Histol Histopathol (2013) 28: 61-78
DOI: 10.14670/HH-28.61

http://www.hh.um.es

Histology and Histopathology
Cellular and Molecular Biology

Expression pattern of the endoplasmic reticulum stress protein GP96 in monophasic and chronic relapsing form of experimental autoimmune encephalomyelitis in rats

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Summary. Gp96 is the endoplasmic reticulum (ER)-resident molecular chaperone, which is involved in the correction of unfolded proteins, in the activation of proteasome-dependent ER-associated degradation of the misfolded proteins, and in activation of the protein translation that modulates polypeptide traffic into the ER. Furthermore, owing to its peptide chaperone capacity and ability to interact with professional antigen-presenting cells, as well as with growth factors, integrins and Toll-like receptors, it is also endowed with crucial immunological functions acting as a "danger signal" to the innate and adaptive immunity.

Considering these properties, in the present study the tissue expression of gp96 was examined during the monophasic and chronic relapsing form of experimental autoimmune encephalomyelitis (CR-EAE), induced in genetically susceptible DA rats by subcutaneous injection of myelin basic protein (MBP) or bovine brain homogenate in complete Freund's adjuvant (CFA). Immunohistochemical analyses were done in periods of attacks and remissions of EAE, and the results were compared with findings in intact rats and those treated only with CFA. The data revealed that the constitutive gp96 expression, found in several neurons and glial cells in the brain and spinal cord of intact animals, significantly diminished during the attacks of CR-EAE. On the contrary, the remission of disease was followed by high upregulation of gp96, mainly in the oligodendrocytes within the white matter, in the neurons of the hippocampal area, as well as in the motoneurons of lumbar spinal cord, suggesting that gp96 might be involved in proteostasis and immune-related pathways linked with the reparative processes in the CNS.

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Key words: Endoplasmic reticulum resident heat shock protein, gp96, Monophasic and chronic relapsing experimental autoimmune encephalomyelitis, DA rats

Introduction

A variety of physiological and pathological processes in endoplasmic reticulum (ER) activate a signaling pathway, termed the unfolded protein response (UPR) (Ma and Hendershot, 2004; Nishikawa et al., 2005; Yang and Li, 2005; Wu and Kaufman, 2006). It is characterized by the coordinated transcriptional up-regulation of ER chaperones and folding enzymes, which participate in the processes of proper folding and assembly of newly synthesized secretory and membrane proteins and in those that prevent the aggregation of unfolded and incompletely folded proteins in the ER. In physiological processes, such as cell cycle and differentiation, ER molecular chaperones are upregulated owing to an increase of the secretory protein synthesis, while in conditions of ER stress, such as ischemia, fever, inflammation and infection, their requirement increases owing to the presence of proteins that are unable to fold properly (Ma and Hendershot, 2004; Nishikawa et al., 2005; Yang and Li, 2005; Wu and Kaufman, 2006). Furthermore, when the UPR is perturbed or is not sufficient to deal with the stress conditions, the ER employs a mechanism termed ER-associated degradation (ERAD) to clear the aggregated misfolded or unassembled proteins (Nishikawa et al., 2005). During ERAD, the ER chaperones target the misfolded or aberrant proteins for degradation by the ubiquitin-proteasome system, often stimulating the apoptosis of cells (Morishima et al., 2002), showing that UPR signaling may result both in cell protective responses as well as in induction of mechanisms that

lead to cell death (Morishima et al., 2002; Ma and Hendershot, 2004; Nishikawa et al., 2005; Yang and Li, 2005; Wu and Kaufman, 2006; Lai et al., 2007). Furthermore, since folding pathways integrated by the ER are linked to general cell physiology through intracellular and extracellular signaling pathways, stress-inducible and constitutively expressed HSPs are involved in a plethora of cellular functions that regulate processes of differentiation and apoptosis (Lanneau et al., 2007; Stetler et al., 2010).

As proteins that assist other proteins to fold, refold and travel to specific cell compartments and across membranes, molecular chaperones bind the client polypeptides and interact with other complexes that integrate the chaperoning network, also regulating antigen presentation and inflammatory signaling pathways. Owing to this, some members of the heat shock (HSP) family, such as hsp60, hsp70, hsp90 and gp96, are able to stimulate cells of the innate immune system directly, acting as "danger"- signaling molecules (Wallin et al., 2002), or as adjuvants that elicit potent antigen-specific immunity to bound peptides of microbial or mammalian origin (Srivastava, 2002b; Tsan and Gao, 2004; Javid et al., 2007).

Among HSPs, particularly, ER-resident heat shock protein gp96, also known as HSPC4/glucose-regulated protein 94, has been shown to act as a natural adjuvant for priming innate and adaptive immunity, owing to its ability to actively chaperon MHC class I-restricted epitopes into the cross-presentation pathway of professional antigen-presenting cells (APC) and induce the activation and maturation of these cells to allow priming of cognate CD8+ T cell effector responses (Srivastava et al., 1994; Singh-Jasuja et al., 2000; Srivastava, 2002a; Srivastava, 2002b; Podack and Raez, 2007). It also facilitates the *in vivo* presentation of a class II-restricted peptide (Doody et al., 2004) and acts as an extracellular mediator of inflammation, which through several receptors induces the secretion of inflammatory cytokines from different APC (including macrophages, monocytes, and myeloid-derived dendritic cells). Moreover, since it was found that mutant macrophages from specific gp96-deficient mouse failed to respond to ligands of both cell-surface and intracellular Toll-like receptors (TLRs) including TLR2, TLR4, TLR5, TLR7 and TLR9 it was proposed that gp96 might be the master chaperone for TLRs in macrophages, which are the dominant sources of proinflammatory cytokines during endotoxemia and Listeria infections (Harding, 2007; Yang et al., 2007). However, there is also the possibility that gp96 participates in restraining the immune response, since it was found that through CD91 triggering it might activate tolerogenic plasmacytoid dendritic cells (pDC) (De Filippo et al., 2008).

Phylogenetically conserved stress proteins are constitutively also expressed in normal CNS tissues, in a variety of cell types (oligodendrocytes, astrocytes and neurons) implying that they may be critical during nervous system development and during the exposure of cells to stresses, such as hypoxia, anoxia, and excessive excitatory stimulation (Birnbaum, 1995; Stetler et al., 2010; Voisine et al., 2010). Moreover, since it was found that antibodies to mycobacterial stress proteins bind to normal human myelin and to oligodendrocytes in regions of demyelination, it was hypothesized that an immune response to the stress proteins of an infectious agent could result in a cross-reactive immune response to CNS myelin, participating in the induction of multiple sclerosis (MS) (Birnbaum, 1995; Birnbaum and Kotilinek, 1997) and experimental allergic autoimmune encephalomyelitis (EAE) (Galazka et al., 2006, 2007). Furthermore, in conditions of neurodegeneration and neuroinflammation it was proposed that the alterations in ATP-binding and ATPase domains of some HSP can disrupt the chaperoning process and cause the accumulation of insoluble protein aggregates (Lindholm et al., 2006; Macario and Conway de Macario, 2007). In this context, however, essential functions in CNS also have other family members of HSP superfamilies, showing that HSPs are attractive therapeutic targets for "chaperonopathies" (van Noort, 2008) and several other neurological diseases (Lindholm et al., 2006). The most studied HSPs with neuro-protective function are the HSPA/HSP70, HSPB (small HSPs) and HSPC/HSP90 family of chaperones, but for a detailed review of the cellular and molecular mechanisms of specific chaperone subtypes, please see Stetler et al., 2010).

Since the mechanisms and the functions of ER-located molecular chaperones are still being discussed in this study we investigated the tissue expression of gp96 (HSPC4/GRP94) in the brain and spinal cord of rats, using the models of monophasic and chronic relapsing (CR)-EAE, trying to correlate the results with the clinical symptoms and kinetics of disease. The data showed that constitutive gp96 expression in several neurons and glial cells in the brain and spinal cord diminished during the attacks in the CR-EAE model, and augmented during remission phases of both forms of disease, affecting particularly the oligodendrocytes within the white matter, neurons in the hippocampal area, and motoneurons in the spinal cord, suggesting that gp96 might support cell survival, participating in the control of protein synthesis and/or in immunological pathways occurring during reparative phases in the CNS.

Material and methods

Animals

Dark Agouti (DA) 2-3 month-old male rats were used for these experiments. They were bred and maintained according to the guide for Institutional Animal Care and used with approval of the local Ethical committee. Four to five rats were kept per cage in standard housing conditions with a light/dark cycle of 12

h and free access to food and water. All experiments were done in accordance with the European Communities Council Directives (86/609/EEC) and the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80 23, revised 1978).

EAE induction and evaluation

Monophasic and CR-EAE was induced in genetically susceptible DA rats by a standard, previously described method (Mostarica-Stojkovic et al., 1982; Jakovac et al., 2011). Immunization was performed by myelin basic protein (MBP; Sigma, St. Louis, Mo., USA) or by bovine brain white matter homogenate emulsion (BBH) in PBS (50%, w/v) mixed with an equal volume of complete Freund's adjuvant (CFA) (Sigma, St. Louis, Mo., USA). Each animal received MBP (100 μg) or 2 x 0.1 ml of BBH emulsion (containing 0.033 g of brain white matter), which was injected subcutaneously in each hind footpad. Rats in the control groups received the same dose of CFA. The severity of the disease was clinically assessed according to the following criteria: 0 - no symptoms; 1 - flaccid paralysis of tail; 2 - hind legs paresis; 3 - hind legs paralysis with incontinence and 4 - death.

Tissue preparation

Rats were anesthetized with chloral hydrate (400 mg/kg i.p.) and sacrificed by exsanguination. The brain and lumbar spinal cord were rapidly removed and fixed in 10 % buffered formalin solution for a minimum of 24h. Tissue samples were then embedded in paraffin wax and 4 μm sections were cut using a HM 340E microtome (Microtom, Germany). Heat induced epitope retrival was done prior to staining procedures by heating tissue slides in boiled citrate buffer pH 6.0 four times, each 5 minutes, using a microwave steamer.

