Acute axonal damage in multiple sclerosis is most extensive in early disease stages and decreases over time

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Summary
Multiple sclerosis is characterized morphologically by the key features demyelination, inflammation, gliosis and axonal damage. In recent years, it has become more evident that axonal damage is the major morphological substrate of permanent clinical disability. In our study, we investigated the occurrence of acute axonal damage determined by immunocytochemistry for amyloid precursor protein (APP) which is produced in neurones and accumulates at sites of recent axon transection or damage. The numbers of APP-positive axons in multiple sclerosis lesions were correlated with the disease duration and course. Most APP-positive axons were detected within the first year after disease onset, but acute axonal damage was also detected to a minor degree in lesions of patients with a disease duration of 10 years and more. This effect was not due to the lack of active demyelinating lesions in the chronic disease stage. Late remyelinated lesions (so-called shadow plaques) did not show signs of axon destruction. The number of inflammatory cells showed a decrease over time similar to that of the number of APP-positive axons. There was a significant correlation between the extent of axon damage and the numbers of CD8-positive cytotoxic T cells and macrophages/microglia. Our results indicate that a putative axon-protective treatment should start as early as possible and include strategies preventing T cell/macrophage-mediated axon destruction and leading to remyelination of axons.

Keywords: multiple sclerosis; axon damage; APP

Abbreviations: APP = amyloid precursor protein; MBP = myelin basic protein; MOG = myelin oligodendrocyte glycoprotein; MRS = magnetic resonance spectroscopy; NAA = N-acetylaspartate; NAWM = normal-appearing white matter; PPMS = primary progressive multiple sclerosis; PPWM = periplaque white matter; RRMS = relapsing–remitting multiple sclerosis; SPMS = secondary progressive multiple sclerosis

Introduction
Multiple sclerosis is a disease of the CNS characterized by multifocal inflammation, demyelination, gliosis and axonal loss. In recent years, the significance of axonal degeneration for permanent neurological deficits in multiple sclerosis patients has been emphasized. Magnetic resonance spectroscopy (MRS) of multiple sclerosis plaques revealed a correlation of disability and reduced levels of N-acetylaspartate (NAA) (Matthews et al., 1998), which is a biochemical marker found exclusively in neurones and their processes (Birken and Oldendorf, 1989). Also the volume of hypointense lesions (‘black holes’) in T1-weighted MRI and the extent of brain atrophy, both putative markers of axonal...
damage, correlated with the degree of disability (Grimaud et al., 1999; Fisher et al., 2000; Paolillo et al., 2000; Pelletier et al., 2001).

Axonal damage can be determined histopathologically by the extent of axonal loss relative to the normal-appearing white matter (NAWM). In addition, acute axonal damage can be detected by immunohistochemistry for the amyloid precursor protein (APP) (Li et al., 1995; Bramlett et al., 1997; Yam et al., 1997; Pesini et al., 1999). APP is found in neurones and undergoes anterograde axonal transport. In the case of an axonal transection, the transport is interrupted and APP accumulates in the proximal axonal ends. As a consequence, so-called APP-positive spheroids are formed that persist for <30 days. A variety of different histopathological studies exist that investigated acute axonal damage in multiple sclerosis (Ferguson et al., 1997; Trapp et al., 1998; Bitsch et al., 2000; Kornek et al., 2000). However, to the best of our knowledge, so far no report has been published that correlates axonal damage and disease duration as carried out in our study. We focused on acute axonal damage within the lesions and the periplaque white matter (PPWM) and correlated it with disease duration, inflammation and clinical course.

Our data demonstrate that acute axonal damage occurs early during disease and lesion formation. APP-positive axons can be detected in all stages of demyelinating activity and also in the PPWM. Acute axonal damage is most prominent within the first year after disease onset. In relapsing–remitting (RRMS) and secondary progressive multiple sclerosis (SPMS), acute axonal damage is significantly higher in early stages of the disease than after a disease duration of 10 years or more. In contrast, in primary progressive multiple sclerosis (PPMS) there are no significant differences over time. Our data indicate that a putative neuroprotective treatment could be most effective if started at the beginning of the disease during the relapsing–remitting stage.

