proposed that neuropathy can occur as a result of a direct toxic effect on peripheral nerves in patients with chronic alcoholism but no thiamine deficiency.[19]

In this study, we compared clinicopathological features of neuropathies associated with chronic alcoholism and thiamine deficiency in a series of consecutive patients who underwent careful determination of thiamine status and assessment of daily alcohol consumption to assess whether neuropathies caused by ethanol and thiamine deficiency were identical, and how thiamine deficiency was related to alcoholic neuropathy.

Patients and Methods

Patients

Consecutive patients with neuropathy who were referred to Nagoya University Hospital and its affiliated institutions between 1990 to 2002 and fulfilled the following criteria were included. Patients were assigned to one of three groups according to cause of neuropathy: pure alcoholic neuropathy without thiamine deficiency (ALN), alcoholic neuropathy with thiamine deficiency (ALN-TD), or pure, nonalcoholic thiamine-deficiency neuropathy (TDN). All patients with "alcoholic neuropathy" (ALN or ALN-TD) had chronic alcoholism, defined by regular intake of more than 100gm of ethanol daily for at least 10 years before onset of neuropathic symptoms. Individuals with lesser but daily consumption of alcohol were excluded from the TDN group. Four subjects not excluded had only occasional intake of alcohol, not exceeding 20gm per occasion. The remaining patients in the TDN group were total abstainers. The ALN group consisted of 36 men ranging from 31 to 70 years of age (mean ± standard deviation [SD], 50.7 ± 10.0); the ALN-TD group, 23 men and 5 women ranging from 27 to 68 years of age (mean ± SD, 51.1 ± 11.2); and the TDN group, 26 men and 6 women ranging from 18 to 81 years of age (mean ± SD, 54.5 ± 15.5; see Table 1). Causes of thiamine deficiency in patients with TDN were dietary imbalance in 12 patients and previous gastrointestinal surgery to treat ulcer or neoplasm in 20 patients.[20–22] Clinicopathological features of 17 patients in the TDN group who had undergone gastrectomy have been reported previously.[22] Of 28 patients in the ALN-TD group, 10 had a history of gastrectomy. Patients who had undergone operations to treat morbid obesity were excluded. A detailed history was obtained from each patient as well as their families concerning lifestyle, occupation, diet, and amount of daily consumption of alcohol. All patients underwent clinical and neurological assessment, routine blood and urine studies, blood thiamine determinations, cranial magnetic resonance imaging or computed tomography, and nerve conduction studies. Sural nerve biopsies were performed in 66 of 96 patients. A major manifestation of thiamine deficiency apart from peripheral neuropathy, Wernicke's encephalopathy, had occurred in 32 and 22% of patients in the ALN-TD and TDN groups, respectively. In the ALN group, no patients manifested this syndrome. Signs of heart failure possibly related to thiamine deficiency (cardiomegaly evident from chest radiographs, or pitting edema in the distal lower limbs) were observed in 50 and 69% of patients in the ALN-TD and TDN groups but no ALN patients. Patients with diabetic neuropathy, chronic inflammatory demyelinating polyneuropathy, Guillain–Barré syndrome, familial amyloid polyneuropathy, or other neuropathies unrelated to alcohol or thiamine deficiency were excluded. Patients' functional status was assessed at the peak phase according to modified Rankin score.[23]

Results of the various assessments in the three neuropathy groups defined above are described in the following order to facilitate comprehension: ALN, TDN, and ALN-TD.

Assessment of Thiamine Status

Thiamine status was determined at the time of the first referral to the hospital in all patients as previously described.[19,22] No patient had received thiamine at the time of

Table 1. Backgrounds of the Patients with Alcoholic Neuropathy and Nonalcoholic Thiamine-Deficiency Neuropathy

Characteristic	Alcoholic Neuropathy		Nonalcoholic Thiamine-Deficiency Neuropathy, n = 32 (%)
	Without Thiamine Deficiency, n = 36 (%)	With Thiamine Deficiency, n = 28 (%)	
Age (yr)	50.7 ± 10.0	51.1 ± 11.2	54.5 ± 15.5
Men/women	36/0	23/5	26/6
Duration of neuropathic symptoms (mo), mean ± SD	26.1 ± 39.3	16.5 ± 32.3	11.4 ± 23.5
Gastrointestinal tract operation			
Gastrectomy	0 (0)	10 (36)	18 (56)
Others	0 (0)	0 (0)	2 (6)
Associated symptoms			
Wernicke's encephalopathy	0 (0)	9 (32)	7 (22)
Heart failure	0 (0)	14 (50)	22 (69)
Total thaimine concentration (ng/ml), mean ± SD	38.5 ± 12.0	12.6 ± 4.0	11.0 ± 4.3

Normal values for whole-blood concentration of total thiamine were established in 100 normal volunteers (mean age ± SD, 29.7 ± 5.0 yr; male/female ratio, 50:50).
Normal thiamine concentration (mean ± 2 SD for normal control) is 20–50ng/ml.

SD = standard deviation.

this determination. Thiamine concentrations were measured by high-performance liquid chromatography as described previously.[13–15] Normal thiamine status was defined by a whole-blood concentration of total thiamine between 20 and 50ng/ml (the mean ± 2 SD for normal control subjects) and normal erythrocyte transketolase activity (between 123.8 and 206.2U/L, representing the mean ± 2 SD for normal controls). Thiamine deficiency was defined as total thiamine concentrations in whole blood below 20ng/ml and decreased erythrocyte transketolase activity less than 123.8U/L. Normal values for total thiamine concentration were established in 100 normal volunteers.[19,22] Normal values for erythrocyte transketolase activity were adopted from previous report.[24]

Electrophysiological Assessment

Motor and sensory conduction was measured in the median, ulnar, tibial, and sural nerves in all patients during their initial clinical assessment at the hospital, using a standard method with surface electrodes for stimulation and recording.[25,26]

Pathological Assessment of Sural Nerve Specimens

Sural nerve biopsy was performed in 29 patients with ALN, 18 patients with ALN-TD, and 19 patients with nonalcoholic thiamine-deficiency neuropathy, as described previously,[27–31] in most cases biopsy was performed before administration of thiamine. Informed consent was obtained beforehand. Specimens were divided into two portions. The first of these was fixed in 2.5% glutaraldehyde in 0.125M cacodylate buffer (pH 7.4) and embedded in epoxy resin for morphometric and ultrastructural study. Density of myelinated fibers was assessed in toluidine blue–stained semithin sections using a computer-assisted image analyzer (Luzex FS; Nikon, Tokyo, Japan), and densities of small and large myelinated fibers were calculated as described previously.[28–31] The extent of subperineurial edema was assessed by measuring total endoneurial area surrounded by perineurial cells and also determining endoneurial area after subtracting the increased subperineurial space representing edema. The latter then was subtracted from the former by the image analysis system.[22,32] Clusters of two or more small myelinated fibers enclosed by one basement membrane were designated as an instance of axonal sprouting.[19,33] Numbers of axonal sprouting were estimated as those of myelinated fibers. Cases with more than 5% of regenerating myelinated fibers (fibers which constitute axonal sprouting) in the total myelinated fibers were designated to be abundant with regenerating fibers.

For electron microscopic study, epoxy resin–embedded specimens were cut into ultrathin transverse sections and stained with uranyl acetate and lead citrate. To assess the density of unmyelinated fibers, we took electron microscopic photographs at a magnification of ×4,000 in a random fashion to cover the area of ultrathin sections as described previously.[28,30,31] Density of unmyelinated fibers was estimated from these electron micrographs. For determination of G-ratios (axon diameter/fiber diameter),[34] electron micrographs of up to 200 randomly selected fibers photographed at a magnification of ×8,000 were used. Numbers of neurofilaments were counted in systematically sampled squares of an overlying transparency placed upon the electron microscopic photographs at a final magnification of ×50,000. The first square was selected using a random table, and subsequent squares were sampled systematically. At least 30% of the axon area was counted, including subaxolemmal and central regions. At least 30 fibers were examined to calculate the mean density of neurofilaments in each case. Cases with abundant regenerating fibers were excluded for determination of the mean G-ratio and neurofilament density. Control values for G-ratio and neurofilament density were obtained from nine normal controls

A fraction of the glutaraldehyde-fixed sample was processed for teased-fiber study, in which at least 100 single fibers were isolated; their pathological condition was assessed microscopically according to criteria described previously.[28,35,36]

The second portion of the specimen was fixed in 10% formalin solution and embedded in paraffin. Sections were cut by routine methods and stained with hematoxylin and eosin as well as by the Klüver–Barrera and Masson trichrome methods.

Statistical Analyses

Quantitative data were presented as the mean ± SD and compared with previously described control values.[19,22,28,32] Statistical analyses were performed using the χ^2 test or the Mann–Whitney U test as appropriate. p values less than 0.05 were considered to indicate significance.

Results

Alcoholic Neuropathy without Thiamine Deficiency

All patients in this group, as well as in the other two groups, showed symmetric polyneuropathy with greater involvement of the lower than upper limbs. The initial symptom of neuropathy was pain or a painful burning sensation in the toes and/or ankle in all patients (see Table 2). This symptom gradually ascended to include the proximal part of the lower extremities, and occasionally to the lower trunk. In severely affected patients, the distal part of the upper limbs also were involved. Progression was mostly slow, occurring over months to years. Nineteen patients (53%) showed weakness in the lower extremities but with a sensory-dominant pattern; the remaining 17 patients (47%) showed a pure sensory pattern without any weakness in the limbs. Weakness in the upper extremities was seen in six patients (17%). Sensory disturbance was present in the lower limbs in all patients and also present in the upper limbs and trunk in 17 (47%) and 7 (19%) of patients, respectively. Almost all patients (97%) reported a painful sensation in the affected limbs and/or trunk. As for the modalities of sensation affected, loss of superficial sensation, particularly nociception, was predominant. Yet, involvement of all sensory modalities was seen in severely affected patients. Biceps, patellar, and Achilles tendon reflexes were reduced or absent in 10 patients (28%), 17 (47%), and 31 (86%),

Table 2. Neuropathic Symptoms of the Alcoholic Neuropathy and Nonalcoholic Thiamine-Deficiency Neuropathy

	Alcoholic Neuropathy			*p*		
	1. Without Thiamine Deficiency, n = 36 (%)	2. With Thiamine Deficiency, n = 28 (%)	3. Nonalcoholic Thiamine-Deficiency Neuropathy, n = 32 (%)	1 vs 2	2 vs 3	1 vs 3
Initial symptom						
Sensory disturbance	36 (100)	16 (57)	16 (50)	<0.0001	NS	<0.0001
Muscle weakness	0 (0)	12 (43)	16 (50)			
Progression						
<1 mo	2 (6)	11 (39)	18 (56)	0.0006	NS	0.001
1 mo to 1 yr	18 (50)	4 (14)	8 (25)			
>1 yr	16 (44)	13 (46)	6 (19)			
Type						
Motor-dominant	0 (0)	15 (54)	27 (84)	<0.0001	0.02	<0.0001
Sensory-dominant	19 (53)	11 (39)	3 (9)			
Pure sensory	17 (47)	2 (7)	2 (6)			
Presence of weakness						
Upper limbs	6 (17)	14 (50)	26 (81)	0.004	0.01	<0.0001
Lower limbs	19 (53)	26 (93)	30 (94)	0.0002	NS	<0.0001
Presence of sensory disturbance						
Upper limbs	17 (47)	19 (68)	25 (78)	NS	NS	0.03
Trunk	7 (19)	7 (25)	9 (28)	NS	NS	NS
Lower limbs	36 (100)	28 (100)	32 (100)	NS	NS	NS
Painful sensation	35 (97)	16 (57)	7 (22)	0.0001	0.005	<0.0001
Modality of sensory deficit						
Superficial sensation-dominant	22 (61)	7 (25)	3 (9)	0.005	NS	<0.0001
All modalities	13 (36)	15 (54)	20 (63)			
Deep sensation-dominant	1 (3)	6 (21)	9 (28)			
Deep tendon reflexes						
Biceps; reduced or absent	10 (28)	13 (46)	26 (81)	NS	0.005	<0.0001
Patellar; reduced or absent	17 (47)	23 (82)	29 (91)	0.004	NS	0.0001
Achilles; reduced or absent	31 (86)	28 (100)	32 (100)	0.04	NS	0.03
Functional status						
Unable to walk	9 (25)	15 (54)	27 (84)	0.02	0.009	<0.0001
Able to walk	27 (75)	13 (46)	5 (16)			
Modified Rankin score	2.1 ± 0.6	2.8 ± 0.9	3.6 ± 1.0	0.04	0.003	<0.0001

Patients' functional status was assessed at the peak phase according to modified Rankin score.[23] 0, asymptomatic; 1, nondisabling symptoms not interfering with lifestyle; 2, minor disability from symptoms leading to some restriction of lifestyle but not interfering with patients' capacity to look after themselves; 3, moderate disability from symptoms significantly interfering with lifestyle or preventing totally independent existence; 4, moderately severe disability from symptoms clearly precluding independent existence, though not requiring 24-hour attention from a caregiver; and 5, severe disability and total dependence, requiring constant attention day and night.

NS = not significant.

respectively. Plantar responses were flexor in all patients. Autonomic symptoms including urinary retention, constipation, impaired sweating, and orthostatic hypotension were not prominent in patients of this group. In all patients, sensory disturbance compromised activities of daily living significantly, but 27 patients (75%) could walk unaided at the time of first referral to the hospital. Functional status determined by the modified Rankin score was 2.1 ± 0.6.

Nerve conduction studies showed more profound abnormalities in the lower than upper limbs (see Table 3). Moderate reduction of compound muscle action potentials (CMAPs) in the tibial nerves and severe reduction of sensory nerve action potentials in the sural nerves were seen. In contrast, CMAPs in the median nerves were relatively preserved, and sensory nerve action potentials in the median nerves were only moderately decreased. Mild to moderate slowing of motor nerve conduction velocity in the median and tibial nerves and of sensory nerve conduction velocities in the median and sural nerves also was observed. Distal latencies in the median and tibial nerves also were mildly prolonged.

