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Widespread Demyelination in the Cerebellar Cortex in Multiple Sclerosis

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Neocortical demyelination in the forebrain has recently been identified as an important pathological feature of multiple sclerosis (MS). Here we describe that the cerebellar cortex is a major predilection site for demyelination, in particular in patients with primary and secondary progressive MS. In these patients, on average, 38.7% of cerebellar cortical area is affected, reaching in extreme examples up to 92%. Cerebellar cortical demyelination occurs mainly in a band-like manner, affecting multiple folia. The lesions are characterized by primary demyelination with relative axonal and neuronal preservation, although some axonal spheroids and a moderate reduction of Purkinje cells are present. Although cortical demyelination sometimes occurs together with demyelination in the adjacent white matter (leukocortical lesions), in most instances, the cortex was affected independently from white matter lesions. We found no correlation between demyelination in the cortex and the white matter, and in some cases, extensive cortical demyelination was present in the near absence of white matter lesions. Our data identify cortical demyelination as a potential substrate of cerebellar dysfunction in MS.

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INTRODUCTION

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system, which leads to large confluent demyelinated plaques in the white matter (15). However, recent studies show that in addition to focal white matter plaques, there is also widespread damage in the gray matter and a diffuse and global injury in the normal appearing white matter (6, 9, 11). With the introduction of new and sensitive staining techniques for myelin, extensive cerebral cortical demyelination in MS brains recently became apparent (2, 17). Cortical lesions are prominent in patients with primary and secondary progressive MS (PPMS and SPMS, respectively) (11), and the majority of cortical demyelination occurs in the form of widespread and band-like subpial lesions, which appear to be related to an inflammatory process within the meninges (3, 11). Cortical demyelination has so far only been investigated in the forebrain. Here we describe that cortical demyelination is even more prominent in the cerebellum.

PATIENTS AND METHODS

Autopsy material. The study was performed on autopsy material from 40 MS patients, eight neurologically normal patients without cerebellar pathology and 34 patients with focal or diffuse cerebellar hypoxia (Tables 1 and 2). The MS cohort consisted of patients with acute MS (AMS; Marburg's type, n = 5), relapsing/remitting MS (RRMS; n = 3), SPMS (n = 19), PPMS (n = 10) and progressive MS, where clinical records were insufficient to differentiate between SPMS and PPMS (UPMS; n = 3). Patient demographics are given in Table 1. Clinical courses were determined by retrospective chart reviews, performed by a neurologist (PSS, CFL, HR and MS) blinded to the outcome of the neuropathological analysis.

Neuropathological evaluation. Autopsy material was fixed in buffered formalin. Because of the archival nature of the material, information on post-mortem and fixation time was not available for the majority of the cases. Hemispheric or double hemispheric tissue blocks of the cerebellum and brain stem were routinely embedded in paraffin and large sections were stained with hematoxylin and eosin, Luxol fast blue myelin stain and Bielschowsky silver impregnation for visualization of axons and neurons. For each case, at least one double hemispheric or two single hemispheric sections of the cerebellum were available. To determine the extent of demyelination in the cerebellar cortex, sections were immunocytochemically stained for proteolipid protein (PLP; 17).

Immunocytochemistry. For immunocytochemistry, sections were dewaxed and antigen retrieval was performed by exposing the sections in a steamer for 1 h in citrate buffer (pH 6.0). After blockage of nonspecific antibody binding with 10% fetal calf serum, the sections were incubated with primary antibodies against the following targets: PLP (polyclonal; Serotec, Oxford, UK) and neurofilament (Chemicon, Temecula, CA). The quality of immunocytochemistry for these two antibodies is not affected by post-mortem and fixation time. Bound primary antibodies were visualized by a biotin–avidin technique as described previously (1). For control we used sections incubated in the absence of

