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Differential Involvement of the Periaqueductal Gray in Multiple System Atrophy

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Abstract

The periaqueductal gray (PAG) consists of distinct columns that participate in the integrated control of autonomic function. We sought to determine whether the PAG is affected in multiple system atrophy (MSA), a disorder characterized by prominent autonomic failure. Brains were obtained at autopsy from 13 MSA patients (10 M, 3 F, age 61 ± 3 years) and 13 controls (8 M, 5 F, age 67 ± 4 years). Transverse formalin-fixed 50 μ m sections were obtained throughout the PAG and immunostained for the vesicular transporter 2 (VGLUT-2), nitric oxide synthase (NOS), or α -synuclein and co-stained with thionin. Some sections were processed for myelin or astrocyte staining. Stereological quantitation was performed separately in the ventrolateral, lateral, dorsolateral, and dorsomedial columns of the PAG. In MSA cases, there was a decrease in the total estimated number of VGLUT-2 immunoreactive neurons in the ventrolateral, lateral, and dorsomedial and to a lesser extent dorsolateral PAG compared to controls (ventrolateral PAG: $16,299 \pm 1,612$ vs. $27,906 \pm 2,480$ respectively, $p < 0.01$; lateral PAG: $11,004 \pm 1,401$ vs. $16,078 \pm 1,140$ respectively, $p < 0.05$; and dorsomedial PAG: $8,847 \pm 1,052$ vs. $15,412 \pm 1,097$ respectively, $p < 0.001$). The number of NOS immunoreactive neurons in the dorsolateral PAG was similar to controls. In all columns, the number of non-immunolabelled Nissl-stained cells was similar between groups. There was accumulation of glial cytoplasmic inclusions in all PAG columns in MSA. Our findings indicate involvement of the PAG columns in MSA, which may contribute to autonomic disturbances in this disorder.

Keywords

Central gray; VGLUT-2; nitric oxide synthase; glial cytoplasmic inclusions

Introduction

The periaqueductal gray (PAG) provides an anatomical and functional interface between the forebrain and the lower brain stem and has a major role in integrated autonomic responses to stress, pain modulation, and other adaptive functions. The PAG is subdivided into different longitudinal columns, ventrolateral, lateral, dorsolateral, and dorsomedial, which have

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different cortical and brainstem connections, and coordinate different types of behaviors. Different neuronal groups in the PAG participate in integrated cardiovascular responses associated with pain modulation, thermoregulation, respiration, airway control, micturition, arousal, and rapid eye movement (REM) sleep.

These functions involve extensive PAG interconnections with the prefrontal and anterior cingulate cortex, amygdala, hypothalamus, and brainstem nuclei involved in autonomic, motor, and pain modulatory responses. The PAG is affected in neurodegenerative disorders, such as Alzheimer disease and Parkinson disease.

Multiple system atrophy (MSA) is a neurodegenerative disorder characterized by prominent autonomic failure associated with parkinsonism, cerebellar ataxia, or both. Features of MSA include orthostatic hypotension, neurogenic bladder, respiratory disturbances, and disorders of arousal and REM sleep. The prominent role of the PAG in the control of cardiovascular, micturition, respiration and sleep, and its connections with brainstem areas that are neuropathologically affected in MSA, such as the rostral ventrolateral medulla and medullary raphe, suggest that PAG involvement may contribute to the clinical manifestations of this disorder. We have reported that there is loss of tyrosine hydroxylase (TH)-immunoreactive neurons in the ventrolateral PAG in MSA. Whereas TH cell loss may contribute to disorder of arousal, there is no clear evidence that these neurons are involved in cardiovascular, respiratory, or other functions affected in MSA. Many PAG neurons of the different columns projecting to brainstem autonomic and respiratory nuclei are glutamatergic, and neurons in the dorsolateral PAG, which do not project directly to brainstem autonomic regions, express nitric oxide synthase (NOS). The possible involvement of glutamatergic or nitroxydergic cell groups in the different PAG columns, and the relationship between neuronal involvement and the degree of accumulation of α -synuclein immunoreactive glial cytoplasmic inclusions (GCIs) and other neuropathological markers of MSA, such as astrocytic gliosis have not yet been explored. This information could provide insight into the pathodynamics and potential contributory anatomical substrates for the autonomic manifestations of MSA. To address these questions, we performed a stereological quantitative analysis of the glutamatergic and nitroxydergic cell groups as well as the burden of GCIs in the different functional columns of the PAG in patients with neuropathologically confirmed MSA.

