Original paper

Folia Neuropathologica

Expression of components involved in cholesterol homeostasis maintenance during experimental autoimmune encephalomyelitis in rat spinal cord

Smilja Todorovic*, Katarina Milosevic*, Ana Milosevic, Marija M. Janjic, Srdjan J. Sokanovic, Danijela Savic, Irena Lavrnja

Institute for Biological Research "Siniša Stanković" – National Institute of Republic of Serbia, Department for Neurobiology, University of Belgrade, Serbia

Smilja Todorovic and Katarina Milosevic equally contributed to this work.

Folia Neuropathol 2024; 62: *DOI: https://doi.org/10.5114/fn.2024.141376*

Abstract

Dysregulations in cholesterol homeostasis contribute to the pathogenesis of multiple sclerosis (MS) and its best described animal model, experimental autoimmune encephalomyelitis (EAE). Cholesterol is an important component of myelin, which is necessary for signal transmission between neurons. Demyelination leads to the formation of oxysterols, degradation products of cholesterol that are ligands for nuclear liver X receptors (LXRs). Genes regulated by LXRs are involved in cholesterol efflux, absorption, transport, and excretion, which we investigated in this study. In this study, we detected changes in gene expression of Srebf1, Ldlr, Soat1, Abca1, Lrp1, and Npc1, all of which are important in the regulation of cholesterol homeostasis, during the course of EAE in male and female rats. In particular, differential expression of Srebf1, Ldlr, and Soat1 was observed in the spinal cord of male and female rats during EAE. Moreover, these genes are altered during EAE. In contrast, the expression of Abca1 and Lrp1 was significantly affected only by sex. In male animals, the expression of Npc1 is conspicuously reduced in EAE pathology.

Thus, our study confirms the involvement of enzymes of cholesterol metabolism in the pathophysiology of EAE, with sex and disease progression affecting the expression of these genes. These findings may improve the understanding of neurodegenerative diseases associated with impaired lipid metabolism in the brain, such as MS/EAE.

Key words: *experimental autoimmune encephalomyelitis, cholesterol, genes.*

Introduction

Multiple sclerosis (MS) is a chronic, immune-mediated, neurodegenerative disease that causes motor and cognitive deficits, and its etiology remains unclear. In general, MS is thought to be a multifactorial and polygenic disease in which there is a complex interaction between the immune system, genetic/epigenetic, and environmental factors. Experimental autoimmune encephalomyelitis (EAE) is the best characterized and most widely used animal model to study the pathogenesis of MS. The main features of MS/EAE pathology include inflammation, myelin loss, reactive astrogliosis/microgliosis, axonal loss, dysbiosis of the gut microbiome, and metabolic changes. Lipid dysregulation has been suggested to be associated with MS immunopathology [11]. Cholesterol is an essential component of myelin membranes. In the central nervous system

Communicating author:
Irena Lavrnja, PhD, Institute for Biological Research "Siniša Stanković" – National Institute of Republic of Serbia, University of Belgrade, Bulevar Despota Stefana 142, 11060 Belgrade, Serbia, phone: +381 11 2078 340, fax: +381 11 2761 433, e-mail: irenam@ibiss.bg.ac.rs
Received: 13.10.2023, **Accepted:** 23.11.2023, **Online publication:** 2024

(CNS), cholesterol originates from neurons and glial cells [18], with the highest rate of cholesterol biosynthesis observed in astrocytes [37,62]. Cholesterol homeostasis is achieved through sophisticated and intricate regulation of synthesis, transport, and excretion from the brain. High cholesterol levels have been shown to be associated with chronic neurological diseases [54], in which excess cholesterol must be eliminated from the brain to maintain cholesterol homeostasis. The major route of cholesterol excretion is through conversion to oxidized cholesterol derivatives, oxysterols. The enzyme cholesterol 24S hydroxylase (CYP46A1), whose gene expression was altered in EAE [31], converts cholesterol to the most abundant oxysterol in the brain, 24(S)-hydroxycholesterol (24-OHC) [7].

Oxysterols can cross the blood-brain barrier (BBB) and play a specific role in autoimmunity, inflammation, and neuroinflammation [20,65]. It has been suggested that they may fine-tune immune responses through antiviral effects and modulation of inflammasomes [20]. During MS/EAE, the BBB is damaged and increased influx/efflux of oxysterol molecules is observed [41]. Several oxysterols, including 24(S)-OHC, 25-OHC, and 27-OHC, may be potential markers for specific disease progression in EAE/MS [22,53,65]. Oxysterols are agonists for the liver X receptor (LXR), an important nuclear metabolic receptor involved in the regulation of cholesterol, fatty acid, and glucose homeostasis [36]. Although the exact role of the LXR pathway during MS/EAE remains to be investigated, its involvement in reduction of neuroinflammation has been reported, and it is suggested that modulation of the LXR pathway and its target genes may be a potential therapy in MS/EAE [6,50,57,58]. The genes regulated by the LXR pathway are involved in the maintenance of cholesterol homeostasis, i.e., efflux, absorption, transport, and excretion of cholesterol [67]. At the transcriptional level, LXRs regulate ATP-binding cassette protein A1 (ABCA1), which is encoded by the *Abca1* gene and is involved in cholesterol transport and efflux [20,55], and the transcription factor SREBP, which is encoded by the *Srebf1* and *Srebf2* genes. Two isoforms of SREBP-1 (SREBP-1a and SREBP-1c) and SREBP-2 undergo similar proteolytic activation and overlap in function. They are traditionally considered modulators of cholesterol homeostasis and fatty acid synthesis [27,47,48]. SREBP-1 is involved in lipid and cholesterol production by inducing the synthesis or uptake of cholesterol [51], whereas SREBP-2 primarily targets genes of the cholesterol biosynthesis pathway [35]. Moreover, in the CNS, SREBPs regulate neurite growth, synaptogenesis, and synaptic function [9].

