Proteomic changes during experimental de- and remyelination in the corpus callosum

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Abstract

Background
In the cuprizone model of multiple sclerosis, de- and remyelination can be studied without major interference from the adaptive immune responses. Since previous proteomic studies did not focus on the corpus callosum, where cuprizone causes the most pronounced demyelination, we performed a bottom up proteomic analysis on this brain region.

Methods
Eight week-old mice treated with 0.2% cuprizone, for 4 weeks and controls (C) were sacrificed after termination of the treatment (4wD), and 2 (2dR) or 14 (2wR) days later. Homogenates of dissected corpus callosum were analysed by quantitative proteomics. For data processing, clustering, gene ontology analysis, and regulatory network prediction, we used Perseus, PANTHER and Ingenuity Pathway Analysis softwares, respectively.

Results
We identified 4886 unmodified, single- or multi phosphorylated and/or gycosylated (PTM) proteins. Out of them, 191 proteins were differentially regulated in at least one experimental group. We found 57 proteins specific for demyelination, 27 for early- and 57 for late remyelination while 36 proteins were affected in two, and 23 proteins in all three groups. Phosphorylation represented 92% of the post translational modifications among differentially regulated modified (PTM) proteins with decreased level, while it was only 30% of the PTM proteins with increased level. Gene ontology analysis could not classify the demyelination specific proteins into any biological process category, while allocated the remyelination specific ones to nervous system development and myelination as the most specific subcategory. We also identified a protein network in experimental remyelination, and the gene
orthologues of the network were differentially expressed in remyelinating multiple sclerosis brain lesions consistent with an early remyelination pattern.

**Conclusion**
Proteomic analysis seems more informative for remyelination than demyelination in the cuprizone model.

**Introduction**
Multiple sclerosis is the most common chronic inflammatory demyelinating disease that affects mainly young adults [1]. The disease is progressive, and impacts the central nervous system with a complex pathomechanism involving both neurodegenerative and inflammatory characteristics [1]. The current disease modifying therapies aim to prevent relapses by suppressing inflammation in the relapsing–remitting form of the disease, but limited options are available to prevent demyelination or axonal degeneration [2]. Therefore, intensive research is going on to identify novel therapeutic targets.

The complexity and heterogeneity of multiple sclerosis pathology cannot be replicated by a single animal model; the most commonly used experimental autoimmune encephalomyelitis, and toxin- and/or virus-induced demyelination models capture only certain clinical and pathological features of the disease. The neurotoxin cuprizone (bis-cyclohexanoneoxaaza-dihydrazone, CPZ) causes reproducible, anatomically selective and reversible demyelination [3] that is not affected by the absence of T and B cells while the blood–brain barrier is considered to be intact [4]. Therefore, and in contrast to other multiple sclerosis models, de- and remyelination can be studied without interference from the contribution of adaptive immune responses [5].

Proteomic approach was successfully applied for studying pathomechanism [6, 7] of or finding new drug targets [8, 9] for various diseases. Interestingly, we found only three previous studies utilizing this approach for analysing CPZ-induced reversible demyelination [10–12]. None of them measured proteomic changes in the corpus callosum, where CPZ-induces the most pronounced demyelination [13]. Accordingly, in the present study, we assessed proteomic changes during de- and remyelination in the corpus callosum of CPZ treated mice.

**Materials and methods**

**Materials**

Cuprizone (CPZ) was from Sigma-Aldrich (Budapest, Hungary) The Protease inhibitor mix without EDTA and PhosSTOP phosphatase inhibitor cocktail were from Roche Applied Science (Meylan, France). Benzonase was from Merck (Darmstadt, Germany). Lysyl endopeptidase (Lys-C) was from Wako Pure Chemical Industries (Osaka, Japan). Modified trypsin was from Promega (Madison, WI, USA). iTRAQ 4-plexTM was from Applied Biosystem (Foster City, CA, USA). Titanium dioxide beads were from GL Science (Japan). Poros Oligo R3 reversed phase chromatographic materials were from Applied Biosystems (Framingham, MA, USA). PHOS-select™ metal chelate beads were from Sigma-Aldrich (St. Louis, MO, USA). TSK amide-80 HILIC 3 µm from Tosoh Bioscience (Stuttgart, Germany). 3M Empore C8 disk was from 3M Bioanalytical Technologies (St. Paul, MN, USA). All other reagents used in the experiments were of sequencing grade, and the water was from a Milli-Q system (Millipore, Bedford, MA).
Ethic statement and cuprizone treatment

The animal experiments were performed according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health, and the protocol was approved by the Animal Research Review Committee, University of Pecs, Hungary. All animal experiments were controlled by trained personnel, and all efforts were made to minimize animal suffering.

C57BL/6 male mice were purchased from Charles River, Innovo Kft (Isaszeg, Hungary) and kept under standardized circumstances (controlled temperature, humidity and 12:12 h light-dark cycles.) Food and water were freely available.

Starting at 8 weeks of age, 20 animals were randomly assigned into 4 groups. Three groups were nourished with powdered rodent chow (1324 Altromin, Germany) containing 0.2% CPZ for 4 weeks ad libitum to induce demyelination, as described previously [14]. Control (C) group received the same without CPZ. To follow the methodical effect of CPZ treatment, the weights of the mice were measured twice a week [14], extent of demyelination was assessed by MRI imaging [15] at the end of CPZ treatment and before sacrificing the animals. The mice were sacrificed with cervical dislocation in deep isoflurane anaesthesia after termination of the treatment (4wD), and 2 (2dR) or 14 (2wR) days later. Brains were excised and the corpus callosums were dissected and snap-frozen on dry-ice. The samples were stored at −80˚C until further processing.

Sample preparation

Dissected corpus callosums were homogenised in buffered solution (pH 7.5) consisting of protease and phosphatase inhibitors, 10mM DTT, 10mM sodium orthovanadate and benzonase (0.05%) using Dounce homogenizer, followed by probe sonication. Proteins were collected by centrifugation at 20000g after being precipitated by addition of 8 volumes of -20˚C acetone and 1 volume of trichloroacetic acid. Protein pellets were solubilized with 6 M urea, 2 M thiourea, 10 mM DTT, followed by alkylation with iodoacetamide and digesting with lysyl endopeptidase and trypsin. Peptides were purified using homemade RP columns (both C8 and C18 resins were used) and labelled by isobaric tags (iTRAQ 4-plex™) for relative quantitation. Based on amino acid composition analysis, equal amounts of samples were labelled as follow: Control, iTRAQ-114; 4 weeks demyelination, iTRAQ-115; 2 days remyelination, iTRAQ-116; and 2 weeks remyelination, iTRAQ-117 and combined in 1:1:1:1 ratio. In order to enrich for phosphopeptides and glycosylated peptides, the TiSH (TiO₂-SIMAC-TiO₂) protocol [16] was applied. Prior to the nano LC-MSMS identification, the phosphorylated, deglycosylated and nonmodified peptides were fractionated by HILIC as described previously [17].