Immunohistochemistry

As previously described (Jakovac et al., 2011), immunohistochemical studies were performed on paraffin embedded tissues using DAKO EnVision+System, Peroxidase (DAB) kit according to the manufacturer's instructions (DAKO Corporation, USA). Slides were incubated with peroxidase block to eliminate endogenous peroxidase activity. After washing, monoclonal rat anti-Grp94 antibody (Clone 9G10; Stressgen, Canada) diluted 1:30 in phosphate-buffered saline supplemented with bovine serum albumin was added to tissue samples and incubated overnight at 4°C in a humid environment, followed by 45 minutes incubation with peroxidase labeled polymer conjugated to goat anti-mouse immunoglobulins containing carrier protein linked to Fc fragments to prevent nonspecific binding. The immunoreaction product was visualized by adding substrate-chromogen (DAB) solution. Tissues were counterstained with hematoxylin and 37 mM ammonia water, dehydrated in a gradient of alcohol and mounted with mounting medium. The specificity of the reaction was confirmed by substitution of anti-Grp94 antibody with mouse irrelevant $IgG_{2\alpha}$ immunoglobulin (clone DAK-G05; Dako, USA), used in the same conditions and dilutions as the primary antibody. For double immunohistochemical labeling the DakoCytomation EnVision Doublestain System was used, according to the manufacturer's instructions (DakoCytomation, USA) as previously described (Jakovac et al., 2011). Anti-Grp94 antibody (Clone 9G10; Stressgen, Canada) and anti-glial fibrillary acidic protein (monoclonal anti-GFAP; Becton Dickinson, USA), were used as primary antibodies, respectively. Binding of the first primary antibody (anti-Grp94) was visualized by peroxidase labeled polymer conjugated with secondary antibodies, using DAB as a tracer, resulting in brown staining at the first antigen site. Upon completion of the first reaction, slides were incubated with double stain block to remove any potential cross-reactivity between the reactions, along with blocking any endogenous alkaline phosphatase that may be present. Afterwards, the second primary antibody (anti-GFAP) was added, followed by incubation with alkaline phosphatase labeled polymer containing secondary antibodies. The second antigen staining was finalized by incubation with the Fast Red substrate-chromogen, which results in a red-colored precipitate at the sites of the second immunoreaction. Reddish brown coloration indicated the presence of both labeled antigens. Control slides were identically treated, but with the omission of primary antibodies. The slides were examined on an Olympus BX51 photomicroscope (Olympus, Tokyo, Japan).

Using the same protocol, the CD45RO isoform of the leukocyte common antigen was detected by mouse monoclonal anti-CD45RO antibody [UCHL-1] (Abcam), diluted 1:100 in phosphate-buffered saline supplemented with bovine serum albumin

Immunohistochemical staining quantification

Immunohistochemical, cell-based staining quantification was performed using Cell F v3.1 software (Olympus Soft Imaging Solutions). Captured images were subjected to intensity separation. They were subsequently inverted, resulting in grey scale images with different intensity range, depending on the strength of immunohistochemical signals. Regions of interest were set up to cover the cytoplasm of immunopositive cells to measure gray intensity. Twenty regions of interest were analyzed per field (400 x) in the ten fields per microscopic slide of tissue samples, obtained from rats treated with BBH+CFA and those treated with CFA. Means were analyzed by Student's t-test at a significance level of 5%.

Results

Clinical course of EAE in DA rats

Genetically susceptible DA rats immunized with MBP in CFA developed symptoms typical for monophasic EAE (MF-EAE), while those treated with BBH in CFA developed a chronic-relapsing form of EAE (Fig. 1). In both groups of rats the disease started on the 10th postimmunization day with tail paresis, resulting subsequently in one peak of clinical symptoms with a mean clinical score of 3 (with hind legs paralysis and incontinence) on day 12 (MF-EAE) or in two peaks of disease on days 12 and 22 postimmunization (CR-EAE). Remissions of symptoms occurred on day 20 or on days 18 and 22, respectively. Rats in the control

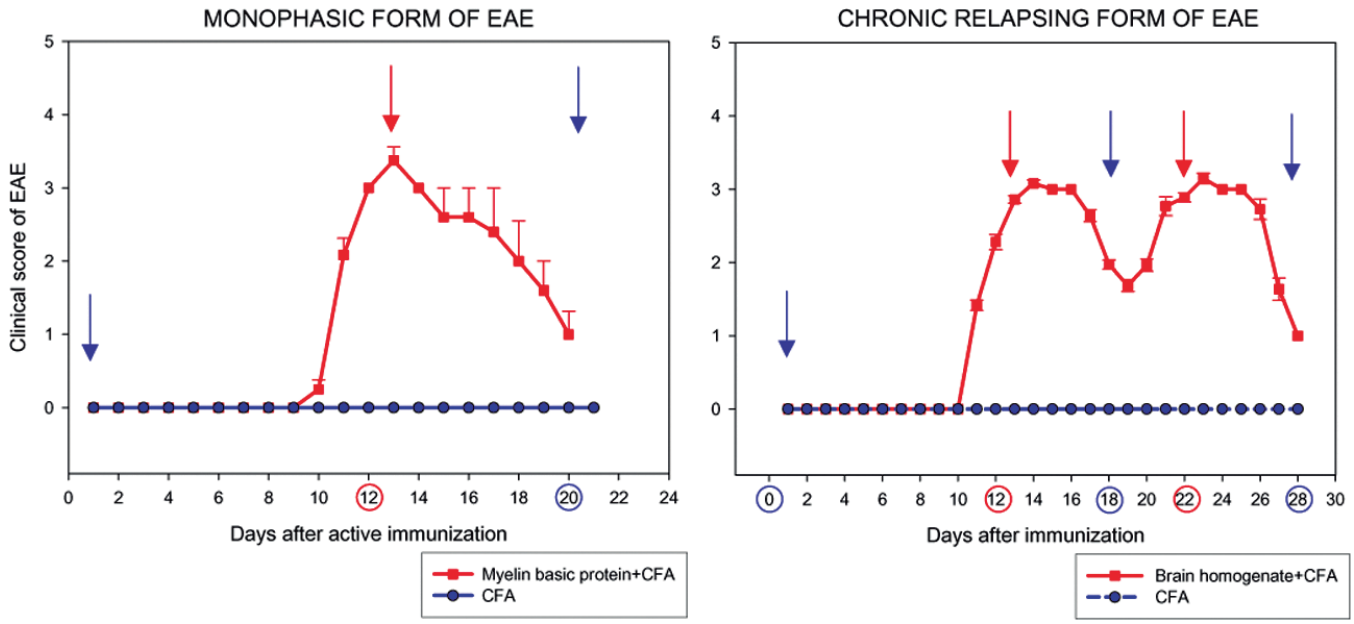

Fig. 1. Clinical score of monophasic (MF) and chronic relapsing (CR) form of EAE induced in Dark Agouti (DA) rats (n=14) by injection of myelin basic protein in complete Freund's adjuvant (CFA) and by bovine brain homogenate in CFA, respectively. Rats in control group (n=14) were injected with CFA alone. Arrows point to the time when tissue samples for detection of gp96 expression were taken (i.e. in intact rats and at the time of attacks-red or remission-blue). Data are mean ± SE.

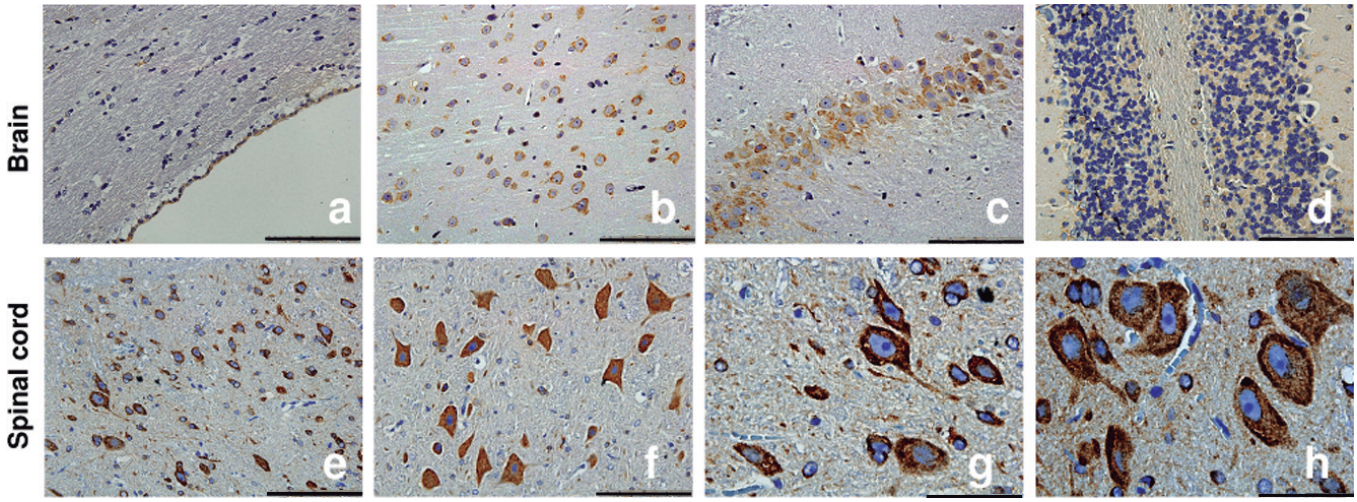

Fig. 2. Expression of gp96 in different parts of brain and spinal cord in intact DA rats: subventricular region of the brain **(a);** brain cortex **(b)**; hippocampus **(c)**; cerebellum **(d)**, and lumbar spinal cord **(e-h)**. The results are representative findings of 3 rats. Scale bars: a-g, 100 μm; h, 10 μm.

