Synaptophysin Is a Reliable Marker for Axonal Damage

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INTRODUCTION

Neurons are highly specialized and polarized CNS cells with discrete cell bodies, numerous dendrites, and normally a single axon that can reach to a meter in length in humans; they can be several meters long in other mammals such as whales. Axons serve not only as “electrical mains” but also as “highway networks” to move different cargos such as mitochondria, synaptic neurotransmitters, and various peptides throughout the CNS. Proteins synthesized in the neuronal cell body (anterograde transport) or absorbed via endocytosis at distal sites (retrograde transport) are delivered to their target places on microtubule “roads” via motor proteins such as kinesin (1, 2) or dynein (3, 4) by energy-dependent axonal transport (5, 6). Fast axonal transport (100–400 mm/day, 1–5 μm/s) mainly moves vesicular cargos whereas slow axonal transport (0.2–5 mm/day, 0.0002–0.05 μm/s) conveys mostly cytosolic and cytoskeletal proteins, such as microtubules, neurofilaments, and actin (5–8).

Axonal transport impairment and/or axonal damage are prominent features of CNS neuroinflammatory and neurodegenerative diseases, such as multiple sclerosis (MS) (9, 10). In neuropathological investigations of MS and CNS trauma, axonal damage is most often identified using immunohistochemical staining for the β-amyloid precursor protein (APP), a ubiquitously expressed 110–135 kDa integral glycoprotein type 1 with an extracellular N-terminus (11–13). The precise biological function(s) of APP is(are) still not well defined but different biological roles including regulation of growth pattern, differentiation, cell adhesion, and apoptosis in different cell types have been proposed. Synaptotrophic and neuroprotective functions have also been postulated (14, 15). APP undergoes kinesin-dependent, predominantly anterograde fast axonal transport (16, 17). Axonal damage/transaction results in disturbances of this transport leading to accumulation of vesicles and subsequent formation of APP-positive spheroids or bulbs (18, 19).

The antibody SMI-32, which detects non-phosphorylated neurofilaments, is another established marker for axonal damage (20–23). Neurofilaments are major components of the neuronal cytoskeleton; their phosphorylation is tightly linked to axonal transport and myelination (24–27). Neurofilaments are moved via slow axonal transport (8, 28, 29). Because of aberrant phosphorylation or impaired transport, neurofilaments also accumulate in affected axons in various pathological conditions.

Abstract

Synaptophysin is an abundant membrane protein of synaptic vesicles. The objective of this study was to determine the utility of identifying synaptophysin accumulations (spheroids/ovoids/bulbs) in CNS white matter as an immunohistochemical marker of axonal damage in demyelinating and neuroinflammatory conditions. We studied the cuprizone toxicity and Thiel’s murine encephalomyelitis virus (TMEV) infection models of demyelination and analyzed CNS tissue from patients with multiple sclerosis (MS). Synaptophysin colocalized with the amyloid precursor protein (APP), a well-known marker of axonal damage. In the cuprizone model, numerous pathological synaptophysin/APP-positive spheroids/ovoids were identified in the corpus callosum at the onset of demyelination; the extent of synaptophysin/APP-positive vesicle aggregates correlated with identified reactive microglia; during late and chronic demyelination, the majority of synaptophysin/APP-positive spheroids/ovoids resolved but a few remained, indicating persistent axonal damage; in the remyelination phase, scattered large synaptophysin/APP-positive bulbs persisted. In the TMEV model, only a few large- to medium-sized synaptophysin/APP-positive bulbs were found in demyelinated areas. In MS patient tissue samples, the bulbs appeared exclusively at the inflammatory edges of lesions. In conclusion, our data suggest that synaptophysin is a reliable marker of axonal damage in the CNS in inflammatory/demyelinating conditions.

Key Words: Axonal damage, Demyelination, Inflammation, Synaptophysin.
(21, 30–32). The pathological mechanisms of axonal damage are not completely understood and additional markers to detect axonal disturbances are needed.

Synaptophysin is a 38-kDa integral transmembrane protein with 4 transmembrane domains (cytosolic C and N termini) and a major small synaptic vesicle protein comprising 7%–10% of the total synaptic vesicle proteins (33–35). It is a major Ca\(^{2+}\)- and cholesterol-binding protein of synaptic vesicles (36–38), and it interacts with different synaptic vesicle and motor proteins including v-SNARE vesicle-associated, membrane protein 2/synaptoprevin II (VAMP2), vesicular proton pump V-ATPase, myosin V, dynamin I, and adaptor protein 1 (AP-1) (39–44). Synaptophysin is involved in different steps of synaptic biogenesis, vesicle protein sorting, vesicle priming, synapse formation, and exo- and endocytosis (37, 38, 45–48). It is transported in the fast axonal component in both anterograde and retrograde directions (6, 49, 50).

In this study, we investigated the potential for synaptophysin to serve as a marker for CNS axonal damage using 2 animal models that mimic different aspects of the complex processes in MS and in brain tissue from patients with MS.