Liquid Chromatography–Mass Spectrometry (LC–MS) analysis

The peptides were separated by reverse phase chromatography using homemade C18 column (3 μm, 75 μm x 150 mm) and gradient elution at a flow rate of 250 nl/min with an aid of Thermo EASY-nLC 1000 HPLC. Approximately 1 μg of peptide sample was loaded on the column. The buffer system consisted of solvent A (aqueous formic acid solution (0.1%) and solvent B (acetonitrile/formic acid (99.9/0.1%v/v)). Gradient elution varied depending on the complexity of samples and was either 60 or 130 min from 0–34% solvent B, then 5–15 min for reaching 100% B and 10 min at 100% B. Eluted peptides were analysed by MS instruments (Orbitrap Velos and Q-Exactive, ThermoFisher). For the Velos instrument: scanning range was set to 400–1500 m/z for MS¹ scans and the first mass fixed at 110 m/z for following 7 data dependent MS² scans. Peptides were fragmented by HCD at 35 NCE. For the Q-Exactive instrument: scanning range was set to 350–1600 m/z for MS¹ scans and the first mass fixed at
110 m/z for following 12 data dependent MS² scans. Peptides were fragmented by HCD at 30 NCE. Raw datafiles were processed by Thermo Proteome Discoverer software v1.4. Protein identification was carried out by searching for Mus musculus taxonomically restricted in the databases of the NCBI and the Swiss-Prot using Mascot V2.4.1. Search parameters were set to allow two missed cleavage site, we accepted 15 ppm mass tolerance at MS¹ and 0.02 Da at the MS² mode and we searched for variable modifications including methionine oxidation, deamidation of Asn, phosphorylation on Ser/Thr/Tyr, peptide N-terminal iTRAQ labelling and lysine iTRAQ labelling and carbamidomethylation on cysteine as a fixed modification. The relevance threshold was set to >20 MASCOT score. Data from ProteomeDiscoverer was further processed using Excel (Microsoft).

The mass spectrometry proteomics data have been deposited to the MassIVE data repository (Mass Spectrometry Interactive Virtual Environment) ftp://MSV000083506@massive.ucsd.edu

**Immunohistochemistry**

We performed immunohistochemistry utilising protein phospho (p)-Ser, p-Thr and p-Tyr specific primary antibodies (Santa Cruz Biotechnology) on brain sections of the animals (n = 3) from all four treatment groups. Briefly, the chilled brains were removed, formalin-fixed, paraffin-embedded and sectioned (8 μm). The sections were dewaxed, exposed to 500 W microwave for 3 x 5 min (antigene exposure) in 0.1 M citrate buffer pH 6.0, then the endogenous peroxidase activity was blocked in 0.1 M phosphate buffered saline (PBS) pH 7.4 containing 3% hydrogen peroxide for 20 min. The sections were blocked in PBS containing 0.2 10% bovine serum albumin (BSA) for 30 min, then were exposed to said primary and horseradish peroxidase conjugated anti-mouse IgG (Sigma) secondary antibodies in PBS containing 1% BSA overnight and for 1 h, respectively. Antibodies were diluted according to the manufacturer’s recommendation, and between all steps a 3 x 5 min washing in PBS was performed. Antibodies were visualised by exposing the sections to 0.067% diaminobenzidine solution in PBS containing 0.02% hydrogen peroxide for 5–15 min against negative controls lacking primary and/or secondary antibody exposure. Nuclei were counterstained by Meyer’s hematoxylin for 2 min then the sections were dehydrated and Balsam Canada (Sigma) mounted. The sections were scanned by Panoramic midi slide scanner at 1200 dpi resolution. For quantitative analysis, all nuclei and staining artefacts were eliminated from the sections and staining intensities of corpus callosums were normalised to that of left and right retrosplenial area, ventral part, layer 1 by an expert blind to the experiment by using Molecular Devices’ MetaXpress® image analyser software.

**Gene Ontology (GO), pathway and functional correlation analyses**

Deregulated proteins were categorised into protein classes using Protein Analysis Through Evolutionary Relationships (PANTHER) classification system software (http://www.pantherdb.org) and the general annotation from UniProt (http://uniprot.org). For clustering, we used the Perseus software platform (http://www.perseus-framework.org) developed by the Max Planck Institute of Biochemistry (https://maxquant.net/perseus/). Gene ontology (GO) analysis of biological processes and cellular components was performed with PANTHER software. Regulatory network prediction was performed by Ingenuity Pathway Analysis (IPA) software (Qiagen Inc. https://digitalinsights.qiagen.com/products-overview/discovery-insights-portfolio/analysis-and-visualization/qiagen-ipa/) utilising Ingenuity Knowledge Base, a highly structured repository of biological interactions and functional annotations.
Statistical analyses

The protein and phosphopeptide intensities within each condition were normalised to the total peptide amount. The fold changes of the different conditions were estimated using the average control abundances from five biological replicates. The log2 transformed protein ratios and differential expression were analysed using limma and stats packages [18]. Phosphopeptide abundances were further normalized against the abundance of the non-modified proteins. The ratios of proteins were regarded as being significantly changed between conditions compared if the q value was less than 0.05.

For comparing multiple groups, one-way ANOVA was performed followed by Tukey’s post-hoc test. Groups were considered to be significantly different when p value was less than 0.05.

Differential expression of IPA predicted regulatory genes among human multiple sclerosis lesion types vs. nonaffected white matter (NAWM) was identified by using the edgeR package (3.8) software [19] from the database created previously [20]. Adjusted p value filtering using the procedure of Benjamini and Hochberg was used to establish significant differences.

Results

De- and remyelination affected about 5.5% of the proteins identified

In an attempt to identify key elements regulating de- and remyelination, we isolated and homogenized the corpus callosum from mice exposed to CPZ, and performed liquid-chromatography mass-spectrometry analysis from the homogenates. Following the same protocol we have used for studying transcriptome changes and the effect of microRNA-146a on CPZ-induced demyelination [14, 21], we induced demyelination in all mice except the controls (C) for 4 weeks with 0.2% CPZ (4wD), then allowed remyelination by terminating CPZ supplementation for 2 days (2dR) and 2 weeks (2wR). From the 20 samples (5 mice per group) we could identify altogether 3183 unmodified proteins. In addition to the unmodified ones, we detected 6017 single- or multi phosphorylated and/or gycosylated (PTM) peptides. Based on them, we identified 1703 PTM proteins. We performed clustering of these proteins for visualization how their level changed during de- and remyelination. Initially, pattern of protein level changes became markedly more coherent as the number of clusters was increased. However, the coherency did not increase significantly by increasing the number of clusters from 7 to 8. Therefore, we selected 7 clusters for both the unmodified (Fig 1A) and PTM proteins (Fig 1B). However, the proportional distribution (Fig 1A and 1C) of the proteins among the clusters differed. So did the directions of the protein level changes (Fig 1C and 1D) among the different experimental groups within the given clusters. Furthermore, while for the unmodified proteins, demyelination was found to be more closely related to late remyelination (Fig 1A) by the Perseus algorithm, it was early remyelination for the PTM proteins (Fig 1B). All these data indicated that clustering the whole protein population, although provided a basic visualisation of the changes the proteins followed during de- and remyelination, did not provide any clear lead toward finding proteins that conceivably regulate de- or remyelination.

For further analysis, we used those 1970 unmodified and 1255 PTM proteins only that were present in at least 80% of the samples. Furthermore, we considered the protein level to be significantly altered when (i) the protein’s identification was based on at least two unique peptides, (ii) it differed from the control significantly (q<0.05) and (iii) level of the unique peptides changed parallel to each-other. We identified 161 such unmodified proteins (Table 1); 93 of increased and 68 of decreased level during de- and remyelination, respectively (Fig 2A). Out of the 1255 single- or multi phosphorylated and/or gycosylated proteins, 40 were
significantly affected by de- and remyelination (Table 2 and Fig 2B). Interestingly, these protein populations did not overlap; we could not find any protein that significantly changed one way by the CPZ treatment and the other way when CPZ treatment was discontinued. We found only nine proteins that were identified both as PTM and unmodified ones (Tables 1 and 2; grey shading). Among the PTM proteins of elevated level, 30% were phosphorylated.

