sensitive assays for anti-human AQP4 antibody using human AQP4-transfected human embryonic kidney 293 cells as substrates of the indirect immunofluorescence assay. The highly specific NMO-IgG and anti-AQP4 antibody for NMO should allow early detection of longitudinally extensive myelitis (LEM) without optic involvement, or optic neuritis without spinal cord involvement, with stringent diagnostic accuracy.

A few patients with LEM and seropositivity of anti-AQP4 antibody without optic involvement, corresponding to a limited form of NMO with myelitis (limited NMO [MY]), have been described previously.[8-11] However, details of the clinicopathologic features and long-term course remain elusive. We therefore investigated 8 patients with limited NMO (MY) in comparison with 9 patients showing the definite form of NMO (definite NMO) using clinical, immunologic, radiologic, and pathologic data from biopsy and autopsy specimens. We clarified the homogeneity of characteristic features of NMO through long-term entire courses consisting of both the limited and definite form.

**METHODS Patients and diagnostic criteria.** We retrospectively reviewed the medical records of 306 consecutive patients (219 women, 87 men) between 1980 and 2008 at the multiple sclerosis (MS) clinic in the Department of Neurology at Niigata University Hospital. We stringently defined definite NMO cases as fulfilling all items of the 2006 NMO criteria[12] and limited NMO as either 1) optic neuritis with seropositivity for anti-AQP4 antibody, but without brain, brainstem, or spinal cord lesions (limited NMO [ON]); or 2) myelitis with seropositivity of anti-AQP4 antibody, but without optic nerve involvement (limited NMO [MY]). As a disease control, MS (n = 13) was defined as clinically definite MS according to the criteria of Poser et al.[13] and International Panel criteria for MS,[14,15] excluding definite NMO and limited NMO, between 2006 and 2008 at the MS clinic. The present study was approved by the institutional review board of the Niigata University School of Medicine, Niigata, Japan. Written informed consent was obtained from all patients or guardians of patients participating in the study.

**MRI examinations.** MRI was performed with 5-mm-thick slices using a 1.5-T scanner (GE Medical Systems, Milwaukee, WI). MRI scans were performed at the time of clinical relapse.

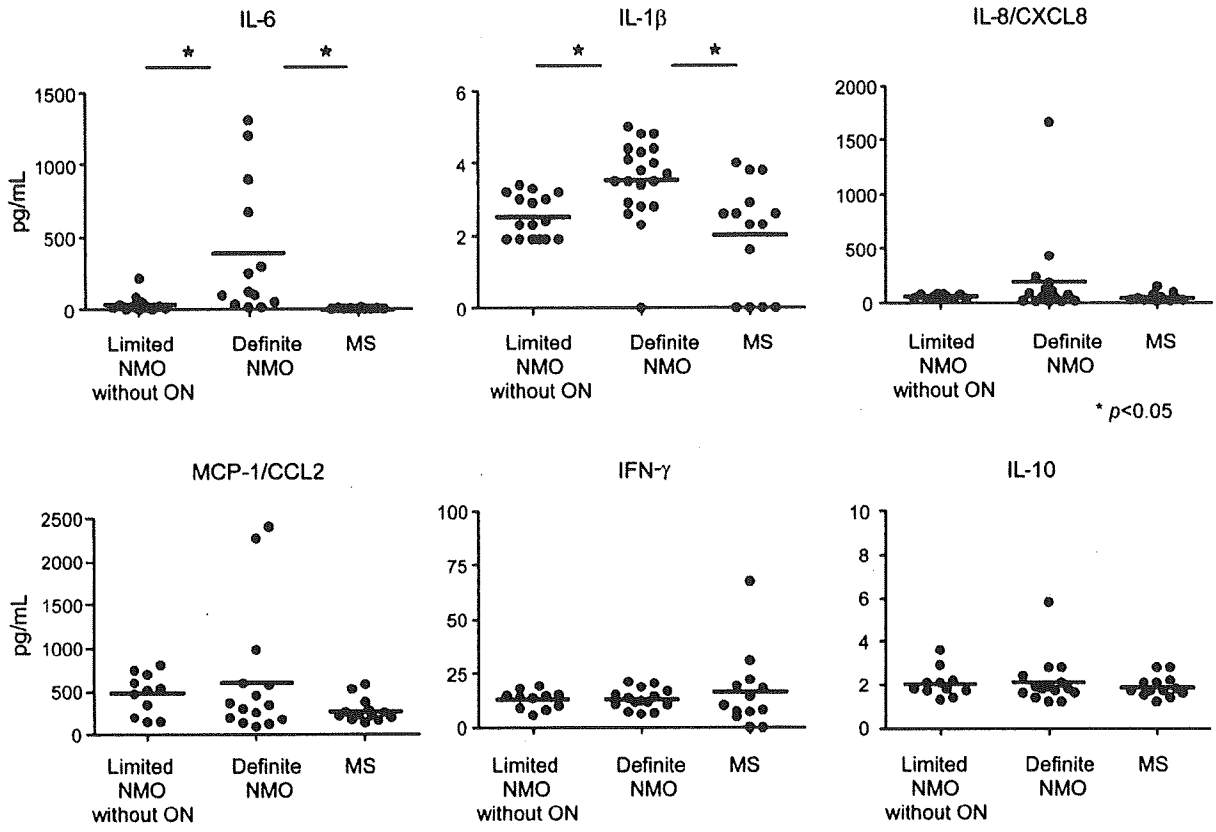
**Anti-AQP4 antibody assay in sera and profiles of cytokines and chemokines in CSF.** We examined anti-AQP4 antibody using the method described in our previous report[3] and titrated specimens in sera. CSF supernatants were analyzed simultaneously for 14 different cytokines and chemokines, namely, interleukin (IL)-1β; IL-2; IL-4; IL-5; IL-6; IL-8/CXCL8; IL-10; IL-12p70; interferon (IFN)-γ; tumor necrosis factor (TNF)-α; regulated on activation, normal T-cell expressed, and secreted (RANTES)/CCL5; monokine induced by IFN-γ (MIG)/CXCL9; monocyte chemoattractant protein (MCP)-1/CCL2; and IFN-inducible protein (IP)-10/CXCL10, using BD™ cytometric bead arrays (BD Pharmingen, San Diego, CA) according to the instructions from the manufacturer.

**Neuropathologic techniques and immunohistochemistry.** The study was performed on brain, optic nerve, and spinal cord materials from 1 patient (case 15) with definite NMO at autopsy and 1 patient (case 2) with limited NMO (MY) at diagnostic spinal cord biopsy. Materials were processed for 4-μm-thick, paraffin-embedded slides. Sections were stained with hematoxylin & eosin and Klüver-Barrera (KB). Immunohistochemistry was performed without modification using avidin-biotinylated enzyme complex (Vectastain; Vector Laboratories, Burlingame, CA). Primary antibodies were specific for myelin basic protein (MBP; DAKO, Glostrup, Denmark), neurofilament protein (NF/SMI-31; Sternberger Monoclonals, Baltimore, MD), glial fibrillary acidic protein (GFAP; DAKO) for astrocytes, and AQP4 (Chemicon, Temecula, CA). Diaminobenzidine was used as the chromogen. Selected sections were counterstained with a filtered solution of hematoxylin (blue).

**Statistical analyses.** We compared clinical features of the diagnostic categories and subgroups, such as patients with limited NMO and definite NMO. Statistical analyses between the 2 subgroups of limited NMO and definite NMO were performed using the Mann-Whitney $U$ test or Fisher exact probability test, as appropriate. Statistical analyses among 3 subgroups of 1) limited NMO, 2) early phase with myelitis exclusively, and 3) progressive phase with both myelitis and optic neuritis (with 2 and 3 extracted from definite NMO) were performed using analysis of variance, the Kruskal-Wallis H test, or the $\chi^2$ test. When significant results were obtained, multiple comparisons between each subgroup were performed using the Bonferroni, Bonferroni-Dunn, or Tukey multiple comparison test. Changes in Extended Disability Status Scale (EDSS) score at from relapse to remission were analyzed by the Wilcoxon signed-ranks test. All statistical analyses were considered significant for values of $p < 0.05$.

**RESULTS Demographics, age at onset, disease duration, and index events.** All patients were Japanese in our series. A female predominance was seen for both limited NMO (MY) (male/female = 0/8) and definite NMO (male/female = 1/8) (table e-1 on the *Neurology*® Web site at www.neurology.org). The median age at disease onset in the limited NMO (MY) cohort (44.5 years) was older than that in definite NMO cohort (24.0 years). In our series, the duration of the disease for limited NMO (MY) (mean ± SD, 4.8 ± 4.5 years) was significantly shorter than that for definite NMO (16.7 ± 10.5 years), and the number of exacerbations of limited NMO (MY) (2.1 ± 1.3) was significantly fewer than that of definite NMO (9.1 ± 5.5). Limited NMO (MY) showed either a recurrent (n = 4) or a monophasic (n = 4) course, whereas all cases of definite NMO had recurrent courses (n = 9) (figure e-1). Meanwhile, annual relapse rates did not differ between limited NMO (MY) (0.5 ± 0.3/y) and definite NMO (0.6 ± 0.3/y). Even if we retrospectively

**Figure 1** Cytokine and chemokine levels in CSF from patients with limited NMO (MY), definite NMO, and MS at the nadir of attacks, assessed by the multiplexed fluorescent bead-based immunoassay