**MATERIALS AND METHODS**

**Animals**

Mice underwent routine cage maintenance once a week and were microbiologically monitored according to the Federation of European Laboratory Animal Science Associations recommendations (51). All research procedures were approved by the Review Board for the Care of Animal Subjects of the district government (Lower Saxony, Germany) and performed according to international guidelines on the use of laboratory animals. Male C57BL/6 mice were purchased from Charles River Laboratories (Sulzfeld, Germany); female SJL/JHanHsd mice were purchased from Harlan (Rossdorf, Germany). Control animals did not display any abnormalities during experiments.

**Induction of Demyelination via Cuprizone and Tissue Processing**

Experimental toxic demyelination was induced by feeding 8-week-old male C57BL/6 mice with 0.2% (w/w) cuprizone (bis-cyclohexanone oxalidihydrazone, Sigma-Aldrich, St. Louis, MO) mixed into a ground standard rodent chow (maintenance diet, rats/mice, Altromin, Lage, Germany) (52). The drinking water was prepared in the animal facility of the Hanover Medical School by demineralizing, filtering (5 \(\mu\)m) and bottling with a bottle filling system (Scanbur BK, Denmark). Cuprizone treatment was maintained for 5 weeks to investigate acute demyelination (53). Chronic demyelination was induced by feeding cuprizone for 12 weeks (54). After withdrawal of cuprizone, remyelination was analyzed 2 and 3 weeks later (ie week 7 and 8 after onset of experiments). After the 12-week period of cuprizone feeding, remyelination was investigated 2 and 4 weeks after withdrawal of the toxin (week 14 and 16 after onset of experiments). Tissue processing was performed as previously described (55, 56). At different time points, the mice under deep anesthesia were perfused transcardially with 4% paraformaldehyde (PFA), Merck) for immunohistochemistry or with phosphate-buffered saline for real-time polymerase chain reaction (PCR) analyses.

For immunohistochemistry, brains were post-fixed in 4% PFA and paraffin-embedded. Seven-\(\mu\)m serial coronal sections were cut on a bright rotary microtome (RM2245, Leica) from –0.82 mm bregma to –1.70 mm bregma. A group size of 4–6 animals was evaluated at each time point along with control animals.

**Theiler’s Murine Encephalomyelitis Virus Infection and Tissue Processing**

For induction of Theiler’s murine encephalomyelitis virus (TMEV) infection, 5-week-old female SJL/JHanHsd mice were intracerebrally infected with 1.63 \(\times\) 10\(^{6}\) PFU/mouse of the BeAn strain of TMEV or mock infected (tissue culture medium) in age-matched controls, as previously described (57, 58). Spinal cords were removed, post-fixed in 10% formalin for 24 hours, decalcified in 25% ethylenediamine tetraacetic acid (EDTA) for 48 hours and embedded in paraffin. For light microscopy, 3-\(\mu\)m transverse sections of the thoracic spinal cord were prepared on a microtome. A group size of 4–6 animals was evaluated at each time point (1 section/animal) (control, 42, 98, 147, 196, and 245 days post-infection [dpi]). Previous studies revealed significant inflammation, as demonstrated by immunohistochemistry targeting CD3 (T-lymphocytes), CD45R (B-lymphocytes) and CD107b (microglia/macrophages), as well as axonal damage in the spinal cord of susceptible SJL-mice during the chronic phase of TME, including the time points 42, 98, 147, 196, and 245 dpi (57, 71).

**MS Patient Tissue**

Paraffin-embedded postmortem human brain tissue was obtained through a rapid autopsy protocol from 6 patients with different MS progression (ie 3 patients with secondary progressive MS, 2 patients with primary progressive MS, and 1 patient with not-characterized MS) in collaboration with VUMedical Center, Amsterdam, The Netherlands. MS patients represented both genders (2 female and 4 male MS patients). The ages of the patients ranged from 49 to 80 years. In addition, 3 control brains from patients without evidence of neurological disease or neuropathological alterations were included. This study was carried out accordingly to the applicable national ethical guidelines and legal regulations regarding the use of archival postmortem material.

**Immunohistochemistry**

For immunohistochemistry, paraffin-embedded sections were dewaxed and heat-unmasked in 10mM citrate buffer (pH 6.0). The following primary antibodies were used: for myelin, anti-myelin proteolipid protein (PLP) mouse monoclonal IgG2a, 1:500, Serotec); and -myelin basic protein (MBP) mouse monoclonal IgG2b, 1:500, Covance); for microglia, anti-ionized calcium-binding adaptor molecule 1 (Iba-1) rabbit polyclonal IgG, 1:1000, Wako); for neurons, anti-NeuN...
For immunofluorescent single/double staining, slides were incubated with respective secondary antibodies (Alexa 488 or biotinylated secondary antibodies, followed by 555 conjugated goat IgG, 1:500, Invitrogen). Slides were counterstained either with hematoxylin and eosin for DAB or with 3,3′-diaminobenzidine (DAB Dako). For immunofluorescent single/double staining, slides were incubated with respective secondary antibodies (Alexa 488 or 555 conjugated goat IgG, 1:500, Invitrogen). Slides were counterstained either with hematoxylin and eosin for DAB or with 4′,6-diamidino-2-phenylindole (DAPI) for immunofluorescence staining.