Number	Age (years) /gender	Disease duration (months)	MS type	CDM	WMDM	Clinical signs of possible cerebellar dysfunction
1	28/M	1	AMS	0	33.4	NA
2	35/M	1.5	AMS	0.3	9.4	Nystagmus, ataxic gait
3	46/F	0.5	AMS	0	0	NA
4	46/F	7	AMS	0	0	NA
5	51/F	7	AMS	2.2	24.1	Nystagmus, dysmetria
6	20/F	48	RRMS	0	0	NA
7	58/M	120	RRMS	0.3	0.9	None
8	67/M	NA	RRMS	7.5	3.1	Unsteady gait
9	28/M	96	SPMS	7.7	0	Ataxic gait
10	31/M	132	SPMS	18.0	0	Ataxia
11	33/F	108	SPMS	71.4	8.8	Ataxic gait
12	35/F	144	SPMS	81.6	23.7	Ataxia, severe intention tremor, scanning speech, spontaneous nystagmus
13	38/F	168	SPMS	21.2	28.3	Ataxia, tremor, scanning speech, dysarthria
14	41/M	156	SPMS	78.3	3.1	Ataxia
15	45/F	240	SPMS	17.1	37.2	NA
16	45/M	360	SPMS	92.0	30.0	Ataxia, intention and postural tremor, scanning speech, spontaneous nystagmus
17	46/F	84	SPMS	41.5	2.9	Tremor, dysdiadochokinesis
18	46/F	264	SPMS	63.4	24.2	+?
19	47/F	276	SPMS	41.0	17.4	Scanning speech, dysarthria
20	53/F	241	SPMS	38.5	8.6	Ataxia
21	55/M	366	SPMS	89.6	21.6	Nystagmus, scanning speech
22	56/F	408	SPMS	5.3	39.2	+?
23	60/M	234	SPMS	2.4	0.4	None
24	62/F	288	SPMS	7.9	6.1	Ataxia, intention and postural tremor, dysarthria, nystagmus
25	66/M	396	SPMS	16.4	55.9	Ataxia
26	66/F	96	SPMS	39.3	3.5	Ataxia, tremor, dysmetria, dysdiadochokinesis
27	70/F	NA	SPMS	4.9	1.2	NA
28	36/M	61	PPMS	14.3	0	Ataxia; "cerebellar syndrome"
29	44/F	180	PPMS	75.4	3.3	NA
30	52/F	30	PPMS	0	0	Ataxic gait, dysdiadochokinesis, dysmetria
31	54/M	108	PPMS	71.3	28.4	Ataxia (first symptom), nystagmus, sacchadic eye movements
32	55/F	60	PPMS	34.8	1.6	Ataxia, dysmetria
33	56/F	132	PPMS	86.7	18.4	Ataxia
34	68/F	336	PPMS	26.9	11.5	+?
35	71/F	252	PPMS	25.7	0.8	Nystagmus
36	72/M	411	PPMS	7.9	1.9	Ataxia, dysarthria
37	75/F	314	PPMS	26.0	7.1	+?
38	34/M	NA	UPMS	44.6	32.0	NA
39	46/F	NA	UPMS	7.64	39.2	+?
40	57/F	28	UPMS	80.4	28.6	NA

Table 1. Demographic data in relation to pathology of the MS cases included in this study. Abbreviations: CDM = percentage of cerebellar cortical area affected by demyelination; WMDM = percentage of cerebellar white matter area affected by demyelination; M = male; F = female; AMS = acute multiple sclerosis (Marburg's type); RRMS = relapsing/remitting MS; SPMS = secondary progressive MS; PPMS = primary progressive MS; UPMS = progressive MS with insufficient clinical information to separate between PPMS and SPMS; NA = data not available; (in AMS, focal neurological symptoms are frequently masked because of the fulminant disease evolution); +? = clinical reports only mention cerebellar involvement without specifications.

primary antibody or with irrelevant antibodies of the same immunoglobulin class.

