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Differential expression between synaptic vesicle proteins and presynaptic plasma membrane proteins in the anterior horn of amyotrophic lateral sclerosis

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Abstract This study concerns the immunohistochemical investigation of synaptic proteins in the anterior horn of amyotrophic lateral sclerosis (ALS). Antibodies against synapsin 1 and synaptophysin (i.e. synaptic vesicle proteins), and those against syntaxin and the synaptosomal-associated, 25 kDa protein, SNAP25 (i.e. presynaptic plasma membrane proteins) were used for immunostaining, respectively. Lumbar spinal cords from five ALS and eight control patients were examined. In the controls, all four synaptic proteins exhibited fine granular immunoreactivities, distributed throughout the spinal gray matter almost uniformly. In contrast, in all five ALS patients, two of the synaptic vesicle proteins examined decreased in the anterior horn neuropil diffusely, while in the same lumbar segments of these cases the immunoreactivities of the two presynaptic plasma membrane proteins showed no apparent decrease, or were only mildly diminished in the same gray matter area. These results indicate that, during the presynaptic terminal degeneration in the anterior horn of ALS, synaptic vesicle involvement may precede that of the presynaptic plasma membrane.

Keywords Amyotrophic lateral sclerosis · Presynaptic terminal · Spinal cord · Synaptic protein

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Introduction

Recent progress in neuropathological techniques has provided increasing knowledge concerning the synapse pathology of neurodegenerative disorders. As indicated in Alzheimer's disease studies, the investigation of the synaptic changes has revealed that synapse loss may predominate over loss of large neurons in the cerebral cortices [28], and has been reported as the major correlate of cognitive impairment in this disease [44].

As for amyotrophic lateral sclerosis (ALS), immunohistochemical examinations of synaptophysin [15, 17, 18, 19, 29, 36], a component protein of the synaptic vesicle membrane [30, 47], and ultrastructural studies on the anterior horn synapses [35, 37] have indicated an extensive degeneration of the presynaptic terminals on the surfaces of the anterior horn cells (AHCs), and presumably some plastic changes of these axonal terminals [18, 19, 35, 37]. In addition, investigations on the growth-associated protein 43 (GAP43) in the ALS spinal cord have shown an increase in this protein [22, 34], and have suggested that a plastic change may occur, to a certain extent, in the axonal terminals attached to AHCs [16] as well as those of the neuromuscular junctions [45].

We examined, immunohistochemically, a set of synaptic proteins in the ALS anterior horn. The proteins examined were synapsin 1, synaptophysin, syntaxin, and the synaptosomal-associated, 25 kDa protein, SNAP25. All of these are related to the synaptic vesicle cycle that consists of docking and fusion of the vesicles, and subsequent exocytosis and neurotransmission [2, 7, 41, 43, 50]. As reported previously, synaptophysin decreases in the anterior horn areas in ALS [15, 17, 18, 19, 29, 36], and this change may be correlative to the AHC degeneration [18, 36]. However, no information is available about the other synaptic proteins, especially those located on the presynaptic plasma membrane, in this disorder. Hence, we aimed to provide more details about the synaptic changes in the ALS anterior horn.

Materials and methods

Patients examined

The lumbar spinal cords from five ALS and eight control patients obtained at autopsy were examined. Diagnoses of all the ALS patients were finally confirmed histopathologically. All of them were adult-onset, had the sporadic form of the disease, and exhibited both anterior horn cell (AHC) and corticospinal tract degenerations. All the control patients who showed no definite AHC loss nor corticospinal tract involvement in their spinal cords, as determined by conventional methods including hematoxylin-eosin, luxol fast blue, and modified Bielschowsky's stainings, were selected from our autopsy case file. The mean age of the patients was 57.0±9.1 years in the ALS group and 70.6±13.2 years in the control group, respectively (Table 1).

Primary antibodies

The following primary antibodies were used for the immunohistochemical assays: rabbit antiserum to synapsin 1 of bovine brain, which crossreacts with rat, mouse, cow and human synapsin 1 (Alexis Biochemicals; diluted 1:1500); rabbit affinity-isolated antibody to human synaptophysin (DAKO, Glostrup, Denmark; diluted 1:50); mouse monoclonal antibody to synaptosomal-associated protein of 25 kDa (SNAP25) (SMI 81; Sternberger Monoclonals Incorporated, Lutherville, Md., USA; diluted 1:1000) [33, 42]; Mouse monoclonal antibody to syntaxin (mouse IgG1 isotype), which recognizes an epitope located in the extracellular domain of the 35 kDa membrane protein syntaxin (Sigma Chemical Company, Saint Louis, Mo., USA; diluted 1:200).