**Determination of De- and Remyelination**

The extent of cuprizone-induced demyelination and initial remyelination was assessed as previously described (56, 59). Sections immunostained for myelin proteins were scored in the midline of the corpus callosum using a light microscope (Olympus BX61) on a scale from 0 (complete demyelination) to 3 (normal myelin); scores 1 and 2 represented the different grades of partial demyelination/ remyelination (53, 60). To determine the extent of demyelination in the thoracic spinal cord segment of TMEV infected mice, paraffin sections were stained with PLP for identification of white matter and synaptophysin for mapping grey matter. Demyelinated PLP-negative areas were manually outlined using the Cell’s software® from Olympus BX6. The percentage of demyelination in the thoracic spinal cord segment of TMEV infected mice was determined by calculating the percentage of PLP-negative white matter areas to the total white matter area. The same set of threshold values was used for all animals.

Human material was processed for anti-PLP immunostaining as published previously to stage lesional activity (61). Murine and human control tissue did not display any myelin abnormalities.

**Quantification of Synaptophysin- and APP-Positive Spheroids and Glial Reactions**

Synaptophysin- and APP-positive spheroids were counted in the central corpus callosum using a magnification of ×400 (Olympus BX61, Cell’s software®) or in the total white matter of thoracic spinal cord sections at a magnification of ×200. The sizes of synaptophysin- and APP-positive spheroids (small [<2 μm], medium [2–7.5 μm], and large [8–15 μm]) were measured using Cell’s software®. The results are presented as a mean ± SEM of spheroid numbers per mm² (n = 4–6). Quantitation of microglia accumulation was performed for immunoreactivity of Iba-1 staining (62). Data are presented as a number of cells per mm² (mean ± SEM; n = 4–6). Human material was processed for anti-MHC-II (LN3) staining as published previously to stage lesional activity (61). No evidence of inflammation was detected in age-matched control mice. Sporadic age-related aggregations of activated microglia were observed in single control human samples (data not shown).

**Real-Time PCR**

 Corpus callosum and cerebral cortex were dissected from whole brains under a light microscope. The total RNA was extracted from the tissue using the RNeasy® Mini Kit (Qiagen), as previously described (55, 63). CDNA was synthesized according to the manufacturer’s recommendations using the High Capacity cDNA Reverse Transcription Kit (Life Technologies). Quantitative real-time PCR analysis was performed using the StepOne™ Real-Time PCR System and appropriate TaqMan probes (Life Technologies). The ΔΔCt method was applied to determine differences in the expression of Synaptophysin, IL-1β, TNF-α, Kif1a, Kif1b, Kif1c, Kif5a, Kif5b, Kif5c, and dyngein genes between cuprizone-treated and age-matched control animals (n = 4–5/group). Changes in mRNA expression levels were calculated after normalization to the arithmetic mean of HPRT-I and GAPDH. (1) Ct target treated − Ct target control = ΔCt target treated. 
(2) Ct target control − Ct control = ΔCt control. 
(3) ΔCt control + (n − 1)ΔΔCt control + nΔΔCt target = Σ ΔCt control + (n − 1) ΔCt control + n ΔΔCt control = ΔΔCt control. 
(4) ΔCt treated − Δ ΔCt control mean = ΔΔCt treated. 
(5) Fold change treated = 2^−ΔΔCt treated. 
(7) Fold change control = 2^−ΔΔCt control.

**Statistical Analysis**

Statistical analysis was performed using two-way analysis of variance (ANOVA) or one-way ANOVA followed by the Tukey’s multiple comparison post hoc test. All data are given as arithmetic means ± SE. P values of the ANOVAs are given in the Results section; group comparisons derived from post hoc analysis are provided in the figures. Correlation and linear regression analysis was performed by applying the Pearson correlation test. The normality of all values was evaluated using Kolmogorov–Smirnov test. In all cases, significant effects are indicated by asterisks (compared to the preceding time point) or hash marks (compared to controls) (*p < 0.05; **p < 0.01; ***p < 0.001).

**RESULTS**

**Numbers and Sizes of Synaptophysin-Positive Spheroids Change during Cuprizone-Induced Demyelination**

To investigate the distribution of synaptophysin under both physiological and pathological conditions in the CNS,
immunohistochemical staining of coronal brain sections from control and cuprizone challenged mice were performed. We used 2 different antibodies for synaptophysin: a mouse monoclonal IgG from Serotec and rabbit monoclonal from Abcam. Both antibodies showed similar staining patterns (Supplementary Data Fig. S1). The grey matter of the cerebrum and cerebellum in controls displayed a widespread fine granular synaptophysin-positive signal (Fig. 1A, B), whereas a few small physiological synaptophysin-positive dots were found in the white matter of the corpus callosum (Fig. 1C).

During cuprizone-induced demyelination, the sizes of synaptophysin-positive dots and their amounts increased (Fig. 1E, F; p < 0.0001 for small and medium-sized spheroids during both an acute and chronic demyelination; p < 0.001 for large spheroids/ovoids in both acute and chronic demyelination). Numbers of small dots decreased significantly during cuprizone-induced demyelination reaching the lowest numbers at the time point week 5 (which corresponds to nearly complete demyelination in the corpus callosum in this model) and remained at a low level during chronic demyelination of 12 weeks. When animals were allowed to remyelinate after 5-week cuprizone treatment, the small synaptophysin spheroids recovered already after 2 weeks of remyelination. In contrast, after chronic demyelination, small physiological synaptophysin-positive spheroids recovered with a delay and a significant increase occurred after 4 weeks of remyelination.

