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Expression Profiles of Metallothionein I/II and Megalin in Cuprizone Model of De- and Remyelination

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Abstract—Copper chelator cuprizone (CPZ) is neurotoxicant, which selectively disrupts oligodendroglial respiratory chain, leading to oxidative stress and subsequent apoptosis. Demyelination is, however, followed by spontaneous remyelination owing to the activation of intrinsic CNS repair mechanisms. To explore the participation of metallothioneins (MTs) in these processes, in this study we analyzed the expression profiles of MT-I/II and their receptor megalin (low-density lipoprotein receptor related protein-2) in the brain of mice subjected to different protocols of CPZ feeding. Experiments were performed in female C57BL/6 mice fed with 0.25% CPZ during 1, 3 and 5 weeks. They were sacrificed immediately after feeding with CPZ or 2 weeks after the withdrawal of CPZ. The data showed that CPZ-induced demyelination was followed by high astrogliosis and enhanced expression of MTs and megalin in white (corpus callosum and internal capsule) and gray matter of the brain (cortex, hippocampus, and cerebellum). Moreover, in numerous cortical neurons and progenitor cells the signs of MT/megalin interactions and Akt1 phosphorylation was found supporting the hypothesis that MTs secreted from the astrocytes might directly affect the neuronal differentiation and survival. Furthermore, in mice treated with CPZ for 5 weeks the prominent MTs and megalin immunoreactivities were found on several neural stem cells and oligodendrocyte progenitors in subgranular zone of dentate gyrus and subventricular zone of lateral ventricles pointing to high modulatory effect of MTs on adult neuro- and oligodendrogenesis. The data show that MT I/II perform important cytoprotective and growth-regulating functions in remyelinating processes activated after toxic demyelinating insults.

Key words: adult neurogenesis, Akt1 phosphorylation, cuprizone, endocytosis, low-density lipoprotein receptor-related protein-2, metallothioneins I/II.

INTRODUCTION

The complex molecular mechanisms contributing to the de- and remyelination in multiple sclerosis (MS) and other inflammatory and neurodegenerative diseases are often investigated in the cuprizone (bis-cyclohexanone-oxalyldihydrazone, CPZ) model of intoxication, since it leads to reproducible induction of acute or chronic demyelinating lesions followed by spontaneous remyelination after withdrawal of cuprizone feeding (Kipp et al., 2009; Bénardais et al., 2013; Gudi et al., 2014; Praet et al., 2014). It is assumed that induced pathology and the selective vulnerability of mature oligodendrocytes (OLGs) to apoptosis originate from copper deficiency due to Cu chelation or from Cu entrapment within the cell (Messori et al., 2007), since this leads to detrimental effects on mitochondrial function and induce the disturbance of energy metabolism in oligodendroglia that require vast amounts of energy for myelin synthesis (reviewed by (Torkildsen et al., 2008; Kipp et al., 2009, 2012; Skripuletz et al., 2011; Zendedel et al., 2013; Praet et al., 2014). Subsequently, demyelinated axons became more vulnerable to attacks by brain intrinsic and extrinsic immune cells and inflammatory mediators owing to the lack of trophic support by myelin sheaths and higher energy demand for the conduction of action potentials in the presence of higher $Na+$ channel density (Smith and Lassmann, 2002; Craner et al., 2004; Zendedel et al., 2013). Demyelination is, however, transitory and followed by spontaneous remyelination which depends on numerous factors that protect injured cells and/or stimulate the proliferation of remaining oligodendrocyte progenitor cells (OPCs) and their differentiation into mature OLGs that interact with the denuded axons (Franklin and French-Constant, 2017). In this context it was also emphasized that the remyelination might be diminished not only due to precursor cell depletion, but also owing to the presence of a nonpermissive local environment and defective activation of signaling pathways

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Abbreviations: CPZ, copper chelator cuprizone; DG, dentate gyrus; GCL, granular cell layer; LDL-R, low-density lipoprotein receptor; MTs, metallothioneins; OLGs, oligodendrocytes; OPCs, oligodendrocyte progenitor cells; PBS, phosphate-buffered saline; PLA, proximity ligation assay; ROIs, regions of interest; SVZ, subventricular zone.

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that provide protective effects following CPZ intoxication (Kipp et al., 2009; Praet et al., 2014).

A large body of evidence implies that to the later events also contribute cysteine-rich metallothioneins (MTs), which during CNS injury show both neuroprotective and neuroregenerative properties (Hidalgo et al., 2001; Aschner and West, 2005; Penkowa, 2006; Fitzgerald et al., 2007; West et al., 2008; Pedersen et al., 2009). Their function has been attributed not only to intracellular free radical scavenging and to zinc and copper regulation in injured cells (Coyle et al., 2002; Maret, 2011) but also to the ability of secreted MT to bind on surface receptors belonging to the family of low-density lipoprotein receptor (LDL-R)-related proteins (LRP), such as LRP-2/megalin and LRP-1, which in turn activate the signal transduction pathways that support neurite outgrowth and survival (Ambjørn et al., 2008; Chung et al., 2008b; Asmussen et al., 2009; West et al., 2011; Auderset et al., 2016). In this regard it was shown that MT and its synthetic analog EmtinB might directly stimulate neurite outgrowth and promote survival of cerebellar granule neurons *in vitro* (Ambjørn et al., 2008), as well as that native MT-2A might block copper-mediated deposition of insoluble extracellular β-amyloid (Aβ) plaques and toxicity in rat cortical neurons (Chung et al., 2010). Accordingly, it was suggested that MTs might have a significant therapeutic potential in megalin-mediated mechanisms, which are involved in the pathogenesis of Alzheimer's (AD) and other neuropathological diseases (Ambjørn et al., 2008; Chung et al., 2008b; Asmussen et al., 2009; West et al., 2011; Auderset et al., 2016). Moreover, implying that similar anti-oxidant and anti-inflammatory mechanisms might be activated also in CPZ model of demyelination, in glial cells and in OLGs and OPCs were found an overexpression of MT-I/II mRNA and proteins, elevated levels of TGF-β and IL-10 and increased activities of NAD-linked cytoplasmic oxidoreductase and glycerolphosphate-3 dehydrogenase (Biancotti et al., 2008).

In an attempt to provide further insights into the potential neuroprotective function of MTs, in the present study we made an immunohistochemical spatio-temporal profiling of MT-I/II expression and distribution throughout CNS tissue during acute and chronic CPZ demyelination, induced by different CPZ-feeding protocol, with a focus on possible interaction of MT-I/II with megalin/ LRP-2 and their involvement in processes of de- and remyelination.

EXPERIMENTAL PROCEDURES

Experimental animals

Experiments were performed on female C57BL/6 mice (9–10 weeks of age). They were housed 4–5 per cage under standard conditions of light, temperature and humidity with unlimited access to food and water. Experimental procedures involving animals complied with Croatian laws and rules (NN 135/06; NN 37/13; NN 125/13; NN 055/2013) and with the guidelines set by European Community Council Directive (86/609/EEC). Experimental protocol was approved by the Ethics Committee of the University of Rijeka.

Cuprizone administration

The cuprizone model was performed according to the previously described protocols (Torkildsen et al., 2008; Kipp et al., 2009; Zendedel et al., 2013). To induce demyelination mice were fed with a 0.25% (w/w) cuprizone/CPZ) [finely powdered oxalic bis (cyclohexylidene-hydrazide); Sigma–Aldrich C9012-25G; Germany] homogeneously blended in the standard food for laboratory animals. Feeding lasted 1, 3 or 5 weeks and mice were sacrificed next day after the expiration of the dietary intoxication protocol or 2 weeks after the withdrawal of CPZ given for 5 weeks (group 5 + 2 weeks). Control mice received the same chow without cuprizone. At the end of the experiment mice were anesthetized by combination of Ketamine (80 mg/kg) and Xylasine (5 mg/kg), given by intraperitoneal (i.p.) injection, transcardially perfused with cold phosphate-buffered saline (PBS, 10 mM, pH 7.4) and 4% paraformaldehyde (PFA, SIGMA, Germany) and subsequently sacrificed by exsanguinations in deep anesthesia, according to the guidance of European Community Council Directive (86/609/EEC) and recommendation of National Centre for the Replacement, Refinement and Reduction of Animals in Research (www.nc3rs.org.uk).

Tissue preparation for paraffin slices

Tissue samples of the brain were rapidly removed from six animals/group, coronally or sagittally dissected after perfusion and fixed in 4% paraformaldehyde solution (Sigma, Germany) during 24 h. Tissue was embedded in paraffin wax and cut using HM 340E microtome (Microtom, Germany). The tissue sections (4 μm) were deparaffinized, rehydrated and subjected to heat-induced antigen retrieval (0.01 M Sodium Citrate pH 6.0).

Immunohistochemistry

Immunohistochemical labeling of MT I + II and megalin proteins was performed on paraffin embedded tissues using DAKO EnVision + System, Peroxidase (DAB) kit according to the manufacturer's instructions (DAKO Cytomation, USA), as previously described (Jakovac et al., 2011). Briefly, slices were incubated with peroxidase block to eliminate endogenous peroxidase activity. After washing, mouse monoclonal anti-MT I + II IgG1 (clone E9; Dako Cytomation, USA; diluted 1:50 with 1% BSA in PBS), rabbit polyclonal anti-megalin IgG (H-245, Santa Cruz Biotechnology, USA; diluted 1:200 with 1% BSA in PBS), rabbit polyclonal anti-GFAP IgG (Abcam, UK; diluted 1:5000 with 1% BSA in PBS) or rabbit polyclonal anti-myelin PLP IgG antibodies (Abcam, UK, diluted 1:1000 with 1% BSA in PBS) were added to tissue samples and incubated overnight at 4 °C in a humid environment, followed by a 45-min incubation with peroxidase-labeled polymer conjugated to goat anti-mouse or anti-rabbit immunoglobulins containing carrier protein linked to Fc fragments to prevent nonspecific binding. The immunoreactions' product was visualized by adding

substrate chromogen (DAB) solution. Tissues were counterstained with hematoxylin, dehydrated through graded ethanol, and mounted using Entelan (Sigma–Aldrich, Germany). The photomicrographs were taken and examined under Olympus BX51 light microscope (Olympus, Japan).

Immunofluorescence

Immunofluorescent labeling was performed on paraffin embedded brain tissue slides. Tissue sections were submitted to heat-induced antigen retrieval and nonspecific binding was blocked by a one-hour incubation with 1% BSA in PBS containing 0.001% NaN3 at room temperature. Different combinations of double immunofluorescence labeling were then carried out combining suitable primary and secondary antibodies, to detect expressions of MTs and megalin on astrocytes (GFAP+ cells), neural and glial progenitors cells and neuroblasts (GFAP+, nestin+, DCX+, NG2+, O4+ cells) and mature neuronal cells (NeuN+ cells), as well as their co-expression in different regions of the brain. For that purpose the following primary antibodies were used: mouse anti-MT I + II IgG1 (clone E9; Dako Cytomation, USA; diluted 1:50), rabbit anti-megalin IgG (H-245, Santa Cruz Biotechnology, USA; diluted 1:50), rabbit anti-GFAP IgG (Abcam, UK; 1:5000), rabbit anti-myelin PLP IgG (Abcam, UK, 1:1000), rabbit anti-nestin IgG (Sigma–Aldrich, USA, 1:100), rabbit anti-NG2 IgG (Merckmillipore, USA, 1:200), rabbit anti-NeuN IgG (Abcam, UK, 1:100), rabbit anti-doublecortin IgG (Abcam, UK, 1:500), mouse anti-O4 IgM (R&D Systems, USA, 1:200), mouse anti-PCNA IgG2a (Abcam, UK, 1:1000) and mouse anti-AKT1 (phospho T308) IgG1 (Abcam, UK, 1:50).

