Differential involvement of hypothalamic vasopressin neurons in multiple system atrophy

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We sought to determine whether there is differential involvement of different groups of hypothalamic arginine-vasopressin (AVP) synthesizing neurons in multiple system atrophy (MSA). Hypothalamus was obtained from five subjects with clinical diagnosis of MSA confirmed neuropathologically and five age-matched controls. Sections were immunostained for AVP, and cells with visible nuclei were counted in the posterior portion of the paraventricular nucleus (PVNp), supraoptic nucleus (SON), magnocellular PVN and suprachiasmatic nucleus (SCN). Sections of the hypothalamus and medulla were also immunostained for tyrosine hydroxylase (TH). There was a significant loss of AVP neurons in the PVNp in MSA compared with controls (17 ± 6 versus 59 ± 10 cells/section, P < 0.01). There was preservation of AVP- and TH-immunoreactive neurons in the SON and magnocellular PVN in all MSA cases. In contrast, there was marked depletion of TH-immunoreactive fibres innervating these magnocellular AVP neurons, coincident with a loss of neurons in the A1 area (6 ± 1 versus 13 ± 1 cells/section, P < 0.01). There was loss of AVP neurons in the SCN in MSA compared with control cases (14 ± 3 versus 71 ± 16 cells/section, P < 0.02). Our results indicate that, in MSA, loss of AVP neurons in the PVNp may contribute to sympathetic failure, whereas loss of catecholaminergic input from the brainstem to the magnocellular AVP neurons may contribute to impaired AVP secretion in response to orthostatic stress. Loss of AVP neurons in the SCN may contribute to impaired circadian regulation of endocrine and autonomic functions.

Keywords: suprachiasmatic nucleus; supraoptic nucleus; paraventricular nucleus; arginine vasopressin; tyrosine hydroxylase

Abbreviations: AVP = arginine vasopressin; MSA = multiple system atrophy; PVNp = posterior portion of the paraventricular nucleus; SON = supraoptic nucleus; SCN = suprachiasmatic nucleus; TH = tyrosine hydroxylase


Introduction

Multiple system atrophy (MSA) is a sporadic, progressive, adult-onset disorder characterized by early and severe autonomic failure, associated with parkinsonism (MSA-P), cerebellar ataxia (MSA-C), or both (Wenning et al., 2004). Although involvement of brainstem areas involved in autonomic control is consistently found in MSA (Benarroch, 2003), there is also evidence of hypothalamic dysfunction in this disorder. Abnormal circadian fluctuations of plasma arginine vasopressin (AVP) (Ozawa et al., 2001) and cortisol (Ozawa et al., 1999), as well as impaired physiological nocturnal fall in body temperature (Pierangeli et al., 2001) have been reported in MSA. Ozawa et al. (1998) reported depletion of AVP neurons in the suprachiasmatic nucleus (SCN), the circadian pacemaker, and preservation of magnocellular AVP neurons in a single case of MSA. However, it is still uncertain whether this is a consistent finding in this disorder. For example, the number of AVP neurons in the SCN varies with age, sex and time of the year (Swaab et al., 1992), and expression of AVP in magnocellular hypothalamic neurons varies with the state of hydration (Swaab, 2003; Panayotacopoulou et al., 2005).

Consistent with preservation of magnocellular AVP neurons in the supraoptic (SON) and paraventricular (PVN) nuclei, there is preservation of AVP response to dehydration in MSA patients (Puritz et al., 1983). In contrast, there is impaired release of AVP in response to orthostatic stress in these patients (Puritz et al., 1983; Williams et al., 1985; Kaufmann et al., 1992; Deguchi et al., 2004). This indicates that, although there may be sparing of magnocellular AVP neurons, there is an impairment in the reflex regulation of AVP secretion in response to haemodynamic stimuli in MSA. This may reflect loss of inputs from the brainstem,
involving those from A1 noradrenergic neurons of the caudal ventrolateral medulla, which control reflex release of AVP (Williams et al., 1985; Blessing and Willoughby, 1987; Day and Sibbald, 1989). Although previous studies from our laboratory indicate that there is a reduction in the number of A1 neurons in MSA (Benarroch, 1998), there is not yet direct evidence of loss of catecholaminergic innervation of magnocellular AVP neurons in this disorder.