Subjects and Methods

Brains were obtained at autopsy from 13 patients (10 men, 3 women; age 61 ± 3 years) with clinically probable MSA according to current criteria, and 13 controls with no history of neurologic disease (8 men and 5 women; age 67 ± 4 years) (Table 1). All subjects had given informed consent for autopsy according to the guidelines of the Institutional Review Board. For the MSA cases, symptom duration was 7 ± 0.8 years; range (2–14); parkinsonism was the prominent finding in 8 (MSA-P) and cerebellar ataxia in 5 (MSA-C). All MSA cases had history of orthostatic hypotension and neurogenic bladder; and all men had erectile dysfunction. Seven cases had history of excessive diurnal somnolence; 7 had REM sleep behavior disorder; 3 had laryngeal stridor; 5 had documented sleep apnea; and 2 had periodic leg movements of sleep.

Tissue Processing and Immunocytochemistry

Post-mortem delay was similar in controls (17 ± 1 hour) and in MSA cases (12 ± 2 hours). One half of the brain was separated for routine neuropathological examination and the other half for our present study. All cases had neuropathological criteria for MSA. Of the 13 MSA cases, 10 had severe involvement of the putamen and substantia nigra pars compacta and 6

had moderate to severe involvement of the pons, inferior olivary nucleus, and cerebellum. All cases had moderate to severe GCI accumulation in these regions. One MSA case had mild to moderate and two had only rare associated Lewy body pathology. The presence of neurofibrillary tangles and senile plaques was classified in each case according to the stages of Alzheimer disease as described by Braak and Braak (B&B) and defined by the *Consortium to Establish a Registry for Alzheimer's disease* (CERAD). Twelve MSA cases had B&B staging 0-II, and one had stage III; and none fulfilled CERAD criteria for Alzheimer disease.

A block containing the midbrain from 33–47 mm rostral to the obex to include the whole extent of the PAG was separated, immersion fixed in 5% formalin for 24 hours at 4°C and cryoprotected in buffered 30% sucrose for 5 to 7 days prior to processing. Serial transverse 50 µm cryostat sections were obtained from one half of the midbrain and every eighth section was processed for immunoreactivity for vesicular glutamate transporter-2 (VGLUT-2, guinea pig polyclonal, 1:2000, Millipore, Temecula, CA.); nitric oxide synthase 1 (NOS1, rabbit polyclonal, 1:2000, Santa Cruz Biotechnologies, Santa Cruz, CA.); tyrosine hydroxylase (TH, mouse monoclonal, 1:3000, Immunostar, Hudson, WI.), or α -synuclein (goat polyclonal, 1:400, Santa Cruz Biotechnologies, Santa Cruz, CA). Diaminobenzidine/glucose oxidase solution with nickel enhancement (SIGMA, St. Louis, MO.) 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. Omission of the primary antibody, use of appropriate blocking peptide, 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. Selected paraffin embedded 12 µm sections were processed for Luxol Fast Blue for myelin staining, or double immunofluorescence for α -synuclein (Santa Cruz Biotechnology Inc., Santa Cruz, CA) which incorporated a fluorescein-conjugated (donkey anti-rabbit IgG, 1:200, Millipore, Temecula, CA), and glial fibrillary acidic protein (GFAP) (rabbit polyclonal, 1:400, Dako, Glostrup, Denmark) followed by a Cy-3 conjugated (donkey anti rabbit IgG, 1:200, Millipore, Temecula, CA).

