Extensive Hippocampal Demyelination in Multiple Sclerosis

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Abstract
Memory impairment is especially prominent within the spectrum of cognitive deficits in multiple sclerosis (MS), and a crucial role for hippocampal pathology may therefore be expected in this disease. This study is the first to systematically assess hippocampal demyelination in MS. Hippocampal tissue samples of 19 chronic MS cases and 7 controls with non-neurologic disease were stained immunohistochemically for myelin proteolipid protein. Subsequently, number, location, and size of demyelinated lesions were assessed. Furthermore, the specimens were stained for HLA-DR to investigate microglia/macrophage activity. An unexpectedly high number of lesions (n = 37) was found in 15 of the 19 MS cases. Mixed intrahippocampal-perihippocampal lesions, which were more often found in cases with cognitive decline, were large and did not respect anatomical borders. Moderate microglial activation was frequently observed at the edges of these mixed lesions. Isolated intrahippocampal lesions were also frequently found. These were smaller than the mixed lesions and had a specific anatomical predilection: the cornu ammonis 2 subregion and the hilus of the dentate gyrus were consistently spared. Microglial activation was rare in isolated intrahippocampal lesions. Our results indicate that hippocampal demyelination is frequent and extensive in MS and that anatomical localization, size, and inflammatory activity vary for different lesion types.

Key Words: Cognitive decline, Demyelination, Hippocampus, Inflammation.

INTRODUCTION
Multiple sclerosis (MS) is a chronic, inflammatory demyelinating disease of the CNS, affecting many young adults each year. Apart from a variety of neurologic symptoms, such as motor and sensory disturbances, ataxia, speech, and visual abnormalities, the disease is also characterized by cognitive decline in up to 65% of cases (1, 2), beginning in the earliest phases of the disease (3, 4). Within the spectrum of cognitive impairment, memory dysfunction seems to be especially prominent (1, 5–8).

The hippocampus plays a pivotal role in memory processing (9, 10). However, apart from a few sporadic observations (11–13), no systematic (postmortem) studies have evaluated hippocampal demyelination in MS. This may be due to the fact that it has long been difficult to detect demyelinated gray matter (GM) lesions histopathologically, because conventional myelin stainings such as Luxol fast blue (LFB) visualize GM myelin poorly and the focus on white matter (WM) pathology has long predominated the field of MS research.

Although the occurrence of GM demyelination in MS was described in early studies (14, 15), it was long neglected until modern immunohistochemical staining methods incited a renewed interest in the field of GM lesions in MS (16–18). It has been shown that cortical lesions are abundant (16, 18), they may extend over several gyri (17, 19), they show a histopathologic pattern different from that of WM lesions (18, 20), and they are generally missed by conventional magnetic resonance imaging (MRI) methods (21). In vivo quantitative MRI and atrophy studies also found abnormalities in “normal-appearing” GM (22–26) and showed that these were related to physical and cognitive deficits (27, 28). It was even suggested that a separate cortical variant of MS may exist, with distinct cortical symptoms (dysphasia, dysgraphia, and dyslexia), depression, and memory impairment (29).

MRI and histopathology studies found atrophy and demyelination not only in the cortical GM but also in deep GM structures (21, 26, 30, 31). For example, hypothalamic lesions were found to be numerous in a postmortem MS dataset (32), and higher inflammatory activity of these hypothalamic lesions was associated with a more unfavorable disease course (33).

Because imaging studies have suggested that structural (34) and functional/metabolic (35–38) abnormalities may occur in the MS hippocampus, we expected to find demyelinated lesions in this structure, as in other (deep) GM areas in the MS brain. Therefore, in the current study, archived hippocampal tissue samples of 19 patients with chronic MS and 6 non-neurologic controls were immunohistochemically assessed. Furthermore, the specimens were stained for HLA-DR to investigate microglia/macrophage activity. An unexpectedly high number of lesions (n = 37) was found in 15 of the 19 MS cases. Mixed intrahippocampal-perihippocampal lesions, which were more often found in cases with cognitive decline, were large and did not respect anatomical borders. Moderate microglial activation was frequently observed at the edges of these mixed lesions. Isolated intrahippocampal lesions were also frequently found. These were smaller than the mixed lesions and had a specific anatomical predilection: the cornu ammonis 2 subregion and the hilus of the dentate gyrus were consistently spared. Microglial activation was rare in isolated intrahippocampal lesions. Our results indicate that hippocampal demyelination is frequent and extensive in MS and that anatomical localization, size, and inflammatory activity vary for different lesion types.

