Distribution of a calcium channel subunit in dystrophic axons in multiple sclerosis and experimental autoimmune encephalomyelitis

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Summary
Multiple sclerosis and experimental autoimmune encephalomyelitis (EAE) are immune-mediated diseases of the CNS. They are characterized by widespread inflammation, demyelination and a variable degree of axonal loss. Recent magnetic resonance spectroscopy studies have indicated that axonal damage and loss are a reliable correlate of permanent clinical disability. Accordingly, neuropathological studies have confirmed the presence and timing of axonal injury in multiple sclerosis lesions. The mechanisms of axonal degeneration, however, are unclear. Since calcium influx may mediate axonal damage, we have studied the distribution of the pore-forming subunit of neuronal (N)-type voltage-gated calcium channels in the lesions of multiple sclerosis and EAE. We found that α1b, the pore-forming subunit of N-type calcium channels, was accumulated within axons and axonal spheroids of actively demyelinating lesions. The axonal staining pattern of α1b was comparable with that of β-amyloid precursor protein, which is an early and sensitive marker for disturbance of axonal transport. Importantly, within these injured axons, α1b was not only accumulated, but also integrated in the axoplasmic membrane, as shown by immune electron microscopy on the EAE material. This ectopic distribution of calcium channels in the axonal membrane may result in increased calcium influx, contributing to axonal degeneration, possibly via the activation of neutral proteases. Our data suggest that calcium influx through voltage-dependent calcium channels is one possible candidate mechanism for axonal degeneration in inflammatory demyelinating disorders.

Keywords: voltage-gated calcium channels; multiple sclerosis; experimental autoimmune encephalomyelitis; axon degeneration

Abbeviations: APP = amyloid precursor protein; CNPase = cyclic nucleotide phosphodiesterase; CO = control cases; EA = early active multiple sclerosis lesions; EAE = experimental autoimmune encephalomyelitis; IA = inactive multiple sclerosis lesions; LA = late active multiple sclerosis lesions; MOG = myelin oligodendrocyte glycoprotein; NWM = normal white matter in multiple sclerosis; PLP = proteolipid protein; PP = periplaque white matter in multiple sclerosis; RM = remyelinated multiple sclerosis lesions; VGCC = voltage-gated calcium channel

Introduction
Multiple sclerosis is an immune-mediated disease of the CNS. It is characterized by widespread inflammation, focal demyelination and a variable degree of axonal loss (Charcot, 1868; Cuzner and Wekerle, 1996; Lassmann, 1998a). The disease usually follows a chronic disease course, resulting in persistent neurological deficits in most patients (Weinshenker et al., 1989).

A number of recent studies have provided evidence that axonal damage and loss within and around multiple sclerosis plaques are the major correlates for permanent clinical
disability (Davie et al., 1995; Losseff et al., 1996; De Stefano et al., 1998; Lee et al., 2000; Reddy et al., 2000). Moreover, it has been demonstrated convincingly that the highest incidence of acute axonal injury occurs during active demyelination (Ferguson et al., 1997; Trapp et al., 1998; Bitsch et al., 2000; Kornek et al., 2000). Repeated episodes of demyelination and persistent chronic inflammation as well as other unknown mechanisms contribute further to ongoing fibre degeneration, leading to 75% and more of axonal loss in inactive demyelinated lesions (Lassmann; 1998b; Mews et al., 1998; Ganter et al., 1999; Evangelou et al., 2000; Lovas et al., 2000).

Axonal damage and loss have been described further in different models of experimental autoimmune encephalomyelitis (EAE) (Raine et al., 1989; Storch et al., 1998; Kornek et al., 2000; McGavern et al., 2000). Among these models, myelin oligodendrocyte glycoprotein (MOG)-induced EAE appears to be most suitable, since it reflects both clinical and pathological features of multiple sclerosis and leads to—in comparison with other EAE models—large plaques of demyelination (Storch et al., 1998). Similarly to multiple sclerosis, in MOG-induced EAE the highest incidence of acute axonal injury occurs during active demyelination, leading to a pronounced reduction of axonal density in all stages of demyelination (Kornek et al., 2000).