Image analysis and quantitation

For each MSA and control case, we analyzed between 14–18 sections obtained 800 µm apart to span the length of the PAG. The sections were examined under bright-field microscopy using a modified light microscope (Zeiss Axioimager A-1; Zeiss, Thornwood, NY) equipped with a motorized specimen stage for automated sampling (Ludl Electronics; Hawthorne, NY, USA), CCD color video camera (Microfire; Optronics, Goleta, CA, USA) and stereology software (Stereo Investigator, v9.2; MBF Bioscience, Williston, VT, USA). Cell counts were performed separately in the ventrolateral, lateral, dorsolateral, and dorsomedial PAG defined according to the atlas of Paxinos and Huang. In each of the columns, we counted separately the VGLUT-2, TH, and NOS immunoreactive neurons as well as, and thionin stained cells that were not immunoreactive for any of the markers. We also counted separately the number of GCIs in each column. Stereological analysis of the total number of neurons or GCIs was performed using the optical fractionator method. All systematic unbiased random samples in each section were analyzed according to established criteria. In addition to the total estimated numbers, the density of neurons or GCIs in each PAG column was calculated using the Cavalieri volumetric method. The same investigator, blinded to the clinical and final neuropathological diagnoses, performed all counts.

Statistical analysis

All results are expressed as mean $\pm$ SEM. Statistical analysis was performed using the software SPSS 14.0 for Windows. Multivariate analysis of variance was used to determine if age, gender, disease phenotype, symptom duration, or post-mortem delay had any effect on cell counts. For cell counts, density and sizes in each PAG column, comparison of control vs. MSA cases was performed using unpaired t-test. Comparison of the density of GCIs among the different columns was made using univariate analysis of variance. Post-hoc analysis was then conducted using Dunnett's and Bonferroni's formulas. A p value of <0.05 was considered significant.

Results

Estimated total number of neurons in the different PAG columns in MSA compared to controls

The distribution and morphology of VGLUT-2 and NOS immunoreactive neurons in the ventrolateral and dorsolateral PAG, respectively, was consistent with observations in experimental animals (Figure 1). Compared to controls, there was loss of VGLUT-2 immunoreactive neurons in the ventrolateral, lateral, and dorsomedial and to a lesser extent dorsolateral column of the PAG. Cell loss in the ventrolateral and dorsomedial PAG was relatively more severe than in the lateral PAG. There was a mild VGLUT-2 cell loss in the dorsolateral PAG compared to other columns (Table 2). For the ventrolateral PAG the total estimated cell number was $16,299 \pm 1,612$ in MSA and $27,906 \pm 2,480$ in controls ($p < 0.01$); for the lateral PAG, it was $11,004 \pm 1,401$ in MSA and $16,078 \pm 1,140$ in controls ($p < 0.05$); and in the dorsomedial PAG it was $8,847 \pm 1,052$ in MSA and $15,412 \pm 1,097$ in controls ($p < 0.001$). The degree of VGLUT-2 cell loss did not correlate with symptom duration, age, sex, disease phenotype (MSA-P or MSA-C), presence or absence of laryngeal stridor, or post-mortem delay. In contrast, there was no significant difference in the number of NOS immunoreactive cells in the dorsolateral PAG compared to controls (Table 2) (Figure 1). Consistent with our previous study, there was also a significant reduction in the numbers of TH immunoreactive cells in the ventrolateral PAG in MSA compared to controls (Table 2). The loss of VGLUT-2 or TH-immunoreactivity could not be explained by down-regulation of the marker, as assessed using Nissl staining. The total estimated numbers and cell density of Nissl-stained, non VGLUT-2, non-NOS, non-TH immunoreactive neurons was similar between MSA and controls in each of the PAG columns (Figure 2). There was no difference in cell size between MSA and controls in any of the columns (Table 2).