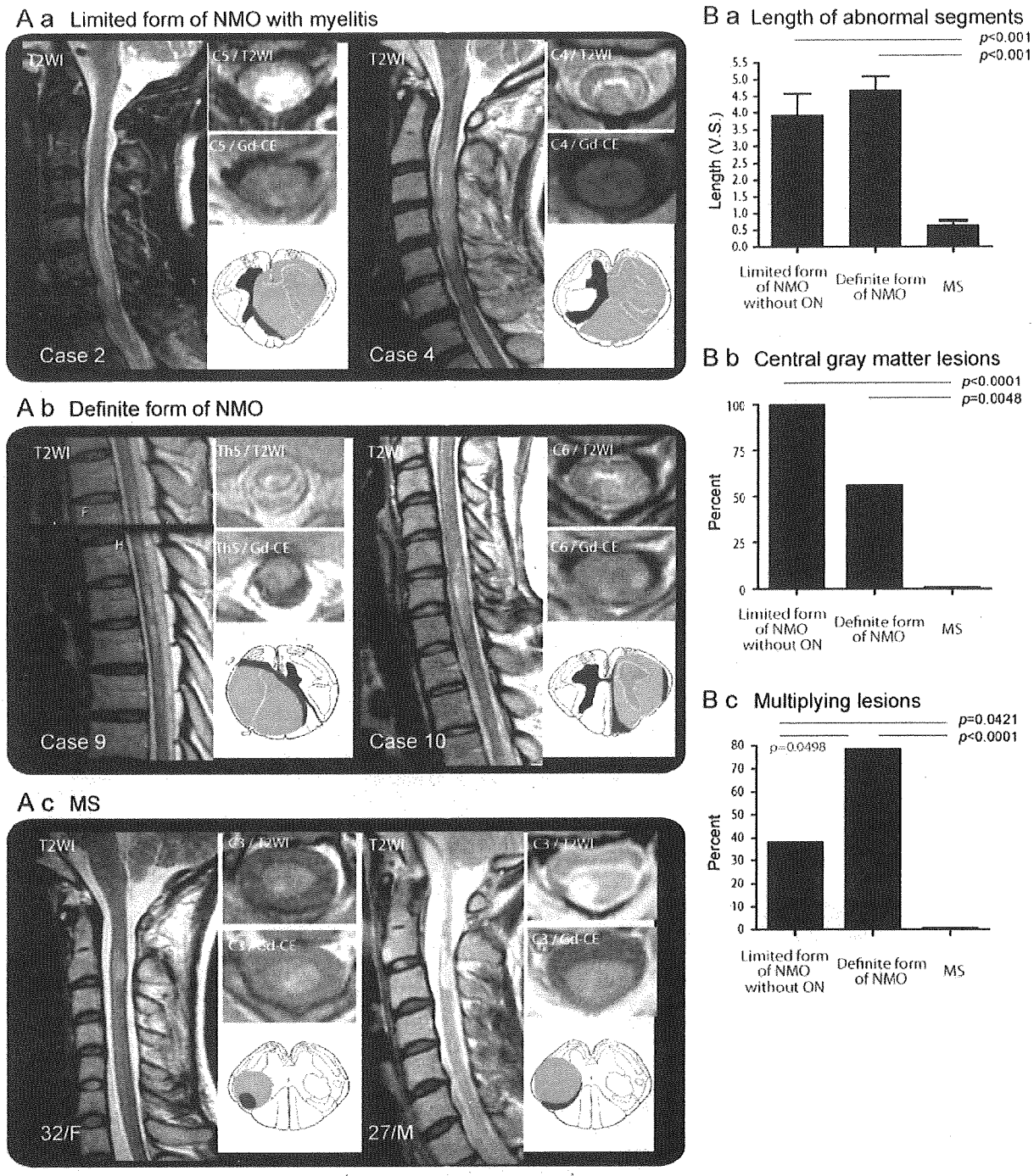

We determined that definite neuromyelitis optica (NMO) at the nadir of relapses showed significantly higher levels of interleukin (IL)-1β and IL-6 inflammatory cytokines in CSF than the limited form of NMO with myelitis (limited NMO [MY]) and multiple sclerosis (MS). The definite form of NMO (definite NMO) also tended to display higher levels of IL-8/CXCL8 in CSF than limited NMO (MY) and MS, but not significantly so. Other cytokine and chemokine levels, including IL-2; IL-4; IL-5; IL-10; IL-12p70; interferon (IFN)-γ; tumor necrosis factor (TNF)-α; regulated on activation, normal T-cell expressed, and secreted (RANTES)/CCL5; monokine induced by IFN-γ (MIG)/CXCL9; and IFN-inducible protein (IP)-10/CXCL10 did not differ between groups (data not shown). Bars indicate the mean for each group. ON = optic neuritis; MCP = monocyte chemoattractant protein.

categorized the definite NMO group as early phase with only myelitis or complete phase with both myelitis and optic neuritis, no differences in annual relapse rates were recognized depending on disease phase, and no subjects showed a secondary progressive clinical course in limited NMO (MY) or definite NMO. Relapses and disease progression phenotypes of limited NMO (MY) and definite NMO were similar.

**Serologic and CSF findings.** All cases of limited NMO (MY) (100.0%) and most cases of definite NMO (88.9%) showed seropositivity for anti-AQP4 antibodies in sera (table e-1). No patients with limited NMO (MY) or definite NMO had marked pleocytosis including neutrophilia in CSF. Immunoglobulin (Ig) G index and albumin leakage in CSF[16] were slightly increased in both limited NMO (MY) and definite NMO, compared with disease control subjects. Moreover, we determined that definite NMO at the nadir of relapses displayed significantly higher amounts of the inflammatory cytokines IL-1β and IL-6 in CSF than limited NMO (MY) and MS (figure 1).

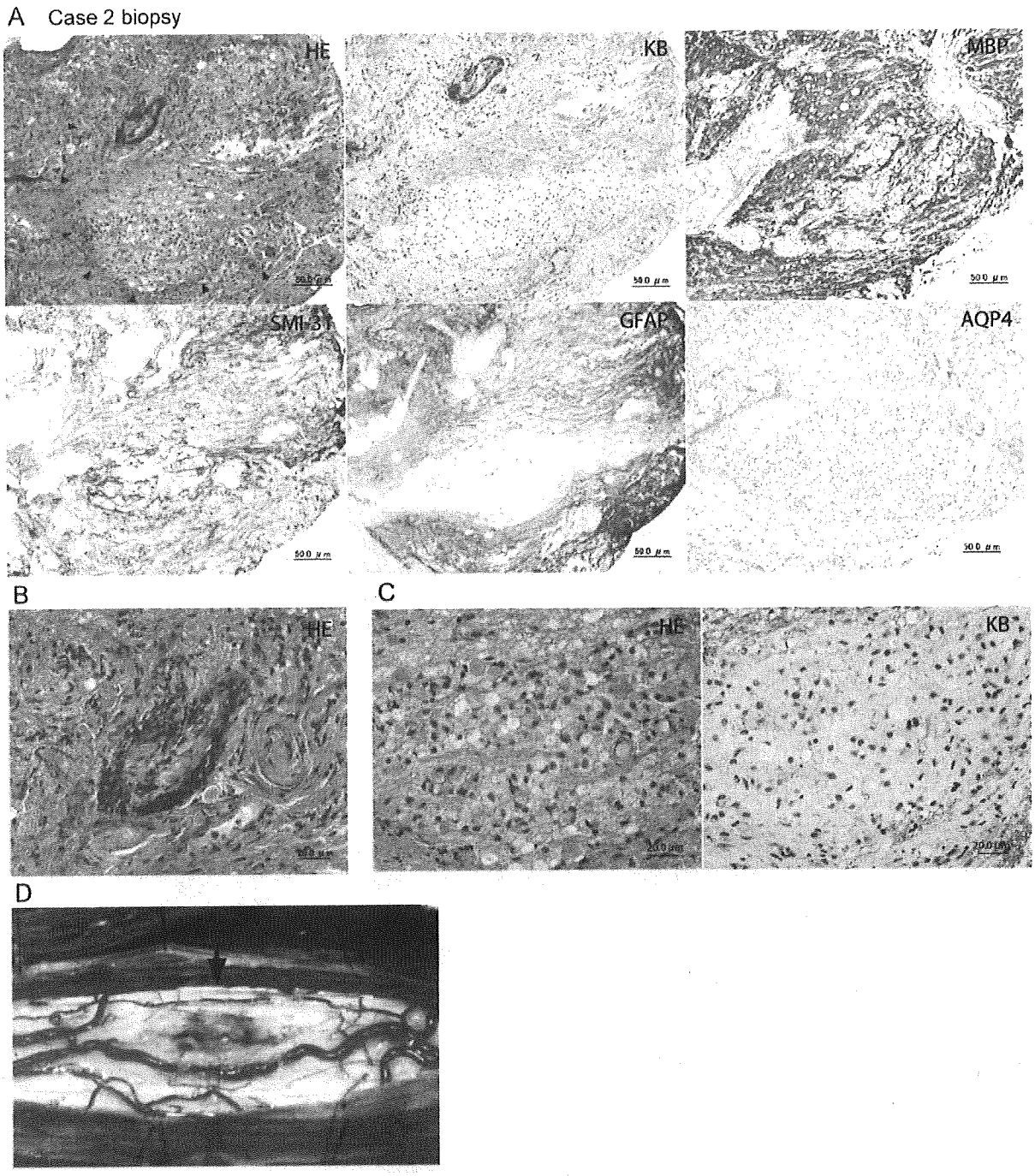
**MRI findings.** No patients with MS, 75.0% of patients with limited NMO (MY), and 77.8% of patients with definite NMO showed LEM (≥3 vertebral segments) on MRI (table e-2). In regard to characteristic figures on axial images, a central gray matter–predominant lesion was revealed as an H-shape on T2-weighted axial imaging, based on the anatomic structure of central gray matter in the spinal cord (figure 2, A and B, and figure e-2). All limited NMO (MY) cases (100.0%) and half of definite NMO cases (55.6%) had this H-shaped sign at the nadir of attacks, whereas no cases of classic MS showed the sign. Moreover, we also recognized a subpial peripheral white matter lesion in both limited and definite NMO on axial images (figures 2A and

Figure 2 MRI findings for the limited form of NMO with myelitis, definite NMO, and MS



