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## Loss of A5 noradrenergic neurons in multiple system atrophy

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### Abstract

The A5 noradrenergic group of the pons projects on the medulla and spinal cord and contributes to control of sympathetic activity and respiration. Because these functions are affected in multiple system atrophy (MSA), we sought to determine whether there was involvement of A5 neurons in this disorder. We counted tyrosine-hydroxylase (TH) immunoreactive neurons in the A5 area and locus ceruleus in the pons obtained from six patients with clinical and neuropathological diagnoses of MSA and six age-matched controls using stereological methods. There was a severe loss of A5 neurons in MSA (total cell number  $3,215 \pm 258$  in controls and  $321 \pm 62$  in MSA,  $P < 0.001$ ). The severity of cell loss was comparable to that of locus ceruleus neurons in these cases. Our results indicate that A5 neurons are severely affected in MSA and this may contribute to some of the manifestations of this synucleinopathy.

### Keywords

Norepinephrine; Cardiorespiratory; Glial cytoplasmic inclusions

### Introduction

The A5 neurons of the pontine tegmentum provide noradrenergic innervation to the preganglionic sympathetic neurons of the spinal cord [24] and elicit sympathoexcitation [12]. The A5 neurons are activated during hypotension [4] and hypoxia [6,9], and their activity is modulated during respiration [9]. Although A5 neurons do not appear to be involved in tonic maintenance of arterial pressure, they may play an important role in sympathoexcitatory responses to hypoxia [14]. They modulate the activity of respiratory neurons [11] and project onto central chemoreceptor areas of the ventral medullary surface [20]. Thus, A5 neurons appear to participate in complex cardiorespiratory interactions.

Multiple system atrophy (MSA) may manifest with prominent impairment of sympathetic cardiovascular and respiratory control [8,26]. Whereas MSA is associated with loss of noradrenergic neurons in both the locus ceruleus [1,13] and A1 area of the caudal ventrolateral medulla [2], involvement of neurons of the A5 group has not been systematically explored. In

this study, we sought to determine whether the A5 neurons are involved in MSA and to compare their involvement with that of the locus ceruleus neurons in these cases.

## Methods

### Subjects

Brains were obtained at autopsy from 12 subjects (Table 1). All the patients had signed informed consent for autopsy according to the Institutional Review Board guidelines. Six subjects (five women, one man, age  $70 \pm 4$  years) had no history of neurological or psychiatric disease, and six subjects (four men, two women, age  $65 \pm 4$  years) had the clinical diagnosis of MSA confirmed neuropathologically. The clinical and neuropathological diagnoses of MSA were made according to current consensus criteria [7].

Of the six MSA cases, all had predominant parkinsonism and four also had cerebellar ataxia. All the MSA cases had clinical and laboratory evidence of severe autonomic failure, manifested by orthostatic hypotension and impaired heart rate response to deep breathing indicating impaired vagal control of the sinus node (Table 1). Unfortunately, none of our cases had undergone evaluation for ventilatory or blood pressure responses to hypercapnia or hypoxia.

### Tissue processing and immunocytochemistry

Postmortem delay was similar in control ( $17 \pm 2$  h) and MSA cases ( $18 \pm 5$  h). The left half of the brain was examined for routine neuropathological studies. The six control brains, including those from patients with mild memory complaints, were macroscopically normal and were not studied for Braak and Braak staging. Of the six MSA cases, all had evidence of severe striatonigral degeneration. Five also had evidence of olivopontocerebellar atrophy that was severe in two and moderate in three cases. All the MSA cases had evidence of  $\alpha$ -synuclein immunostained glial cytoplasmic inclusions (GCIs). The MSA cases had mild Braak and Braak Alzheimer-type pathology (Braak and Braak stages 0–III), none of which satisfied the NIA-Reagan or CERAD criteria for AD [3].

A block containing the pons from 15 to 22 mm rostral to the obex was separated for the purposes of this study. Brains were immersion-fixed in 5% formalin for 24 h at 4°C and cryoprotected in buffered 30% sucrose for 5–7 days prior to processing. Serial 50  $\mu$ m cryostat sections were obtained and every eighth section was processed for tyrosine hydroxylase (TH) immunoreactivity (mouse monoclonal, 1:3,000, Immunostar, Hudson, WI, USA). Diaminobenzidine/glucose oxidase solution with nickel enhancement (SIGMA, St Louis, MO, USA) was used for the substrate reaction. Immunoreactive neurons were identified under bright-field illumination by a characteristic dark brown to black reaction product that densely fills the perikarya and their processes. The distribution of immunoreactive neurons in the pons identified using this TH antibody was similar to that reported in earlier studies [18]. Omission of the primary antibody or incubation with normal sera resulted in a lack of immunostaining. All sections were co-stained with thionin to identify surrounding structures and to determine whether loss of immunoreactivity reflected neuronal loss or lack of expression of the antigen. Paraffin-embedded 6- $\mu$ m sections obtained from the contralateral pons at the same level were immunostained for  $\alpha$ -synuclein (goat polyclonal, 1:400, Santa Cruz Biotechnologies, Santa Cruz, CA, USA).