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Table 1. Concentrations of unmodified proteins significantly affected by de- and remyelination.

| access # | Protein                                             | 4wD     | q       | 2dR     | Q      | 2wR     | q       |
|----------|-----------------------------------------------------|---------|---------|---------|--------|---------|---------|
| O35639   | Annexin A3                                          | 1.129   | 0.000   | 0.877   | 0.004  | 0.613   | 0.027   |
| O89017   | Legumain                                            | 1.591   | 0.000   | 1.025   | 0.002  | 0.708   | 0.009   |
| P03995   | Glial fibrillary acidic protein                     | 1.465   | 0.000   | 1.333   | 0.000  | 1.733   | 0.000   |
| P07356   | Annexin A2                                          | 0.947   | 0.001   | 0.741   | 0.016  | 0.567   | 0.017   |
| P14106   | Complement C1q subcomponent subunit B               | 1.126   | 0.000   | 0.792   | 0.007  | 1.578   | 0.000   |
| P16110   | Galectin-3                                          | 2.181   | 0.002   | 1.616   | 0.002  | 1.632   | 0.003   |
| P18242   | Cathepsin D                                         | 1.262   | 0.000   | 0.753   | 0.003  | 0.729   | 0.000   |
| P20152   | Vimentin                                            | 1.562   | 0.000   | 1.107   | 0.000  | 1.275   | 0.000   |
| P24452   | Macrophage-capping protein                          | 1.196   | 0.000   | 0.815   | 0.017  | 1.457   | 0.000   |
| P26041   | Moesin                                              | 0.885   | 0.007   | 0.841   | 0.000  | 0.627   | 0.000   |
| P31786   | Acyl-CoA-binding protein                            | 0.700   | 0.026   | 0.792   | 0.006  | 0.505   | 0.001   |
| Q61233   | Plastin-2                                           | 1.009   | 0.007   | 0.735   | 0.011  | 0.709   | 0.000   |
| Q8BTM8   | Filamin-A                                           | 0.701   | 0.002   | 0.434   | 0.031  | 0.276   | 0.049   |
| Q99L04   | Dehydrogenase/reductase SDR family 1                | 1.024   | 0.001   | 0.698   | 0.011  | 0.712   | 0.000   |
| Q9DAW9   | Calponin-3                                          | 0.949   | 0.002   | 0.570   | 0.027  | 0.752   | 0.000   |
| Q9WU7    | Cathepsin Z                                         | 1.538   | 0.001   | 0.926   | 0.006  | 1.181   | 0.004   |
| P20060   | Beta-hexosaminidase subunit beta                    | 1.168   | 0.000   | 0.503   | 0.895  | 0.000   |
| P29758   | Ornithine aminotransferase, mitochondrial           | 0.404   | 0.032   | 0.385   | 0.304  | 0.040   |
| Q9JLF6   | Gamma-glutamyltransferase K                        | 1.605   | 0.006   | 1.204   | 1.714  | 0.004   |
| Q97O9    | Peroxiredoxin-3                                    | 0.598   | 0.014   | 0.448   | 0.759  | 0.000   |
| P10605   | Cathepsin B                                        | 0.749   | 0.003   | 0.137   | 0.511  | 0.004   |
| P16045   | Galectin-1                                         | 1.062   | 0.002   | 0.508   | 0.749  | 0.002   |
| P68033   | Actin, alpha cardiac muscle 1                       | 1.886   | 0.002   | 0.986   | 1.404  | 0.004   |
| P84075   | Neuron-specific calcium-binding protein             | 0.775   | 0.012   | 0.390   | 0.806  | 0.000   |
| P97371   | Proteosome activator complex subunit 1              | 0.630   | 0.019   | 0.337   | 0.480  | 0.003   |
| Q02105   | Complement C1q subcomponent subunit C              | 1.102   | 0.001   | 0.504   | 1.725  | 0.000   |
| Q61599   | Rho GDP-dissociation inhibitor 2                    | 0.805   | 0.001   | 0.420   | 0.387  | 0.026   |
| Q8BMS1   | Trifunctional enzyme subunit alpha                  | 0.437   | 0.030   | 0.307   | 0.318  | 0.026   |
| P48036   | Annexin A5                                         | 0.349   | 0.464   | 0.030   | 0.396  | 0.004   |
| P61205   | ADP-ribosylation factor 3                           | 1.228   | 0.920   | 0.043   | 0.779  | 0.004   |
| P58771   | Tropomyosin alpha-1 chain                           | 0.231   | 0.671   | 0.039   | 1.172  | 0.004   |
| P47955   | 60S acidic ribosomal protein P1                     | 0.541   | 0.414   | 0.011   | 0.315  | 0.027   |
| P56564   | Excitatory amino acid transporter 1                 | 0.390   | 0.232   | 0.451   | 0.012  |
| P98086   | Complement C1q subcomponent subunit A              | 0.820   | 0.596   | 1.510   | 0.000  |
| Q9EQ5    | Protein SET                                        | 0.423   | 0.160   | 0.477   | 0.004  |
| Q6890    | Clusterin                                          | 0.039   | 0.346   | 0.779   | 0.000  |
| O35658   | Complement component 1 Q-binding protein            | 0.431   | 0.265   | 0.536   | 0.047  |
| P03921   | NADH-ubiquinone oxidoreductase chain 5              | 0.469   | 0.528   | 0.393   | 0.029  |
| P08226   | Apolipoprotein E                                   | 0.518   | 0.397   | 0.846   | 0.000  |
| P27898   | Granulins                                          | 0.926   | 0.080   | 1.449   | 0.004  |
| P29416   | Beta-hexosaminidase subunit alpha                   | 0.578   | 0.496   | 0.516   | 0.026  |
| P4225    | Signal transducer/transcription activator 1        | 0.482   | 0.512   | 0.732   | 0.034  |
| P51880   | Fatty acid-binding protein, brain                  | 0.810   | 0.291   | 0.880   | 0.000  |
| P55264   | Adenosine kinase                                   | 0.013   | -0.182  | 0.321   | 0.038  |
| P56565   | Protein S100-A1                                    | 0.582   | 0.614   | 0.664   | 0.001  |
| P62082   | 40S ribosomal protein S7                            | 0.335   | 0.190   | 0.271   | 0.048  |
| P63040   | Complexin-1                                        | 0.482   | -0.019  | 0.656   | 0.003  |