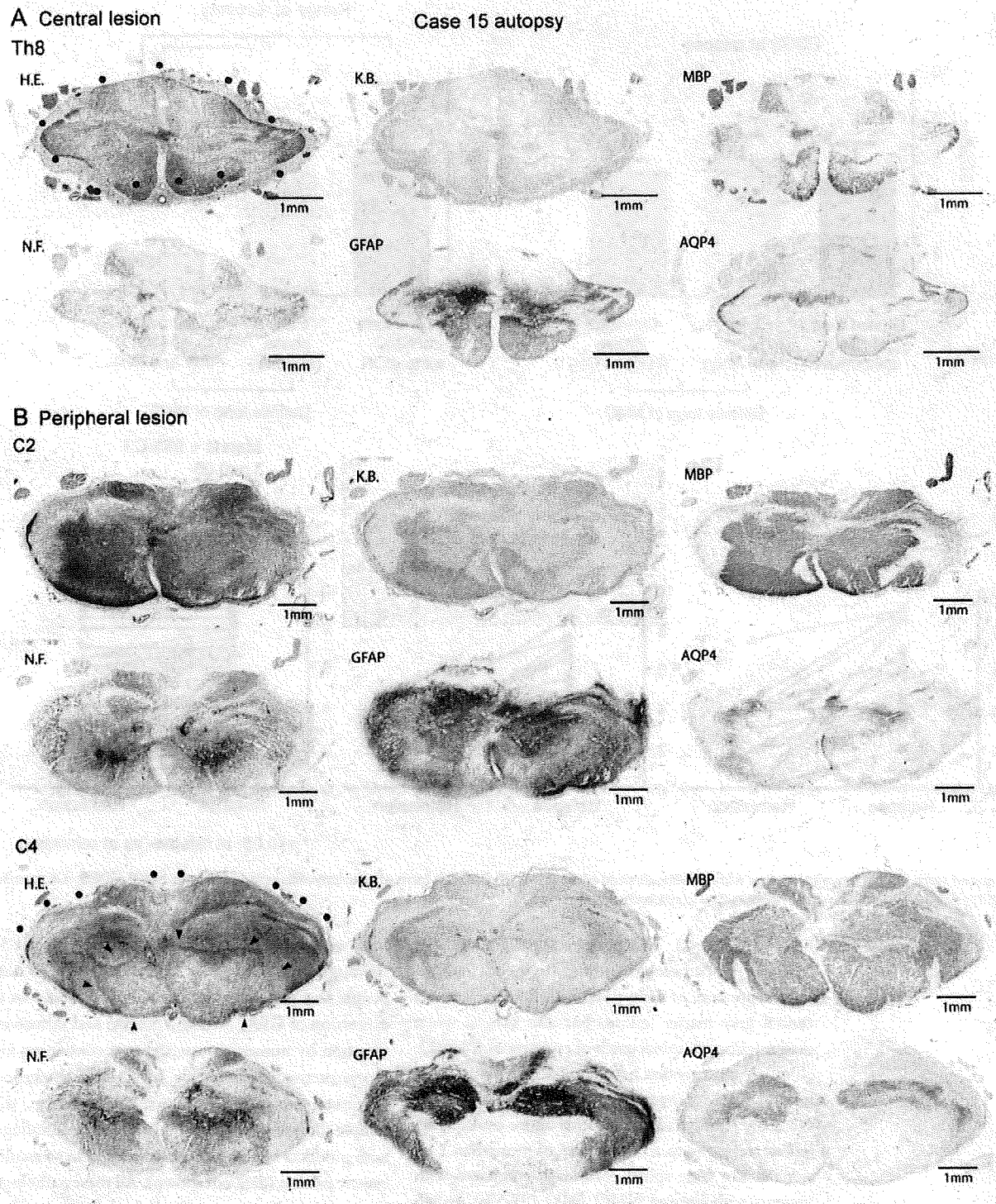

(A) MRI findings for the limited form of neuromyelitis optica (NMO) with myelitis (cases 2 and 4; A, a), definite neuromyelitis optica (cases 9 and 10; A, b), and multiple sclerosis (MS; a 32-year-old woman and a 27-year-old man; A, c). Regions in lower right corners: red indicates abnormal gadolinium-enhancement; yellow indicates abnormal hyperintensity on T2-weighted imaging (T2WI) with subpial peripheral white matter lesions; and blue indicates central gray matter-predominant legions, showing an H-shape on T2-weighted axial imaging, based on the anatomic structure of central gray matter of the spinal cord. These MRI images were representative for each group. (B) Summary of MRI findings for the limited form of NMO with myelitis (limited NMO [MY]; n = 8), definite NMO (n = 9), and MS (n = 13). Patients with limited or definite NMO had significantly longer length of abnormal segments (B, a) than patients with MS. In regard to characteristic figures on axial images, a central gray matter-predominant lesion (B, b) was revealed as an H-shape on T2-weighted axial imaging, based on the anatomic structure of central gray matter in the spinal cord (A, a–c). All cases of limited NMO (MY) (100.0%) and half of definite NMO cases (55.6%) had this H-shaped sign at the nadir of attacks, whereas no cases of classic MS showed the sign. Moreover, we confirmed that definite NMO (77.8%) had significantly more multiplying lesions (B, c), referring to accumulation of more than 2 lesions at the same spinal cord level, compared with patients with limited NMO (MY) (37.5%) or MS (0%).

Figure 3 Macroscopic and pathologic findings from a patient with limited form of neuromyelitis optica with myelitis



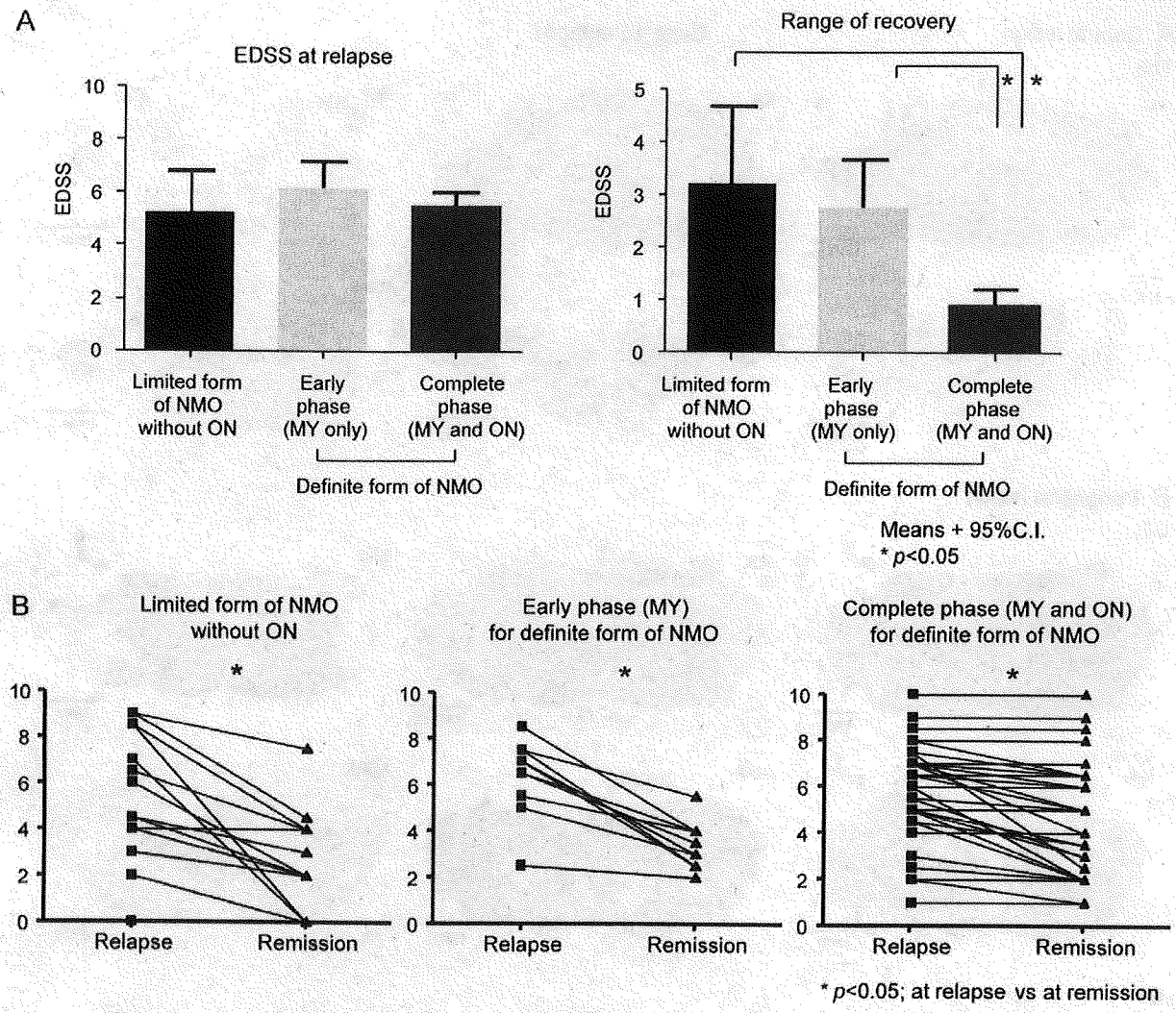

A woman (case 2; A) with Brown-Séquard syndrome at onset, who was 65 years old at onset, presented with progressive neurologic deficits involving both gray and white matter of the spinal cord at the C4 level over 17 days. Because such a progressive course over several weeks was unusual for neuromyelitis optica (NMO),[17] the possibility of spinal cord tumor with malignant features needed to be excluded, and she underwent surgery for biopsy of spinal cord lesions at the posterior funiculus. Macroscopically, the posterior funiculus of the spinal cord was edematous with brownish coloration at the open biopsy (D, arrow). Spinal cord biopsy specimens from a patient with limited NMO with myelitis demonstrated early active demyelinating lesions (arrowheads), with demyelination of a plaque (Klüver-Barrera [KB] staining) and relatively preserved axons (SMI-31 staining), extensive loss of aquaporin-4 (AQP4) expression in both plaques and periplaque white matter, limited loss of expression of glial fibrillary acidic protein (GFAP) within plaques, and diffuse infiltration by numerous macrophages (C) containing immunoreactive products for myelin basic protein (MBP) with thickened, hyalinized blood vessels (B). These findings from biopsy specimens of limited NMO with myelitis mirror the definite form of NMO (figure 4). Subsequently, anti-AQP4 antibody showed positive results in serum. Limited NMO (MY) was diagnosed. The patient was able to walk with a cane after repeated high-dose IV methylprednisolone therapy and tacrolimus (3 mg/d) without relapses. HE = hematoxylin & eosin.

**Figure 4** Pathologic findings from a patient with definite form of neuromyelitis optica




Demyelinating lesions exist not only in central gray matter lesions (A) but also in peripheral white matter lesions (B) in definite neuromyelitis optica (NMO), although most previous reports have emphasized only central spinal cord lesions in NMO. This finding mirrored MRI findings in both limited and definite forms of NMO (figure 2, A and B, and table e-2). Furthermore, we recognized selective loss of both aquaporin-4 (AQP4) and glial fibrillary acidic protein (GFAP) immunoreactivity in early active demyelinating NMO lesions with preserved myelin basic protein (MBP) staining (arrows in B). However, loss of GFAP immunoreactivity was always considerably smaller than loss of AQP4 in our series (arrows). Inactive NMO lesions (dots) indicate selective loss of AQP4 immunoreactivity, although GFAP staining was inconsistent. These data suggest loss of AQP4 immunoreactivity as a prominent feature through all NMO stages. HE = hematoxylin & eosin; KB = Klüver-Barrera; NF = neurofilament protein.

**Figure 5** Responsiveness to immunotherapy for patients with limited and definite forms of neuromyelitis optica




(A) Limited form of neuromyelitis optica with myelitis (limited NMO [MY]). (B) Definite form of neuromyelitis optica (definite NMO). EDSS = Expanded Disability Status Scale; ON = optic neuritis; CI = confidence interval.

e-2 and table e-2). This peripheral pattern on MRI reflected the pathologic findings (figure 4), indicating the presence of demyelinating lesions in not only central gray matter lesions, but also subpial white matter lesions in spinal cords of patients with NMO, although most reports have emphasized only central lesions.[17,18] Moreover, we confirmed that definite NMO (77.8%) had significantly more multiplying lesions, referring to accumulation of more than 2 lesions at the same spinal cord level, compared with patients with limited NMO (MY) (37.5%) or MS (0%) (figure 2B and table e-2).