Immunohistochemical procedures

The lumbar segments of the spinal cords from the 13 patients were fixed for 2 days in 4% paraformaldehyde, and subsequently transferred to 20% sucrose in 0.1 M phosphate buffer, pH 7.4. Sections were cut transversely in a cryostat to 30 μm, and stored in a maintenance solution of 0.1 M phosphate buffered saline (PBS), pH 7.4, containing 0.1% NaN_3. Immunostaining was performed on the free-floating sections. The sections were incubated with 0.5% H_2O_2 for 30 min to quench the endogenous peroxidase activity. Five percent normal horse serum was used to block the non-specific binding in the SNAP25 and syntaxin immunostainings, and normal goat serum of the same concentration for synapsin 1 and synaptophysin. The sections were incubated with the diluted primary antibodies overnight at 4 °C. Each primary antibody was diluted in PBS containing 0.3% Triton X 100 (PBST). The sections incubated with 5% normal horse or goat serum instead of primary antibodies served as negative reaction controls. After rinsing with PBST, the sections for SNAP25 or syntaxin immunostaining were incubated with biotinylated anti-mouse IgG (Vector Laboratories, Burlingame, Calif.; diluted 1:1000 in PBST) for 60 min, and those for synapsin 1 or synaptophysin with biotinylated anti-rabbit IgG (Vector Laboratories, Burlingame, Calif.; diluted 1:1000 in PBST) for 60 min respectively. Subsequently they were reacted with an avidin–biotin–immunoperoxidase complex (Vector Laboratories, Burlingame, Calif.) for 60 min; 3,3′-diaminobenzidine tetrahydrochloride (Nacalai Tesque, Kyoto, Japan) was used as the final chromogen.

For scoring of the intensity of loss of immunoreactivity in the anterior horn of a lumbar segment, every section stained for the synaptic proteins in both ALS and control groups was rated from 0 to 3 (0, no decrease of immunoreactivity in the anterior horn; 1, minimum decrease by microscopic evaluation; 2, between 1 and 3; 3, immunoreactivity of anterior horn neuropil is mostly lost). Subsequently, mean value±standard deviation (SD) was calculated for each synaptic protein examined in each patient (Table 2). A Mann–Whitney test was performed when comparing scores between two groups of synaptic proteins, i.e. synaptic vesicle proteins (synapsin 1 and synaptophysin) versus presynaptic plasma membrane proteins (SNAP25 and syntaxin), in each ALS patient (Table 2).

Table 1 Patient profiles (*?* no information available, *LBHI* Lewy body-like hyaline inclusion, *AHC* anterior horn cell, *NA* not applicable)

	Age at death (years)	Sex	ALS duration	ALS criteria[a]	Cause of death	Post-mortem delay (h)	Cytological features of AHCs
ALS 1	51	F	5 years 11 months	Suspected	Respiratory failure	5	Not specific
2	43	M	10 months	Probable (laboratory-supported)	Respiratory failure	6.5	Bunina body, LBHI
3	58	F	3.5 months	Definite	Respiratory failure	3.5	Bunina body
4	65	M	12 years	Probable	Pneumonia	5	Bunina body, LBHI
5	68	M	4 years	?	Respiratory failure	1	Not specific
			Clinical diagnosis				
Control 1	74	F	Cerebral infarction	NA	Ileus	<6	NA
2	84	M	Dementia	NA	Acute renal failure	3	NA
3	63	F	Respiratory failure	NA	Respiratory failure	1.5	NA
4	71	M	Polyneuropathy	NA	Sepsis	<34	NA
5	87	F	Cerebral infarction	NA	Cerebral herniation	2.5	NA
6	52	M	Binswanger's disease	NA	Pneumonia	<24	NA
7	51	F	Epilepsy	NA	Acute heart failure	<26	NA
8	83	M	Cerebral infarctions	NA	Pneumonia	<24	NA

[a]According to the revised El Escorial Criteria for the Diagnosis of ALS [48]

Table 2 Immunoreactivity changes of synaptic proteins in ALS anterior horn. (Degree of immunoreactivity decrease of synaptic proteins examined: *0* no decrease of immunoreactivity in the anterior horn, *1* minimum decrease by microscopic evaluation, *2*, between 1 and 3, *3* immunoreactivity of anterior horn neuropil is mostly lost. Scores are expressed as mean value±SD for each synaptic protein in each ALS patient. All of the sections in control patients were scored zero for any synaptic protein)