Material and methods

Patients

We retrospectively investigated brain tissue from 39 patients (23 biopsies from 21 patients and 18 autopsies). Two patients underwent two biopsies from different CNS sites at consecutive time points during progression of disease. Biopsies had been performed for diagnostic reasons to exclude neoplastic or infectious diseases. Informed consent had been obtained from each patient. None of the study authors was involved in decision making with respect to biopsy. When the biopsies were taken, none of the patients fulfilled the criteria of clinically definite multiple sclerosis according to the Poser criteria (Poser et al., 1983). The study was approved by the Ethics Committee of The University of Göttingen.

During clinical follow-up, 19 of the 21 biopsied patients fulfilled the Poser criteria for clinically definite multiple sclerosis (two PPMS, 17 RRMS; for details see Table 1). One patient had two relapses with similar symptoms, thus fulfilling the criteria for clinically probable multiple sclerosis according to Poser. Another patient showed all characteristics of PPMS (Thompson et al., 2000). However, with the help of longitudinal MRI data in some cases, all 21 patients fulfilled the new diagnostic criteria for multiple sclerosis (McDonald et al., 2001). Three patients entered a secondary progressive disease course years after biopsy, but had a relapsing–remitting course at biopsy. Therefore, these patients were classified as RRMS patients in the study. Biopsies were performed between 0.5 and 168 months (median: 10 months) after occurrence of the first symptoms. The biopsied patients included in the present study were selected from a larger group of patients in whom an inflammatory demyelinating CNS process was diagnosed at biopsy. The 21 biopsied patients included in the present study had been the subject of an earlier investigation in which the extent of acute axonal damage was correlated with the stage of demyelinating activity within the lesions (Bitsch et al., 2000).

Among the 18 autopsy cases, eight had PPMS, five had a relapsing–remitting disease course and five had SPMS (for details see Table 1). The patients who were autopsied had a disease duration between 30 and 336 months (median: 108 months). No patients with a fulminant variant of multiple sclerosis (Type Marburg) leading to early death were represented in either the biopsy or autopsy group in the study. The patient’s clinical characteristics are summarized in Table 1.

Histopathology

Biopsies were performed in different centres all over Germany and sent to the Department of Neuropathology in Berlin after completion of routine analyses. Autopsies were also collected in Berlin’s Department of Neuropathology. Specimens were fixed in 4% paraformaldehyde and embedded in paraffin. Slices 4 µm thick were stained with haematoxylin and eosin (HE), Luxol-fast blue (LFB), peri-
Acute axonal damage, inflammation and disease duration

Patients were divided into four groups according to disease duration (time from first symptoms to biopsy/autopsy): group I, 0–1 year (12 biopsies); group II, 1–5 years (four autopsies, five biopsies), group III, 5–10 years (seven autopsies, three biopsies); and group IV, >10 years (seven autopsies, three biopsies).

Acute axonal damage in multiple sclerosis lesions was determined in sections stained for APP (Fig. 1). The highest number of APP-positive axons was found in group I (Fig. 2A). Significantly more axons stained for APP in group I than in group II (\( P < 0.001 \)), group III (\( P < 0.01 \)) and group IV (\( P < 0.001 \)). There were also significantly more APP-positive axons in group II compared with group III (\( P < 0.05 \)).