(Continued)
Table 1. (Continued)

| access # | Protein                                                                 | 4wD    | q       | 2dR    | Q       | 2wR    | q       |
|----------|-------------------------------------------------------------------------|--------|---------|--------|---------|--------|---------|
| P70202   | Latexin                                                                | 0.359  | 0.233   | 0.446  | 0.003   |        |         |
| P97372   | Proteasome activator complex subunit 2                                 | 0.634  | -0.456  | 0.905  | 0.014   |        |         |
| P99027   | 60S acidic ribosomal protein P2                                        | 0.357  | 0.244   | 0.544  | 0.006   |        |         |
| Q61207   | Sulfated glycoprotein 1                                                 | 0.444  | 0.299   | 0.612  | 0.006   |        |         |
| Q8BGC4   | Zinc-binding ADH domain-containing protein 2                            | 0.243  | -0.274  | 0.479  | 0.032   |        |         |
| Q9Z127   | Large neutral amino acids transporter 1                                 | 0.519  | 0.310   | 0.689  | 0.004   |        |         |
| Q9Z1T2   | Thrombospondin-4                                                        | -0.661 | 0.211   | 1.371  | 0.016   |        |         |
| P17225   | Polypyrrimidine tract-binding protein 1                                 | 0.681  | 0.009   | 0.473  | 0.021   | 0.221  |         |
| Q62417   | Sorbin and SH3 domain-containing protein 1                              | 0.530  | 0.016   | 0.447  | 0.034   | 0.212  |         |
| Q9JM63   | ATP-sensitive inward rectifier K⁺ channel 10                            | 0.730  | 0.024   | 0.940  | 0.004   | 0.222  |         |
| P62849   | 40S ribosomal protein S24                                               | 0.830  | 0.007   | 0.246  | 0.593   |        |         |
| Q00915   | Retinol-binding protein 1                                               | 0.622  | 0.009   | 0.166  | 0.431   |        |         |
| Q880Y6   | 10-formyltetrahydrofolate dehydrogenase                                 | 0.331  | 0.038   | 0.164  | 0.270   |        |         |
| Q9D379   | Epoxide hydrolase 1                                                     | 0.904  | 0.002   | 0.396  | 0.430   |        |         |
| Q9DCN2   | NADH-cytochrome b5 reductase 3                                          | 0.714  | 0.001   | 0.368  | 0.343   |        |         |
| Q9JJU8   | SH3-binding glutamic acid-rich-like protein                             | 0.480  | 0.036   | 0.311  | 0.280   |        |         |
| Q9WV32   | Arp 2/3 complex subunit 1B                                              | 0.691  | 0.046   | 0.344  | 0.409   |        |         |
| O08677   | Kininogen-1                                                             | 0.818  | 0.042   | 0.302  | -0.039  |        |         |
| O88958   | Glucosamine-6-phosphate isomerase 1                                    | 0.614  | 0.040   | 0.314  | 0.374   |        |         |
| P11352   | Glutathione peroxidase 1                                                | 0.500  | 0.019   | 0.260  | -0.022  |        |         |
| P17047   | Lysosome-associated glycoprotein 2                                       | 0.612  | 0.006   | 0.346  | 0.218   |        |         |
| P26039   | Talin-1                                                                 | 0.646  | 0.012   | 0.396  | 0.244   |        |         |
| Q05816   | Fatty acid-binding protein, epidermal                                   | 0.939  | 0.007   | 0.053  | 0.468   |        |         |
| Q3UHB1   | 5'-nucleotidase domain-containing protein 3                             | 0.504  | 0.036   | 0.339  | 0.167   |        |         |
| Q62348   | Translin                                                                | 0.524  | 0.046   | 0.333  | 0.103   |        |         |
| Q8VDD5   | Myosin-9                                                                | 0.535  | 0.035   | 0.301  | 0.230   |        |         |
| Q9D0S9   | Histidine triad nucleotide-binding protein 2                             | 0.403  | 0.046   | 0.251  | 0.082   |        |         |
| Q9Z110   | Delta-1-pyruroline-5-carboxylate synthase                               | 0.550  | 0.020   | 0.313  | 0.013   |        |         |
| Q9Z1E4   | Glycogen [starch] synthase                                              | 0.966  | 0.046   | -0.055 | 0.331   |        |         |
| O54983   | Ketimine reductase mu-crystallin                                        | 0.578  | 0.489   | 0.026  | 0.193   |        |         |
| Q08331   | Calreptin                                                               | 0.695  | 1.042   | 0.038  | 0.179   |        |         |
| Q9DB73   | NADH-cytochrome b5 reductase 1                                           | 0.326  | 0.404   | 0.039  | 0.130   |        |         |
| Q9Z0F7   | Gamma-synuclein                                                         | 0.709  | 0.696   | 0.014  | -0.224  |        |         |
| P03888   | NADH-ubiquinone oxidoreductase chain 1                                  | 0.977  | 0.839   | 0.039  | 0.583   |        |         |
| P07309   | Transthyretin                                                           | 0.249  | 0.743   | 0.004  | -0.047  |        |         |
| P07724   | Serum albumin                                                           | 0.364  | 0.572   | 0.019  | 0.167   |        |         |
| P10126   | Elongation factor 1-alpha 1                                             | 0.102  | 0.555   | 0.011  | -0.072  |        |         |
| P23492   | Purine nucleoside phosphorylase                                         | 0.248  | 0.539   | 0.011  | 0.055   |        |         |
| P34884   | Macrophage migration inhibitory factor                                  | 0.882  | 0.502   | 0.026  | 0.158   |        |         |
| P63038   | 60 kDa heat shock protein, mitochondrial                               | 0.233  | 0.412   | 0.039  | 0.152   |        |         |
| Q6PE15   | Mycophenolic acid acyl-glucuronide esterase                             | 0.520  | 0.477   | 0.039  | 0.202   |        |         |
| Q9CQI6   | Coacisin-like protein                                                   | 0.129  | 0.554   | 0.039  | 0.228   |        |         |
| Q9D1I5   | Methylmalonyl-CoA epimerase                                             | 0.121  | 0.695   | 0.039  | 0.050   |        |         |
| Q9DBS2   | Tumor p63-regulated gene 1-like protein                                 | 0.548  | 0.544   | 0.030  | 0.172   |        |         |
| Q9QYG0   | Protein NDRG2                                                           | 0.731  | 0.595   | 0.038  | 0.380   |        |         |
| Q9RP09   | Ubiquitin hydrolase L1                                                 | 0.399  | 0.624   | 0.011  | 0.151   |        |         |
| P97315   | Cysteine and glycine-rich protein 1                                     | -0.704 | 0.007   | -0.667 | 0.004   | -0.390 | 0.014   |