Although the kinetics and the extent of axonal damage have been described in detail in multiple sclerosis, the mechanisms of axonal degeneration are as yet unclear. Understanding these mechanisms, however, is necessary for the development of axon-protective strategies aimed at reducing permanent clinical disability in multiple sclerosis.

A number of recent studies have emphasized the involvement of calcium ions in the development of axonal disturbance in trauma (George et al., 1995; Büki et al., 1999) and ischaemia (Stys et al., 1991; Fern et al., 1995). These studies suggested that, under pathological conditions, calcium may enter the axon via ion-specific transport mechanisms such as voltage-gated calcium channels (VGCCs) and/or reverse operation of the Na+/Ca2+ exchanger (Stys et al., 1991, 1992; Stys and Lopachin, 1998; Fern et al., 1995; George et al., 1995).

Increased levels of intra-axonal calcium then lead to activation of neutral proteases, e.g. calpains, which are known to degrade major components of the cytoskeleton (Schlaepfer and Zimmermann, 1985; Banik et al., 1993; Kampfl et al., 1997). Furthermore, pharmacological blockade of the respective channels can reduce axonal damage in trauma (George et al., 1995) and ischaemia (Stys et al., 1992; Fern et al., 1995). In order to determine a possible role of calcium channels in axonal degeneration in inflammatory demyelinating disorders, we have studied the distribution of the pore-forming subunit of N-type VGCCs in the lesions of multiple sclerosis and EAE by immunohistochemistry.

| Table 1 Number and characteristics of patients included in the study |
|--------------------------|----------------|----------------|----------------|
| Patient | Age/sex (years) | Disease duration | Disease course |
| 1 | 47/F | 3.5 months | Acute |
| 2 | 46/F | 12 days | Acute |
| 3 | 29/M | 1.5 months | Acute |
| 4 | 51/F | 7 months | Acute |
| 8 | 45/M | 3 weeks | Acute |
| 9 | 20/F | 4 years | RR/acute |
| 10 | 35/M | 1.5 months | Acute |
| 13 | 39/F | 3 years | SP |
| 14 | 43/F | 20 years | SP |
| 16 | 42/F | 11 years | RR |
| 17 | 45/F | 16 years | SP |
| 18 | 37/F | 2 years | PP |
| 20 | 33/F | 10 years | SP |

F = female; M = male; RR = relapsing–remitting multiple sclerosis; SP = secondary progressive multiple sclerosis; PP = primary progressive multiple sclerosis; Acute = acute multiple sclerosis, leading to significant disability or even to death within 1 year (Lublin and Reingold, 1996).

Material and methods

Multiple sclerosis patients and controls

The study was performed on autopsy tissue from 13 patients. In addition, two control brains were included in this series from patients without evidence of neurological disease or neuropathological alterations. Clinical background data are summarized in Table 1. All material was fixed in 4% paraformaldehyde and embedded in paraffin wax.

EAE material

Lewis,1N rats were kindly provided by Professor H. Hedrich (Hedrich, 1990). EAE was induced in 18 rats by active sensitization with 20–100 µg of recombinant MOG as described in detail previously (Storch et al., 1998). In addition, three healthy rats of the same strain served as controls. Animals were weighed and examined daily for clinical signs of EAE. At various time points after sensitization (days 11–61), animals were sacrificed and perfused via the aorta with 4% paraformaldehyde. Brains and spinal cords were dissected and routinely embedded in paraffin wax. All experimental procedures were approved by the Bavarian and Swedish governments and performed in compliance with international animal welfare standards.

Neuropathology

Sections 2–4 µm thick were cut on a microtome and stained with haematoxylin–eosin, Luxol fast blue and periodic acid–Schiff, and Bielschowsky silver impregnation to assess inflammation, demyelination and axonal loss, respectively.