Myelinated fiber density in the sural nerve was significantly reduced (see Table 4). Densities of large myelinated fibers were 1307 ± 864 fibers/mm^2 (43% of normal control); those of small myelinated fibers were 1381 ± 1278 fibers/mm^2 (27% of normal control). In

Table 3. Nerve Conduction Studies, Mean ± SD

	Alcoholic Neuropathy						
	1. Without Thiamine Deficiency, n = 36	2. With Thiamine Deficiency, n = 28	3. Nonalcoholic Thiamine-Deficiency Neuropathy, n = 32	*p* 1 vs 2	*p* 2 vs 3	*p* 1 vs 3	Controls (n = 121 ~ 191)
Median nerve							
MCV (m/sec)	51.2 ± 4.7[a]	50.4 ± 5.2[a]	52.3 ± 5.9[a]	NS	NS	NS	57.8 ± 3.7
DL (msec)	4.2 ± 0.7[a]	4.0 ± 0.6[a]	3.6 ± 0.6	NS	0.02	0.005	3.4 ± 0.4
CMAP (mV)	8.4 ± 4.2[b]	8.7 ± 5.0[a]	6.7 ± 4.5[a]	NS	NS	NS	10.7 ± 3.5
Not elicited	None	None	None				
SCV (m/sec)	48.0 ± 7.2[a]	46.9 ± 8.4[a]	47.9 ± 9.3[a]	NS	NS	NS	57.8 ± 4.7
SNAP (μV)	8.7 ± 6.4[a]	6.8 ± 4.9[a]	7.8 ± 8.4[a]	NS	NS	NS	23.5 ± 8.4
Not elicited	2 cases (6%)	1 case (4%)	8 cases (25%)				
Tibial nerve							
MCV (m/sec)	40.6 ± 4.2[a]	40.8 ± 5.6[a]	42.8 ± 4.5[a]	NS	NS	NS	46.9 ± 3.5
DL (msec)	5.3 ± 0.8[a]	5.3 ± 1.8[c]	4.9 ± 1.0[c]	NS	NS	NS	4.5 ± 0.8
CMAPs (mV)	4.6 ± 3.5[a]	5.4 ± 5.1[a]	2.6 ± 2.4[a]	NS	0.045	0.002	10.9 ± 3.8
Not elicited	None	3 cases (11%)	3 cases (10%)				
Sural nerve							
SCV (m/sec)	38.8 ± 6.9[a]	40.5 ± 6.3[a]	41.0 ± 11.8[c]	NS	NS	NS	51.0 ± 5.1
SNAP (μV)	2.4 ± 3.3[a]	1.5 ± 2.3[a]	2.0 ± 3.5[a]	NS	NS	NS	11.5 ± 4.7
Not elicited	14 cases (39%)	17 cases (61%)	21 cases (66%)				

[a] $p < 0.001$, [b] $p < 0.005$, and [c] $p < 0.05$ (Mann–Whitney *U* test) for the control values.

SD = standard deviation; MCV = motor nerve conduction velocity; DL = distal latency; CMAP = compound muscle action potential; SCV = sensory nerve conduction velocity; SNAP = sensory nerve action potential; NS = not significant.
Control values are based on previously published reports.[19,22]

Table 4. Pathology of the Sural Nerves, Mean ± SD

	Alcoholic Neuropathy						
	1. Without Thiamine Deficiency, n = 29	2. With Thiamine Deficiency, n = 18	3. Nonalcoholic Thiamine-Deficiency Neuropathy, n = 19	*p* 1 vs 2	*p* 2 vs 3	*p* 1 vs 3	Controls (n = 9)
Total MFD (no./mm^2)	2,687 ± 1,875[a]	2,727 ± 1,649[a]	2,367 ± 1,868[a]	NS	NS	NS	8,190 ± 511
Large MFD (no./mm^2)	1,307 ± 864[a]	928 ± 764[a]	663 ± 693[a]	NS	NS	0.005	3,068 ± 294
Small MFD (no./mm^2)	1,381 ± 1,278[a]	1,800 ± 1,245[a]	1,704 ± 1,310[a]	NS	NS	NS	5,122 ± 438
Axonal sprouting (no./mm^2)	137.6 ± 264.8	157.3 ± 271.4	2.8 ± 9.5	NS	0.01	0.046	
Small/large							
All cases	1.8 ± 2.9 (n = 29)	4.4 ± 8.0 (n = 18)	13.6 ± 27.0 (n = 19)	NS	0.02	<0.0001	1.7 ± 0.2
Cases with abundant RMFs are excluded	0.7 ± 0.3[a] (n = 20)	4.7 ± 9.6 (n = 12)	13.6 ± 27.0 (n = 19)	0.03	0.02	<0.0001	1.7 ± 0.2
G-ratio	0.58 ± 0.07[a]	0.54 ± 0.06[a]	0.56 ± 0.06[a]	NS	NS	NS	0.73 ± 0.03
Neurofilamant density (no./μm^2)	187.0 ± 44.1[a]	188.2 ± 41.2[a]	193.5 ± 43.8[a]	NS	NS	NS	108.3 ± 25.9
UMFD (no./mm^2)	7,029 ± 4,153[a]	11,194 ± 4,386[a]	10,585 ± 4,867[a]	0.004	NS	0.006	29,913 ± 3,457
Subperineurial edema (%)	6.6 ± 2.9[b]	7.3 ± 4.2[c]	10.3 ± 4.5[a]	NS	0.02	0.002	4.6 ± 1.0
Teased fiber study							
Myelin irregularity (%)	17.3 ± 11.3	9.2 ± 7.7	5.7 ± 5.2	0.01	NS	0.0003	
De/remyelination (%)	9.0 ± 5.2	10.3 ± 8.2	3.4 ± 4.8	NS	0.003	0.001	9.5 ± 8.8
Axonal degeneration (%)	30.9 ± 19.7[a]	45.3 ± 30.7[a]	57.8 ± 25.3[a]	NS	NS	0.001	1.7 ± 1.4

[a] $p < 0.001$, [b] $p < 0.005$, and [c] $p < 0.05$ (Mann–Whitney *U* test) for the control values.

SD = standard deviation; MFD = myelinated fiber density; small/large = ratio of small myelinated fibers to large myelinated fibers; UMFD = unmyelinated fiber density; RMFs = regenerating myelinated fibers, NS = not significant.
Cases with abundant regenerating fibers were excluded for determination of the mean *G*-ratio and neurofilament density.
Control values are based on previously published reports,[28,32] and control values for *G*-ratio and neurofilament density are obtained from nine normal volunteers.

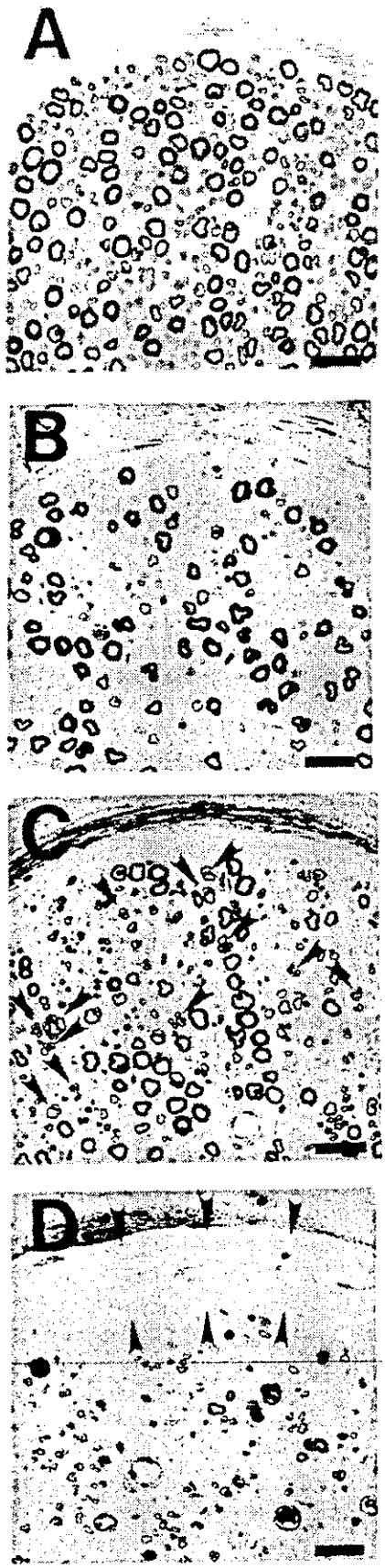
nine cases (31%) axonal sprouting was abundant (more than 5% of regenerating fibers in the total myelinated fibers; see Fig 1C). The duration of neuropathic symptoms of these nine cases was extremely long (70.5 ± 49.5 months), and the regenerating myelinated fibers increased the proportion of small myelinated fibers (small/large, 4.3 ± 4.4; control, 1.7 ± 0.2). In the remaining 20 cases (69%), duration of neuropathic symptoms was much shorter (9.7 ± 11.6 months). Reduction of small myelinated fibers in these 20 cases was more profound than reduction of large myelinated fibers (small/large, 0.7 ± 0.3), hence small-fiber-predominant loss was clearly evident (see Figs 1B and 2). Axonal shrinkage with increased neurofilament density accompanied by a redundant loop of myelin was observed in some myelinated fibers. Decreased G-ratio (0.58 ± 0.07; control, 0.73 ± 0.03) and increased neurofilament density (187.0 ± 44.1 filaments/μm^2; control, 108.3 ± 25.9) indicated axonal atrophy. Reduction of unmyelinated fiber density also was profound (7029 ± 4153 fibers/mm^2). Clusters of small unmyelinated fibers, suggestive of regenerating fibers, were seen in cases with abundant axonal sprouting of myelinated fibers. Varying degrees of subperineurial edema was seen. In teased-fiber preparations, the frequency of axonal degeneration was prominent (30.9 ± 19.7%), and myelin irregularity was conspicuous in the remaining fibers (17.3 ± 11.3%). The proportion of segmental de/remyelination was 9.0 ± 5.2%. This demyelination consisted of widening of consecutive nodes of Ranvier resulting from attenuation of the internodes of the myelin sheath (see Fig 3).

Nonalcoholic Thiamine Deficiency Neuropathy

All patients manifested symmetric polyneuropathy with more involvement in the lower than upper limbs, showing a centripetal pattern of progression. The initial symptom of neuropathy was variable, in contrast with alcoholic neuropathy without thiamine deficiency (ALN); this was weakness in the lower extremities in 16 patients (50%) and numbness in the distal lower limbs in 16 patients (50%; see Table 2). Progression rate also varied; acute progression within 1 month was seen in 18 patients (56%), whereas slow progression over more than 1 year was seen in 6 patients (19%). On average, progression was more rapid than in ALN ($p = 0.001$). Impairment usually was motor dominant, as in 27 patients (84%), which contrasted to the sensory-dominant pattern characteristic of ALN. Some patients whose motor weakness progressed over days initially were thought to have Guillain–Barré syndrome. Motor symptoms were more predominant in the lower than upper extremities; even so, 26 patients (81%) showed weakness in the upper limbs. Sensory disturbance was present in the lower limbs in all patients and also was present in the upper limbs and trunk in 25 patients (78%) and 9 patients (28%), respectively. Varying degrees of numbness with or without painful sensations were noted in all patients, but painful sensations were reported by only 7 patients (22%). Involvement of all sensory modalities was a common feature in TDN, in contrast with predominant affection of nociception in ALN; superficial sensation was most affected in only 3 patients (9%), deep sensation was most involved in 9 patients (28%), and both modalities were equally affected in 20 patients (63%). Biceps, patellar, and Achilles tendon reflexes were reduced or absent in most patients. Plantar responses were flexor in all patients. Autonomic symptoms were absent or only mildly present in most patients, but six patients who had severe thiamine deficiency manifested flaccid bladder requiring urethral catheterization or severe intestinal gas retention that mimicked ileus. Activities of daily living were significantly more impaired than in ALN ($p < 0.0001$) mainly because of rapid progression of muscle weakness. Only five patients (16%) could walk unaided at the time of initial examination. Functional status determined by modified Rankin score was 3.6 ± 1.0.

Findings of nerve conduction studies were similar to those in ALN (see Table 3). Only distal latency of the median nerve was significantly prolonged ($p = 0.005$), whereas CMAPs in the tibial nerves were significantly smaller ($p = 0.002$) in ALN than in TDN.

Myelinated fiber density was significantly reduced (see Table 4). Reduction of large meylinated fibers was greater than in ALN ($p = 0.005$). Densities of large myelinated fibers were 663 ± 693 fibers/mm^2 (22% of normal control), whereas those of small myelinated fibers were 1704 ± 1310 fibers/mm^2 (33% of normal control). The mean ratio of small to large myelinated fibers was 13.6 ± 27.0 (control, 1.7 ± 0.2), significantly higher than in ALN ($p < 0.0001$). Axonal sprouting was scarce in all cases. In contrast with ALN, all cases showed more loss of large myelinated fibers than loss of small mylinated fibers except one (see Figs 1D and 2). The mean G-ratio was 0.56 ± 0.06 (control, 0.73 ± 0.03) and the density of neurofilaments was 193.5 ± 43.8 filaments/μm^2 (control, 108.3 ± 25.9), not differing significantly from those in ALN. Reduction of unmyelinated fibers also was seen but was less profound than in ALN ($p = 0.006$). Regeneration of unmyelinated fibers was scarce. Subperineurial edema was more severe than in ALN ($p = 0.002$). In teased-fiber preparations, significantly more axonal degeneration was seen than in ALN ($p = 0.001$). The proportion of fibers showing segmental de/remyelination was small compared to ALN ($p = 0.001$). Myelin irregularity was observed in 5.7 ± 5.2% of fibers, significantly less often than in ALN ($p = 0.0003$).