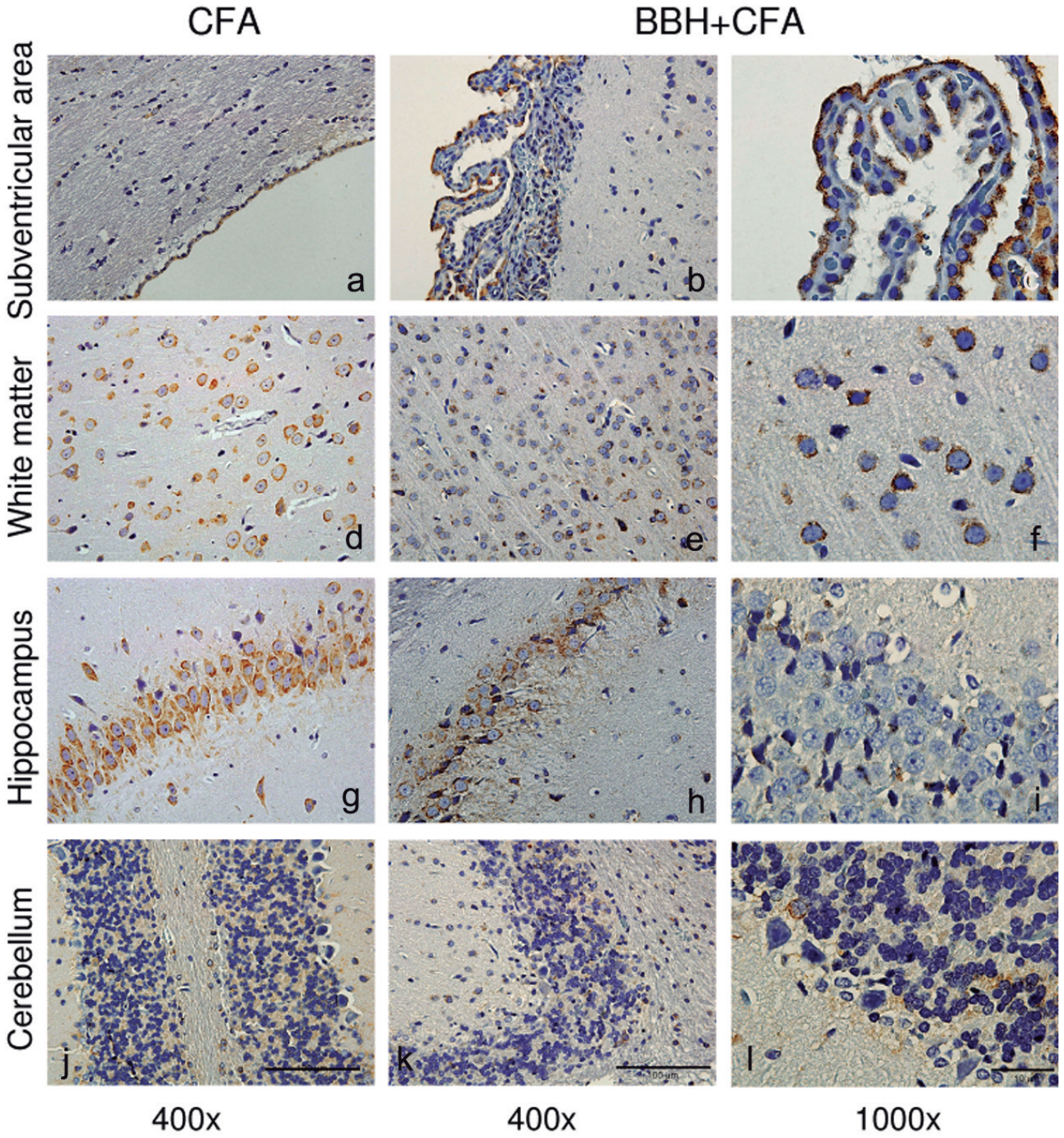

Fig. 3. Expression of gp96 protein during the first attack of CR-EAE (on the 12th day after immunization with bovine brain white matter homogenate in the CFA; clinical score 3) in the subventricular area **(b, c)**, white matter of the brain **(e, f)**, hippocampus **(h, i)** and cerebellum **(k, l)**. First column **(a, d, g, j)** shows gp96 expression found in analogous paraffin-embedded tissue samples, obtained at the same post-immunization day from control rats, injected with CFA alone. The results are representative findings of 3 rats. Scale bars: a, b, d, e, g, h, j, k, 100 μm; c, f, i, l, 10 μm.

group, which received CFA only, did not develop clinical symptoms of EAE (Fig. 1).

Tissue samples of the brain and lumbar spinal cord for investigation of gp96 expression were taken at the times of attacks and remissions and the data were compared with findings in intact DA rats, and with analogue tissue samples, obtained on the same post-inoculation day from rats treated only with CFA. The latter was of particular interest because, in spite of the fact that CFA alone did not induce the clinical symptoms of EAE, the heat-killed mycobacteria in CFA might activate a variety of APCs in the draining lymph node and in CNS through innate immunity receptors, such as TLR and NOD-like receptors (Lassmann, 2010; Mix et al., 2010).

Expression of Gp96 in the brain and spinal cord of intact DA rats

As shown on Figure 2, moderate gp96 expression was found in ependymal cells in the subventricular region (Fig. 2a), in multiple oligodendrocytes within the white matter (Fig. 2b), and in hippocampal CA1 area (Fig. 2c) in the brain samples, obtained from intact DA rats. In the cerebellum gp96 immunoreactivity was low (Fig. 2d). A high level of constitutively expressed gp96 was, however, found on numerous motor neurons in the lumbar spinal cord (Fig. 2e-h).

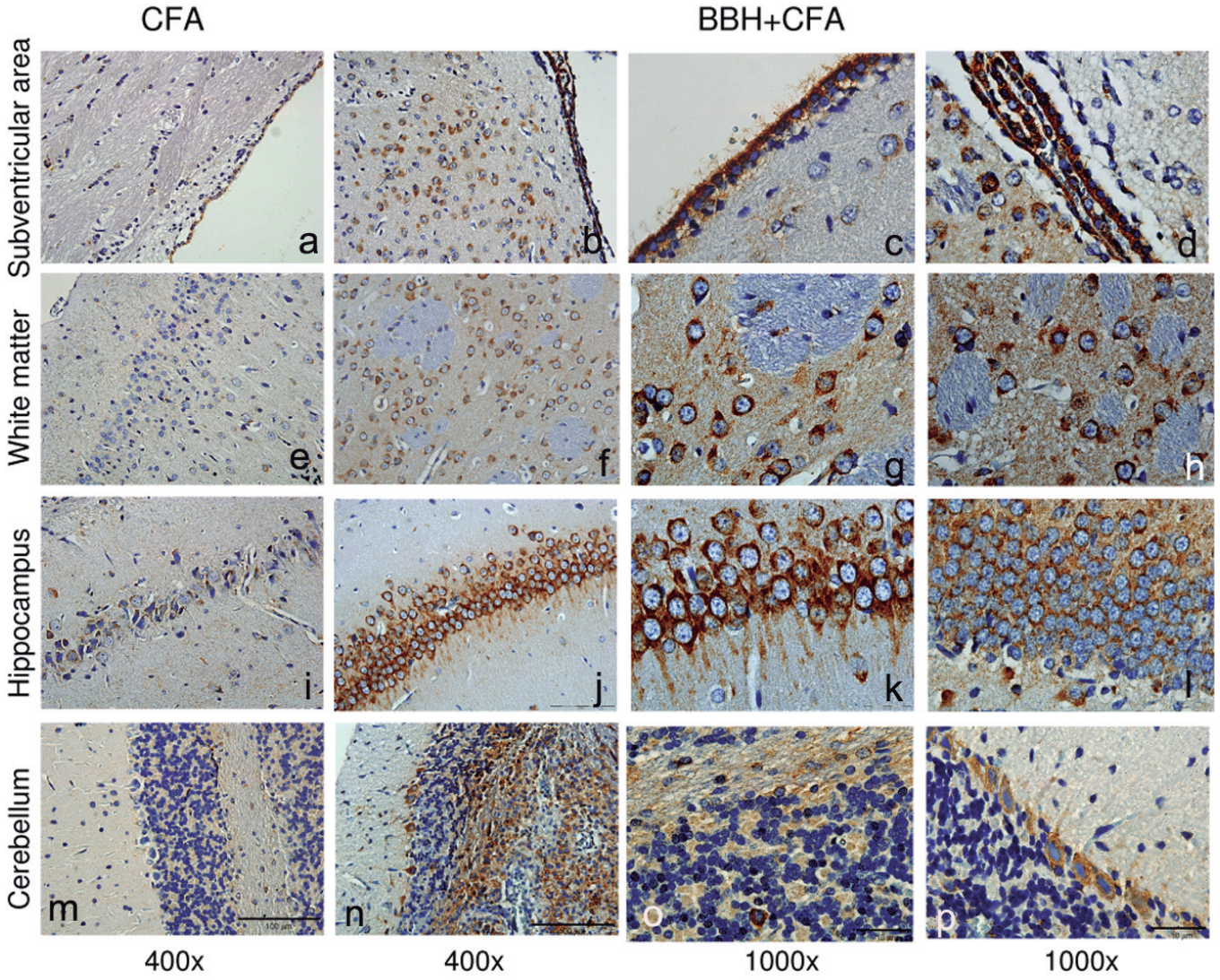

Fig. 4. Expression of gp96 protein during the first remission of CR-EAE (on the 18th day after immunization with bovine brain white matter homogenate in the CFA) in subventricular area **(b, c, d),** white matter of the brain **(f, g, h)**, hippocampus **(j, k, l)** and cerebellum (**n, o, p)**. First column (**a, e, i, m**) shows gp96 expression found in analogous paraffin-embedded tissue samples, obtained at the same post-immunization day as control rats, injected with CFA alone. The results are representative findings of 3 rats. Scale bars: a, b, e, f, i, j, m, n, 100 μm; c, d, g, h, k, l, o, p, 10 μm.

Expression of Gp96 during EAE

Changes in the brain

First attack of CR-EAE and MF-EAE. As shown in Figure 3, the expression of gp96 was found in the subventricular region (Fig. 3a), in multiple oligodendrocytes within the white matter of brain parenchyma (Fig. 3d) and in the hippocampal CA1 area (Fig. 3g) in the samples of brain from control rats (obtained on the 12th day after treatment with CFA). Immunoreactivity for gp96 in the cerebellum was mostly negative (Fig. 3j). The findings in BBH+CFA rats were comparable to those of rats that received CFA only. A moderate gp96 expression was still present in choroid plexus (Fig. 3b,c) and on some oligodendrocyte-like cells in white matter (Fig. 3e and f). In the granular layer of dentate gyrus (DG), however, several mononuclear cells were seen (Fig. 3i), while gp96-immunoreactivity in the CA1 field was slightly less expressed, in comparison with findings in CFA-treated rats (Fig. 3h).