Alpha-Synuclein inclusions and Other Neuropathological Changes in the PAG

There were abundant GCIs in all columns in the PAG in the MSA cases (Figure 3). We found no convincing evidence of neuronal inclusions in our samples. Although there was large variability among cases, GCIs appeared to be more abundant in the ventrolateral than in the other columns (Figure 4). There was no correlation between the number of GCIs and the patients' age or symptom duration and no differences between cases with clinical MSA-P or MSA-C. There was loss of myelin and astrocytic gliosis in the area of GCI accumulation, as assessed with double immunocytochemistry in all columns of the PAG (Figure 5).

Discussion

Our results indicate that the PAG is involved in MSA. Our main findings are that in MSA (1) there involvement of selected cell groups in the different columns of the PAG; and (2) there may be dissociation between GCI accumulation and cell loss. Loss of glutamatergic

neurons was observed in the ventrolateral, lateral and dorsomedial, and to a lesser extent the dorsolateral PAG; in contrast, there was preservation of NOS immunoreactive neurons in the dorsolateral PAG. Involvement of the PAG may contribute to some of the manifestations of MSA. Experimental evidence indicates that the ventrolateral PAG has a major role in integrated cardiovascular and pain modulatory responses to visceral and somatic pain, control of micturition, respiration (Zhang et al., 2007) and upper airway resistance. The ventrolateral PAG projects to the nucleus of the solitary tract and may thus contribute to modulation of the baroreflex. The ventrolateral PAG is a critical relay nucleus in the micturition reflex and, via its connections with the nucleus retroambiguus, inhibits muscle tone in the upper airway. Therefore, loss of glutamatergic neurons in the ventrolateral PAG may contribute to impaired baroreflex, abnormal micturition, and laryngeal stridor in MSA. The lateral PAG column mediates sympathoexcitatory responses via its projections to the rostral ventrolateral medulla (Bandler et al., 2000) and cell loss in this column may contribute to impaired sympathetic cardiovascular control in MSA. The dorsomedial PAG has a major role in mediating anxiety responses in experimental animals, but the clinical significance of neuronal loss in this region in MSA is uncertain. The affected PAG columns receive inputs from the medial preoptic area, and their involvement may contribute to abnormal thermoregulation in MSA patients.

Our results also show abundant GCI accumulation in all columns of the PAG. The preservation of Nissl-stained non-VGLUT-2 immunoreactive neurons in all PAG columns and the preservation of NOS immunoreactive neurons in the dorsolateral PAG are consistent with previous studies showing a lack of a direct correlation between GCI density and severity of neuronal loss in MSA. This supports the hypothesis that oligodendrocyte pathology is an early feature preceding neuronal loss in MSA, and that the degeneration of cell bodies is not a prerequisite for the formation of GCIs. There was myelin loss and astrocytic gliosis in association with GCI accumulation in all columns of the PAG. This is also consistent with previous observations and suggests that functional disconnection, abnormal local microenvironment, or both may affect PAG neuronal function in MSA. Further studies are required to further address these critical issues.

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Figure 1.

Representative 50 μ m transverse hemisection at the level of the periaqueductal gray (PAG) showing the characteristics of vesicular glutamate transporter-2 (VGLUT-2) immunoreactive neurons in the ventrolateral (vl) column (left panel), and nitric oxide synthase (NOS) immunoreactive neurons in the dorsolateral (dl) column in a 75 year-old man with no neurological history (A,C, post mortem delay 18 hrs), and in a 65 year-old woman with multiple system atrophy (B,D, post mortem delay 10 hrs). There was loss of VGLUT-2 immunoreactive neurons in the vlPAG but preservation of NOS neurons in the dlPAG in the MSA compared to the control case. Bar = 25 μ m.

Figure 2.

Total estimated numbers of VGLUT-2 (left) and non immunoreactive Nissl stained neurons in the ventrolateral, (vl), lateral (l), dorsolateral (dl), and the dorsomedial (dm) columns of the PAG (right). In MSA cases, there was a reduction in the total estimated number of VGLUT-2 immunoreactive neurons in the ventrolateral, lateral and dorsomedial with a trend to decrease in the dorsolateral PAG. There was no significant difference between MSA and controls in the total estimated cell counts or cell density of Nissl-stained, non-VGLUT-2, non-TH, or non-NOS immunoreactive neurons in any of the PAG columns.

