Detection of galanin receptors in the spinal cord in experimental autoimmune encephalomyelitis

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Aims. The neuropeptide galanin is a widely distributed neurotransmitter/neuromodulator that regulates a variety of physiological processes and also participates in the regulation of stress responses. The aims of the present study were to investigate the expression of galanin receptors (GalR1, GalR2, GalR3) in the spinal cords in a murine model of multiple sclerosis, experimental autoimmune encephalomyelitis (EAE) using qPCR analysis and to determine GalR1 cellular localization (oligodendrocytes, microglia, astrocytes, ependymal cells, and endothelial cells in the capillaries) by immunohistochemistry.

Methods. Twelve samples from the EAE group and 14 samples from the control group were analyzed. Spinal cords samples were obtained at the peak of the EAE disease.

Results. The GalR1 mRNA level was significantly decreased in the EAE mice compared with the controls (P=0.016), whereas the mRNA levels of GalR2 and GalR3 were not significantly different for the EAE and the control mice. No significant correlations were found between the severity of the EAE disease and the mRNA levels of GalR1, GalR2 and GalR3. Immunochemical detection of the GalR1 revealed its expression in the ependymal and endothelial cells. Additionally, a weak GalR1 immunoreactivity was occasionally detected in the oligodendrocytes.

Conclusion. This study provides additional evidence of galanin involvement in EAE pathophysiology, but this has to be further investigated.

Key words: multiple sclerosis, experimental autoimmune encephalomyelitis, mRNA, galanin, GalR1, GalR2, GalR3, immunohistochemistry

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INTRODUCTION

Multiple sclerosis (MS) is an autoimmune neurological disease characterized by chronic inflammation of the central nervous system (CNS), resulting in a range of physical, mental and psychiatric symptoms[1,2]. The pathophysiology of MS is not fully elucidated. It is generally accepted that MS arises from a combination of genetic susceptibility, epigenetic events, and various environmental factors such as chemical and microbiological agents, smoking and diet[3]. The past decade of multiple sclerosis research has been marked by important advances in the understanding of the disease. This progress has made personalized medicine in MS a realistic possibility. Despite outstanding progress in the development of novel therapeutic agents for MS in recent years, we are still far from discovering an ultimate drug for MS. Current disease-modifying therapies (DMT) aim at prevention of inflammatory damage to the central nervous system (CNS), but their severe adverse effects are pushing for new, safer therapeutic approaches.

A possible drug candidate for MS may be found within the group of neuropeptides. The neuropeptide galanin is a widely distributed neurotransmitter/neuromodulator that regulates a variety of physiological processes and also participates in the regulation of the stress response[4,5]. The effects of galanin are mediated via three types of receptors (GalR1, GalR2 and GalR3) that are widely expressed in the CNS and peripheral nervous system (PNS), as well as in extra-neuronal tissues[4,5]. Galanin acts as a co-transmitter in the CNS and plays an important role in several physiological and pathophysiological conditions such as epilepsy, depression, Alzheimer's disease, and memory[4,5]. So far, the presence of the galanin receptors has been detected in endothelial cells in the capillaries, as well as in all glial cells (oligodendrocytes, astrocytes, microglia), with the exception of ependymal cells[6-10]. Galanin was found to be a potent modulator of cytokine and chemo-

kine expression in macrophages[7]. All galanin receptors seem to be involved in the immune response modulation but specifically GalR3 was found to be crucial for the pathophysiology of different inflammatory diseases. Namely, GalR3 deletion worsened the disease severity in experimental murine models of arthritis, psoriasis, pancreatitis, and colitis[11-14]. Furthermore, galanin was reported to be a protective factor against oxidative stress in rat astrocytes *in vitro* and these effects were mediated by GalR2 (ref.[15]). Moreover, galanin receptors GalR1 and GalR2 were detected in the endothelial cells of the eye and skin[6,8] and may be involved in the process of angiogenesis[6,16-18]. In this case, galanin might actually have a detrimental role, as angiogenesis seems to contribute to the progression of the disease in MS (ref.[19]).