In rats treated with MBP+CFA, during the attack of disease (on the 12th postimmunization day) the intensity of gp96 expression was similar to that found in BBH+CFA inoculated EAE rats (not shown).

First remission of CR-EAE and MF-EAE. In contrast to the previous findings and the absence of gp96 expression in rats treated with CFA (Fig. 4a,e,i,m) in rats recovering from the first attack of EAE (on the 18th post-inoculation day) in the brain of rats immunized with BBH+CFA, a marked upregulation of gp96 was noticed in the subventricular region (Fig. 4b,c,d) and on multiple oligodendrocyte-like cells in the white matter of brain

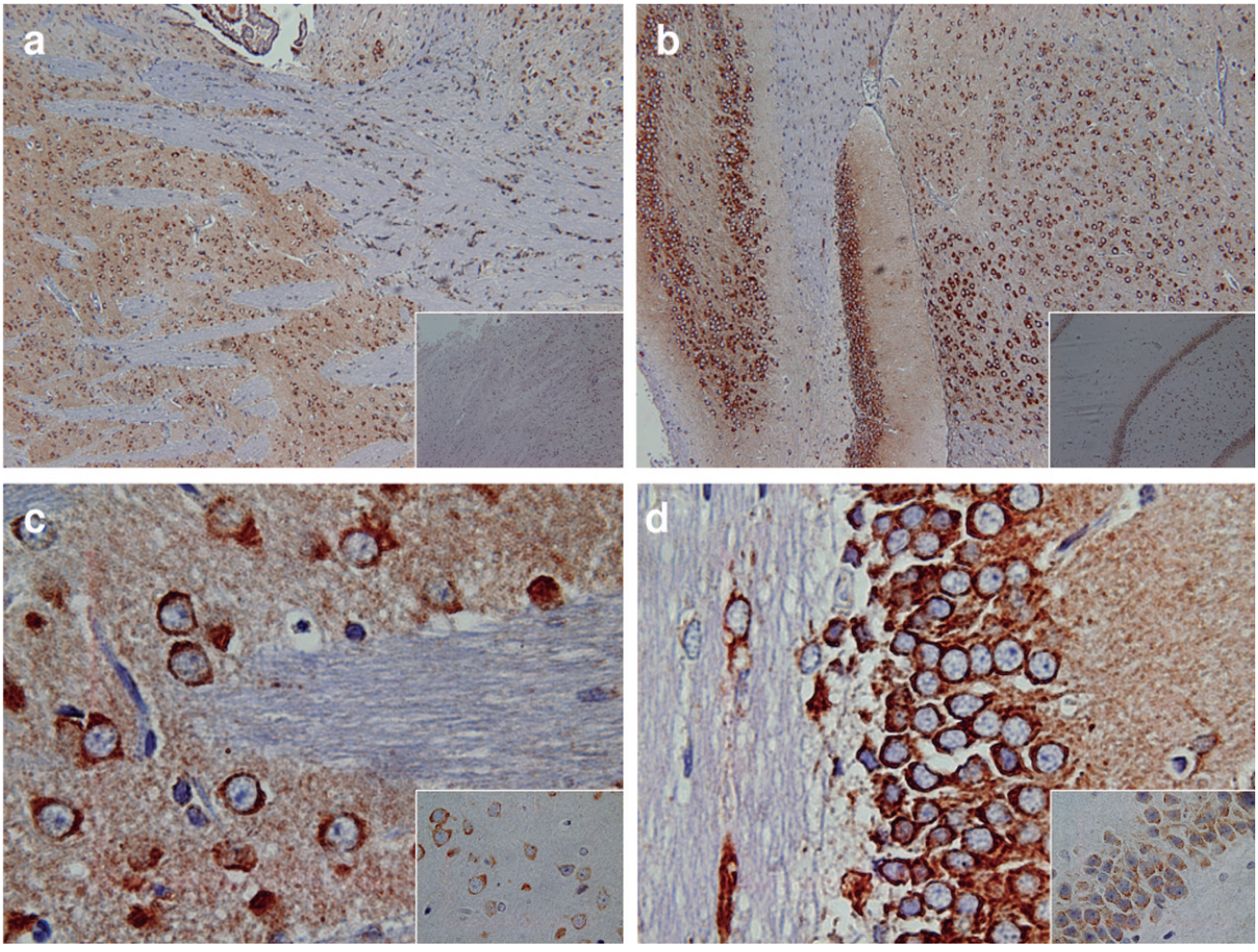

Fig. 5. Expression of gp96 protein during the remission phase of MF-EAE (on the 20th day after immunization with myelin basic protein in CFA) in white matter of the brain (**a, c** and hippocampal area (**b, d**). Imbedded pictures show gp96 expression found in analogous paraffin-embedded tissue samples, obtained at the same post-immunization day as control rats, injected with CFA alone. The results are representative findings of 3 rats. a, b, x 100; c, d, x 400

parenchyma (Fig. 4f,g,h). Moreover, high gp96 immunoreactivity was found in the hippocampal CA1 area (Fig. 4j,k) and in DG (Fig. 4l). Furthermore, expression of gp96 was highly upregulated in the cerebellum, on several cells in the granular layer (Fig. 4n,o). Some Purkinje cells also became Gp96 positive

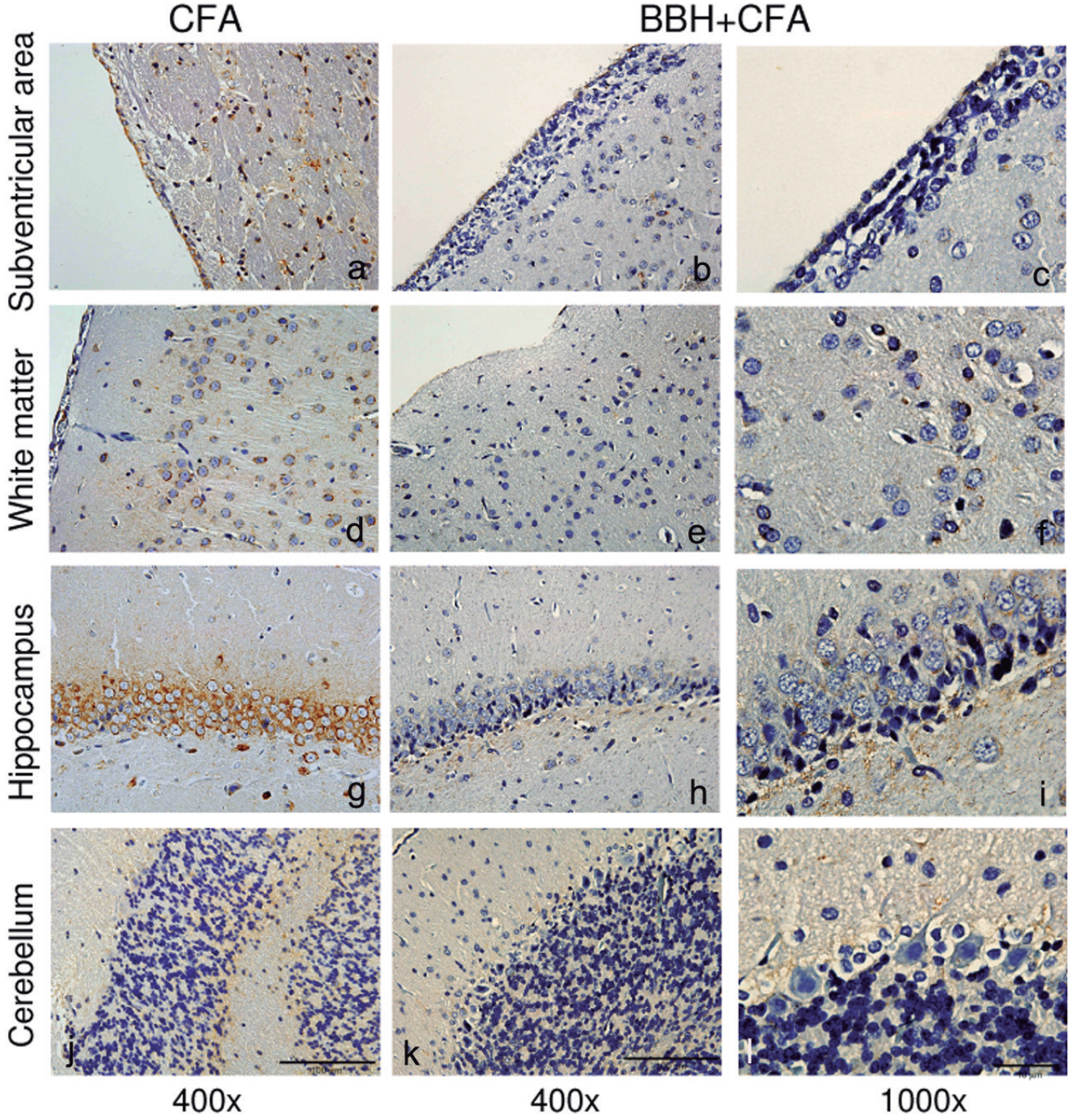

Fig. 6. Expression of gp96 protein during the second attack of CR-EAE (on the 22nd day after immunization with bovine brain white matter homogenate in the CFA at clinical score 3) in subventricular area (**b, c**), white matter of the brain (**e, f**), hippocampus (**h, i**) and cerebellum (**k, l**). First column (**a, d, g, j**) shows gp96 expression found in analogous paraffin-embedded tissue samples, obtained at the same post-immunization day as control rats, injected with CFA alone. The results are representative findings of 3 rats. Scale bars: a, b, d, e, g, h, j, k, 100 μm; c, f, i, l, 10 μm.

(Fig. 4n,p).

Moreover, confirming the link of gp96 expression with the remission phase of disease in rats treated with MBP+CFA on the 20th postimmunization day, a high upregulation of gp96 expression was also found in the white matter of brain parenchyma (Fig. 5a,c) and in the hippocampal area (Fig. 5b,d).