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Key Words: Cognitive decline, Demyelination, Hippocampus, Inflammation.
stained for myelin, and hippocampal lesion occurrence, extent, and localization were studied. Furthermore, tissue sections were immunohistochemically stained for human leukocyte antigen (HLA) to investigate whether hippocampal lesions, like hypothalamic and spinal cord GM lesions but unlike purely intracortical lesions, are inflammatory.

MATERIALS AND METHODS

**Autopsy Procedure and Brain Material**

Between 1992 and 2006, tissue samples were selected from 10-mm-thick coronal brain slices after rapid autopsy (mean postmortem delay: 7 hours, 35 minutes). Retrospectively, 19 hippocampal samples from 19 chronic MS cases (14 women, mean age 65.6 years) were selected for this study. Also, 7 healthy control samples were collected from 6 different patients with no neurologic or psychiatric disease (Table 1). Only hippocampi that were cut coronally were selected, and the anatomical origin in the rostrocaudal direction was similar for all samples to enable accurate and systematic scoring of possible demyelination within different anatomical subregions of the hippocampus (Fig. 1).

**Tissue sampling and autopsy procedures were extensively described earlier** (21, 39, 40). Because of the retrospective nature of this study, postmortem MRI data were, unfortunately, not available.

The tissue was obtained from the Netherlands Brain Bank, Netherlands Institute for Neuroscience, Amsterdam. All material was collected from donors from whom a written informed consent for brain autopsy and the use of the material and clinical information for research purposes had been obtained by the Netherlands Brain Bank. Clinical files were studied to describe general demographics, disease type, cause of death, and cognitive decline (Table 1).

**Immunohistochemistry**

The selected tissue samples were fixed in 10% formalin and embedded in paraffin for 30 days. The embedded samples

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**TABLE 1. Patients With MS and Controls**

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<th>Case</th>
<th>Sex</th>
<th>Age</th>
<th>PMD</th>
<th>DD</th>
<th>Type</th>
<th>Mixed Lesions</th>
<th>Cognitive Decline</th>
<th>COD</th>
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**Control**

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<th>PMD</th>
<th>DD</th>
<th>Type</th>
<th>Mixed Lesions</th>
<th>Cognitive Decline</th>
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<td>MI</td>
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<td></td>
<td>MI</td>
</tr>
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<td>6</td>
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AD, cognitive deterioration was more likely to be attributed to Alzheimer disease; COD, cause of death; cognitive decline, as reported by patients’ medical charts (+; cognitive problems were explicitly reported [mostly (severe) memory impairment]); —, no report of cognitive problems was found in the charts; CVA, cerebrovascular accident; DD, disease duration; type, disease type (either secondary progressive [SP], primary progressive [PP], or relapsing remitting [RR]); MI, myocardial infarct; MS, multiple sclerosis; PMD, postmortem delay; mixed lesions, number of mixed hippocampal-perihippocampal lesions present in the individual cases; VAD, vascular dementia was most likely the cause for cognitive decline.
were cut into 8-μm-thick tissue sections, deparaffinized in xylene, and rehydrated through a series of 100%, 96%, and 70% ethanol and distilled water. Endogenous peroxidase activity was blocked by incubating the sections in methanol.