### Image analysis and quantitation

The sections were examined under bright-field microscopy using the Axiovision 4.2 image analysis system (Carl Zeiss, Inc., Thornwood, NY, USA) and cell numbers were estimated using design-based stereology [16,21]. Digital images were acquired using the AxioCam MRc, (Carl Zeiss, Inc., Thornwood, NY, USA) attached to a microscope (Leica, Diaplan; Wetzlar,

Germany) fitted with a motorized stage (Ludl Electronic Products, Hawthorne, NY, USA) and stage position encoder. Sections were studied using the Analyze 8.1 three-dimensional stereology software developed in the Biomedical Imaging Resource Department, Mayo Clinic, Rochester, MN, USA [15]. We systematically sampled the whole extent of the pons by obtaining 50  $\mu\text{m}$  sections at intervals of 400  $\mu\text{m}$  apart. The total number of sections studied was  $18 \pm 1$  (average 17.5) per case. The total number of TH immunoreactive neurons was determined using a systematic and uniformly random sampling of the A5 area or locus ceruleus. The A5 and locus ceruleus boundaries were determined as defined on the basis of the atlas by Paxinos and Huang [17]. We only counted the immunoreactive neurons that could be clearly identified by the presence of nucleus, dendritic processes, or both in a single focal plane within the total cross section of each 50  $\mu\text{m}$  section. All immunoreactive neurons that fulfilled this criterion were counted within each cross section. We also measured the diameter and area of the soma (without including the processes) of all highly immunoreactive cells that could be identified by these criteria. For neuronal size measurements, we selected, for each case, five sections obtained 1.5 mm apart. We measured 20 neurons per section in each of the six controls (total 600 neurons) and four neurons per section in each MSA case (total 120 neurons).

The same investigator who blinded to the clinical and final neuropathological diagnoses performed all counts.

### Statistical analysis

The numbers of TH-immunoreactive cells (mean  $\pm$  SEM) in the A5 or locus ceruleus regions were compared between control and MSA cases. Results were analyzed using the analysis of covariance (ANCOVA) with multivariate and univariate analyses with the SPSS version 10.1 software (SPSS Inc., Chicago, IL, USA). Groups (controls and MSA), age, and disease duration were used as the covariates, and cell count was the dependent variable. Post hoc analyses for a pairwise comparison were also performed using Student's *t* test. A *P* value of less than 0.05 was considered to be significant.

### Results

There was severe loss of TH immunoreactive neurons in the A5 region in all the MSA cases compared to controls (Fig. 1). As assessed in Nissl-co-stained sections, this could not be explained by lack of antigen expression in surviving cells. The total number of neurons counted was  $414 \pm 21$  in controls and  $41 \pm 7$  in MSA ( $P < 0.001$  with respect to controls). The total estimated cells was  $3,215 \pm 258$  in controls and  $321 \pm 62$  in MSA ( $P < 0.001$  with respect to controls; Fig. 2). There was no difference in A5 cell size between control and MSA cases. In controls, the cell size was  $28.3 \pm 0.5$   $\mu\text{m}$  in diameter and  $545 \pm 20$   $\mu\text{m}^2$  in area ( $n = 100$  per case, total 600 cells); in MSA, the cell size was  $28.8 \pm 0.4$   $\mu\text{m}$  in diameter and  $551 \pm 15$   $\mu\text{m}^2$  in area ( $n = 20$  per case, total 120 cells). There was no relationship between the degree of A5 neuronal depletion and age (age effect  $P = 0.09$ ).