Primary antibodies were diluted in blocking solution and incubated with tissue sections overnight at 4 °C in a humid environment. To visualize immunocomplexes, the following secondary antibodies were used: Alexa Fluor goat anti-mouse IgG1 555 nm (Molecular probes, USA, 1:500), Alexa Fluor donkey anti-rabbit IgG 488 nm (Molecular Probes, USA, 1:300), Alexa Fluor donkey anti-rabbit IgG 594 nm (Molecular Probes, USA, 1:500), Alexa Fluor goat anti-mouse IgG2 488 nm (Molecular probes, USA, 1:300), Alexa Fluor goat anti-mouse IgM 488 nm (Molecular probes, USA, 1:300) and Alexa Fluor goat anti-mouse IgG1 488 nm (Molecular probes, USA, 1:300).

Secondary antibodies were diluted in blocking solution and incubated with tissue sections in dark for 1 h at room temperature in a humid environment. Nuclei were visualized with 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI, Molecular Probes, USA). Images were captured on Olympus imaging system BX51 equipped with DP71CCD camera (Olympus, Tokyo, Japan) and CellF imaging software was used.

Proximity ligation assay

To determine metallothionein I + II and megalin interaction, we performed proximity ligation assay (PLA) on paraffin embedded tissue sections using Duolink PLA *in Situ* Fluorescence kit (Sigma–Aldrich, Germany) according to the manufacturer's protocol with custom blocking and antibody diluent solution. Briefly, after dewaxing and washing with PBS, slides were incubated for 1 h at room temperature with the same blocking solution as used for immunofluorescence studies (1% BSA in PBS) but without NaN3. Subsequently, slides were incubated overnight at 4 °C in a humid environment with mouse anti-MT I + II IgG1 (clone E9; Dako Cytomation, USA) and rabbit anti-megalin IgG (H-245, Santa Cruz Biotechnology, USA), both diluted 1:50 in blocking solution. After washing in PBS, anti-rabbit MINUS and anti-mouse PLUS PLA probes diluted 1:5 with 1% BSA in PBS and sited at room temperature for 20 min were added to tissue slides and incubated in a pre-heated humidity chamber for 1 h at +37 °C. Afterward, slides were washed with 1× Wash Buffer A (prepared according to enclosed instructions) and the ligation process was performed by incubating slides with Ligation-Ligase solution (Ligation stock diluted 1:5 in nuclease-free water with immediately added ligase at a 1:40 dilution) in a pre-heated humidity chamber for 30 min at +37 °C. After washing in 1× Wash Buffer A, amplification was done by incubating samples with Amplification-Polymerase solution (Amplification stock diluted 1:5 in nuclease free water with immediately added Polymerase at a 1:80 dilution) in a pre-heated humidity chamber for 100 min in the dark at +37 °C. Tissue slides were subsequently washed twice in 1× Wash Buffer B (prepared according to enclosed instructions) for 10 min, and then twice in 0.01× Wash Buffer B for 1 min, continuously protected from the light. Nuclei were visualized by DAPI staining (1:1000 in PBS for 5 min; Invitrogen, USA). Slides were afterward washed in PBS and mounted with Mowiol (Sigma–Aldrich, Germany). The microphotographs were taken under fluorescent microscope equipped with DP71CCD camera (Olympus, Japan) and CellF imaging software.

Immunohistochemical staining quantification

Immunohistochemical staining quantification of MT, GFAP, PLP and megalin expression in different regions of the brain was performed using Image J software. Captured images were converted to 16-bit images based on gray-scale with different gray intensity range, depending on the strength of immunohistochemical signals. Digital background subtraction was done and intensities were inverted in order to achieve positive correlation of staining intensity and brightness as a gray intensity. Withal, threshold was manually set so that any background brightness was considered as the value 0, and background signals were completely excluded from the calculation. Fifteen regions of interest (ROIs) were arranged to cover the area being analyzed and mean gray values were measured. ROI surface size always was equal for each analyzed area. The measurements were made on three separate slides per animal, in five animals/group. The data were expressed as mean gray value ± SE.

Statistical analysis

The statistical analyses were performed using Statistica software version 12 (StatSoft Inc., Tulsa, OK, USA). The distribution of data was tested for normality using the Kolmogorov–Smirnov test. Differences between groups were assessed with a one-way analysis of variance (ANOVA) followed by the post hoc Scheffé test. All data are given as arithmetic mean ± standard error of the mean (SEM). The level of significance was set at $p < 0.05$.

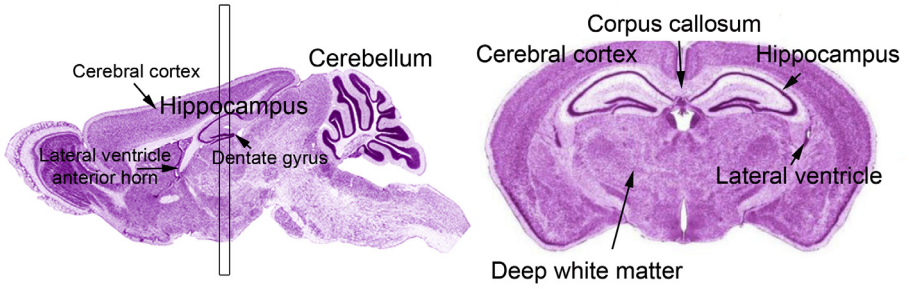
RESULTS

Expression of metallothionein-I + II and megalin in the brain of mice subjected to different cuprizone feeding protocols

Expression profiling of metallothionein-I + II and megalin was made in female C57BL/6 mice fed by 0.25% CPZ (w/w) during 1, 3 and 5 weeks and in those fed by CPZ during 5 weeks and sacrificed 2 weeks after CPZ withdrawal. The results were compared with findings in mice maintained on normal chow (Fig. 1).

MT-I/II immunoreactivity. In mice maintained on normal diet the expression of MT-I/II proteins in the brain was low and restricted mostly to ependymal cells and subventricular zone (SVZ) (Fig. 2A a). CPZ-supplemented diet induced, however, a high MT overexpression. It was observed already one week after feeding with CPZ (Fig. 2B a–f), but the greatest upregulation of MT immunoreactivity was found in mice fed with CPZ for five weeks and sacrificed immediately after the treatment with CPZ (Fig. 2B m-s; $p < 0.001$ in comparison with mice fed by standard food; Fig. 2C). In all groups the expression of MTs arose in white matter (corpus callosum and capsula interna (Fig. 2B a, g, m and, b, h, n, respectively) and in hilus of cerebellum (s), but also in the cortex (c, i, o) and in granular and molecular layer of cerebellum (f, l, s, w). Besides, strong MT immunostaining was found particularly in SVZ of lateral ventricles and in SGZ and molecular layer of the dentate gyrus (DG) (Fig. 2B d, j, p, z and k, l, e, y, respectively), pointing to the involvement of MTs in the processes of adult neurogenesis. Most of the MT-positive cells in white matter and cortex had astrocyte-like features and around many of them a strong extracellular MT immunoreactivity was found, suggesting that MT-I/II were secreted from these cells (Fig. 2B n, o, y). The semi-quantitative analysis also showed that MT-I/II immunostaining in the brain of CPZ-fed mice was in all regions significantly greater than that found in mice maintained on normal chow (Fig. 2C).

Megalin immunoreactivity. Feeding with CPZ-supplemented diet induced also the upregulation of the megalin, a suggested receptor for MTs. As shown in Fig. 3B (g–l) megalin immunoreactivity started to rise in



Group /Week	1	2	3	4	5	6	7
Cuprizone 0.25% (w/w)	CPZ						
	CPZ	CPZ	CPZ				
	CPZ	CPZ	CPZ	CPZ	CPZ		
	CPZ	CPZ	CPZ	CPZ	CPZ	Standard food	Standard food
Control	Standard food	Standard food	Standard food	Standard food	Standard food	Standard food	Standard food

Fig. 1. Study design. Female C57BL/6 mice were fed with a 0.25% cuprizone (CPZ) (w/w) supplemented diet during 1, 3 and 5 weeks. They were sacrificed next day after the expiration of the dietary intoxication protocol or 2 weeks after the withdrawal of CPZ given for 5 weeks (group 5 + 2 weeks). Control mice received the same chow without CPZ. At the end of the experiment six animals/group were deeply anaesthetised with a combination of Ketamine and Xylasine, transcardially perfused with cold phosphate-buffered saline (PBS, 10 mM, pH 7.4) and 4% paraformaldehyde and sacrificed by exsanguinations. Tissue samples of the brain were rapidly removed, coronally or sagittally dissected and fixed in 4% paraformaldehyde solution during 24 h. Paraffin slices were prepared for immunohistochemical and immunofluorescent determination of metallothioneins and megalin expression and phenotypic analysis of affected cells. On representative coronal and sagittal microphotographs of the mouse brain (Sidman et al., 2004) http://www.hms.harvard.edu/research/brain/atlas.html) the subfields examined in this study are shown (cerebral cortex, deep white matter, corpus callous, hippocampus, lateral ventricle, cerebellar cortex and medulla). The line on the sagittal view denotes the area of the coronal sectioning.

