Depletion of ventromedullary NK-1 receptor-immunoreactive neurons in multiple system atrophy

Eduardo E. Benarroch,1 Ann M. Schmeichel,1 Phillip A. Low1 and Joseph E. Parisi2

Departments of1Neurology and 2Anatomic Pathology, Mayo Foundation, Rochester, MN, USA

Summary
We sought to determine whether there are neurokinin-1 receptor-like-immunoreactive (NK-1R-LI) neurons in human ventrolateral medulla and whether these neurons are more severely involved in multiple system atrophy (MSA) than in Parkinson’s disease. Brains were obtained at autopsy from six control subjects, six subjects with clinical diagnosis of MSA and four with Parkinson’s disease, both confirmed neuropathologically. Serial 50 μm cryostat sections were obtained throughout the medulla, and every eighth section was processed for NK-1R-LI neurons. Some sections were processed simultaneously for tyrosine hydroxylase or choline acetyltransferase. Abundant NK-1R-LI neurons were identified in the ventrolateral medulla. These neurons were distinct from local cholinergic or catecholaminergic neurons. There was a severe depletion of these NK-1R-LI neurons in all MSA cases compared with controls (6 ± 1 cells/section versus 49 ± 2 cells/section in controls). Although there was also a reduction in Parkinson’s disease (20 ± 2 cells/section), this was significantly less severe than in MSA. Our findings suggest that the human ventrolateral medulla contains NK-1R-LI neurons, and the more severe depletion in MSA than in Parkinson’s disease may explain the higher incidence of respiratory and cardiovascular abnormalities in the former condition.

Keywords: parkinsonism; ventrolateral medulla; substance P receptors

Abbreviations: CAT = choline acetyltransferase; MSA = multiple system atrophy; MSA-C = MSA with cerebellar ataxia; MSA-P = MSA with parkinsonism; NK-1R = neurokinin-1 receptor; NK-1R-LI = neurokinin-1 receptor-like-immunoreactive; OH = orthostatic hypotension; pre-BötC = pre-Bötzinger complex; TH = tyrosine hydroxylase

Introduction
The intermediate reticular formation of the medulla, including the ventrolateral medulla, has a critical role in tonic and reflex control of respiratory, cardiovagal and sympathetic vasomotor functions (Spyer, 1994; Guyenet et al., 1996). This region receives innervation by substance P-immunoreactive fibres (Huang and Paxinos, 1995). Local administration of substance P into the ventrolateral medulla stimulates ventilation (Chen et al., 1990) and elicits an increase in arterial pressure (Sun and Guyenet, 1989; Urbanski et al., 1989); and administration into the nucleus ambiguous elicits bradycardia (Massari et al., 1996). These respiratory and cardiovascular effects are mediated by neurokinin-1 receptors (NK-1Rs). The NK-1R is also a marker of neurons of the pre-Bötzinger complex (pre-BötC), a component of the ventral respiratory group of the medulla that plays a critical role in respiratory rhythmogenesis (Smith et al., 1991; Bianchi et al., 1995; Gray and Feldman, 1998; Wang et al., 2001). In experimental animals, selective destruction of NK-1R-containing neurons in the pre-BötC results in atactic breathing patterns with prolonged periods of apnoea (Gray et al., 2001) and reduces both the tachypnoeic and depressor effect of chemical stimulation of the ventral respiratory group (Wang et al., 2002). Thus, NK-1R-containing neurons in the ventrolateral medulla may have an important role in respiratory as well as cardiovascular control. Whether NK-1R-containing neurons are present in the human ventrolateral medulla has not been explored systematically.

Multiple system atrophy (MSA) is a neurodegenerative condition characterized by impaired sympathetic vasomotor control, manifested with orthostatic hypotension (OH) (Bannister, 1993), cardiovagal impairment (Sandroni et al., 1991) and respiratory abnormalities, including sleep apnoea, sleep hypopnoea, dysrhythmic breathing and nocturnal stridor due to obstruction of the upper airway, which may
underlie sudden death during sleep (Bannister et al., 1981; Chokroverty, 1988). These manifestations are more frequent and severe than in Parkinson’s disease (Sandroni et al., 1991). In MSA, impaired ventilatory responses to hypoxia are more prevalent than in Parkinson’s disease (Tsuda et al., 2002).

