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Changes in total cell numbers of the basal ganglia in patients with multiple system atrophy — A stereological study

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ABSTRACT

Total numbers of neurons, oligodendrocytes, astrocytes, and microglia in the basal ganglia and red nucleus were estimated in brains from 11 patients with multiple system atrophy (MSA) and 11 age- and gender-matched control subjects with unbiased stereological methods. Compared to the control subjects, the MSA patients had a substantially lower number of neurons in the substantia nigra ($p = 0.001$), putamen ($p = 0.001$), and globus pallidus ($p < 0.001$), and, to a lesser extent in the caudate nucleus ($p = 0.03$). A significantly lower number of oligodendrocytes were only observed in the putamen ($p = 0.04$) and globus pallidus ($p = 0.01$). In the MSA brains the total number of astrocytes was significantly higher in the putamen ($p = 0.04$) and caudate nucleus ($p = 0.01$). In all examined regions a higher number of microglia were found in the MSA brains with the greatest difference observed in the otherwise unaffected red nucleus ($p = 0.001$). The results from the stereological study were supported by cell marker expression analyses showing increased markers for activated microglia. Our results suggest that microgliosis is a consistent and severe neuropathological feature of MSA, whereas no widespread and substantial loss of oligodendrocytes was observed. We have demonstrated significant neuronal loss in the substantia nigra, striatum, and globus pallidus of patients with MSA, while neurons in other basal ganglia nuclei were spared, supporting the region-specific patterns of neuropathological changes in MSA.

Introduction

Multiple system atrophy (MSA)[1] is a sporadic, adult-onset neurodegenerative disorder. It is clinically characterized by a combination of autonomic failure, parkinsonism, cerebellar ataxia, and corticospinal dysfunction with varying severity, causing progressive disability and death, usually within 7–9 years (O'Sullivan et al., 2008). Patients with MSA are clinically classified into one of two subtypes (parkinsonian or cerebellar) based on their predominant motor presentation (Gilman et al., 2008). Multiple system atrophy is an important differential diagnosis for Parkinson's disease due to a considerable overlap between the clinical presentations (Poewe and Wenning, 2002).

The defining neuropathology in MSA is the deposition of insoluble alpha-synuclein-positive glial cytoplasmic inclusions (GCIs) located in oligodendrocytes together with the degeneration of striatonigral or olivopontocerebellar structures (Trojanowski and Revesz, 2007). The pathogenesis of MSA is unknown. However, previously reported neuropathological findings suggest that alpha-synuclein accumulation precedes neuronal degeneration (Fujishiro et al., 2008; Wenning et al., 1994). The formation of GCIs is presumed to cause oligodendroglial dysfunction and myelin degeneration, which lead to axonal damage and neurodegeneration (Ahmed et al., 2012; Ishizawa et al., 2008; Wakabayashi and Takahashi, 2006; Yoshida, 2007). Variable degrees of neuronal loss have been reported in previously published non-stereological studies of brains from patients with MSA. In general, marked neurodegeneration with astrogliosis was found in the substantia nigra, putamen, and olivopontocerebellar structures (Dickson, 2012; Jellinger et al., 2005; Ozawa et al., 2004; Wenning et al., 1997, 2002). Involvement of the caudate nucleus and globus pallidus has also been described (Dickson, 2012; Jellinger et al., 2005; Ozawa et al., 2004; Wenning et al., 1997, 2002). In contrast, the subthalamic nucleus is described as unaffected or relatively spared (Dickson, 2012; Wenning et al., 1997), and the red nucleus, which is a part of the cerebellar output system and located in the midbrain, is

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[1] Multiple system atrophy (MSA), glial cytoplasmic inclusions (GCIs), quantitative real-time polymerase chain reaction (qRT-PCR), coefficient of error (CE), coefficients of variation (CVs).

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described as unaffected (Dickson, 2012). In addition, similar distributions of oligodendroglial apoptosis and microgliosis have been described (Ishizawa et al., 2004; Probst-Cousin et al., 1998). However, to our knowledge this study is the first to report the total cell numbers of the basal ganglia and red nucleus in MSA measured by methods based on unbiased stereological principles.

Stereology is a reliable method for the quantitative estimation of biological structures providing unbiased and precise estimates of structural features (Boyce et al., 2010; West, 2012). A major advantage of stereology is obtaining total quantities and stereology plays an important role in comparative studies of the human brain (Fabricius et al., 2013; Karlsen and Pakkenberg, 2011; Pakkenberg et al., 1991; Pakkenberg and Gundersen, 1997; Pedersen et al., 2005). In this study, we used stereology to quantify the total numbers of neurons and glial cells (subdivided into oligodendrocytes, astrocytes, and microglia) in the basal ganglia (substantia nigra [only pigmented neurons], putamen, caudate nucleus, and globus pallidus) and red nucleus in brains from 11 patients with MSA and 11 age- and gender-matched control subjects. Moreover, we employed a quantitative real-time polymerase chain reaction (qRT-PCR) approach to compare mRNA levels of specific markers for neurons, oligodendrocytes, astrocytes, and activated microglia measured in tissue samples from the substantia nigra and the striatum of 10 MSA brains and 14 control brains.