In humans, AVP is also expressed by a group of neurons in the posterior subnucleus of the PVN (PVNp); these neurons are thought to be homologous to those projecting to autonomic and respiratory centres in the brainstem and spinal cord in experimental animals (Koutcherov et al., 2000). In these regions, AVP exerts a sympathoexcitatory effect (Coote, 2004) and increases inspiratory drive (Kc et al., 2002). Thus, loss of AVP neurons in the PVNp may contribute to sympathetic and respiratory impairment in MSA. The involvement of AVP neurons in the PVNp in MSA has not yet been explored. In this study, we sought to determine whether, in MSA patients, (i) there is involvement of putative autonomic AVP neurons in the PVNp; (ii) there is evidence of catecholaminergic denervation of magnocellular AVP neurons; and (iii) there is consistent involvement of AVP neurons in the SCN.

Methods

Subjects

We examined the hypothalamus and medulla in five cases with neuropathologically confirmed diagnosis of MSA (three males and two females, age 63 ± 4 years) and five age-matched controls (two males and three females, age 70 ± 4 years) with no neurological disease (Table 1). Three MSA patients had parkinsonism (MSA-P) and two had parkinsonism and ataxia (MSA-M). All had evidence of sympathetic failure with severe orthostatic hypotension and blunted nocturnal fall of arterial pressure with supine hypertension. All patients had neurogenic bladder, constipation and anhidrosis, and one had laryngeal stridor. No MSA patient had history of excessive urination or thirst during the day, but three of them had history of nocturnal polyuria. Unfortunately, none of the MSA patients or controls had determinations of plasma AVP during life. The time of autopsy was similar between the control and the MSA group. In general, death occurred between July and December in both groups. Post-mortem delay was similar in MSA cases (14 ± 2 hours) and controls (15 ± 3 h).

Tissue processing and immunocytochemistry

The left half of the brain was processed for routine neuropathologival studies. All MSA cases were confirmed neuropathologically and showed various degrees of striatonigral and olivopontocerebellar degeneration, associated with accumulation of glial cytoplasmic inclusions. Two blocks were obtained from the right half of the brain; one included the hypothalamus from the level 2 mm anterior to the optic chiasm to the posterior border of the mammillary bodies, and a second included the medulla. These blocks were immersion fixed in 5% formalin for 24 h at 4°C and cryoprotected in buffered 30% sucrose for 5–7 days and then frozen in dry ice. Serial 50 μm cryostat sections were obtained throughout the hypothalamus and every eighth section was immunostained for TH immunostaining was also performed in sections of the medulla obtained between 3 mm caudal and 2 mm rostral to the obex, corresponding to the distribution of AVP (Chemicon, Temecula, CA, USA). Sections of the anterior hypothalamus that included the SON and magnocellular PVN were also immunostained for tyrosine hydroxylase (TH, Immunostar, Hudson, WI, USA), which is co-expressed with AVP in hypothalamus and every eighth section was immunostained for