Alcoholic Neuropathy with Thiamine Deficiency

Neuropathic symptoms in alcoholic neuropathy with thiamine deficiency (ALN-TD) were variable, showing characteristics of both ALN and TDN (see Table 2). The initial symptom was numbness or painful paresthesias in the lower limbs in 16 patients (57%) but was weakness in 12 others (43%). Progression varied from acute (within 1 month) in 11 patients (39%) to chronic (occurring over 1 year) in 13 (46%). Relative degrees of motor and sensory deficits also were variable; 15 patients (54%) showed a motor-dominant pattern, whereas the remaining 13 (46%) showed a sensory-dominant or purely sensory pattern. Muscle weakness was present in the lower limbs in 26 patients (93%) and in the upper limbs in 14 (50%). Sensory disturbance was present in the lower extremities in all patients extending to the trunk and distal portion of the upper extremities in 7 patients (25%) and 19 patients (68%), respectively. Painful paresthesias were reported by 16 patients (57%), significantly less often than in ALN ($p = 0.005$). Modaliy of sensory deficit also was variable; superficial and deep sensations were affected equally in 15 patients (54%), deep sensation predominated slightly in 6 (21%), and predominant involvement of nociception associated with painful paresthesias as in ALN was seen in 7 (25%). Deep tendon reflexes were reduced in the biceps, patellar, and Achilles tendons in 13 patients (46%), 23 (82%), and 28 (100%), respectively. Plantar responses were flexor in all patients. The modified Rankin score was 2.8 ± 0.9, intermediate between ALN and TDN scores.

Findings of nerve conduction studies were similar to those in ALN and TDN (see Table 3).

Sural nerve biopsy specimen findings also were variable occupying a range between ALN and TDN (see Table 4). Densities of large myelinated fibers were 928 ± 764 fibers/mm^2 (30% of normal control) and those of small myelinated fibers were 1,800 ± 1,245 fibers/mm^2 (35% of normal control). The mean ratio of small to large myelinated fibers was 4.4 ± 8.0,

Fig 1. Light microscopic observations of the sural nerve. (A) Transverse section of a sural nerve specimen from a control case. (B) A specimen from a patient with a 4-month history of alcoholic neuropathy without thiamine deficiency. Small myelinated fibers show more loss than large myelinated fibers. Subperineurial edema is slight. (C) A specimen from a patient with a 5-year history of alcoholic neuropathy without thiamine deficiency does not show small-fiber-predominant axon loss as in B, because of the presence of abundant axonal sprouting (arrowheads), *which indicates regeneration of axons. (D) A specimen from a patient with nonalcoholic thiamine-deficiency neuropathy. In contrast with B, large myelinated fibers show more loss than small myelinated fibers, although both types of myelinated fibers are significantly reduced. Subperineurial edema is marked (between* arrowheads). *Bar = 30μm.*

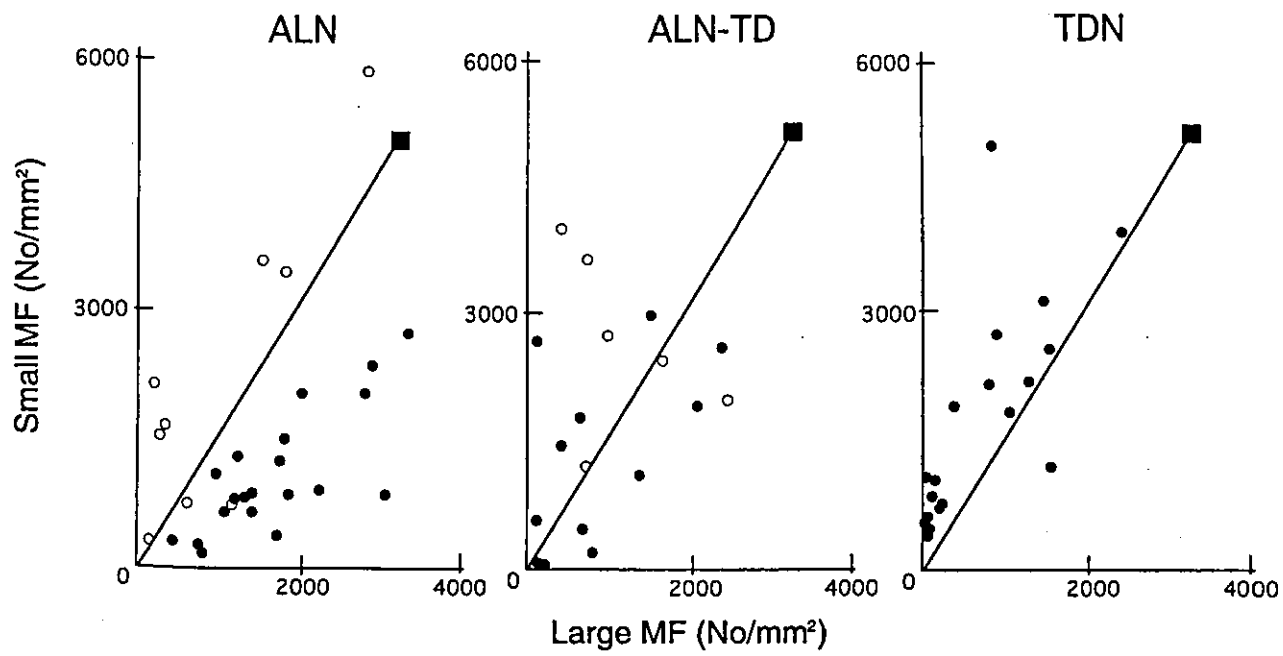


Fig 2. Relationships between large and small myelinated fibers. Boldface lines represent the normal ratio of small to large myelinated fibers (small/large = 1.7). Black boxes indicate the mean value in control cases. Circles located below the boldface lines indicate predominance of large myelinated fibers, whereas those above the boldface lines indicate predominance of small myelinated fibers. Black circles represent cases with few regenerating myelinated fibers (<5% of total myelinated fibers), whereas white circles represent cases with abundant regenerating myelinated fibers (> 5% of total myelinated fibers). Abundant regeneration of myelinated fibers increase the number of small myelinated fibers. In the pure alcoholic neuropathy (ALN) group, all cases with few regenerating fibers showed predominance of large myelinated fibers, reflecting predominantly small-fiber loss; all except one nonalcoholic thiamine-deficiency neuropathy (TDN) cases showed predominance of small myelinated fibers, reflecting predominantly large-fiber loss.

which was intermediate between ratios in ALN and TDN. Axonal sprouting was abundant in six cases (33%). The proportions of large and small myelinated fibers were highly variable between cases (see Fig 2). The mean G-ratio was 0.54 ± 0.06 and the density of neurofilaments was 188.2 ± 41.2 filaments/μm^2, not differing significantly from those in ALN or TDN. In teased-fiber preparations, the frequency of axonal degeneration was 45.3 ± 30.7%. Myelin irregularity was conspicuous in the remaining fibers (9.2 ± 7.7%). The proportion of segmental de/re-myelination was 10.3 ± 8.2%. Values for the teased-fiber preparations were intermediate between the range of ALN and TDN.

Discussion

The pathogenesis of alcoholic neuropathy, especially its relationship to thiamine deficiency, has remained unclear. Recent studies indicated a direct neurotoxic effect of ethanol or its metabolites, involving ethanol-induced glutamate neruotoxicity,[18,37] decreased production of neurofilament protein or its phosphorylated form,[38,39] or impairment of fast axonal transport.[40] Axonal degeneration has been documented in animals receiving ethanol while maintaining normal thiamine status.[41] Human studies also have suggested a direct toxic effect, because a dose-dependent relationship has been observed between severity of neuropathy and amount of ethanol consumed.[17] In addition to this direct toxic effect, thiamine deficiency is closely related to chronic alcoholism[4] and also can induce neuropathy in alcoholic patients. Ethanol diminishes thiamine absorption in the intestine and reduces hepatic stores of thiamine.[42,43] Ethanol also decreases phosphorylation of thiamine, reducing availability of the active form of the vitamin.[44–46] In addition, patients with chronic alcoholism tend to have dietary imbalance. These relationships make chronic alcoholism a risk factor for thiamine-deficiency neuropathy.

Clinicopathological features of alcoholic neuropathy have remained obscure despite its wide prevalence, in large part because of incomplete differentiation from beriberi neuropathy. Although sometimes attributed to inadequate nutritional assessment in reported cases, technical limitations of thiamine status assessment contributed greatly to the problem. Only in the 1960s were assays of erythrocyte transketolase activity intro-

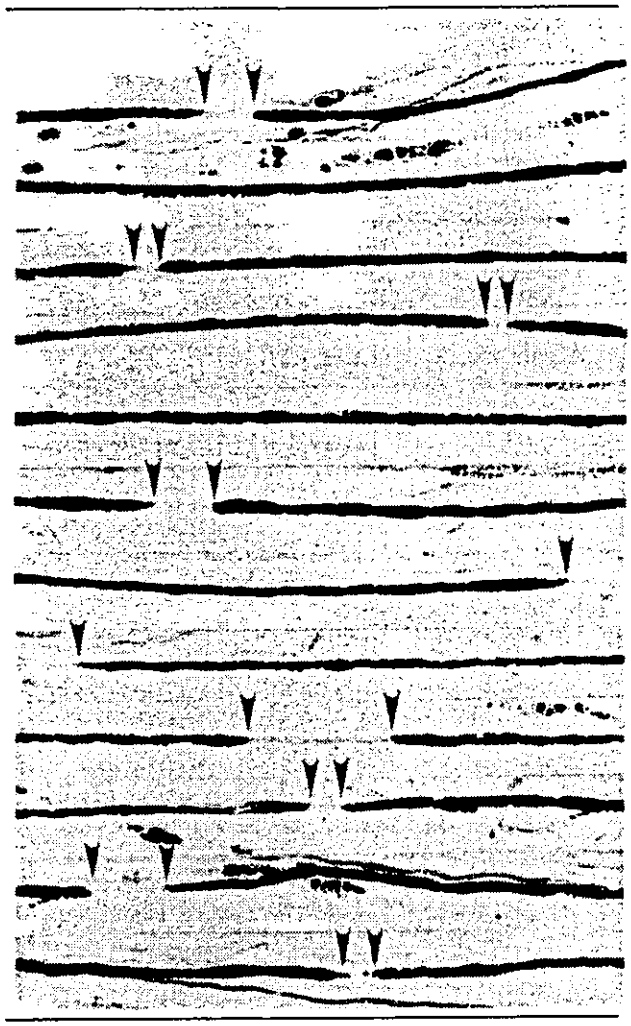


Fig 3. Consecutive portions along the length of a single teased fiber from a patient with alcoholic neuropathy. Note marked irregularity of myelin. Segmental demyelination has resulted from widening of consecutive nodes of Ranvier (arrowheads).

duced,[47] and this method assesses thiamine only indirectly. In the 1980s, a direct, highly sensitive, and convenient high-performance liquid chromatographic assay for thiamine became widely available.[13–15] When we assess clinicopathological features of "alcoholic neuropathy," both direct toxicity of ethanol or its metabolites and concomitant effects of thiamine deficiency need to be considered. In addition, clinicopathological features of pure thiamine-deficiency neuropathy need to be studied in nondrinkers.

In this study, we divided consecutively recruited patients with "alcoholic neuropathy" into two groups based on thiamine status. Alcoholic neuropathy without thiamine deficiency (ALN) was considered to be attributable solely to direct toxicity of ethanol or ethanol metabolites. Characteristics of ALN-TD were considered to reflect combined cause of ethanol toxicity and thiamine deficiency. On the other hand, patients with nonalcoholic (ie, "pure") thiamine-deficiency neuropathy (TDN), identical to previously reported "beriberi neuropathy," were assessed for comparison. We thus were able to differentiate the distinct clinicopathological features of the individual neuropathies and confirm that effects of thiamine deficiency can modify the features of ALN.

As for clinical features, ALN in our series uniformly showed slowly progressive, sensory-dominant symptoms. Painful paresthesias were major complaints and that often limited daily activities in these patients. In pure TDN, in contrast, many patients manifested an acutely progressive, motor-dominant pattern leading to loss of ambulation, although variation including slow progression or sensory-dominant pattern was apparent in some patients. Clinical features of ALN-TD were particularly variable, constituting a spectrum raging from a picture of ALN to that of TDN among individual patients.

Major electrophysiological and histopathological findings in our three groups of patients indicated axonal neuropathy, in agreement with previous descriptions of both alcoholic neuropathy and beriberi neuropathy.[6–11] The electrophysiological features that we observed were similar in ALN and TDN. Like the clinical findings, these studies showed predominant lower limb involvement in both neuropathies. Lower amplitude of CMAPs in TDN than in ALN were reflected by more severe muscle weakness in TDN. Electrophysiological findings commonly associated with myelin damage (slowing of MCV and SCV as well as prolongation of DL) also were observed in patients with ALN and those with TDN, even though axonal damage was dominant histopathologically.

Sural nerve specimens showed more clear-cut differences between these neuropathies than did electrophysiological studies. ALN showed loss of mainly small fibers, less subperineurial edema, and more frequent myelin irregularity and segmental de/remyelination. In contrast, TDN showed predominantly large-fiber loss, more subperineurial edema, and less myelin irregularity and segmental de/remyelination. In ALN, small-fiber-predominant axonal loss (small-fiber axonopathy) was most evident in cases with recent onset. In long-standing cases, abundant regenerating fibers obscured some small-fiber loss. The finding of small-fiber-predominant loss was in accord with previous descriptions of painful alcoholic neuropathy.[19] Relative preservation of deep tendon reflexes in ALN reflected relative sparing of the large fibers that mediate them. ALN-TD showed an extensive range of pathology from small-fiber-predominant loss to large-fiber-predominant loss with features of both ALN and TDN. Axonal sprouting in long-standing cases and large-fiber-predominant loss in some ALN-TD cases may have

obscured the characteristic feature of small-fiber-predominant loss in ALN in some previous reports describing loss of nerve fibers throughout the entire range of fiber diameter.[7,8,10] Axonal atrophy as determined by the G-ratio and the density of neurofilaments did not differ between the three groups, but irregularity of myelin, which also indicates axonal atrophy,[48] was significantly more frequent in ALN than in TDN. Segmental de/remyelination in teased-fiber preparations also was more frequent in ALN than in TDN. This change is consisted of the widening of consecutive nodes of Ranvier. Irregularity of the myelin sheath also was seen in these fibers; so these findings are supposed to reflect axonal atrophy.[48,49] Differences in relative frequency of these changes are supposedly caused by different mechanism of axonal atrophy in ALN from TDN.