	Synapsin 1	Synapto-physin	SNAP25	Syntaxin	*P*
ALS 1	3.0±0	3.0±0	1.3 ±0.4	1.3 ±0.4	0.006
2	2.5±0.5	2.0±0	0.0 ±0	0.40±0.5	<0.001
3	1.5±0.5	1.5±0.5	0.11±0.3	0.57±0.5	<0.02
4	3.0±0	3.0±0	1.5 ±0.5	2.3 ±0.5	0.047
5	3.0±0	3.0±0	1.0 ±0	1.0 ±0	0.028

Results

Control

At a low magnification of the spinal cord of the control patients, the immunoreactivity of synapsin 1 was distributed throughout the gray matter areas almost uniformly, and no apparent immunoreactivity was seen in the white matter areas except for that along the neuronal processes adjacent to the gray matter (Fig. 1A). The anterior horn neuropil exhibited numerous fine granular immunoreactivities diffusely (Fig. 1E), and the individual normal AHC had fine granular or some dot-like immunoreactivity on the surface of the cell body while there was no apparent positivity within the perikaryon (Fig. 1F). As reported previously [15, 17, 18, 19, 29, 36], the immunoreactivity of synaptophysin showed a similar pattern to that of synapsin 1 (Fig. 1B).

In contrast, the immunoreactivity of SNAP25 was distributed throughout the spinal gray matter almost uniformly. However some immunoreactivity was seen in the white matter, especially evident in almost the entire areas of the anterior and lateral columns (Fig. 1C). This immunoreactivity in the white matter was in the axons. Similar to the synaptic vesicle proteins, fine immunoreactive granules were scattered diffusely throughout the anterior horn neuropil. Individual AHC exhibited fine granular immunoreactivity on the surface of the cell body and proximal processes (Fig. 1G). However, no apparent positive materials were seen within the perikaryon of these cells (Fig. 1G). Syntaxin showed a similar immunoreactive pattern to that of SNAP25 (Fig. 1D, H).

All of the sections in control patients were scored zero for any synaptic protein.

ALS

Immunohistochemical examination of the ALS spinal cords at a low magnification showed that all the ALS patients had a decreased density of synapsin 1 immunoreactivity in the anterior horn area diffusely, while there were no obvious changes in the other areas of the lumbar spinal cord (Fig. 2A). A similar decrease in synaptophysin immunoreactivity in the anterior horn area was seen in the adjacent sections of the same lumbar segment in each ALS patient examined (Fig. 2B).

SNAP25 and syntaxin showed no significant changes in immunoreactivity in the anterior horn area, in addition to the other spinal cord areas in the same lumbar segment, even when the sections adjacent to those showing obvious decrease in synapsin 1 or synaptophysin immunoreactivity were examined immunohistochemically (Fig. 2C, D). However, a slight decrease in these presynaptic plasma membrane proteins in the anterior horn neuropil could be seen in those segments with severe AHC loss. The immunoreactivities in the other spinal cord areas exhibited no significant changes compared to those of the controls, including axonal immunoreactivity in the white matter (Fig. 2C, D).

Examination of these sections at a higher magnification, showed that synapsin 1 (Fig. 2E) or synaptophysin immunoreactive granules diminished in the anterior horn area diffusely, while in the same areas on adjacent sections stained for SNAP25 (Fig. 2F) or syntaxin, the decrease in immunoreactive granules was milder.

Scoring of the intensity of loss of immunoreactivity exhibited significant differences between former two (i.e. synapsin 1 and synaptophysin) and latter two (i.e. SNAP25 and syntaxin) in all ALS patients (Table 2).