**Pathologic findings.** Spinal cord biopsy specimens of limited NMO (MY) (case 2) with early active demyelinating lesions, classified as previously described,[19] demonstrated demyelination of a plaque (KB staining) with relatively preserved axons (NF/SMI-31 staining), extensive loss of AQP4 expression in both plaque and periplaque white matter, limited loss of expression of GFAP within a plaque, and diffuse infiltration by numerous macrophages containing immunoreactive products for MBP with thickened, hyalinized blood vessels (figure 3). Moreover, this sample showed deposits of IgG and IgM colocalizing with products of complement activation in a vasculocentric pattern (data not shown). All these pathologic findings in a biopsy sample derived from a patient with limited NMO (MY) (figure 3) were consistent with autopsy materials derived from patients with definite NMO (case 15) (figure 4). Although the duration from initial symptoms of a patient with definite NMO (case 15) was 28 years at the autopsy, he

still kept both a high amount of inflammatory cytokines in CSF (figure 1) and a lot of active inflammatory demyelinating lesions in the spinal cord (figure 4), optic nerves, and brain. These findings indicated that NMO has continuous pathogenic effector immune reactions until the terminal stage.

**Impairment through the entire course of limited NMO.** As previously reported,[2,17,20] 80% to 90% of patients with definite NMO had relapsing episodes of optic neuritis and myelitis, and most attacks of definite NMO worsened over several days and then slowly and incompletely improved in the weeks or months after reaching maximum clinical deficit. However, treatment response, disease course, and long-term impairment in limited NMO (MY) with stringent diagnostic criteria or definite NMO in the earlier phase remain elusive. First, we compared variations in EDSS scores before and after high-dose IV methylprednisolone (HIMP; 1 g/d for 3 days) at each acute attack between limited NMO (MY) and definite NMO. EDSS scores at the nadir of relapse did not differ between limited NMO (MY) (mean ± SD, 5.2 ± 2.8) and definite NMO (5.3 ± 2.2) (figure 5 and table e-1). However, EDSS scores at remission after repeated HIMP differed significantly between limited NMO (MY) (2.5 ± 2.2) and definite NMO (4.2 ± 2.4). Degrees of EDSS score change before and after repeated HIMP in patients with limited NMO (MY) (3.2 ± 2.4) were higher than in definite NMO (1.2 ± 1.4). Moreover, we retrospectively divided the phase of definite NMO into early phase with only myelitis and complete phase with both myelitis and optic neuritis. EDSS score at relapse of early-phase definite NMO with only myelitis (6.2 ± 1.6) was similar to that in complete phase with both myelitis and optic neuritis (5.5 ± 2.1). However, degrees of EDSS score change before and after repeated HIMP in patients with definite NMO with only myelitis at the early phase (2.8 ± 1.4) were higher than with definite NMO at complete phase (0.9 ± 1.1). These data suggest that limited NMO (MY) or early-phase definite NMO with only myelitis shows better prognosis with treatment using IV steroid therapy than definite NMO.

**DISCUSSION** Our findings of clinical, pathologic, radiologic, and immunologic features indicated that NMO at the early stage shows a common pathogenesis to NMO at the complete stage, contrasting with large population-based classic MS natural history cohorts,[21-23] as described below.

First, with regard to clinical features, the extreme predominance of females, annual relapsing rates, and a uniform relapsing-remitting course through the entire disease course, but not a secondary progressive course, and concomitant serology of several autoimmune conditions showed the common features of both limited and definite NMO. In particular, extreme uniform disease courses for both limited and definite NMO, as previously reported for definite NMO,[20] differed considerably from the courses seen with large population-based MS natural history cohorts, because more than two-thirds of patients with relapsing-remitting MS (inflammatory phase with autoimmune reactions) eventually experience secondary progression as defined by gradual, unremitting clinical deterioration of neurologic function (degenerative phase with degeneration of both myelin sheath and underlying axon), with or without superimposed relapses.[21-23]

Second, all pathologic findings for both limited and definite NMO showed common features in terms of demyelinating lesions with myelin degradation products within numerous macrophages, pronounced perivascular deposition of immunoglobulins and complement,[24] pattern-specific loss of AQP4 immunoreactivity,[25,26] and relatively preserved MBP-stained myelinated fibers despite the loss of AQP4 and GFAP staining, as previously reported.[26] These findings thus strongly emphasized that limited NMO (MY) shows the same pathologic findings as definite NMO, independent of disease duration, on the basis of a common pathogenesis for complement-activating AQP4-specific autoantibodies as the initiator of the NMO lesion.[25]

Third, in regard to immunologic conditions, limited and definite NMO showed no differences in CSF abnormalities of pleocytosis, IgG index, albumin leakage, oligoclonal IgG bands, MBP, and serum abnormalities of seropositivity (anti-AQP4 antibodies) and other autoimmune status, including antinuclear antibodies, consistent with previous reports.[27,28] These data suggest that definite NMO and limited NMO (MY) throughout the entire course are associated with broad activation of humoral immunity including B-cell and plasma cell activations without the consumption of any complement in sera at the peak of disease activity.

Finally, in terms of radiologic findings, existence of LEM and the H sign on axial images, suggesting preferential involvement of spinal central gray matter, were consistent with previous pathologic and radiologic studies,[17,18] and subpial peripheral white matter lesions represent a common feature between limited NMO and definite NMO. Subpial peripheral white matter lesions in spinal cords on MRI (figure 2, A and B, table e-2, and figure e-2) and pathologic findings (figure 4) were marked for definite and limited NMO, although this finding has never been stressed in previous reports.[29] Such sub-

pial peripheral white matter lesions might be created by the prominently high expression of AQP4 in not only central gray matter,[26] but also white matter[25] within spinal cord tissues in contrast with brain. This finding might be caused by direct invasion of lymphocytes and macrophages derived from meningeal infiltration in NMO, and broader spectrum of immunogenic specificities for not only strict and limited target antigens such as AQP4, but also other antigens such as myelin.[29-32]

All these findings could be why NMO displays homogeneity of pathogenic effector immune mechanisms through the long-term course, while MS should be recognized as a heterogeneous 2-stage disease that could switch from inflammatory to degenerative phase.

In NMO, good prognosis after immunotherapy for patients with limited NMO or definite NMO in the early stage was suggested to be produced as follows. First, in terms of the results of accumulated lesions at the same spinal cord level, cases of definite NMO could display greater axonal injury and incrementally increased disability compared with limited NMO (figure 2B and table e-2). Second, we supposed that the most crucial points were large inflammatory responses on the basis of the same immunologic pathogenesis in definite NMO. Definite NMO at the nadir of relapses showed significantly higher amounts of IL-1$\beta$ and IL-6 in CSF, compared with limited NMO (MY) and MS (figure 1). These cytokines are reportedly important inflammatory cytokines influencing not only antigen-specific immune responses and inflammatory reactions, but also differentiation/induction of the $T_H$-17 lineage.[33] Definite NMO might have more inflammatory responses with more IL-17–producing cells, such as $T_H$-17 cells, and could more easily disrupt and pass through the blood-brain barrier by higher anti-AQP4 antibody titers compared with limited NMO. Larger inflammatory responses might produce larger lesions with more axonal damages and cavity formations in definite NMO compared with limited NMO.

Even though we analyzed limited and definite NMO using a uniform shorter period of <5 years (table e-3 and figure e-3), all findings including immunologic and other characteristic features for limited and definite NMO were consistent with data for the entire disease courses (figures 1, 2A, and 2B and tables e-1 and e-2). We therefore concluded that limited and definite NMO as described above exhibit distinct characteristic features, irrespective of disease duration.

In our series, IV corticosteroid therapy was commonly used as the initial treatment for acute attacks of NMO. Maintenance immunosuppressive therapy for reducing relapses of NMO was performed using a combination of oral prednisone and immunosuppressive agents, including azathioprine and tacrolimus. Recent reports have described long-term treatment with oral agents such as azathioprine[34] or mycophenolate mofetil[35] for patients with relatively mild disease, and rituximab[36] for those with treatment-refractory disease. We agree with the previously proposed concept[8] that patients with LEM and seropositivity for NMO-IgG should be treated for a minimum of 5 years, because the risk of "conversion" to NMO seems highest in the first 5 years based on a prior clinic-based series of NMO,[2] consistent with our data indicating that conversion from limited NMO with myelitis to definite NMO happened at a mean of 2.9 ± 1.5 years from onset (table e-1). Early detection and start of immunotherapy at limited NMO would be key to the long term-prognosis of patients with NMO.

We have documented 2 basic characteristic phenotypes in the NMO spectrum: a limited form of NMO as an early type and a definite form of NMO as a complete type. Based on not only high specificity of NMO-IgG but also characteristic homogeneity of pathogenic effector immune mechanism, we emphasized that NMO should be recognized as a distinct pathologic entity with a fundamentally different etiology from MS with heterogeneous 2-stage disease.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# Depletion of medullary serotonergic neurons in patients with multiple system atrophy who succumbed to sudden death

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**Multiple system atrophy (MSA) is a neurodegenerative disorder characterized by prominent autonomic failure with ataxia and/or parkinsonism. The leading cause of death in MSA is sudden death. We have shown that the early development of autonomic failure is an independent risk factor for sudden death. The depletion of sympathetic preganglionic neurons in the spinal intermediolateral cell column (IML) and its afferent medullary catecholaminergic and serotonergic neurons has been proposed to be partly responsible for autonomic failure in MSA. In this study, we investigated whether the depletion of neurons in any of these autonomic neuron groups contributes to sudden death in MSA. Out of 52 autopsy-proven patients with MSA, we selected 12 individuals who had died within 3.5 years after disease onset to define the accurate levels of slices and identify early neuropathological changes of autonomic nuclei in MSA. Four patients succumbed to sudden death and eight patients died through established causes. Serial 10 μm sections were obtained from the 8th segment of the thoracic cord and the rostral medulla oblongata. Sections from the medulla oblongata were immunostained for thyrosine hydroxylase and tryptophan hydroxylase. The total cell number in the five sections was computed for comparison. Compared with the control, the MSA group showed a marked depletion of neurons in the IML ($38.0 \pm 7.1$ versus $75.2 \pm 7.6$ cells, $P < 0.001$), thyrosine hydroxylase-immunoreactive neurons in the ventrolateral medulla (VLM) ($17.4 \pm 5.1$ versus $72.8 \pm 13.6$ cells, $P < 0.01$) and tryptophan hydroxylase-immunoreactive neurons in the VLM ($15.6 \pm 9.2$ versus $60.8 \pm 17.0$ cells, $P < 0.01$), nucleus raphe obscurus ($19.3 \pm 4.4$ versus $75.3 \pm 8.6$ cells, $P < 0.001$), nucleus raphe pallidus ($2.1 \pm 2.7$ versus $9.0 \pm 3.4$ cells, $P < 0.03$), and arcuate nucleus ($0.4 \pm 0.8$ versus $2.3 \pm 1.5$ cells, $P < 0.05$). Moreover, in patients who succumbed to sudden death, when compared with patients who had established causes of death, we found a marked depletion of tryptophan hydroxylase-immunoreactive neurons in the VLM ($7.3 \pm 3.5$ versus $21.8 \pm 6.5$ cells, $P < 0.02$) and nucleus raphe obscurus ($15.0 \pm 2.0$ versus $22.5 \pm 2.1$ cells, $P < 0.01$). The results indicate that the spinal IML and medullary catecholaminergic and serotonergic systems are involved even in the early stages of MSA, and the dysfunction of the medullary serotonergic system regulating cardiovascular and respiratory systems could be responsible for sudden death in patients with MSA.**