Medium-sized spheroids (2–7.5 μm) were only rarely found in controls. During cuprizone-induced demyelination, the numbers of medium-sized synaptophysin-positive bulbs increased dramatically reaching a peak at the time point week 4, which corresponds to the peak of microglial activation in this model. Thereafter, the numbers of medium-sized spheroids decreased even during chronic demyelination at 12 weeks.

Large synaptophysin-positive spheroids (8–15 μm) were also not found in the corpus callosum in controls. During demyelination, their numbers increased but did not reach significant levels during the short 5-week period of demyelination. During chronic demyelination, significantly, numbers of large spheroids were detected at the time point week 6 and remained increased during the later demyelination phase. During remyelination, large spheroids were still present in low numbers.

Summing up, we hypothesized that small synaptophysin-positive dots represent physiological synaptic vesicle terminals transported through the axonal network, whereas medium- and large-sized synaptophysin-positive bulbs/ovoids/spheroids reflect pathological vesicle accumulations due to axonal transport disturbances. Similar synaptophysin patterns were observed in other prominent white matter tracts such as the internal capsule or hippocampal white matter (Fig. 1G, H). Double staining demonstrated that synaptophysin did not colocalize with the oligodendrogial marker Nogo-A (Fig. 1I), astrocytic marker GFAP (Fig. 1J), or the microglia marker Iba-1 (Fig. 1K).

### Synaptophysin as a Marker of Axonal Transport Disturbances in the Cuprizone Model

The next step aimed to verify synaptophysin as a marker to study axonal damage and/or axonal transport disturbances. We performed immunohistochemical double staining for synaptophysin and APP, a widely accepted marker of axonal damage. For quantification, only synaptophysin-positive spheroids >2 μm were considered as pathological vesicles because small synaptophysin-positive dots were considered to be physiological (Figs. 1C, 4A, C, 5G).

Double staining for APP/synaptophysin revealed that synaptophysin-positive spheroids colocalized with APP during de- and remyelination in the corpus callosum (Fig. 2A–H). However, during the time point of strong microglia activation and demyelination, some APP-positive structures that were not positive for synaptophysin were found. Because it is known that APP may colocalize with reactive astrocytes (64), additional double staining with anti–GFAP was performed. GFAP-positive astrocytes were also found to express APP (Fig. 2I, K, M), whereas there were not GFAP/synaptophysin double-positive cells found (Fig. 2L, N). Thus, for colocalization studies only APP-positive spheroids without nuclear co-staining were counted (Fig. 2A, B, D, G).

Colocalization studies for synaptophysin and SMI-32 (another established marker of axonal damage) were next performed. In untreated control animals neurofilaments were highly phosphorylated, as shown by immunostaining with the marker SMi-312, which stained highly phosphorylated axonal epitopes on neurofilaments M (medium) and H (heavy) (Fig. 3B). Over the course of demyelination, neurofilaments substantially lost their phosphorylation. However, SMi-312-positive swellings/traces could be detected in the affected corpus callosum (Fig. 3D, F, H). SMi-32 visualizes non-phosphorylated neurofilament H. In controls, large axons were not marked with SMi-32 in the corpus callosum (Fig. 3A). Numerous non-phosphorylated SMi-32-positive spheroids were, however, identified at the time point week 4, which corresponded to the peak of microglial activation and strong demyelination (Fig. 3C, E). During early and complete demyelination (weeks 3–5), SMi-32- or SMi-312-positive spheroids only rarely colocalized with synaptophysin (Fig. 3C, 3E, example for colocalization: 3F, F1–F3). In contrast, at later time points beginning at week 6 and further on during chronic demyelination SMi-32 and SMi-312 were predominantly located in large synaptophysin-positive spheroids (Fig. 3G, H); these were also-positive for APP (data not shown). Nevertheless, mainly single synaptophysin-positive spheroids and some single SMi-32 or SMi-312-positive spheroids were detected.

The expression and distribution of neurofilament medium chain (neurofilament M) was also analyzed (Fig. 4A–E). In control animals neurofilament M was observed in cortical neuronal bodies and in callosal axons; physiological synaptophysin-positive vesicles were located inside of neurofilament-positive axons (Fig. 4A, C). Upon demyelination the intensity of neurofilament M staining decreased and few neurofilament M-positive swellings appeared in the affected corpus callosum (Fig. 4B, D, E). Colocalization studies with synaptophysin (Fig. 4B, D, E), and APP (data not shown) revealed aligned patterns similar to those of SMi-312 and SMi-32.