Quantitative determination of cortical and white matter demyelination. All sections were scanned, and camera lucida maps of demyelinated lesions in the cortex and white matter were inserted into the scans. Within the cortex, three lesion types were distinguished: subpial band-like lesions, intracortical perivenous lesions and leukocortical lesions. The sections were then overlaid by a morphometric grid, and the total area of the cortex and white matter, as well as the area of demyelination,

	Number of cases	% DM cortex	Range	% DM WM	Range
AMS	5	0	0–2.2	9.4	0–33.4
RRMS	3	2.8	0–7.5	1.0	0–3.1
SPMS	19	38.2	2.4–92.0	8.8	0–55.9
PPMS	10	26.5	0–86.7	2.6	0–28.4
UPMS	3	44.6	7.6–80.4	32.0	28.8–39.2
Normal controls	8	0		0	
Diff. hypoxia acute	7	0		0	
Diff. hypoxia chronic	13	0		0	
Focal hypoxia acute	5	0		0	
Focal hypoxia chronic	9	0		0	
Global		$P < 0.005$		$P < 0.05$	
Ac&RR/Progressive		$P < 0.001$		NS	
SPMS/PPMS		NS		NS	

The values given in the table represent the median percentage and the range.
% DM cortex: percentage of cortical area which is demyelinated. % DM WM: percentage of white matter area which is demyelinated. Diff. hypoxia: diffuse hypoxic damage of the cerebellum.
Statistics: Global: global group differences between MS subgroups and controls; Ac&RR/PP and SP: differences between acute and relapsing vs. secondary and primary progressive MS patients; SPMS/PPMS: differences between secondary and primary progressive MS patients; Diff. hypoxia: global hypoxic/ischemic brain damage; acute: <1 week; chronic: >1 month; Focal hypoxia: focal infarct affecting the cortex.
Abbreviations: AMS = acute MS; RRMS = relapsing/remitting MS; SPMS = secondary progressive MS; PPMS = primary progressive MS; NS = not significant.

Table 2. Demyelination in the cerebellar cortex and white matter in multiple sclerosis (MS) and controls.

were determined by counting the intersections of the grid, located within the respective areas. The values given in Table 1 represent the percentage of demyelinated area in comparison with the total area of cortex and white matter, respectively.

Quantitative evaluation of Purkinje cell density. For the quantification of Purkinje cells in the cerebellar cortex, we selected 17 cases with extensive cortical demyelination (PPMS: n = 9; SPMS: n = 8) and eight control cases without cerebellar pathology. The selection criterion for MS cases was the presence of demyelinated cortical lesions, which extended over several adjacent folia. Purkinje cell nuclei were counted in the microscope at a magnification of 200× (40 microscopic fields) in an area of 10 mm^2 of myelinated and demyelinated cerebellar cortex per case. The microscopic fields were positioned in a way that the Purkinje cell band spanned the field in a longitudinal direction.

Quantification of synaptophysin immunoreactivity. For the analysis of synapse density in demyelinated and normal portions of MS cerebellar, cortex sections were stained by immunocytochemistry for synaptophysin (A-0010, Dako, Glostrup, Denmark). For this substudy, seven cases were selected, which contained within the same cerebellar section large areas of demyelination side by side with large areas of normal cortex and showed optimal staining for synaptophysin in the normal cortical areas. Paraffin sections were examined under a BX51 Olympus microscope with DP50 CCD camera (Olympus optical LTD, Tokyo, Japan). At 40× magnification, three digital images (1392 × 1040 pixels, 0.073 mm^2) from synaptophysin-stained normal and demyelinated areas from the granular layer of the cerebellar cortex were taken and binarized using AnalySis 3.2 software (Olympus Soft Imaging Solutions, Münster, Germany). Demyelinated areas were recognized in consecutive sections stained for PLP. Next, quantification of the density of synaptophysin staining in the various areas was carried out with Image J (version 1.36b, freeware downloaded from http://rsb.info.nih.gov/ij/, an image processing and analysis program developed at the National Institutes of Health, USA). The density of synaptophysin was measured in the binarized images using the Voxel counter plugin developed by Wayne Rasband. For each case, the average density in the nondemyelinated and demyelinated areas was calculated. Finally, an overall average density of synaptophysin in both the nondemyelinated and demyelinated areas of all cases was calculated.