Second attack of CR-EAE. At the time of second attack of EAE (22nd day after immunization with BBH+CFA) in rats immunized with BBH+CFA, gp96 expression was found to be lower than that in the CFA-treated rats, sacrificed on the same p.i. day, particularly in the hippocampal CA1 area (Fig. 6h,i versus g).

Second remission of CR-EAE. As shown in Figure 7 the second remission of CR-EAE (tissue taken on the on 28th p. i. day from rats that survived both attacks of EAE) was characterized by a new upregulation of gp96 in the subventricular area (Fig. 7b,c), in choroid plexus (Fig. 7d) and in brain parenchyma (Fig. 7f,g,h). Furthermore, a high gp96 expression was found again in the hippocampal area in multiple cells in CA1 region (Fig. 7j,k) and in the granular and subgranular layer of DG (Fig. 7l). Gp96 positive cells also reappeared in the cerebellum (Fig. 7n,o,p).

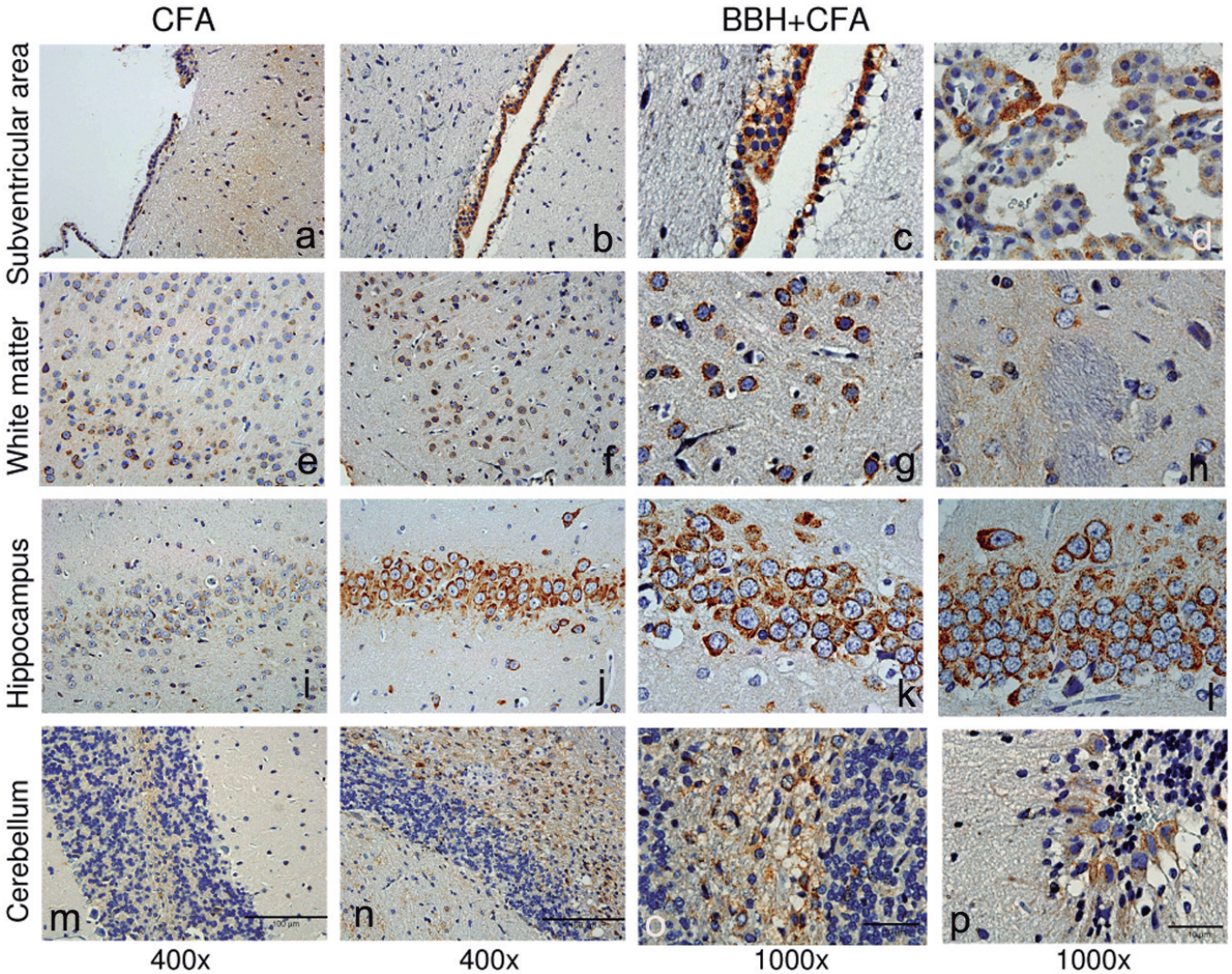

Fig. 7. Expression of gp96 protein during the second remission of CR-EAE (on the 28th day after immunization with bovine brain white matter homogenate in the CFA) in subventricular area (**b, c, d)**, white matter of the brain (**f, g, h**), hippocampus (**j, k, l)** and cerebellum **(n, o, p**). First column (**a, e, i, m**) shows gp96 expression found in analogous paraffin-embedded tissue samples, obtained at the same post-immunization day as control rats, injected with CFA alone. The results are representative findings of 3 rats. Scale bars: a, b, e, f, i, j, m, n, 100 μm; c, d, g, h, k, l, o, p, 10 μm.

Changes in the spinal cord

Tissue samples of lumbar spinal cord were taken from experimental and control rats, at the same time intervals after immunization with BBH+CFA or CFA, respectively (i.e. on 12th, 18th, 22nd and on the 28th p. i. day). As shown in Fig. 8, in CFA-treated rats gp96 expression was found at all time intervals on multiple

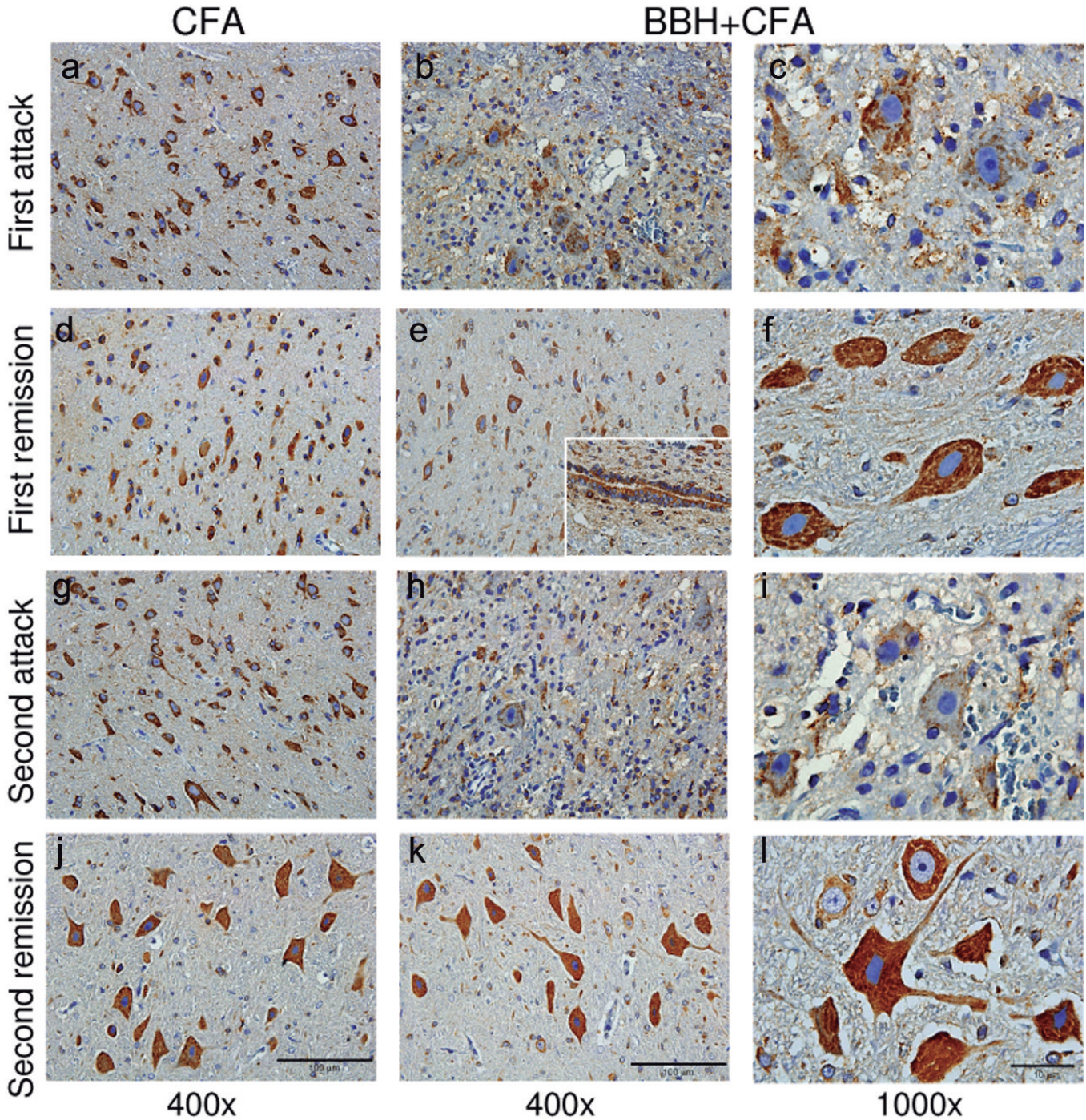

Fig. 8. Expression of gp96 protein in lumbar spinal cord at different clinical stages of CR-EAE. Analyses were done on paraffin-embedded sections of tissue obtained from DA rats injected with bovine brain white matter homogenate (BBH) in CFA at the time of first attack (**b, c)**; first remission (**e, f;** imbedded picture shows gp96 expression around the central canal); second attack (**h, i**); and second remission (**k, l)**. First column **(a, d, g, j**) shows gp96 expression found in analogous paraffin-embedded tissue samples, obtained at the same post-immunization day as control rats, injected with CFA alone. The results are representative findings of 3 rats. Scale bars: a, b, d, e, g, h, 100 μm; c, f, i, l, 10 μm.

motoneurons, as homogenous cytoplasmic immunoreactivity (Fig. 8a,d,g,j). In experimental rats, however, a highly time-dependent fluctuation of gp96 expression was noticed. Thus, in both attacks, most neurons exhibited inhomogeneous, spotty cytoplasmic gp96 staining (Fig. 8c,i), while in the parenchyma lymphatic infiltration was seen (Fig. 8b,h). At both remissions, however, these changes disappeared, implying that neurons had re-expressed their cytoplasmic gp96 reactivity (Fig. 8f,k,l). Simultaneously, at first remission a diffuse, extracellular gp96 staining and upregulation of gp96 was found on epithelial cells around the central canal (imbedded picture on Fig. 8e).

