

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 μ m sections were obtained from the 8th segment of the thoracic cord and the rostral medulla oblongata. Sections from the medulla oblongata were immunostained for tyrosine 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 ± 7.1 versus 75.2 ± 7.6 cells, $P < 0.001$), tyrosine hydroxylase-immunoreactive neurons in the ventrolateral medulla (VLM) (17.4 ± 5.1 versus 72.8 ± 13.6 cells, $P < 0.01$) and tryptophan hydroxylase-immunoreactive neurons in the VLM (15.6 ± 9.2 versus 60.8 ± 17.0 cells, $P < 0.01$), nucleus raphe obscurus (19.3 ± 4.4 versus 75.3 ± 8.6 cells, $P < 0.001$), 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, $P < 0.05$). 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 ± 3.5 versus 21.8 ± 6.5 cells, $P < 0.02$) and nucleus raphe obscurus (15.0 ± 2.0 versus 22.5 ± 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.

Keywords: multiple system atrophy; sudden death; medulla oblongata; serotonin; catecholamine

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- μ m 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; 1:10000) 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.

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 \pm 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

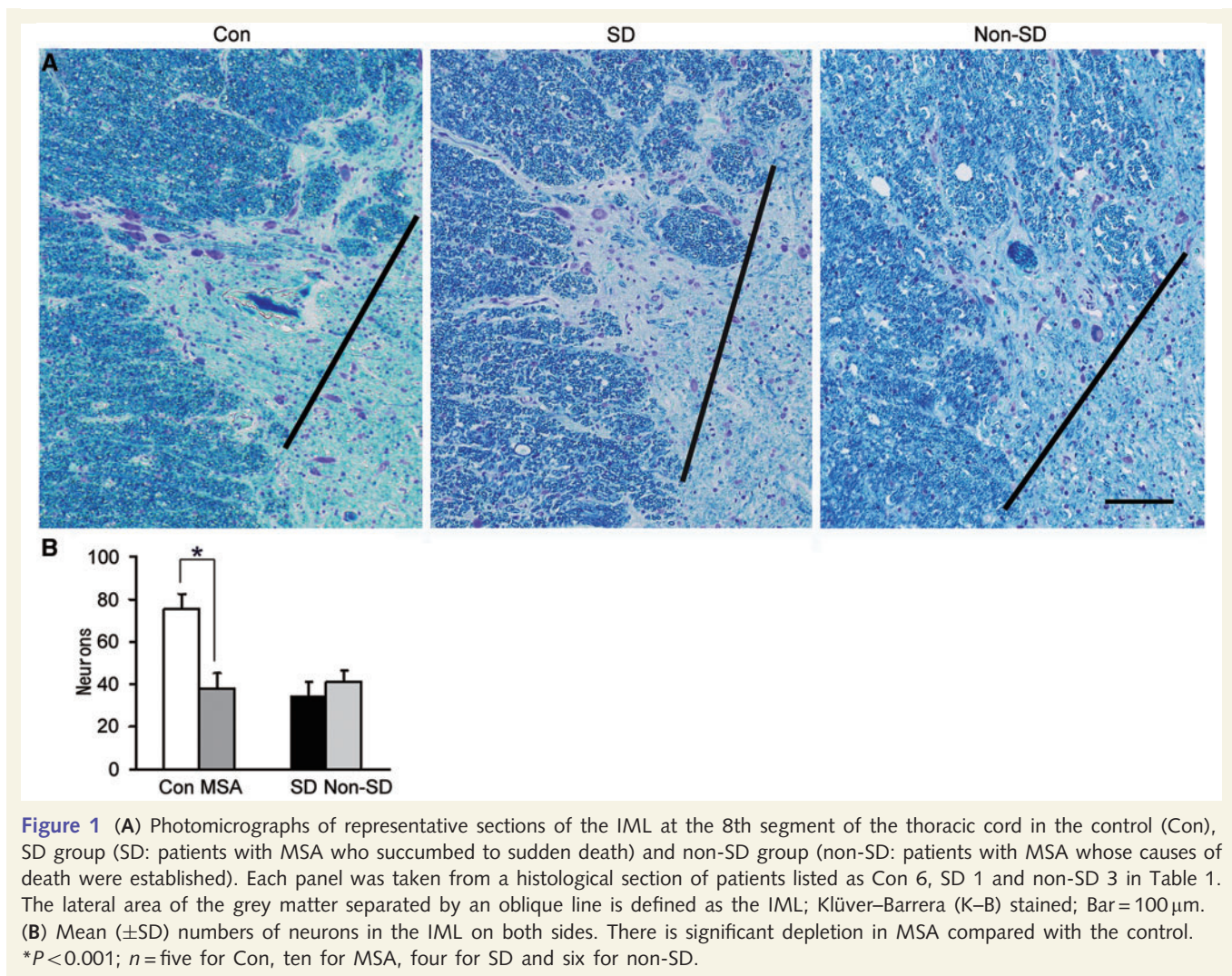
In MSA, atrophy of various degrees in the IML was observed (Fig. 1A). There were significantly fewer neurons in the IML in the MSA group than in the control (38.0 ± 7.1 versus 75.2 ± 7.6 cells, $P < 0.001$) (Fig. 1B). This finding was common in all the patients with MSA, with a short disease duration. 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).