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Abbreviations: IML = intermediolateral cell column; MSA = multiple system atrophy; TH = tyrosine hydroxylase; TrOH = tryptophan hydroxylase; VLM = ventrolateral medulla

# Introduction

Multiple system atrophy (MSA) is a progressive and incurable neurodegenerative disorder characterized by prominent autonomic failure with ataxia and/or parkinsonism (Wenning *et al.*, 2004). The average disease duration is within 9 years (Watanabe *et al.*, 2002; Tada *et al.*, 2007; O'Sullivan *et al.*, 2008; Schrag *et al.*, 2008), which is shorter than that of idiopathic Parkinson's disease and hereditary ataxias (Klockgether *et al.*, 1998). The leading cause of death in patients with MSA is sudden death, which has been documented in over a quarter of patients with MSA, and frequently occurs during sleep (Papapetropoulos *et al.*, 2007; Tada *et al.*, 2007; Shimohata *et al.*, 2008). Sudden death can occur even in the early stages of MSA (Munschauer *et al.*, 1990). Although continuous positive airway pressure or tracheostomy has been proposed as a therapeutic approach to prevent the obstruction of the upper airway, it is still unclear whether these approaches are effective in preventing sudden death (Iranzo *et al.*, 2004; Shimohata *et al.*, 2006; Jin *et al.*, 2007; Tada *et al.*, 2007). The pathogenesis underlying the episode of MSA should be elucidated to help develop an appropriate therapeutic strategy for preventing sudden death.

Laryngeal stridor is an important and frequently observed clinical manifestation in MSA (Isozaki *et al.*, 1996). The obstruction of the upper airway due to impaired laryngeal function results in laryngeal stridor, and has been suggested to cause sudden death in patients with MSA. Sudden death, however, also occurs in patients who underwent tracheostomy to remove upper airway obstruction (Papapetropoulos *et al.*, 2007; Tada *et al.*, 2007; Shimohata *et al.*, 2008). Furthermore, we have shown that the presence of stridor is not a predictive factor for either sudden death or poor prognosis in patients with MSA (Tada *et al.*, 2007). These results indicate that the upper airway obstruction might not fully explain the mechanism of sudden death in patients with MSA.

Dysregulation of the autonomic function for the respiratory and cardiovascular systems is another hypothesis in the pathogenesis of sudden death. Patients with MSA exhibit impaired autonomic respiratory function, including central sleep apnea (Cormican *et al.*, 2004; Shimohata *et al.*, 2007), reduced chemosensitivity to hypoxia (Tsuda *et al.*, 2002) and dysrhythmic breathing (Shimohata *et al.*, 2007). In addition, autonomic cardiovascular dysregulation, including orthostatic hypotension, low RR variability and denervation supersensitivity of the vessels and heart, has been observed in patients with MSA (Sakakibara *et al.*, 2000; Deguchi *et al.*, 2004; Wenning *et al.*, 2004). Subclinical cardiovascular abnormalities have been observed even in the early stages of MSA (Sakakibara *et al.*, 2000). Pathologically, sympathetic ganglia and cardiac sympathetic nerves are well-preserved in MSA (Orimo *et al.*, 2008), and autonomic cardiovascular dysregulation may be caused by central rather than peripheral autonomic failure (Deguchi *et al.*, 2004; Wenning *et al.*, 2004). Along with other researchers, we have demonstrated that the early development of autonomic failure is an independent risk factor for poor prognosis or sudden death in patients with MSA (Watanabe *et al.*, 2002; Tada *et al.*, 2007; O'Sullivan *et al.*, 2008). From these results, it is possible that the dysfunction of the central autonomic nervous system regulating the cardiovascular and respiratory systems might result in sudden death among patients with MSA.

Neuropathological investigations of MSA have shown widespread neuronal cell loss in the central autonomic nuclei, including neurons in the spinal intermediolateral cell column (IML) (Oppenheimer, 1980; Gray *et al.*, 1988; Sakajiri *et al.*, 1996; Benarroch, 1999), catecholaminergic neurons in the ventrolateral medulla (VLM) (Benarroch *et al.*, 1998, 2005) and serotonergic neurons in the nucleus raphe magnus, nucleus raphe obscurus, nucleus raphe pallidus and VLM (Benarroch *et al.*, 2004, 2005). These medullary autonomic neurons project to the neurons in the IML and mediate sympathetic autonomic function (Strack *et al.*, 1989). However, it is still unclear whether the depletion of these neurons is responsible for sudden death in patients with MSA. This study seeks to determine whether neuronal cell loss in any or both of these groups contribute to sudden death among patients with MSA. We evaluated the number of these autonomic neurons in patients suffering from MSA, and made a comparison between patients who succumbed to sudden death and those who died through established causes.

# Materials and Methods

## Subjects

We reviewed the medical records of the 52 consecutive patients with pathologically confirmed MSA [31 male and 21 female: 33 patients with MSA with predominant cerebellar ataxia (MSA-C) and 19 patients with MSA with predominant parkinsonism (MSA-P) (Gilman *et al.*, 2008): age 66.6 ± 5.9 years] who were referred to the Brain Research Institute, University of Niigata, between 1970 and 2003. The relative predominance of the phenotype MSA-C over the MSA-P in this series was consistent with other large-scale clinical studies on the Japanese population (Watanabe *et al.*, 2002; Yabe *et al.*, 2006). Since the medulla oblongata of patients with long disease duration is severely atrophic, it was difficult to establish accurate medullary levels in the histological sections for quantification. Therefore, we retrieved 12 individuals (six male and six female: age 65.1 ± 5.9 years) in whom the disease duration was ≤3.5 years, corresponding to half the median survival period for the patients with MSA in general (7.0 years, range 1–19 years) or less, i.e. those with short disease duration. The prognosis of our patients seems relatively poor in comparison with some clinical studies on MSA (Wenning *et al.*, 1994; Testa *et al.*, 1996; Watanabe *et al.*, 2002; O'Sullivan *et al.*, 2008; Schrag *et al.*, 2008). This may reflect a case

**Table 1 Patient population**

| Case | Age/ sex | PMD (h) | Clinical phenotype | Initial manifestations | Autonomic manifestations | Disease duration (years) | Cause of death | Pathological diagnosis (phenotype) |
|---|---|---|---|---|---|---|---|---|
| Con 1[a,b] | 80/M | 3.0 | NA | NA | None | NA | Abdominal haemorrhage | Abdominal haemorrhage |
| Con 2[a,b] | 49/F | 3.0 | NA | NA | None | NA | Hepatic failure | Congenital myopathy |
| Con 3[a,b] | 76/M | 3.0 | NA | NA | None | NA | GI bleeding | Rhabdomyolysis |
| Con 4[a] | 57/F | 2.0 | NA | NA | None | NA | Pulmonary embolism | Dermatomyositis |
| Con 5[b] | 42/F | 6.5 | NA | NA | None | NA | Tetanus | Muscle necrosis |
| Con 6[b] | 70/F | 4.0 | NA | NA | None | NA | Cerebral embolism | Cerebral infarcts |
| SD 1[a,b] | 63/F | 4.5 | MSA-P | Parkinsonism | OH,[c] NB dyshidrosis | 3.0 | Sudden death | MSA (SND type) |
| SD 2[a,b] | 61/M | 4.0 | MSA-P | OH | OH,[c] NB, dyshidrosis | 2.0 | Sudden death | MSA (SND type) |
| SD 3[a,b] | 68/M | 2.5 | MSA-P | impotence | OH,[c] NB, impotence, dyshidrosis | 3.0 | Sudden death | MSA (SND type) |
| SD 4[b] | 68/F | 3.0 | MSA-P | OH | OH, NB | 3.0 | Sudden death | MSA (SND type) |
| Non-SD 1[a] | 69/M | 3.0 | MSA-C | Ataxia | None | 2.0 | GI bleeding | MSA (OPCA type) |
| Non-SD 2[a,b] | 70/F | 5.0 | MSA-C | Ataxia | OH, NB, dyshidrosis | 2.5 | Bronchopneumonia | MSA (SND = OPCA type) |
| Non-SD 3[a,b] | 71/M | 16.0 | MSA-P | Parkinsonism | NB | 1.0 | Bronchopneumonia | MSA (SND type) |
| Non-SD 4[a,b] | 65/F | 40.0 | MSA-P | Axial dystonia | None | 3.0 | Complete A-V blockade | MSA (SND type) |
| Non-SD 5[b] | 71/F | 2.0 | MSA-C | Ataxia | NB | 3.0 | Bronchopneumonia | MSA (OPCA type) |
| Non-SD 6[b] | 63/M | 3.5 | MSA-C | Ataxia | NB | 3.5 | Suffocation due to misswallowing | MSA (OPCA type) |
| Non-SD 7[b] | 50/M | na | MSA-C | NB | OH, NB, impotence, dyshidrosis | 3.0 | Malnutrition | MSA (OPCA type) |
| Non-SD 8 | 62/F | 2.0 | MSA-P | Parkinsonism | OH, NB, dyshidrosis | 3.0 | Bronchopneumonia | MSA (SND = OPCA type) |

Con = control; SD = group of patients who succumbed to sudden death; non-SD = group of patients whose causes of death were established; PMD = post-mortem delay; na = not available; NA = non-applicable; OH = orthostatic hypotension; NB = neurogenic bladder; GI = gastrointestinal.
a Cases adopted for quantitative analyses of tyrosine or tryptophan hydroxylase-immunoreactive cells in the medulla oblongata.
b Cases adopted for quantitative analyses of neurons in the spinal IML.
c With recurrent syncope.

collection bias. The present study was conducted on autopsied patients only, who were examined over the past three decades.