Samples were stained for proteolipid protein (PLP) and human leukocyte antigen (HLA-DR). For HLA-DR staining only, sections were microwaved for 10 minutes in a 10 mM sodium citrate buffer (pH 6.0) to allow antigen retrieval. Sections were allowed to cool to room temperature and were rinsed with 0.01 M PBS (pH 7.4). Subsequently, they were incubated with the primary antibodies, diluted in PBS containing 1% bovine serum albumin (Boehringer Mannheim, Mannheim, Germany). Dilution was 1:3000 for PLP (clone plpc1; Serotec Ltd, Oxford, UK) and 1:100 for HLA-DR (generous gift from Dr. Hilgers, Department of Obstetrics and Gynaecology, VU University Medical Center, Amsterdam, The Netherlands). To prevent nonspecific binding, sections were preincubated with normal rabbit serum (1:50; DAKO, Glostrup, Denmark) in PBS containing 1% bovine serum albumin for 10 minutes at room temperature. Incubation with biotinylated polyclonal rabbit anti-mouse immunoglobulins (1:500; DAKO) for 30 minutes at room temperature followed, and sections were incubated for 60 minutes with streptavidin-avidin-biotin complex, coupled to horseradish peroxidase (1:200; DAKO). Peroxidase activity was demonstrated by adding Envision 3,3-diaminobenzidine tetrahydrochloride (DAB Envision Kit, 1:50 dilution; DAKO) for 10 minutes, which led to a brown reaction product. Finally, sections were counterstained with hematoxylin, dehydrated through a series of ethanol and xylene, and mounted (Entellan; Merck, Darmstadt, Germany).

**Analysis**

Demyelinated areas were scored in consensus (J.J.G.G., L.B., and P.vd.V.) using the PLP-stained hippocampal tissue sections. Scoring was performed blindly (i.e. the scorers were unaware of clinical information). According to their anatomical localization (Fig. 1), lesions were classified as follows: 1) mixed intrahippocampal-perihippocampal lesions, involving both (parts of) the hippocampus and of the perihippocampal WM; 2) isolated intrahippocampal lesions, subclassified anatomically as being localized to subiculum, cornu ammonis (CA) 1, CA2, and CA3, hilus of the dentate gyrus, or molecular layer of the dentate gyrus; 3) perihippocampal GM lesions located either within the entorhinal cortex or the parahippocampal gyrus (hippocampus not involved) (lesions in the entorhinal cortex and the parahippocampal gyrus were not scored separately in this study); and 4) perihippocampal WM lesions (hippocampus not involved).

Demyelination was defined as total absence of myelin (no single thread of myelin left). This strict definition was maintained because myelin density is intrinsically low in several GM areas, both intra- and extrahippocampally (e.g. in the molecular layer of the dentate gyrus and the CA1 subfield: Fig. 1). Lesions within the entorhinal cortex and parahippocampal gyrus were classified according to the standard classification system for cortical GM lesions (lesion types I, II, III, and IV), as described in previous studies (18, 21). Briefly, type I lesions are mixed cortical GM-subcortical WM lesions; type II lesions are small intracortical lesions, often surrounding a blood vessel; type III lesions are large subpial lesions, representing the most frequently occurring cortical lesion type in MS; type IV lesions form a separate category of subpial lesions, that span the entire cortex. No lesions were scored beyond the collateral sulcus. Total lesion numbers per lesion category were calculated.

HLA-DR-stained sections were used to assess the presence and activity of microglia/macrophages (also in consensus; J.J.G.G., I.H., P.vd.V., and L.B.) in normally myelinated and demyelinated peri- or intrahippocampal areas. Additionally, all lesions were manually outlined on digital images of the PLP-stained sections using ImageJ software (U.S. National Institutes of Health, Bethesda, MD, http://rsb.info.nih.gov/ij/, 1997–2006), and resultant lesion sizes were expressed in mm² after appropriate calibration. The mean lesion area (square millimeters) ± SD was calculated per lesion category. Because of low statistical power, results are presented descriptively.

**RESULTS**

**Cases**

A total of 19 hippocampal samples were selected from 19 different patients with MS. Nine of the patients had a
secondary progressive (SP), 7 a primary progressive (PP), and 1 a relapsing-remitting (RR) disease course. One progressive case was clinically poorly documented, especially at the onset of the disease and was therefore denoted as SP/PP (Table 1). In 1 case, the disease course could not be retrospectively determined.