From earlier studies, it is known that there are more APP-positive axons in active lesions than in demyelinated or remyelinating plaques. To test whether the significant higher accumulation of APP-positive axons in group I is the result of an unbalanced distribution of active lesions, we compared active, demyelinated and remyelinating lesions of the groups I–IV. As shown in Fig. 3A, active lesions of group I revealed the highest number of APP-positive axons, indicating that the high level of acute axonal damage in group I is, in fact, caused by an increased number of transected axons rather than an unbalanced distribution of active demyelinating lesions (Fig. 1A–C). Numbers of APP-positive axons were significantly higher in group I than in group III (\( P < 0.05 \)) and in group IV (\( P < 0.01 \)). In demyelinated and remyelinating lesions, the number of APP-positive axons was relatively low in all groups, and no significant differences between the groups were observed (Fig. 3B and C). In late remyelinating lesions, with few exceptions, only little APP staining was seen (Figs 1D–F and 3D). These results demonstrate that acute axonal damage is (i) an early event during multiple sclerosis; (ii) highest at the beginning of the disease; and (iii) low in remyelinated lesions.

In a second series of analyses, we investigated the composition of the inflammatory infiltrates in multiple sclerosis plaques in correlation to disease duration. Lesions were infiltrated mainly by macrophages and CD3-positive T cells (Fig. 2B–D). Significantly more CD3 cells were seen in group I compared with all other groups (Fig. 2B; group I versus II, \( P < 0.05 \); group I versus III, \( P < 0.01 \); I versus III, \( P < 0.01 \)). Most CD8-positive lymphocytes were found in group I (group I versus II, \( P < 0.05 \); I versus IV, \( P < 0.05 \)). The highest number of macrophages was observed in group I, whereas no significant differences were found between groups II, III and IV (Fig. 2D; group I versus III, \( P < 0.05 \)).
Interestingly, only few plasma cells were seen, and the number of plasma cells was similar in all groups (Fig. 2E).

**Acute axonal damage and inflammation within the periplaque white matter**

Numerous APP-positive axons were detected within the PPWM of multiple sclerosis lesions. We studied the number of APP-positive axons in correlation to disease duration. Highest numbers of APP-positive axonal spheroids were identified in the PPWM of patients who were biopsied within 1 year after onset of first symptoms (group I) (Fig. 2F; group I versus IV, \( P < 0.05 \)). In the other groups, only few APP-positive axons were observed in the PPWM. Inflammatory infiltrates were seen perivascularly and diffusely distributed through the otherwise normal appearing PPWM. They consisted mainly of macrophages and T cells (Fig. 2G–I). The number of macrophages and CD3-positive lymphocytes within the PPWM was similar in all groups (Fig. 2G and H), whereas most CD8-positive T cells were found in group I patients (Fig. 2H; group I versus II, \( P < 0.05 \); I versus IV, \( P < 0.05 \)).

![Fig. 1 APP-positive axons in active demyelinating (A–C) and remyelinating (D–E) lesions. (A) Demyelinated lesion with an active demyelinating border of a patient with a disease duration longer than 10 years (group IV). The area of active demyelination is marked by arrows. (B) Numerous LFB-positive macrophages are seen (LFB staining, see arrows). (C) In the area of active demyelination, many APP-positive axons were found (immunohistochemistry for APP, see arrows). (D) Late remyelinating lesion (shadow plaque) of a patient with a disease duration of 5–10 years (group III). (E) Thin, irregularly formed myelin sheaths that are characteristic for remyelinating lesions are shown, see arrows (immunohistochemistry for MBP). (F) In these lesions, only a few APP-positive axons were found, see arrow (immunohistochemistry for APP).](image-url)
Fig. 2 Acute axonal damage and inflammation in lesions and periplaque white matter. Number of (A and F) APP-positive axons, (B and G) CD3- and (C and H) CD8-positive T cells, (D and I) macrophages/microglia and (E) plasma cells in lesions and in periplaque white matter of patients with different disease duration (I, 0–1 year; II, 1–5 years; III, 5–10 years; IV, >10 years). Values are given as means ± SD.
Correlation of acute axonal damage and inflammation