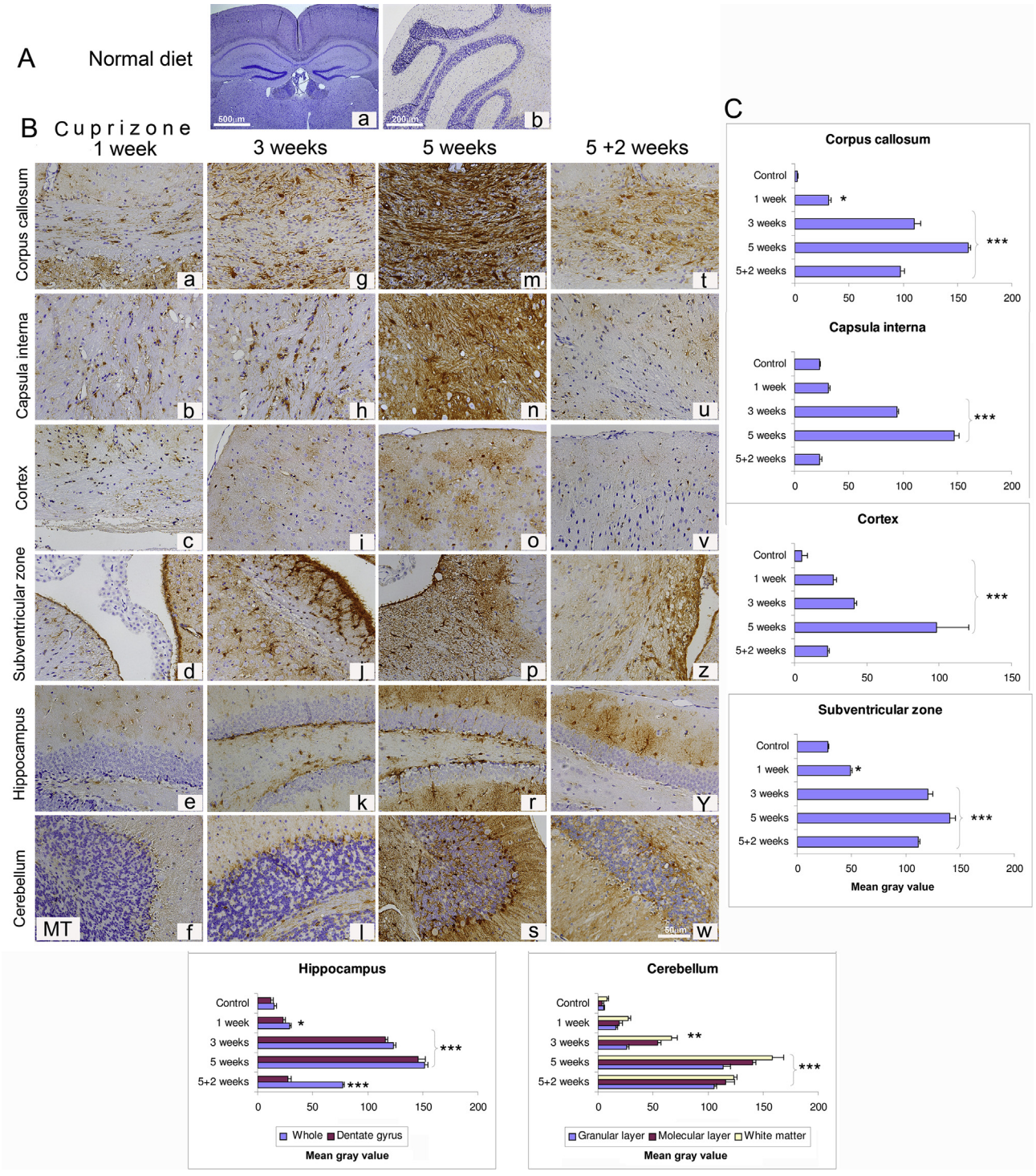


Fig. 2. Cuprizone-supplemented diet induces the upregulation of MT-I/II immunoreactivity in the brain. Representative immunohistochemical pictures show staining with anti-MT-I/II antibody in paraffin-embedded sections of the brain tissue obtained from C57BL/6 mice fed: (A) by normal chow and (B) by 0.25% CPZ (w/w) during the one, three and five weeks and sacrificed immediately after CPZ feeding or after 7 weeks (5 weeks of CPZ feeding +2 weeks of normal diet). (C) MT-I/II immunoreactivity in different brain regions. The measurements were made by ImageJ software analysis on 15 regions of interest (3 ROI/mouse × 5 animals/group). Values are expressed as mean gray value ± SE ($N = 15$). $^{*}p < 0.05$. $^{**}p < 0.01$ and $^{***}p < 0.001$ in comparison with mice fed by normal diet.

mice fed with CPZ for 3 weeks but, similarly as found for MTs, its expression was maximally upregulated in tissues of mice treated with CPZ for 5 weeks (Fig. 3B m–s). At that time a high number of megalin-positive cells were found in corpus callosum (Fig. 2B m) and in capsula interna (Fig. 3B n), but particularly in frontal cortex, where megalin immunoreactivity was found mainly in the nuclei of these cells (Fig. 3B o). Besides, high megalin immunoreactivity was found in choroid plexus (Fig. 3B p), in SVZ and SGZ of DG (Fig. 3B r),

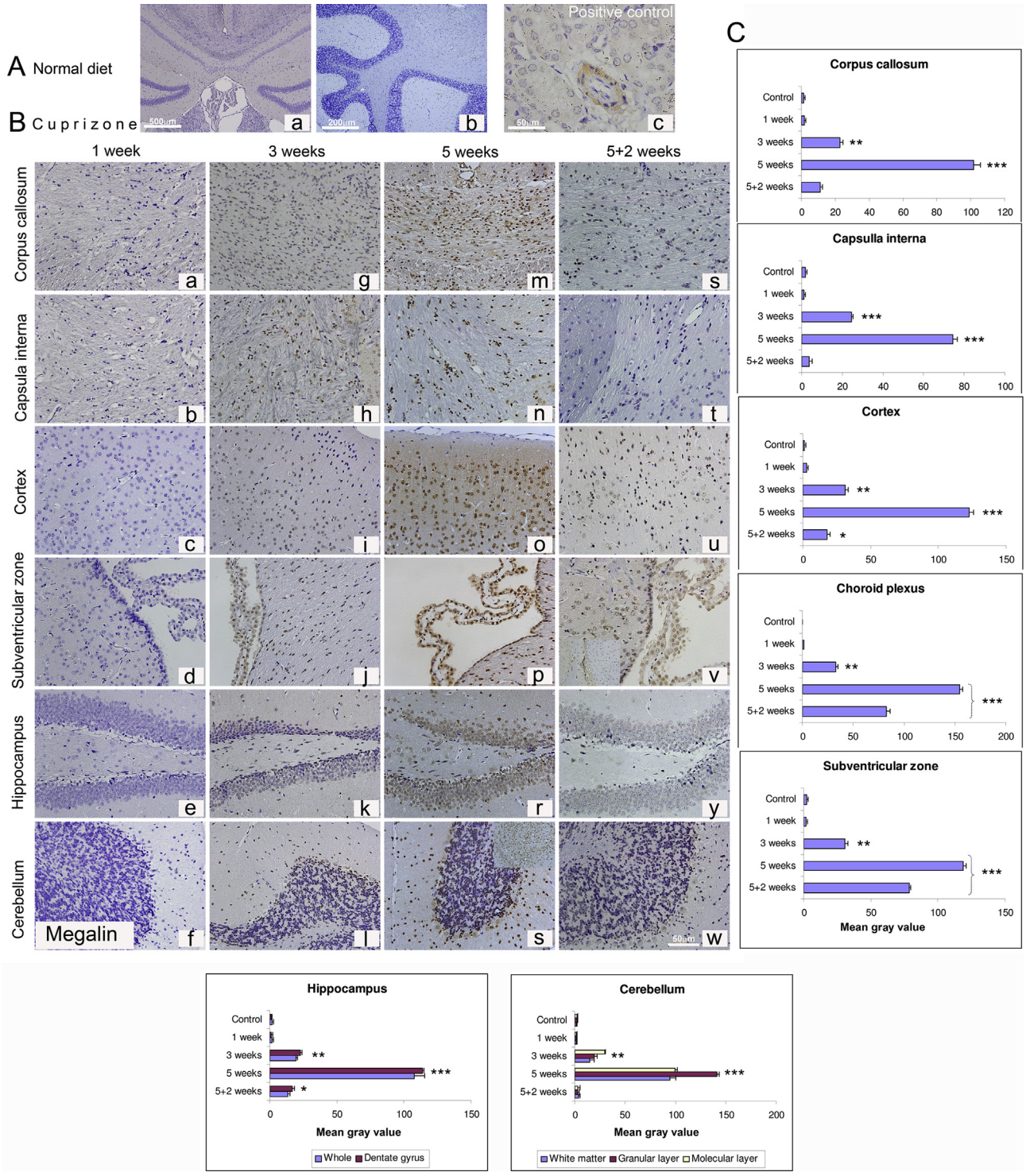


Fig. 3. Cuprizone-supplemented diet induces the upregulation of megalin/LRP-2 immunoreactivity in the brain. Representative immunohistochemical pictures show staining with anti-megalin antibody in paraffin-embedded sections of the brain tissue obtained from C57BL/6 mice fed: (A) by normal chow and (B) by 0.25% CPZ (w/w) during the one, three and five weeks and sacrificed immediately after CPZ feeding or after 7 weeks (5 weeks of CPZ feeding +2 weeks of normal diet). (C) Megalin immunoreactivity in different brain regions. The measurements were made by ImageJ software analysis on 15 regions of interest (3 ROI/mouse × 5 animals/group). Values are expressed as mean gray value ± SE ($N = 15$). $^{*}p < 0.05$. $^{**}p < 0.01$ and $^{***}p < 0.001$ in comparison with mice fed by normal diet.

as well as in cerebellar cortex (granular and molecular layers) and cerebellar white matter (Fig. 3B s). The quantitative analysis also showed that megalin immunostaining in the brain of CPZ-fed mice was significantly greater than that in mice maintained on normal chow (Fig. 3C).

The described data clearly showed that intoxication with CPZ led to upregulation of both MT-I/II and their

receptor, as well as that the feeding with CPZ for 5 weeks could be used as the most suitable protocol for the characterization of phenotype of involved cells and further analysis of relationship between the MTs and megalin in different brain areas.

Relationship of MT-I/II to processes of demyelination and astrogliosis induced by CPZ

The data obtained by immunohistochemical determination of proteolipid protein (PLP) (Fig. 4A) and GFAP (Fig. 4B) showed that CPZ-supplemented diet after 5 weeks led to high demyelination and marked astrogliosis in white matter of cerebrum and cerebellum. PLP immunoreactivity was predominantly reduced in corpus callosum (Fig. 4A a, b versus e, f) and cerebellar medulla (d versus h), but also in hippocampus (Fig. 4A c versus d). Staining with anti-GFAP antibodies also showed that in these zones were present in numerous hypertrophic astrocytes (Fig. 4B e–l). The semi-quantitative analysis of PLP and GFAP expression in these brain regions also showed that changes induced by CPZ feeding were significantly different from those found in untreated mice (Fig. 4C $p < 0.01$ and $p < 0.001$), confirming that chronic CPZ intoxication led to high demyelination in the brain and subsequent massive astrogliosis (Torkildsen et al., 2008; Kipp et al., 2009; Zendedel et al., 2013). Besides, the data obtained by double immunofluorescent labeling with anti-PLP and anti-MT + I + II antibodies clearly showed that in demyelinating zones of corpus callosum and capsula interna were present numerous astrocyte-like cells that expressed high cytoplasmic MT immunoreactivity (Fig. 5a–d). In addition, the granular expression of MT on astrocytic or neuronal filaments in white matter was found (Fig. 5 g, h, arrows).