Previous studies have found a neuroprotective role of galanin in the murine models of MS, experimental autoimmune encephalomyelitis (EAE) and cuprizone-induced demyelination[9,10,20]. These studies mostly focused on the galanin impact on oligodendrocytes and its ability to promote remyelination; however, there are no data on the distribution of galanin receptors on other glial cells. Namely, galanin was reported to be a survival factor for oligodendrocytes *in vitro*[20]. In oligodendrocyte cell cultures, the mRNA levels of GalR2 were 100-fold higher than GalR3 and 1,000-fold higher than GalR1 (ref.[20]). In the cuprizone-induced MS model, GalR1 and GalR2 were found to be upregulated in the oligodendrocytes during demyelination and remyelination, respectively[10]. Transgenic mice with chronically elevated galanin levels (10-fold higher than in wild-type (WT) mice) were shown to be relatively resistant to the cuprizone-mediated corpus callosum (CC) demyelination[10]. The authors observed a 2.5-fold increase in the number of callosal mature oligodendrocytes at the time of maximal demyelination. Considering new findings on neuroprotective and immunomodulatory effects of galanin in the CNS, the aim of the present study was to investigate the expression of galanin receptors in the spinal cords in the EAE and to determine localization of GalR1 on different cells (oligodendrocytes, microglia, astrocytes, ependymal cells, and endothelial cells in the capillaries) by immunohistochemistry.

MATERIALS AND METHODS

Animals and induction of EAE

EAE was actively induced in conventional C57Bl/6 mice (9–13 weeks old) by immunization with MOG35-55 peptide (Prospec, Rehovot, Israel) and complete Freund's adjuvant containing *Mycobacterium tuberculosis* H37Ra (Sigma-Aldrich, Prague, Czech Republic). The MOG/CFA emulsion was made by connecting two glass syringes with a 3-way connector and passing the solutions through the connector. The emulsion was injected subcutaneously in two 50 µL doses. In total, 100 µg of MOG peptide to the conventional mice was administered. To facilitate the transfer of lymphocytes into the CNS, 300 ng pertussis toxin (List Biologicals, Campbell, USA) in 200 µL PBS
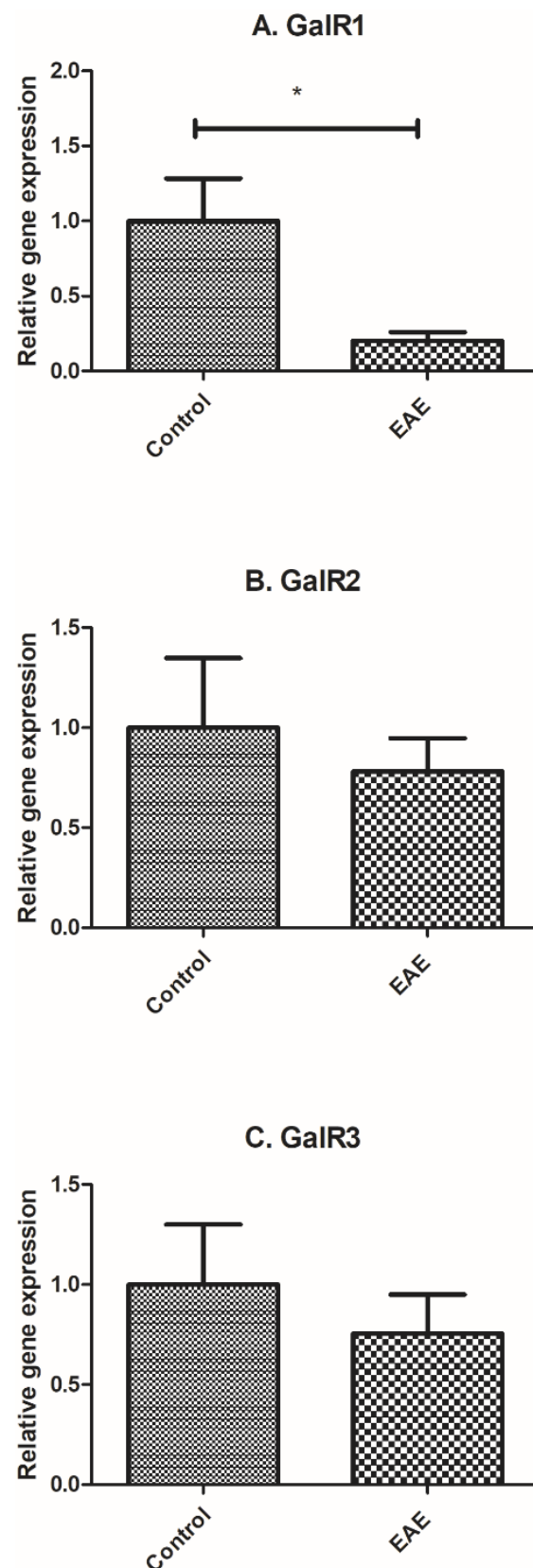