The extent of acute axonal damage in the entire group of lesions correlated significantly with the number of CD8-positive T cells \( (P < 0.0005) \) and macrophages/microglia \( (P < 0.0001) \). In the PPWM, a significant correlation was only found for CD8-positive T cells \( (P < 0.0001) \). However, differences were observed when grouping the lesions according to the disease duration. In lesions obtained early in the disease (groups I and II, disease duration <5 years), a positive correlation was found between the number of APP-positive axons and macrophages/microglia \( (P = 0.005) \), but not for CD8-positive T cells. The opposite was found in the PPWM at this disease stage. Acute axonal damage significantly correlated with the number of CD8-positive T cells \( (P = 0.0001, r = 0.79) \). In the late disease stages (groups III and IV, disease duration >5 years), in both the lesions and the PPWM, a significant correlation was found between acute axonal damage and numbers of CD8-positive T cells \( (P < 0.01) \) and macrophages/microglia \( (P < 0.005) \). These data suggest that different components of the inflammatory infiltrate may contribute differentially to axon damage in the demyelinating lesions and the PPWM.

Acute axonal damage and disease course

We also studied the number of APP-positive axons in patients of different clinical courses and disease durations. In patients with a relapsing–remitting disease course, there were significantly more APP-positive axons at the beginning of the disease than after 10 years (Fig. 4A; group I versus IV, \( P < 0.001 \)). In autopsies from patients with SPMS, all lesions were older than 1 year. Again, APP expression was more pronounced in early lesions (Fig. 4B; group III versus IV, \( P < 0.001 \)). In PPMS, there were no significant differences between different disease durations after correction for multiple comparisons (Fig. 4C). We also investigated whether there was a difference between male and female patients with respect to acute axonal damage, but no significant variations between the sexes were detected.

Discussion

Our present data show that acute axonal damage is an early event during the development of multiple sclerosis lesions. We observed the highest number of APP-positive axons, a marker of acute axonal damage, in lesions of patients with a
disease duration of <1 year. Our data also suggest that axonal damage is a continuous process that can be found in patients who suffer from multiple sclerosis for >10 years independently of the clinical course. The number of inflammatory cells runs through a decrease over time similar to the number of APP-positive axons. In addition, acute axonal damage can also be seen in the PPWM with a similar time course to that inside the lesions. The extent of axon damage correlates with inflammation. However, especially in the early disease evolution, different components of the immune system (CD8-positive T cells and macrophages/microglia) seem to be involved differentially in this process in the lesions and the NAWM.

Axonal loss can be detected indirectly by MRS (Arnold et al., 1990; Matthews et al., 1998) and MRI techniques (Brück et al., 1997; van Walderveen et al., 1998) in vivo. In MRS, axonal dysfunction or loss is associated with reduced levels of NAA (De Stefano et al., 1995) that is located mainly in neurones and axons (Birken and Oldendorf, 1989). It is therefore a suitable marker of axonal loss (Simmons et al., 1991). Hypointense T1 lesions and a decreased magnetization transfer ratio (MTR) are also associated with tissue destruction and axonal loss (Brück et al., 1997; van Walderveen et al., 1998; van Waesberghe et al., 1999). T1 lesion hypointensity (Grimaud et al., 1999; Paolillo et al., 2000), decreased MTR and reduced NAA levels all correlate with an increased disability (Gass et al., 1994; De Stefano et al., 1995, 1998; Davie et al., 1995, 1999). Some studies show abnormal low NAA/Cr (creatine) values in early stages of multiple sclerosis and a reduction of the NAA/Cr level that even precedes significant disability (De Stefano et al., 2001). However, MR techniques are not able to differentiate between acute and long-standing axonal damage. MRS, MRI and MTR can only reflect the accumulation of axonal damage/dysfunction. In contrast, histopathological studies are able to distinguish between acute axonal damage and permanent loss of axons. The latter can be measured by the number of axons counted after Bielschowsky’s stain or immunohistochemical neurofilament stainings. In contrast, APP is a marker of acute axonal damage. It persists in transected axons for a period of <30 days (Li et al., 1995; Pierce et al., 1996; Bramlett et al., 1997; Yam et al., 1997).