In the A5 region, there were abundant  $\alpha$ -synuclein immunoreactive GCIs in all the MSA cases. We did not detect  $\alpha$ -synuclein immunoreactive inclusions in the cytoplasm of remaining A5 neurons (Fig. 3). The magnitude of A5 cell loss (approximately 90%) was comparable to that observed in the adjacent locus ceruleus (LC) of the same cases (Fig. 4). The total LC cells counted was  $1,590 \pm 111$  in controls and  $220 \pm 59$  in MSA ( $P < 0.001$  with respect to controls). The total estimated numbers of LC cells was  $29,612 \pm 2,548$  in controls and  $3,960 \pm 613$  in MSA ( $P < 0.001$  with respect to controls). There was no difference in locus ceruleus cell size between controls and MSA cases. In controls, the cell size was  $31.8 \pm 1$   $\mu\text{m}$  in diameter and  $703 \pm 42$   $\mu\text{m}^2$  in area ( $n = 100$  per case, total 600 cells); in MSA, the cell size was  $32.3 \pm 1$   $\mu\text{m}$  in diameter and  $706 \pm 33$   $\mu\text{m}^2$  in area ( $n = 20$  per case, total 120 cells). There was no relationship between the degree of locus ceruleus neuronal depletion and age (age effect  $P =$

0.8). As in the A5 region, there were abundant  $\alpha$ -synuclein immunoreactive GCIs in the locus ceruleus of all the MSA cases. We did not detect  $\alpha$ -synuclein immunoreactive inclusions in the cytoplasm of locus ceruleus neurons.

## Discussion

Our results indicate, for the first time to our knowledge, that there is severe loss of A5 neurons in MSA. The magnitude of A5 neuronal loss in MSA is comparable to that of locus ceruleus neurons in the same cases. Although the clinical implications of A5 neuronal loss in MSA are uncertain, it is conceivable that A5 cell loss may contribute to some of the manifestations of this disorder.

The A5 neurons provide noradrenergic innervation to preganglionic sympathetic neurons of the spinal cord [24] and elicit sympathoexcitation [12]. The A5 neurons are activated during baroreceptor unloading [4] but do not appear to have a major contribution in maintaining resting sympathetic tone or arterial pressure [10]. However, A5 neurons are also activated by hypoxia and stimulation of the carotid chemoreceptors [6,9] and their activity is modulated during respiration [9]. It has been proposed that A5 neurons may play an important role in the carotid sympathetic chemoreflex triggered by hypoxia [14]. Respiratory modulation of A5 neurons during hypoxia coincides with episodes of blood pressure drop [19]. Although A5 neurons are activated by hypoxia [6,9,19], they do not appear to be a direct component of the hypoxia chemosensitive circuit, as defined by Phox2b gene expression [23]. However, A5 neurons modulate the activity of respiratory neurons [11] and studies in the rat indicate that there is a projection from A5 neurons to central chemoreceptor areas of the ventral medullary surface [20]. The A5 neurons also modulate the cardiorespiratory responses evoked by activation of the parabrachial nucleus [5], which is a critical component of the brainstem respiratory network required for eupnea [22]. Thus, A5 neurons appear to participate in complex cardiorespiratory interactions, including those associated with sympathetic responses to hypoxia. Therefore, it is conceivable that involvement of these neurons may contribute to respiratory [8,26] or cardiovascular [25] manifestations of MSA. However, given that the role of A5 neurons is not fully characterized by experimental studies, our conclusions regarding their potential contribution to cardiorespiratory manifestations of MSA are speculative at this point.

In summary, our study indicates that there is a severe loss of A5 neurons in MSA. Involvement of A5 neurons may at least have a contributory role in the cardiovascular and respiratory manifestations of this disorder. Unfortunately, none of our cases had undergone assessment of cardiovascular responses to hypoxia. These studies are currently being performed in our laboratory.