Fig. 1. Expression of galanin receptors in spinal cord in the control and experimental autoimmune encephalomyelitis (EAE) mice.
Relative gene expression of A. galanin receptor 1; B. galanin receptor 2; C. galanin receptor 3. Results are presented as mean ± SD (n = 12 samples in the EAE and 14 samples in the control group), * $P<0.05$

Table 1. Primer sequences for target gene detection.

Gene	Reverse	Forward
GalR1	GTGATTCTGAAGAAGGAAG	AAAAGTCTGAAGCATCCAAG
GalR2	GACTGTAGTAGCTCAGGTAG	CGTTCATTTCCTCATCTTCC
GalR3	CCAGGTACCTATCCACTG	ATCTACACACTGGATGCC
Beta-actine	TCGTCATCCATGGCGAACTGG	CTGTCGAGTCGCGTCCACC

was injected intraperitoneally two hours and two days after the EAE induction[21].

The signs of EAE were scored in the following way: 0 – no signs of clinical disease; 1 – limp tail; 2 – weakness of hind legs; 3 – complete paralysis of hind legs; 4 – hind and forelimb paralysis; 5 – dead. Pilot study before the main experiments has indicated that the peak of the EAE disease in our lab is on the 5th day after the beginning of the clinical symptoms. On day 5 after the induction (the severity disease peak, Fig. 1), the mice were euthanized, decapitated and the spinal cords were removed. Twelve samples from the EAE and 14 samples from the control group were analyzed, respectively. All experiments were approved by the Ministry of Education, Youth and Sports of the Czech Republic under the number MSMT-9445/2018-8. The mice were supplied with a standard granulated diet and water *ad libitum* and were housed in standard environmental conditions: light (i.e. 12 h light and 12 h dark); temperature (22 ± 2 °C); relative humidity ($50 \pm 10\%$).

qPCR for gene expression

Spinal cords and brains were stored in RNAlater™ Stabilization Solution at -80°C for later qPCR analysis. Total RNA was extracted using TRI reagent (Sigma-Aldrich, Prague, Czech Republic). cDNA was synthesized from RNA using a M-MLV Reverse Transcriptase (Top Bio, Prague, Czech Republic). cDNA served as a template for amplification of target genes, as well as the house-keeping gene β-actin (Actb gene) by the quantitative real-time PCR with SsoAdvanced™ Universal SYBR® Green Supermix (Biorad, Prague, Czech Republic). cDNA was analyzed by CFX96 Touch Real-Time PCR Detection System (Biorad, Prague, Czech Republic). Target genes were GalR1, GalR2, and GalR3 (the primer sequences are presented in Table 1). The PCR cycling program was as follows:

95 °C for 30 s and 40 cycles of 95 °C for 15 s and 60 °C for 30 s. The melting curve program was as follows: 65 °C to 95 °C at 0.5 °C/5s. The expression of the target genes was calculated by comparison of relative levels after normalization to β-actin expression[22].

Immunohistochemistry

Samples of the spinal cords were immediately fixed in 4% paraformaldehyde, cryoprotected with sucrose, embedded into the optimal cutting temperature compound, frozen at -20 °C and stored until further use. For the indirect immunofluorescence method, 7 μm thick cryosections were used. After thawing and washing in the Phosphate Buffered Saline (PBS), non-specific antibody binding
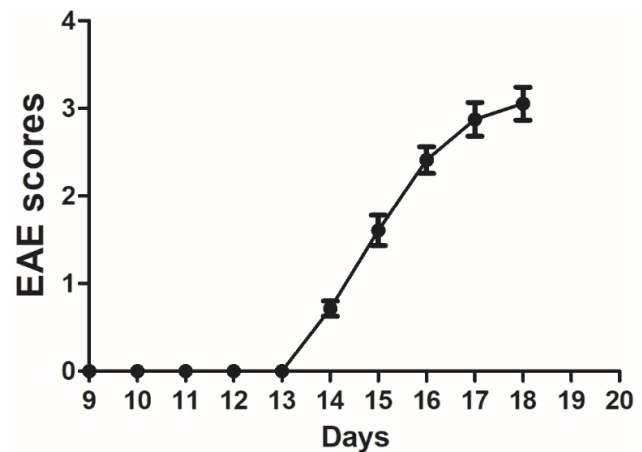
Fig. 2. Development of EAE in the experimental group.