Statistical analysis. In a first step, group differences between all different courses of MS and controls were analyzed. In a second step, the values for AMS and RRMS as well as those for progressive MS (SPMS, PPMS and UPMS) were pooled and the two groups were compared. For analysis, nonparametric group tests (Kruskal–Wallis) were used. Spearman's rank correlations were used to identify interdependence of variables. *P*-values <0.05 were considered significant.

RESULTS

Structural features of demyelinated lesions in the cerebellar cortex. In the normal cerebellar cortex, only few and thin myelin sheaths were present in the molecular layer. However, there was a prominent band of myelinated fibers just above and below the layer of the Purkinje cells (supra- and infraganglionic layer). A moderate myelin density was also present in the granular layer of the cerebellum (Figure 1F and L).

In areas of cortical demyelination in MS, all myelin sheaths were lost (Figure 1D, E, G and H). In rare instances, cortical demyelination was present in small intracortical perivenous areas (intracortical lesions; Figure 1G). The vast majority of cortical demyelination affected all layers of the cortex in a band-like manner, affecting the molecular layer, the infra- and supraganglionic myelinated fibers and extending in variable depths into the granular layer (Figure 1A–C). Some demyelinated plaques in the cortex were also found in continuity with demyelination in the subcortical white matter (leukocortical lesions, Figure 1B). These leukocortical lesions were either associated with large white matter plaques or only affected a small strip of subcortical white matter, just underneath the zone of cortical demyelination (Figure 1B). Within the demyelinated area, neurons and axons were largely intact, although some axonal spheroids were present (Figure 1H and I).

A detailed quantification showed a moderate, but significant, reduction of Purkinje cell density in areas of cortical demyelination, when demyelinated plaques were

	Multiple Sclerosis (n = 17)	Controls (n = 8)
Normal cortex	12.2 (8.3–21.4)	13.2 (9.0–15.7)
Demyelinated cortex	10.2 (4.4–16)*	None

The numbers of Purkinje cells were determined according to material and methods. Values given in the table are medians and range (in brackets).
*$P < 0.02$ (demyelinated cortex compared with normal cortex in multiple sclerosis or controls).

Table 3. Number of Purkinje cells per mm^2 of cerebellar cortex.

compared with normal cerebellar cortex of MS patients and controls (Table 3). No difference in the density of synaptophysin immunoreactivity was seen between demyelinated and normal areas of the granular layer of the cerebellar cortex in MS tissue (Figure 1J and K; average density of immunoreactivity in normal areas: 12.35 ± 3.06 and in demyelinated areas: 12.50 ± 5.17).

To control for ischemic damage in the cerebellum as a possible cause for cortical demyelination, we analyzed the tissue of 34 patients with hypoxic cerebellar damage. In diffuse cerebellar hypoxia, myelin sheaths were well preserved in the cerebellar cortex, in spite of extensive neuronal death or loss in the Purkinje cell layer and the granular cell layer (Figure 1M and N). In focal infarcts, myelin was lost in the necrotic infarct core, but not in the adjacent cortical tissue (Figure 1O and P).

Incidence of cortical and white matter plaques in the cerebellum of MS. Demyelinated plaques in the cerebellar white matter were present in patients with all clinical courses of MS. They were more frequent in patients with Marburg's type of acute MS and in patients with secondary progressive MS than in patients who died during relapsing MS and in those with primary progressive MS (Table 1). However, these differences did not reach statistical significance.

In contrast, a high incidence of cerebellar cortical lesions and large cortical demyelinated areas were the hallmark of patients with primary or secondary progressive MS. On average, in these patients, 38.8% (SPMS) and 36.9% (PPMS) of the total cerebellar cortical area was demyelinated. In extreme cases, however, complete loss of myelin was present in up to 92% of the cortical areas analyzed (Table 1). Only sparse cortical demyelination was seen in patients with AMS and RRMS (Tables 1 and 2).