| Table 1 Patient population |
|---------------------------|------------------|------------------|------------------|------------------|------------------|
| Case | Age/sex | Motor manifestation | Autonomic manifestation | Disease duration (years) | Month/year of death | PMD (hours) | Pathological diagnosis |
| Con 1 | 64/F | None | None | N/A | 11/02 | 20 | COPD |
| Con 2 | 68/M | None | None | N/A | 7/03 | 16 | Hepatitis |
| Con 3 | 57/F | None | None | N/A | 8/03 | 9 | Ovarian carcinoma |
| Con 4 | 84/M | None | Constipation | N/A | 12/03 | 6 | CAD |
| Con 5 | 77/F | Gait instability | Constipation | N/A | 8/04 | 22 | CAD |
| MSA 1 | 68/M | Parkinsonism | OH, NB, impotence, constipation, anhidrosis | 14 | 11/02 | 23 | MSA (SND–OPCA) |
| MSA 2 | 54/F | Parkinsonism, ataxia | OH, NB, constipation, anhidrosis | 8 | 10/03 | 9 | MSA (SND–OPCA) |
| MSA 3 | 53/M | Parkinsonism, ataxia | OH, NB, constipation | 6 | 12/03 | 10 | MSA (SND–OPCA) |
| MSA 4 | 57/M | Parkinsonism | OH, NB, constipation, anhidrosis | 7 | 8/04 | 14 | MSA (SND–OPCA) |
| MSA 5 | 81/F | Parkinsonism | OH, NB, constipation, anhidrosis | 5 | 12/03 | 13 | MSA (SND–OPCA) |

CAD = coronary artery disease; COPD = chronic obstructive pulmonary disease; Con = control; MSA = multiple system atrophy; NB = neurogenic bladder; OH = orthostatic hypotension; OPCA = olivopontocerebellar atrophy; PMD = post-mortem delay; SND = striatonigral degeneration.
A1 neurons (Paxinos and Huang, 1995) to assess involvement of these cells, which innervate the magnocellular AVP neurons. Although dopamine-beta-hydroxylase (DBH) would have been a more selective marker of A1 noradrenergic neurons than TH, we chose TH instead of DBH for two reasons: (i) in our hands TH provides a much better immunostaining of catecholaminergic fibres than DBH and (ii) TH could also be used as an additional marker of magnocellular neurons in the SON and PVN. Since TH expression correlates with that of AVP in these magnocellular neurons and is affected by the state of hydration (Panayotacopoulou et al., 2002, 2005), this immunocytochemical marker would provide an internal control for the potential effects of this variable on AVP expression in these cells.

Antibodies against AVP or TH were diluted in a 0.1 M phosphate buffer, pH 7.4. To block endogenous peroxidase, the sections were preincubated in a 3% hydrogen peroxide solution for 30 min at room temperature. Sections were then incubated in the primary antibody for 3 days at 4°C. To block non-specific binding, the tissue was incubated with 5% normal serum. Omission of the primary antibody or incubation with normal sera resulted in a lack of immunostaining. Sections were then rinsed and reacted in a diaminobenzidine (DAB)/glucose oxidase for 5–10 min. Sections were then mounted, co-stained with thionin, dehydrated and coverslipped in DPX (Fluka, Rokonhoma, NY, USA).

**Image analysis and quantification**

The sections were examined under bright field microscopy. Cells immunolabelled for AVP or TH were counted every 400 μm. Every immunoreactive cell identified by the presence of a nucleus was mapped and counted. Sections were viewed on a Diaplan Axiophot microscope (Carl Zeiss Microimaging, Inc. Thornwood, NY, USA), equipped with a 2.5x/0.075 NA objective lens. Image analysis was performed using the Carl Zeiss Axiocam and Axiovision Interactive Measurement software (Carl Zeiss Inc.). Images were digitized and stored. AVP-immunoreactive neurons were counted separately in the SCN, SON, ventrolateral (magnocellular) portion of the PVN and PVNp. For the purpose of this study, the PVNp was defined as the portion of the nucleus located between 4 and 8 mm posterior to the plane of the anterior commissure, according to Koutcherov et al. (2000). TH-immunoreactive neurons and fibres were examined in the SON and ventrolateral (magnocellular) PVN, and TH-immunoreactive neurons were counted in the ventrolateral medulla between 3 mm caudal and 2 mm rostral to the obex, corresponding to the A1 group (Paxinos and Huang, 1995). Fibre density was estimated qualitatively. The Abercrombie’s correction factor for nuclear counts was 0.9. The investigator performing the cell counts was blinded to the clinical and neuropathological diagnosis. Cell numbers [mean ± standard error of measurement (SEM)] were compared using analysis of variance. A P-value of <0.05 was considered significant.