sites were blocked with 5% goat serum in 0.1 % Triton X-100 - Phosphate Buffered Saline (TPBS). Sections were incubated with rabbit anti-human galanin receptor 1 diluted 1:500 (Alomone Labs, Jerusalem, Israel) in TPBS + 1.5% normal goat serum overnight at 4 °C. For visualization, a secondary antibody goat anti-rabbit Cy3 (Jackson Immunoresearch, Cambridge, United Kingdom) diluted 1:500 in TPBS + 5% normal goat serum was applied to sections for 30 min at room temperature. The nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). Imaging was performed on the LSCM Olympus FluoView 1000 (Olympus, Tokyo, Japan). The control slides, where the primary antibody was omitted, gave a negative result.

Histological analyses

Samples of the spine with spinal cords were fixed with 4% formaldehyde and spinal cords were carefully removed from the vertebral canal. Material was embedded into paraffin wax. Sections (7μm thick) were stained with hematoxylin-eosin staining. Images were captured at Leica DMLB microscope with MC170 HD camera (Leica Microsystems, Wetzlar, Germany). The inflammation was scored as previously described[23]. Briefly, 12 sections per animal were evaluated. The inflammation extent was scored as follows: 0 = no inflammation evident; 1 = small number of inflammatory cells; 2 = numerous infiltrating cells; 3 = extension of perivascular cuffing into adjacent tissues (widespread infiltration).