In histopathological studies, a significant reduction of axon density was found in multiple sclerosis lesions (Lovas et al., 2000). An average axonal loss of 59–82% was observed in demyelinated and remyelinated multiple sclerosis plaques compared with the PPWM (Mews et al., 1998). The acute axonal damage determined by APP staining or axonal spheroids was investigated in acute (Ferguson et al., 1997; Kornek et al., 2000), early (Bitsch et al., 2000) and late chronic (Kornek et al., 2000) multiple sclerosis cases. From these studies, it is well known that axonal damage is an early event during development of multiple sclerosis plaques and that the highest degree of acute axonal damage is associated with active demyelination (Ferguson et al., 1997; Bitsch et al., 2000; Kornek et al., 2000).

Our data confirm these investigations. We see the highest numbers of APP-positive axons in active demyelinating plaques. However, APP-positive axons are also present in early remyelinating and to a much less degree in demyeli-
Acute axonal damage in multiple sclerosis

In our material, the extent of acute axonal damage depends on disease duration and clinical course. The highest number of APP-positive axons was detected in active lesions of patients with a disease duration <1 year. To exclude that the significant higher accumulation of APP in group I is the result of an unbalanced distribution of active lesions, we compared the number of APP-positive axons in active lesions of groups I–IV. Active lesions are defined by the presence of macrophages with myelin debris within their cytoplasm. In early active lesions, macrophages contain MOG- and MBP-positive debris, and in late active lesions only MBP. The digestion of the minor myelin protein MOG lasts about a week, whereas MBP is removed completely after 2–3 weeks (Lassmann, 1983; Brück et al., 1995), indicating that the age of active lesions is similar in all patients. In active lesions of group I, a significantly higher number of APP-positive axons is found compared with groups II–IV, suggesting that acute axonal damage is highest at the beginning of the disease and does not depend on an unbalanced distribution of active lesions or different lesion ages. One could argue that there may be a selection bias of cases included in our study, since brain biopsies form a major part of our study and these patients might suffer from an atypical clinical course at the beginning of the disease. However, in our case collection, there are no patients with a very fulminant disease course leading to early death (Type Marburg), and all biopsied patients developed a classical chronic multiple sclerosis during follow-up. Additionally, biopsies were included in all groups (I–IV), thus largely excluding a selection bias. Our findings suggest that an axon-protective treatment, if available, should start immediately after disease onset and should be continued during the following years, especially in patients with a relapsing–remitting disease course. This is in agreement with clinical studies that showed a benefit of immunomodulatory treatment with β-interferon starting early after disease onset (Jacobs et al., 2000; Comi et al., 2001). Treatment in later disease stages resulted only in a limited benefit [Secondary Progressive Efficacy Clinical Trial of Recombinant Interferon-beta-1a in MS (SPECTRIMS) Study Group, 2001]. According to our data, acute axonal damage becomes less prominent in the chronic disease stage. As acute axonal damage and inflammatory infiltrates are associated with each other, the reduced number of APP-positive axons may be due to the mild inflammatory reaction in completely demyelinated or late remyelinating lesions.

In particular, patients with a relapsing–remitting disease course demonstrate prominent acute axonal damage in early disease stages, whereas the amount of acute axonal damage in patients with PPMS is generally lower and does not change significantly over time. This is in line with earlier studies from our group in which the lowest number of APP-positive axons was also found in PPMS (Bitsch et al., 2000). Additionally, in MRI, the hypointense T1 lesion load is lowest in patients with PPMS (Filippi et al., 1999a; van Walderveen et al., 2001). These results indicate that acute axonal damage in the lesions may not be the only cause of progressive clinical disability in PPMS. However, more studies comparing axon density and loss in different clinical courses are required to confirm this conclusion.