Immunohistochemical quantification of Gp96 expression in hippocampus and spinal cord during the CR-EAE

Since during the course of CR-EAE the expression of gp96 varied, particularly in the hippocampal areas and in lumbar spinal cord, the immunohistochemical staining quantification of gp96 expression was done in these zones in comparable slides obtained from experimental and control rats (Figs. 9, 10). The data showed that the previously described changes of gp96 expression in DG during the remissions (Fig. 9B) and in spinal cord during all estimated phases (Fig. 10B) in BBH+CFA-treated rats were statistically different from those found in rats treated by CFA ($p<0.001$), pointing to specificity of treatment. Furthermore, the expression of gp96, found in BBH+CFA-treated rats at remissions of EAE in the hippocampal area and in the spinal cord was significantly greater than that found in attacks of disease (Figs. 9B, 10B; $p<0.001$), implying that changes in gp96 expression were more related to the recovery processes.

Double labeling with anti-GFAP and anti-gp96 antibodies at the 28th p.i. day in the hippocampus also revealed that multiple GFAP-positive astrocytes were present among the gp96 positive cells in the granular layer of DG, which extended their processes around the vessels (Fig. 9C). It is interesting to note that large

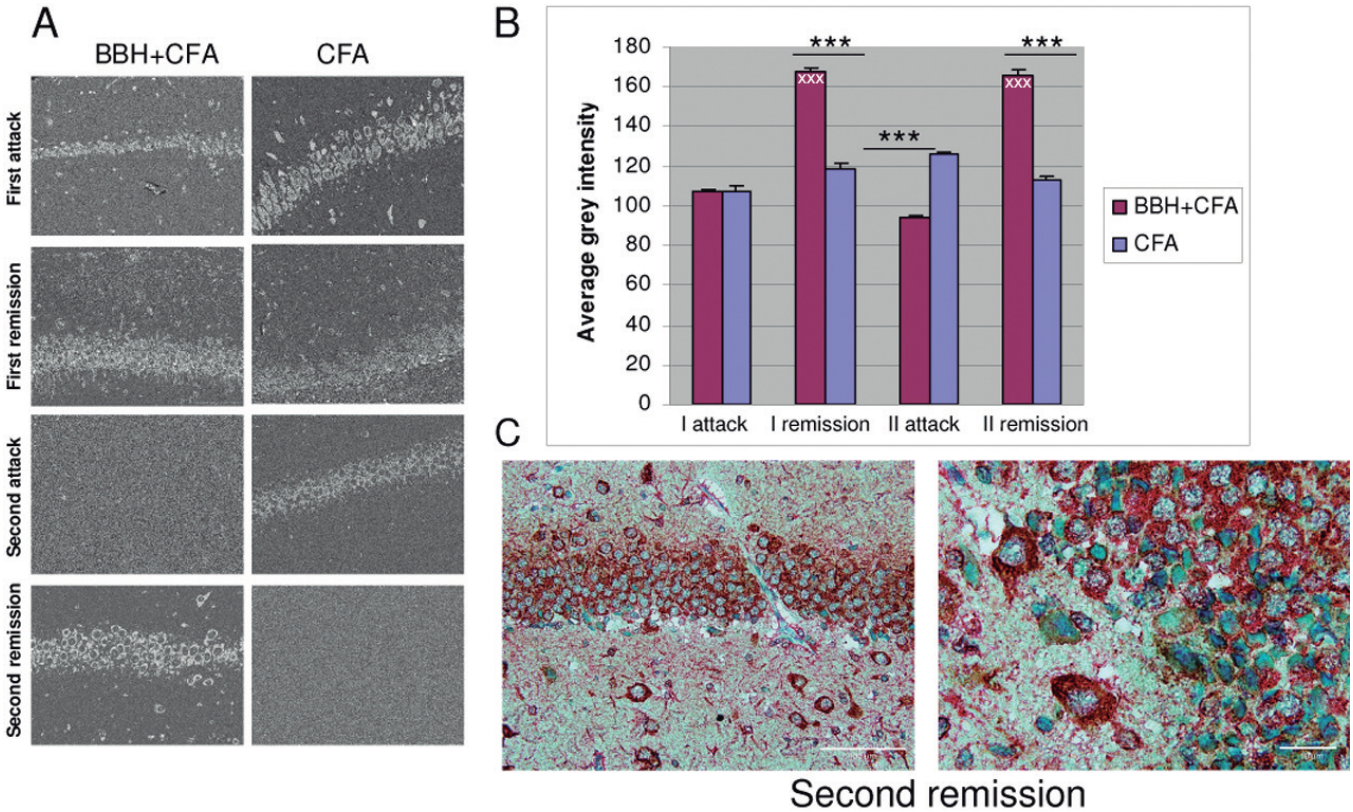

Fig. 9. A. Immunohistochemical, cell-based staining quantification of gp96 expression in hippocampal dentate gyrus found in analogous paraffin-embedded sections obtained from experimental (BBH+CFA) and control (CFA)-treated rats in different clinical stages of CR-EAE. **B.** Corresponding average grey distribution of gp96 expression (B) was calculated from twenty regions of interest (magnification 400 x per field) in the ten fields per microscopic slides of tissue samples. Results are mean ± SE. *** $p<0.001$ represents a statistically significant difference between BBH+CFA and CFA treated rats, xxx $p<0.001$ represents a statistically significant difference between findings in remissions and attacks in BBH+CFA and CFA treated rats. **C.** Single and double immunohistochemical labeling of gp96 and GFAP in hippocampal dentate gyrus during the second remission phase of CR-EAE. Brown staining indicates a single positivity for gp96; a red-colored precipitate indicates positivity for GFAP, while reddish brown (russet) coloration indicates the presence of both gp96 and GFAP. The results are representative findings of 3 rats. Scale bars: left, 100 μm; right, 10 μm.

GFAP-positive cells and some Gp96 positive cells were also present in the subgranular zone (SGZ) of the dentate gyrus, which is characterized by significant neurogenesis.

Single labeling with anti-GFAP antibodies and antibodies against the CD45RO isoform of the leukocyte common antigen at the 12th p.i. day, also showed that during the first attack of EAE, GFAP positive astrocytes were present in the spinal cord (Fig. 10Cb) and several cells with the T cell "memory" phenotype, located around the blood vessels (Fig. 10Ca). Besides, double labeling with anti-GFAP and anti-gp96 antibodies at the 28th p.i. day revealed that during the second remission numerous gp96-positive neurons were present among the multiple GFAP-positive astrocytes (Fig. 10c).

Discussion

Using highly susceptible DA rats and immunization with BBH or MBP in combination with CFA as animal models for the induction of chronic relapsing or monophasic form of EAE, respectively (Mostarica-Stojkovic et al., 1982; Vukmanovic et al., 1990; Jakovac et al., 2011), here we point to large temporal variations in the expression of ER-resident molecular chaperone gp96 in the brain and spinal cord, emphasizing its important functions in the maintenance of cell homeostasis during an autoimmune disorder. Overall, the data imply that gp96 was downregulated in several cells during attacks and selectively upregulated in some regions in the remission phases of the disease, but we can only speculate about the causative relationship between this chaperon and the pathogenesis of disease, since the induction of HSPs involves complex regulatory pathways, which ultimately lead to differential responses on stress signals in different types of cells (Lindholm et al., 2006; van Noort, 2008; Stetler et al., 2010). However, since this time-related study is, to our