Another important characteristic of "alcoholic neuropathy" is a presence of postgastrectomy patients; 36% of ALN-TD patients but no ALN patients. This finding suggests that gastrectomy is a risk factor for thiamine deficiency in patients with chronic alcoholism and establishes thiamine deficiency as a cause of postgastrectomy polyneuropathy.[19]

In conclusion, the nature of "alcoholic neuropathy" has been unclear because of an often undetected or overestimated influence of thiamine deficiency, whereas the clinical picture of thiamine-deficiency neuropathy (ie, beriberi neuropathy) can be distorted by concomitant effects of ethanol. We compared these two neuropathies with careful consideration of interactions, confirming that the two neuropathies are clinically and pathologically distinct. Not only thiamine deficiency but also direct toxic effects of ethanol or its metabolites can cause alcoholic neuropathy. Although clinicopathological features of pure alcoholic neuropathy are remarkably uniform, extensive variation results when thiamine deficiency is present.

This study was supported by grants from the Ministry of Health and Welfare of Japan (G.S.).

We thank Drs Y. Takeuchi, M. Hirayama, and S. Mitake for the provision of clinical data.

References

1. Shattuck GC. Relation of beriberi to polyneuritis from other causes. Am J Trop Med 1928;8:539–543.
2. Victor M, Adams RD. On the etiology of the alcoholic neurologic diseases. Am J Clin Nutr 1961;9:379–397.
3. Novak DJ, Victor M. The vagus and sympathetic nerves in alcoholic polyneuropathy. Arch Neurol 1974;30:273–284.
4. Darnton-Hill I, Truswell AS. Thiamine status of a sample of homeless clinic attenders in Sydney. Med J Aust 1990;152:5–9.
5. Pekelharing CA, Wrinkler C. Mittheilung über die Beri-Beri. Dtsch Med Wochenschr 1887;13:845–848.
6. Takahashi K. Thiamine deficiency neuropathy, a reappraisal. Int J Neurol 1981;15:245–253.
7. Walsh JC, McLeod JG. Alcoholic neuropathy; an electrophysiological and histopathylogical study. J Neurol Sci 1970;10: 457–469.
8. Tredici G, Minazzi M. Alcoholic neuropathy; an electronmicroscopic study. J Neurol Sci 1975;25:333–346.
9. Takahashi K, Nakamura H. Axonal degeneration in beriberi neuropathy. Arch Neurol 1976;33:836–841.
10. Behse F, Buchthal F. Alcoholic neuropathy: clinical, electrophysiological, and biopsy findings. Ann Neurol 1977;2:95–110.
11. Ohnishi A, Tsuji S, Igisu H, et al. Beriberi neuropathy: morphometric study of sural nerve. J Neurol Sci 1980;45:177–190.
12. Denny-Brown DE. The neurological aspects of thiamine deficiency. Fed Proc 1958;17(suppl 2):35–39.
13. Kimura M, Fujita T, Itokawa Y. Liquid-chromatographic determination of the total thiamine content of blood. Clin Chem 1982;28:29–31.
14. Warnoch LG. The measurement of erythrocyte thiamine pyrophosphate by high- performance liquid chromatography. Anal Biochem 1982;126:394–397.
15. Froridi A, Pupita M, Palmerini CA, et al. Thiamine pyrophosphate determination in whole blood and erythrocyts by high performance liquid chromatography. Int J Vitam Nutr Res 1984;54:165–167.
16. Claus D, Eggers R, Engelhardt A, et al. Ethanol and polyneuropathy. Acta Neurol Scand 1985;72:312–316.
17. Monforte R, Estruch R, Valls-Solé J, et al. Autonomic and peripheral neuropathies in patients with chronic alcoholism; a dose-related toxic effect of alcohol. Arch Neurol 1995;52: 45–51.
18. Ikonomidou C, Bittigau P, Ishimaru MJ, et al. Ethanol-induced apoptotic neurodegeneration and fetal alcohol syndrome. Science 2000;287:1056–1060.
19. Koike H, Mori K, Misu K, et al. Painful alcoholic polyneuropathy with predominant small-fiber loss and normal thiamine status. Neurology 2001;56:1727–1732.
20. Markkanen T. Metabolic disturbance after gastro-oesophageal resection. Int J Vitam Nutr Res 1973;43:549–554.
21. Shimomura T, Mori E, Hirono N, et al. Development of Wernicke-Korsakoff syndrome after long intervals following gastrectomy. Arch Neurol 1998;55:1242–1245.
22. Koike H, Misu K, Hattori N, et al. Postgastrectomy polyneuropathy with thiamine deficiency. J Neurol Neurosurg Psychiatry 2001;71:357–362.
23. van Swieten JC, Koudstaal PJ, Visser MC, et al. Interobserver agreement for the assessment of handicap in stroke patients. Stroke 1988;19:604–607.
24. Herve C, Beyne P, Lettéron P, et al. Comparison of erythrocyte transketolase activity with thiamine and thiamine phosphate ester levels in chronic alcoholic patients. Clin Chim Acta 1995; 234:91–100.
25. Kimura J. Principles of nerve conduction studies. In: Kimura J, ed. Electrodiagnosis in diseases of nerve and muscle: principles and practice. 2nd ed. Philadelphia: F.A, Davis, 1989:78–102.
26. Kimura J. Assessment of individual nerves. In: Kimura J, ed. Electrodiagnosis in diseases of nerve and muscle: principles and practice. 2nd ed. Philadelphia: F.A, Davis, 1989:103–138.
27. Sobue G, Yasuda T, Mitsuma T, et al. Expression of nerve growth factor receptor in human peripheral neuropathies. Ann Neurol 1988;24:64–72.
28. Sobue G, Hashizume Y, Mukai E, et al. X-linked recessive bulbospinal neuronopathy: a clinicopathological study. Brain 1989; 112:209–232.
29. Sobue G, Nakao N, Murakami K, et al. Type I familial amyloid polyneuropathy. A pathological study of the peripheral nervous system. Brain 1990;113:903–919.

30. Hattori N, Ichimura M, Nagamatsu M, et al. Clinicopathological features of Churg- Strauss syndrome-associated neuropathy. Brain 1999;122:427–439.
31. Misu K, Hattori N, Nagamatsu M, et al. Late-onset familial amyloid polyneuropathy type I (transthyretin Met 30-associated familial amyloid polyneuropathy) unrelated to endemic focus in Japan; clinicopathological and genetic features. Brain 1999;122: 1951–1962.
32. Nagamatsu M, Terao S, Misu K, et al. Axonal and perikaryal involvement in chronic inflammatory demyelinating polyneuropathy. J Neurol Neurosurg Psychiatry 1999;66:727–733.
33. Vital A, Ferrer X, Lagueny A, et al. Histopathological features of X-linked Charcot-Marie-Tooth disease in 8 patients form 6 families with different connexin32 mutations. J Peripher Nerv Syst 2001;6:79–84.
34. Llewelyn JG, Gilbey SG, Thomas PK, et al. Sural nerve morphometry in diabetic autonomic and painful sensory neuropathy. Brain 1991;114:867–892.
35. Sobue G, Li M, Terao S, et al. Axonal pathology in Japanese Guillain-Barré syndrome; a study of 15 autopsied cases. Neurology 1997;48:1694–1700.
36. Dyck PJ, Giannini C, Lais A. Pathologic alterations of nerves. In: Dyck PJ, Thomas PK, Griffin JW, Low PA, Poduslo JF, eds. Peripheral neuropathy. 3rd ed. Philadelphia: Saunders.: 1993:514–595.
37. Ikonomidou C, Bosch F, Miksa M, et al. Blockade of NMDA receptors and apoptotic neurodegeneration in the developing brain. Science 1999;283:70–74.
38. Guru SC, Shetty KT, Shankar SK. Effect of chronic ethanol ingestion on phosphate content of neurofilament proteins and neurofilament associated protein phosphatase in rat spinal cord. Neurochem Res 1991;16:1193–1197.
39. Saunders DE, DiCerbo JA, Williams JR, et al. Alcohol reduces neurofilament protein levels in primary cultured hippacampal neurons. Alcohol 1997;14:519–526.
40. McLane JA. Decreased axonal transport in rat nerve following acute and chronic ethanol exposure. Alcohol 1987;4:385–389.
41. Bosch EP, Pelham RW, Rasool CG, et al. Animal models of alcoholic neuropathy: morphologic, electrophysiologic, and biochemical findings. Muscle Nerve 1979;2:133–144.
42. Hoyumpa AM Jr, Breen KJ, Schenker S, et al. Thiamine transport across the rat intestine. II. Effect of ethanol. J Lab Clin Med 1975:803–816.
43. Dancy M, Evans G, Gaitonde MK, et al. Blood thiamine and thaimine phosphate ester concentrations in alcoholic and non-alcoholic liver diseases. Brit Med J 1984;289:79–82.
44. Hoyumpa AM Jr. Mechanism of thiamine deficiency in chronic alcoholism. Am J Clin Nutr 1980;33:2750–2761.
45. Paladin F, Russo Perez G. The haepatic thiamine level in the course of alcoholic neuropathy. Eur Neurol 1987;26:129–133.
46. Poupon RE, Gervaise G, Riant P, et al. Blood thiamine and thiamine phosphate concentrations in excessive drinkers with or without peripheral neuropathy. Alcohol Alcoholism 1990;25: 605–611.
47. Dreyfus PM. Clinical application of blood transketolase determinations. N Eng J Med 1962;267:596–598.
48. Dyck PJ, Lais AC, Karnes JL, et al. Permanent axonotomy, a model of axonal atrophy and secondary segmental demyelination and remyelination. Ann Neurol 1981;9:575–583.
49. Ohi T, Kyle RA, Dyck PJ. Axonal attenuation and secondary segmental demyelination in myeloma neuropathies. Ann Neurol 1985;17:255–261.

American Journal of Pathology, Vol. 163, No. 2, August 2003

Dorfin Localizes to the Ubiquitylated Inclusions in Parkinson's Disease, Dementia with Lewy Bodies, Multiple System Atrophy, and Amyotrophic Lateral Sclerosis

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In many neurodegenerative diseases, the cytopathological hallmark is the presence of ubiquitylated inclusions consisting of insoluble protein aggregates. Lewy bodies in Parkinson's disease and dementia with Lewy bodies disease, glial cell inclusions in multiple system atrophy, and hyaline inclusions in amyotrophic lateral sclerosis (ALS) are representative of these inclusions. The elucidation of the components of these inclusions and the mechanisms underlying inclusion formation is important in uncovering the pathogenesis of these disorders. We hypothesized that Dorfin, a perinuclearly located E3 ubiquitin ligase, participates in the formation of ubiquitylated inclusions in a wide range of neurodegenerative diseases. Here, we report that affinity-purified anti-Dorfin antibody labeled ubiquitylated inclusions of Parkinson's disease, dementia with Lewy bodies disease, multiple system atrophy, and sporadic and familial ALS. A double-immunofluorescence study revealed that Dorfin shows a distribution pattern parallel to that of ubiquitin. Furthermore, by a filter trap assay, we detected that Dorfin is present in the ubiquitylated high-molecular weight structures derived from these diseases. These results suggest that Dorfin plays a crucial role in the formation of ubiquitylated inclusions of α-synucleinopathy and ALS. However, because we failed to show the direct binding of α-synuclein with Dorfin, future investigations into the binding partner(s) of Dorfin will be needed to deepen our understanding of the pathophysiology of α-synucleinopathy and ALS. *(Am J Pathol 2003, 163:609–619)*

Protein aggregates are formed when the cell fails to further degrade misfolded or mutated proteins. Protein aggregates are generally difficult to unfold or degrade; their formation in cells is related to the pathogenesis of several common aging-related neurodegenerative diseases including Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), polyglutamine disease (Huntington's disease and spinocerebellar ataxias resulting from an expanded CAG repeat in their causative gene), and Alzheimer's disease.[1,2] These group of disorders are called conformational diseases, in which the underlying protein aggregation results from β-sheet linkages.[1] Furthermore, the characteristic intracellular inclusions composed of aggregated ubiquitylated protein surrounded by disorganized filaments are the common histopathological hallmark of many neurodegenerative diseases.[3] Lewy bodies (LBs) in PD and dementia with Lewy bodies (DLB), glial cell inclusions (GCIs) in multiple system atrophy (MSA), and hyaline and skein-like inclusions in ALS are representative of such inclusions.[4–8] To elucidate the mechanisms underlying inclusion body formation and neurodegeneration, it is important to know which protein components are involved.

We have reported previously that Dorfin is predominantly localized in neuronal hyaline inclusions found in familial ALS with *SOD1* mutation and in *SOD1*^{G93A}-transgenic mice.[9] Dorfin is a gene product we cloned from the anterior horn tissues of the human spinal cord.[10] Its mRNA is ubiquitously expressed through the central nervous system, including the spinal cord. Dorfin contains a RING-finger/IBR (in-between ring-finger) domain[11–13] at its N-terminus and mediates E3 ubiquitin (Ub) ligase activity.[10] Dorfin physically binds and ubiquitylates various SOD1 mutants derived from familial ALS patients and enhances their degradation, but it has no effect on the stability of wild-type SOD1.[9] Overexpression of Dorfin protects neural cells against the toxic effects of mutant SOD1 and reduces SOD1 inclusions.[9] Our previous results indicate that Dorfin protects neurons by recognizing

Supported by grants from the Ministry of Education, Culture, Sports, Science, and Technology, and from the Ministry of Health, Labor, and Welfare of Japan.

Accepted for publication April 24, 2003.

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and then ubiquitylating mutant SOD1 proteins, subsequently targeting them for proteasomal degradation.