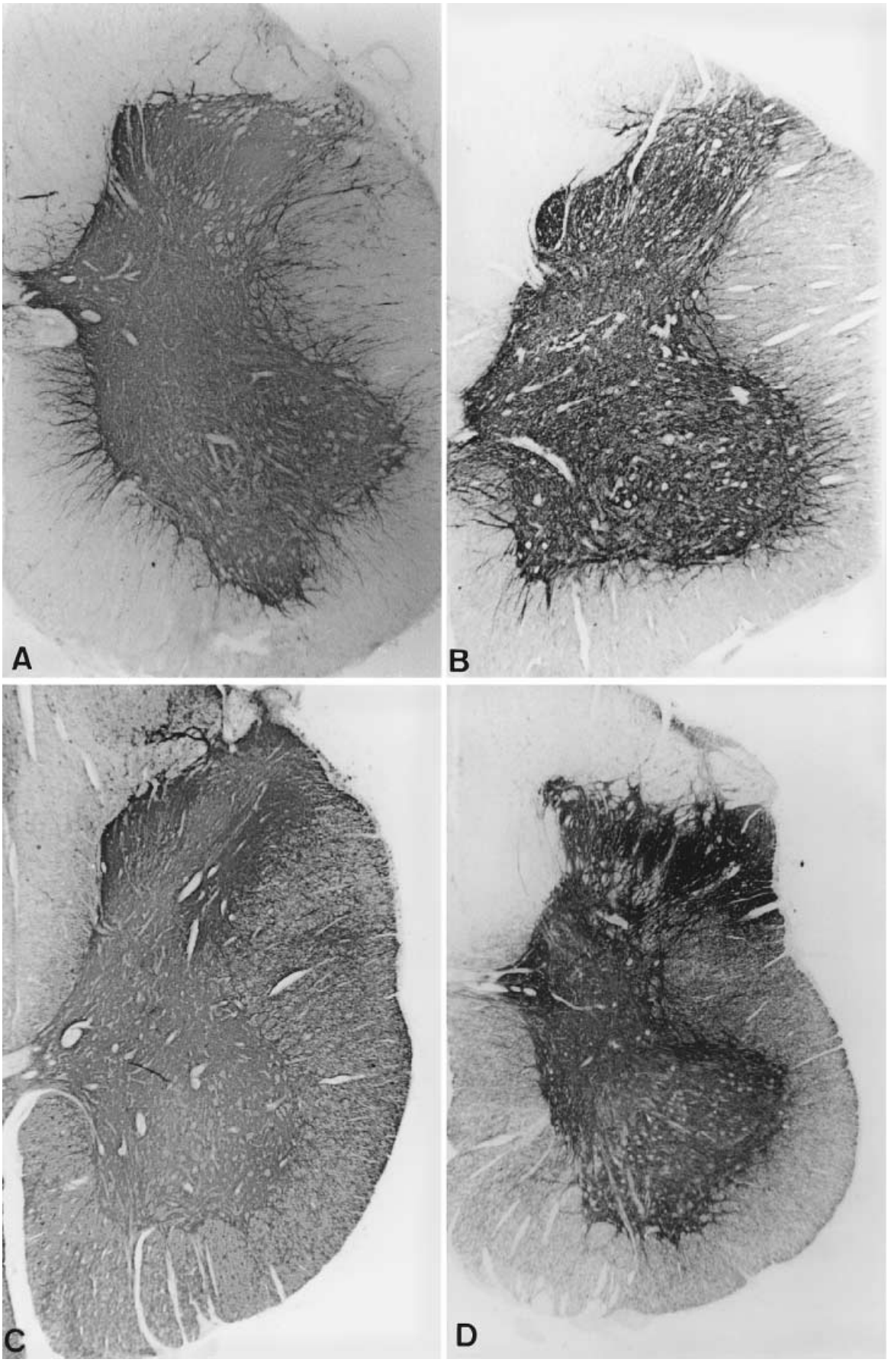
There were some remaining AHCs with densely accumulated synapsin 1 immunoreactivity on the surface of the cell body and proximal processes on the sections showing a decreased density in the immunoreactivity of the neuropil (Fig. 2G), as similarly reported for synaptophysin [15, 18, 19, 29]. A similar, but somewhat milder, accumulation of immunoreactivity on the AHC was seen on the sections stained for SNAP25 (Fig. 2H), or syntaxin (Fig. 2I).

No reaction products were observed when the sections of the ALS and control patients were incubated with normal horse or goat serum instead of the primary antibody.

Discussion

The four synaptic proteins examined here have been reported to play essential roles in the synaptic vesicle cycle, which mainly consists of the processes of docking, fusion, and exocytosis of the synaptic vesicles [40, 43]. These processes are regulated by the complex interaction of a number of different proteins localized in the cytosome, synaptic vesicles, or presynaptic plasma membranes in the presynaptic terminals [7, 43, 50].