Immunohistochemistry was performed in adjacent serial sections using an avidin–biotin or an alkaline phosphatase–anti-alkaline phosphatase technique. The following primary
antibodies were used: MOG (anti-MOG; Department of Biochemistry, Cardiff, UK), proteolipid protein (anti-PLP; Serotec, Oxford, UK), 2',3'-cyclic nucleotide phosphodiesterase (anti-CNPase; Affiniti Research Products, Ilkleton, UK), myelin basic protein (anti-MBP; BioGenex, San Ramon, USA), macrophages/activated microglia in rats (anti-ED 1; Serotec), early activated human haematopoietic macrophages (anti-27E10; BMA Biomedicals), late activated macrophages (anti-MRP 14; BMA Biomedicals, Augst, Switzerland), late activated macrophages (anti-27E10; BMA Biomedicals), β-amyloid precursor protein (anti-APP; Boehringer, Mannheim, Germany), α1B, the pore-forming subunit of N-type VGCCs (anti-α1B; Alomone Laboratories, Jerusalem, Israel), and SMI 33 (anti-non-phosphorylated neurofilament M;H; Affiniti Research Products Ltd, Exeter, UK). Control sections were incubated in the absence of primary antibody. Staining was omitted when the α1B antibody was incubated together with a specific anti-α1B-peptide (data not shown).

In situ hybridization for PLP mRNA was performed according to Breitschopf and colleagues (Breitschopf et al., 1992).

Laser scanning confocal microscopy

For double staining of APP and α1B, essentially the same immunocytochemistry protocol was used as for normal light microscopy. Briefly, sections were incubated with both primary antibodies overnight, then incubated with secondary antibodies (Cy3 goat anti-rabbit; Jackson ImmunoResearch Laboratories, West Grove, Pa., USA) and biotinylated sheep anti-mouse (Amersham, UK) for 2 h at room temperature. In a third step, sections were incubated with streptavidin-conjugated Cy2 (Jackson) for 1 h at room temperature. After rinsing with PBS (phosphate-buffered saline), sections were embedded in PBS/glycerol (1 : 9) with 3% DABCO (Sigma) and placed on a coverslip. Fluorescent preparations were examined using a Zeiss laser scan microscope as described previously (Akassoglou et al., 1998) (Cy2 signal green, Cy3 signal red, overlay yellow).

Electron microscopy

Immune electron microscopy was performed according to Vass and colleagues (Vass et al., 1986). Briefly, spinal cord tissue of EAE animals was fixed by perfusion with 4% paraformaldehyde. Tissue sections 50–100 µm thick were incubated with the primary antibody against the α1B subunit of VGCCs for 18 h at 4°C. The blocks were then washed extensively in PBS, incubated in biotinylated anti-rabbit immunoglobulin and in avidin peroxidase subsequently. The peroxidase reaction product was developed with di-amino benzidine reagent. The material was then routinely osmicated and embedded in resin. For control, primary antibody was omitted.

Selection of demyelinated plaques and definition of lesional staging

In multiple sclerosis and EAE, areas of normal white matter, periplaque white matter, actively demyelinating plaques, inactive demyelinated plaques and remyelinated shadow plaques were selected for further analysis of axonal pathology. Normal white matter was defined as an area which showed no evidence of demyelination by macroscopic inspection and histology within the area and the surrounding tissue. In multiple sclerosis, periplaque white matter represented a strip of tissue of 5 mm adjacent to the border of active or inactive plaques. In EAE, periplaque and normal white matter were not analysed separately for the evaluation of axonal pathology.

The following categories for demyelinated plaques were defined (Brück et al., 1995)

Early active lesions (EA)

These lesions were heavily infiltrated by T cells and macrophages. Myelin sheaths were being disrupted and macrophages contained degradation products, which were stained by Luxol fast blue and immunoreactive for all myelin proteins, including MOG and CNPase.

Late active lesions (LA)

In these lesions, myelin was already destroyed and removed from axons. Macrophages contained degradation products reactive for major myelin proteins, such as PLP, but were negative for MOG and CNPase.

Inactive lesions with active border (IA + A)

The inactive centre of radially expanding lesions still showed pronounced inflammation and macrophage infiltration. The macrophages revealed empty vacuoles and showed no immunoreactivity for myelin proteins.

Inactive lesions (IA)

These lesions showed no evidence for ongoing myelin destruction at their borders. Although these lesions also contained some T cells and macrophages, their number was much lower compared with active lesions. However, microglia activation was prominent.