Mean ages ± SD of patients with MS and controls were similar (65.6 ± 12.4 vs 62.2 ± 14.9 years, respectively) and mean disease duration for the patients with MS was 25.4 ± 12.1 years. Cause of death of the controls was cardiovascular disease in 4 of 6 cases (Table 1).

**Hippocampal Demyelination**

All PLP and HLA-DR stainings analyzed were of high quality, and sections could be scored consistently. Control samples showed differing myelin densities throughout the hippocampus and whereas little myelin was inherently present in the molecular layer of the dentate gyrus and in CA1, it was denser in the hilus of the dentate, in CA3 and CA2, and in the subiculum and parahippocampal gyrus (Fig. 1). These findings are consistent with the literature (42).

No demyelinated lesions were found in any of the control samples. Similarly, in 4 of the 19 MS cases, no lesions were found in (proximity to) the hippocampus. However, in the remaining 15 MS cases, a total of 37 lesions were found in and around the hippocampus. Of these lesions, 10 were mixed (27.0% of the total lesion number), involving both the hippocampus and the perihippocampal WM. Thirteen lesions (35.1%) were restricted entirely to the hippocampus; i.e., 6 lesions (16.2%) in the molecular layer of the dentate gyrus, 1 (2.7%) in CA3, 4 (10.8%) in CA1, and 2 (5.4%) in the subiculum. No isolated lesions were detected in the hilus of the dentate gyrus in any of the cases, and CA2 was also spared entirely. This anatomical predilection of isolated intrahippocampal lesions contrasted with the mixed lesions, which did not seem to respect any anatomical boundaries and therefore also occasionally involved the hilus of the dentate and CA2 (Fig. 2).

Furthermore, a total of 14 lesions (37.8%) were found in close proximity to the hippocampus: 3 type II cortical GM lesions (8.1%) and 8 type III subpial cortical lesions (21.6%) in the parahippocampal gyrus (including entorhinal cortex), and 3 lesions (8.1%) in the perihippocampal WM. Type IV intracortical lesions were not observed in the parahippocampal gyrus nor were type I mixed GM-WM lesions that did not involve the hippocampus.

As for the size of the lesions, the mixed hippocampal GM-perihippocampal WM lesions constituted by far the largest lesion type (46.60 ± 39.93 mm²), followed by lesions in the perihippocampal WM (3.95 ± 1.87 mm²) and by type III subpial cortical lesions in the parahippocampal gyrus (3.22 ± 2.42 mm²). Lesions within the hippocampus (CA1, CA3, dentate gyrus, and subiculum) were generally small (mean area of intrahippocampal lesions: 1.10 ± 1.58 mm²). Total lesion numbers within the different anatomical areas, numbers of patients in which lesions were found, and mean ± SD lesion sizes are shown in Table 2 and in Figures 2 and 3.

**Microgliia/Macrophages**

Within isolated hippocampal (CA1 and CA3) and dentate lesions, HLA-DR-positive activated microglial cells were sparse. HLA-DR immunopositivity was predominantly found in areas where the myelin was still relatively intact, and 8 of 10 mixed intrahippocampal-perihippocampal lesions showed activated microglia at the lesion borders (Fig. 3), both in the GM and the WM parts of the lesions. Immunopositive cells were more abundant in the perihippocampal WM lesions, where HLA-DR-positive cells in various activation stages were found; foamy macrophages...
were observed in 2 WM lesions, although without apparent intracellular PLP products (Fig. 3). In the control material, HLA-DR staining was marginal (far less pronounced than in the MS cases) and the vast majority of these immunopositive cells had "resting" (nonactivated) microglial morphology. The number of positive cells varied slightly between control cases, probably because of differences in causes of death.

Cognitive Decline and Hippocampal Demyelination

Interestingly, cognitive deficits (sometimes explicitly defined as memory impairment) were found in the medical files of 9 of 19 MS cases. The files of the other 10 cases did not report on cognitive decline, which may indicate either that it was not found in the patients or that it was not noted (many of the patients with chronic MS spent their last years at home or in nursing homes where specialized neuropsychologic assessment is uncommon).