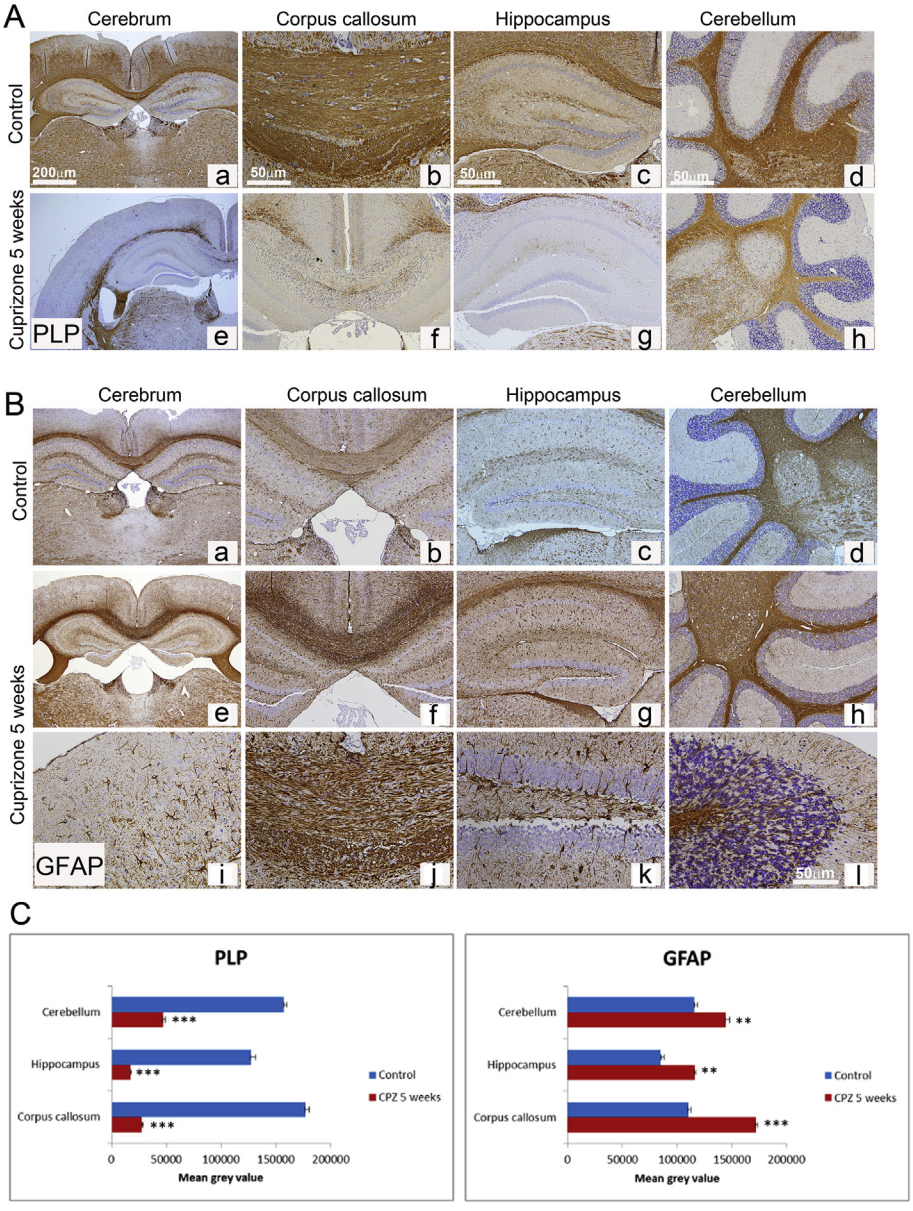


Fig. 4. Cuprizone-supplemented diet induces acute demyelination and massive astrogliosis in the brain. Representative immunohistochemical pictures show: (A) staining with anti-PLP antibodies and (B) staining with anti-GFAP antibodies in paraffin-embedded sections of the brain tissue obtained from C57BL/6 mice fed by normal chow (a–d) and from mice fed by 0.25% CPZ (w/w) during the five weeks and sacrificed immediately after CPZ feeding (e–l). (C) PLP and GFAP immunoreactivity in corpus callosum, hippocampus and cerebellum. The measurements were made by ImageJ software analysis on 15 regions of interest (3 ROI/mouse × 5 animals/group). Values are expressed as mean gray value ± SE ($N = 15$). $^{**}p < 0.01$ and $^{***}p < 0.001$ in comparison with mice fed by normal diet.

MT/Megalin interactions in CPZ-affected brain regions

Owing to current postulation that astrocyte-derived MTs may modulate cell signaling and neuronal growth and repair through direct contact with megalin (Ambjørn et al., 2008; Chung et al., 2008a; West et al., 2011) we attempted to visualize also the possible interaction between the cells expressing MT and megalin in brain regions that were highly affected by CPZ intoxication using double immunofluorescence and PLA.

Corpus callosum and capsula interna. In this context double staining with anti-GFAP and anti-MT antibodies confirmed that feeding with CPZ for five weeks induced a high astrogliosis in corpus callosum and appearance of numerous hypertrophic GFAP-positive astrocytes that co-expressed MT (Fig. 6A a–f). Moreover, around many of them a high extracellular MT immunoreactivity was clearly seen

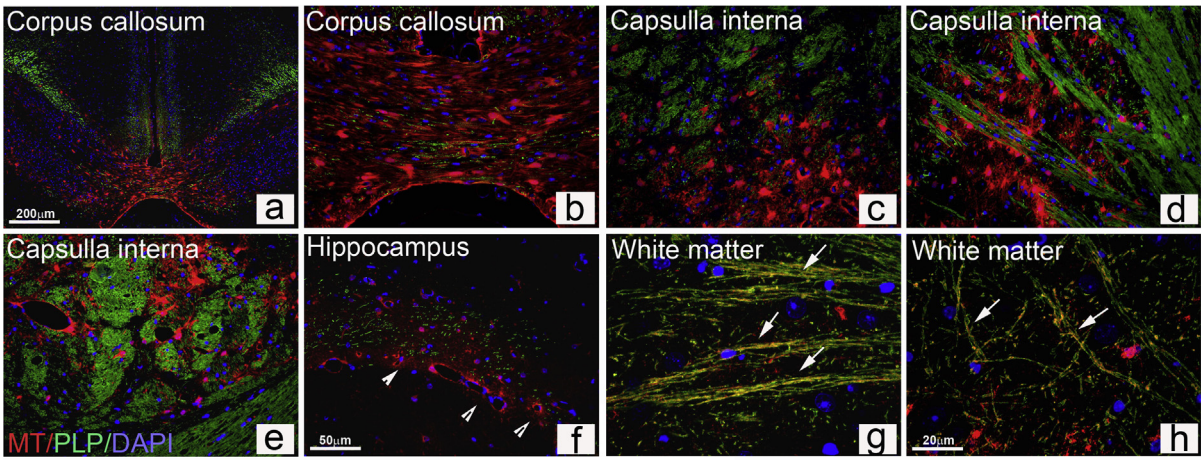


Fig. 5. In CPZ-fed mice the MT-I/II overexpression is induced on astrocyte-like cells in areas of demyelination. Representative immunofluorescent pictures show staining with anti-PLP and anti-MT-I/II antibodies in paraffin-embedded sections of the brain tissue obtained from C57BL/6 mice fed by 0.25% CPZ (w/w) during the five weeks and sacrificed immediately after CPZ feeding (e–l).

(Fig. 6A f, arrows). The data also implied that in white matter of CPZ-treated mice were present oligodendrocyte progenitors, since numerous cells were stained by monoclonal antibody against NG2 chondroitin sulfate proteoglycan and O4 antigen (Fig. 6A g–i). Only some of them co-expressed MT-I/II (Fig. 6A g–h, arrows), but according to the staining with anti-PCNA antibodies many of MT+ cells were surrounded by mitotically active cells (Fig. 6D).

Double staining with anti-MT and anti-megalin antibodies also showed that in corpus callosum and white matter were present numerous small (Fig. 6B a–c) and some large megalin+/MT- cells, located near the cortical zone (Fig. 6B d). Staining with NeuN antibodies visualized that beneath corpus callosum were present some megalin-positive neurons (Fig. 6C, arrows).

Cortex. Cuprizone feeding during 5 weeks increased also the number of GFAP+/MT+ astrocytes in cortical gray matter and similarly as it was found in the white matter, around many of them the extracellular MT immunoreactivity was seen (Fig. 7A a–c, arrows). Besides, implying that in this area the secreted MTs were recognized by their receptors and internalized into the cell, in the cortex were found numerous megalin-positive cells, which showed MTs immunoreactivity in their membranes or in cytoplasm (Fig. 7B a–c, inserts). The neuronal phenotype of these cells was confirmed by co-staining with NeuN (Fig. 7C a–c). Moreover, implying that MT was internalized also in some oligodendrocyte progenitors the spots of MTs immunoreactivity were found in NG2+ cells localized near the white matter (Fig. 7A d, e) and on numerous O4 cells (Fig. 7A f).

The described data, obtained by double immunofluorescence, clearly showed that MT-I/II and megalin co-localize in the NeuN+ cortical neurons and in some oligodendrocyte progenitors (Fig. 7), implying that in CPZ-intoxicated mice extracellular MTs might *via* the megalin/LRP2 modulate functional state of target cells. To proof this possibility and to visualize the protein–protein interactions we used the PLA, which enables *in situ* recognition of two potentially interacting proteins using a pair of specific antibodies conjugated to a matched pair of short single-stranded oligonucleotides (Söderberg et al., 2008). Besides, since numerous studies have shown that ligation of megalin/LRP2 receptor may activate AKT1/Protein kinase B (PKB, RAC-alpha serine threonine-protein kinase), which through phosphorylation and inhibition of a range of downstream substrates regulates multiple signaling pathways, including neuronal survival (Noshita et al., 2001; Li et al., 2001a,; Endo et al., 2006; May et al., 2007; Ambjørn et al., 2008; Auderset et al., 2016), in megalin-positive and NeuN-positive cells we estimated also the presence of phospho-AKT1 (pAKT1) as a result of MT-I/II/megalin interaction. The data obtained by PLA and with antibody pair against MT-I/II and megalin clearly confirmed the MT-I/II not only co-localized with megalin (Figs. 7B a–c and 8A a–c), but also interacted with its receptor in cortical tissue of mice fed by CPZ-supplemented diet for 5 weeks (Fig. 8A c–d). Moreover, double immunofluorescence analysis with antibodies against megalin and pAKT1 (phospho threonine 308), and those against NeuN and pAKT1 revealed that in numerous megalin-positive cells was upregulated the expression of phospho-Akt (Fig. 8B a–c), as well as that it was localized particularly in nucleus of cortical NeuN-positive neurons (Fig. 8C a–c). Since similar changes were not observed in untreated mice (Fig. 8B d–f and C d–f), the data imply that in CPZ-treated mice highly expressed megalin/LRP2 contributed not only to the internalization of extracellular MT-I/II into neurons, but also to triggering of signal transduction pathways that protect the cell against the toxic effects of CPZ.