Given the experimental evidence that NK-1R-containing neurons in the ventrolateral medulla are critical for respiratory rhythmogenesis (Gray et al., 2001) and that NK-1R may mediate the effects of substance P on sympathetic (Sun and Guyenet, 1989; Urbanski et al., 1989) and cardiovagal (Massari et al., 1996) functions, we postulated that NK-1R may be a marker of populations of ventrolateral medullary neurons that are more vulnerable in MSA than in Parkinson’s disease. In the present study, we sought to determine (i) whether the human ventrolateral medulla contains NK-1R-like-immunoreactive (NK-1R-LI) neurons; and (ii) whether there is NK1R-LI neuronal loss in MSA and, if so, if it is more severe than in Parkinson’s disease.

### Methods

#### Subjects

Brains were obtained at autopsy from six subjects (three men and three women, age 63 ± 7 years) with no history of neurological disease, six subjects (four men and two women, age 62 ± 3 years) with clinical diagnosis of MSA and four subjects (three men and one woman, age 75 ± 4 years) with clinical diagnosis of Parkinson’s disease (Table 1). Three of the MSA patients had predominant parkinsonism (MSA-P) and three had cerebellar ataxia (MSA-C). All MSA patients had OH, neurogenic bladder and a history of obstructive sleep apnoea, documented polysomnographically in three of the six subjects. Three MSA patients had cardiovagal failure documents in autonomic laboratory testing. Of the four patients with Parkinson’s disease, one had OH and another had both OH and obstructive sleep apnoea. These manifestations were not present in any of the controls. None of the MSA or Parkinson’s disease patients had a history of dementia.

#### Neuropathological assessment

Post-mortem delay was similar among controls (15.3 ± 3 h), MSA (14.2 ± 2 h) and Parkinson’s disease (13.3 ± 3 h) cases. The lower brainstem was separated for the purpose of this study. The rest of the brain was processed for routine neuropathological assessment. In all cases, the diagnosis of MSA or Parkinson’s disease was confirmed neuropathologically. All MSA cases showed neuronal loss in the substantia nigra pars compacta, putamen, basis pontis and cerebellum in various combinations and the presence of α-synuclein-immunoreactive oligodendroglial cytoplasmic inclusions. There were no Lewy bodies in the brainstem, basal ganglia or cerebral cortex. Four of the MSA cases had scarce Lewy bodies in the neocortex, and three had sparse to moderate diffuse plaques, neuritic plaques or neurofibrillary tangles, in different combinations, in the neocortex, hippocampus or both.

For the purpose of this study, tissue was immersion fixed in 2% buffered paraformaldehyde for 24 h and cryoprotected in sucrose for 24 h. Serial 50 μm cryostat sections of the medulla were obtained between −4 and +7.6 mm with respect to the obex.
**Immunohistochemistry**

Every eighth section was processed for NK-1R-immunoreactivity (polyclonal rabbit antibody 1:1000; Novus Biologicals, Littleton, CO). Antibody specificity was assessed by western blot, which showed a single band migrating at ~53 kDa. This is consistent with previous results on this antibody (Mournir and Parent, 2002).

Diaminobenzidine-glucose oxidase solution with nickel enhancement (Sigma, St Louis, MO) was used for the substrate reaction. Omission of the primary antibody, incubation in the presence of a blocking peptide or incubation with normal sera resulted in a lack of immunostaining.

After NK-1R immunoreactivity was detected and analysed, sections were co-stained with thionin. To determine whether NK-1R-LI corresponded to catecholaminergic or cholinergic neurons in the ventrolateral medulla, some sections were processed for both NK-1R and either tyrosine hydroxylase (TH; rabbit polyclonal antibody 1:750, Chemicon, Temecula, CA) or choline acetyltransferase (CAT; polyclonal goat antibody, 1:100, Chemicon). For this purpose, we utilized the double antigen immunohistochemistry method described by Levey et al. (1986). Briefly, DAB (3,3'-diaminobenzidine) was employed as the first chromogen to produce a diffuse brown staining of the TH- or CAT-immunoreactive neurons. This was followed by incubation with the NK-1R as the second primary antibody. Sections were then reacted with benzidine hydrochloride (BDHC) (Sigma) to produce a blue granular reaction product. Negative controls were generated by omission of the primary antibodies or substitution of normal serum. While there was a small amount of non-specific BDHC product, it was easily differentiated from specific neuronal staining.

