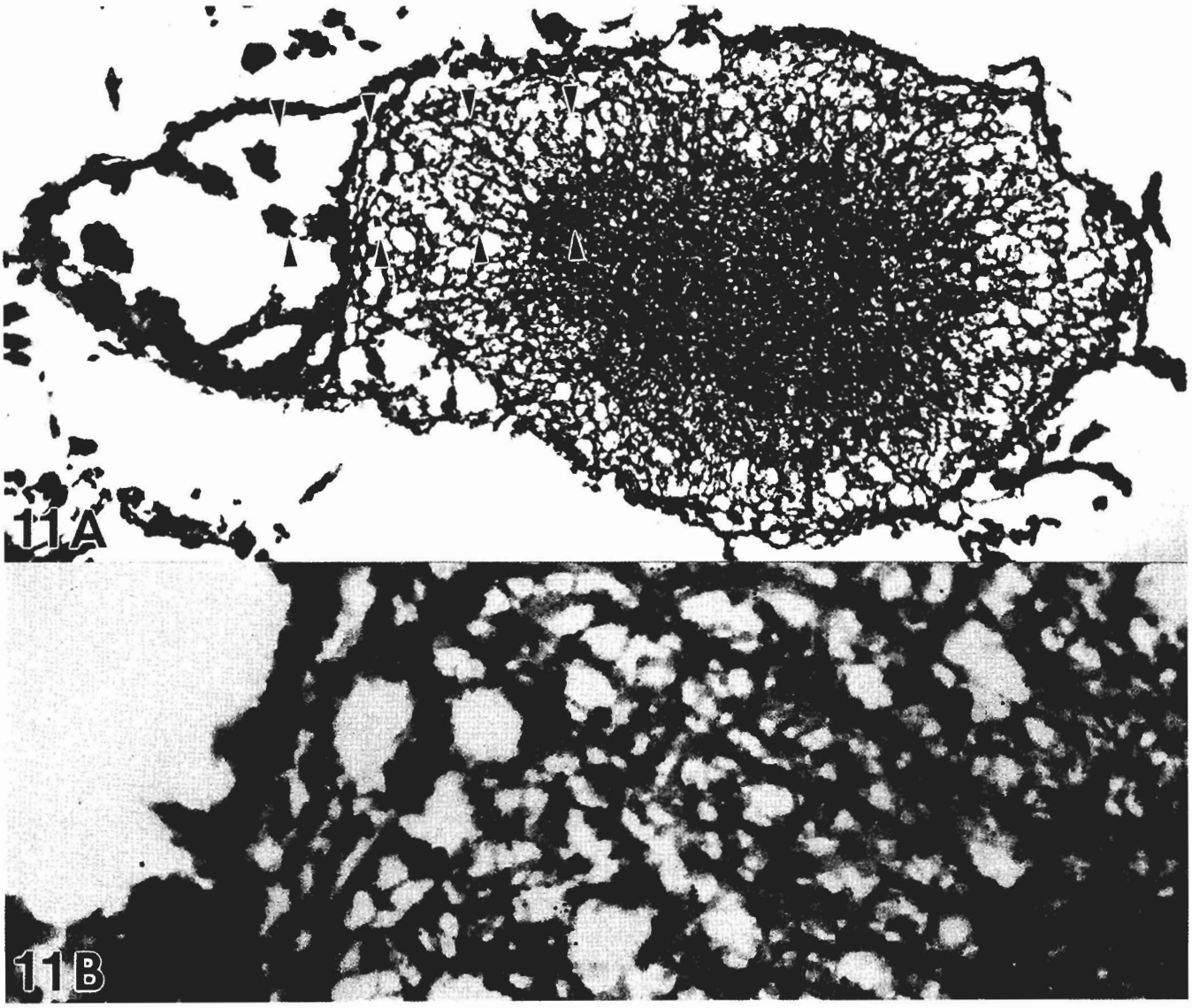

Fig. 11. Immunoelectron micrographs of indirect immunogold labeling of a core and halo type of Ast-HI by the antibody against SOD1. **A.** Low-power magnification of an Ast-HI. x 7,000. **B.** At higher magnification (portion indicated by arrowheads in A), colloidal gold particles are only present on the surface of the granule-coated fibrils shown in Fig. 10B. x 32,000. From Kato et al. (1997). Reproduced with permission from American Journal of Pathology.

(Fig. 11).

It is another important immunohistochemical finding that both neuronal LBHIs and Ast-HIs are highly immunostained by the antibody to ubiquitin (Figs. 4C, 8D). Moreover, the granule-coated fibrils have the epitope of ubiquitin, as seen immunoelectron microscopically (Fig. 12). As for the relationship between ubiquitin, and SOD1, oxidative stress-inducible ubiquitin which participates in the ATP-dependent proteolytic system responsible for the degradation of abnormal cell proteins (Hershko, 1983; Ciechanover et al., 1984; Schlesinger, 1990), would have a role in SOD1 degradation. Therefore, both proteins could interact with each other and be involved in the formation of the granule-coated fibrils.