Mutant SOD1 protein is fairly unstable compared to its wild-type, and toxic gain of function is thought to be related to this unstable conformation.[14,15] Recently, CHIP (carboxyl terminus of Hsc70-interacting protein), U-box type E3, has been shown to interact with Hsp90 or Hsp70 and to ubiquitylate unfolded proteins trapped by these molecular chaperones, thus acting as a quality control E3.[16–18] The physiological role of Dorfin remains unknown, but it may be regarded as another quality control E3 because it can discriminate between the normal and abnormal status of SOD1 proteins.[9] In cultured cells, Dorfin resides in the perinuclear region and forms aggresome-like structures.[10] Aggresomes are perinuclear cytoplasmic inclusions containing misfolded ubiquitylated proteins that appear when the cell fails to further degrade such proteins.[19,20] Thus, an important and interesting question in this context is whether Dorfin plays a role in neurodegenerative diseases with cytosolic ubiquitylated inclusions other than familial ALS with SOD1 mutations through ubiquitylation of target proteins. To address this question, using immunohistochemical analysis of Dorfin, we examined various neurodegenerative diseases with ubiquitylated inclusion bodies, including α-synucleinopathy (sporadic PD, DLB, and MSA) as well as motor neuron disease (sporadic and familial ALS). We here report that Dorfin co-localizes to the ubiquitylated inclusion bodies in these neurodegenerative diseases, and we suggest that Dorfin plays an important role in the disease process.

Materials and Methods

Tissue Samples

The participants of this study were five PD patients (age, 67 to 79 years; four men and one women), five cases of DLB (age, 65 to 78 years; four men and one women), five with MSA (age, 60 to 72 years; three men and two women), two men with sporadic ALS (SALS) (age, 68 and 69 years), one man with familial ALS (FALS) (57 years), and five controls without neurological disease (C; age, 61 to 78 years; four men and one women). Diagnoses of all cases were confirmed by clinical and pathological diagnostic criteria for each disease.[21–23] The brain and spinal cord were removed at autopsy performed 4 to 12 hours postmortem. The midbrain for PD, cerebral cortex of the temporal lobe for DLB, putamen and midbrain for MSA, and the spinal cords for SALS and FALS were excised and subjected to extensive study for each disease. These tissues were fixed in 20% buffered formalin and embedded in paraffin.

Characterization of Anti-Dorfin Antibody

Polyclonal rabbit antiserum (Dorfin-30) was raised against a C-terminal portion (amino acids 678 to 690) of Dorfin as described.[10] A synthetic peptide, RKIHNRYEGKDVSKHKRN (corresponding to amino acid sequence of residues 396 to 413 of Dorfin), was used for immunization in rabbit and affinity-purified to raise another polyclonal antiserum against Dorfin (Dorfin-41). Brain (cerebral cortex and putamen) and spinal cord tissues from normal controls without neurological disease were homogenized in sodium dodecyl sulfate (SDS) lysis buffer (10 mmol/L Tris, pH 8.0, 150 mmol/L NaCl, 2% SDS) with a protease inhibitor mixture (Complete; Roche Diagnostics, Basel, Switzerland) and were fractionated by centrifugation at 16,000 × *g*. The protein concentration was determined with a DC protein assay kit (Bio-Rad, Hercules, CA), and supernatants were used for Western blotting analysis. Construction of an N-terminal Xpress-tagged Dorfin expression vector (pcDNA4/HisMax-Dorfin) and Myc-tagged Ub expression vector (pcDNA3.1Myc-Ub) was reported elsewhere.[10] A C-terminal Myc-tagged Dorfin expression vector was constructed from cDNA containing the entire coding region of Dorfin inserted in-frame into the *Kpn*I and *Xba*I site of pcDNA3.1/MycHis(+) vector (Invitrogen, Carlsbad, CA). A N-terminal FLAG-tagged Dorfin expression vector was constructed from cDNA containing the entire coding region of Dorfin inserted in-frame into the *Cla*I and *Kpn*I site of pFLAG-CMV-2 vector (Sigma, St. Louis, MO). pcDNA3.1(+)FLAG-CHIP and pcDNA3.1(+)FLAG-parkin were kind gifts from Dr. Keiji Tanaka (Tokyo Metropolitan Institute of Medical Science) and Dr. Nobutaka Hattori (Juntendo University School of Medicine), respectively. Human embryonic kidney 293 (HEK293) cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal calf serum. Transfections were performed using the Effectene transfection reagent (Qiagen, Hilden, Germany). Cells were cultured for 24 hours and lysed in TNES lysis buffer (50 mmol/L Tris, 150 mmol/L NaCl, 1% Nonidet P-40, and 0.1% SDS) with a protease inhibitor mixture (Roche Diagnostics). The protein concentration was determined with a DC protein assay kit (Bio-Rad), and lysates were electrophoresed by SDS-polyacrylamide gel electrophoresis and transferred to Hybond-P polyvinylidene difluoride membrane (Amersham Pharmacia, Piscataway, NJ) for Western blotting. The membranes were blocked in 5% milk in Tris-buffered saline (TBS) containing 0.1% Tween-20, and incubated overnight at 4°C with Dorfin antiserum (1:5000 dilution). The blots were then washed three times for 10 minutes each in TBS with 0.1% Tween 20, followed by a 1-hour incubation in horseradish peroxidase coupled to secondary antibody (1:5000 dilution, Amersham Pharmacia). The blots were then washed three times for 10 minutes each in TBS with 0.1% Tween 20 before incubation in enhanced chemiluminescence reagent (Amersham Pharmacia) and exposure to film.

Immunohistochemistry and Immunoelectron Microscopy

Immunohistochemistry was performed as described previously.[24–26] Four-μm-thick sections were obtained from the paraffin-embedded midbrain, cerebral cortex, putamen, hippocampus, and spinal cord of the patients with PD, DLB, MSA, SALS, FALS, and controls, respectively. These sections were immunostained using the avidin-

biotin-peroxidase complex method with 3.3-diaminobenzidine tetrahydrochloride (Wako, Osaka, Japan) as a chromogen. The immunolabeled sections were lightly counterstained with hematoxylin. Dorfin antiserum (1:200 dilution in both Dorfin-30 and Dorfin-41), and anti-Ub (P4D1, 1: 400 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) antibodies were used. For staining with anti-Dorfin antiserum, the sections were pretreated with 99% formic acid (Wako) for 5 minutes at room temperature. Specificity of anti-Dorfin antibody on immunostaining for human tissue was assessed by the preabsorption of antibody with peptide antigen. To assess the co-localization of Dorfin and Ub, a double-labeling immunofluorescence study was performed on the selected sections with a combination of anti-Dorfin and anti-Ub antibodies. Anti-Dorfin antibody was visualized by anti-rabbit goat IgG coupled with Alexa Fluor 568 (Molecular Probes, Eugene, OR), and anti-Ub antibody was visualized with anti-mouse sheep IgG coupled with Alexa Fluor 488 (Molecular Probes), and observed under a LSM-510 confocal microscope (Carl Zeiss, Gottingen, Germany). To assess Dorfin immunoreactivity in ubiquitylated inclusions, serial sections were prepared for every two serial sections; one was stained with anti-Dorfin antibody and the other with anti-Ub antibody. The ratio of Dorfin-positive inclusion bodies among Ub-positive inclusions was evaluated by assessing 40 to 120 Ub-positive inclusion bodies in each case of PD, DLB, MSA, and at least 10 inclusion bodies in FALS and SALS.

Immunoelectron microscopy was performed as previously described.[24,25] The selected deparaffinized sections were immunostained with Dorfin-30 antibody in the same manner, then washed in phosphate buffer, post-fixed with 1% osmium tetroxide, dehydrated in a graded series of ethanol, and embedded in epoxy resin. Ultrathin sections were prepared and examined under an H-7000 electron microscope (Hitachi, Tokyo, Japan). Each immunohistochemical reaction was ascertained by substituting normal rabbit or mouse sera for the primary antibodies, and no specific peroxidase reactions occurred in these control experiments.

Filter Trap Assay

For this assay, ~100- to 200-mg tissues from the cerebral cortex of DLB, putamen of MSA, and the cerebral cortex and spinal cord of ALS and control were used. A filter trap assay was performed as previously described.[27] Tissues were homogenized in 10 vol of TBS. Homogenates were centrifuged at 800 × *g* for 10 minutes at 4°C and the supernatants were diluted with 10 vol of TBS with 0.1% SDS. Protein concentrations were determined with a DC protein assay kit (Bio-Rad) and, using a slot blot device (Bio-Rad), the supernatants were filtered under vacuum through 0.22-μm cellulose acetate membranes (Sartorius, Gottingen, Germany) followed by two washes in TBS. The membranes were then incubated in 5% dry milk in TBS at room temperature for 1 hour, followed by an overnight incubation at 4°C with Dorfin-30 (1:5000 dilution), anti-Ub (1:1000 dilution; Zymed, San Francisco, CA) or anti-α-synuclein (LB509, 1:1000 dilution; Zymed) antibody in TBS with 0.1% Tween 20. Horseradish peroxidase-conjugated second antibodies (1:5000, Amersham Pharmacia) were used and detected with enhanced chemiluminescence reagent (Amersham Pharmacia). To confirm equal loading of proteins, the same samples were filter trapped using 0.45-μm nitrocellulose membranes (Bio-Rad) and were probed with anti-α-tubulin antibody (1:1000 dilution, Sigma).

Fractionation of Normal and Diseased Brain Tissues

Approximately 100- to 200-mg tissues of cingulate gyrus from normal or DLB brains, 200-mg tissues of putamen from MSA brains, or 400-mg ALS spinal cord were homogenized in 10 vol of lysis buffer A (50 mmol/L Tris-HCl at pH 7.5, 500 mmol/L NaCl, 5 mmol/L ethylenediaminetetraacetic acid, and 10 mmol/L NaF) with a protease inhibitor mixture (Complete, Roche Diagnostics) and centrifuged at 16,000 × *g* for 30 minutes at 4°C. Resulting pellets were sequentially extracted by homogenization in Triton X-100 (buffer A containing 1% Triton X-100), and urea (50 mmol/L Tris-HCl, 8 mol/L urea, 1 mmol/L EGTA) followed by centrifugation at 100,000 × *g*.

Immunoprecipitation

α-Synuclein cDNA was amplified by polymerase chain reaction from human brain cDNAs and cloned into the *Eco*RV site of pcDNA3.1/MycHis(+) (Invitrogen). To generate the mutant α-synuclein expression vector, A30P and A53T mutations were introduced into the pcDNA3.1/MycHis-α-synuclein with a QuickChange site-directed mutagenesis Kit (Stratagene, La Jolla, CA) according to Lee and colleagues.[28] Construction of pcDNA3.1/MycHis-wild-type and G85R mutant SOD1 vector was previously described.[9] Xpress-tagged Dorfin (pcDNA4/HisMax-Dorfin) and Myc-tagged α-synuclein or SOD1 were transiently expressed using the Effectene transfection reagent (Qiagen) in HEK293 cells. Cells were lysed in TNES lysis buffer with a protease inhibitor mixture (Roche Diagnostics). To inhibit cellular proteasome activity, cells were treated with 0.5 μmol/L MG132 (Z-Leu-Leu-Leu-al; Sigma) for 16 hours after overnight posttransfection. Immunoprecipitation from the transfected cell lysates was performed with 2 μg of anti-Xpress antibody (Invitrogen) and protein A/G Plus agarose (Santa Cruz Biotechnology), and then washed four times in lysis buffer. Immunoprecipitates were analyzed by Western blotting with enhanced chemiluminescence detection reagents (Amersham Pharmacia).

Results

Specificity of Anti-Dorfin Antibody

To examine the pathophysiological role of Dorfin, we raised affinity-purified antisera to two separate regions of human Dorfin (Figure 1A) and characterized their speci-

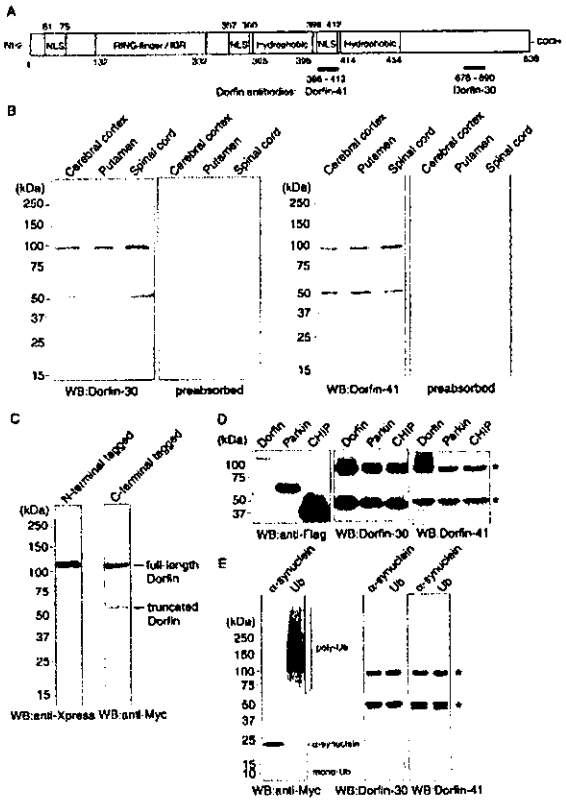

Figure 1. Characterization of affinity-purified antibody to human Dorfin. **A:** Schematic diagram of human Dorfin and peptide antibodies. IBR, in-between ring-finger; NLS, nuclear localization signal-like sequence. **B:** SDS-polyacrylamide gel electrophoresis and Western blotting analysis of the extracted protein from human central nervous tissues. Soluble extracts from normal adult brains (cerebral cortex and putamen) and spinal cord (50 μg each) were used and probed with antibodies to Dorfin. Note that antiserum preabsorbed with excess peptide antigen shows no staining. **C:** SDS-polyacrylamide gel electrophoresis and Western blotting analysis of lysates of HEK293 cells expressing Xpress-Dorfin (**left**) or Dorfin-Myc (**right**). Note that only C-terminal Myc-tagged Dorfin shows truncated fragment. **D:** Specific binding of anti-Dorfin antibodies to Dorfin proteins. Lysates of HEK293 cells expressing FLAG-tagged Dorfin, parkin, and CHIP were analyzed by Western blotting with antibodies to Dorfin. Both Dorfin-30 and Dorfin-41 recognize only Dorfin fusion proteins. **E:** Anti-Dorfin antibodies do not cross-react with α-synuclein and Ub. Lysates of HEK293 cells expressing Myc-tagged α-synuclein and Ub were analyzed by Western blotting with antibodies to Dorfin. Both Dorfin-30 and Dorfin-41 recognize only endogenous Dorfin. **Asterisks** on the **right** indicate endogenous Dorfin.