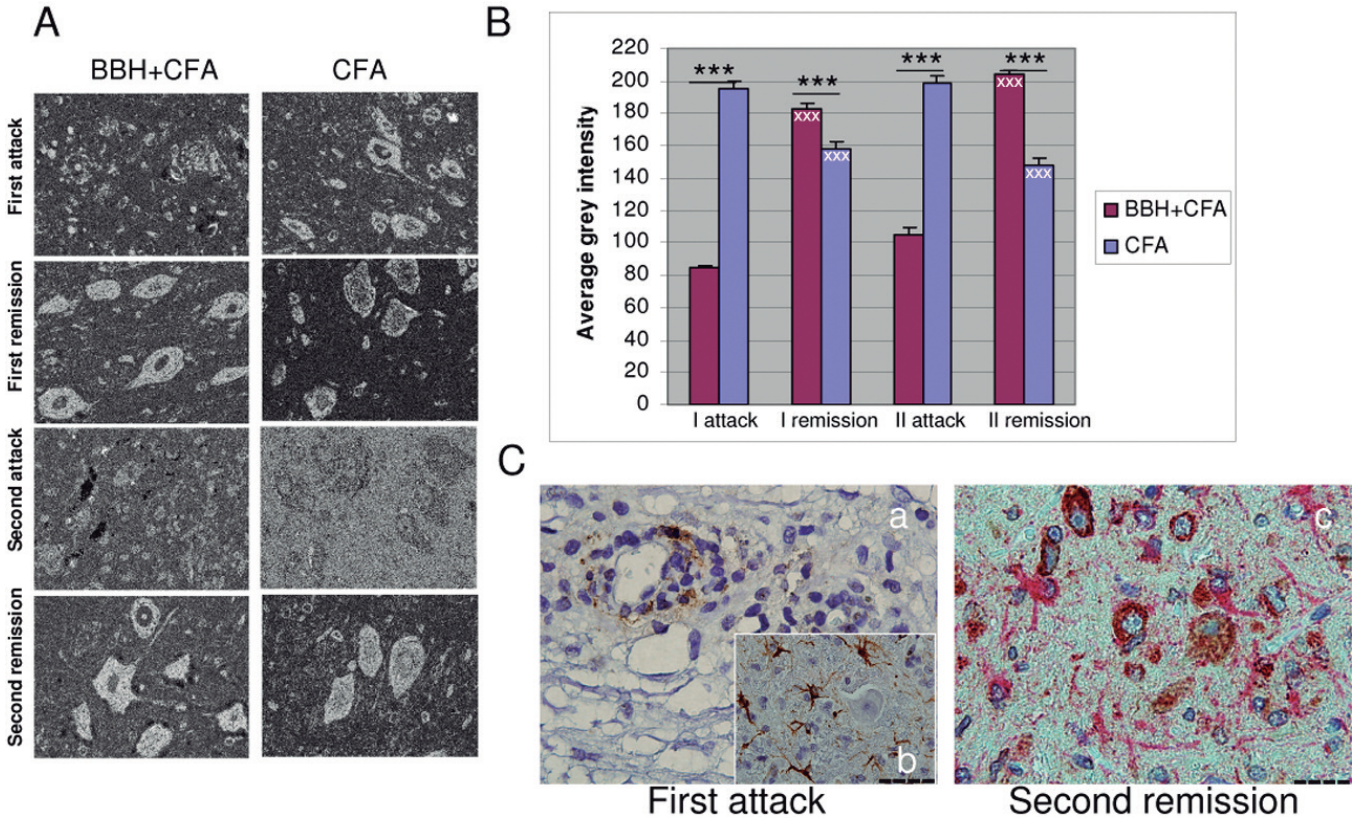

Fig. 10. A. Immunohistochemical, cell-based staining quantification of gp96 expression in lumbar spinal cord found in analogous paraffin-embedded sections obtained from experimental (BBH+CFA) and control (CFA)-treated rats in different clinical stages of CR-EAE. **B.** Corresponding average grey distribution of gp96 expression in spinal cord was calculated from twenty regions of interest (magnification 400 x per field) in the ten fields per microscopic slide of tissue samples. Results are mean ± SE. *** $p<0.001$ represents statistically significant difference between BBH+CFA and CFA treated rats, xxx $p<0.001$ represents statistically significant difference between findings in remissions and attacks in BBH+CFA and CFA treated rats. **C.** Single immunohistochemical labeling of CD45RO **(a)** and GFAP **(b)** in samples of spinal cord during the first attack of CR-EAE; Single and double immunohistochemical labeling of gp96 and GFAP in spinal cord during the second remission phase of CR-EAE (c). Brown staining indicates a single positivity for gp96; a red-colored precipitate indicates positivity for GFAP, while reddish brown (russet) coloration indicates the presence of both gp96 and GFAP. The results are representative findings of 3 rats. Scale bar: 10 μm.

knowledge, the first description of the kinetics of gp96 expression in EAE-susceptible DA rats, we will try to discuss our data in the context of known facts about the pathogenesis of autoimmune processes in this strain of rats and on the role of HSPs in EAE, trying also to compare these results with our recently published data about the expression of metallothioneins (MTs) in the same experimental model (Jakovac et al., 2011).

EAE induced in rats by BBH+CFA belongs to a widely employed old animal model, which induces the relapsing-remitting course of disease, similar to multiple sclerosis (for a detailed review of the immunogenetic and pathogenetic features of various experimental models please see (Krishnamoorthy and Wekerle, 2009; Batoulis et al., 2010; Lassmann, 2010; Mix et al., 2010). Generally, the response to encephalitogenic determinants is governed by MHC class II and T cell receptor repertoire available to the animal. CD4/TH1 paradigm states that the major pathogenic component of the disease are the CD4+ T cells that display a TH1 polarized cytokine profile, but new evidence shows that CNS lesion topography depends on TH17:TH1 ratio of cells that enter the CNS via the epithelium of the plexus choroideus in several other cells (TH9, Treg cells, B cells, NK and NKT cells, microglial cells, astrocytes), as well as on a variety of local pathogenic and physiological mechanisms that modulate the autoimmune responses at many levels (Batoulis et al., 2010). The autoimmune reaction against myelin components in the CNS leads to axonal loss and permanent clinical disability, but the interactions between inflammation, demyelination and neurodegeneration are still being investigated (Peterson and Fujinami, 2007; Lassmann, 2010).

Clinical course and development of CNS lesions in DA rats depends on the choice of adjuvant/myelin protein used for immunization and the timing of their administration. It is, however, well known that EAE-susceptible DA rats and EAE-resistant AO rats differ significantly in their peripheral and central response to immunization with encephalitogenic emulsion (BBH or spinal cord homogenate in CFA) (Mostarica-Stojkovic et al., 1985; Markovic et al., 2009). They recruit more CD4+ and CD17+ T cells and produce higher levels of pro-inflammatory cytokines, including IFN-γ, IL-17, IL-6, IL-12 and IL-23 in draining lymph nodes (Miljkovic et al., 2006; Markovic et al., 2009). Furthermore, recent reports emphasize that the susceptibility of these rats to the induction of EAE markedly depends on the target tissue, since they had less active mechanisms for the elimination of autoreactive effector cells by apoptosis, as well as for the chemokine-induced suppression of autoimmune processes (Miljkovic et al., 2011).

Well known regulators of the immune responsiveness of the CNS milieu are MTs and HSPs, which are constitutively expressed or induced by various stressful stimuli in the CNS parenchyma (for review please see (Hidalgo et al., 2006; Penkowa, 2006; Manso et al., 2011) and (Lindholm et al., 2006; van Noort, 2008; Stetler et al., 2010) respectively. In this sense, our previously reported data confirmed significant participation of MT-1/MT-2 isoforms in pathogenesis of EAE in DA rats, showing also that upregulation of MTs was followed by marked changes in the concentrations of zinc and copper in the brain and spinal cord, as well as in the liver (Jakovac et al., 2011). Interestingly, the dynamics and distribution of MT-1/MT-2 expression during CR-EAE was found to be different in comparison with gp96 expression, since MT I-II were more expressed in attacks of disease (in subarachnoid regions and perivascular space on ependymal cells, epithelial cells of choroid plexus, endothelial cells, astrocytes, microglia and spinal neurons), while gp96 was upregulated in the resolution phases of CR-EAE (particularly on oligodendrocyte-like cells in the brain white matter and in hippocampal regions), suggesting that these stress proteins might be activated by various mechanisms and/or that all brain structures were not equally susceptible to inflammatory and other types of stress that result in neuroinflammation and neurodegeneration.

Regarding the latter, it is well known that the heat shock response represents an essential reaction primarily to acute and chronic proteotoxic damage (Gidalevitz et al., 2011). ER-stress may arise in highly secretory cells, such as oligodendrocytes by an increase in protein load, or can be induced by conditions that disrupt protein folding in the ER, such as hypoxia, inhibition of protein glycosylation and perturbation of the redox potential of the ER and Ca^{2+} depletion from the ER lumen (Ma and Hendershot, 2004; Nishikawa et al., 2005; Yang and Li, 2005; Wu and Kaufman, 2006; Treglia et al., 2012). The response is highly dependent on the intensity of stress and involves the activities of heat shock factors 1 (HSF1) (for recent review please see Neef et al., 2011), and three classes of ER resident transmembrane proteins - inositol-requiring protein-1 (IRE1), activating transcription factor-6 (ATF6), and protein kinase RNA (PKR)-like endoplasmic reticulum kinase (PERK), which sense the state of protein folding and regulate the outcome of UPR signaling pathways, leading to transcriptional activation or repression of genes that regulate cell survival, death or differentiation (for review please see Treglia et al., 2012).

For our data it might be relevant that stress-dependent regulation of HSF1 is a multistep, time-regulated process that is controlled by a feedback loop in which the newly synthesized HSP90, HSP70, HSP40 inhibit HSF1 activity (Neef et al., 2011). Thus, under basal conditions HSF1 exists largely as an inactive monomer in the cytoplasm, repressed through the interaction with HSP90, HSP70 and HSP40 and its phosphorylation on Ser 303 and ser 307 residues. During the proteotoxic stress HSP90 is, however, dissociated from HSF1, owing to its binding to misfolding proteins. This allows HSF1 to form homotrimers that accumulate in the nucleus and bind to heat shock elements in the promoters of stress-responsive genes, resulting in the

transcriptional activation of genes that encode chaperone proteins and other components of the ER protein folding and degradation machinery, also regulating several other aspects of cell survival, such as ion transports, energy generation, vesicular transport, cytoskeleton formation etc. (Lindholm et al., 2006; Stetler et al., 2010; Neef et al., 2011). UPR includes, therefore, the repression of translation, upregulation of ER chaperons and shuttling of misfolded proteins out of the ER to the proteasome for degradation, or activation of alternative mechanisms of protein degradation, such as lysosomal autophagy. For retrotranslocation of misfolded proteins from the ER to the proteasome and activation of ubiquitin-proteasome pathway the crosstalk of HSPC with the cytosolic members of the HSPA and DNAJ families is required, pointing to high functional interrelationship between diverse classes of molecular chaperons (for reviews please see Sherman and Goldberg 2001; Stetler et al., 2010).

Preventing the aggregation of insoluble misfolded or mutant proteins and promoting the fusion of sequestrosomes to autophagic vacuoles and lysosome, molecular chaperones also enhance the cell's capacity to prevent the formation of "aggresomes", which in neurons may have toxic effects. The significance of these and other ER–mitochondria interactions for the pathogenesis of various human neuronal diseases has been comprehensively reviewed elsewhere (Lindholm et al., 2006; Stetler et al., 2010; Gidalevitz et al., 2011; Neef et al., 2011).

Besides, it should be emphasized that HSPs released from stressed cells also act extracellularly, as factors that stimulate both pro- and anti-inflammatory effects, affecting cytokines, chemokines, and cytolytic immune cells (Srivastava, 2002b; Gidalevitz et al., 2011). In this sense, it was found that in particular the extracellular presence of Hsp70 and Hsp27 may be used as valuable biomarkers of CNS ischemia, since their quantitative, measurement in the cerebrospinal fluid may even predict the risk of perioperative paralysis (Hecker and McGarvey, 2011).