Correlation between cerebellar cortical demyelination and clinical parameters. We found no significant association between cortical demyelination in the cerebellum and age, gender or disease duration. Thus, demyelination in the cerebellar cortex was similar to or even more profound than that previously described in forebrain cortical areas most affected by demyelination, such as the insular or the cingulate cortex (10, 11). There was, however, a highly significant correlation ($P < 0.0005$) between cortical demyelination in the cerebellum and the forebrain, previously determined in the same patient cohort (11). This was not the case for white matter lesions. Most extensive cortical demyelination was found in patients who died between 35 and 57 years of age, while in older patients with very long disease duration, cortical demyelination was less prominent (Table 1).

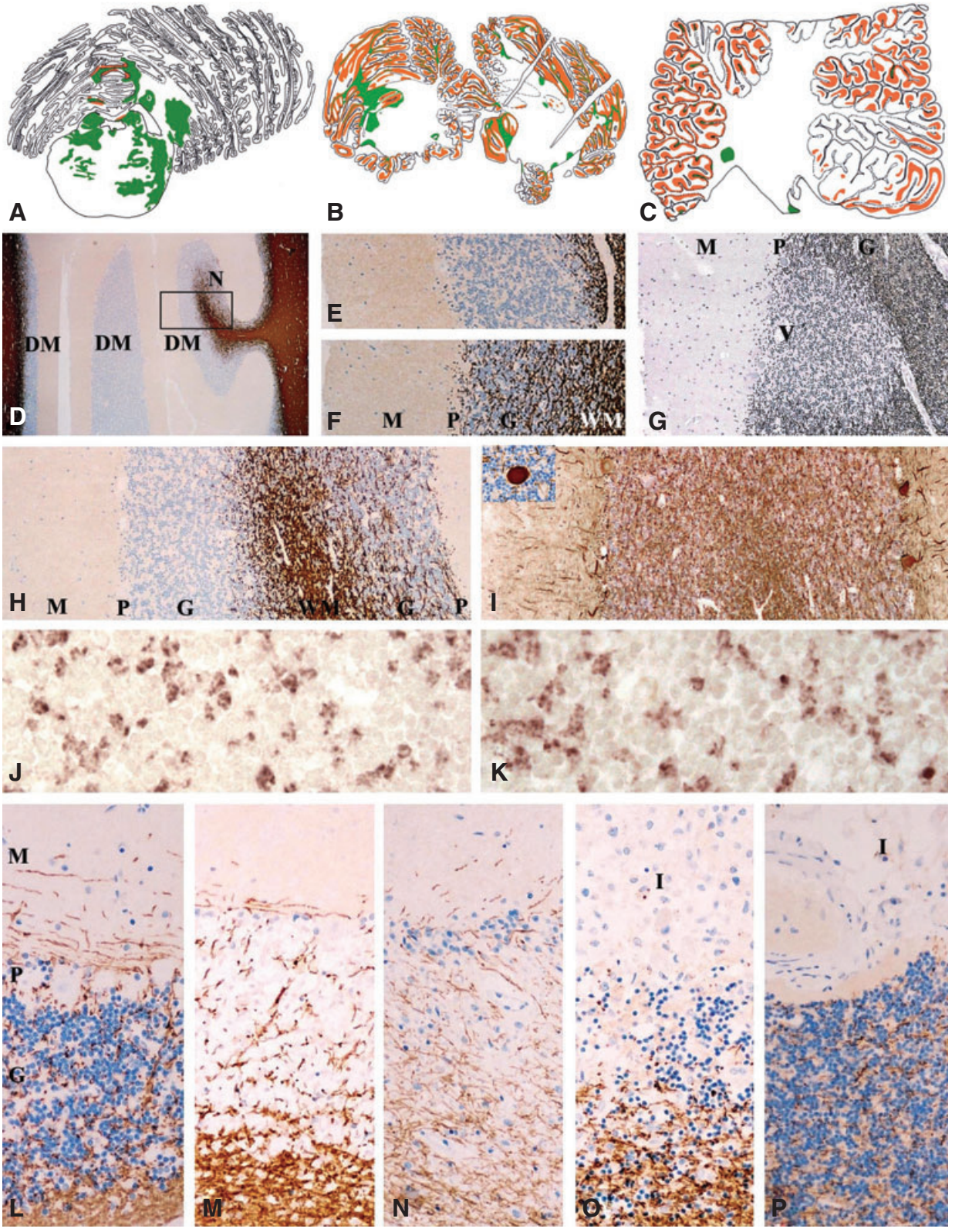
Retrospective analysis of clinical records revealed signs of possible cerebellar involvement in most of the patients. All patients with extensive cerebellar cortical demyelination showed clinical signs of possible cerebellar involvement, regardless of the presence or absence of white matter lesions (Table 1).