ficity. First, we tested Dorfin antisera by Western blotting under reduced conditions against brain and spinal cord tissues from a control subject without neurological disease. In this assay, both Dorfin-30 (amino acids 678 to 690) and Dorfin-41 (amino acids 396 to 413) recognized these tissues' cognate antigens in a specific manner, and recognized ~98-kd and ~52-kd protein of human brain extracts as well as those of spinal cord extracts (Figure 1B, left). Their reactivities were abolished by preabsorption with the excess of peptide antigens (Figure 1B, right). The ~98-kd protein probably corresponds to the full-length Dorfin protein because the calculated molecular weight of Dorfin is ~91 kd. The ~52-kd band is thought to be a truncated C-terminal fragment of Dorfin recognized by Dorfin antiserum. To confirm this inference, we next examined the size of N-terminal Xpress-tagged and C-terminal Myc-tagged Dorfin fusion proteins expressed in the cultured cell line HEK293 by Western blotting. Both anti-Xpress and anti-Myc antibody recognized ~102-kd full-length proteins, but only anti-Myc antibody to the C-terminal tag revealed the ~60-kd truncated C-terminal fragment (Figure 1C). Similar results have been reported by another laboratory, using various mouse tissues with anti-XYbp antiserum (*XYbp* is a mouse orthologue of *Dorfin*) in Western blotting analysis.[29] Furthermore, we determined the selective specificity of our antibodies to Dorfin. We assessed whether our antibodies recognize other E3 ligases, although two epitope regions, we have chosen to create anti-Dorfin antibodies, show no homology to other proteins with BLASTp searches in the database of National Center for Biotechnology Information. We chose E3 ligases, parkin, and CHIP as controls, because parkin has RING-finger/IBR domain,[11] localizes in the LBs,[30] and ubiquitylates unfolded protein(s)[31] as well as Dorfin, and CHIP is also E3 ligase for abnormal proteins as the so-called protein quality control ligase[16–18] and interacts with parkin to enhance its activity.[32] N-terminal FLAG-tagged fusion proteins of Dorfin, parkin, and CHIP were examined by Western blotting with Dorfin-30 and Dorfin-41 antibodies. Both anti-Dorfin antibodies recognized ~102-kd full-length fusion proteins and endogenous Dorfin, but did not recognized parkin and CHIP at all (Figure 1D). In addition, we confirmed that anti-Dorfin antibodies do not cross-react with α-synuclein and Ub (Figure 1E).

Dorfin Localizes to Ubiquitylated Inclusion Bodies of PD, DLB, MSA, and ALS

To assess Dorfin immunoreactivity in the involved lesions of the central nervous system of neurodegenerative diseases, we examined the inclusion body-rich regions of the brain and spinal cord sections from sporadic PD, DLB, MSA, sporadic and familial ALS, and normal controls by light and electron microscopic immunohistochemistry.

In PD, both anti-Dorfin antisera, Dorfin-30 and Dorfin-41, labeled LBs of various types existing inside and outside the substantia nigra. More intense immunoreactivity was observed in LBs with Dorfin-41 antibodies. The peripheral rims of typical LBs, either round or elongated, in neuronal cell bodies and in processes were strongly stained, whereas the central cores remained unstained or only weakly stained (Figure 2; A, B, and M). The pale body, Lewy neuritis, axonal spheroids in substantia nigra, and the glial inclusions were also immunostained by both anti-Dorfin antibodies (Figure 2, C and N). Ubiquitin was predominantly seen in rims of LBs, but sometimes in the core of LB. Most, if not all, α-synuclein-positive LBs are also ubiquitin-positive[33] and we have previously shown that Dorfin co-localized with ubiquitylated hyaline inclusions in ALS.[9] Thus, we counted Dorfin-positive inclusions in comparison with ubiquitin. Serial sections stained with anti-Dorfin and anti-Ub antibodies showed that 40.9 ± 12.1% of Ub-positive LBs were positive for Dorfin-30, and 92.2 ± 11.8% of them were positive for Dorfin-41

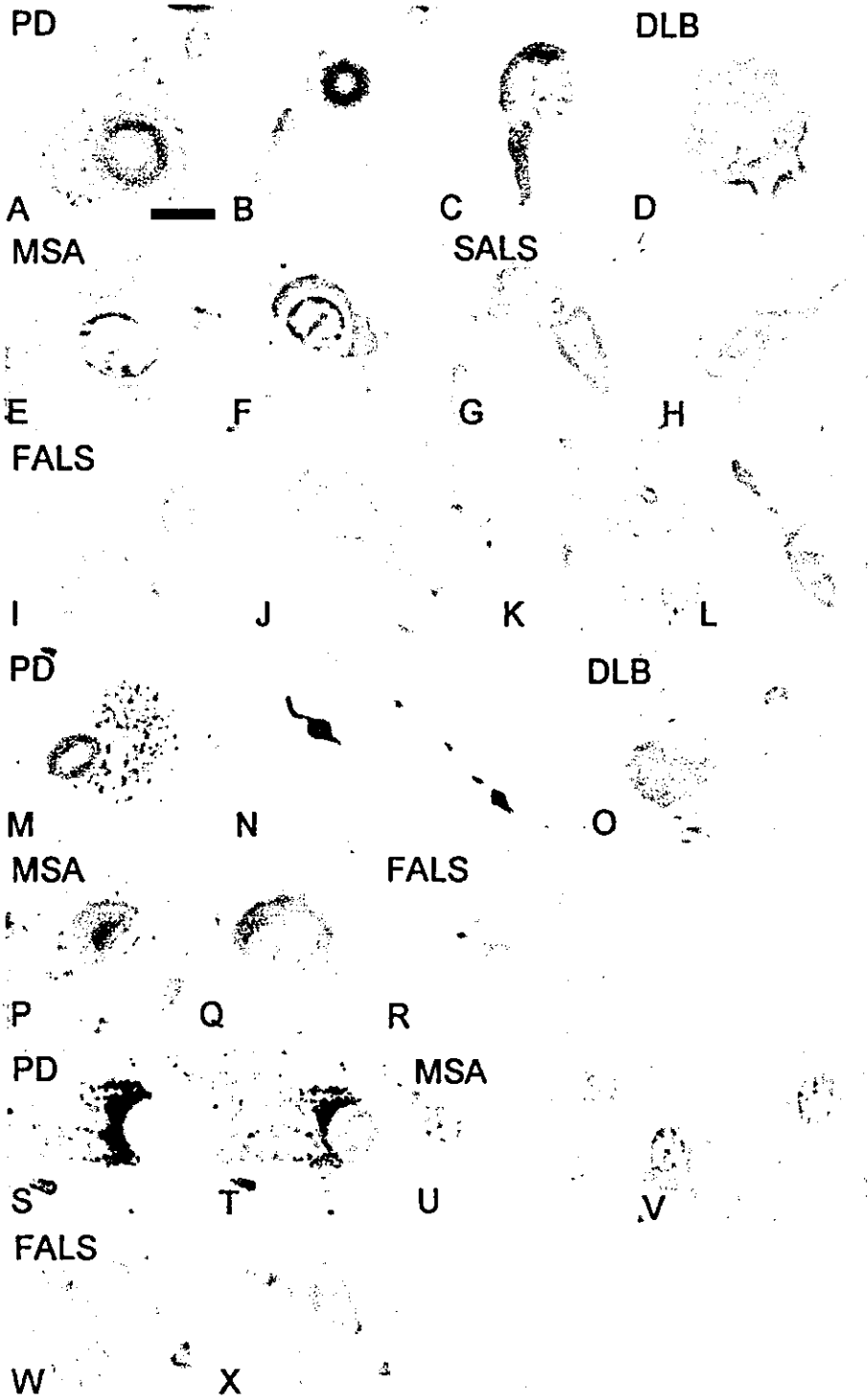

Figure 2. Light microscopic Dorfin immunohistochemistry in neuronal and glial inclusions of PD, DLB, MSA, SALS, and FALS. Immunostainings with Dorfin-30 antibody (**A–L, P–X**) and Dorfin-41 antibody (**M–O**) are shown. LBs in the substantial nigral neurons (**A** and **M**) and in the Edinger-Westphal nucleus (**B**) of PD patients are heavily Dorfin-immunoreactive. GCIs (**C**) and Lewy neuritis (**N**) of PD patients are also Dorfin-immunoreactive. Cortical LBs in the temporal cortex of the DLB patient are Dorfin-positive (**D** and **O**). Glial inclusions of the oligodendroglia in MSA patients in the putamen are Dorfin-immunoreactive (**E, F, P,** and **Q**). Dorfin is also localized in the cytoplasmic inclusions of the spinal motor neurons of SALS patients (**G** and **H**). Dorfin is localized in the LB-like inclusions (**I, J,** and **R**) and skein-like inclusions (**K** and **L**) in the remaining motor neurons of the FALS spinal cord. Each inclusion was strongly immunostained by Dorfin (**T, V,** and **X**), whereas preabsorbed antibody abolished most immunoreactivity (**S, U,** and **W**). **S** and **T:** LBs in pigmented neuron of the substantia nigra in PD. **U** and **V:** GCIs of the putamen in MSA. **W** and **X:** LB-like inclusion of hypoglossal neuron of a FALS case. Scale bar in **A** is equivalent to: 20 μm (**A, B, G, J, L,** and **M**); 10 μm (**C, D, I,** and **O**); 5 μm (**E, F, P,** and **Q**); 16 μm (**H, K,** and **R**), 52 μm (**N**), 12 μm (**S–V**); 8 μm (**W** and **X**).

Table 1. Dorfin and Ubiquitin Immunoreactivity in Neuronal and Glial Inclusions of PD, DLB, MSA, and ALS

Antibody	Dorfin (+) inclusions (assessed number)	Ubiquitin (+) inclusions (assessed number)	Dorfin (+)/ubiquitin (+) (%)
PD (n = 5)			
Dorfin-30	33.8 ± 11.4	68.6 ± 10.5	40.9 ± 12.1
Dorfin-41	52.4 ± 6.5	57.2 ± 4.1	92.2 ± 11.8
DLB (n = 5)			
Dorfin-30	34.2 ± 12.8	53.6 ± 17.8	62.6 ± 13.7
Dorfin-41	52.4 ± 4.3	64.0 ± 8.0	85.4 ± 9.2
MSA (n = 5)			
Dorfin-30	85.0 ± 18.5	125.0 ± 28.9	70.9 ± 20.5
Dorfin-41	69.0 ± 13.5	73.8 ± 9.2	92.9 ± 7.5
SALS (n = 2)			
Dorfin-30	7	17	41.2
Dorfin-41	4	17	23.5
FALS (n = 1)			
Dorfin-30	15	31	48.4
Dorfin-41	14	31	45.2

(+), Immunoreactive.
Numbers of Dorfin- and ubiquitin-immunoreactive inclusions were assessed on 10 consecutive sections. Values are shown as mean ± SD for samples indicated in parentheses.

(Table 1). In DLB, Dorfin-30 and Dorfin-41 labeled the cortical type LB (Figure 2, D and O). Lewy neurites in the hippocampal CA2-3 region and glial inclusions were also immunolabeled by anti-Dorfin antibody as they were in PD (data not shown). The 62.6 ± 13.7% of the Ub-positive cortical LBs were immunoreactive for Dorfin-30, and the 85.4 ± 9.2% of them were immunoreactive for Dorfin-41 (Table 1). In MSA, GCIs observed in the oligodendroglia were immunostained by anti-Dorfin antibody. Dorfin-immunoreactive inclusions appear as flame-shaped (Figure 2, E and P) or sickle-shaped (Figure 2, F and Q) in glial cells. The 70.9 ± 20.5% of ubiquitin-positive GCIs were labeled by Dorfin-30 antibody, and 92.2 ± 11.8% were by Dorfin-41 antibody (Table 1). Only a few of the neuronal cytoplasmic inclusions and neuronal and glial nuclear inclusions were positive for Dorfin (data not shown). In SALS and FALS, eosinophilic, Ub-positive cytoplasmic inclusions and skein-like inclusions were found in motor neurons of the spinal cord.[7] LB-like hyaline inclusions (LBHIs) were also ubiquitylated.[7] Dorfin-30 and Dorfin-41 labeled some of these inclusions, such as the ring-, round-, irregular round-, filamentous rod-, and loose ball-shaped types (Figure 2; G to L and R). Dorfin immunoreactivity was strongly present in the central core of the round- or irregular round-shaped inclusions, and in the middle layer of the ring-shaped inclusions, whereas the outer layers of these inclusions were less immunoreactive (Figure 2; H to J). The Bunina body, which was negative for anti-Ub antibody, was not stained with Dorfin-30 and Dorfin-41 antibody (data not shown). Dorfin-30 was positive at 48.4% of Ub-positive inclusions in SALS and 41.2% in FALS, and Dorfin-41 was positive at 23.5% in SALS and 45.2% in FALS (Table 1). These inclusions were only weakly stained by Dorfin-41. Preabsorbed anti-Dorfin antibody abolished most immunoreactivity (Figure 2; S to X), indicating that our anti-Dorfin antibody specifically recognizes Dorfin-epitopes on the inclusions of these tissues. In normal control, no Dorfin-immunoreactive structure was detected (data not shown).

A double-labeled immunofluorescence study revealed that Dorfin-30 and Ub were co-localized in LBs (Figure 3; A to C). Pale bodies, which have been considered to be the precursors of LB,[34] and Lewy neurites were also immunolabeled by anti-Dorfin and anti-Ub antibodies (Figure 3; D to F). GCIs in MSA (Figure 3; G to I), and the hyaline inclusions in FALS (Figure 3; J to L) were also Dorfin- and Ub-immunoreactive.

At the immunoelectron microscopic level, Dorfin-30 immunoreactivity was localized on filamentous structures, particularly the halo of the LBs that was composed of the radially arranged intermediate filaments associated with granular materials and vesicular structures. These radial filaments and associated structures were strongly immunostained by anti-Dorfin antibody, whereas the central core was not stained (Figure 4, A and B). Thus, the staining profile of Dorfin was very similar to that of Ub and α-synuclein,[33] but different from that of parkin, which localizes predominantly to the core of LBs.[30] Dorfin immunoreactivity in GCI of MSA was composed of randomly arranged tubules or filamentous structures associated with granular materials (Figure 4, C and D). In FALS, the thicker, granule-coated filaments were decorated by the Dorfin immunoreactive deposit and formed the core, whereas the thinner filaments without a granular coating were not recognized by anti-Dorfin antibody and formed the halo (Figure 4, E and F).