Materials and methods

Patients

We assessed brain hemispheres from 11 patients with MSA and 11 neurologically healthy control subjects. The included hemispheres were obtained from autopsied individuals following the Danish laws on autopsied human tissue and all individuals gave their informed consent. Several of the control brains have been included in previous studies (Karlsen et al., 2014; Karlsen and Pakkenberg, 2011; Walloe et al., 2014). The cause of death of one control subject was an aortic aneurysm, while the remaining 10 control subjects died from acute myocardial infarctions. Demographic and autopsy-related data for the two groups are summarized in Table 1, and clinical characteristics for the patients with MSA are listed in Table 2. All patients were followed by a movement disorder specialist at the Movement Disorders Clinic, Department of Neurology, Bispebjerg Hospital, Copenhagen, and were clinically diagnosed with a probable diagnosis of MSA (either the parkinsonian or the cerebellar subtype) according to accepted clinical consensus criteria (Gilman et al., 2008). The clinical data were retrospectively obtained from the patients' medical records.

Tissue processing

The protocol for tissue processing and stereological cell counting of whole hemispheres is previously described in details (Karlsen et al., 2014; Karlsen and Pakkenberg, 2011; Sigaard et al., 2014).

In short, the hemispheres were fixed for at least 5 months in 0.1 M sodium phosphate-buffered (4% formaldehyde, pH 7.2) before being sliced into 3-cm thick slabs starting from a random position within the first 3 cm. Each slab was embedded in paraffin using a Leica ASP300 S tissue processor (Leica, Wetzlar, Germany) followed by exhaustive coronal sectioning on a Leica SM2400 sliding microtome with a setting of 40-μm. A known and predetermined fraction of the sections were sampled and stained with Giemsa's azure eosin methylene blue solution (Merck, Darmstadt, Germany) and $KHPO_4$ at a 1:4 ratio.

Stereological design

The sections were sampled according to systematic uniform random sampling (Gundersen et al., 1999; Gundersen and Jensen, 1987). The six different regions were outlined in each section based on the cytoarchitectonic landmarks using a ×1.25 objective (Fig. 1). Volume estimates were obtained by employing Cavalieri's principle (Gundersen and Jensen, 1987). The numerical densities of neurons and glial cells were estimated with the optical disector (Gundersen et al., 1988). The neurons and glial cells were counted in systematically randomly placed counting frames using the optical disector principle, and the numerical density for one defined region was estimated. The total number of particles was calculated by multiplying the reference volume (uncorrected for shrinkage) with the numerical density. Multiplication by two was performed to obtain bilateral numbers. The sampling scheme for each region is provided in the online Supplementary material. The counting was performed using a ×60 (caudate nucleus, putamen, and globus pallidus) or ×100 (substantia nigra, red nucleus, and subthalamic nucleus) oil immersion objective, resulting in final magnifications of ×2650 (caudate nucleus, putamen, and globus pallidus) and ×1650 (substantia nigra, red nucleus, and subthalamic nucleus). The upper and lower guard zones were set at 5 and ~15 μm, respectively. The section thickness was measured in every disector to an average of 40 μm. No systematic variance in cell density was observed in the z-axis. All sections were coded during the stereological quantification process, and the investigator was blind to the disease status.

Cell identification

The different cell types were differentiated by morphological criteria and spatial distribution as illustrated in Fig. 2a (Pelvig et al., 2008). Neurons were identified by their large nuclei containing a single dark-stained nucleolus and their visible cytoplasm. Oligodendrocytes are often situated in groups and in close proximity to neurons or blood vessels. They have small, round, dark nuclei. Astrocytes have round pale nuclei with granulated heterochromatine and visible nuclear membranes. Microglial cell have comma-shaped nuclei and are visibly smaller than the other cell types.

As the degenerative pathology of MSA may influence the morphology of cells, immunolabeling for astrocytes (anti-glial fibrillary acidic protein antibody, M0761, 1:100; DAKO, Glostrup, Denmark) and neurons

Table 1
Demographic and autopsy-related data.[a]

	Putamen Caudate nucleus Globus pallidus		Substantia nigra Subthalamic nucleus Red nucleus		Cell markers qRT-PCR	
	Controls n = 11	MSA n = 11	Controls[a] n = 10	MSA[a] n = 10	Controls n = 14	MSA n = 10
Age (years)	68 [60–75]	66 [61–73]	69 [60–75]	66 [61–73]	74 [63–86]	64 [54–74]
Male/female	5/6	5/6	4/6	4/6	8/6	8/2
PMI (hours)	31.7 [10–96]	46.4 [22–115]	19 [10–96]	48 [22–115]	34.9 [21–73]	35.9 [12–80]
Hemisphere (left/right)	6/5	5/6	6/4	5/5	NA	NA
Hemisphere weight (g)	571.4 [466–730]	611.6 [533–703]	578.8 [466–730]	601.4 [533–688]	NA	NA

Values are mean and [range].
MSA = multiple system atrophy, n = number of subjects, PMI = postmortem interval, qRT-PCR = quantitative real-time polymerase chain reaction, NA = not available.
[a] Some samples were omitted from different assessments due to technical artifacts or lack of tissue.