### Axonal Disturbances during Microglia Activation in the Cuprizone Model

To investigate the reasons for the axonal transport disturbances we first investigated the gene expression of different
FIGURE 1. (A–C) Representative sections illustrate immunohistochemical staining for synaptophysin (Syp) in the cerebral cortex and hippocampus (A), cerebellum (B), and corpus callosum (C) of untreated control mice. (D) Accumulation of synaptophysin-positive vesicles = spheroids/ovoids/bulbs in the corpus callosum of a cuprizone-treated mouse. Arrows indicate the different sizes of synaptophysin-positive-spheroids; small [S], <2 μm (as in panel C); middle-sized [M], 2–7.5 μm; large [L], 8–15 μm, pathological size. (E, F) Graphs show numbers of different sized synaptophysin-positive spheroids in the corpus callosum during acute (5 weeks, E) and chronic (12 weeks, F) demyelination followed by remyelination after stopping cuprizone. Significant effects between different time points were calculated separately for each type/size of bulbs (*p < 0.05, **p < 0.01, ***p < 0.001). Significant effects for different cuprizone time treatments in comparison to the untreated control: #p < 0.05, ##p < 0.01, ###p < 0.001), n = 4–6. (G, H) Representative images show synaptophysin-positive bulbs in the demyelinated hippocampus (G) and internal capsule (H). (I–K) Representative images show that synaptophysin does not colocalize with the oligodendroglial marker Nogo-A (I), the astrocytic marker GFAP (J), or with the microglial marker Iba-1 (K). Scale bar in (K) = 100 μm, applies to (I) and (J).
FIGURE 2. (A, B) Graphs represent the quantitative analysis of APP, synaptophysin (Syp), and double-positive spheroids in the corpus callosum of cuprizone-treated mice with acute demyelination (A) and chronic demyelination (B). Two-way ANOVA analysis identify that there were no significant differences between numbers of APP- and synaptophysin-positive spheroids during
kinesins and dynein, which are important motor proteins responsible for anterograde and retrograde vesicle transport in cortical neurons. Hares et al. reported reduced axonal motor protein expression in non-lesional grey matter in MS patients (65). In our study, apart from kinesin 1b, which was upregulated at week 4 of demyelination (Supplemental Fig. 2D), we did not identify significant alterations of mRNA expression for either kinesins 1a, 1c, 5a, b, and c nor for dynein in the cortical grey matter at all time points investigated (Supplementary Data Fig. S2B–I). Synaptophysin mRNA expression was also not altered in cortical grey matter during the course of acute demyelination (Supplementary Data Fig. S2I).

We next tested whether myelin loss might be responsible for axonal transport disturbances. Thus, we correlated the callosal expression of myelin proteins MBP and PLP to the appearance of pathological medium-sized and large synaptophysin-positive axonal swellings in the corpus callosum (Figs. 5G-L, 6A). There was a positive correlation for MBP during acute demyelination (p = 0.0051; R = 0.8856; r = −0.9410) (Fig. 6A, D), but not for PLP (p = 0.1920; R = 0.3807; r = −0.617) (Fig. 6A, C). For chronic demyelination we did not see any correlation for either MBP (p = 0.0738; R = 0.3867; r = −0.6218) or PLP (p = 0.3164; R = 0.1426; r = −0.3776) (Fig. 6F, G). During severe chronic demyelination the entire amount of pathological synaptophysin-positive spheroids (medium- and large-sized) did not increase (Fig. 6B). Moreover, medium-sized bulbs even decreased in their numbers while the amount of large bulbs rose slightly (Figs. 1F). These large bulbs persisted after 4 weeks of remyelination. During remyelination some synaptophysin-positive bulbs were surrounded by PLP-positive myelin, suggesting that disturbed axons can be remyelinated (Fig. 5O, P). Finally, we investigated the relationship of microglia infiltration/activation and the appearance of medium- and large-sized synaptophysin-positive ovoids. We found a strong correlation between these 2 parameters during both acute and chronic demyelination (acute: p = 0.0188; R = 0.7849; r = 0.8860; chronic: p = 0.0075; R = 0.6635; r = 0.8146) (Figs. 5A–F, M, N, 6A, B, E, H).

Changes in mRNA expression for the inflammatory cytokines IL-1β and tumor necrosis factor (TNF) were found in the cortex (area of neuronal cell bodies) and the corpus callosum (axonal area). The expression of these cytokines peaked after week 4/5 weeks of cuprizone diet and matched with the maximum of synaptophysin amount (Fig. 7B, C, E, F).

Thus, because we found a strong correlation between microglia accumulation and synaptophysin-positive spheroid numbers, we conclude that inflammation might be the strongest factor leading to acute axonal transport disturbances. In addition, the amount of synaptophysin aggregations decreased during chronic demyelination, when inflammation abated.

**Synaptophysin as a Marker for Axonal Damage in Theiler’s Murine Encephalomyelitis Virus-Induced Demyelination**

To confirm synaptophysin as a reliable marker to detect axonal transport damage during demyelinating conditions, a second model of CNS neuroinflammation was studied. Immunohistochemical analyses were performed in thoracic spinal cord sections after TMEV-induced demyelination. Infiltration of microglia/macrophages was visualized using Iba-1 staining. As shown in Figure 8, a scattered accumulation of activated microglia/macrophages could be observed already after 42 dpi. Microglial activation peaked at 98 and 147 dpi with a subsequent decline on days 196 and 245 (p < 0.001) (Fig. 8A). Microglia/macrophage infiltration followed not only a temporal sequence but also a special spatial pattern. First, microglia/macrophages appeared in the ventral part of the spinal cord (42 dpi) and then spread to the lateral parts (98, 147 dpi). At the latest time point analyzed, activated microglia/macrophages were observed predominantly in the dorsal parts between the dorsal horns of the spinal cord (Fig. 8E–H).