Cerebellum. The data obtained by double immunofluorescence confirmed that intoxication with CPZ induced a high MT-I/II upregulation in both cerebellar hilus (Fig. 9A a) and cerebellar cortex (Fig. 9A d), as well as that majority of these cells were GFAP+ astrocytes (Fig. 9A b, c) and nestin+ Purkinje cells (Fig. 9 e, f). Moreover, the data showed that in

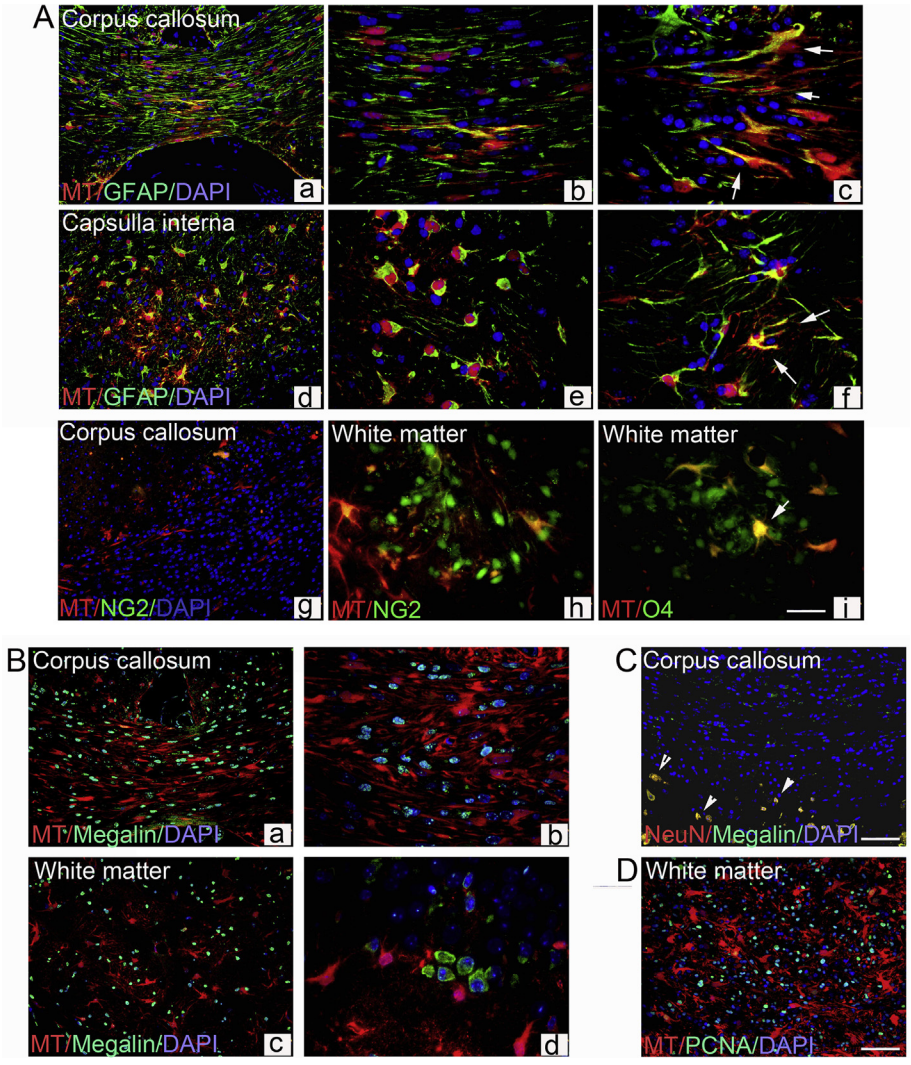


Fig. 6. In cerebral white matter of mice feed by cuprizone-supplemented diet are present astrocytes that abundantly express MT I + II and juxtapositioned cells that express NG2, megalin and PCNA Representative immunofluorescent pictures show: (A) relationship of MT+ cells to GFAP+ astrocytes (a–f) and NG2+ or O4+ cells (g–i), B) relationship of MT+ cells to megalin-positive cells (a–d); C) NeuN+ cells co-expressing megalin and D) relationship of MT+ cells to PCNA+ cells. Brain tissue was obtained from C57BL/6 mice fed by 0.25% CPZ (w/w) during the five weeks and sacrificed immediately after CPZ feeding.

granular layer were present numerous megalin+ cells Fig. 9B a–f), that were in direct contact with MT+ cells in molecular layer (Fig. 9B b, c), as well as that several megalin+ cells were NeuN+ neurons (Fig. 9B d–f).

Hippocampus. Double immunofluorescence with anti-MT-I/II antibodies and antibodies against stage-specific markers typical for neurogenic and gliogenic stem/progenitor cells (Fig. 10A) clearly demonstrated that MTs might have a high influence on processes of self-renewal in neurogenic niches located in SGZ of hippocampus and in SVZ of the lateral ventricle.

In the SGZ of DG of mice fed by CPZ for 5 weeks we, thus, found MT immunoreactivity in numerous radial glia-like cells expressing GFAP and on their protrusions extending into the granular cell layer (GCL) (Fig. 10B a–c), on some cells expressing nestin (Fig. 10B g–i), as well as in several DCX+ cells (Fig. 10B, j–l), implying that MT had affected type I cells, non-radial precursors (type II cells) and intermediate progenitor cells or neuroblasts (type 2b or type 3 cells) (Kempermann et al., 2004; Ming and Song, 2011). Moreover, MT-immunoreactivity was found in several NG2+ oligodendrocyte progenitors in molecular layer of DG (Fig. 10B m–o), pointing to the potential influence of MTs on cells destined to oligodendroglial lineage. In addition, we found MTs expression in vascular and GFAP+ cells in the hippocampal hilus (Fig. 10B f), in some large GFAP+ cells in CA1 region of hippocampus (Fig. 10B, insert on f), as well as in some mature NeuN+ neurons in GCL (Fig. 10B p–s).

The data also showed that in SGZ numerous cells co-expressed MT-I + II and megalin immunoreactivities (Fig. 10C a–c). According to the phenotypic markers some of them were nestin + cells, located in GCL and in the hilus (Fig. 10C d–f) and DCX+ neuroblasts migrating into GCL (Fig. 10C g–i). Megalin immunoreactivity was, however, also found in some NeuN+ interneurons in the hilus and in all mature NeuN+ neurons in GCL of hippocampus (Fig. 10C j–l). The data obtained with PLA, also clearly showed that MT interacted with megalin in several cells located in subgranular, granular and molecular layer, as well as in CA1 region of hippocampus (Fig. 10D).

Subventricular zone. Similar analysis made in SVZ in anterior horn of lateral ventricle clearly showed that MT-I/II were also extensively expressed in this neurogenic niche, which generate new neurons that migrate through the rostral migratory stream (RMS) to the olfactory bulb to become different subtypes of interneurons (reviewed by (Zhao et al., 2008; Ming and Song, 2011; Gonzalez-Perez, 2012) (Fig. 11A). As shown on Fig. 11 high MT immunoreactivity was found in GFAP+ cells (Fig. 11B (a–c) and in ependymal cells lining the lateral ventricle, which surrounded the neurogenic niche containing numerous PCNA+ (Fig. 11B g–i) and DCX+ cells (Fig. 11B j–l), implying that in SVZ MT-I/II affected the radial-glia like cells (B cells) and contributed to the survival of transient amplifying cells (C cells) and neuroblasts

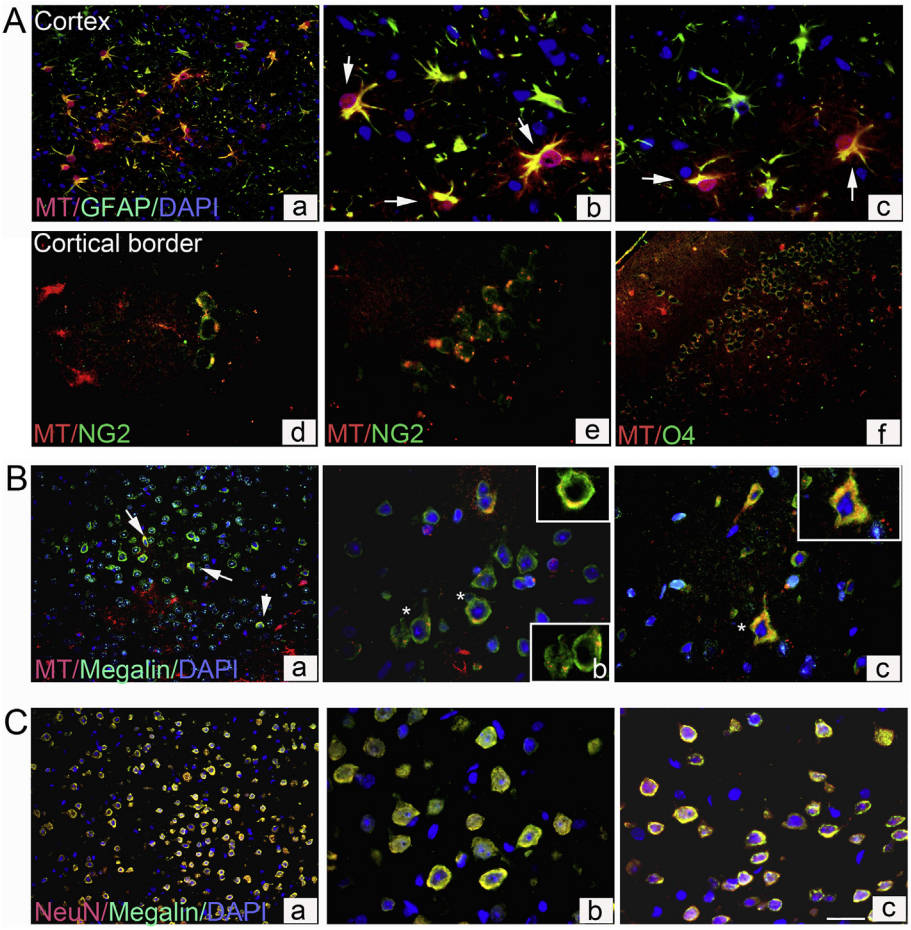


Fig. 7. In cerebral cortex of mice feed by cuprizone-supplemented diet are present astrocytes that abundantly express MT-I/II, NG2+ and O4+ oligodendrocyte progenitors that express MTs and NeuN+ neurons that express megalin and MTs A Representative immunofluorescent pictures show: (A) GFAP+ astrocytes (A a–c) and NG2+ or O4+ oligodendrocyte progenitors (A d–f) co-expressing MTs; (B) megalin+ cells co-expressing MTs (B, a–c); C) NeuN+ neurons co-expressing megalin (a–c). Brain tissue was obtained from C57BL/6 mice fed by 0.25% CPZ (w/w) during the five weeks and sacrificed immediately after CPZ feeding (e–l).

(A cells). DCX+ cells, however, did not co-express MT, similarly to mature NeuN+ cells located around the lateral ventricle (Fig. 11A p–s). In contrast, an extensive MT immunoreactivity was found in small and large nestin+ cells in layers adjacent to the lateral ventricle (Fig. 11B d–f), as well as in numerous NG2+ oligodendrocyte progenitors (Fig. 11B m–o), suggesting that MT might contribute to oligodendrocyte regeneration. The data also showed that several MT+ cells co-expressed the megalin (Fig. 11C a–c), as well as that this receptor was present on numerous nestin+ cells (Fig. 11B d–e) and on some DCX+ neuroblasts that formed a chain of migrating cells (Fig. 11C g–i). Importantly, the data obtained by PLA confirmed that in SVZ MT interacted with megalin in numerous cells (Fig. 11D).

DISCUSSION

In agreement with a number of previous reports in the current study we confirm that feeding of mice with CPZ leads to a robust demyelination and profound astrogliosis in different white and gray matter regions of the brain (Matsushima and Morell, 2001; Kipp et al., 2009; Skripuletz et al., 2011; Zendedel et al., 2013; Gudi et al., 2014; Praet et al., 2014), as well as to a high upregulation of MTs (Zatta et al., 2005; Biancotti et al., 2008). However, as a novelty we describe here the relationship of MT + I/II to their receptor megalin/LRP-2 and temporal and spatial distribution of these molecules during the CPZ-intoxication lasting 1, 3 or 5 weeks, as well as during reparatory, cuprizone-free period.

In this context our data show that MT expression in the brain correlated with the duration of CPZ intoxication (Fig. 2), as well as that in mice fed by CPZ for 5 weeks the intracellular MT immunoreactivity might be found particularly on hypertrophic and hyperplastic astrocytes in the demyelinating areas of white matter and in the cortex (Figs. 6 and 7) and in ependymal and epithelial cells surrounding the stem cells niche in SGZ and SVZ (Figs. 10 and 11). Moreover, supporting the hypothesis that some effects of MTs might be mediated by direct interaction of secreted MTs with their receptors (Aschner and West, 2005; Ambjørn et al., 2008; West et al., 2008; Chung et al., 2008b), we show herein that in several megalin-positive cortical neurons (Figs. 7 and 8) and in glial and neural stem/progenitors cells (Figs. 10 and 11) the membranous or cytosolic fluorescent immunostaining of MT-I/II might be found. Importantly, in these areas the direct MT-I/II/megalin interaction was also confirmed by PLA (Figs. 8A d–f, 10D and 11D). Moreover, in megalin-positive cortical neurons an increased phospho-Akt1 (Thr308) expression was shown (Fig. 8B, C a–c), indicating that in CPZ-fed mice the accelerated phosphorylation of Akt1/PKB might be associated with activation of cellular survival pathways.