When the other proteins as constituents of the Ast-HIs are investigated, approximately 50% of the Ast-HIs are labeled by antibodies against metallothionein (MT), glutamine synthetase (GS), αB-crystallin and tubulin (α and ß). Even though the proportion of stained inclusions is less, about 10-30% of them react with antibodies to tau protein, S-100 protein (S-100) and heat-shock protein 27 (HSP 27) (Table 2). MT, an approximately 6 kDa protein containing 6-8% metals (Bühler and Kägi, 1974), has been identified in the liver, small intestine and kidneys (Nishimura et al., 1989). This protein is rich

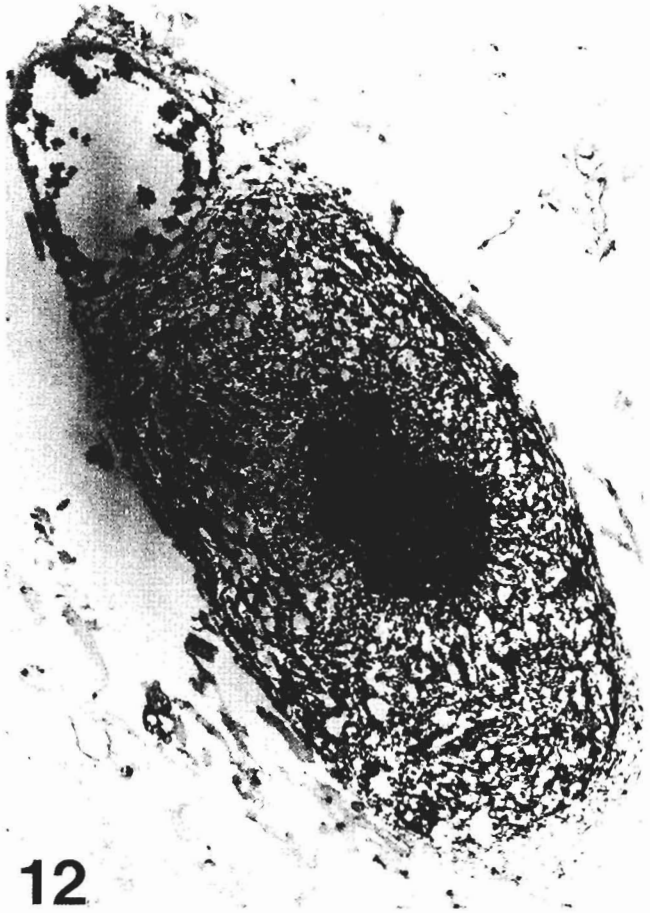

Fig. 12. Immunoelectron micrograph of a core and halo type of Ast-HI stained with the antibody to ubiquitin. Electron-dense immunoreaction product deposits are located on Ast-HI constituents. Nuclear staining is also seen because ubiquitin is constitutively found in the nucleus (Bonner et al., 1988). Indirect immunoperoxidase decoration, contrast-stained with uranyl acetate. x 3,100

Table 2. Immunoreactivity with astrocytic hyaline inclusions (Ast-HIs) and neuronal Lewy body-like hyaline inclusions (LBHIs) using paraffin sections. Modified from Kato et al. (1997). Reproduced with permission from the American Journal of Pathology.

ANTIBODY AGAINST	Ast-HIs	NEURONAL LBHIs
SOD1	+	+
Ubiquitin	+	+
CML	+	+
GFAP	-	-
MT	+/-	-
GS	+/-	-
αB-Cry	+/-	-
α-Tubulin	+/-	+
ß-Tubulin	+/-	+
Tau protein	-/+	-/+
S-100 protein	-/+	-
HSP27	-/+	-
pNFP(SMI31)	-	+
npNFP(SMI32)	-	+
Sypt	-	+/-
NSE	-	+/-
MBP	-	-
Leu7	-	-
CNP	-	-
SOD2	-	-
Actin	-	-
Vimentin	-	-
Desmin	-	-
Cytokeratin (52.5kDa)	-	-
MAP1(1A)	-	-
MAP2	-	-
MAP5(1B)	-	-
PHF(Ab39)	-	-
nNOS	-	-
iNOS	-	-
HSP60	-	-
HSP72	-	-
HSP90	-	-
Chromgr A	-	-
Cath D	-	-
AACT	-	-
AAT	-	-
Lysozyme	-	-
EMA	-	-