Figure 3.

Glial cytoplasmic inclusion (GCI) distribution throughout the periaqueductal gray (PAG) in a 66 year-old man with multiple system atrophy (MSA). (Case MSA-9 in Table 1). Insert shows the approximate level of the sections.

Figure 4.

A: Schematic representation of the periaqueductal gray (PAG) showing the topography ventrolateral (vl), lateral (l), dorsolateral (dl), and the dorsomedial (dm) columns and B, composite of the thirteen sections (from Figure 3) showing the distribution of glial cytoplasmic inclusions (GCIs) in one MSA case. C. Density of GCIs in the different PAG columns in all MSA cases. GCIs appeared to be more abundant in the ventrolateral than in other columns. (** $p < 0.01$ vl vs dm, † $p < 0.05$, vl vs dl). DRN, dorsal raphe nucleus. Bar = $20\mu\text{m}$.

Figure 5.

Representative 12 μm sections of the ventrolateral PAG stained for Luxol fast blue for myelin (A, B), and double-label immunocytochemistry for α -synuclein (FITC) and glial fibrillary acidic protein (GFAP) (Cy3). There was loss of myelin and astrocytic gliosis in the area of GCI accumulation. Bar = 25 μm .

Table 1

Patient Population

Case	Age/Gender	PMD (hrs)	SxD (yrs)	Motor phenotype	Autonomic, manifestations	Stridor	Sleep disorder	Pathological diagnosis
C 1	51/M	22	N/A	None	None	N/A	None	CHF
C 2	57/F	9	N/A	None	None	N/A	None	Ovarian carcinoma
C 3	58/M	9	N/A	None	None	N/A	None	Lymphoma
C 4	64/F	20	N/A	None	None	N/A	None	COPD, Cardiac arrest
C 5	66/M	22	N/A	None	None	N/A	None	AAA
C 6	66/F	16	N/A	None	None	N/A	None	Cholestatic hepatitis
C 7	67/F	15	N/A	None	None	N/A	None	Gastrointestinal hemorrhage
C 8	67/M	20	N/A	None	None	N/A	None	Respiratory failure
C 9	68/M	16	N/A	None	None	N/A	None	Pulmonary fibrosis
C 10	73/M	18	N/A	None	Constipation	N/A	None	CHF
C 11	75/M	18	N/A	None	None	N/A	None	Renal disease
C 12	77/M	11	N/A	None	None	N/A	None	CHF
C 13	77/F	22	N/A	None	Constipation	N/A	None	Scleroderma, CAD
MSA 1	46/M	17	5	MSA-C	OH, NB, ED, Constipation, Anhidrosis	Yes*	EDS, OSA, PLMS, RBD *	MSA
MSA 2	46/M	3	6	MSA-C	OH, NB, ED, Dysphagia, Anhidrosis	No	None	MSA
MSA 3	53/M	10	6	MSA-C	OH, NB, ED, Constipation, Anhidrosis	No	None	MSA
MSA 4	56/M	10	12	MSA-P	OH, NB, ED, Dysphagia, Anhidrosis	Yes*	EDS, OSA, RBD *	MSA
MSA 5	56/M	12	14	MSA-P	OH, NB, ED, Constipation, Anhidrosis	No	EDS	MSA
MSA 6	59/M	26	5	MSA-P	OH, NB, ED, Constipation, Anhidrosis	Yes*	EDS, OSA, RBD *	MSA
MSA 7	63/F	11	6	MSA-P	OH, NB, Dysphagia, Anhidrosis	No	RBD	MSA
MSA 8	65/F	10	7	MSA-C	OH, NB, Constipation, Anhidrosis,	No	EDS, OSA, PLMS, RBD *	MSA
MSA 9	66/M	5	4	MSA-P	OH, NB, ED, Constipation, Anhidrosis,	No	None	MSA
MSA 10	67/M	1	2	MSA-P	OH, NB, Constipation	No	OSA	MSA
MSA 11	68/M	23	14	MSA-P	OH, NB, ED	No	RBD	MSA
MSA 12	70/M	20	10	MSA-C	OH, ED, Constipation, Anhidrosis	No	EDS, RBD	MSA
MSA 13	75/F	8	4	MSA-P	OH, NB, Dysphagia, Constipation	No	EDS	MSA