DISCUSSION

The main finding of our study is that the cerebellum is a major predilection site for cortical demyelination in MS. Thus, the extent of demyelination in the cerebellar cortex is similar to or even more pronounced than cortical regions in the forebrain most severely affected by demyelination (10, 11). Although cortical pathology in the cerebellum so far escapes detection by magnetic resonance imaging, it may represent an important pathological substrate for functional deficits. Similar types of cortical lesions, as described in the forebrain (2, 5, 9, 12, 17), are also seen in the cerebellum: intracortical, leukocortical and subpial lesions. Cerebellar cortical lesions are also characterized by complete demyelination with relative preservation of neurons, axons and synapses. There is, however, an additional destructive component within the lesions, as shown before in neocortical plaques (17, 21). This is reflected by the presence of scattered axonal swellings and end bulbs as well as a moderate degree of neuronal loss, seen in quantitative studies on Purkinje cell density. For the evaluation of synaptic density, we chose the granular layer of the cerebellar cortex. As the main synaptic input into the granular layer comes from the white matter, it allows the determination of whether there is a direct effect of the demyelinating process on synapses. Similarly, as described by Vercellino et al (21) in the forebrain, we did not detect abnormalities in the distribution of synaptophysin in these areas. This, however, does not exclude that cortical lesions may be associated with secondary synaptic loss in other regions, caused by loss of intrinsic cortical neurons.

Cortical demyelination was most pronounced in patients who died in the 4th to 6th decade of their life, while it was less abundant in patients dying at old age. Death at old age may indicate a benign course of MS. In addition, remyelination may occur in cortical lesions in a similar way as in white matter plaques (19) (M. Albert, W. Brück and C. Stadelmann, unpub. obs.), which may be more pronounced at late stages of the disease, when inflammatory and demyelinating activity in plaques subsides (16).

In the forebrain, cortical lesions are mainly located at sites of restricted cerebrospinal fluid circulation, such as the gyral sulci, the insular cortex, the basolateral cortex and the cingulate cortex (2, 10, 11). Active cortical lesions in progressive MS only show very mild lymphocytic infiltrates in the lesion parenchyma, while T-cells and B-cells are mainly present within the meninges (3, 11). Active demyelination is associated with profound microglia activation. This scenario suggests that soluble factors are produced within the meningeal infiltrates, which diffuse into the cortex and initiate myelin destruction either directly or indirectly through microglia activation (11). Specific antibodies against surface components of myelin could be such candidates; however, evidence for antibody and complement-mediated demyelination in cortical lesions of MS patients is so far lacking (4). Cortical lesions with features reminiscent of those found in MS patients



can be reproduced in animal models with antibody-mediated demyelination (13, 14, 18). The deep meningeal infoldings of the cerebellar cortex, which may form niches for persistent inflammation, could explain the abundance of cortical damage on the basis of the mechanism described above. However, because most of the cerebellar cortical lesions in our sample were inactive, we were not able to show a topographical association between active demyelination and meningeal inflammation in the present study.