Remyelinated shadow plaques (RM)

These lesions were characterized by myelin pallor, due to abnormally thin myelin sheaths and a pronounced expression of PLP mRNA in oligodendrocytes. Similarly to inactive lesions, residual inflammation and microglia activation were present.
Fig. 1 Distribution of α1B, the pore-forming subunit of N-type VGCCs in the CNS. (A) Immunohistochemistry for α1B shows its localization in the spinal cord of a healthy rat with a uniform distribution in the ventral and dorsal horn and pronounced staining of regions of high synaptic density, whereas white matter immunoreactivity is absent. (B) Higher magnification of A reveals intense staining of neurones and the surrounding neuropil in the ventral horn of the spinal cord. (C) Motor neurone with strong punctuate surface staining along the cell body and dendrites, consistent with the presence of nerve terminals forming synapses on them. (D) Axons are not reactive for α1B in normal white matter areas. Co-localization of α1B (E), APP (F) and SMI 33 (G) is found in injured axons of multiple sclerosis in adjacent serial sections (arrows). Magnifications: A, ×45.1; B, ×223; C, ×1361; D, ×1540; E–G, ×240. Confocal microscopy reveals numerous damaged axons immunoreactive for APP (green) and α1B (red) as well as for both (yellow, arrows) in multiple sclerosis (K–M) and EAE (H–J). α1B reactivity seems to be enhanced along the plasma membrane of acutely injured axons (J, L and M). Magnifications: H, ×321; I, ×849; J, ×641; K, ×214; L, ×340; M, ×1188.
Fig. 2 Distribution of $\alpha_{1B}$ in injured axons of multiple sclerosis and EAE. (A–G) Actively demyelinating multiple sclerosis lesion, typically located around a vessel with multiple layers of inflammatory cells. (A) Luxol fast blue reveals a demyelinated plaque which is sharply delineated against the surrounding area of apparently normal myelin. (B) Bielschowsky silver impregnation for axons shows reduction of axonal density within the lesion in comparison with the surrounding white matter. (C) Immunohistochemistry for APP stains numerous axons, indicating a high incidence of acute axonal injury within the demyelinating plaque. (D) Immunoreactivity for $\alpha_{1B}$ is also found within a high number of axons and axonal spheroids in the lesion. (E–G) Higher magnification of the border of the plaque as indicated by the rectangle in A. (E) Luxol fast blue myelin stain reveals macrophages containing myelin degradation products (arrows), indicating active demyelination. Numerous axons immunoreactive for APP (F) and $\alpha_{1B}$ (G) are found within the plaque; some are also found in the periplaque white matter. (H–J) Actively demyelinating EAE plaque, located around a dilated venule. (H) Luxol fast blue reveals the absence of normal myelin within the plaque and the presence of myelin degradation products with macrophages (arrows). Similarly to the multiple sclerosis lesion, a high density of axons immunoreactive for APP (I) and $\alpha_{1B}$ (J) is found with the demyelinated area. (K) Alternating dilatations and constrictions along an $\alpha_{1B}$-immunopositive axon, as taken from an multiple sclerosis plaque. (L) Swollen axon immunoreactive for $\alpha_{1B}$, as seen within an EAE lesion. Magnifications: A–D, $\times$23; E–J, $\times$278; K and L, $\times$752.
**Quantitative determination of α_{1B} reactivity**

Camera lucida drawings of demyelinating lesions were made in order to define precisely the pattern of myelin destruction for each lesional area. In the selected area, the demyelinating activity was determined by the presence or absence of myelin degradation products within macrophages immunoreactive for MOG, PLP and CNPase as described above. On adjacent serial sections, the number of α_{1B}-positive elements stained per square unit of tissue was counted as described previously (Kornek et al., 2000). A 0.01 mm² wide field, defined by an ocular morphometric grid, was selected in the centre of each lesional area. In this field, α_{1B}-positive fibres were counted under a ×100 objective. In inactive demyelinated lesions, shadow plaques, periplaque and normal white matter for each lesional area, the average number of α_{1B}-positive axons in 10 adjacent fields of 0.01 mm² were taken for quantification because of the low density of injured axons.

**EAE**

Total number of lesional areas analysed: n = 53; among those, n = 11 early active (EA), n = 11 late active (LA), n = 11 inactive plus active border (IA + A), n = 4 remyelination with concomitant demyelinating activity.
Multiple sclerosis

Total number of lesional areas analysed: \( n = 91 \); among those, \( n = 16 \) EA, \( n = 10 \) LA, \( n = 15 \) IA + A, \( n = 7 \) inactive (IA), \( n = 7 \) remyelination (RM), \( n = 20 \) PP, \( n = 16 \) normal white matter in multiple sclerosis brains (NWM), \( n = 8 \) white matter of controls (CO).