AAA=abdominal aortic aneurysm, C=control, CAD=coronary artery disease, CHF= congestive heart failure, COPD= chronic obstructive pulmonary disorder, ED= erectile dysfunction, EDS=excessive diurnal somnolence, MSA= multiple system atrophy, MSA-C= multiple system atrophy cerebellar, MSA-P= multiple system atrophy parkinsonism, N/A=not applicable, NB=neurogenic bladder, OH=orthostatic hypotension, OSA=obstructive sleep apnea, PLMS=periodic leg movements sleep, PMD=post-mortem delay, RBD=REM behavior sleep disorder, SxD=symptom duration,

Table 2

Cell counts, density and sizes in the different columns of the PAG

Column	Cell type	Variable	Control (n=13) (mean±SEM)	MSA (n=13) (mean±SEM)
Ventrolateral	VGLUT-2	Total estimated number	27,906 ± 2,480	16,299 ± 1,612
		Density/mm ³	1,413 ± 59	836 ± 114
		Size (μm ²) (n=100)	349 ± 18	323 ± 19
	TH	Total estimated number	21,895 ± 2,248	11,348 ± 1,634
		Density/mm ³	1,006 ± 147	523 ± 72
		Size (μm ²) (n=100)	617 ± 61	709 ± 87
	Nissl (non-VGLUT-2, non-TH)	Total estimated number	58,073 ± 7,302	57,165 ± 4,612
		Density/mm ³	2,266 ± 135	2,168 ± 201
		Size (μm ²) (n=100)	195 ± 9	197 ± 8
Lateral	VGLUT-2	Total estimated number	16,078 ± 1,140	11,004 ± 1,401
		Density/mm ³	2,266 ± 135	1,081 ± 159
		Size (μm ²) (n=100)	218 ± 10	240 ± 19
	Nissl (non-VGLUT-2)	Total estimated number	38,283 ± 4,481	36,743 ± 3,700
		Density/mm ³	2,760 ± 263	2,384 ± 344
		Size (μm ²) (n=100)	175 ± 13	177 ± 6
Dorsolateral	VGLUT-2	Total estimated number	14,080 ± 1,107	10,306 ± 1,455
		Density/mm ³	1,296 ± 63	1,007 ± 169
		Size (μm ²) (n=100)	187 ± 14	200 ± 9
	NOS	Total estimated number	27,576 ± 1,653	27,484 ± 2,626
		Density/mm ³	1,627 ± 182	1,575 ± 204
		Size (μm ²) (n=100)	202 ± 8	192 ± 9
	Nissl (non-VGLUT-2, non-NOS)	Total estimated number	48,852 ± 3,996	47,382 ± 5,101
		Density/mm ³	2,813 ± 98	2,753 ± 194
		Size (μm ²) (n=100)	197 ± 14	188 ± 9
Dorsomedial	VGLUT-2	Total estimated number	15,412 ± 1,097	8,847 ± 1,052
		Density/mm ³	1,277 ± 74	796 ± 101
		Size (μm ²) (n=100)	197 ± 11	212 ± 10
	Nissl (non-VGLUT-2)	Total estimated number	42,611 ± 3,142	48,148 ± 5,714
		Density/mm ³	2,549 ± 141	2,468 ± 181
		Size (μm ²) (n=100)	169 ± 18	175 ± 9

NOS=nitric oxide synthase, TH=tyrosine hydroxylase, VGLUT-2=vesicular transporter 2