The functional consequences of demyelination in the cerebellar cortex are so far

Figure 1. *Cortical pathology in MS and hypoxia*. **A–C.** Schematic drawing of demyelination in the cerebellar cortex (red) and white matter (green) in different MS courses. **A.** AMS with massive demyelination in the brain stem and cerebellar white matter, but only minor demyelination in the cortex mainly associated with white matter lesions. **B.** SPMS with massive cortical demyelination and moderate white matter demyelination; some of the cortical lesions are in continuity with large white matter plaques, while others show only a rim of white matter involvement adjacent to cortical demyelination; most of the cortical demyelination is not associated with white matter lesions. **C.** PPMS: massive cortical demyelination with only very small areas of white matter involvement. **D.** Low power view of cerebellar cortex in SPMS shows large areas of complete demyelination (DM) adjacent to areas of preserved myelin (N); the rectangle marks the area shown in Figure 1**H–K**). Immunocytochemistry for PLP; ×15. **E.** Higher magnification of demyelinated cortical area, showing complete loss of myelin in the cortical layers, but preservation of myelin in the subcortical white matter. Immunocytochenistry for PLP; ×120. **F.** Adjacent nondemyelinated cortical areas of the same section with normal myelin in the lower parts of the molecular layer (M), the Purkinje cell layer (P) and the granular layer (G); WM: subcortical white matter. Immunocytochemistry for PLP; ×120. **G.** Small focal intracortical demyelinated plaque in a patient with SPMS; V: central vein. Immunocytochemistry for PLP; ×120. **H,I.** Neurons and axons in demyelinated plaques in the cerebellar cortex of SPMS; serial sections are stained by immunocytochemistry for PLP (**H**), neurofilament (**I**). Cerebellar cortex on the left side is demyelinated, while myelin is preserved in the cortex at the right side (**H**); staining for neurofilament shows no major differences between demyelinated and myelinated areas, although there is some reduction of Purkinje cells (**I**). In addition, few dystrophic axonal spheroids are present (**I**; insert); ×150. **J,K.** Synaptic immunoreactivity in normal (**J**) and demyelinated (**K**) area of the granular layer reveals no visible difference in synaptic density; immunocytochemistry for synaptophysin; ×900. **L.** Normal distribution of myelinated fibers in the cerebellar cortex of a normal control patient. Immunocytochemistry for PLP; ×300. **M.** Acute diffuse hypoxia of the cerebellar cortex with massive reduction of granule cell nuclear staining, but regular distribution of myelin sheaths. Immunocytochemistry for PLP; ×300. **N.** Chronic diffuse hypoxia of the cerebellar cortex shows loss of Purkinje cells and granule cells and reactive gliosis; myelin sheaths, however, are preserved. Immunocytochemistry for PLP; ×300. **O.** Acute cerebellar cortical infarct with loss of neurons and myelin sheaths in the infarct area (I). Immunocytochemistry for PLP; ×300. **P.** Chronic cystic infarct of the cerebellar cortex; the myelin sheaths in the adjacent granular layer are well preserved. Immunocytochemistry for PLP; ×300. Abbreviations: MS = multiple sclerosis; AMS = acute MS (Marburg's type); SPMS = secondary progressive MS; PPMS = primary progressive MS; PLP = proteolipid protein.

not clear. Magnetic resonance imaging studies suggest a major clinical relevance of cortical pathology in the forebrain (7, 8, 20). However, because myelin density is very low in the normal cerebellar cortex, it may be argued that loss of these myelin sheaths may be functionally insignificant. On the other hand, demyelination may induce subtle functional changes, which may pass the clinical threshold, in particular when additional neuronal loss is present. In this context, it is interesting that clinical signs of possible cerebellar involvement were noted in our series in patients with extensive cortical demyelination in the near absence of white matter plaques. However, we cannot exclude that in these patients lesions in other brain areas have contributed to the clinical deficit.

In conclusion, we have shown that the cerebellar cortex is extensively involved in MS patients, particularly in those with primary or secondary progressive disease. It will be important for future studies to determine the clinical correlate of this newly discovered pathological aspect of MS.

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