**Quantitation and data analysis**

The sections were examined under bright field microscopy. The numbers of sections examined were 23, 29, 28, 27, 29 and 21, respectively, for the six controls; 22, 29, 29, 29 and 34, respectively, for the six MSA cases; and 22, 23, 29 and 29, respectively, for the four Parkinson’s disease cases. NK-1R-LI cells were counted in the region corresponding to the intermediate reticular zone as defined by Huang and Paxinos (1995), and in the cuneate nucleus. The nuclei were identified on the basis of the atlas of Paxinos and Huang (1995).

Image analysis was performed using a KS400 image analysis system (Carl Zeiss, Inc., Thornwood, NY). There was no significant difference in size between the surviving NK-1R-LI neurons between the MSA cases (22 ± 1 μm, n = 15), control cases (21 ± 1 μm, n = 15) and Parkinson’s disease cases (20 ± 2 μm, n = 15). Although a split cell would not affect the results given the similar cell size in controls and MSA cases, a correction factor was calculated and was 0.95. Cell numbers (mean ± SEM) were compared among control, MSA and Parkinson’s disease groups using ANOVA (analysis of variance). A P value < 0.05 was considered significant.

**Results**

**Characteristics of NK-1R-LI neurons in human ventrolateral medulla**

NK-1R-LI neurons were identified in the ventrolateral medulla, trigeminal nucleus, cuneate nucleus, nucleus tracti solitarii, dorsal vagal nucleus, hypoglossal nucleus, and medial and inferior vestibular nuclei. Many NK-1R-LI neurons were identified in the ventrolateral medulla, with a pattern of staining similar to that of the adjacent trigeminal nucleus (Fig. 1). The ventrolateral medullary NK-1R-LI neurons had a polygonal shape and a diameter between 17 and 25 μm, and were more abundant between 0 and 5 mm above the obex. As assessed in double-labelled sections, most NK-1R-LI neurons appeared to be distinct from neighbouring TH- or CAT-immunoreactive neurons (Fig. 2), although the possibility of double labelling could not be excluded in some cells.

**Depletion of NK-1R-LI neurons in the ventrolateral medulla in MSA**

There was a significant reduction in the numbers of NK-1R-LI neurons in the ventrolateral medulla in both MSA and Parkinson’s disease cases (Fig. 3). This could not be explained by lack of immunoreactivity, as supported by consistent loss of Nissl staining. Neuronal loss was consistent in all MSA cases and in all sections examined, and was of similar degree in the three MSA-P and the three MSA-C cases (Fig. 4). Although there was a reduction of NK-1R-LI ventrolateral medullary neurons in the Parkinson’s disease cases, this was significantly less severe than in MSA. The degree of reduction in the Parkinson’s disease cases was similar in the two patients with and the two patients without OH. In contrast to the findings in the ventrolateral medulla, the numbers of NK-1R-LI neurons in the cuneate nucleus was similar in MSA, Parkinson’s disease and control subjects (Fig. 5).

**Discussion**

Our results indicate that the human ventrolateral medulla contains NK-1R-LI neurons and that these neurons are more severely depleted in MSA than in Parkinson’s disease.

**NK-1R-LI neurons in the human ventrolateral medulla**

The distribution of NK-1R-LI neurons in the human medulla is consistent with that described in the rat (Nakaya et al., 1994). In human ventrolateral medulla, the distribution of NK-1R-LI neurons corresponds to that previously shown for substance P-immunoreactive fibres (Huang and Paxinos, 1995) and includes the area of the intermediate reticular zone presumably containing the rostral sympathoexcitatory and caudal sympathoinhibitory ventrolateral medullary groups.
the ventral respiratory group and the ventrolateral portion of the nucleus ambiguus. Therefore, for the purpose of this study, our analysis was focused on this intermediate reticular zone.