A summary of the clinical characteristics of each subject is shown in Table 1. Among the 12 selected patients, we identified four (two male and two female: age 65.0 ± 3.6 years) who succumbed to sudden death. Sudden death was defined as death occurring suddenly and unexpectedly in patients who had been stable before the event (Groh *et al.*, 2008), and when the cause of death could not be clarified by clinical examinations and general autopsy. Although detailed gross- and histopathological-examinations were performed in all patients, we failed to find any evidence indicating possible causes of death, such as suffocation, dissecting aneurysm of the aorta, severe pneumonia, septicemia, acute myocardial infarction, pulmonary embolism and subarachnoid haemorrhage. We classified these patients as the SD (sudden death) group. The other eight patients (four male and four female: age 65.1 ± 7.1 years) were defined as the non-SD group. There was no significant inter-group difference in the age at onset, gender or disease duration. All the patients except one had been included in a previous clinical study (Tada *et al.*, 2007). We also identified nine patients (six male and three female: age 68.7 ± 5.1 years) who had died suddenly and unexpectedly, in whom the disease duration was over 3.5 years; however, we did not include them in the present study.

Quantitative analyses of the IML neurons were performed in 10 patients with MSA (five male and five female: age 65.0 ± 6.4 years) and five age-matched controls (two male and three female: age 63.4 ± 16.9 years). Similarly, quantitative analyses of the medullary autonomic nuclei were performed in seven patients with MSA (four male and three female: age 66.7 ± 3.8 years) and four age-matched controls (two male and two female: age 65.5 ± 14.9 years), as indicated in Table 1. All the subjects in the control group had no history of neurological symptoms and had well-established causes of death.

## Pathologic methods

All brains and spinal cords were fixed in formalin. Tissue blocks from the frontal, temporal, parietal and occipital neocortices, basal ganglia, thalamus, amygdaloid nucleus, hippocampus, midbrain, pons, medulla oblongata and cerebellum were cut and subsequently embedded in paraffin. All the cases were assessed for neuronal and glial synuclein pathology using polyclonal rabbit antibody against α-synuclein, and fulfilled the pathologic criteria for MSA (Trojanowski and Revesz, 2007). Sections of 4-μm thickness stained with haematoxylin and eosin were used for the semi-quantitative analysis of neuronal cell loss in the striatonigral and olivopontocerebellar regions, using the method described previously (Ozawa *et al.*, 2004). The semi-quantitative analysis was carried out by one of the authors (M.T.), and reviewed by two other investigators (T.O. and A.K.) to ensure the consistency of evaluation.

For the quantitative analyses, 5 mm-thick transverse slices of the 8th segment of the thoracic cord and medulla oblongata at the level of the Olszewski and Baxter plate XIV (Olszewski and Baxter, 1982)

were prepared. The sections were embedded in paraffin, and serial 10 μm-thick sections were cut. Five sections, each separated by 100 μm, were subjected to Klüver–Barrera (K–B) staining. The other sets of five sections, each separated by 100 μm, were subjected to immunohistochemistry to identify catecholaminergic and serotonergic neurons in the medulla oblongata.

### Immunohistochemistry

Paraffin-embedded sections of the medulla oblongata were immunostained using primary monoclonal antibodies against tyrosine hydroxylase (TH) (clone TH-16, Sigma, Saint Louis, MO, USA; 1:2000) and tryptophan, tyrosine, and phenylalanine hydroxylases (clone PH8, PharMingen, San Diego, CA, USA; 1:500). A monoclonal antibody against phosphorylated α-synuclein (clone pSy#64, Wako, Osaka, Japan; 1:10 000) was also used. As PH8 binds to tryptophan hydroxylase (TrOH), but not to TH in paraffin-embedded human tissue, it can be used to identify serotonergic neurons (Haan *et al.*, 1987). Tissue sections were pre-treated in a microwave oven for 18 min in a 10-mM citrate buffer (pH 6.0) for TH and with formic acid for phosphorylated α-synuclein. Immunolabelling was detected using the avidin–biotin–peroxidase complex method (Vector, Burlingame, CA, USA), and visualized with diaminobenzidine/$H_2O_2$ solution. Counterstaining was carried out with Mayer's haematoxylin.

### Cell quantification and mapping

An investigator, who was blinded to the clinical and neuropathological diagnosis, performed the cell counts.

The IML was defined as a triangular area of grey matter in accordance with the method described by Oppenheimer (1980). Neurons were identified by the presence of Nissl substance. The number of neurons with nuclei in the bilateral IML was counted, and the total number in the five sections was computed for comparison.

TH-immunoreactive (ir) cells in the VLM and TrOH-ir cells in the VLM, nucleus raphe obscurus, nucleus raphe pallidus and arcuate nucleus were also counted. These areas were identified on the basis of the atlas of Paxinos and Huang (1995). Only immunolabelled cells with nuclei were mapped and counted to avoid the duplication of single cells in the count. At the level of the medulla oblongata adopted in the study, TH-ir cells were subdivided into two groups according to their spatial relationship with the nucleus ambiguus. Although tyrosine hydroxylase does not allow the discrimination of epinephrine-synthesizing neurons from the norepinephrine neurons, most of the TH-ir neurons located ventrolaterally to the nucleus ambiguus were considered as epinephrine-synthesizing neurons, and were treated as the 'C1' group (Pearson *et al.*, 1990). Only the number of neurons observed in this region was counted. We counted TH- or TrOH-ir cells in medulla oblongata bilaterally, and the total number in the five sections was computed for comparison. To demonstrate the distribution patterns of the TH- or TrOH-ir cells, all the cells observed in both the right and left sides of medulla oblongata in the five sections were mapped for three cases in each group (SD and non-SD groups and control).

### Statistical analysis

Data were analysed using SPSS version 11.5 software (SPSS Inc., Chicago, IL, USA). Cell numbers [mean ± standard deviation (SD)] were compared between the control and patients with MSA, and between the MSA SD and non-SD groups. To compare the neurons in the IML and TH- or TrOH-ir cells in the VLM and nucleus raphe obscurus, the Student's *t*-test was used; for TrOH-ir cells in the nucleus raphe pallidus and arcuate nucleus, the Mann–Whitney U-test was performed. The value of $P < 0.05$ was considered as significant.

# Results

## Clinicopathological features

All the four SD-group patients exhibited the clinical phenotype of MSA-P with severe autonomic dysfunction. In the non-SD group, five patients exhibited MSA-C phenotype and three patients exhibited MSA-P phenotype (Gilman *et al.*, 2008). Two of them showed no autonomic symptoms during life (Table 1). Two patients in the SD group underwent tracheostomy.

All the four brains in the SD group exhibited striatonigral degeneration (SND) type, whereas in the non-SD group, four of the eight patients showed olivopontocerebellar atrophy (OPCA) type, two showed the SND type, and two were considered to have similarly severe pathology in both the systems (SND = OPCA type). In all the cases, regardless of the clinical and pathological phenotypes, many α-synuclein-immunolabelled glial cytoplasmic inclusions were encountered in the medullary autonomic nuclei, and only a few were found in the IML (data not shown), consistent with the features of MSA.

## Neurons in the IML

In MSA, atrophy of various degrees in the IML was observed (Fig. 1A). There were significantly fewer neurons in the IML in the MSA group than in the control (38.0 ± 7.1 versus 75.2 ± 7.6 cells, $P < 0.001$) (Fig. 1B). This finding was common in all the patients with MSA, with a short disease duration. There was no significant difference in the number of IML neurons between the SD (33.8 ± 7.5 cells) and the non-SD group (40.8 ± 5.8 cells) (Fig. 1B).

## Neurons in the medulla oblongata

Examples of the medullary sections adopted are shown in Fig. 2A.

## Catecholaminergic neurons

TH-ir neurons were distributed in the intermediate reticular zone (IRt) (Fig. 2B and C), and the pattern was consistent with earlier descriptions (Paxinos *et al.*, 1990; Benarroch *et al.*, 1998). There were significantly fewer TH-ir neurons in the VLM in the MSA group than in the control (17.4 ± 5.1 versus 72.8 ± 13.6 cells, $P < 0.01$) (Fig. 2D). A marked loss of TH-ir neurons was also observed consistently in all patients with MSA, regardless of the age at death or post-mortem delay. There was no significant difference in the number of TH-ir neurons in the VLM between the SD and non-SD groups (14.7 ± 6.7 versus 19.5 ± 3.1 cells) (Fig. 2D).

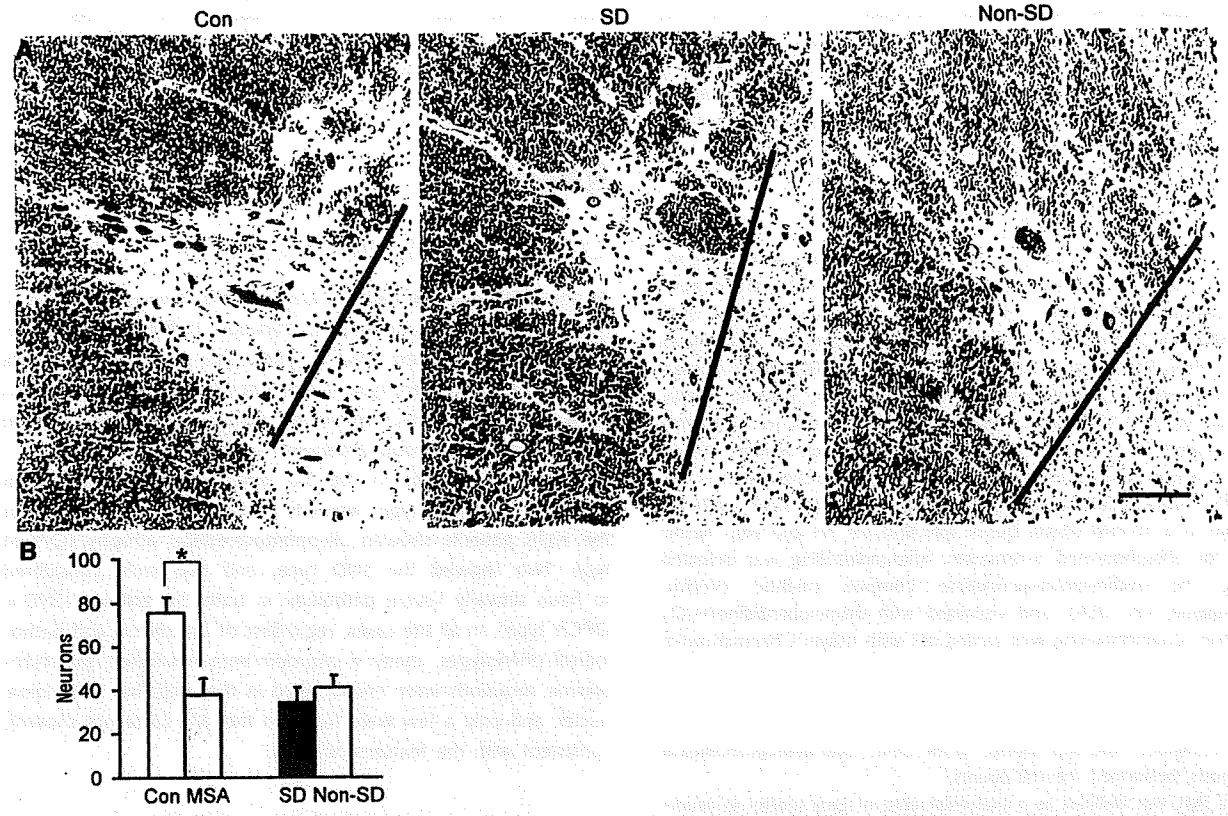


**Figure 1** (**A**) Photomicrographs of representative sections of the IML at the 8th segment of the thoracic cord in the control (Con), SD group (SD: patients with MSA who succumbed to sudden death) and non-SD group (non-SD: patients with MSA whose causes of death were established). Each panel was taken from a histological section of patients listed as Con 6, SD 1 and non-SD 3 in Table 1. The lateral area of the grey matter separated by an oblique line is defined as the IML; Klüver–Barrera (K–B) stained; Bar = 100 μm. (**B**) Mean (±SD) numbers of neurons in the IML on both sides. There is significant depletion in MSA compared with the control. *$P < 0.001$; $n$ = five for Con, ten for MSA, four for SD and six for non-SD.