The appearance of demyelinating areas showed a similar distribution pattern. Demyelination began at 42 dpi and progressed gradually during the next weeks (p < 0.001). Finally, at 245 dpi, nearly 30% of all spinal cords white matter was demyelinated (Fig. 8B). We detected pathological medium- and large-sized synaptophysin-positive spheroids in the white matter during TME-induced demyelination (p = 0.0022) (Fig. 8C). These synaptophysin-positive formations colocalized with APP-positive spheroids (Fig. 8L–N, S–U), and were predominantly observed in demyelinated (Fig. 8L, O) and microglia-enriched areas (Fig. 8J, K, P). Pathological synaptophysin/APP-positive bulbs were not present in untreated animals.

**Synaptophysin as a Marker for Axonal Damage in CNS Tissue of MS Patients**

Synaptophysin immunostaining revealed a fine granular pattern in synapse rich human grey matter areas (Fig. 9T). Ten chronic active demyelinating lesions from 6 MS patients were analyzed. At the edges of 4 MS lesions we observed numerous synaptophysin-positive spheroids (Fig. 9A–K [lesion 1]; Fig. 9L–O [lesion 2]). These areas were characterized by accumulation of LN3-positive microglia/monocytes and diffuse myelin loss (Fig. 9A, B, I, L–O). Some single synaptophysin-positive spheroids were also found in completely demyelinated areas (data not shown). In both cases, synaptophysin-positive spheroids colocalized with APP-positive bulbs (Fig. 9C-H, J, K, N). Synaptophysin-positive/APP-positive bulbs were not detected in the human control brain tissue.

**FIGURE 2.** Continued

Both acute and chronic demyelination or during the remyelination periods. Significant effects between different time points are indicated by asterisks (*p < 0.05, **p < 0.01, ***p < 0.001). Significant effects for different cuprizone time treatments in comparison to the untreated control were indicated by hatch marks (#p < 0.05, ##p < 0.01, ###p < 0.001), n = 4–6. (C–H) Representative pictures show colocalization in the corpus callosum of both markers at 4- and 12-week time points. Arrows in (D) and (E) indicate astrocytes expressing APP in the corpus callosum. (I–N) Representative images show that APP (MAB 348) was also expressed in reactive astrocytes in the hippocampus; GFAP and synaptophysin are not colocalized.
FIGURE 3. Immunohistochemical staining of the corpus callosum evaluating phosphorylation of neurofilaments and their colocalization with synaptophysin (Syp) during cuprizone-induced demyelination. (A–H) SMI-32 detecting non-phosphorylated heavy neurofilaments are shown in panels (A, C, E, G). SMI-312 detecting phosphorylated neurofilaments H and M are...
DISCUSSION

Vesicular axonal transport disturbances and axonal damage are hallmarks of numerous neurological disorders, including MS (9, 10). Thus, reliable markers for the detection of axonal transport disturbances are required to study the underlying pathomechanisms. Recently, a calpain-derived alpha-II spectrin N-terminal fragment (SNTF) was identified as a new, highly sensitive marker to study different axonal transport disturbances in traumatic brain injury (66). Synaptophysin presents another possible marker to detect axonal damage under different neuropathological conditions such as MS (67–69). Here, we established synaptophysin, an abundant vesicle membrane protein, as a reliable marker for the detection of axonal damage by performing detailed synaptophysin expression studies in 2 animal models of demyelination and in brain tissue from MS patients. The accumulation of synaptophysin during pathological conditions was compared with the well-established axonal damage markers APP (12, 70, 71) and SMI-32 (21, 72).

APP is synthetized in the neuronal cell soma and undergoes fast axonal transport mediated by conventional kinesin (16, 17, 73). Disruptions of axonal transport or axon dissection lead to accumulation of APP-positive vesicles that can be identified using immunohistochemistry (18, 74). During its transport along the axon, the N-terminus of APP is located inside and the C-terminus outside of the vesicles. Previously, it was reported that antibodies against the C-terminus are even more sensitive to detect the accumulation of APP-positive...
FIGURE 5. (A–P) Representative sections show immunostaining in the corpus callosum for synaptophysin/lba-1 (A–F) and synaptophysin/PLP (G–L) during acute and chronic cuprizone induced demyelination and subsequent remyelination. Inserts in (A–F) show the morphology/shape of microglia identifying their activation state. Inserts in (G–L) show higher magnification of synaptophysin accumulations. Panels (M) and (N) are confocal images from RCA-1 (microglia activation marker, green) and synaptophysin (red) during inflammation in cuprizone-treated mice. Panels (O) and (P) show remyelinated axons (PLP, green) with internal synaptophysin accumulation (red).
vesicles at the site of axonal damage (19, 75). Thus, the choice of an appropriate anti-APP antibody seems important because APP becomes post-transcriptionally processed by glycosylation, sulfation, and undergoes a complicated post-translational proteolytic processing (14, 76, 77). Moreover, APP is not exclusively expressed in neurons but may occur in other cell types, including reactive astrocytes (64, 78–80). Different splice variants are known, which are predominantly expressed by neurons (APP-695) or other cell types (APP-751, APP-770, L-APP, and APP-639) (81–84). In our study, we detected APP in some reactive astrocytes during cuprizone-induced demyelination, which is in line with previous publications (64).