Our double labelling study indicates that most of these NK-1R-LI neurons lack immunoreactivity for TH or CAT, although the possibility of co-existence in some neurons could not be absolutely excluded. The lack of immunoreactivity for TH or CAT and their distribution in relation to other cell groups in the ventrolateral medulla suggest that a subpopulation of NK-1R-LI neurons identified in our study may correspond to the pre-BöC (Smith et al., 1991; Wang et al., 2002). However, this functionally defined neuronal group has not yet been identified in humans, and the number of NK-1R-LI neurons found in our study was higher than would be expected if they were only restricted to the pre-BöC, based on results in experimental animals (Wang et al., 2002).

There is experimental evidence that NK-1R-expressing cells in the rostral ventrolateral medulla control both respiratory rhythm and blood pressure. Chemical stimulation of the area corresponding to the rostral ventral respiratory group elicits both tachypnoea and depressor responses, and both effects are reduced ipsilaterally to the site of selective loss of NK-1R neurons induced by local injection of a conjugate of saporin with a selective NK-1R agonist (Wang et al., 2002). Most NK-1R-containing neurons in this region appear to be glutamatergic, but other neuronal populations may also be involved. It has been shown that the NK-1R is present on a small percentage of bulbospinal C1 neurons (Makeham et al., 2001) and in a minimal fraction of GABAergic neurons in the caudal ventrolateral medulla (Wang et al., 2001). Functional studies suggest that NK-1Rs may also be present in cardiovagal neurons of the rostral ventrolateral portion of the nucleus ambiguus (Massari et al., 1996) and that substance P activates sympathoexcitatory neurons in the ventrolateral medulla (Sun and Guyenet, 1989; Urbanski et al., 1989). Thus, loss of NK-1R-LI neurons in the ventrolateral medulla could manifest with both respiratory and cardiovascular disturbances.

**Significance of the loss of NK-1R-LI neurons in MSA**

We found consistent and severe depletion of NK-1R-LI neurons in the ventrolateral medulla in MSA. Neighbouring NK-1R-LI neuronal groups in the cuneate nucleus were spared. NK-1R-LI neurons also appeared to be spared in the trigeminal, vestibular and hypoglossal nuclei, but quantitative analysis was not performed. This finding suggests that NK-1R-LI neurons in the ventrolateral medulla may constitute a selectively vulnerable population in MSA. MSA is characterized by severe sympathetic, cardiovagal and respiratory abnormalities (Bannister, 1993). Loss of NK-1R-LI neurons

---

**Fig. 1** A 50 μm section of the mid medulla at the level of the nucleus ambiguus processed for immunohistochemistry for the neurokinin-1 receptor (NK-1R). Upper panel: distribution of NK-1R-LI neurons in human medulla. The box identifies the area of the medulla utilized for the quantitative studies. Lower panel: (A) NK-1R-LI neurons of the ventrolateral medulla (VLM) had a typical polygonal shape and a size of 17–25 μm. (B) NK-1R-LI neurons of the trigeminal nucleus. Bar = 50 μm.
in the ventrolateral medulla may contribute to these manifestations. Although MSA is characterized by a profound loss of catecholaminergic neurons in the C1 and A1 areas (Benarroch et al., 1998), studies in experimental animals (Makeham et al., 2001) and our present results indicate that only a small percentage of catecholaminergic neurons contain NK-1R immunoreactivity. Thus, loss of NK-1R-LI neurons cannot be attributed solely to loss of these catecholaminergic cells. Since most NK-1R-LI-containing neurons in the ventrolateral medulla are glutamatergic (Wang et al., 2001) and glutamate mediates the tonic sympathoexcitatory effect of the rostral ventromedullary neurons projecting to the intermediolateral cell column (Morrison et al., 1989), loss of NK-1R-LI neurons may reflect depletion of this sympathoexcitatory population and thus contribute to vasoconstrictor failure in MSA patients. If some of the NK-1R-LI neurons identified in this study correspond to depressor GABAergic ventrolateral medullary neurons mediating the baroreflex (Wang et al., 2001), their loss may contribute to baroreflex failure, presumably contributing to supine hypertension in MSA.