Synapsins, including synapsin 1, are synaptic vesicle-associated proteins which bind to proteins of the cytoskeleton. Synapsin phosphorylation liberates the synaptic vesicle from the cytoskeleton, which results in free movement of the vesicle in the presynaptic terminal [3, 9, 10, 13, 43, 46]. Synaptophysin, a main component of synaptic vesicle membrane proteins [30, 47], is thought to control exocytosis [12, 21]. SNAP25 has been implicated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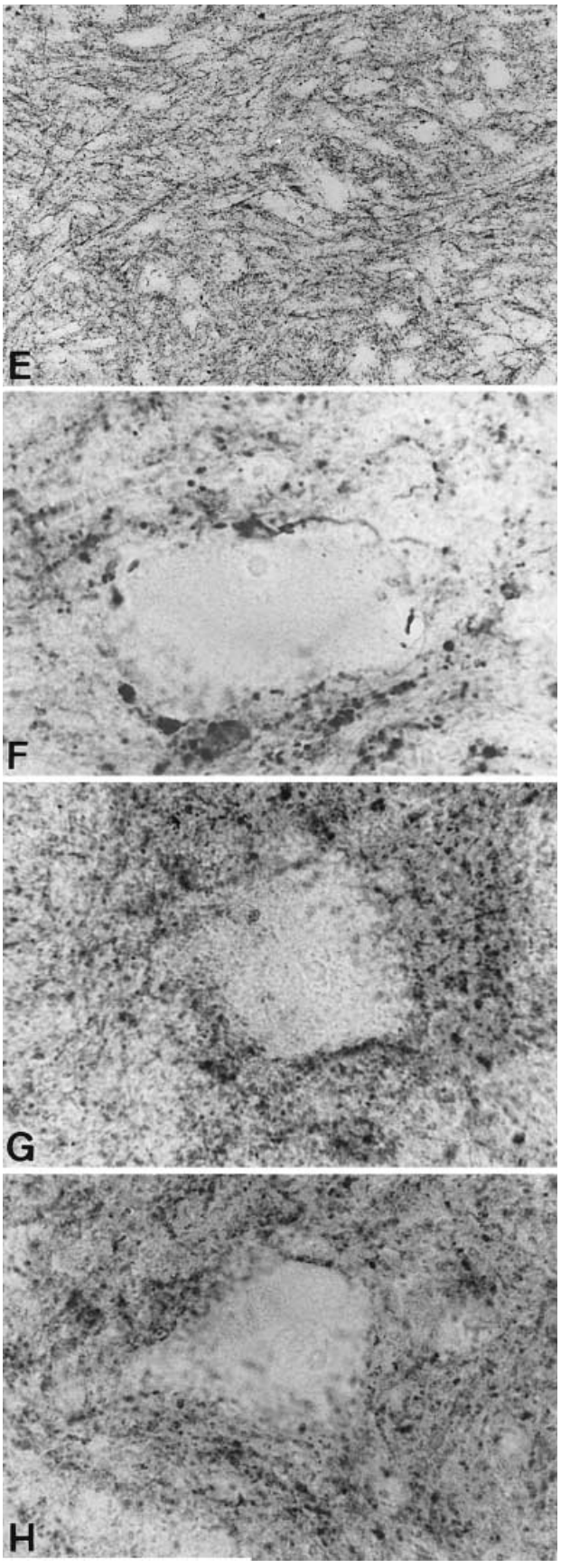
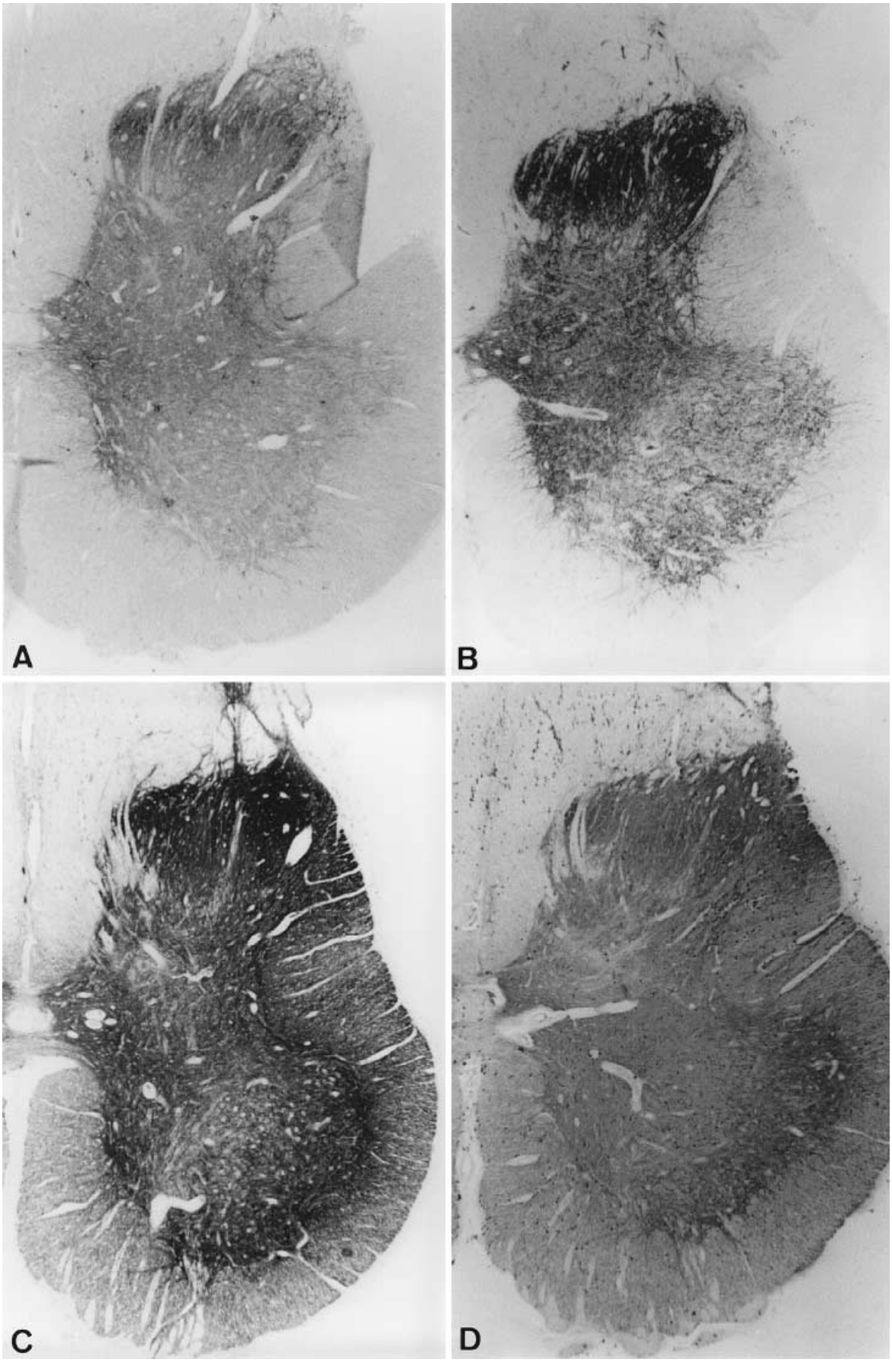