Dorfin Accumulation in Ubiquitylated High-Molecular Weight Complexes

In studies of polyglutamine disorders, it has been demonstrated that high-molecular weight aggregates of mutant proteins are retained by filtration through cellulose acetate.[35,36] In a *SOD1*-transgenic mice ALS model, this assay is also applied to detect mutant SOD1 aggregation.[27] Cellulose acetate membranes usually bind protein very poorly and are used to filter high-molecular weight structures from complex mixtures.[35] Thus we investi-

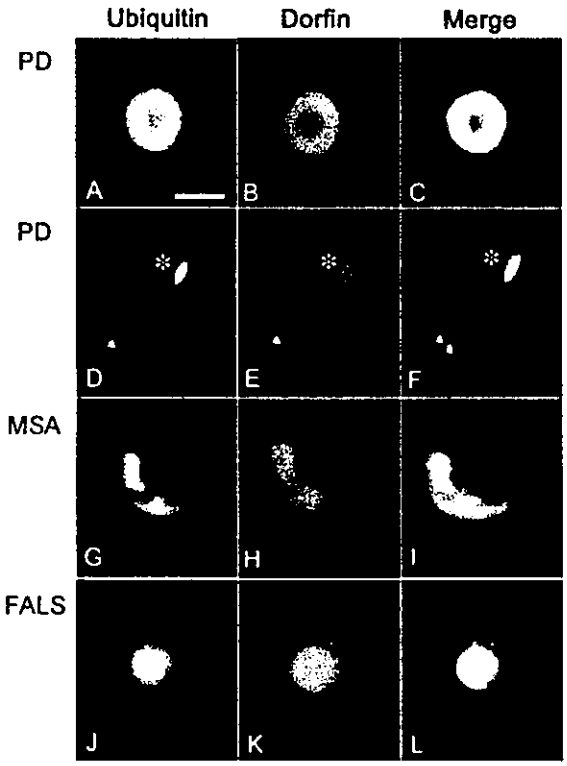

Figure 3. Co-localization of Dorfin-30 immunoreactivity with Ub in neuronal and glial inclusions. Sections were doubly labeled with Dorfin antiserum and an antibody against Ub and analyzed with a laser-scanning confocal microscope. Panels at **left (green)** correspond to Ub, **middle** panels (**red**) correspond to Dorfin, and panels at **right** correspond to merged images; structures in yellow indicate co-localization. Dorfin is co-localized with Ub in ubiquitylated inclusions in the nigral neurons of PD (**A–F**), in glial cells in the putamen of MSA (**G–I**), and in spinal motor neurons of FALS (**J–L**). Co-localization of Dorfin and Ub is also seen in the pale body (**arrow**) and Lewy neurite (**asterisk**) in (**D–F**). Scale bar in **A** is equivalent to: 20 μm (**A-C** and **J–L**); 100 μm (**D–F**); 5 μm (**G–I**).

gated whether Dorfin is retained in high-molecular weight aggregates from α-synucleinopathy and ALS tissues by cellulose acetate filter trap assay. Homogenates of brain and spinal cord tissues from DLB, MSA, SALS, and controls were solubilized in TBS with 0.1% SDS, then filtered through a 0.22-μm cellulose acetate membrane. We have chosen the cerebral cortex for DLB, putamen for MSA, and the cerebral cortex and spinal cord for SALS for this assay, because these regions exhibit the most prominent pathology in each disease. Subsequent staining with Dorfin antiserum revealed trapped proteins (Figure 5A, top), which were also recognized by antibodies to Ub (Figure 5A, top middle). Furthermore, in DLB and MSA brains, α-synuclein was trapped, as expected (Figure 5A, bottom middle). Interestingly, high-molecular weight aggregates were difficult to detect in extracts of the cerebral cortex of SALS, in which pathological changes are less severe compared to those in the spinal cord. No high-molecular weight aggregate was present in either brain or spinal cord samples from normal control.

Sequential detergent extraction methods have been successfully applied to detect high-molecular weight complexes in brain of patients with α-synucleinopathies.[37,38] We used this method to detect insoluble Dorfin molecules in inclusion-rich tissues. Buffer- and Triton X-100-soluble ~98-kd and ~52-kd Dorfin were detected in brains from normal control as well as from DLB, MSA, and ALS patients (Figure 5B). In contrast to urea extracts of normal brain that were devoid of Dorfin immunoreactivities, ~98-kd full-length and ~52-kd truncated Dorfin were found in urea extracts from DLB, MSA, and ALS patients (Figure 5B). Bands, ~200 kd, ~45 kd and ~35 kd bands, were also detected (Figure 5B). These Dorfin-immunoreactive bands were not recognized by anti-Ub antibody (data not shown), and higher molecular weight bands may represent dimeric forms of Dorfin and smaller molecular weight species may be processed products of Dorfin.

These observations indicated that Dorfin is present in high-molecular weight structures in the tissues of α-synucleinopathy and ALS, suggesting that Dorfin is a major component of aggregated and ubiquitylated proteins forming inclusion bodies in these neurodegenerative disorders. Full-length Dorfin antigen is present in abnormal inclusions, but Triton-insoluble-urea-soluble higher molecular weight Dorfin and processed fragments seem to be major building blocks for inclusion bodies.

Dorfin Does Not Bind to Wild-Type and Mutant α-Synuclein

α-Synuclein is the main structural component of the insoluble protein aggregates that form the LBs of PD and DLB as well as the GCIs of MSA[37–39] and it has been shown to be ubiquitylated by an E3 Ub ligase, parkin.[40] As demonstrated, Dorfin is another major component of inclusion bodies in α-synucleinopathy, and because it has E3 activity,[9,10] we examined whether it interacts with α-synuclein *in vivo*. To this end, Xpress-tagged Dorfin was co-expressed with Myc-tagged wild-type or mutant forms of α-synuclein in HEK293 cells (Figure 6). In our experimental system, exogenously expressed α-synuclein was not phosphorylated (data not shown). Western blotting analysis after immunoprecipitation revealed that Dorfin binds with neither wild-type nor mutant α-synuclein. However, it strongly bound with mutant SOD1 (Figure 6), as we reported previously.[9] *In vitro* ubiquitylation assay using immunoprecipitated α-synuclein from transformed HEK293 cells, Dorfin did not ubiquitylate wild-type and mutant α-synuclein (data not shown).

Discussion

In the present study, we showed that Dorfin co-localizes to the ubiquitylated inclusions in common neurodegenerative diseases, including LBs in PD and DLB, GCIs in MSA, and hyaline and skein-like inclusions in ALS. Moreover, filter-trapped high-molecular weight structures contained Dorfin, indicating that it is a major constituent of these inclusions irrespective of the different disease etiologies and different morphological features of these inclusion bodies.

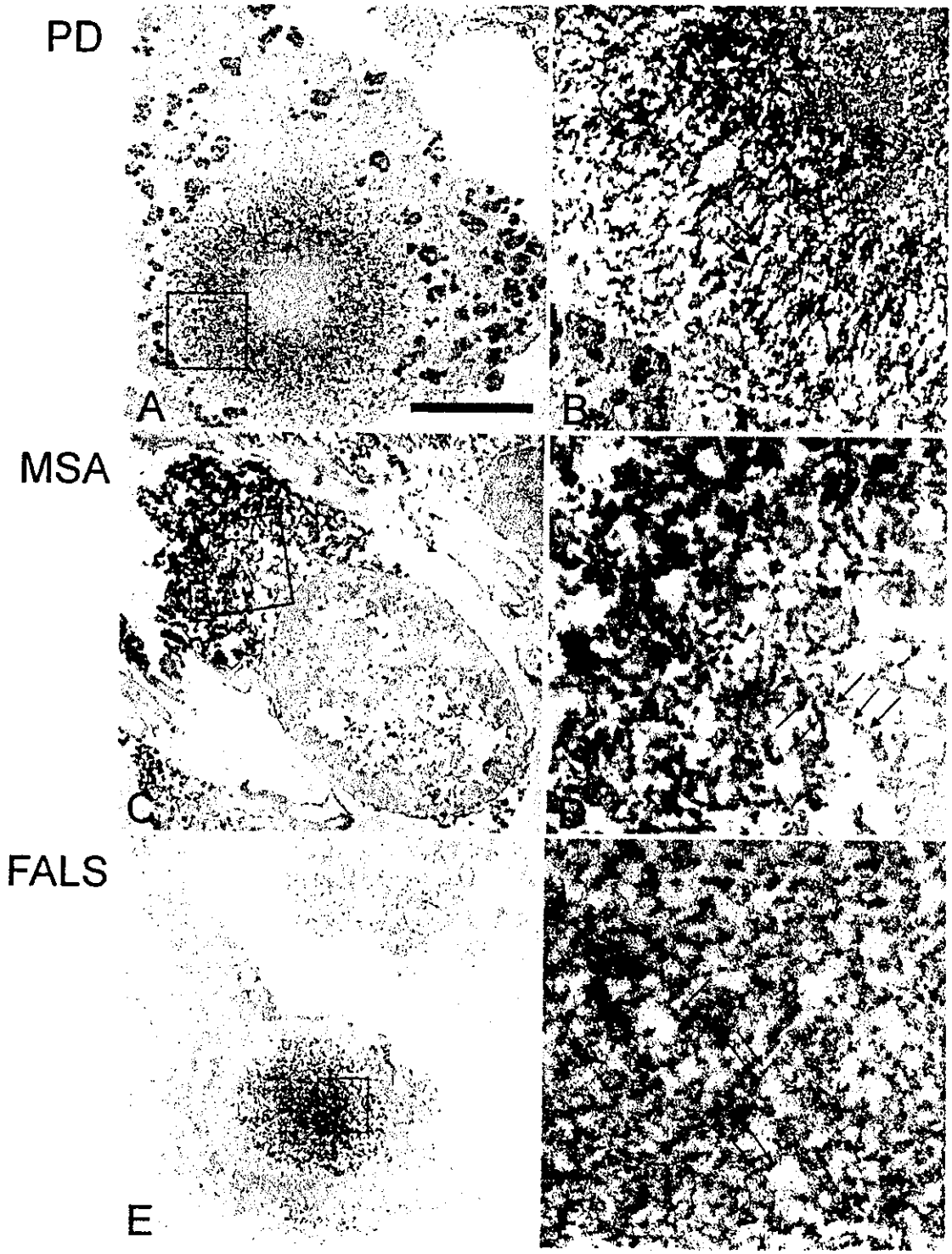

Figure 4. Immunoelectron microscopy of the neuronal and glial inclusions of PD, MSA, and FALS using the Dorfin-30 antibody. **A** and **B:** Typical LB immunostained with anti-Dorfin antibody in a pigmented neuron of substantia nigra in PD. The peripheral radiating filaments are strongly stained, and the central core is not Dorfin-immunoreactive. The immunoreactive filaments labeled with anti-Dorfin-30 antibody in **A** are shown at higher magnification (**arrows** in **B**) from area in the **square** in **A**. **B** shows that granular and fibrous materials are strongly positive for Dorfin, and are arranged radially. **C** and **D:** GCI of the oligodendrocyte is positive for Dorfin immunoreactivity in MSA. The granular materials (**arrowheads** in **D**) and the fibrous structures (**arrows** in **D**) are strongly stained with the Dorfin antibody from area in the **square** in **C**. **E** and **F:** LB-like hyaline inclusion in the spinal motor neurons in FALS. The filamentous structures (**arrows** in **F**) in the central core are strongly stained with anti-Dorfin antibody, but the filaments in the halo are not. **F** shows higher magnification of the central core region (**square**) in **E**. The granule-associated thick filaments are decorated. Scale bar in **A** is equivalent to: 5.5 μm (**A** and **E**); 2.5 μm (**C**); 0.8 μm (**B**); 0.83 μm (**D**); 0.85 μm (**F**).

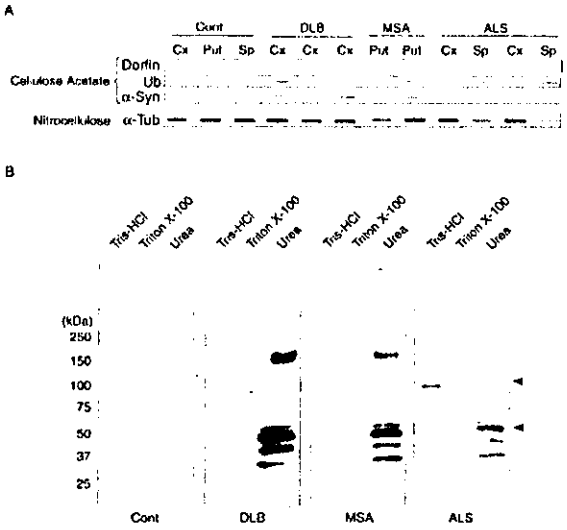

Figure 5. Dorfin accumulation in ubiquitylated high-molecular weight complexes. **A:** Detection of Dorfin in high-molecular weight structures in DLB, MSA, and SALS. Homogenates from brain and spinal cord of these diseases were filtered through cellulose acetate membranes and detected with Dorfin-30 antibody (**top**), anti-Ub antibody (**top middle**), and anti-α-synuclein antibody (**bottom middle**). Cx, cerebral cortex; Put, putamen; Sp, spinal cord. Nitrocellulose slot-blot probed with anti-α-tubulin antibody was used to confirm loading of equal amounts of samples (**bottom**). These results were replicated in duplicate experiments with different DLB, MSA, SALS, and control cases. **B:** Sequential extraction of Dorfin. Brains (normal, DLB, and MSA) and spinal cord (ALS) samples were sequentially extracted based on their solubility into Tris-HCl, Triton X-100, and urea buffers. Dorfin immunoreactivities were recovered in Tris and Triton fractions as ~98-kd and ~52-kd bands. In addition to these bands, Dorfin-30 antibody detected ~200-kd, ~45-kd, and ~35-kd bands in DLB, MSA, and ALS urea extracts, but not in normal brain urea extract. The **arrowheads** indicate ~98-kd full-length and ~52-kd truncated Dorfin bands.