The results are in high agreement with current knowledge showing that neuroprotective effects of MTs might be attributed to both intracellular and extracellular forms of MTs (Hidalgo et al., 1991; Aschner and West, 2005; Penkowa, 2006; Nielsen et al., 2007; West et al., 2008; Chung et al., 2008a; Pedersen et al., 2009), as well as with the postulation that binding of MTs to surface receptors of the LRP family might regulate the processes of MT endocytosis and activate signal transduction pathways that promote neurite outgrowth and survival (Ambjørn et al., 2008; Chung et al., 2008b; West et al.,

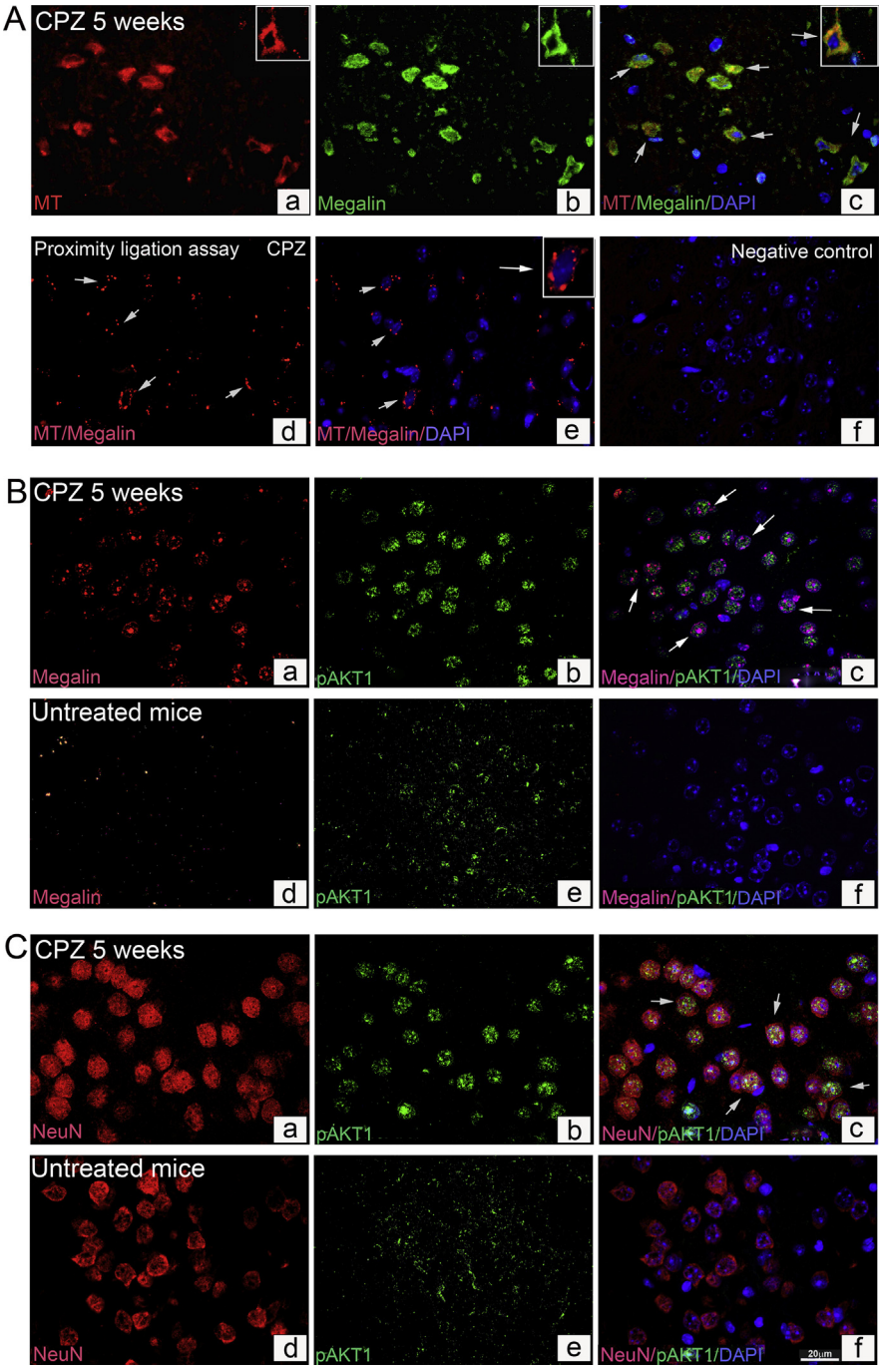


Fig. 8. In mice fed by cuprizone MT-I/II interacts with megalin in cortical neurons and enhances the phosphorylation of AKT1/Protein kinase B (RAC-alpha serine threonine-protein kinase). Representative images show: (A) Co-localization (orange) of MT-I/II (red) and megalin (green) in cortical neurons detected by double-immunofluorescent staining (a–c) and interaction between the MT-I/II and megalin (red fluorescence) detected by proximity ligation assay (c, d). Blue fluorescence (DAPI) is used as counterstain. (B) Upregulation of phospho-AKT1/Protein kinase B (phospho Thr308) (green) in megalin-positive cortical cells (red) detected by double immunofluorescence in the brain tissue of mice fed by CPZ during the five weeks (a–c) and in those maintained on normal chow (d–f). (C) Upregulation of phospho-AKT1/Protein kinase B (phospho Thr308) (green) in NeuN-positive cortical neurons (red) detected by double immunofluorescence in mice fed by CPZ (a–c) and in those maintained on normal chow (d–f). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2011) or affect other neuroprotective processes (Li et al., 2001a; May et al., 2007; Spuch et al., 2012; Auderset et al., 2016).

The mechanisms leading to MT upregulation during CPZ intoxication is still unclear, but it is well known that the transcription of this family of evolutionarily highly conserved small (6–7 kDa), metal-binding proteins is regulated by factors that affect the promoter region of the multigene complex containing metal-responsive regulatory elements (MREs), controlled by the metal regulatory transcription factor (MTF)-1, as well as antioxidant (or electrophile) response elements (AREs), signal transducer and activator of transcription 3 (STAT-3) and glucocorticoid response elements (GREs) that respond to redox status, cytokine signaling and stressful conditions, respectively. This leads to the protection of cells against the toxicity of divalent heavy metal cations and oxidative stressors, such as nitric oxide, peroxynitrite, hydrogen peroxide and superoxide providing thus resistance against apoptosis/necrosis induced by cytokines and other noxious insults (Andrews, 2000; Miles et al., 2000; Hidalgo et al., 2001; Coyle et al., 2002; Sato and Kondoh, 2002; Inoue et al., 2009; Maret, 2011).

Based on this knowledge we may assume that during CPZ intoxication the transcription of MT genes and synthesis of MT-I/II proteins were induced by combination of various triggers. One of the initial might be the induction of oxidative stress and mitochondrial damage since CPZ, as bidentate ligand with two hydrazides, selectively binds cupric ($Cu2+$) ions or stabilizes $Cu3+$ oxidation state in the cells (Faizi et al., 2016). This leads to *in situ* Cu chelation and alterations in proper functioning of metalloenzymes, such as Cu/Zn-superoxide dismutase, cytochrome c oxidase, ceruloplasmin, or dopamine beta-monooxygenase and to the dysfunction of oxidative phosphorylation and energy metabolism (Matsushima and Morell, 2001; Zatta et al., 2005; Gudi et al., 2014; Praet et al., 2014). Consequently,

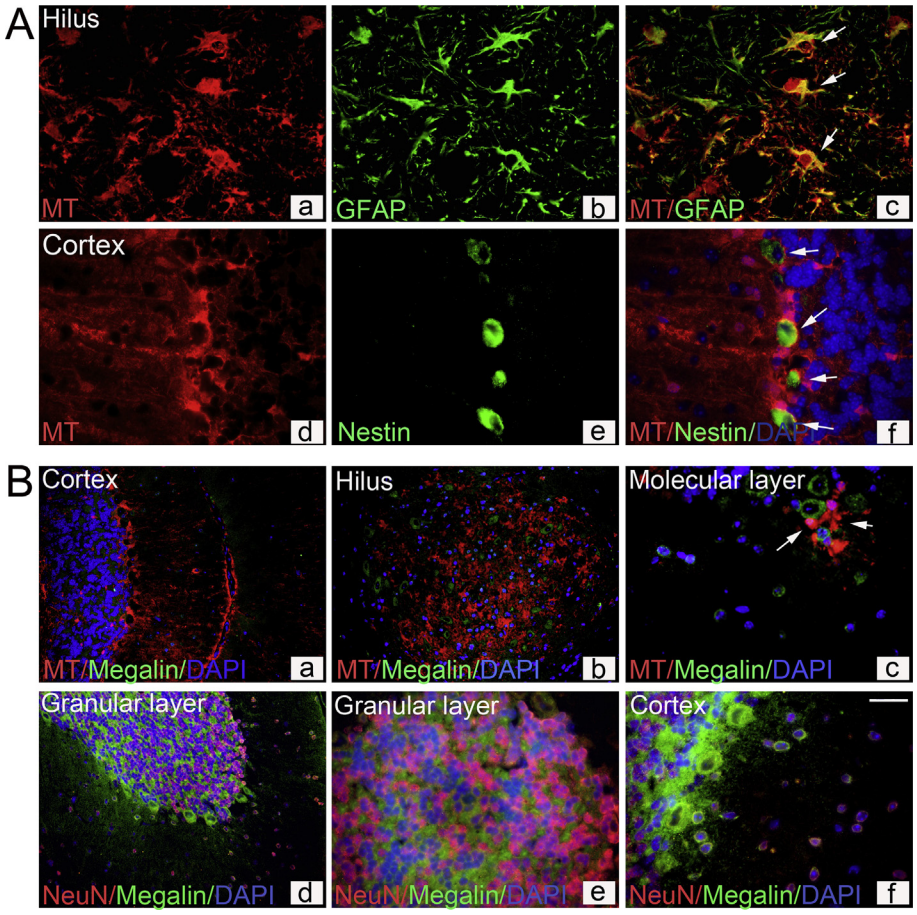


Fig. 9. Effect of cuprizone-supplemented diet on cerebellum. Representative immunofluorescent pictures show: (A) MT-I/II expression on GFAP+ astrocytes (a–c) and nestin+ cells (d–f). (B) Relationship of megalin+ cells to MT+ cells (a–c) and to NeuN+ neurons (d–e). Brain tissue was obtained from C57BL/6 mice fed by 0.25% CPZ (w/w) during the five weeks and sacrificed immediately after CPZ feeding (e–l).

primarily are attacked differentiated mature OLGs, which require high energy for myelin production, while other glial cells, such as microglia, astrocytes and OPC, as well as neuronal cells are less vulnerable to oxidative stress (Bénardais et al., 2013). Our findings imply that MTs contributed to this protection, similarly as the reports showing that following CPZ treatment OLGs express only low amounts of MTs, in contrast to astrocytes (Biancotti et al., 2008).