Serotonergic neurons

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, 2007a), we have demonstrated that the depletion

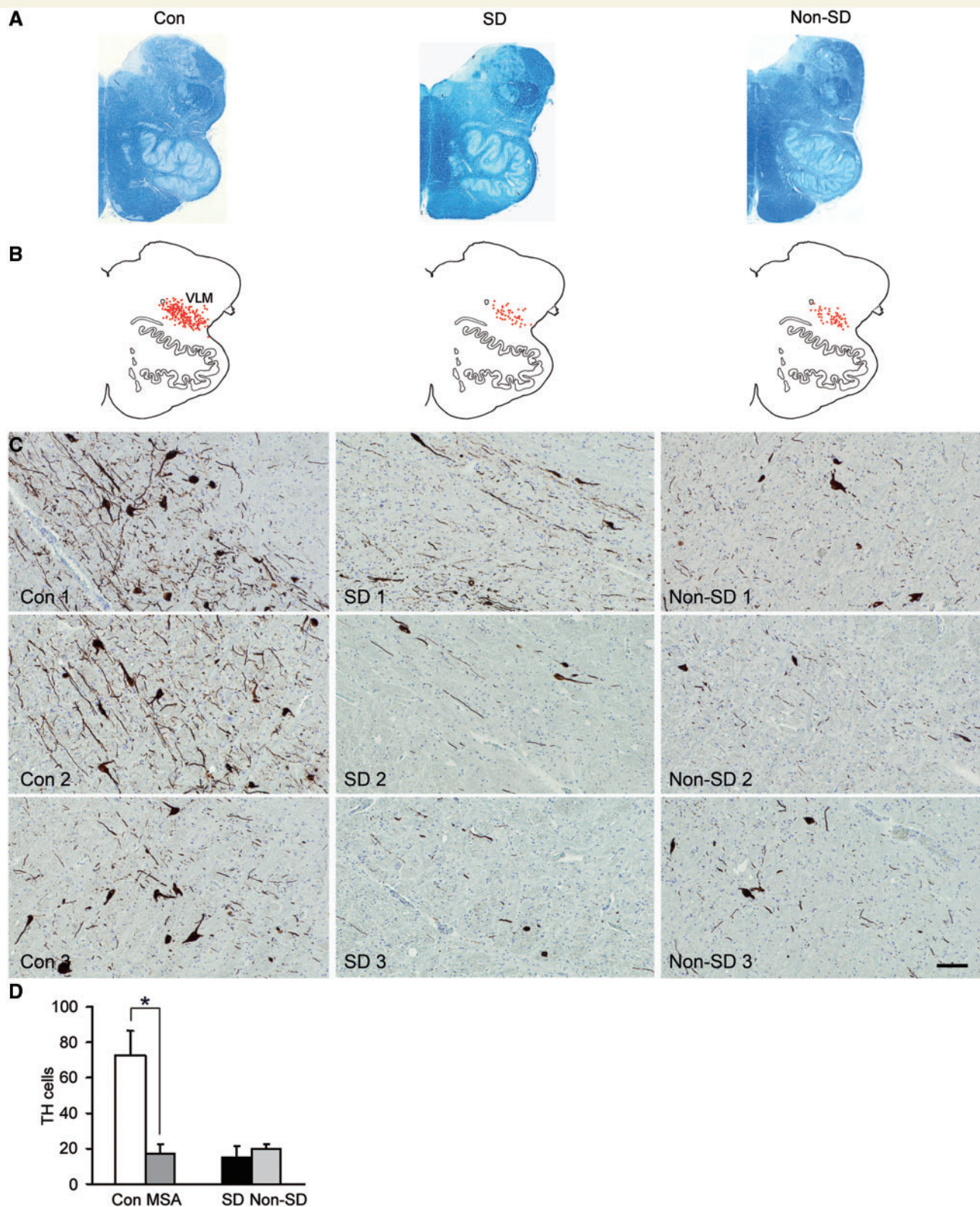


Figure 2 (A) Photographs of representative medullary sections of the control, SD and non-SD groups; K–B stained. (B) Schematic representation of the distribution of TH-immunoreactive neurons in the VLM in the control, SD and non-SD groups. Each dot represents the approximate position of a single neuron. 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 μ m. (D) Mean (\pm 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.