**Results**

**Posterior subnucleus of the PVN**

AVP-immunoreactive cells were counted in the PVNp, defined in this study by the portion of the PVN located between 4 and 8 mm posterior to the anterior commissure (Koutcherov et al., 2000). The mean diameter of AVP-immunoreactive neurons in this region was 22 ± 2.6 μm in MSA and 21 ± 3.4 μm in controls (n = 20 each). There was a significant reduction of the number of AVP-immunoreactive neurons in the PVNp in MSA cases compared with controls (17 ± 3 versus 59 ± 10 cells/section; P < 0.01) (Fig. 1).

**Magnocellular groups in the supraoptic and paraventricular nuclei**

Abundant medium-to-large AVP-immunoreactive neurons were observed in the SON and ventrolateral (magnocellular) portions of the PVN (Fig. 2). The average diameter for these neurons in the SON was 24.5 ± 0.2 μm in controls and 26.9 ± 0.2 μm in MSA (n = 100 each); and in the ventrolateral PVN it was 24.5 ± 0.2 μm in controls and 24.6 ± 0.2 μm in MSA (n = 100 each). Consistent with previous studies (Ozawa, 1998) there was no significant difference in cell counts between the MSA and the control groups either in the SON (188 ± 26 cells/section in MSA versus 161 ± 20 cells/section in controls) or in the ventrolateral PVN (173 ± 18 cells/section in MSA versus 160 ± 11 cells/section in controls). There was no significant difference in TH cell counts in these regions between the MSA or control groups (for the SON: 102 ± 21 cells/section in MSA versus 86 ± 9 cells/section in controls; for the magnocellular PVN: 62 ± 11 cells/section in MSA versus 73 ± 10 cells/section in controls).

In contrast, there was a marked reduction in density of TH-immunoreactive fibres in the SON and ventrolateral PVN in the MSA cases (Fig. 3). In all these cases, there was a significant loss of TH-immunoreactive neurons in the A1 region of the medulla (6 ± 1 versus 14 ± 1 cells/section, P < 0.01) (Fig. 3).

**Suprachiasmatic nucleus**

AVP-immunoreactive neurons were identified in the dorsal aspect of the SCN, consistent with previous studies (Swaab et al., 1992) (Fig. 4). There was no significant difference in the cell diameter between the MSA (15 ± 1 μm, n = 20) and the control group (16 ± 1 μm, n = 20). In all MSA cases, there was significant AVP cell loss in the SCN (14 ± 3 in MSA versus 71 ± 16 cells/section in controls, P < 0.02). Whereas in the control group cell numbers appeared to be higher in men (mean number of cells/section was 66 for the three females and 90 for the two males); cell loss was similar in all MSA cases, regardless of sex (the mean number of cells/section was 14 for the two females and 14 for the three males).

**Discussion**

Our findings indicate that, in MSA, (i) there is loss of putative autonomic AVP neurons in the PVNp, whereas there is preservation of magnocellular AVP neurons in the SON and PVN; (ii) there is loss of catecholaminergic innervation of magnocellular SON and PVN neurons; and (iii) there is consistent loss of AVP neurons in the SCN.
Involvement of AVP neurons in the PVNp may contribute to autonomic failure in MSA. Chemoarchitectonic studies indicate that these neurons correspond to PVN neurons that project to brainstem and spinal autonomic areas in the rat (Koutcherov et al., 2000). Inputs from these neurons to the intermediolateral cell column, either directly or via a relay in the rostral ventrolateral medulla, have been implicated in sympathoexcitatory responses (Coote, 2004). Since AVP