Majority of the TrOH-ir neurons were identified at four locations, corresponding to the nucleus raphe obscurus, nucleus raphe pallidus, arcuate nucleus and VLM (Fig. 3A–C). There were significantly fewer TrOH-ir neurons in the VLM in the MSA group than in the control (15.6 ± 9.2 versus 60.8 ± 17.0 cells, $P < 0.01$), which was also observed in the nucleus raphe obscurus (19.3 ± 4.4 versus 75.3 ± 8.6 cells, $P < 0.001$) (Fig. 3D). Again, a marked loss of TrOH-ir neurons was also observed consistently in all patients with MSA, regardless of the age at death or post-mortem delay. Intriguingly, a comparison of the number of TrOH-ir neurons in the SD group with that in the non-SD group demonstrated a significant reduction in the former, both in the VLM (7.3 ± 3.5 versus 21.8 ± 6.5 cells, $P < 0.02$) and the nucleus raphe obscurus (15.0 ± 2.0 versus 22.5 ± 2.1 cells, $P < 0.01$) (Fig. 3D). Similarly, a marked reduction in the number of TrOH-ir neurons was observed in the MSA group compared with the control, both in the nucleus raphe pallidus (2.1 ± 2.7 versus 9.0 ± 3.4 cells, $P < 0.03$) and arcuate nucleus (0.4 ± 0.8 versus 2.3 ± 1.5 cells, $P < 0.05$) (Fig. 3D), although the TrOH-ir neurons in both the nuclei were sparse. Because of the scarcity of TrOH-ir neurons in patients with MSA, it is difficult to draw a statistical significance between the SD and non-SD groups based on the numbers of the nucleus raphe pallidus and arcuate nucleus. However, there is a tendency that TrOH-ir neurons are more reduced in the SD group than in the non-SD group, both in the nucleus raphe pallidus (0.7 ± 0.6 versus 3.3 ± 3.3 cells) and arcuate nucleus (0 versus 0.8 ± 1.0 cells) (Fig. 3D).

# Discussion

This study indicates a significant depletion of the serotonergic neurons in the VLM and nucleus raphe obscurus in patients with MSA who succumbed to sudden death, when compared with patients who died through well-established causes. On the contrary, neurons in the IML and the catecholaminergic neurons in the VLM are affected in close severity in both groups of patients with MSA. Although a depletion of medullary serotonergic neurons has already been reported in MSA (Benarroch *et al.*, 2004, 2005, 2007*a*), we have demonstrated that the depletion

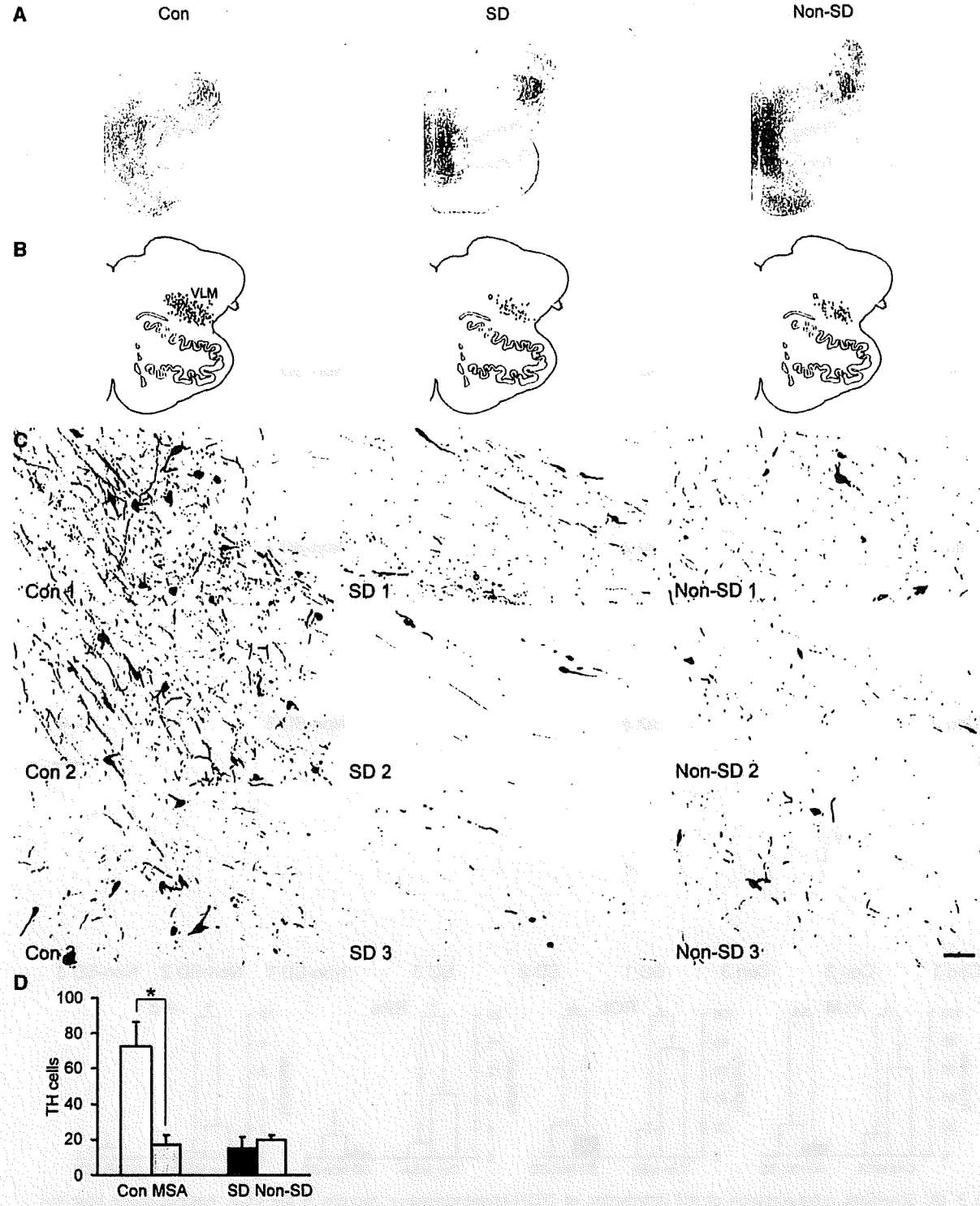


Figure 2 (A) Photographs of representative medullary sections of the control, SD and non-SD groups; K–B stained. (B) Schematic representation of the distribution of TH-immunoreactive neurons in the VLM in the control, SD and non-SD groups. Each dot represents the approximate position of a single neuron. To demonstrate the distribution patterns and to facilitate comparison, the image of the left half was inverted then placed on the right. (C) Examples of the VLM area in three patients each from the control, SD and non-SD groups (left, middle and right columns, respectively). The case number shown in each panel corresponds to that in Table 1. Note the apparent loss of immunolabelled neurons and fibres in patients with MSA. Bar = 100 μm. (D) Mean (±SD) numbers of TH-labelled neurons. The VLM catecholaminergic neurons in MSA are significantly fewer than those in the control. *$P < 0.01$; $n = 4$ for Con, seven for MSA, three for SD and four for non-SD.