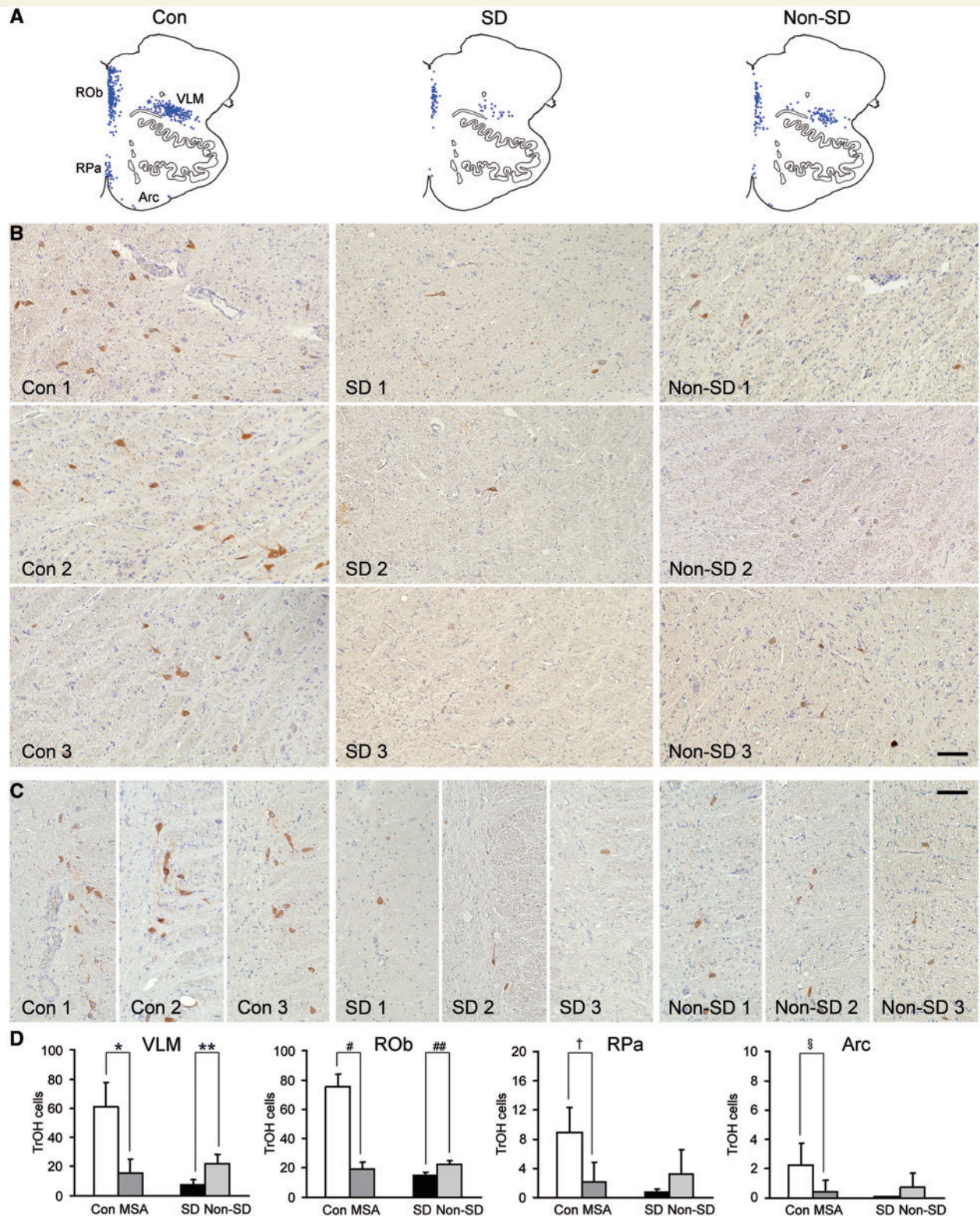


Figure 3 (A) Schematic representation of the distribution of TrOH-immunoreactive neurons in the VLM, nucleus raphe obscurus (ROb), nucleus raphe pallidus (RPa) and arcuate nucleus (Arc) in the control, SD and non-SD groups. Each dot represents the approximate position of a single neuron. To demonstrate 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 μ m. (D) Mean (\pm 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. * $P < 0.01$, ** $P < 0.02$, # $P < 0.001$, ## $P < 0.01$, † $P < 0.03$, § $P < 0.05$; $n = 4$ 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 precise 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.*, 2006a, b, 2007b, 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, 2006b, 2007a). 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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