Unfortunately, in some cases it is difficult to distinguish between an APP-positive damaged axon and APP-positive astrocytic processes without co-staining with an astrocytic marker. Thus, additional markers for axonal damage might be helpful to resolve these difficulties. Like APP, synaptophysin is also moved via the fast axonal transport mostly in an anterograde direction. Here, we found that small synaptophysin-positive dots appear in very high numbers in the normal CNS grey matter; fewer are seen in normal white matter. In the cuprizone-induced demyelination model, the amounts of small physiological synaptophysin dots decreased and larger spheroids, which can be considered as pathological accumulations of synaptic

FIGURE 6. The appearance of synaptophysin (Syp)-positive pathological (medium-and large-sized) spheroids was compared and correlated with the extent of demyelination (proteolipid protein: PLP, myelin basic protein: MBP) and microglia infiltration (Iba-1) induced by cuprizone feeding (acute or chronic). (A, B) Graphs show the extent of microglia accumulation, appearance of pathological synaptophysin bulbs and the extent of de- and remyelination as judged by scoring of MBP and PLP immunostaining. Score of 3 represents complete myelination; score of 0 represents complete demyelination. (C–H) Correlation analyses of synaptophysin-positive bulbs with the extent of de- and remyelination and microglia numbers (n = 5–6).
vesicles in damaged axons, were found in the corpus callosum and in other white matter structures. Colocalization studies revealed that these spheroid structures are immunopositive for both synaptophysin and APP. This suggests that the entire fast axonal transport mechanism is impaired during cuprizone-induced demyelination because APP and synaptophysin are known to be moved in different vesicular compartments (50).

Further, we compared the expression pattern of synaptophysin with the marker SMI-32, which detects non-phosphorylated neurofilaments (20–23, 85). In the normal white matter, neurofilaments are mostly phosphorylated whereas in demyelinated axons neurofilaments become dephosphorylated (21, 72, 86). Neurofilaments are moved via slow axonal transport (8, 28, 29). As a consequence of aberrant phosphorylation or transport, neurofilaments accumulate in the affected axons (30, 31, 68, 87). In the present work we detected SMI-32-positive bulbs during the acute microglia activation and demyelination phase in the cuprizone model. At this time, most SMI-32-positive spheroids did not colocalize with synaptophysin or APP. This is probably due to the different transport mechanisms, i.e. slow axonal transport for SMI-32 and fast axonal transport for synaptophysin and APP. A similar result was observed in another study that investigated axonal transport disturbances in immature rat brain injury and detected compacted neurofilament (RM014 immunoreactivity) and APP (31). However, we observed some large double-positive SMI-32/synaptophysin or SMI-32/APP bulbs at later time points of cuprizone-induced demyelination and even during remyelination. We hypothesize that the large SMI-32/APP/synaptophysin-positive bulbs might represent permanent axonal damage or terminally transected axons. In contrast, different medium-sized spheroids, which are either APP/synaptophysin double-positive or SMI-32 single-positive, might detect reversible fast or slow axonal transport impairments, i.e. “vesicle jams”, that can disintegrate when inflammation is resolved. This concept was proposed by Ferguson et al. in 1997 (74), and later corroborated by others (88), particularly by the group of Kerschensteiner investigating axonal transport disturbances in experimental autoimmune encephalomyelitis (EAE) using in vivo imaging (89). In our study, the amount of synaptophysin-positive bulbs correlated poorly with the loss of myelin proteins during acute demyelination, as judged by PLP or MBP immunostaining. The mRNA expression of kinesins 1a, 1b, 1c, 5a, 5b, 5c, and dynein, which represent main motor proteins responsible for the transport of synaptic vesicles (90, 91), was also not affected in our study. Thus, we hypothesize that inflammation in particular may be the key factor that impairs axonal transport. This assumption is in line with the correlation between the extent of microglia accumulation and the amount of synaptophysin-positive spheroids in the corpus callosum. Moreover, the appearance of synaptophysin-positive bulbs showed a similar temporal pattern as the mRNA expression of the inflammatory cytokines IL-1β and TNF. Stagi et al. also showed that microglial NO significantly inhibited axonal transport of synaptophysin enhanced green fluorescent protein-marked synaptic vesicles in hippocampal neuronal cultures (92).

Kornek et al. investigated the distribution of voltage-gated calcium channels in samples from MS patients and animals with EAE. They showed that axonal transport was impaired and the α1b subunit of N-type calcium channels accumulated in dystrophic axons of active demyelinating MS brain.
lesions and spinal cord lesions in EAE similar to APP (93). Frischer et al performed correlative studies between synapto-
physin reactivity and accumulation of reactive T cell and mac-
rophage infiltrates in different MS lesions and provided direct
evidence that axonal injury is consistently associated with
inflammation (67). Nevertheless, the precise sequence of
the degenerative events remains an open and intensively dis-
cussed question, i.e. whether axonal damage/axonal transport
collapse is caused by inflammation or if it is a consequence
of demyelination or both (13, 74, 94–99). In a previous
study, we showed less microglial activation after astrocyte
depletion using the cuprizone model, which led to delayed
myelin debris removal. In those mice, the number of
synaptophysin-positive spheroids was not changed, suggest-
ing that microglial accumulation is at least not the sole rea-
son for axonal damage (63).