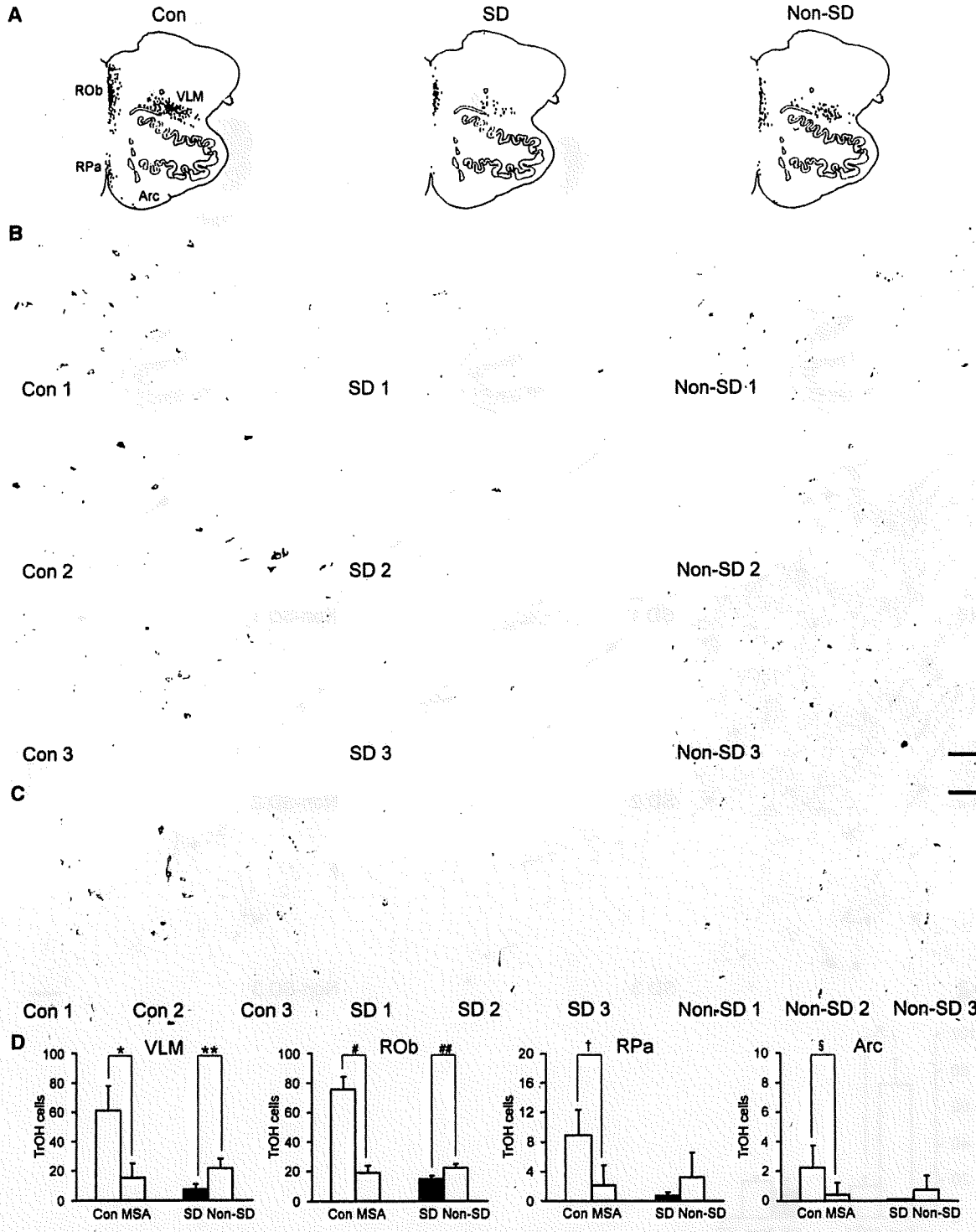


Figure 3 (A) Schematic representation of the distribution of TrOH-immunoreactive neurons in the VLM, nucleus raphe obscurus (ROb), nucleus raphe pallidus (RPa) and arcuate nucleus (Arc) in the control, SD and non-SD groups. Each dot represents the approximate position of a single neuron. To demonstrate the distribution patterns and to facilitate comparison, the image of the left half was inverted then placed on the right. (B and C) Image of areas including the VLM (B) or nucleus ROb (C), taken from three patients each in the control, SD and non-SD groups (left, middle and right columns, respectively), showing marked loss of immunolabelled cells in MSA. The remaining immunolabelled cells are atrophic. The case number shown in each panel corresponds to that in Table 1. Bar = 100 μm. (D) Mean (±SD) numbers of the TrOH-labelled neurons in the VLM, nucleus ROb, nucleus RPa and Arc. The numbers of serotonergic neurons in these areas in MSA are significantly lower than those in the control. A comparison between SD and non-SD groups shows that the numbers in the VLM and nucleus ROb are significantly smaller in the former than in the latter. $^{*}P<0.01$, $^{**}P<0.02$, $^{\#}P<0.001$, $^{\#\#}P<0.01$, $^{\dagger}P<0.03$, $^{\S}P<0.05$; $n=4$ for Con, seven for MSA, three for SD and four for non-SD.

of these neurons could be responsible for sudden death in patients with MSA.

It remains to be elucidated how the depletion of medullary serotonergic neurons causes sudden death. In experimental animals, it has been demonstrated that medullary serotonergic neurons project to many autonomic nuclei in the medulla oblongata and spinal cord, which then influence the sympathetic outflow as well as thermal, respiratory and cardiovascular regulation (Manaker and Tischler, 1993; Sun *et al.*, 2002; Raul, 2003; Jordan, 2005; Nason and Mason, 2006; Hodges *et al.*, 2008). Thus, the marked depletion of medullary serotonergic neurons could result in the impairment of these autonomic functions. In humans, although the precious functions of serotonergic neurons in each nucleus are still unclear, serotonergic neurons in the medullary raphe nuclei and arcuate nucleus have been suggested to be responsible for automatic breathing by enhancing respiratory response to hypercapnea and hypoxia (Feldman *et al.*, 2003; Richerson, 2004). Moreover, sudden infant death syndrome (SIDS) cases show a significantly lower density of 5-$HT_{1A}$ receptor binding sites as well as higher medullary serotonergic neuron count, indicating that the serotonin pathway plays a key role in the pathogenesis of SIDS (Paterson *et al.*, 2006). These findings support the notion that the dysfunction of medullary serotonergic neurons could be important in the pathogenesis of sudden death in patients with MSA.

However, we cannot exclude the possibility that other neuronal groups regulating the autonomic, respiratory and cardiovascular function might also be responsible for sudden death in patients with MSA. For example, the loss of neurokinin-1 receptor-like immunoreactive (NK-1R-LI) neurons in the VLM, which may correspond to the pre-Bötzinger complex, has been known in MSA (Benarroch *et al.*, 2003). The neurons have been proposed to play a critical role in respiratory rhythmogenesis in experimental animals (Smith *et al.*, 1991; Gray *et al.*, 1999). A possible association between the loss of NK-1R-LI neurons and respiratory dysfunction has been reported also in a patient with Perry syndrome, autosomal dominant parkinsonism associated with depression, weight loss and central hypoventilation (Tsuboi *et al.*, 2008). The loss of putative chemosensitive glutamatergic neurons in the arcuate nucleus has also been reported in MSA (Benarroch *et al.*, 2007a). Moreover, A5 noradrenergic neurons of the pons, cholinergic neurons in the dorsal motor nucleus of vagus and ambiguous nucleus, and hypothalamic vasopressin and hypocretin neurons, which are all connected with autonomic homeostasis, are severely affected in MSA (Ozawa *et al.*, 1998; Benarroch *et al.*, 2006*a*, *b*, 2007*b*, 2008). Thus, the loss of these autonomic neurons could also be responsible for sudden death in patients with MSA. Therefore, investigating the relevance of these nuclei to sudden death requires further analysis of a large data set.

While it has been reported that the depletion of neurons in the IML and the medullary catecholaminergic and serotonergic neurons can occur during the course of MSA (Oppenheimer, 1980; Gray *et al.*, 1988; Sakajiri *et al.*, 1996; Benarroch *et al.*, 1998, 2005), we found that these findings are consistent even in the early stages of MSA, irrespective of remarkable autonomic failure. As we focused on patients in the early stages, we were able to determine the early neuropathological change of medullary autonomic nuclei in MSA.

Concerning the methodology, Benarroch *et al.* have provided a detailed report on the morphometric alterations in the medullary autonomic nuclei in MSA and Lewy body disease by performing extensive sampling methods (Benarroch *et al.*, 1998, 2003, 2004, 2005, 2006*b*, 2007*a*). Compared with these methods, the methods used in this study appear to be somewhat simple. However, we consider that the medullary slices used in this study (Fig. 2A) are at the adequate level for evaluating neurons in the VLM and nucleus raphe obscurus, because the adopted medullary level apparently corresponds well to panel 'c' in Fig. 5 for catecholaminergic neurons, and panel 'c' in Fig. 9 for serotonergic neurons, which has been demonstrated in a previous study by Halliday *et al.* (1988).

In this study, it was extremely important to prepare medullary slices that were cut at almost identical levels. The slices prepared for routine histopathological examinations in our large MSA series were oriented somewhat differently in relation to the rostro-caudal axis of the medulla, especially those cut from the severely atrophic medulla of patients with long disease duration. Our examination was therefore restricted to the medulla oblongata of patients who had died after short disease duration. We also examined additional patients with longer disease duration. We selected two SD group patients (one male and one female; age: 65 and 68 years, respectively; disease duration: 6 and 7 years, respectively; 1 MSA-C and 1 MSA-P) and two non-SD group patients (two female; age: 72 and 52 years, respectively; disease duration: 7 and 7 years, respectively; 1 MSA-C and 1 MSA-P), whose medullary levels were suitable for this study. The number of the serotonergic neurons in both the VLM and nucleus raphe obscurus was much smaller than those in the present study. However, these neurons tended to be more reduced in the SD group patients than in the non-SD group patients, both in the VLM (7 and 9 versus 12 and 16 cells) and nucleus raphe obscurus (12 and 13 versus 17 and 18 cells). This evidence is consistent with the results in patients with a short disease duration.

In summary, our findings indicate that the loss of medullary serotonergic neurons contributes to cardiorespiratory failure followed by sudden death in MSA. This information may be crucial when considering a new therapeutic strategy for MSA. Recently, the serotonin reuptake inhibitor, paroxetine, has been proposed to improve motor performance in patients with MSA (Friess *et al.*, 2006). Such serotonin-modifying drugs could also be useful in preventing sudden death related to this disease. Further investigation on the serotonergic autonomic system in a larger series of patients with MSA is needed to clarify this issue.

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BRAIN
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# Reduced O-GlcNAcylation links lower brain glucose metabolism and tau pathology in Alzheimer's disease

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It has been established for a long time that brain glucose metabolism is impaired in Alzheimer's disease. Recent studies have demonstrated that impaired brain glucose metabolism precedes the appearance of clinical symptoms, implying its active role in the development of Alzheimer's disease. However, the molecular mechanism by which this impairment contributes to the disease is not known. In this study, we demonstrated that protein O-GlcNAcylation, a common post-translational modification of nucleocytoplasmic proteins with β-*N*-acetyl-glucosamine and a process regulated by glucose metabolism, was markedly decreased in Alzheimer's disease cerebrum. More importantly, the decrease in O-GlcNAc correlated negatively with phosphorylation at most phosphorylation sites of tau protein, which is known to play a crucial role in the neurofibrillary degeneration of Alzheimer's disease. We also found that hyperphosphorylated tau contained 4-fold less O-GlcNAc than non-hyperphosphorylated tau, demonstrating for the first time an inverse relationship between O-GlcNAcylation and phosphorylation of tau in the human brain. Downregulation of O-GlcNAcylation by knockdown of O-GlcNAc transferase with small hairpin RNA led to increased phosphorylation of tau in HEK-293 cells. Inhibition of the hexosamine biosynthesis pathway in rat brain resulted in decreased O-GlcNAcylation and increased phosphorylation of tau, which resembled changes of O-GlcNAcylation and phosphorylation of tau in rodent brains with decreased glucose metabolism induced by fasting, but not those in rat brains when protein phosphatase 2A was inhibited. Comparison of tau phosphorylation patterns under various conditions suggests that abnormal tau hyperphosphorylation in Alzheimer's disease brain may result from downregulation of both O-GlcNAcylation and protein phosphatase 2A. These findings suggest that impaired brain glucose metabolism leads to abnormal hyperphosphorylation of tau and neurofibrillary degeneration via downregulation of tau O-GlcNAcylation in Alzheimer's disease. Thus, restoration of brain tau O-GlcNAcylation and protein phosphatase 2A activity may offer promising therapeutic targets for treating Alzheimer's disease.

Keywords: tau phosphorylation; O-GlcNAcylation; glucose metabolism; protein phosphatase 2A; neurofibrillary degeneration

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