**Fig. 1** Upper panel: Fifty micrometre sections of the posterior hypothalamus at the level of PVNp immunostained for AVP either alone (A and B) or co-stained with thionin (C and D). Left: 64-year-old woman with no neurological disease, post-mortem delay 20 h. Right: 54-year-old woman with clinical and neuropathological diagnosis of MSA, post-mortem delay 9 h. There was a marked loss of AVP-immunoreactive neurons in the PVNp in the MSA case. Bar = 20 μm. Lower panel: Number of AVP cells/section in the PVNp in five controls and five MSA cases. There was a consistent loss of AVP neurons in the PVNp in MSA cases. *P < 0.01.
activates premotor sympathoexcitatory neurons of the rostral ventrolateral medulla (Yang and Coote, 2003) and intermediodorsal cell column (Kolaj and Renaud, 1998), involvement of AVP-containing autonomic PVNp neurons may contribute to sympathetic failure in MSA. The evidence that PVN vasopressinergic input may not be critical for maintenance of tonic activity of neurons in the rostral ventrolateral medulla (Yang and Coote, 2003) is consistent with the presence of a residual basal sympathetic activity in MSA patients (Parikh et al., 2002). There is also evidence that the PVNp projects to respiratory groups of the medulla and spinal cord, where AVP exerts a stimulating effect on respiratory drive (Kc et al., 2002). Thus loss of AVP input from the PVNp to medullary respiratory centres may potentially contribute to failure of automatic ventilation, which manifests as sleep apnoea or respiratory arrhythmia in MSA patients.

Our study shows that whereas putative autonomic AVP neurons in the PVNp are consistently involved in MSA there is preservation of magnocellular AVP neurons in the SON and ventrolateral PVN. Although preservation of magnocellular AVP neurons has been reported in a single MSA case (Ozawa et al., 1998), to our knowledge, our study is the first to show consistent preservation of both AVP- and TH-immunoreactive magnocellular neurons in MSA. Our findings are supported by evidence that TH expression correlates with that of AVP in magnocellular neurons.
according to the state of hydration (Panayotacopoulou et al., 2005). One limitation of our study is that AVP levels and their changes in response to dehydration had not been assessed in our MSA cases. However, there is consistent evidence that AVP responses to dehydration are preserved in MSA (Puritz et al., 1983; Williams et al., 1985). Although three of our MSA subjects had history of nocturnal polyuria, no patient had history of excessive thirst or polyuria during the day. Our findings, together with previous observations (Ozawa et al., 1998), explain the preservation of magnocellular AVP cell responses to dehydration in MSA.

Our results provide a mechanistic explanation for the impairment of AVP responses to orthostatic hypotension in MSA. These impaired responses have been reported in many studies (Puritz et al., 1983; Williams et al., 1985; Kaufmann et al., 1992; Deguchi et al., 2004). Whereas a limitation of our study is that AVP response to orthostatic stimuli had not been assessed in our MSA cases, the bulk of previous evidence indicates that this is likely to have also

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**Fig. 3** Upper panel: Fifty micrometre sections of the anterior hypothalamus at the level of SON (A and B) or ventrolateral medulla (C and D) immunostained for TH. Left: 68-year-old man with no history of neurological disease, post-mortem delay 16 h. Right: 54-year-old woman with clinical and neuropathological diagnosis of MSA, post-mortem delay 9 h. There was severe loss of TH-immunoreactive axons despite the preservation of TH-immunoreactive neurons in the SON in the MSA case (A and B). In this case, there was also loss of TH-immunoreactive neurons in the A1 area of the caudal ventrolateral medulla (C and D). Bar = 20 μm. Lower panel: Number of TH cells/section in the A1 area in five controls and five MSA cases. **p < 0.01.
been the case in our subjects. There is experimental evidence that reflex AVP secretion in response to unloading of the baroreceptors and cardiac receptors is, in part, activated by noradrenergic inputs from A1 neurons of the ventrolateral medulla (Blessing and Willoughby, 1987; Day and Sibbald, 1989). Neurochemical studies have shown that there are reduced norepinephrine levels in the hypothalamus in MSA (Williams et al., 1985), and our previous study showed a reduced number of A1 neurons in this condition (Benarroch, 1998). However, our present results provide, for the first time, morphological evidence that there is reduced catecholaminergic innervation of magnocellular