## Acknowledgments

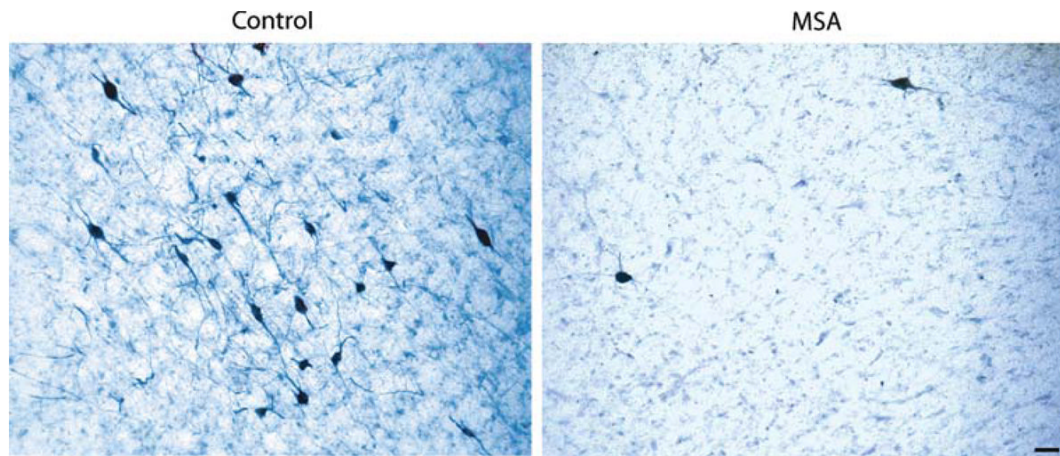
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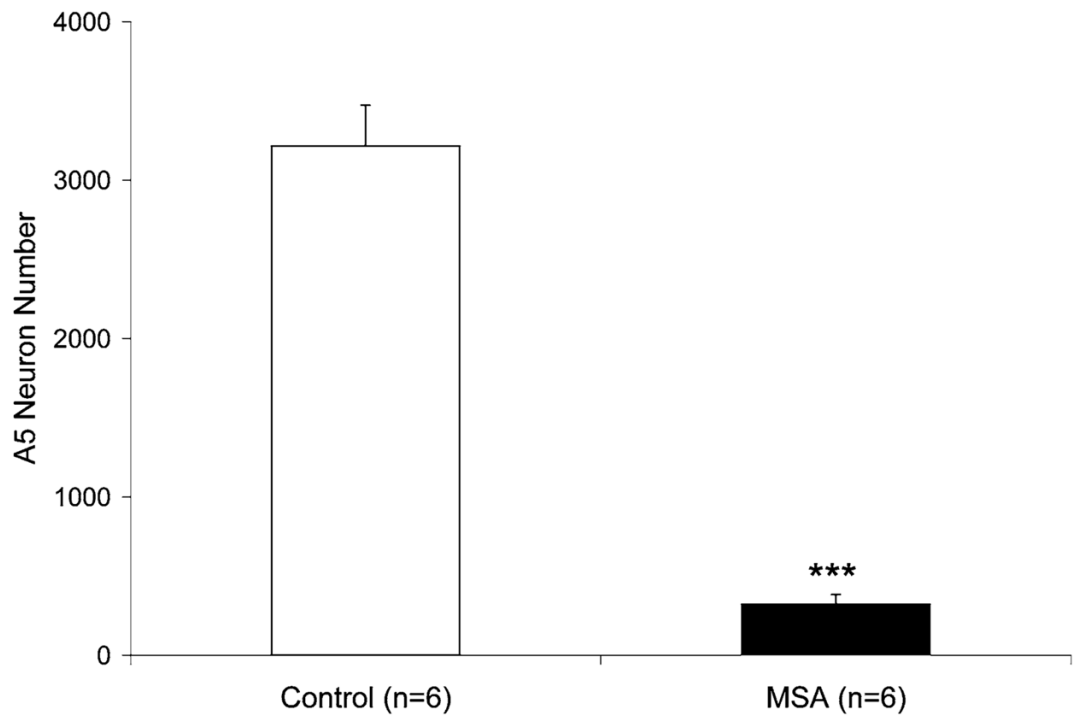
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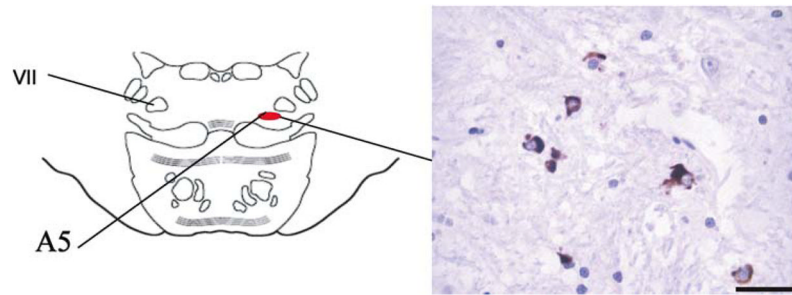


**Fig. 1.** Tyrosine hydroxylase (TH) immunoreactive neurons (co-stained with thionin) in the A5 area in 50  $\mu\text{m}$  sections of a 77-year-old woman with no history of neurological disease (postmortem delay 22 h) and a 59-year-old man with pathological diagnosis of multiple system atrophy (MSA, postmortem delay 26 h). *Bar* 50  $\mu\text{m}$