(Continued)
| access # | Protein                                     | 4wD  | q    | 2dR  | Q   | 2wR  | q    |
|---------|--------------------------------------------|------|------|------|-----|------|------|
| QSEBJ4  | Ermin                                       | -1.053 | 0.000 | -0.818 | 0.033 | -0.641 | 0.020 |
| Q8K298  | Actin-binding protein anillin               | -0.681 | 0.008 | -0.912 | 0.011 | -0.902 | 0.000 |
| Q8R3P0  | Asparoacylase                               | -0.617 | 0.016 | -0.883 | 0.000 | -0.839 | 0.000 |
| Q8RR63  | Protein FAM177A1                            | -0.646 | 0.037 | -0.698 | 0.046 | -0.846 | 0.002 |
| P16330  | 2',3'-cyclic-nucleotide 3'-phosphodiesterase | -0.492 | 0.042 | -0.537 | 0.059 | -0.933 | 0.004 |
| P23927  | Alpha-crystallin B chain                    | -0.836 | 0.011 | -0.446 | 0.072 | -0.727 | 0.029 |
| Q05BC3  | Echinoderm microtubule-associated 1         | -0.707 | 0.016 | -0.467 | 0.042 | -0.422 | 0.012 |
| Q9CRB6  | Tubulin polymerization-promoting protein 3  | -0.658 | 0.007 | -0.388 | 0.070 | -0.702 | 0.000 |
| Q9D8B7  | Junctional adhesion molecule C              | -1.109 | 0.003 | -0.577 | 0.065 | -0.665 | 0.027 |
| Q7M750  | Opalin                                      | -0.577 | -0.803 | 0.007 | -0.937 | 0.000 |
| Q9D154  | Leukocyte elastin inhibitor A               | -0.442 | -0.606 | 0.006 | -0.596 | 0.003 |
| Q5SYD0  | Unconventional myosin-Id                    | -0.251 | -0.505 | 0.016 | -0.566 | 0.006 |
| O54988  | STE20-like serine/threonine-protein kinase | -0.290 | -0.238 | -0.401 | 0.007 | -0.271 | 0.032 |
| Q8CAY6  | Acetyl-CoA acetyltransferase, cytosolic     | -0.711 | 0.074 | -0.407 | 0.007 | -0.902 | 0.000 |
| Q8R3P0  | Asparto acylase                             | -0.617 | 0.016 | -0.883 | 0.000 | -0.839 | 0.000 |
| P16330  | 2',3'-cyclic-nucleotide 3'-phosphodiesterase | -0.492 | 0.042 | -0.537 | 0.059 | -0.933 | 0.004 |
| P23927  | Alpha-crystallin B chain                    | -0.836 | 0.011 | -0.446 | 0.072 | -0.727 | 0.029 |
| Q05BC3  | Echinoderm microtubule-associated 1         | -0.707 | 0.016 | -0.467 | 0.042 | -0.422 | 0.012 |
| Q9CRB6  | Tubulin polymerization-promoting protein 3  | -0.658 | 0.007 | -0.388 | 0.070 | -0.702 | 0.000 |
| Q9D8B7  | Junctional adhesion molecule C              | -1.109 | 0.003 | -0.577 | 0.065 | -0.665 | 0.027 |
| Q7M750  | Opalin                                      | -0.577 | -0.803 | 0.007 | -0.937 | 0.000 |
| Q9D154  | Leukocyte elastin inhibitor A               | -0.442 | -0.606 | 0.006 | -0.596 | 0.003 |
| Q5SYD0  | Unconventional myosin-Id                    | -0.251 | -0.505 | 0.016 | -0.566 | 0.006 |
| O54988  | STE20-like serine/threonine-protein kinase | -0.290 | -0.238 | -0.401 | 0.007 | -0.271 | 0.032 |
| Q8CAY6  | Acetyl-CoA acetyltransferase, cytosolic     | -0.711 | 0.074 | -0.407 | 0.007 | -0.902 | 0.000 |
| Q05BC3  | Echinoderm microtubule-associated 1         | -0.707 | 0.016 | -0.467 | 0.042 | -0.422 | 0.012 |
| Q9CRB6  | Tubulin polymerization-promoting protein 3  | -0.658 | 0.007 | -0.388 | 0.070 | -0.702 | 0.000 |
| Q9D8B7  | Junctional adhesion molecule C              | -1.109 | 0.003 | -0.577 | 0.065 | -0.665 | 0.027 |

(Continued)

Table 1. (Continued)
In contrast, 92% of the PTM proteins of decreased level were at least monophosphorylated (Table 2; yellow shading).

As we [15] and others [13, 22] demonstrated previously, 4 weeks of cuprizone treatment caused massive demyelination in the corpus callosum that was followed by rapid remyelination when the treatment was discontinued [13, 22]. Accordingly, we expected proteins specific for demyelination-, early- and late remyelination, i.e. proteins with levels that differed from the C group respectively in the 4wD, 2dR and 2wR groups only. Based on this definition, we identified 39 demyelination specific, 24 early- and 48 late remyelination specific unmodified (Fig 2A), and 18, 3 and 9 PTM proteins (Fig 2B), respectively. On the other hand, although in different extent, 30 unmodified and 6 PTM proteins were affected in two, while 20 unmodified and 3 PTM proteins in all three groups (Fig 2).

Immunohistochemical analysis of phosphorylation during de- and remyelination

Many cellular functions are regulated by phosphorylation, and the observed uneven distribution of phosphorylation among up- and downregulated PTM proteins raised the possibility of a specific role for phosphorylation during de- and remyelination. Accordingly, we performed immunohistochemistry utilising p-Ser, p-Thr and p-Tyr specific primary antibodies on brain sections of the animals from all four experimental groups. We observed intermediate to weak specific staining that was localised to the nerve fibres rather than the cell bodies or nuclei (Fig 3A). Seldom, staining of a nucleus or a cell body was found, however, no specific pattern or (Table 2; yellow shading), the rest were N-glycosylated. In contrast, 92% of the PTM proteins of decreased level were at least monophosphorylated (Table 2; yellow shading).
staining characteristic for a certain cell type could be identified (Fig 3A). In an attempt to correlate phosphorylation with either de- or remyelination, we measured staining intensities among the brain sections by using an image analyser software. We found up to about 9, 17 and 26% differences among the groups for Ser, Thr and Tyr phosphorylation, respectively (Fig 3B). Also, the staining intensities were lower in early remyelination than in the control and the demyelination groups for Thr and Tyr phosphorylation, respectively (Fig 3B).

**GO analysis of proteins affected by de- and remyelination**

In order to identify specific biological processes, we performed over-representation test on the proteins affected by de- and remyelination. This test determines representation of the proteins in question in molecular function, biological process or cellular component categories selected as significant ones by the software over estimated representation of the mouse proteome in the same categories. We pooled the differentially regulated 192 PTM and unmodified proteins, and performed the test for biological processes. All the proteins but two were classified into 76 categories, an extract of which is presented in Table 3. The biological process categories included glial and neuronal function, metabolism, cell death, inflammatory response, protein
Table 2. Concentrations of single- or multi phosphorylated and/or glycosylated (PTM) proteins significantly affected by de- and remyelination.