Statistical analysis

All statistical analyses were performed using GraphPad Prism 8.2.1 software (GraphPad Inc., La Jolla, USA).
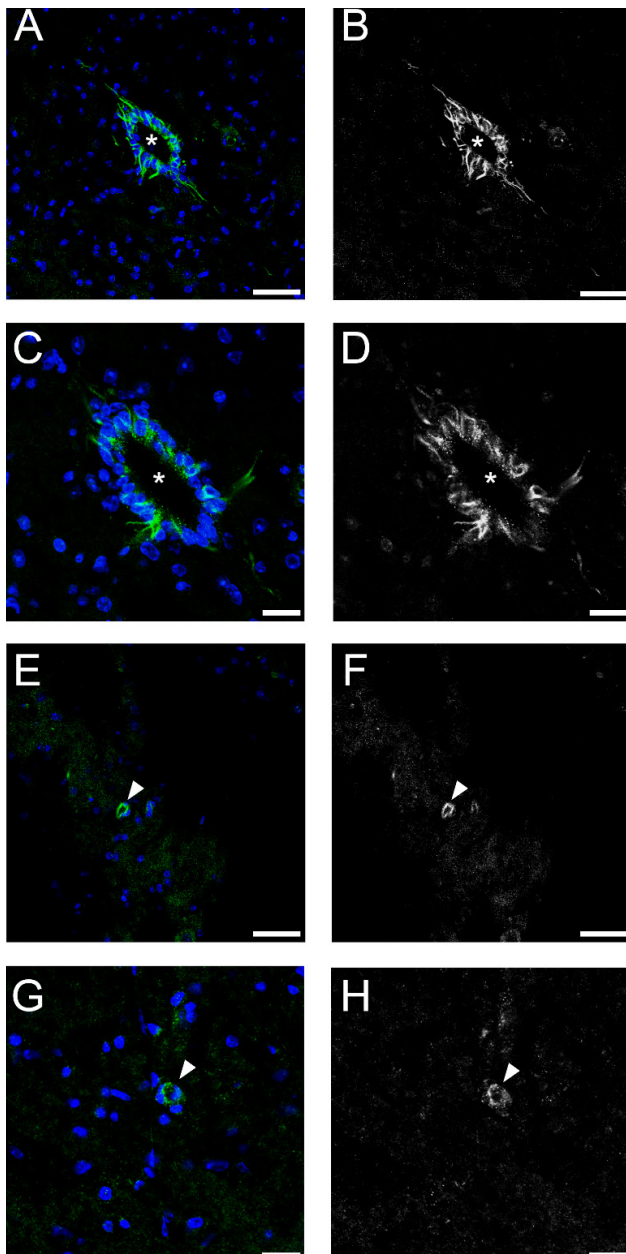
Fig. 3. Immunohistochemical detection of GalR1 in the spinal cord. The intensity of GalR1 immunoreactivity was comparable between the samples from the EAE mice and the control mice. Confocal images showing results of immunofluorescence labeling for GalR1 in the spinal cord of mice with EAE (A, B, G, H) and healthy control mice (C–F). (A, C) GalR1 is expressed in ependymal cells (green) around the central canal of the spinal cord (*). Nuclei are stained with DAPI (blue). (B, D) A single channel from (A, C) corresponding to GalR1 immunoreactivity. (E, G) GalR1 (green) is expressed in the endothelium of capillaries in the spinal cord (arrowhead). Nuclei are stained with DAPI (blue). (F, H) A single channel from (E, G) showing GalR1 expression. Scale bar in A, B, E, F =50 μm; in C, D, G, H = 20 μm.
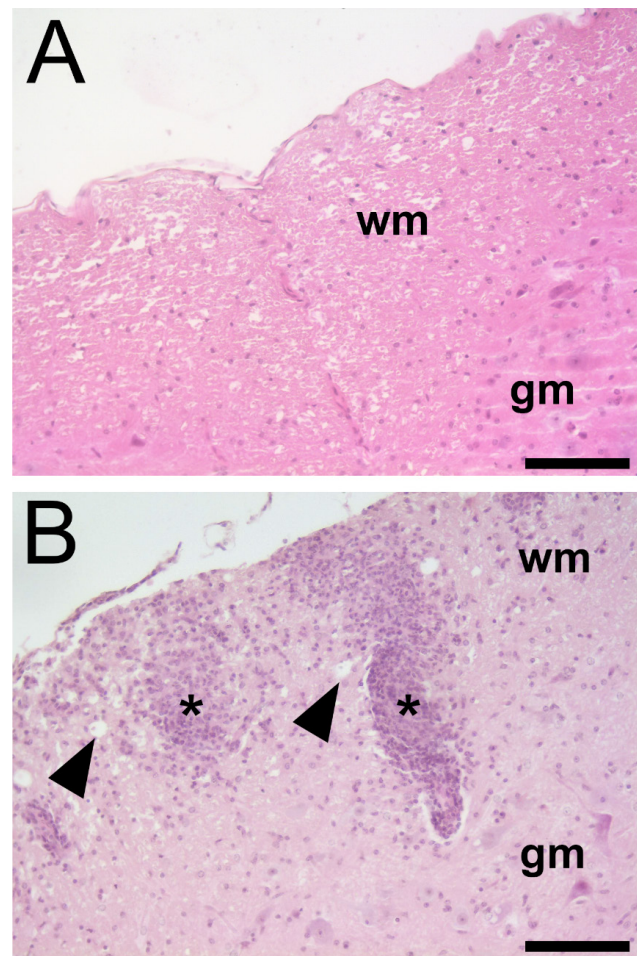

Fig. 4. Morphology of the spinal cord in the control and experimental autoimmune encephalomyelitis (EAE) mice.
A. Image of white matter of the spinal cord from the control healthy animal. Structure of the spinal cord is well-preserved, and no inflammatory infiltration is present. B. Image of white matter of the spinal cord from an animal with EAE. Two inflammatory foci are visible (asterisk) together with multiple swollen axons (arrowheads). Hematoxylin-eosin staining. Scale bars in A-B=50 μm. wm = white matter; gm = gray matter

The normality of the data was checked using the Shapiro-Wilk test. Because the data were normally distributed, an unpaired T test was used to test for differences between the levels of galanin receptor mRNA in the EAE and control group. The strength of the correlation between two investigated parameters (disease severity score and mRNA expression) is expressed by the Pearson's correlation coefficients. $P<0.05$ was considered significant. The results for the variables' data are expressed as mean and standard deviation (SD).

RESULTS

Gene expression

On day 5 after the EAE induction, at the peak of the disease (severity disease measured by the EAE scores was 3.07 ± 0.70, Fig. 1), the GalR1 mRNA level was significantly decreased in the EAE mice compared with the con-

trols (*P*=0.016). However, the mRNA levels of GalR2 and GalR3 were not significantly different for the EAE and the control mice. No significant correlations were found between the severity of disease and the GalR1 mRNA level (r = -0.391, *P*=0.083), the GalR2 mRNA level (r = 0.127, *P*=0.01) and the GalR3 mRNA level (r = 0.408, *P*=0.074).