Our data, showing high temporal variation in the expression of gp96 during chronic relapsing and monophasic form of EAE are, therefore, consistent with the current knowledge. Generally, they confirm that the susceptibility to protein misfolding disorders and other types of ER-stress depends on variations in the constitutive expression of HSPs (Chen and Brown, 2007), on the differentiation state of cells (Hatayama et al., 1997; Oza et al., 2008), as well as on the regional distribution of genes related to inflammation, immune response, apoptosis and repair (Zeier et al., 2011) that regulate the sensitivity of cells to pathophysiological stressors (Kato et al., 1993).

The data found in untreated rats are, thus, in agreement with evidence showing that gp96 (Duzhak et al., 2003) and several other HSPs (Lindholm et al., 2006; van Noort, 2008; Stetler et al., 2010) may be constitutively expressed in almost all neural cells and oligodendrocytes, where they play key roles in routine protein folding and transport, as well as in secretory pathways for lipids in myelinating cells and those involved in the biosynthesis of steroids (Smith and Toft, 2008) or participate in the regulation of cellular calcium, which is mainly stored in the ER lumen (Lin and Popko, 2009).

Besides, our data showing a great fluctuation of gp96 expression in areas that are vulnerable to autoimmune injury and responsible for neurogenesis (Dayer et al., 2003; Doetsch, 2003) imply that the late up-expression of gp96 in the hippocampal area, in white matter, and in spinal neurons might be an index of the remyelination and neuronal reorganization that occur after the immune attack in these areas. The hypothesis needs to be proven, but seems to be supported by findings of Ziehn and coworkers (2010) in myelin oligodendrocyte glycoprotein (MOG)-induced EAE, in which they have clearly demonstrated that the process of hippocampal neurodegeneration and inflammation was an early sign of developing EAE. It was characterized by a marked decrease in CA1 pyramidal volume, a significant loss of GABA-ergic interneurons, a reduction in pre-synaptic puncta and synaptic protein expression that contributed to the development of cognitive deficits and memory dysfunction, similar to that seen in MS patients. Moreover, in accordance with our findings, Ziehn and coworkers (2010) found that widespread focal demyelinated lesions in the hippocampus were followed by increased apoptosis of pyramidal neurons, interneurons and astrocytes and chronic microglial activation in the later stage of EAE. Besides, supporting the opinion that a CNS antigen-specific immune response might be followed by cellular dedifferentiation and an increased proliferation of immature neurons that may retain the expression of the glial marker (Dayer et al., 2003; Huehnchen et al., 2011), we point herein to extensive immunostaining of gp96 in the granular cell layer of DG and to the presence of several large cells expressing the GFAP during the remission phases of CR-EAE (Figs. 4, 7 and 9).

The significant participation of HSPs in these events are also emphasized by data showing that the vulnerability of neurons in hippocampal areas might depend on expression of gp96/grp94 (Bando et al., 2003), as well as by evidence showing that the expression levels of several heat shock proteins (HSP) including HSP90, HSP70, HSP40, and HSP27 in the three main regions of the hippocampus (the CA1, CA3, DG) might be differently induced in response to environmental and pathophysiological stressors, including aging and caloric restriction and EAE (for review please see (Zeier et al., 2011). Besides, supporting our opinion that gp96 might be related to the ongoing repair, in the model of experimental brain ischemia, it was shown that the late synthesis of hsp72 was linked with compensatory mechanisms, associated with remodeling of dendrites and axons (Fredduzzi, 2001). Similarly, a delayed induction at the mRNA level

was described for HSPA5/GRP78 and HSPC/GRP94 family members of HSPs following global ischemia (Truettner et al., 2009).

Similar involvement of ER chaperones was described in various other experimental conditions and neurodegenerative diseases (van Noort, 2008), also pointing to marked participation of brain areas that control the neuroendocrine stress response, such as dentate gyrus, habenula, hypothalamus, granular layer of the cerebellum and the olfactory area. Noteworthy, most of them react on stressful condition through glucocorticoids receptors that belong to nuclear hormone receptor superfamily (García-Bueno et al., 2008), which is also regulated by HSP (Noguchi et al., 2010; Smith and Toft, 2008).

In agreement with reports showing that in chronic inflammatory disease of the CNS, such as MS, nonprogressive neuronal loss may also occur at cervical and lumbar levels (Schirmer et al., 2009), our data also show that during attacks of CR-EAE model, the expression of gp96 in motoneurons of lumbar spinal cord is markedly decreased (Fig. 8c,i), pointing to their vulnerability to detrimental effects of misfolded and/or aggregated proteins, and its possible involvement in mechanisms regulating antigen presentation and inflammatory signaling pathways and apoptosis (Yenari et al., 2005; van Noort, 2008). In this sense, we can speculate that the presence of inhomogeneous cytoplasmatic gp96 staining in spinal motoneurons in both attacks (Fig. 8b, c and h, i) points to the association of gp96 to misfolded or unfolded proteins and initial neurodegeneration, as was described in motor neurons of ALS patients (Sasaki, 2010). However, the causative link between gp96 downregulation and clinical score of EAE cannot be explained, since axonal and neuronal pathology during EAE involves multiple and different mechanisms (Lassmann, 2010). The hypothesis is, however, consistent with the evidence showing that impaired proteostasis leads to aggregation toxicity (Kikis et al., 2010), as well as with the fact that IFNs trigger ubiquitylation machinery functioning to target defective ribosomal products for degradation by immuno-proteasomes (Seifert et al., 2010). Moreover, since during ERAD the ER chaperones might also stimulate the mechanisms that lead to cell death (Morishima et al., 2002; Ma and Hendershot, 2004; Nishikawa et al., 2005; Yang and Li, 2005; Wu and Kaufman, 2006; Lai et al., 2007), there is a possibility that gp96-peptide complexes, released from injured cells, contributed to activation of CD8+ or CD4+ T-cell responses specific to the chaperoned antigenic peptide (Srivastava, 2002a; Wallin et al., 2002; Tsan and Gao, 2004; Yenary et al., 2005; van Noort, 2008) and/or to activation of dendritic cells (Jockheck-Clark et al., 2010). Our data, which show that during spinal cord injuries in lymphatic infiltrates were present CD45RO positive T cells (Fig. 10Ca), which might belong to circulating myelin-reactive cells with "memory" phenotype, seem to support this possibility, but the hypothesis needs to be proven, although it is in accordance with findings that the association of hsp70 with MBP, myelin proteolipid protein and myelin oligodendrocyte protein might enhance immune recognition of autoantigen in EAE and MS (Cwiklinska et al., 2003).

In animals that have survived the attacks of disease we also found high upregulation of gp96 expression in the brain white matter, implying that in remission phases of EAE it has contributed to the survival of oligodendrocytes, which are responsible for the myelination process. The hypothesis is supported by evidence showing that ER stress in immune-mediated demyelinating disorders might have both beneficial and detrimental effects on oligodendrocyte survival, depending on the developmental status of the cells. As reviewed by Lin and Popko (2009) the outcome is highly dependent on the activation of the PERK-eIF2· pathway and specific for myelinating cells, since they may respond to ER stress in a manner that is somewhat distinct from that observed in other cell types, since CHOP, which is a downstream effecter molecule of the PERKeIF2α pathway does not contribute to UPR-induced apoptosis, but promotes oligodendrocyte survival during ER stress (Lin and Popko, 2009).

Moreover, the evidence shows that gp96 is also able to downregulate the pre-existing pathological autoimmune response through the generation of immunoregulatory CD4+ T cells that prevent myelin basic protein or proteolipid protein-induced EAE in SJL mice, as well as the onset of diabetes in non-obese diabetic mice (Chandawarkar et al., 2004). Interestingly, in the latter report it was shown that the suppression of the immune response by a high-dose of gp96 required as a substrate an ongoing immune response, indicating that gp96 was able to cause the deviation of pre-existing and existing antigen-specific CD4 response into an antigen-specific suppressor CD4 population, or induce the activation of a non-specific super-regulatory suppressor population that could inhibit any activated T cell response (Chandawarkar et al., 2004). Confirming this proposal recent reports also showed that gp96 was able to stimulate the regulatory T (T_{reg}) cells in a dose-dependent manner (Liu et al., 2009), as well as that gp96 might regulate the generation of tolerogenic pDCs that participate in restraining an immune response (De Filippo et al., 2008) and in maintenance of immune self-tolerance in multiple sclerosis (Zozulya and Wiendl, 2008). The important role of molecular chaperones in the development of autoimmune diseases is also emphasized by data showing that Hsp70-peptide complexes (pc) isolated from brains of mice with EAE prevented clinically and morphologically the development of EAE when administered before proteolipid protein 139-151 (Galazka et al., 2006). Moreover, transfer of NK cells from Hsp70-pc-immunized mice to recipients sensitized for EAE abolished the disease development, showing that Hsp70 has the ability to bind to peptides generated during brain inflammation and induce a regulatory NK cell

population that is capable of preventing subsequent autoimmunization for EAE. Additionally, it was shown that the induction of regulatory NK cells by Hsp70-pc was dependent on cross-activation between H60 and NKG2D, leading to altered dendritic cells (Galazka et al., 2007).

In the light of this evidence our data, showing initial downregulation (Figs 3, 6, 8) and marked upregulation of gp96 during remission phases of CR-EAE (Figs.4, 5, 7, 8, 9, 10) imply that it contributed to protein homeostasis and regulation of autoimmune attack during CR-EAE.

Conclusions

Taken together, our data point to a large temporal and regional variability in the expression of gp96 during chronic relapsing EAE, emphasizing its interaction with multiple ER quality control pathways and with processes of demyelination and repair in autoimmune disorders. Although the mechanisms need still to be clarified, our data support the increasing evidence showing that ER chaperones might be induced to ameliorate the accumulation of misfolded proteins in the CNS and protect neuronal cells against autoimmune attack (Chandawarkar et al., 2004; van Noort, 2008; Zozulya and Wiendl, 2008; Stetler et al., 2010; Voisine et al., 2010; Neef et al., 2011).

Acknowledgements. This work was supported by a grant from the Croatian Ministry of Science (Project No 62-0621341-1337). We are grateful to Prof. Dr. Adriana del Rey (Marburg, Germany) for help and critical comments on the manuscript.

Declaration of interest. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Accepted July 6, 2012