| Peptide sequence | access # | protein description | 4wD | q | 2dR | q | 2wR | q | p-value |
|------------------|----------|---------------------|-----|---|-----|---|-----|---|---------|
| AVLVNdNITGGEK    | Q91X2    | Golgi membr. p1     | 1.961 | 0.001 | 1.339 | 0.011 | 1.225 | 0.030 |
| VIFVVPdNHSNIPFSR | Q88G07   | Phospholipase D4    | 1.319 | 0.047 | 1.679 | 0.003 | 1.861 | 0.008 |
| AIPPTALTdTNSSK   | Q07797   | Galectin-3-binding  | 1.994 | 0.001 | 2.113 | 0.000 | 1.993 | 0.001 |
| ALGyEdNATQALQR   |          |                     | 2.109 | 0.001 | 1.984 | 0.000 | 2.046 | 0.003 |
| GLdNLTEdTYKPR    |          |                     | 2.690 | 0.000 | 2.524 | 0.000 | 2.724 | 0.000 |
| dNLTTLGIFGAATNK  | Q80WV3   | Carbohydrate ST2    | 1.601 | 0.003 | 0.729 | 0.079 | 1.305 | 0.035 |
| LdNFtGPePDLSR    | P11835   | Integrin beta-2     | 1.384 | 0.011 | 0.954 | 0.037 | 1.606 | 0.006 |
| VLTNQSPyQINHTGR  | P98086   | Complement C1q      | 1.751 | 1.778 | 0.010 | 2.306 | 0.001 |
| AVdNGSDGEyR      | P08508   | IG-G Fc receptor3   | 2.005 | 0.816 | 0.954 | 1.606 | 0.006 |
| dNdNLTSPEATAK    | OS5026   | EctP D'Pase2        | 0.847 | 0.404 | 0.404 | 0.000 | 1.029 | 0.030 |
| TGEFPDeEgTFrPSSR | Q9Z239   | Phospholemman       | 0.653 | 0.607 | 0.607 | 0.000 | 1.408 | 0.004 |
| SPPDQAVpNPtTSPvPLKQEPTSR | Q9QYC | Alpha-adducin       | 0.387 | -0.489 | -0.489 | 0.000 | 1.455 | 0.030 |
| QELdNDLQVqER     | Q06890   | Clusterin           | -0.284 | 0.052 | 0.052 | 0.000 | 1.655 | 0.014 |
| dNSTGCLK         |          |                     | 0.558 | 0.895 | 0.895 | 0.000 | 1.896 | 0.014 |
| ETISAdpTSPk      | Q3UH99   | Protein shisa-6     | 1.839 | 1.513 | 0.153 | 0.014 | 1.288 | 0.000 |
| VLGKPPKPNdNLEtYPLMMK | P14094 | NA^-K^-ATPase       | 2.710 | 0.001 | 2.268 | 0.000 | 1.588 | 0.001 |
| dNSTFGeSEvFSLDPNK | P56528   | cADP ribose galactosylase | 1.695 | 0.040 | 1.569 | 0.071 |
| YYHGeLSYldNvTrK  | P18242   | Cathepsin D         | 1.540 | 0.046 | 1.021 | 0.000 | 1.168 | 0.014 |
| TPALpSQRPtLTTQpQSGTLK | Q64332 | Synapsin-2          | 1.376 | 0.011 | 0.438 | 0.048 |
| AWGvSLNvPINk     | P31996   | Macrosialin         | 2.719 | 0.003 | 1.881 | 0.074 |
| EAEdNqntQvqQtSIR | P41233   | ATP-B c A 1          | 1.022 | 0.040 | 0.720 | 0.000 | 0.874 | 0.000 |
| dNdNATqEILYHLEK  | Q61207   | Sulf. Glycoprot1    | 1.476 | 0.014 | 1.156 | 0.037 |
| EGEEpTyvGdDEEpk  | Q55022   | Progesterone R      | 2.252 | 0.011 | 1.144 | 0.048 |
| VAAGHeLoPLALdIVdqRqSpSr | P23242 | Gap junction c-1    | 1.228 | 0.041 | 0.961 | 0.049 |
| DLGPALAdNvSSdVvK | P17439   | Ceramidase          | 1.560 | 0.049 | 0.999 | 0.078 |
| pSAEdLTGDSyYdILdNAELqK | Q9D0L7 | AR-containing p10   | 1.147 | 0.049 | 1.093 | 0.081 |
| EdNITAEALdLsLk  | Q61704   | Trypsin inhib.h3    | 3.325 | 0.002 | 1.380 | 0.090 |
| VVLHPdNHdVdIGLk  | Q61646   | Haptoglobin         | 2.244 | 0.000 | 0.720 | 0.000 |
| NLdNdHSeTASAk    | 0.920    |                     | 3.621 | 0.012 | 0.725 | 0.000 |
| EEAkspSpGEEAKpsGEEAk | P19246 | Neurofilament H     | 2.150 | 2.889 | 0.048 | -0.311 |
| GVVTYNGLdpSpAEskKeGdssSDK | P08553 | Neurofilament M     | -6.053 | 0.039 | -1.546 | -3.232 | 0.030 |
| TTHyGlSPKqSpQGHr | P04370   | Myelin basic prot.  | -4.866 | -1.751 | -4.179 | 0.012 |
| FFPsGDGRAPk      | -0.702   |                     | -1.974 | -3.758 | 0.014 |
| NIVpTrpsTppSSQkG | -2.850   |                     | -1.920 | -0.938 |
| NVyVptTRppSSQkG  | -2.498   |                     | -1.810 | -0.900 |
| EAEnTANAGEPeSpQvELQvDsvK | Q6NVE8 | WDR-contain. p44    | -1.297 | -0.542 | -2.456 | 0.014 |
| SHTsEdARldN xpTnPSGatGnNAGpK | Q62433 | Protein NDRG1       | -1.374 | -0.832 | -2.134 | 0.014 |
| DLHESSFpSLdGQdDDHvK | Q9EQF6 | Dihydropyrimidine S | 0.115 | -0.181 | -1.617 | 0.004 |
| KGvpPiHgpSr      | P16330   | cNMP 3'-PDE         | -1.541 | -0.158 | -1.568 | 0.013 |
| HQPApSpVVVR      | Q9dQ97   | Myelin-associ. OBP  | -2.722 | 0.001 | -1.340 | -0.945 |
| EHAEnDAsQAQspApNPsTtSpGkspPpKAk | Q8VDQ8 | Sirtuin-2           | -1.130 | 0.008 | -1.264 | -0.653 |
| EHAEnDAsQAQspApNpsTtSpGKspPpKAk | Q8VDQ8 | Sirtuin-2           | -1.130 | 0.008 | -1.264 | -0.653 |
| QPGFqPSpsDPSlpSpsqPQDr | Q91vC7 | Phosphatase 1       | -1.673 | 0.003 | -1.635 | -1.012 |
| TFSpPEPEpAPGAqK  | Q80Yn3   | BCAS 1 homolog      | -1.072 | 0.019 | -1.225 | -0.539 |
| AVySpPvTsR       | Q7TD2    | Tubulin PPP         | -2.207 | 0.027 | -1.196 | -0.459 |
| VVvHKEtElpTPedGED | Q9WV92   | Band 4.1-like p3    | -4.207 | 0.003 | -0.775 | -0.200 |
| QKFHPdSeGddTteTedYR | Q8k019 | Bcl-2-associ. TF1   | -2.924 | 0.041 | -0.916 | 0.167 |

(Continued)
and cation homeostasis, and cytoskeleton related processes (Table 3). Besides the expected oligodendrocyte compatible ones, the overrepresentation test indicated involvement of astrocyte and microglia related events such as glutamate metabolic process [23] and inflammatory response [24, 25]. These data are consistent with the accepted view that CPZ induced oligodendrocyte loss is accompanied by expansion and activation of microglia and astrocytes [26].

In addition to the two unclassified proteins, we eliminated those that were involved in biological processes of less than 50% over- or under-representation. We divided the remaining 157 proteins (Fig 4) into remyelination only (RO, Fig 4 shaded) and demyelination related (DR, Fig 4 unshaded) groups and performed over-representation test on them. The biological process over-representation test allocated the DR proteins into 9 categories (Table 4). For the RO proteins, the single significant category was nervous system development that included myelination as the most specific subcategory (Table 5). Interestingly, a biological process over-representation test on the 60 demyelination specific proteins of the DR group did not identify any significant process.