**FIGURE 8.** (A–C) Graphs show microglia/macrophage infiltration (A) and the course of demyelination (B) during Theiler’s virus induced encephalomyelitis in the thoracic segment of murine spinal cord. (C) Panel shows the appearance of pathologic accumulation of synaptophysin (Syp). Significant effects between different time points were indicated as: *p < 0.05, **p < 0.01, ***p < 0.001. Significant effects for different time treatments in comparison to the untreated control are indicated as: # p < 0.05, ## p < 0.01, ### p < 0.001), n = 4–6. (D–H) Representative images of thoracic spinal cord segment illustrate infiltration of microglia/macrophage at different days after the injection of Theiler’s murine encephalomyelitis virus. (I–U) Panel (I) shows the appearance of synaptophysin-positive bulbs exclusively in the demyelinated area; (J) and (K) illustrate the appearance of synaptophysin-positive spheroids surrounded by activated microglia/macrophages. (L–N) Colocalization of synaptophysin and APP in the spheroid structures. (O–U) Serial staining of the same spinal cord area during Theiler’s virus induced encephalomyelitis (O, synaptophysin/PLP double staining; P, synaptophysin/iba-1 double staining; R, synaptophysin/APP double staining; S–U, higher magnification of area shown in R).
FIGURE 9. Representative images show the expression of synaptophysin (Syp) in CNS tissue of 2 different patients with MS. (A–H) Different immunohistochemical stainings from a chronic active MS lesion (serial sections). Panels (E) and (F) show the higher magnifications from boxed areas 1 in (C) and (D). Panels (G) and (H) demonstrate higher magnification from boxed areas 2 in (C) and D. (I–K) Representative double immunostaining for synaptophysin and Iba-1 demonstrate microglia accumulation (I) and proving colocalization of APP and synaptophysin (J, K). (L–O) Demyelination/inflammation/axonal damage in another MS lesion. DAB staining in (L) and (M) illustrate the extent of demyelination (PLP) and inflammation (LN3 marks HLA-DR-positive cells: lymphocytes and macrophages) in this MS lesion. Panels N and O show higher magnification of the boxed are from this lesion in (L) and (M) demonstrating double expression of APP and synaptophysin in (N) and double staining of PLP and synaptophysin in (O). (P–U) Corpora amylacea are immunostain-positive for APP (P, S), and NeuN (U), but not synaptophysin (R, T).
In the present study, we found some differences between the animal models and MS patient tissue. In contrast to the cuprizone model, only a few large- or medium-sized APP/synaptophysin-positive bulbs were found in TME. The differences in the amount of APP/synaptophysin bulbs are probably due to different pathomechanisms underlying axonal damage in the 2 models. Tsunoda et al postulated that axonal damage can occur in TME even prior to demyelination due to neuronal cell death caused by TMEV followed by Wallerian degeneration (Inside-Out model (100, 101)). Later this hypothesis was refined and proposed as a concept for axonal damage in demyelinating diseases, i.e. a primary event caused by neuronal cell death and Wallerian degeneration evoking an inflammatory cascade and secondary axonal damage or axonopathy (Inside-Out and Outside-In model) (102). Because in our study, we examined particularly the demyelinating phase of TME, we probably detected secondary axonal damage.

Finally, we investigated the expression of synaptophysin and the presence of axonal damage in MS patient CNS tissue. Synaptophysin-positive spheroids were found to be colocalized with APP, supporting the finding that synaptophysin is a marker for axonal damage in the human CNS. However, this is in conflict with the hypothesis of Schirmer et al postulating synaptophysin as a marker for neuronal regeneration after an inflammatory insult (85). However, similar to the work of Frischer et al (67), synaptophysin/APP-positive bulbs were found predominantly at the edges of demyelinating lesions, areas with the highest amount of infiltrating immune cells. This observation supports the hypothesis of an inflammatory origin of axonal damage. However, these synaptophysin/APP-positive spheroids were not always found in MS lesions and their amounts varied in different lesions, suggesting a high complexity or different mechanisms underlying axonal damage. This could also be due to different stages of MS lesion development because it is not possible to obtain the material at defined time points. Furthermore, anti-APP antibodies also detected large spheroid-like structures, which were faintly stained with hematoxylin and were identified as corpora amylacea (Fig. 9P, S). Interestingly, corpora amylacea were also positive for NeuN, suggesting a neuronal origin of these aggregations (Fig. 9U). This observation is in line with some previous studies (103, 104). In contrast to anti-APP, anti-synaptophysin antibodies did not immunostain corpora amylacea (Fig. 9R, T) or reactive astrocytes. However, there was sometimes a diffuse fine granular background staining. Thus, for better interpretation of immunohistochemical staining identifying axonal damage in MS tissue as well as in the spinal cord during TME we suggest applying both anti-APP and -synaptophysin with nuclear co-staining.

In conclusion, we confirm here that synaptophysin is a reliable marker to study axonal damage in the cuprizone model of demyelination (105) since it colocalized with APP and did not cross-react with neither oligodendrocytes, astrocytes, nor microglia. Synaptophysin detected axonal damage in demyelinating human MS lesions and may also serve as a marker for studying axonal damage in the TMEV model.

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