in the establishment and stabilization of presynaptic nerve terminals during development [8, 31, 32] and is believed to be an essential component of docking and fusion [6, 38, 41, 43]. Syntaxins are integral plasma membrane proteins, which are implicated in the docking of synaptic vesicles at presynaptic active zones [4, 5, 20]. These four proteins are divided into two groups according to their localization in the presynaptic terminals, i.e., synapsin 1 [9, 10, 43] and synaptophysin [30, 47] are components of the synaptic vesicle membrane whereas SNAP25 [33] and syntaxin [1, 49] exist as presynaptic plasma membrane proteins.

We have demonstrated the differential expression between the two groups of synaptic proteins in the ALS anterior horn of the spinal cord. The former group, the synaptic vesicle proteins, were significantly decreased in segments with considerable AHC degeneration, while the latter showed no apparent changes in the same segments, or, if any, slight decreases in the immunoreactivity in those segments with severe AHC loss and neuropil rarefaction. In fact, scores of the intensity of immunoreactivity loss showed significant differences between the former and latter groups in all ALS patients (Table 2).

On the sections stained for the presynaptic plasma membrane proteins in the ALS and control patients, there was some immunoreactivity in the axons in the spinal white matter, which was especially evident in the anterior and lateral columns. The presence of SNAP25 immunoreactivity in axons has been reported in the central and peripheral nervous systems of experimental animals [11], and in the descending fibers of the basis pontis in humans [23]. The detection of SNAP25 in the axons may be associated with the transportation by fast axonal flow [14, 24], and similarly with syntaxin. However, our observations on the differential expression between the synaptic proteins examined in the ALS anterior horn could not be derived from the presence of axonal immunoreactivity, since the immunoreactivity in the white matter, including the corticospinal tract areas, showed no significant differences between the ALS and control groups, and since the