The Mann--Whitney \( U \) test and the \( \chi^2 \)-test were used for statistic analysis.

Results

Distribution of the N-type channel in normal CNS tissue

The pattern of the pore-forming subunit of N-type calcium channels (\( \alpha_{1B} \)) was studied in grey and white matter of the rat spinal cord and in selected areas of the human brain (Fig. 1.).

Corresponding to previous observations (Westenbroek et al., 1992, 1998a; Day et al., 1996), the \( \alpha_{1B} \) staining was prominent throughout the CNS. In the rat spinal cord, immunoreactivity was found along the surface of neurones in the ventral and dorsal horn (Fig. 1A and B) and throughout the laminae of the dorsal horn (Fig. 1A). Immunoreactivity for \( \alpha_{1B} \) was most pronounced in regions of high synaptic density (Fig. 1A).

At the light microscopic level, we observed a strong punctate staining along neuronal cell bodies and dendritic shafts, suggesting the presence of \( \alpha_{1B} \) in nerve terminals forming synapses on the neurones (Fig. 1C). This was confirmed by immune electron microscopy which was performed on rat spinal cord tissue. We found smooth and punctate surface staining consistent with the presence of \( \alpha_{1B} \) in the cell surface of nerve terminals forming synapses (Fig. 3A and B). In addition, some immunoreactive vesicles were found within the neuronal perikarya and in synaptic boutons (Fig. 3A).

No staining for \( \alpha_{1B} \) was observed in axons of the normal white matter at the light microscopic level (Fig. 1D). Yet, at the ultrastructural level, a low density of immunoreactive vesicles, which were not inserted into the axonal plasma membrane (Fig. 3C), was found within the axolemma.

Distribution of \( \alpha_{1B} \) in the lesions of multiple sclerosis and EAE

A prominent reactivity for \( \alpha_{1B} \) was found in axons of actively demyelinating lesions of multiple sclerosis (Fig. 2D, G and K) and EAE (Fig. 2J and L; Tables 3 and 4). Actively demyelinating lesions were frequently located around vessels, both in multiple sclerosis (Fig. 2A) and in EAE (Fig. 2H). Within the multiple sclerosis lesions, axonal density was reduced (Fig. 2B). A prominent axonal staining pattern for APP as a marker for acute axonal injury (Gentleman et al., 1993; Sherriff et al., 1994) was found in multiple sclerosis (Fig. 2C and F) and EAE lesions (Fig. 2I).

Similarly to APP, the immunoreactivity for \( \alpha_{1B} \) was present in large calibre axons reminiscent of axonal spheroids (Fig. 2K), in focal axonal swellings of non-transected fibres (Fig. 2L) as well as in some demyelinated axons with normal calibre.

In addition, many swollen axons displayed immunoreactivity for APP, \( \alpha_{1B} \) and SMI 33, a marker for non-phosphorylated subunits of neurofilaments, in multiple sclerosis lesions, as seen on serial sections (Fig. 1E–G).

Some glial staining was also observed, at both the light (Fig. 2B and C) and electron microscopic level (Fig. 3D and E). However, there was no difference in glial reactivity for \( \alpha_{1B} \) in control tissue or lesional areas, as described previously in diverse forms of brain injury, but also hypomyelination (Westenbroek et al., 1998b). In addition, some endothelial cells displayed immunoreactivity for \( \alpha_{1B} \) (Fig. 2D and J). At the light microscopic level, the accumulation of \( \alpha_{1B} \) in axons was comparable with that of APP. APP is a well-recognized marker for disturbance of axonal transport (Gentleman et al., 1993; Sherriff et al., 1994). As described in our previous study (Kornek et al., 2000), the highest number of APP-positive axons was also found in actively demyelinating lesions of multiple sclerosis and EAE.