SOD1: Cu/Zn-containing superoxide dismutase 1; CML: N^{ε}-carboxy-methel lysine; GFAP: glial fibrillary acidic protein; MT: metallothionein; GS: glutamine synthetase; αB-Cry: αB-crystallin; HSP: heat-shock protein; pNFP: phosphorylated neurofilament protein; npNFP: non-phosphorylated neurofilament protein; Sypt: synaptophysin; NSE: neuron specific enolase; MBP: myelin basic protein; CNP: 2',3'-cyclic nucleotide 3'-phosphodiesterase; SOD2: mitochondrial Mn-containing superoxide dismutase 2; MAP: microtubule-associated protein; PHF: paired helical filament; nNOS: neuronal nitric oxide synthase; iNOS: inducible nitric oxide synthase; Chromgr A: chromogranin A; Cath D: cathepsin D; AACT: α1-antichymotrypsin; AAT: α1-antitrypsin; EMA: epithelial membrane antigen. +: most inclusions showed strongly a positive reaction; +/-: about 50% of inclusions showed a positive reaction; -/+: some inclusions were positively stained; -: negative.

in cysteine residues, binding and detoxifying metals such as copper, zinc, cadmium and mercury (Kägi, 1991). It has been reported that MT is readily detectable in a subgroup of astrocytes of the normal human brain (Blaauwgeers et al., 1993). GS is also present in the astrocytes (Oda et al., 1983) and participates in the metabolism of the neurotransmitters, glutamate and glutamate-aminobutyric acid, as well as in ammonia detoxication (Van den Berg, 1970). S 100 is an acidic calcium-binding protein that is present in the nervous tissues of a wide variety of animals (Moore, 1975), especially as a constituent of astrocytes (Matus and Mughal, 1975). There is evidence that αB-crystallin is a major component of the Rosenthal fibers of astrocytes (Iwaki et al., 1989) and that it is present in astrocytic elements of brain tumors (Iwaki et al., 1991; Kato et al., 1993a, 1995) and in reactive astrocytes (Renkawek et al., 1992). HSP 27, which bears some amino acid sequence homology with αB-crystallin, is present in astrocytic tumor cells (Kato et al., 1992, 1993a, 1995). Taken together, these above-mentioned antibodies recognize certain astrocytic constituents. Therefore, Ast-HIs express several astrocytic markers, and the granule-coated fibrils forming the Ast-HIs contain the astrocyte-associated proteins, in addition to the findings that SOD1 is present as a core protein of the fibrils and ubiquitin is an inducible protein responsible for the degradation of SOD1. Because the granule-coated fibrils are not glial fibrils, anti-GFAP antibody gives a negative reaction to the Ast-HIs themselves.

In contrast, most of the neuronal LBHIs are immunostained for pNFP and npNFP: this reflects the ultrastructural finding that neurofilaments are almost always included in the neuronal LBHIs. In addition, most neuronal LBHIs are also stained by antibodies to tubulin (α and ß) and around 50% are positive for synaptophysin and NSE. Although the proportion of stained inclusions is less, a positive reaction is observed with the antibody against tau protein (Table 2).

On the other hand, both Ast-HIs and neuronal LBHIs express tubulin (α and ß) and tau protein. Tubulin is a constitutive cytosolic protein of neurons and astrocytes, which diffuses passively into Ast-HIs and neuronal LBHIs and is not specifically deposited or otherwise sequestered in granule-coated fibrils. Similar possibilities may be involved in the expression of tau protein, one of the low molecular-weight-microtubule-associated proteins which are part of constituents of microtubules of neurons and astrocytes.

It is another novel immunohistochemical finding that most of neuronal LBHIs and Ast-HIs are intensely positive for N^{ε}-carboxymethyl lysin (CML), one of advanced glycation endproducts (AGEs) (Table 2) (Kato et al., 1999). Granule-coated fibrils which are ultrastructural components of both inclusions, have the epitope of CML (Kato et al., 1999). When considered in relation to the fact that the SOD1 protein itself is susceptible to the AGE reaction (i.e., Mailard reaction) (Arai et al., 1987; Ookawara et al., 1992), CML deposition in the granule-coated fibrils may be associated with the presence of SOD1 integrated into the granule-coated fibrils. Based on the most novel finding that intracellular aggregation of mutant SOD1 independent from wild-type SOD1 (i.e., neuronal LBHIs or Ast-HIs) underlies a portion of mutant-mediated toxicity (Bruijn et al., 1998), the glycated SOD1 in the granule-coated fibrils would be mutant SOD1.

The essential common constituents between Ast-HIs and neuronal LBHIs are SOD1-positive granule-coated fibrils. When granule-coated fibrils are formed as Ast-HIs in astrocytes, the inclusions express several astrocytic markers, and when they are produced as neuronal LBHIs in neurons, the neuronal inclusions express certain neuronal epitopes. Unlike in neurons, however, the formation of granule-coated fibrils in astrocytes requires a long period in certain FALS patients. Although further pathological and molecular analyses of a large number of FALS patients are necessary to elucidate the ultimate significance, in this review, the authors would like to emphasize that the FALS disease process originating from SOD1 gene mutations occurs in not only neurons but also astrocytes and that SOD1 is a component of granule-coated fibrils (namely neuronal LBHIs and Ast-HIs) in mutant SOD1-related FALS with posterior column involvement.

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