It should be noted that neuropathologic investigation revealed Alzheimer-type changes (Braak stage 5) in 1 case and pathologic changes possibly indicative of vascular dementia (severe arteriosclerosis and lacunar infarcts) in another case. Therefore, 7 cases ultimately remained in which the cause of the cognitive problems could be attributed to MS.

In 6 of these 7 cases, a total of 7 large, mixed hippocampal-perihippocampal lesions were found (Table 1; Fig. 2). Furthermore, several smaller, intrahippocampal lesions were found (2 in the molecular layer of the dentate, 1 in CA3, 1 in CA1, and 1 in the subiculum), as well as 2 lesions in the perihippocampal region (1 in layer III of the parahippocampal gyrus and 1 in the perihippocampal WM). Although the intrahippocampal subregions affected by the large, mixed lesions (molecular layer and hilus of the dentate gyrus, CA3, CA2, CA1, subiculum, and parahippocampal gyrus) differed between patients, none of these areas was prone to exclusion from the demyelinating process. One of the MS cases with severe demyelination also suffered from epilepsy. Hippocampal lesions were found in 8 of 10 cases without cognitive decline as well. In these cases, however, only 3 mixed hippocampal-perihippocampal lesions were observed (Table 1). In the remaining patients, 7 intrahippocampal lesions were found (2 lesions in CA1, 4 in the molecular layer of the dentate gyrus, and 1 in the subiculum) as well as 2 lesions in the perihippocampal region (1 in layer II of the parahippocampal gyrus and 1 in the perihippocampal WM).

DISCUSSION

This study is the first to show that hippocampal demyelination is frequent and extensive in a randomly sampled dataset of MS autopsy cases (37 lesions were found in 15 patients with chronic MS). Approximately one fourth (27%) of the demyelinated lesions appeared to be mixed, covering part of the hippocampal formation, as well as part of the perihippocampal WM. Mixed lesions formed the largest (in square millimeters) hippocampal lesion type and HLA-DR-positive microglia were often present at the edges of these lesions, indicating chronic inflammatory activity, although ongoing demyelination (PLP products in the macrophages) was not observed. Apart from mixed lesions, isolated intrahippocampal lesions were also frequently observed (35% of the total number of lesions). Interestingly, whereas the molecular layer of the dentate gyrus and the CA1 subfield were relatively often affected by isolated intrahippocampal lesions, both the hilus of the dentate and the CA2 field were consistently spared in all MS cases. It should be noted that this sparing of CA2 and the hilus of the dentate only applied for isolated intrahippocampal lesions and not for mixed lesions, which were far less anatomically specific.

More research should be performed to investigate possible pathogenetic differences between isolated intrahippocampal and mixed hippocampal-perihippocampal lesion types to determine whether different lesional patterns represent different underlying pathologic processes or whether these patterns are simply different stages of the same pathology. This also holds for the HLA-DR immunopositivity, which was more prominent in mixed lesions compared with isolated intrahippocampal lesions. It is already known that mixed cortical lesions are more inflammatory than purely intracortical lesions (18, 20), although reasons for this difference have so far remained obscure. Nevertheless, similar pathologic patterns may apply for both the cortical GM and the hippocampus in MS.

The CA1 subregion is known to be affected in several neurologic conditions such as medial temporal lobe epilepsy,
Alzheimer disease, and hypoxic/ischemic disease (43–46), and, as mentioned above, both isolated and mixed lesions were found to involve CA1 in the current study. In contrast, CA2 (which is referred to as the “resistant band” in the literature [47]) is usually enigmatically preserved in many of the above-mentioned neurologic conditions (44) and was also consistently spared in our PLP-stained samples. Reasons for the relative survival of neuronal elements in CA2 could be that high concentrations of calcium-binding proteins and growth factors can be found within the pyramidal cells of this region (44, 48, 49), which may protect the cells from damage. How regional differences in susceptibility of myelin arise in the MS hippocampus is, however, not clear. Recent studies have suggested a pathogenetic role for glutamate excitotoxicity in MS (50–53), and it was shown that oligodendrocytes are especially vulnerable to increased tissue glutamate concentrations (52, 54). In an experimental rat model of mesial temporal lobe epilepsy, neuronal glutamate transporter EAAC1 concentrations were relatively unaffected in CA2, as opposed to the other CA fields and the dentate gyrus, indicating that excitotoxic processes may have a limited effect within the CA2 field (55). These regional variations in excitotoxic sensitivity within the hippocampus could contribute to the different sensitivities to demyelination observed in the present study.