Confocal microscopy of actively demyelinating lesions of multiple sclerosis and EAE

When confocal microscopy was performed, the co-localization of APP and \( \alpha_{1B} \) in dilated axons in multiple sclerosis (Fig. 1K, L and M) and EAE lesions (Fig. 1H, I and J) could be demonstrated. Most interestingly, however, in some large, swollen axons, \( \alpha_{1B} \) staining was accentuated along the axonal plasma membrane, suggesting the integration of the channel subunits into the axolemma (Fig. 1J, L and M). In order to discern the distinct localization of \( \alpha_{1B} \) within the axon, immune electron microscopy for \( \alpha_{1B} \) was performed.

Electron microscopy of actively demyelinating lesions of EAE

The axonal accumulation of \( \alpha_{1B} \) in areas of active demyelination was confirmed by electron microscopy, which was performed on the EAE material. In some demyelinated axons and, in particular, in dystrophic axonal swellings, the number of \( \alpha_{1B} \)-positive vesicles was clearly increased (Fig. 3D). Moreover, as suggested by confocal laser scanning, immune electron microscopy revealed that \( \alpha_{1B} \) is not only accumulated, but also integrated in the axonal plasma membrane in areas of active demyelination (Fig. 3D and E).
Quantitative evaluation of $\alpha_{1B}$-positive axons in the lesions of multiple sclerosis and EAE

Actively demyelinating lesions of multiple sclerosis and EAE

While myelinated axons of the normal white matter showed no immunoreactivity for $\alpha_{1B}$ at the light microscopic level (Fig. 1D), some apparently normal axons as well as many axonal swellings of actively demyelinating lesions (Fig. 2D, G and J) were intensely stained by this antibody, for the pore-forming subunit of N-type calcium channels (multiple sclerosis + EAE: CO/EA, $P < 0.0001$; CO/LA, $P < 0.0001$). Both in multiple sclerosis and in EAE, the number of $\alpha_{1B}$-positive axons was slightly higher in early compared with late active lesions; the difference, however, was not statistically significant (multiple sclerosis + EAE: EA/LA, n.s.) (Tables 2 and 3).

Inactive lesions with an actively demyelinating edge

In both multiple sclerosis and EAE, a significant number of $\alpha_{1B}$-positive axons was found in the inactive centre of lesions with ongoing demyelinating activity at the edge. Again, the staining pattern for $\alpha_{1B}$ was comparable with that of APP (multiple sclerosis + EAE: CO/IA + A, $P < 0.0001$) (Tables 2 and 3).

Inactive lesions of multiple sclerosis

In inactive lesions of multiple sclerosis, the number of $\alpha_{1B}$-positive axons was low, but it was significantly increased compared with controls (multiple sclerosis: CO/IA, $P < 0.05$) (Table 2). The EAE sample did not contain entirely inactive lesions.

Remyelinated lesions of multiple sclerosis and EAE

Remyelinated shadow plaques in multiple sclerosis showed no evidence of recurrent demyelinating activity. The number of $\alpha_{1B}$-positive axons was not significantly increased compared with control white matter (multiple sclerosis: CO/RM, n.s.). In contrast to multiple sclerosis, remyelinated EAE plaques, which showed signs of recurrent demyelinating activity, also showed $\alpha_{1B}$-positive axons (EAE: CO/RM-A, $P < 0.05$) (Tables 2 and 3).

Periplaque and normal white matter of multiple sclerosis and EAE

There was a low, but significant increase of $\alpha_{1B}$ in the periplaque white matter of multiple sclerosis lesions (multiple sclerosis: CO/PP, $P < 0.05$). In normal white matter, no significant increase of $\alpha_{1B}$-positive axons was observed (multiple sclerosis: CO/NWM, n.s.). In EAE, no statistically significant increase of $\alpha_{1B}$-positive axons was noted in the periplaque/normal white matter (Tables 2 and 3).

In both multiple sclerosis and EAE, there was a highly significant correlation between the frequency of injured axons immunoreactive for APP and $\alpha_{1B}$ in the respective lesional areas (APP/$\alpha_{1B}$: $P < 0.0001$).