To further study the RO proteins (Fig 4, shaded) for their involvement in mechanisms regulating remyelination, we used Ingenuity Pathway Knowledge Base to select a group of interconnected nodes to assess their cellular level changes during the phases of 2dR and 2wR. The analyses performed on the two remyelination groups resulted the same sole network of eight members (Fig 5A). In early remyelination, two of them were upregulated and the other six members not affected significantly, while the reverse pattern was observed during late remyelination (Fig 5A). We searched occurrence of the eight genes among genes expressed differentially between human multiple sclerosis lesion types vs. NAWM [20]. As we found, four of them were differentially expressed in at least one of the lesion types, and perlecan (HSPG2) gene was the most upregulated in remyelinating lesions (Fig 5B). In remyelinating multiple sclerosis lesions, the gene expression pattern of the identified experimental remyelination network orthologues was more consistent with early remyelination in the CPZ model, i.e. upregulation of HSPG2 and downregulation of signal transducer and activator of transcription 1 (STAT1) and Thrombospondin-4 (THBS4) (Fig 5B).

Discussion

Due to the lack of clear concept for the aetiology of multiple sclerosis, clinical treatment strategies are limited to delaying progression of the disease by suppressing inflammation [27]. Recently, proteomic analysis of CPZ treated mice’s brain samples was suggested as a potentially useful approach for finding therapeutic targets enabling causal treatment of the disease [28]. However, none of the previous studies [10–12] targeted the corpus callosum, where CPZ induces the most pronounced demyelination [13]. Therefore, we assessed proteomic changes during de- and remyelination at different time points in the isolated corpus callosum of CPZ treated mice.
Although all previous proteomic studies used mice of the C57BL/6 genetic background, there were differences between genders of the animals, age of the mice at the start and length of CPZ treatment, and the timing of sample acquisition during de- and remyelination [10–12]. As we demonstrated previously [15], CPZ induced demyelination increases in extent rapidly during the first 4 weeks, then at a much slower rate for the following 2 weeks before the time window for regeneration closes [13]. Accordingly, we followed the same protocol as previously [14, 21], namely, 4 weeks of CPZ treatment (demyelination) followed by 2 (early remyelination) and 14 (late remyelination) days of recovery after termination of the CPZ treatment.

Proteomic analysis of the corpus callosums resulted in altogether 4886 proteins, however, Fig 3. Immunohistochemical analysis of phosphorylation during de- and remyelination. We performed immunohistochemistry utilising phospho (p)-Ser, p-Thr and p-Tyr specific primary antibodies on brain sections of the animals (n = 3) from all four treatment groups. Representative sections are presented (A). For quantitative assessment of phosphorylation during de- and remyelination (B), all nuclei and staining artefacts were eliminated from the sections, and staining intensities of corpus callosum were normalised by an expert blind to the experiment to background i.e. left and right retrosplenial area, ventral part, layer 1 (lightly stained area containing few nuclei left and right to the arrow in A) by using Molecular Devices’ MetaXpress® image analyser software. Data are presented mean ± standard deviation % of the background (n = 3). Filled, dark grey, open and light grey bars denote control, 4-week demyelination, 2-day and 2-week remyelination groups, respectively. * significantly different from C; # significantly different from 4W.

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Table 3. Extract of panther biological process over-representation test for the proteins significantly altered in the experimental groups.

| Biological process                                                | # Mus musculus | Client Text Box Input | Fold Enrich. | q   |
|-------------------------------------------------------------------|----------------|-----------------------|--------------|-----|
| myelin maintenance                                                | 15             | 3                     | 0.13         | 23.47 | 2.73E-02 |
| positive regulation of cholesterol transport                      | 24             | 4                     | 0.2          | 19.56 | 8.12E-03 |
| negative regulation of catalytic activity                         | 671            | 16                    | 5.72         | 2.8  | 1.80E-02 |
| neurofilament bundle assembly                                     | 3              | 2                     | 0.03         | 78.23 | 3.82E-02 |
| negative regulation of amyloid fibril formation                   | 3              | 2                     | 0.03         | 78.23 | 3.80E-02 |
| regulation of postsynaptic membrane organization                  | 21             | 4                     | 0.18         | 22.35 | 5.78E-03 |
| response to calcium ion                                           | 128            | 7                     | 1.09         | 6.42  | 1.22E-02 |
| synapse organization                                              | 258            | 11                    | 2.2          | 5    | 2.69E-03 |
| walking behavior                                                  | 47             | 4                     | 0.4          | 9.99  | 4.64E-02 |
| response to toxic substance                                       | 287            | 9                     | 2.45         | 3.68  | 4.90E-02 |
| positive regulation of apoptotic process                           | 615            | 14                    | 5.24         | 2.67  | 4.88E-02 |
| negative regulation of cell death                                  | 1018           | 22                    | 8.68         | 2.54  | 7.08E-03 |
| inflammatory response                                             | 429            | 12                    | 3.66         | 3.28  | 2.55E-02 |
| negative regulation of protein homooligomerization                | 11             | 4                     | 0.09         | 42.67 | 1.18E-03 |
| positive regulation of sodium ion export across plasma membrane   | 4              | 3                     | 0.03         | 88.01 | 2.76E-03 |
| cellular potassium ion homeostasis                                | 14             | 3                     | 0.12         | 25.15 | 2.45E-02 |
| positive regulation of calcium ion transport                       | 133            | 7                     | 1.13         | 6.18  | 1.44E-02 |
| actin filament organization                                        | 223            | 12                    | 1.9          | 6.31  | 2.43E-04 |
| negative regulation of blood coagulation                           | 45             | 5                     | 0.38         | 13.04 | 6.58E-03 |
| response to caloric restriction                                    | 3              | 2                     | 0.03         | 78.23 | 3.79E-02 |
| Unclassified                                                      | 1873           | 2                     | 15.96        | 0.13  | 2.48E-03 |

Protein list from Tables 1 and 2 were combined and biological process over-representation test was performed by using Protein Analysis Through Evolutionary Relationships (PANTHER) classification system software (http://www.pantherdb.org). # Mus musculus and # denotes the number of proteins in the given category based on the mouse genome and the actual number of proteins in the aforementioned list, respectively. “Expected” indicates the expected number of proteins in the aforementioned list in the case of no over- or under-representation. Fold Enrich. = #/ expected; the fold over- or under-representation. Significance of the analysis is indicated by presenting the q value.

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initial clustering of them did not indicate any group whose concentration changes followed a pattern indicative of de- and remyelination regulators (Fig 1).

We could select altogether 192 proteins whose concentration was significantly different from the control in at least one experimental group (Tables 1 and 2). They represented a high variety of molecular functions, few of which were characteristic of de- and remyelination processes. Out of the altogether 57 demyelination specific proteins, 25 had more than 1.5 fold of increased and 14 of decreased concentration (Tables 1 and 2). Among the latter, myelin basic protein (MBP) decreased to 13.9 while myelin-associated oligodendrocyte basic protein to 20.8% of their respective level in the control group [14]. We identified myelin basic protein based on three of its phosphopeptides; one of them bearing two threonine phosphorylation sites (Table 2). Phosphorylation of these sites by mitogen activated protein kinases (MAPKs) was reported to dramatically reduce the protein’s binding to negatively charged lipid bilayers [29]. Considering that MAPKs are activated during the treatment [15], these phosphorylation changes are consistent with the cuprizone induced demyelination. Additionally, besides 17 proteins involved in lipid metabolism, we found two more myelin-associated proteins; 2’,3’-cyclic-nucleotide 3’-phosphodiesterase [30] and ermin [31]. Interestingly, the latter occurred at decreased concentrations in all three experimental conditions.
Table 4. Panther biological process over-representation test for the shortlisted demyelination related proteins.