Neuroprotection may be based on antioxidant activity of MTs and scavenging of reactive oxygen species (ROS), but since antioxidant effect of MTs is coupled also with release of zinc ions there is a possibility that the activation of multiple Zn-dependent transcription factors and proteins contributed to MT-triggered neuroprotective processes (Mocchegiani et al., 2007; Levenson and Morris, 2011; Maret, 2011; Ruttkay-Nedecky et al., 2013). In addition, it should be taken into account that during CPZ intoxication the redox properties of MTs control also the release of Fe from ferritin and contribute to elimination of Fenton-mediated radical damage (Praet et al., 2014), as well as that MTs may preserve the mitochondrial structural and functional integrity by augmentation of coenzyme Q10, glutathione, ferritin, melatonin, and neuromelanin synthesis in neurons (Sharma et al., 2013). Furthermore, it was found that the autophagocytozed or up-regulated cytosolic MTs might reduce intralysosomal oxidation and lysosomal membrane destabilization induced by oxidative stress (Baird and Kurz, 2006).

Besides, we cannot exclude either the possibility that during CPZ intoxication the synthesis of MTs was stimulated by activation of MRE and STAT-3, since it was found that administration of CPZ in drinking water might increase the concentration of Cu and Zn in the brain and in the peripheral organs (Zatta et al., 2005), as well as that factors released from dying oligodendrocytes led to activation of macrophages and microglia and subsequent secretion of various chemokines, cytokines, and growth factors, which might promote the local inflammation and induce the attraction of astrocytes and OPC in demyelinating area (Pasquini et al., 2007; Scheld et al., 2016). Importantly, in this context it was shown that activation of an inflammatory response by a combination of growth factors led to myelin repair in cuprizone-induced demyelinated brain (Biancotti et al., 2008), as well as that CPZ demyelination might induce a unique inflammatory response particularly in SVZ neurogenic niche, which contributes to remyelination (Hillis et al., 2016).

Taken together these evidences show that during CPZ intoxication the synthesis of MTs might be induced by different triggers and that their cytoprotective and pro-regenerative actions might be related with intracellularly synthesized or receptor-internalized MTs, and with the activities of LRP-induced transduction pathways activated after binding of MTs to their receptors. The later proposal is in high agreement with current knowledge showing that extracellular MTs play an important role in the astrocyte–neuron response to injury (Aschner and West, 2005; West et al., 2008; Chung et al., 2008b) and with the findings that their neuronal effects might be mediated through direct binding to surface receptors belonging to the LDL-R family, such as megalin/LRP2 and LRP1 (Fitzgerald et al., 2007; May et al., 2007; Ambjørn et al., 2008; West et al., 2011; Leung et al., 2012; Auderset et al., 2016; Landowski et al., 2016). These interactions enable the cleavage of a C-terminal intracellular component of megalin after endocytosis of MTs and lead to subsequent activation of signal transduction pathways that promote regeneration, such as extracellular signal-regulated

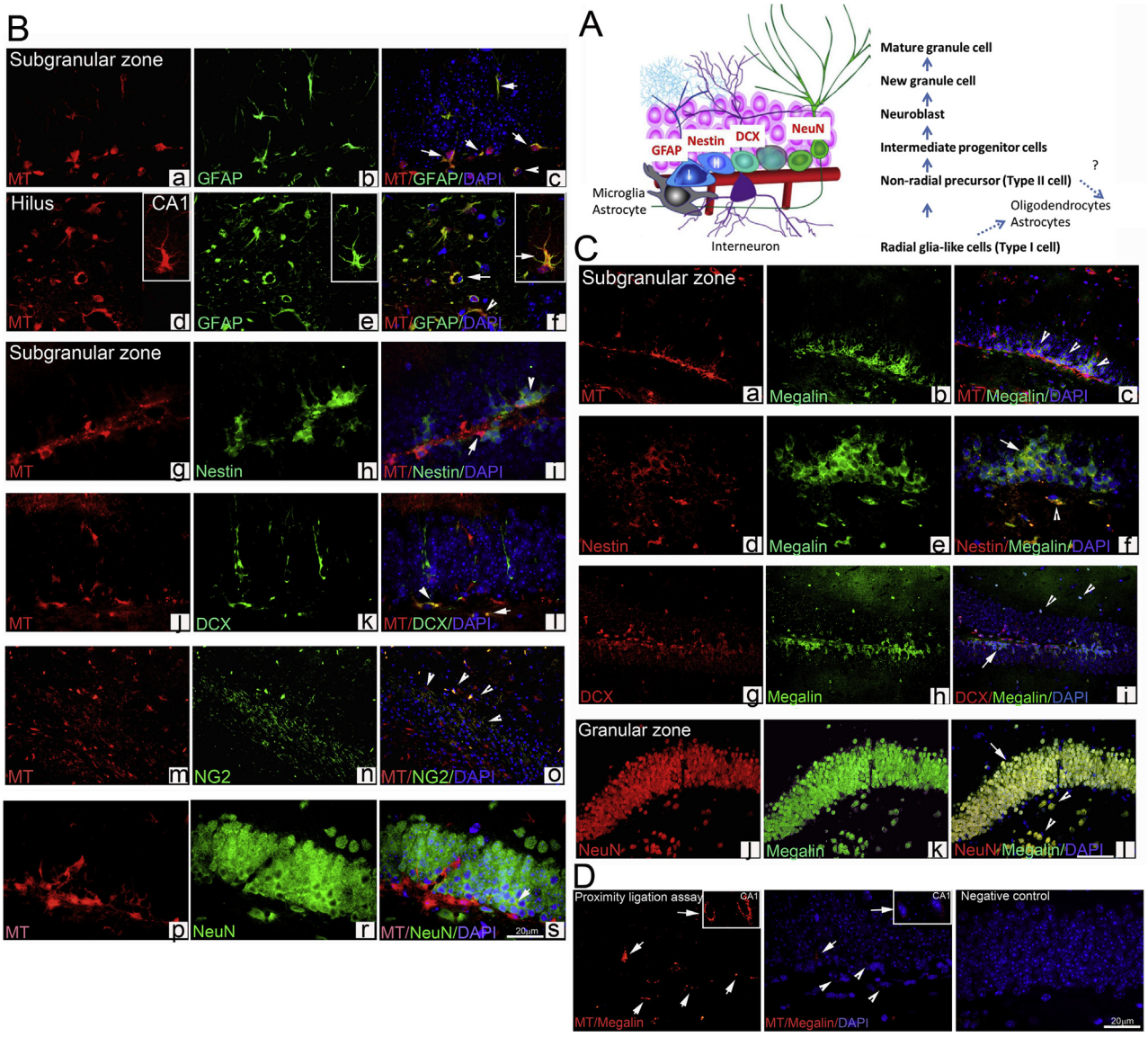


Fig. 10. Effect of cuprizone-supplemented diet on hippocampus. (A) A schematic illustration shows the neural stem cell niche in the subgranular zone (SGZ) of the adult dentate gyrus (based on (Ming and Song, 2011) and cells types that might be detected by monoclonal antibodies used in this study. Representative immunofluorescent pictures show: (B) MT-I/II expression in GFAP+ cells (a–f), in nestin+ stem cells (g–f), in doublecortin (DCX)+ neuroblasts/immature neurons (j–l), in NG2+ oligodendrocyte progenitors (m–o) and in mature NeuN+ neurons (p–s). (C) Expression of megalin in MT+ cells (a–c), in nestin+ cells (d–f), in DCX+ cells (g–i) and in NeuN+ neurons (j–l). (D) Interaction of MT-I/II and megalin in cells of SGZ, granular cell layer (GCL) and CA1, detected by proximity ligation assay. Brain tissue was obtained from C57BL/6 mice fed by 0.25% CPZ (w/w) during the five weeks and sacrificed immediately after CPZ feeding (e–l).

kinase (ERK), protein kinase B, and cAMP response element-binding (CREB) protein (Ambjørn et al., 2008) and mitogen-activated protein kinase (Leung et al., 2012). Furthermore, this may stimulate co-receptors, such as the transthyretin (Fleming et al., 2009) or induce the activation of tropomyosin receptor kinase A and calcium signaling (Landowski et al., 2016).

Our data seem to be in line with the reported evidence, since in the late phase of CPZ intoxication we found the increased MT immunoreactivity inside and around the reactive astrocytes (Fig. 7A a–c), as well as inside numerous megalin-positive cortical neurons (Fig. 7B a–c) and OPCs at the cortico-medullary border (Fig. 7A d, e, f). In addition, we emphasize that MT/megalin interactions contribute also to the complex biochemical signaling in germinal niches, where different intrinsic and extrinsic mechanisms regulate distinct stages of adult neurogenesis (reviewed by (Kempermann et al., 2004; Morrison and Spradling, 2008; Zhao et al., 2008; Kriegstein and Alvarez-Buylla, 2009; Ming and Song, 2011; Zhang and Jin, 2012). Within this context, we show that in mice fed by CPZ for 5 weeks MT-I/II and megalin expressions were upregulated in several stem/progenitor cells (GFAP+, nestin+ cells, DCX + neuroblasts and NG2+ oligodendrocyte progenitors) and in other cellular components that might play a prominent role in regulating cell proliferation in the niches, such as astrocytes, ependymal, perivascular and stromal cells