Some of the NK-1R-LI neurons identified in this study may correspond to the pre-BötC, and presumably their loss may contribute to some of the respiratory abnormalities that occur in MSA. Central apnoea or dysrhythmic breathing are manifestations of selective loss of NK-1R-containing neurons in the pre-BötC in experimental animals (Gray et al., 2001). Although all our MSA cases had a history of sleep apnoea, obstructive sleep apnoea was confirmed polysomnographically in only three of them, and central apnoea or dysrhythmic breathing was not documented. Thus, whether loss of ventrolateral medullary NK-R-LI contributes to respiratory dysfunction in MSA is an intriguing but still unproven possibility. Impaired ventilatory responses to hypoxia are more prevalent in MSA than in Parkinson’s disease (Tsuda et al., 2002). Although the role of the pre-BötC neurons in these responses is undetermined, if a population of these NK-1R-LI neurons corresponds to the pre-BötC, the more severe
Fig. 3 Upper panel: computer reconstruction of NK-1R-LI in the intermediate reticular zone of the ventrolateral medulla of a 73-year-old man with no history of neurological disease (post-mortem delay 18 h); a 74-old man with neuropathological diagnosis of Parkinson’s disease (post-mortem delay 24 h); and a 67-year-old man with clinical and neuropathological diagnosis of MSA and history of sleep apnoea and laryngeal stridor (post-mortem delay 8 h). Lower panels: 50 μm sections of the ventrolateral medulla at the level of the nucleus ambiguus, processed for NK-1R and co-stained with thionin of these same patients. There was a marked depletion of NK-1R-reactive neurons in the ventrolateral medulla of the MSA patient; this was more severe than in the Parkinson’s disease case. This could not be attributed to loss of expression of NK-1R immunoreactivity as demonstrated by counterstaining with thionin. Bar = 50 μm.

Fig. 4 Number of ventrolateral medullary NK-1R-immunoreactive neurons per section in brainstem sections obtained between −2.8 and +6.8 mm with respect to the obex from six controls (C; open bars), four patients with Parkinson’s disease (PD; grey bars) and six patients with MSA (filled bars). There was a consistent, severe depletion of NK-1R-immunoreactive neurons in the intermediate reticular zone of the ventrolateral medulla at all levels analysed in all MSA cases. Although there was a significant reduction in the Parkinson’s disease cases compared with controls, this was much less severe than in MSA. $P < 0.001$ *** MSA compared with controls. †††† MSA compared with Parkinson’s disease.
neuronal loss in MSA than in Parkinson’s disease would be consistent with the higher prevalence of respiratory disturbances in MSA. This possibility should be tested by studying a larger number of cases.

Although, in our study, we found no differences in the degree of NK-1R-LI neuronal loss between the three MSA-P and the three MSA-C cases, studies of a larger number of cases would be necessary to confirm these observations. In summary, our study indicates that the human intermediate reticular zone contains NK-1R-LI neurons that may, in part, correspond to those implicated in respiratory and cardiovascular control. The significantly more severe degree of loss of these neurons may contribute to the higher prevalence and severity of cardiovascular and respiratory abnormalities in MSA than in Parkinson’s disease. Whether these neurons are affected in Lewy body disease, which is occasionally associated with autonomic failure, is an important question that deserves further investigation.

Acknowledgements
This work was supported in part by a grant from the National Institute of Neurological Disorders and Stroke (PO1 NS32352-P2).

References


Bianchi AL, Denavit-Saubie M, Champagnat J. Central control of breathing in mammals: neuronal circuitry, membrane properties, and neurotransmitters. Physiol Rev 1995; 75: 1–45.


Gray PA, Feldman JL. NK-1 and mu opioid receptor expression in


Wang H, Germanson TP, Guyenet PG. Depressor and tachypneic responses to chemical stimulation of the ventral respiratory group are reduced by ablation of neurokinin-1 receptor-expressing neurons. J Neurosci 2002; 22: 3755–64.

Received March 17, 2003. Revised April 25, 2003. Accepted April 26, 2003