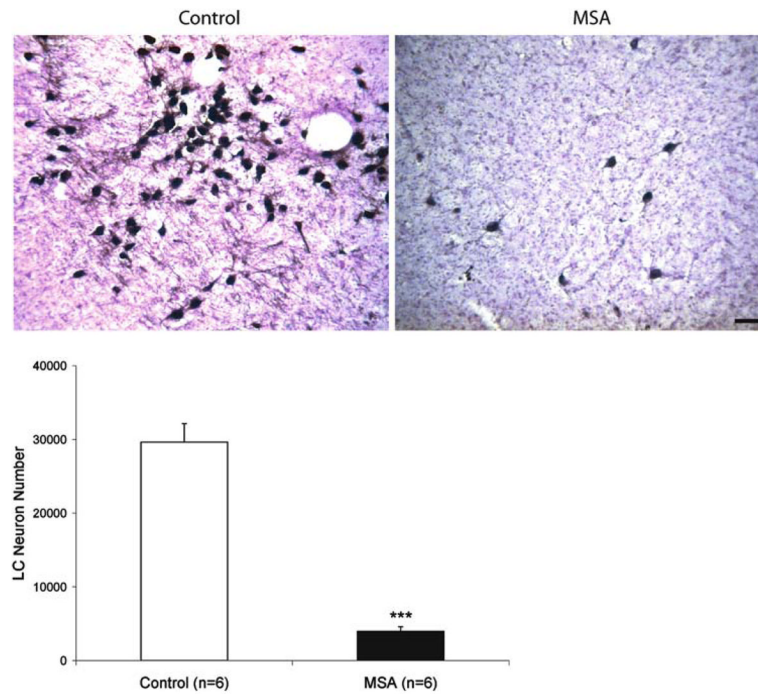


**Fig. 2.** Estimated total number (mean  $\pm$  SEM) of tyrosine hydroxylase (TH) neurons in the A5 area in six control subjects and six cases with pathological diagnosis of multiple system atrophy (MSA). There was a significant reduction of TH positive A5 neurons in all MSA cases. \*\*\*  $P < 0.001$  compared to controls





**Fig. 3.** Six micrometer paraffin-embedded sections of the pons at the level of the A5 area (*left*) stained for  $\alpha$ -synuclein and showing the distribution of glial cytoplasmic inclusions in a 68-year-old man with pathological diagnosis of multiple system atrophy (*right*) (MSA, postmortem delay 23 h). Bar 25  $\mu$ m



**Fig. 4.** *Upper panel* tyrosine hydroxylase (TH) immunoreactive neurons of the locus ceruleus in 50  $\mu\text{m}$  sections of a 77-year-old woman with no history of neurological disease (postmortem delay 22 h) and a 59-year-old man with pathological diagnosis of multiple system atrophy (MSA, postmortem delay 26 h). *Bar* 50  $\mu\text{m}$ . *Lower panel* estimated total number (mean  $\pm$  SEM) of tyrosine hydroxylase (TH) immunoreactive neurons in the locus ceruleus (LC) in six control cases, and six cases with pathological diagnosis of multiple system atrophy (MSA). There was a significant reduction of locus ceruleus neurons in all MSA cases. \*\*\*  $P < 0.001$  compared to controls

Table 1

## Patient population

Case	Age/sex	PMD (h)	Motor/cognitive manifestation	Autonomic manifestations	Disease duration (years)	Diagnosis
Control 1	85/F	16	None	HTN	N/A	MI
Control 2	73/M	18	Mild memory loss	Constipation, HTN	N/A	CHF
Control 3	66/F	18	None	None	N/A	Pulmonary fibrosis
Control 4	57/F	8.5	None	HTN	N/A	Metastatic ovarian carcinoma
Control 5	77/F	22	Mild memory loss	HTN, constipation	N/A	Scleroderma, CAD
Control 6	62/F	20	None	DM, HTN	N/A	COPD
MSA 1	68/M	23	Parkinsonism	OH, NB, impotence, dysphagia	14	MSA-SND-OPCA
MSA 2	67/M	1	Parkinsonism, ataxia	OH, NB, HTN, constipation, CVF	2	MSA-SND
MSA 3	53/M	36	Parkinsonism ataxia	OH; NB, constipation	3	MSA-SND/OPCA
MSA 4	81/F	13	Parkinsonism	OH, NB, constipation, anhidrosis, CVF	5	MSA-SND/OPCA LBD (neocortical)
MSA 5	59/M	26	Parkinsonism ataxia	OH, NB, impotence, constipation, anhidrosis, CVF	5	MSA-SND-OPCA
MSA 6	63/F	6	Parkinsonism ataxia	OH, NB, HTN, anhidrosis, dysphagia	6	MSA-SND-OPCA

CAD coronary artery disease, CHF congestive heart failure, COPD chronic obstructive pulmonary disease, CVF cardiovagal failure, DM diabetes mellitus, HTN hypertension, MI myocardial infarction, MSA multiple system atrophy, NB neurogenic bladder, OH orthostatic hypotension, OPCA olivopontocerebellar atrophy, PMD postmortem delay, SND striatonigral degeneration