Immunohistochemistry

Immunohistochemical detection of GalR1 revealed its expression in the spinal cord of EAE mice, as well as in the healthy control animals. The strongest immunoreactivity was observed in the ependymal cells lining the central canal of the spinal cord (Fig. 3A–3D). Endothelial cells in the capillaries were also immunoreactive for GalR1 (Fig. 3E–3H). A weak GalR1 immunoreactivity was occasionally detected in some oligodendrocytes. The intensity of GalR1 immunoreactivity was comparable between the samples from the EAE mice and the control mice (compare Fig. 3B with Fig. 3D and Fig. 3F with Fig. 3H).

Histological analyses

To verify the degree of neuroinflammation, histological analysis was performed using a tissue section prepared from both the control and EAE group (Fig. 4). Hematoxylin-eosin staining was used to determine the extent of infiltration of mononuclear cells and perivascular cuffing in the spinal cords. In accordance with the clinical results, the EAE group showed massive infiltration of the mononuclear cells into the spinal cords with multiple foci of inflammation (inflammation score was 2.67 ± 0.33).

DISCUSSION

This study aimed to assess the expression of the galanin receptors in the spinal cords of the mice in the EAE model and to determine the distribution of GalR1 in glial cells. GalR1 mRNA level was significantly decreased in the EAE mice compared with the healthy controls, whereas the mRNA levels of other galanin receptors were not changed after EAE induction. Immunohistochemical detection of GalR1 revealed its expression in the ependymal and the endothelial cells. Additionally, a weak GalR1 immunoreactivity was occasionally detected in some oligodendrocytes.

Galanin functions are mediated through 3 galanin receptors (GalR1, GalR2, GalR3), that have substantial differences in their functional coupling to G regulatory proteins using different messenger cascades. The biological activity of GalR1 and GalR3 is associated with the inactivation of adenylate cyclase and cyclic adenosine monophosphate (cAMP) reduction, whereas the activity of the GalR2 receptor is related to the phospholipase C (PLC) activation[5]. In this study, we found decreased levels of GalR1 mRNA in the EAE mice and no changes in expression of GalR2. Interestingly, in the cuprizone-induced MS model, an increase in GalR1 and GalR2 expression during demyelination and remyelination was

found, respectively[10]. There may be several reasons for these discrepancies. Firstly, there are significant pathophysiological differences between the cuprizone-induced MS and the EAE model; these two models may be used for different purposes, as they reflect different aspects of the MS pathophysiology. The EAE model is suitable for mimicking the autoimmune origin of MS, whereas the cuprizone-induced model is preferable for studying fundamental reactions and interactions between the glial cells during demyelination and remyelination, deprived of the immunity-related processes[24]. However, some researchers have raised doubt about the relevance of the cuprizone model for MS, considering that induction of the CNS lesions is not immune-mediated and is quite different from the lesions in MS (ref.[24]). Furthermore, demyelination in the cuprizone-induced model occurs only in the brain of the mice (the most affected region in this model is the caudal segment of the *CC* and not in the spinal cord[25]. Finally, we have investigated GalR3 expression in the spinal cord samples in the EAE model, as recent studies indicate that GalR3 is specifically involved in modulating the immune response. Namely, GalR3 deletion in the experimental models of autoimmune diseases, such as arthritis, psoriasis, pancreatitis, and colitis worsened the disease severity[11-14]. Contrary to our hypothesis, we found no differences in GalR3 expression between the EAE and control mice, therefore, we conclude that GalR3 is not involved in the pathophysiology of EAE.

This is the first study to determine the distribution of GalR1 in the glial cells in the EAE model, as earlier *in vivo* studies have only investigated cellular expression of galanin. Previously, galanin was found to be markedly upregulated in the MS lesions, including shadow plaques in post-mortem brain tissues of patients suffering from chronic MS exclusively in microglia, although not all microglia were galanin-positive[9]. The same authors reported that galanin was upregulated in the spinal cords of the EAE mice, but here exclusively in the oligodendrocytes[9]. We found GalR1 to be expressed in the ependymal cells lining the central canal of the spinal cord and endothelial cells in the capillaries, with an occasional immunoreactivity in the oligodendrocytes in the spinal cords. This was observed in both the EAE group and the healthy control group. These are interesting results, particularly considering that the ependymal and the endothelial cells are largely understudied in the pathogenesis of MS (ref.[26,27]).