An important question is how hippocampal lesions should be placed within the broader spectrum of WM and, especially, GM demyelination in MS. More specifically, the pathologic profile of hippocampal lesions needs to be closely compared with other (GM) lesions in the MS CNS. For example, it was observed that the GM part of spinal cord lesions (56) seems to contain a higher number of activated...
microglia (N. Evangelou and C. P. Gilmore, personal communication, 2007) than subpial intracortical lesions, for example (18). Also, as mentioned before, demyelinated lesions in the hypothalamus were found to express a high inflammatory activity, with numerous foamy macrophages present involved in myelin breakdown (32). In comparison with the microglial activation in spinal cord and with the fulminant hypothalamic inflammatory pattern, HLA-DR immunopositivity within the hippocampal lesions was found to be only marginal in the current study.

In the current retrospective study, we unfortunately had access to only a limited amount of material per case. Further research, using a larger variety of anatomical samples and a more elaborate panel of histopathologic markers, should lead to a more detailed classification of hippocampal lesions. Specifically, it would be interesting to study the relation between demyelination and neuroaxonal damage or gliosis. Increased hippocampal myoinositol concentrations, suggestive of gliosis, were already found in a recent in vivo magnetic resonance spectroscopy study (37). A larger prospective study, with a more diverse patient group, should also shed more light on whether hippocampal demyelination is an early phenomenon, as was reported in MRI studies for cortical GM abnormalities (23, 57) or whether it does not arise until the chronic stage of the disease (58, 59).

In both cognitively affected and cognitively unaffected cases, hippocampal lesions were observed. However, in cases with cognitive decline, a higher number of the large, mixed intrahippocampal-perihippocampal lesions was found. Whether this indicates that the mixed intrahippocampal-perihippocampal lesion type is more likely to cause cognitive problems in MS or whether cognitive decline is simply proportional to the area of hippocampal demyelination is as yet unclear. Moreover, it should be noted that nonhippocampal GM and WM pathology is also known to contribute to cognitive deficits in MS (60–65). Because of the retrospective nature of our study, it was not possible to accurately extract more detailed information regarding the exact type of cognitive deficits (e.g., memory impairment) from the clinical files of our tissue donors. Future researchers performing in vivo studies, therefore, face the challenge of investigating the contribution of hippocampal lesions to memory deficits in MS, and adequate detection of hippocampal lesions with MRI is essential in this perspective. Unfortunately, as previous studies have shown, it is extremely difficult to visualize GM lesions with conventional magnetic resonance techniques (21, 66). The use of 3-dimensional double inversion recovery MRI was reported to improve cortical GM lesion detection (67), and this technique was recently used to image hippocampal lesions (68). In that study, an average of 2.6 lesions per patient was found, which is similar to the numbers presented in the current article. Also, the number of hippocampal lesions detected by 3-dimensional double inversion recovery was shown to be positively correlated with the number of cortical GM lesions. In future studies the use of this sequence may provide more insight into the position of hippocampal demyelination within the general spectrum of GM and WM pathology in MS and into the relative time frames within which hippocampal and cortical lesions develop. Also, the contribution of (uni- and bilateral) hippocampal lesions to cognitive decline can be studied in more detail.

In conclusion, a systematic histopathologic exploration revealed an unexpectedly high number of demyelinated hippocampal lesions in 15 of 19 cases of progressive MS. Several lesion types differing in location and size were found and mild to moderate microglial activation was observed within the different hippocampal lesions. Different hippocampal lesion types were found in both cognitively affected and unaffected cases. However, large, mixed intrahippocampal-perihippocampal lesions were more common in patients who had manifested a cognitive decline during life. The outcome of this study should now further direct attention to the MS hippocampus, which may play a pivotal role in the cognitive decline that is so frequently reported in this disease.

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