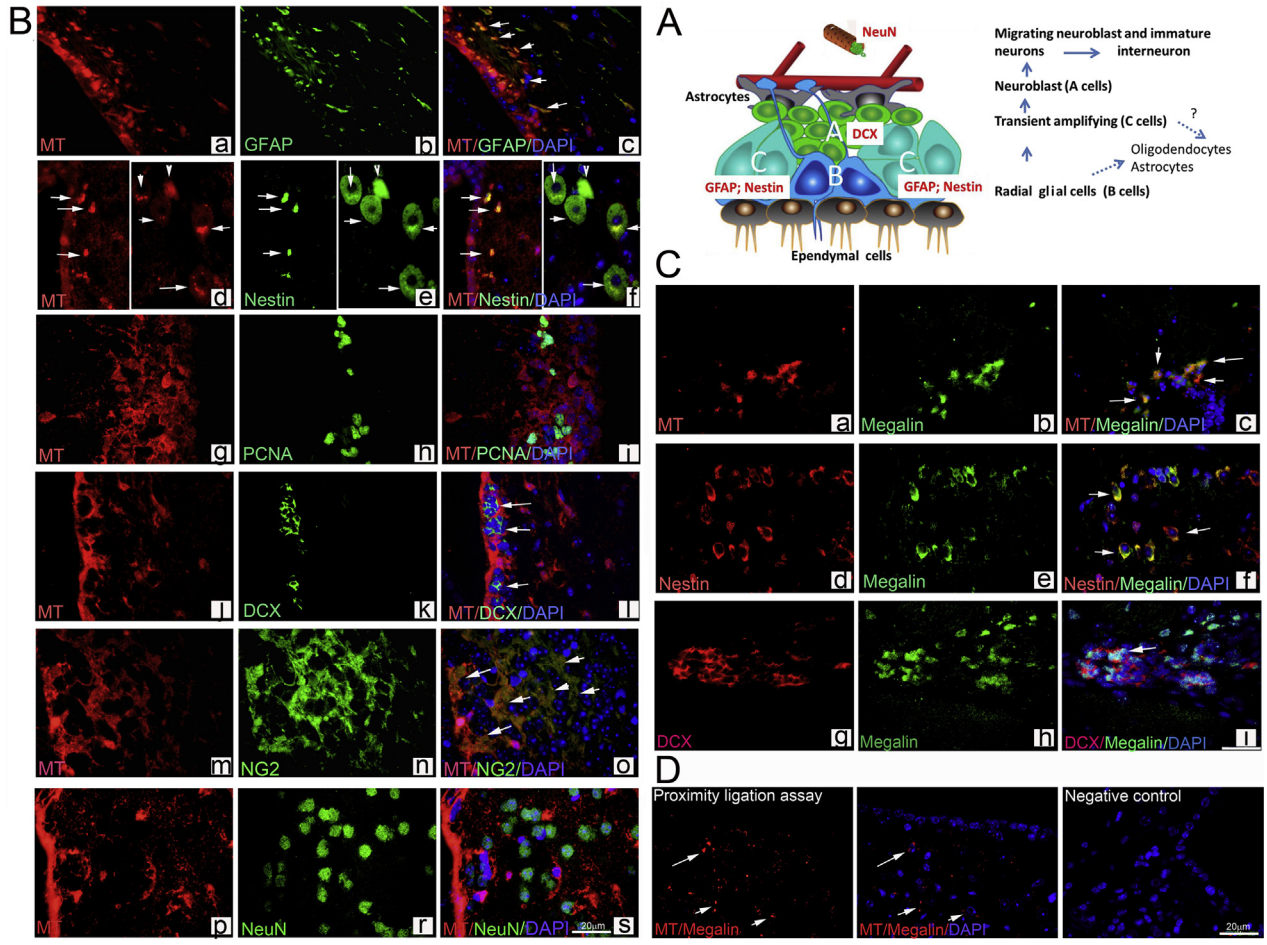


Fig. 11. Effect of cuprizone-supplemented diet on subventricular zone of lateral ventricle. (A) A schematic illustration shows the neural stem cell niche in the adult SVZ (based on (Ming and Song, 2011) and the cells types that might be detected by monoclonal antibodies used in this study. Representative immunofluorescent pictures show: (B) MT-I/II expression in GFAP + cells (a–c), in nestin + stem cells (d–f), in PCNA + cells (g–i), in doublecortin (DCX) + neuroblasts/immature neurons (j–l), in NG2 + oligodendrocyte progenitors (m–o) and in mature NeuN + neurons (p–s). (C) Expression of megalin in MT + cells (a–c), in nestin + cells (d–f), and in DCX + cells (g–i). (D) Interaction of MT-I/II and megalin on cells in SVZ, detected by proximity ligation assay. Brain tissue was obtained from C57BL/6 mice fed by 0.25% CPZ (w/w) during the five weeks and sacrificed immediately after CPZ feeding (e–l).

(Figs. 10A and 11A). The data also imply that during CPZ intoxication the MT/megalin network contributed particularly to the appearance or to the survival of new OPC, since MT-I/II immunoreactivity was noticed in NG2-positive cells located both in SGZ and SVZ (Figs. 10 and 11). We may assume that they belong to proliferating NG2 + OPC, since it was reported that during CPZ treatment these cells might migrate from the SVZ and fornix to the demyelinating corpus callosum and give rise to new OLGs for remyelination (Mason et al., 2000; Matsushima and Morell, 2001; Skripuletz et al., 2011). However, their additional origin from parenchymal progenitor pools and endogenous progenitors within demyelinated lesions in white matter (Franklin and Gallo, 2014) cannot be excluded.

Taken together, the findings imply that in target cells MTs and megalin participated in signaling pathways relevant for self-renewal and multipotential differentiation of stem/progenitors and in processes that provide protection against CPZ-induced oxidative stress. Underlying mechanisms remain to be elucidated, but the findings are in high agreement with hypothesis of Chung, Hidalgo, West and collaborators, who clearly showed that neuroregenerative effects of MT-I/II might be attributed not only to intracellular roles of MT-I/-II, such as metal ions regulation and free radical scavenging, but also to megalin mediated transport of extracellular MTs into neurons (Hidalgo et al., 2001; Chung et al., 2008a,b; West et al., 2011). Besides, the data are in line with a growing number of studies, which emphasize the signaling capacities of the LDL-receptor family (Li et al., 2001b; May et al., 2005; Qiu et al., 2006; Auderset et al., 2016). Regarding the later, we show that binding of MT-I/II to megalin leads to activation of the Akt1/PKB signaling, which as a major effector of the phosphoinositide 3-kinase (PI3-K) pathway may promote the cell survival processes through regulation of genes transcription, protein synthesis and metabolism (Franke et al., 1997; Manning and Cantley, 2007; Hers et al., 2011). It is also of particular interest that in CPZ-fed mice we found that both megalin and pAkt1 (Thr308) were present in the nucleus of cortical neurons (Fig. 8B and C a-c),

since this points to the possibility that pAkt1 reacted with cytosolic C-terminal domain of megalin, which had been released from the plasma membrane by regulated intramembrane proteolysis. Namely, these cytosolic fragments contain critical signaling motifs for interaction with a set of cytoplasmic adaptor and scaffold proteins and in some instances enter the nucleus to regulate transcription of target genes (Brown et al., 2000; Li et al., 2001b; May et al., 2002). In our model, however, the underlying signal transduction mechanisms remain to be clarified, since during CPZ intoxication the Akt signaling cascade might be activated by different mechanisms and pAkt might affect diverse cellular functions through phosphorylation and inhibition of a large number of downstream substrates that regulate transcriptions of genes, protein and fatty acid synthesis, glucose metabolism, cell proliferation and survival, cell migration, and several aspects neural functions (Manning and Cantley, 2007; Hers et al., 2011). Besides, since LRP through its extracellular domain may bind and internalize not only MTs, but at least 40 different ligands ranging from lipoprotein and protease inhibitor complex to growth factors and extracellular matrix proteins, it should be taken into account that this pleiotropism may increase the number of intracellular pathways by which distinct LRP ligands may elicit their effects (Li, 2001; Li et al., 2001a; Kozyraki and Gofflot, 2007; May et al., 2007; Spuch et al., 2012; Wagner and Pietrzik, 2012). As the outcome, structurally and functionally distinct ligands of the LDLR family may be subjected to controlled endocytosis and degradation and involved in signaling mechanisms that control many crucial cell functions.

Importantly, in this context it has been emphasized that LRP1 and LRP2 also highly influence neural stem cells proliferation and differentiation in neurogenic niches and OPC proliferation and migration during development and adulthood (reviewed by (Auderset et al., 2016). Relevant to our data, it was found that megalin expression by ependymal cells of lateral ventricle might regulate neuronal stem cell proliferation in adult neurogenic niche, as well as that megalin-activated pathways in oligodendrocytes might contribute to the maintenance of myelin in embryonic mouse spinal cord and participate in the translocation of signals from the cell membrane to the nucleus (Wicher et al., 2005, 2006). Besides, pointing to importance of MT-I/II/megalin interactions, Leung and colleagues (2012) showed that MT-I/II might affect regenerative axonal sprouting of dorsal root ganglion neurons after physical axotomy and alter neurite outgrowth by activation of ERB and Akt signaling pathways (Leung et al., 2012). They also clearly showed that the effects of exogenous MT-II on neuronal regeneration might be related with its ability to reduce the inflammatory response of microglia following TNFα stimulation and establish *via* LRP1-receptor a supportive environment for axon growth (Leung et al., 2018). Similarly, Landowski and co-authors (2016) reported that MT-II might promote the regeneration of nerve fibers following capsaicin induced denervation in rat skin and found that LRP-1 and LRP-2 receptors might be actively recruited or up-regulated at the membrane toward the MT-II gradient (Landowski et al., 2016).

There is also a high possibility that during CPZ intoxication MTs interacted with multiple other extracellular and intracellular molecular cues that have been implicated in the oligodendrocyte differentiation and (re)myelination and exert permissive/promotional and/or inhibitory effects at the different stages along the OLG lineage (Fuller et al., 2007; Zhao et al., 2008; Ming and Song, 2011; Franklin and Gallo, 2014; Praet et al., 2014; Daynac et al., 2016; Gaesser and Fyffe-Maricich, 2016). However, although it is known that megalin and LRP1 can influence the balance of growth factors and morphogen signaling during neural development (Balordi and Fishell, 2007; Gajera et al., 2010; Daynac et al., 2016), the evidence about the MTs/megalin interactions in these processes are still lacking.

Summarizing, we would like to emphasize that in this study we also found that CPZ intoxication after 3 and 5 weeks induced a significant upregulation of megalin immunoreactivity in choroid plexus (Fig. 3C and B j, p, v). Namely, this points to the possibility that in the later phase of CPZ intoxication the receptor-mediated endocytosis contributed to the uptake of MT-I/II and other neuroprotective ligands, such as apolipoprotein E, cholesterol, vitamin-binding proteins, hormones and immunorelated proteins that were synthesized elsewhere in the body (Gliemann, 1998; Li et al., 2001a; Nuutinen et al., 2009). This would be also a plausible explanation for the selective transport of some substrates across the BBB and blood–cerebrospinal fluid barrier in cuprizone intoxication, since generally it does not induce the breakdown of the blood–brain barrier (BBB) (Hiremath et al., 2008; Skripuletz et al., 2011; Praet et al., 2014). The finding might be of particular interest, because the endogenous processes and therapies that increase megalin expression at the choroid plexus may control also the accumulation of brain Aβ and enhance the repair in the damaged brain, since LDL-R family are involved in trafficking and processing of the amyloid precursor protein (APP) and the uptake of apolipoprotein J/clusterin, a binding protein for the Aβ peptide (Zlokovic et al., 1996; Alvira-Botero and Carro, 2010; Alvira-Botero et al., 2010). In this context our data support the current knowledge pointing to the therapeutic potential of MT-I/II and peptides modeled from MT in AD and MS, as well as in copper and zinc-dyshomeostasis, mitochondrial dysfunctions, aging and other neurodegenerative disorders (Coyle et al., 2002; Mocchegiani et al., 2007; Malavolta et al., 2008; Chung et al., 2008a, 2010; Pedersen et al., 2009; Manso et al., 2011; West et al., 2011; Sharma and Ebadi, 2013; Szewczyk, 2013).

In conclusion the data presented in this study show that CPZ-induced demyelination is followed by high upregulation of MT-I/II and megalin immunoreactivity in astrocytes and epithelial and endothelial cells located in cerebral and cerebellar white and gray matter, as well as in choroid plexus and in numerous constituents of the neurogenic niches (ependymal cells, GFAP+, nestin+, doublecortin (DCX)+ and NG2+ cells) in subgranular zone (SGZ) of DG and in SVZ of lateral ventricles. Moreover, the data point to intercellular transfer of MTs secreted from astrocytes, to direct

binding of MT-I/II to its receptor-megalin in target cells, as well as to the activation of Akt1 phosphorylation cascade in cerebral megalin-positive cortical neurons.

The data imply that MT I/II perform important cytoprotective and growth regulating functions in reparatory/remyelinating processes activated in the brain after toxic demyelinating insults

AUTHORS' CONTRIBUTION

H.J. and T.G.K. performed all in vivo experiments and immunohistochemistry, H.J. and B.R.S. designed the research study and wrote the paper. All authors analyzed and reviewed the results and approved the final version of the manuscript.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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