Discussion

Recent magnetic resonance spectroscopy studies have provided evidence that axonal damage and loss within and around multiple sclerosis lesions are the major correlates of permanent clinical disability (Davie et al., 1995; Truyen et al., 1996; De Stefano et al., 1998; Lee et al., 2000; Reddy et al., 2000). Axonal injury as a common feature of multiple sclerosis pathology was already recognized at the turn of the century (Kornek and Lassmann, 1999). However, only recently have new techniques provided the tools for accurate quantification of acute axonal damage as well as definite axonal loss (Ferguson et al., 1997; Trapp et al., 1998; Mews et al., 1999; Bitsch et al., 2000; Evangelou et al., 2000; Kornek et al., 2000; Lovas et al., 2000). Finally, histopathological correlates of MRI-defined lesions have clarified further when and to what extent axonal injury occurs in multiple sclerosis (Brück et al., 1997; van Walderveen et al., 1998).

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**Table 2 Density of $\alpha_{1B}$-positive axons in multiple sclerosis lesions**

<table>
<thead>
<tr>
<th></th>
<th>Control white matter</th>
<th>Early active</th>
<th>Late active</th>
<th>Inactive + active edge</th>
<th>Inactive</th>
<th>Remyelination</th>
<th>Periplaque white matter</th>
<th>Normal white matter</th>
</tr>
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<tbody>
<tr>
<td>$\alpha_{1B}$-positive axons/0.01 mm$^2$</td>
<td>0.04 ± 0.03</td>
<td>22.25 ± 4.07</td>
<td>16.4 ± 3.28</td>
<td>3 ± 1.37</td>
<td>0.24 ± 0.06</td>
<td>0.11 ± 0.04</td>
<td>0.34 ± 0.1</td>
<td>0.06 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>CO/EA; $P &lt; 0.0001$</td>
<td>CO/LA; $P &lt; 0.001$</td>
<td>CO/IA + A; $P &lt; 0.01$</td>
<td>CO/IA; $P &lt; 0.05$</td>
<td>n.s.</td>
<td>CO/PM; $P &lt; 0.05$</td>
<td>n.s.</td>
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**Table 3 Density of $\alpha_{1B}$-positive axons in EAE lesions**

<table>
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<tr>
<th></th>
<th>Control white matter</th>
<th>Early active</th>
<th>Late active</th>
<th>Inactive + active edge</th>
<th>Remyelination + active demyelination</th>
<th>Periplaque white matter</th>
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<tr>
<td>$\alpha_{1B}$-positive axons/0.01 mm$^2$</td>
<td>0 ± 0</td>
<td>88.18 ± 19.57</td>
<td>47 ± 16.43</td>
<td>2.73 ± 0.71</td>
<td>49.75 ± 32.31</td>
<td>0.12 ± 0.09</td>
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<tr>
<td></td>
<td>CO/EA; $P &lt; 0.0001$</td>
<td>CO/LA; $P &lt; 0.0001$</td>
<td>CO/IA + A; $P &lt; 0.001$</td>
<td>CO/RA; $P &lt; 0.0001$</td>
<td>CO/PP; $P &lt; 0.0001$</td>
<td>n.s.</td>
</tr>
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et al., 1998; Bitsch et al., 1999). The mechanisms of axonal damage, however, are not yet understood.

Axonal degeneration is a common mechanism in a variety of disorders of the CNS (Mohr et al., 1977; Fisher, 1979; McQuin and O’Leary, 1987; Büki et al., 1999). The morphology is very similar under all these circumstances, suggesting a common final pathway in which an increase in the intra-axonal calcium levels appears to be a key step (Ransom et al., 1990; George et al., 1995). External calcium is required for and is sufficient to produce axonal degeneration in ischaemia (Waxman et al., 1993) and in trauma (George et al., 1995), where increased permeability of the axonal membrane may persist for a surprisingly long period (Povlishock and Pettus, 1996).

Under pathological conditions, calcium influx may therefore be mediated through an intact axolemma via calcium-specific transport mechanisms such as voltage gated sodium channels (Agrawal and Fehlings, 1996; Stys and Lopachin, 1998), reverse operation of the Na⁺/Ca²⁺ exchanger (Stys et al., 1991, 1992) or via voltage-dependent calcium channels (Fern et al., 1995; George et al., 1995). Blockade of these channels can inhibit or at least delay axonal degeneration after injury.

While most authors have suggested a primary route of calcium entry through reverse operation of the Na⁺/Ca²⁺ exchanger in experimental white matter anoxic injury (Stys and Lopachin, 1998), Fern and colleagues observed a significantly increased protection when N-type calcium channels were blocked in addition to L-type calcium channels (Fern et al., 1995). They suggested a low density of N-type channels in white matter compared with L-type channels. They concluded that Ca²⁺ influx through N-type channels might only become important during anoxia when L-type channels were blocked.