**Fig. 4** Upper panel: Fifty micrometre sections of the anterior hypothalamus at the level of the SCN immunostained for AVP either alone (A and B) or co-stained with thionin (C and D). Left: 68-year-old man with no neurological disease, post-mortem delay 16 h. Right: 53-year-old man with clinical and neuropathological diagnosis of MSA, post-mortem delay 10 h. There was a marked loss of AVP-immunoreactive neurons in the SCN in the MSA case. Lower panel: Number of AVP cells/section in the SCN in five controls and five MSA cases. There was a consistent loss of AVP neurons in the SCN in all MSA cases. Bar = 20 μm. *P < 0.02.
AVP neurons in the SON in MSA. Although one limitation of our study is that TH cannot distinguish dopaminergic from noradrenergic fibres in the hypothalamus, two additional findings support the fact that TH axonal loss in the magnocellular nuclei probably reflects interrupted noradrenergic input. First, the number of TH-immunoreactive cells in the SON and ventrolateral PVN was similar in MSA and in controls. This indicates that loss of TH-immunoreactive fibres cannot be solely attributable to loss of axons from local TH-immunoreactive cells. Second, in all cases, loss of TH-immunoreactive axons in the SON and magnocellular PVN was associated with neuronal loss in the A1 area. This suggests that loss of A1 neurons may contribute to loss of TH-immunoreactive fibres in the SON and magnocellular PVN. Thus, our present results indicate that impaired reflex AVP secretion in MSA may, in part, reflect loss of noradrenergic input from the A1 area to magnocellular AVP neurons.

Our findings of loss of AVP neurons in the SCN are consistent with previous observations on a single MSA case (Ozawa et al., 1998). However, our results extend these observations by showing that age, sex and time of the year, which are known to affect AVP cell numbers in the SCN (Swaab et al., 1992), cannot account for the differences observed between MSA and control cases. No such conclusions could be drawn from the previous study on a single MSA case (Ozawa et al., 1998). Neuronal loss in the SCN may contribute to abnormalities in circadian regulation in MSA, including loss of diurnal fluctuations of plasma AVP (Ozawa et al., 2001) and cortisol (Ozawa et al., 1999). There is evidence that AVP-containing GABAergic neurons of the SCN control activity of autonomic neurons of the PVN (Saper et al., 2005). For example, the SCN inhibits the PVN neurons that initiate the sympathoexcitatory pathway involved in melatonin secretion (Moore, 1992). Thus, it is conceivable that SCN–PVNp interactions are responsible for circadian variations of other sympathetically regulated functions, including control of body temperature and blood pressure. This may, at least in part, explain the impaired physiological nocturnal fall in body temperature (Fierangel et al., 2001) and contribute to the nocturnal rise in blood pressure that occurs in MSA.

In summary, our findings indicate that there is a differential involvement of hypothalamic cardiovascular control mechanisms mediated by AVP neurons in MSA. Loss of AVP inputs from the PVNp to the brainstem and spinal cord may contribute to sympathetic and perhaps respiratory abnormalities in MSA. Loss of AVP neurons in the PVNp suggests that the ‘system’ degeneration in MSA extends from preganglionic and brainstem neurons to hypothalamic autonomic premotor neurons. Although there is preservation of magnocellular neuroendocrine AVP neurons in MSA, these neurons are functionally disconnected from cardiovascular brainstem reflexes, in part owing to lack of catecholaminergic inputs from the A1 area. Impaired reflex AVP secretion may contribute to orthostatic hypotension in MSA patients. Finally, depletion of AVP neurons in the SCN probably contributes to impaired circadian control of endocrine and autonomic functions, including thermoregulation and blood pressure, in MSA patients. Neuropathological studies of a larger number of cases in whom basal and reflex AVP responses had been assessed during life are necessary to support these possibilities.

Acknowledgements

This study was supported by a grant from the National Institutes of Health (NS32352-P2) and Mayo Funds.

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