Fig. 1A–H The immunoreactivities of the synaptic proteins in the control lumbar cords. **A** synapsin immunoreactivity is seen uniformly throughout the spinal gray matter, while no positivity is seen in the white matter except for the immunoreactivity along the neuronal processes adjacent to the gray matter area (Control 4) ×17. **B** The synaptophysin immunoreactivity in the spinal cord is identical to that of synapsin 1 (Control 4) ×17. **C** The SNAP25 immunoreactivity is distributed densely throughout the gray matter in addition to the mild immunoreactivity in the spinal white matter, especially evident in almost entire areas of the anterior and lateral columns (Control 2) ×17. **D** The syntaxin immunoreactivity in the spinal cord is almost identical to that of SNAP25 (Control 3) ×17. **E** Numerous synapsin 1-positive fine granules are scattered diffusely throughout the anterior horn neuropil (Control 4) ×56. **F** The synapsin 1-positive granules and dots are attached on the surface of a large AHC (Control 3) ×440. **G** The numerous SNAP25-positive fine granules are attached on the surface of a large AHC, surrounded diffusely by numerous immunoreactive granules in the neuropil (Control 2) ×440. **H** The syntaxin immunoreactivity of the neuropil and AHC is almost identical to that of SNAP25 (Control 8) ×440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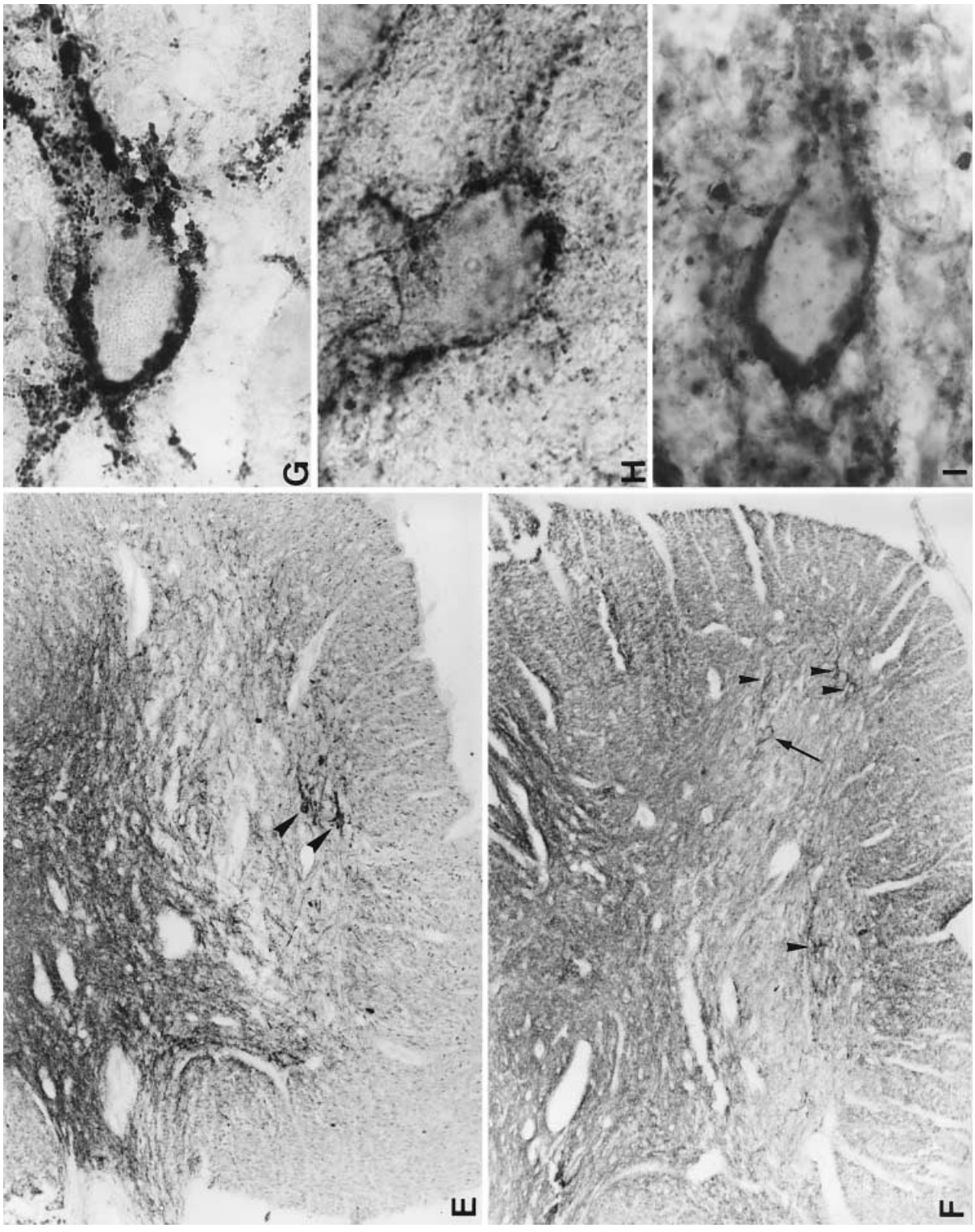