Ependymal cells are ciliated epithelial cells that line the whole ventricular surface of the CNS, including the central canal of the spinal cord[28]. These cells produce cerebrospinal fluid (CSF); moreover, they represent an immunological barrier between the CSF and the CNS, and also control the bidirectional circulation, and metabolism of various molecules in the CSF (ref.[26,28]). Ependymal cells seem to be sensitive to inflammatory mediators; their damage can possibly lead to impaired elimination of cellular waste and may result in an accumulation of detrimental and toxic factors for the CNS (ref.[26]). To our knowledge, this is the first study to detect immunoreactivity of GalR1 in the ependymal cells; so far, the potential

role of galanin in the regulation of ependymal cells has not been identified.

GalR1 were previously detected in the endothelial cells in the eye[6,8], and generally, galanin has been reported to play a role in the process of angiogenesis[17,18]. During the skin granulation tissue formation, GalR1 and GalR2 upregulation in the pericytes, concomitantly with the angiogenesis stimulation, was observed[17]. The role of angiogenesis in the pathophysiology of MS is not completely clear, but recent research indicates that angiogenesis may be detrimental to MS and EAE pathology[19]. Angiogenic factors can cause impairment of the vascular basement membrane, compromise the blood-brain barrier, and trigger immune cells infiltration into the CNS (ref.[29,30]). Pharmacological inhibition of angiogenesis in the EAE mice resulted in the clinical scores amelioration, supporting the hypothesis regarding angiogenesis harmfulness in the EAE (ref.[16,30,31]). Therefore, based on the results of this study, we can assume that galanin may have a role in the brain circulatory system and in promoting angiogenesis in EAE. In this case, the reduction of GalR1 mRNA expression might represent a compensatory mechanism against the harmful angiogenesis. Accordingly, the clinical use of the GalR1 antagonists could potentially lead to beneficial antiangiogenic effects. Because this hypothesis is highly speculative, further research is needed to clarify the possible role of galanin in angiogenesis in EAE and MS.

Although there are rich data on expression of GalR1-3 at the mRNA level, there are insufficient data at the protein level due to the deficiency of available antibodies with an adequate specificity. A recently developed knock-in mice model expressing fluorescently tagged GalR1 and GalR2 has enabled a better understanding of the distribution of these receptors in the CNS (ref.[32]). GalR1 and GalR2 were fluorescently tagged at the C-terminus with, respectively, mCherry or hrGFP (humanized Renilla green fluorescent protein). In the spinal cord, dense Gal1-mCherry immunoreactive processes were detected in the superficial layers of the dorsal horn, as well as at the intrinsic neurons of the lamina III/IV border[32]. Gal2-hrGFP mRNA was also detected in the dorsal root ganglia, but live-cell fluorescence was at the limit of detection. It is concluded that mRNA expression of GalR2 is much lower than the GalR1.

In spite of the fact that galanin ligands hold great promise as CNS therapeutics, specific galanin agonists and antagonists are as yet not available for clinical use. So far, various non-peptide and peptide ligands have been developed or extracted from natural sources (e.g. antifungal metabolite Sch202596 originating from Aspergillus sp.) (ref.[33]). However, none of these compounds has shown a favorable pharmacological profile in terms of metabolic stability, high receptor affinity and selectivity, as well as ability to cross blood-brain barrier[4].

In conclusion, immunohistochemical analyses revealed GalR1 expression in the ependymal and endothelial cells in the spinal cord, with a weak immunoreactivity detected in the oligodendrocytes. GalR1 mRNA expression was decreased in the spinal cords of the EAE mice.

This may be a compensatory mechanism against the galanin-mediated processes which may be detrimental to EAE pathophysiology. However, the gene expression assay and immunohistochemical detection of galanin receptors in this study do not explain the mechanisms of action of galanin in the pathophysiology of the EAE and MS. Further studies are needed to investigate the roles of galanin in EAE and MS pathophysiology.

Author contributions: DM, NKC, OS, IK: conceptualization; DM: draft of the manuscript; TH: methodology: MOG/CFA emulsion preparation; TK: histologic analysis; DM, HKO, TV: PCR analyses; HKO: EAE severity scoring; IK, NKC, MS: samples collection; All authors have read and agreed to the published version of the manuscript.

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