| Biological process                        | # Mus | Client Text Box Input | # expected | Fold Enrich. | q       |
|-------------------------------------------|-------|-----------------------|------------|--------------|---------|
| regulation of synaptic plasticity         | 207   | musculus              | 8          | 0.97         | 8.29    | 1.16E-02 |
| regulation of trans-synaptic signaling    | 506   | 11                    | 2.36       | 4.66         | 2.99E-02 |
| regulation of biological process          | 11120 | 72                    | 51.87      | 1.39         | 4.65E-02 |
| biological regulation                     | 11734 | 77                    | 54.73      | 1.41         | 1.59E-02 |
| regulation of biological quality          | 3830  | 43                    | 17.87      | 2.41         | 1.45E-04 |
| actin filament organization               | 223   | 8                     | 1.04       | 7.69         | 1.62E-02 |
| supramolecular fiber organization         | 444   | 14                    | 2.07       | 6.76         | 2.13E-04 |
| actin cytoskeleton organization           | 462   | 12                    | 2.16       | 5.57         | 4.65E-03 |
| cytoskeleton organization                 | 1004  | 20                    | 4.68       | 4.27         | 2.28E-04 |
| actin filament-based process              | 520   | 13                    | 2.43       | 5.36         | 3.00E-03 |
| cellular metal ion homeostasis            | 551   | 11                    | 2.57       | 4.28         | 3.97E-02 |
| metal ion homeostasis                     | 629   | 12                    | 2.93       | 4.09         | 3.18E-02 |
| cation homeostasis                        | 696   | 13                    | 3.25       | 4             | 2.77E-02 |
| ion homeostasis                           | 777   | 13                    | 3.62       | 3.59         | 4.52E-02 |
| homeostatic process                       | 1588  | 20                    | 7.41       | 2.7          | 3.13E-02 |
| inorganic ion homeostasis                 | 711   | 13                    | 3.32       | 3.92         | 3.02E-02 |
| cellular cation homeostasis               | 610   | 12                    | 2.85       | 4.22         | 2.93E-02 |
| cellular ion homeostasis                  | 624   | 12                    | 2.91       | 4.12         | 3.09E-02 |
| cellular homeostasis                      | 828   | 14                    | 3.86       | 3.62         | 3.02E-02 |
| regulation of cellular component biogenesis| 908   | 16                    | 4.24       | 3.78         | 1.06E-02 |
| regulation of cell migration              | 870   | 14                    | 4.06       | 3.45         | 4.00E-02 |
| regulation of localization                | 2764  | 33                    | 12.89      | 2.56         | 6.69E-04 |
| regulation of transport                   | 1897  | 24                    | 8.85       | 2.71         | 1.03E-02 |
| regulation of cellular component organization| 2479  | 31                    | 11.56      | 2.68         | 8.18E-04 |
| system development                        | 4126  | 37                    | 19.25      | 1.92         | 3.13E-02 |
| positive regulation of biological process | 5897  | 48                    | 27.51      | 1.75         | 2.09E-02 |

Those proteins of Tables 1 and 2 that were PANTHER unclassified or belonged to a biological process of smaller than 50% over- or under-representation were deleted from the list. Demyelination related proteins were classified as those 107 unmodified and single- or multi phosphorylated and/or glycosylated proteins that were significantly different from the control in the cuprizone treated group (Fig 4, unshaded). For the explanation, see Table 3.

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Table 5. Panther biological process over-representation test for the shortlisted remyelination only proteins.

| Biological process                        | # Mus | Client Text Box Input | # expected | Fold Enrich. | q       |
|-------------------------------------------|-------|-----------------------|------------|--------------|---------|
| myelination                               | 103   | 6                     | 0.23       | 25.98        | 2.45E-03 |
| axon ensheathment                         | 105   | 6                     | 0.24       | 25.48        | 9.08E-04 |
| ensheathment of neurons                   | 105   | 6                     | 0.24       | 25.48        | 1.36E-03 |
| nervous system development                | 2178  | 18                    | 4.88       | 3.69         | 2.20E-03 |

Those proteins of Tables 1 and 2 that were PANTHER unclassified or belonged to a biological process of smaller than 50% over- or under-representation were omitted. Remyelination only proteins were classified as those 50 unmodified and single- or multi phosphorylated and/or glycosylated proteins that were significantly different from the control in the early and late remyelination groups only (Fig 4, shaded). For the explanation, see Table 3.

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associated with myelin sheath, neuronal processes, synapse and cytoskeleton organisation (Table 5). However, out of the major oligodendrocyte marker proteins we found PTM form of myelin-associated oligodendrocyte basic protein and MBP only. On the other hand, we found decreased phosphorylated MBP levels (Table 2) as others did previously in multiple sclerosis patients’ and cuprizone mice’s brains [32]. Furthermore, our finding that all but one decreased PTM proteins were phosphorylated is in line with the view that decreased phosphorylation could be part of demyelination pathogenesis [32]. Unfortunately, the immunohistochemical study we performed to assess protein phosphorylation during de- and remyelination proved to be inconclusive. Although we found that Ser, Thr and Tyr phosphorylation was localised to the white matter, it seemed to be associated with the nerve fibres, did not change considerably among the groups, and no clear pattern or cell type specific staining could be identified (Fig 3).

In contrast to the remyelination only proteins, demyelination-related proteins were not associated (Table 4) with their respective expected biological process categories such as oligodendrocyte apoptosis, demyelination, oxidative stress or mitochondrial damage. Instead, a number of DR proteins were categorized into biological processes related to cytoskeletal and organelle reorganization, metal ion homeostasis and migration; processes most probably associated with astrocytosis and microglia activation [26]. Furthermore, the 60 demyelination only DR proteins could not be classified into any biological process category indicating that classification of these proteins with acceptable statistical significance was possible only when they were combined with the proteins that had altered level in two or all three experimental groups.

IPA network construction performed on early and late RO proteins resulted in a sole network, the members of which were activated in an inverse pattern during early vs. late remyelination (Fig 5A). Four out of the eight members were differentially expressed between human multiple sclerosis lesion types vs. NAWM (Fig 5B). Clusterin (CLU) that we found to be upregulated in all lesion types functions as an extracellular chaperone. It prevents aggregation of...
non-native proteins and maintains them in a state appropriate for refolding by ATPase chaperones [33]. In agreement with our results, CLU mRNA levels were reported to be elevated in glial fibrillary acidic protein-positive astrocytes in white matter lesions over NAWM, but not in grey matter of multiple sclerosis patients [34]. HSPG2 that we found to be upregulated in inactive and remyelinating multiple sclerosis lesions is one of the largest extracellular matrix molecules. It was not so far associated with multiple sclerosis, however was suggested to be essential in establishing and patrolling tissue borders [35]. STAT1 acts as a transcription factor for various growth factors. In agreement with other studies [36], we found it to be downregulated in inactive and remyelinating lesions. THBS4 that we found to be downregulated in inactive multiple sclerosis lesions is an adhesive glycoprotein involved in cell-to-cell and cell-to-matrix interactions. Although not in association with multiple sclerosis, it was reported to play a role in tissue remodeling [37]. Taken together all information, a role for this predicted network in regulating remyelination processes does not seem compelling, therefore more studies are needed to identify mechanisms regulating de- and remyelination.

Conclusions

Taken together all aforementioned data, proteomic analysis of the cuprizone treated corpus callosum seems more informative for the processes of remyelination over those of demyelination. In a broader sense, these results may indicate limitations of the cuprizone model in answering demyelination related specific questions in multiple sclerosis research by proteomics.

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