We have investigated a possible involvement of the N-type calcium channel in axonal degeneration in inflammatory demyelination. N-type channels are broadly, if not ubiquitously, expressed in different CNS regions and may be blocked specifically by ω-conotoxin GVIA (Westenbroek et al., 1992, 1998a). They are present on nerve terminals forming synapses on many central neurones (Westenbroek et al., 1992) and play an important role in neurotransmitter release. Like most membrane-bound proteins, calcium channels pass from their sites of synthesis in the endoplasmic reticulum to the Golgi apparatus. From here, packed in membrane vesicles, they are carried by fast axonal transport along tracks formed by microtubules to the synaptic membrane (Alberts et al., 1989). Our study confirmed previous findings of the presence of the α₁B subunit in the presynaptic terminals of neurones by electron microscopy. Furthermore, we could show the presence of α₁B-immuno-reactive vesicles within the axoplasm, showing their way of transport along the axon by the axonal transporting system.

Most interestingly though, we found a prominent axonal accumulation of α₁B-positive vesicles in demyelinated axons and axonal swellings in actively demyelinating lesions of multiple sclerosis and EAE. The pattern was comparable with the axonal accumulation of APP in acutely injured axons of multiple sclerosis and EAE (Ferguson et al., 1997; Kornek et al., 2000). The preferential localization of α₁B and APP in acutely damaged axons has been shown by light and confocal microscopy. We therefore can assume that the axonal accumulation of α₁B in areas of active demyelination is due to the breakdown of axonal transport. In addition, however, α₁B was not only found to be accumulated, but also integrated in the axonal membrane of acutely injured axons, as shown by immune electron microscopy in actively demyelinating EAE lesions. In normally myelinated axons, VGCCs are absent in the internodal axonal plasma membrane (Waxman and Ritchie, 1993).

The abnormal localization of the N-type channel under pathological conditions may have profound consequences for the development of axonal degeneration: Since pore-forming subunits alone can function as intact calcium channels (Westenbroek et al., 1992; Walker and De Waard 1998), an influx of calcium through this particular channel may lead to abnormally high levels of intracellular calcium. Increased levels of intracellular calcium may then potentiate axonal perturbation by the activation of calpain (Banik et al., 1997; Kampfl et al., 1997; Büki et al., 1999) or other unknown mechanisms. These alternative modes of action may evolve over a relatively prolonged temporal framework, leading to delayed secondary axotomy (Büki et al., 1999).

Our study therefore presents the first ultrastructural observation of an abnormal redistribution of N-type calcium channels in acutely injured axons. This may be due to a rearrangement of the axonal membrane after injury. Since sodium channels are known to redistribute along demyelinated axons (Black et al., 1991; Moll et al., 1991), a similar mechanism may also exist for VGCCs. It is unlikely that Ca²⁺ influx through VGCCs is the first step in axonal degeneration in inflammatory demyelinating diseases. In fact, the accumulation of α₁B in axons closely reflecting that of APP suggests that a primary injury, leading to disturbance of axonal transport, must precede the accumulation of VGCCs. Nevertheless, a further increase in calcium ions in partially injured axons through these channels may augment pathological calcium load and further potentiate axonal damage. This may explain why the blockade of VGCCs in trauma and ischaemia reduced but did not completely prevent axonal degeneration.

Since the pattern and timing of axonal injury as seen by the accumulation of APP (Kornek et al., 2000) and of α₁B (this study) was comparable in multiple sclerosis and EAE, MOG-induced EAE appears to be an ideal model for testing axon-protective strategies in inflammatory demyelinating diseases.

Acknowledgements
The authors wish to thank Helene Breitschopf, Angela Kury, Marianne Leisser, Petra Tassotti and Jutta Wakley-Neuninger
for expert technical assistance, and Dr Helmut Rauschka for reading the manuscript and for discussion. The project was supported by the Austrian Science Foundation Project P 12658-MED and the EC Biomed 2-Project BMH 4-97-2027.

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Received July 12, 2000. Revised November 11, 2000.
Accepted February 1, 2001