Fig. 2A–I The immunoreactivities of synaptic proteins in ALS lumbar cords. The expression of synaptic proteins in the same lumbar segment of ALS 3 (**A**–**D**). **A** The synapsin immunoreactivity is mildly decreased in the anterior horn area diffusely, while the other areas are not changed (ALS 3) ×17. **B** The synaptophysin immunoreactivity is decreased in the anterior horn of a lumbar section adjacent to **A** ×17. **C** On a lumbar section adjacent to **A**, the SNAP25 immunoreactivity exhibits no significant changes, including the anterior horn area, compared to the control (**C**) ×17. **D** On a lumbar section adjacent to **A**, the syntaxin immunoreactivity is almost the same as that of the control (**D**) and is preserved in the anterior horn area ×17. The expression of the synaptic proteins in the anterior horn area on the adjacent sections of the same lumbar segment in ALS 5 (**E**, **F**). **E** The synapsin immunoreactivity is obviously reduced in the anterior horn neuropil. The *arrowheads* indicate dense accumulations of immunoreactivity on the remaining AHCs. (The *left* end of the photomicrograph is the median portion of the spinal cord, and the *lower* part is the anterior portion) ×30. **F** The SNAP25 immunoreactivity in the anterior horn neuropil is relatively preserved although a slight sparseness may exist. The *arrowheads* and an *arrow* indicate the accumulations of immunoreactivity on the cell bodies or processes of the AHCs. A remaining AHC indicated by the *arrow* is shown as **H** at a higher magnification (the same area to **E**) ×30. The synaptic protein expressions of individual AHCs (**G**–**I**). Some of the remaining AHCs show densely accumulated immunoreactivity on the surface. **G** Synapsin 1 (ALS 5 ×440), **H** SNAP25 (ALS 5 ×440), **I** Syntaxin (ALS 1 ×440)

immunoreactivity in the anterior horn neuropil in both groups seemed to express basic granular patterns.

Of note is that both the presynaptic plasma membrane proteins, SNAP25 and syntaxin, showed an accumulation on the cell bodies and proximal processes of some of the remaining large AHCs in ALS, even if relatively mild compared to those of synapsin 1 and synaptophysin. Similar observations have been reported in studies on synaptophysin [15, 18, 19, 29] and GAP43 [16] in the spinal cord of this disease. As previously suggested [16, 18, 19], these accumulations may be associated with some plastic changes in the anterior horn presynaptic terminals in ALS. However, further studies would be necessary to elucidate the significance of this change.

Our observation that the ALS synaptic vesicle proteins were decreased more prominently than the presynaptic plasma membrane proteins in the anterior horns, which were degenerated to a similar extent, may indicate that a main element of the presynaptic terminal degeneration in the ALS anterior horn is synaptic vesicle involvement rather than that of the presynaptic plasma membrane, i.e., the former is possibly more vulnerable than the latter to the ALS disease process. In the brain of Alzheimer's disease patients, similar results have been obtained from a western blot analysis [39], which stated that, in temporal cortices, synaptic vesicle proteins such as synaptobrevin and synaptophysin decreased by some 30%, while those of the presynaptic plasma membrane proteins, such as SNAP25 and syntaxin, decreased by only about 10%. Therefore, we suggest that, regarding the presynaptic terminal degeneration, the ALS spinal cord may exhibit a similar pattern of synaptic protein alteration to that of the Alzheimer's-diseased brain. Although some compensatory up-regulation of a synaptic vesicle protein (i.e. synaptophysin) [25] or growth-associated protein [26, 27] has been reported on the cerebral cortex for Alzheimer's disease immunohistochemically, there have been no reports of histological changes of other synaptic proteins studied here. Hence, further immunohistochemical investigations on such substances in this disease might be of interest.

We have mentioned a similarity in synapse pathology between ALS and Alzheimer's disease; however, further investigations of other synaptic proteins are necessary to determine whether synaptic alterations characteristic of ALS exist, or whether they exhibit similar pattern changes among other neurodegenerative disorders. In addition, it still remains to be clarified whether these synaptic proteins are involved individually, or whether the differential expression outlined here results from a difference in vulnerability between the synaptic vesicles and the presynaptic plasma membranes as a whole.

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