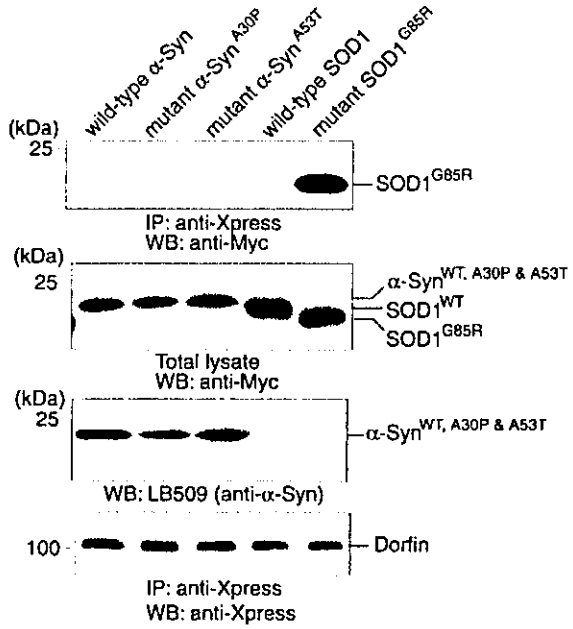

Figure 6. Dorfin binds to mutant SOD1 but not to α-synuclein. Myc-tagged wild-type and mutant α-synucleins were co-transfected with Xpress-tagged Dorfin into HEK293 cells. After immunoprecipitation with anti-Xpress antibody, the resulting precipitates were analyzed by Western blotting. Myc-tagged wild-type SOD1 and mutant $SOD1^{G85R}$ were used for negative and positive controls, respectively.

The following among our reported observations support the view that Dorfin plays an important role in the formation of ubiquitylated inclusion bodies in α-synucleinopathy and ALS: 1) the presence of Dorfin in the inclusion bodies of these diseases, 2) the parallel distribution patterns of Ub and Dorfin, 3) the perinuclear aggresome-like localization of Dorfin in cultured cells,[10] and 4) the E3 Ub ligase function of Dorfin and the resultant generation of mutant SOD1-(Ub)n conjugates.[9,10] The relation of Dorfin to α-synucleinopathies and ALS shows striking similarities to the relation between parkin and PD. Parkin, a gene product responsible for one of the most common forms of familial PD,[41] was shown to have E3 Ub ligase activity.[31,40,42–45] It was recently demonstrated that an O-glycosylated α-synuclein is the substrate of parkin,[40] and that parkin localizes to the LBs of sporadic PD and DLB.[30] A link between sporadic and familial PD through α-synuclein and parkin suggests that common molecular pathogenetic mechanisms underlie PD. The accumulation of toxic or undesired proteins in neurons may result from a primary failure of degradation systems, and could subsequently lead to neurodegeneration. Alternatively, the constant production of high levels of impaired proteins may become a burden on the protein degradation process through the Ub-proteasome pathway, gradually overwhelming the capacity of the proteasome to degrade toxic proteins and subsequently leading to the accumulation of ubiquitylated proteins and eventual neuronal cell death. Such a scenario is consistent with a recent report that impairment of the Ub-proteasome system is caused by protein aggregation.[46]

From this perspective, it is conceivable that familial and sporadic forms of ALS also share a common pathogenetic mechanism with PD involving the dysfunction of the Ub-proteasome pathway. In sporadic ALS, posttranslationally modified unknown substrates of Dorfin other than mutant SOD1 might accumulate in ubiquitylated form and play a role in the pathogenesis of the disease. Therefore, it is important to identify the protein(s) that is (are) the substrate(s) of the E3 activity of Dorfin for an understanding of the pathogenetic mechanism of sporadic ALS. In addition, substrates of Dorfin other than α-synuclein may play an important role in the pathogenesis of sporadic PD, DLB, and MSA, or a posttranslational modification (eg, glycosylation,[40] phosphorylation[47,48]) of α-synuclein may be necessary to be a substrate for Dorfin, because we failed to show the interaction between Dorfin and nonmodified α-synuclein overexpressed in HEK293 cells in this report. Furthermore, our findings raise the possibility that PD, DLB, MSA, and ALS are etiologically distinct, but share a biochemically common metabolic pathway through Dorfin leading to the formation of ubiquitylated inclusion bodies and to neuronal cell degeneration.

The generation of Dorfin knockout mice may determine whether Dorfin is essential to form ubiquitylated inclusion bodies and is indispensable to prevent neurons from the

toxic insult of protein aggregation. It may reveal what specific roles and relationships Dorfin and parkin have with one another as members of an E3 Ub ligase family containing a RING-finger/IBR domain.

References

1. Carrell RW, Lomas DA: Conformational disease. Lancet 1997, 350: 134–138
2. Johnson WG: Late-onset neurodegenerative diseases—the role of protein insolubility. J Anat 2000, 196:609–616
3. Mayer RJ, Lowe J, Lennox G, Doherty F, Landon M: Intermediate filaments and ubiquitin: a new thread in the understanding of chronic neurodegenerative diseases. Prog Clin Biol Res 1989, 317:809–818
4. Forno LS: Neuropathology of Parkinson's disease. J Neuropathol Exp Neurol 1996, 55:259–272
5. McKeith IG: Clinical Lewy body syndromes. Ann NY Acad Sci 2000, 920:1–8
6. Lantos PL: The definition of multiple system atrophy: a review of recent developments. J Neuropathol Exp Neurol 1998, 57:1099–1111
7. Hirano A: Neuropathology of ALS: an overview. Neurology 1996, 47:S63–S66
8. Ince PG, Lowe J, Shaw PJ: Amyotrophic lateral sclerosis: current issues in classification, pathogenesis and molecular pathology. Neuropathol Appl Neurobiol 1998, 24:104–117
9. Niwa J, Ishigaki S, Hishikawa N, Yamamoto M, Doyu M, Murata S, Tanaka K, Taniguchi N, Sobue G: Dorfin ubiquitylates mutant SOD1 and prevents mutant SOD1-mediated neurotoxicity. J Biol Chem 2002, 277:36793–36798
10. Niwa J, Ishigaki S, Doyu M, Suzuki T, Tanaka K, Sobue G: A novel centrosomal ring-finger protein, dorfin, mediates ubiquitin ligase activity. Biochem Biophys Res Commun 2001, 281:706–713
11. Morett E, Bork P: A novel transactivation domain in parkin. Trends Biochem Sci 1999, 24:229–231
12. Moynihan TP, Ardley HC, Nuber U, Rose SA, Jones PF, Markham AF, Scheffner M, Robinson PA: The ubiquitin-conjugating enzymes UbcH7 and UbcH8 interact with RING finger/IBR motif-containing domains of HHARI and H7-AP1. J Biol Chem 1999, 274:30963–30968
13. Ardley HC, Tan NG, Rose SA, Markham AF, Robinson PA: Features of the parkin/ariadne-like ubiquitin ligase, HHARI, that regulate its interaction with the ubiquitin-conjugating enzyme, Ubch7. J Biol Chem 2001, 276:19640–19647
14. Borchelt DR, Lee MK, Slunt HS, Guarnieri M, Xu ZS, Wong PC, Brown Jr RH, Price DL, Sisodia SS, Cleveland DW: Superoxide dismutase 1 with mutations linked to familial amyotrophic lateral sclerosis possesses significant activity. Proc Natl Acad Sci USA 1994, 91:8292–8296
15. Hoffman EK, Wilcox HM, Scott RW, Siman R: Proteasome inhibition enhances the stability of mouse Cu/Zn superoxide dismutase with mutations linked to familial amyotrophic lateral sclerosis. J Neurol Sci 1996, 139:15–20
16. McClellan AJ, Frydman J: Molecular chaperones and the art of recognizing a lost cause. Nat Cell Biol 2001, 3:E51–E53
17. Murata S, Minami Y, Minami M, Chiba T, Tanaka K: CHIP is a chaperone-dependent E3 ligase that ubiquitylates unfolded protein. EMBO Rep 2001, 2:1133–1138
18. Cyr DM, Hohfeld J, Patterson C: Protein quality control: u-box-containing E3 ubiquitin ligases join the fold. Trends Biochem Sci 2002, 27:368–375
19. Kopito RR: Aggresomes, inclusion bodies and protein aggregation. Trends Cell Biol 2000, 10:524–530
20. Johnston JA, Dalton MJ, Gurney ME, Kopito RR: Formation of high molecular weight complexes of mutant Cu,Zn-superoxide dismutase in a mouse model for familial amyotrophic lateral sclerosis. Proc Natl Acad Sci USA 2000, 97:12571–12576
21. McKeith IG, Galasko D, Kosaka K, Perry EK, Dickson DW, Hansen LA, Salmon DP, Lowe J, Mirra SS, Byrne EJ, Lennox G, Quinn NP, Edwardson JA, Ince PG, Bergeron C, Burns A, Miller BL, Lovestone S, Collerton D, Jansen EN, Ballard C, de Vos RA, Wilcock GK, Jellinger KA, Perry RH: Consensus guidelines for the clinical and pathologic diagnosis of dementia with Lewy bodies (DLB): report of the Consortium on DLB International Workshop. Neurology 1996, 47:1113–1124
22. Gilman S, Low PA, Quinn N, Albanese A, Ben-Shlomo Y, Fowler CJ, Kaufmann H, Klockgether T, Lang AE, Lantos PL, Litvan I, Mathias CJ, Oliver E, Robertson D, Schatz I, Wenning GK: Consensus statement on the diagnosis of multiple system atrophy. J Neurol Sci 1999, 163:94–98
23. Brooks BR: El Escorial World Federation of Neurology criteria for the diagnosis of amyotrophic lateral sclerosis. Subcommittee on Motor Neuron Diseases/Amyotrophic Lateral Sclerosis of the World Federation of Neurology Research Group on Neuromuscular Diseases and the El Escorial "Clinical Limits of Amyotrophic Lateral Sclerosis" Workshop Contributors. J Neurol Sci 1994, 124:S96–S107
24. Hishikawa N, Hashizume Y, Yoshida M, Sobue G: Widespread occurrence of argyrophilic glial inclusions in Parkinson's disease. Neuropathol Appl Neurobiol 2001, 27:362–372
25. Li M, Nakagomi Y, Kobayashi Y, Merry DE, Tanaka F, Doyu M, Mitsuma T, Hashizume Y, Fischbeck KH, Sobue G: Nonneural nuclear inclusions of androgen receptor protein in spinal and bulbar muscular atrophy. Am J Pathol 1998, 153:695–701
26. Li M, Miwa S, Kobayashi Y, Merry DE, Yamamoto M, Tanaka F, Doyu M, Hashizume Y, Fischbeck KH, Sobue G: Nuclear inclusions of the androgen receptor protein in spinal and bulbar muscular atrophy. Ann Neurol 1998, 44:249–254
27. Wang J, Xu G, Borchelt DR: High molecular weight complexes of mutant superoxide dismutase 1: age-dependent and tissue-specific accumulation. Neurobiol Dis 2002, 9:139–148
28. Lee M, Hyun D, Halliwell B, Jenner P: Effect of the overexpression of wild-type or mutant α-synuclein on cell susceptibility to insult. J Neurochem 2001, 76:998–1009
29. Parraga M, del Mazo J: XYbp, a novel RING-finger protein, is a component of the XY body of spermatocytes and centrosomes. Mech Dev 2000, 90:95–101
30. Schlossmacher MG, Frosch MP, Gai WP, Medina M, Sharma N, Forno L, Ochiishi T, Shimura H, Sharon R, Hattori N, Langston JW, Mizuno Y, Hyman BT, Selkoe DJ, Kosik KS: Parkin localizes to the Lewy bodies of Parkinson disease and dementia with Lewy bodies. Am J Pathol 2002, 160:1655–1667
31. Imai Y, Soda M, Inoue H, Hattori N, Mizuno Y, Takahashi R: An unfolded putative transmembrane polypeptide, which can lead to endoplasmic reticulum stress, is a substrate of Parkin. Cell 2001, 105:891–902
32. Imai Y, Soda M, Hatakeyama S, Akagi T, Hashikawa T, Nakayama KI, Takahashi R: CHIP is associated with Parkin, a gene responsible for familial Parkinson's disease, and enhances its ubiquitin ligase activity. Mol Cell 2002, 10:55–67
33. Gai WP, Yuan HX, Li XQ, Power JT, Blumbergs PC, Jensen PH: In situ and in vitro study of colocalization and segregation of alpha-synuclein, ubiquitin, and lipids in Lewy bodies. Exp Neurol 2000, 166:324–333
34. Takahashi H, Wakabayashi K: The cellular pathology of Parkinson's disease. Neuropathology 2001, 21:315–322
35. Scherzinger E, Lurz R, Turmaine M, Mangiarini L, Hollenbach B, Hasenbank R, Bates GP, Davies SW, Lehrach H, Wanker EE: Huntingtin-encoded polyglutamine expansions form amyloid-like protein aggregates in vitro and in vivo. Cell 1997, 90:549–558
36. Bailey CK, Andriola IF, Kampinga HH, Merry DE: Molecular chaperones enhance the degradation of expanded polyglutamine repeat androgen receptor in a cellular model of spinal and bulbar muscular atrophy. Hum Mol Genet 2002, 11:515–523
37. Baba M, Nakajo S, Tu PH, Tomita T, Nakaya K, Lee VM, Trojanowski JQ, Iwatsubo T: Aggregation of α-synuclein in Lewy bodies of sporadic Parkinson's disease and dementia with Lewy bodies. Am J Pathol 1998, 152:879–884
38. Dickson DW, Liu W, Hardy J, Farrer M, Mehta N, Uitti R, Mark M, Zimmerman T, Golbe L, Sage J, Sima A, D'Amato C, Albin R, Gilman S, Yen SH: Widespread alterations of α-synuclein in multiple system atrophy. Am J Pathol 1999, 155:1241–1251
39. Spillantini MG, Crowther RA, Jakes R, Hasegawa M, Goedert M: α-Synuclein in filamentous inclusions of Lewy bodies from Parkinson's disease and dementia with Lewy bodies. Proc Natl Acad Sci USA 1998, 95:6469–6473