We also investigated the PPWM with regard to axonal damage and inflammation. Correlating disease duration and the number of APP-positive axons, most APP-positive axons were observed within the first year after disease onset. This was associated with an increased infiltration of the PPWM with CD8-positive T lymphocytes, which may indicate a substantial role for these cells in axonal destruction. We found APP-positive axons in the PPWM without any signs of demyelination. These findings indicate that axonal damage occurs without visible demyelination or even precedes demyelination. These results are in line with earlier studies that investigated acute axonal damage in PPWM of experimental allergic encephalomyelitis (EAE) animals and multiple sclerosis patients (Bitsch et al., 2000; Kornek et al., 2000). Changes in the white matter can be found not only in the PPWM but also in white matter far away from lesions, the so-called NAWM. So far, only few histological studies have investigated the morphological changes that occur in the NAWM. Allen and McKeown (1979) reported on an increased gliosis, small round cell infiltrates and an increased number of macrophages. A recent detailed analysis of the NAWM of a patient with acute multiple sclerosis described axonal changes (Bjartmar et al., 2001). Abnormalities of the NAWM can also be detected with magnetic resonance techniques (for a review see Filippi et al., 1999b). Numerous studies detected decreased NAA levels within NAWM and therefore indicated an axonal loss or dysfunction (Matthews et al., 1996; Davie et al., 1997; Schiepers et al., 1997; Fu et al., 1998). This axonal loss seems to be caused by Wallerian degeneration of axons transected in multiple sclerosis lesions (Evangelou et al., 2000).

The pathogenesis of axonal damage is still a matter of debate. MRS studies demonstrate a temporary decrease of NAA level (Davie et al., 1994). This is either a consequence of phenomena that are reversible in principle, such as oedema, or indicates that axonal damage or dysfunction measured by magnetic resonance techniques is at least partly reversible. Whether the axonal damage that is observed by APP staining is permanent or transient is still unclear (Ferguson et al., 1997). There is a strong interaction between axons and myelin sheaths. Myelin sheaths are essential for maintaining the axonal cytoskeleton. Therefore, it is conceivable that demyelination makes axons vulnerable to axonal damage. However, the occurrence of acute axonal damage in the PPWM makes it unlikely that this pathogenetic pathway is the only one. Other mechanisms such as inflammatory mediators, cellular or antibody-mediated damage may also be responsible for axonal damage. A variety of different inflammatory mediators can be found in multiple sclerosis lesions and in PPWM. The most important are tumour necrosis factor-α (TNF-α), interferon-γ and nitric oxide (NO) (Bitsch et al., 1998; Koebeler and Ball, 1999; Silber and Sharief, 1999). Both TNF-α and interferon-γ
stimulate the release of NO by astrocytes and macrophages. NO may contribute to axonal dysfunction by inducing conduction block (Redford et al., 1997) or axonal degeneration (Smith et al., 2001). The inducible nitric oxide synthetase (iNOS) was identified in multiple sclerosis lesions (Bo et al., 1994; Bitsch et al., 2000), and a correlation between iNOS mRNA expression and axon density was found (Bitsch et al., 2000). Axons can also be damaged directly by a cellular (Gimsa et al., 2000) or antibody-mediated (Rawes et al., 1997; Sadatipour et al., 1998) inflammatory reaction. In our material, acute axonal damage was always accompanied by an inflammatory infiltrate mainly consisting of macrophages, some T cells and only a few plasma cells. The extent of axon damage differentially correlated with the numbers of macrophages/microglia or CD8-positive T cells, suggesting that these cells or their toxic products are the main effector cells in this process. The number of plasma cells was relatively low compared with the number of macrophages and T cells. Additionally, most plasma cells were found in patients with a disease duration >10 years, in which acute axonal damage is relatively rare. Therefore, plasma cells appear less likely to be responsible for induction and maintenance of acute axonal damage.

In summary, we demonstrated in biopsy and autopsy tissue of multiple sclerosis patients that acute axonal damage within multiple sclerosis lesions and in the PPWM is highest during the initial stages of the disease and persists at a lower level during the following years. These findings indicate that a treatment aimed at axon protection should be started as early as possible and should be continued for at least some years.

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References


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Secondary Progressive Efficacy Clinical Trial of Recombinant Interferon-beta-1a in MS (SPECTRIMS) Study Group. Randomized...