Low-density lipoprotein receptor-related protein-1 (LRP1), encoded by the *Lrp1* gene, a major endocytic receptor in the CNS, regulates LXR-mediated gene transcription and is involved in reverse cholesterol transport by regulating cytosolic phospholipase A2 (cPLA2) phosphorylation and ABCA1 expression [66]. Several studies have suggested the role of LRP1 in early oligodendrocyte development and recovery from chemically induced white matter lesions [4,23,43]. Its expression has also been detected in microglia, astrocytes, radial glia, and neurons [3,13,40].

In addition, LXRs regulate the expression of the low-density lipoprotein receptor (LDLR) on the cell membrane [60] which is involved in the myelination process [64]. LDLR in the CNS plays a role in modulating neuronal and glial functions, survival, and regeneration [21]. As a cell surface glycoprotein, LDLR plays a key role in plasma cholesterol homeostasis. The only known ligand for the LDLR in the CNS is ApoE [29], which can influence neurotoxicity and neurodegeneration [17]. Niemann-Pick-C1 protein (NPC1), encoded by the *Npc1* gene, is a late endosomal membrane protein important for the transport of LDL cholesterol into cells. It is involved in cholesterol biosynthesis, uptake, and signal transduction [32]. Mutation in the *Npc1* gene leads to the development of Niemann-Pick type C disease, a rare neurodegenerative disorder that results in abnormal late endosomal/lysosomal lipid storage [14].

Neurons and glial cells can process the excess cholesterol by esterifying it. High levels of free (unesterified) cholesterol are toxic to the cell, and the excess is esterified by sterol O-acyltransferase (SOAT-1), which is encoded by the *Soat1* gene [19], an integral membrane protein localized in the endoplasmic reticulum and a potential target for the treatment of various human diseases [25,46]. This enzyme is involved in the intracellular translocation of unesterified cholesterol to other cytoplasmic compartments and is stored in lipid droplets in the cytoplasm [12]. Previously, SOAT1 inhibitors were shown to have a beneficial effect on Alzheimer's disease [1,46].

Sexual dimorphism in lipid metabolism and its effects on neuroinflammation have been noted previously. Therefore, we wanted to determine whether the expression of some components involved in the maintenance of cholesterol homeostasis correlates with the disabling status of EAE in male and female rats. We found significant sex differences in the expression of *Ldlr1*, *Soat1*, and *Lrp1*, with *Ldlr1* and *Soat1* predominantly expressed by females and *Lrp1* by males. The expression of Srebf1 and Abca1 was also influenced by sex, but to a lesser degree, whereas the expression of *Npc1* was unaffected by sex. In addition, genes whose expression was more pronounced in females were also sensitive to alterations by EAE in this sex. On the other hand, in males only *Npc1* expression was reduced several times in all stages of EAE.

Material and methods

Animals and EAE induction

Experimental autoimmune encephalomyelitis was induced in female and male Dark Agouti (DA; RRID: RGD_21409748) rats from the local colony, weighing about 150-175 g, using a standard protocol. Briefly, the immunization protocol includes rat spinal cord tissue homogenate in complete Freund's adjuvant containing 1 mg/ml of *Mycobacterium tuberculosis* (CFA; Sigma, St. Louis, MO). Each animal received 100 µl of the immunogen in the right hind paws. Unimmunized rats were used as a control group. Two independent observers regularly weighed and examined the rats for neurological signs of EAE. The rats were euthanized by gradual asphyxia in a CO_2 chamber and sacrificed at onset (Eo) – loss of tail tone, 7-8 days postimmunization, peak (Ep) – ataxia or paralysis, 13-15 days postimmunization and end (Ee) – recovered animal without symptoms of EAE, 28 days postimmunization, together with control rats (C). For the experiments, 44 animals were used in total.

To evaluate the severity of EAE, several parameters of the disease were studied.

Clinical severity was classified using following criteria: 0 – asymptomatic, 1 – complete loss of tail tone, 2 – paraplegia of the hindlimb, 3 – complete hindlimb paralysis, 4 – complete hindlimb paralysis/moribund. The human endpoint was set at a score of 4 for two consecutive days. During hindlimb paralysis, rats were given food and water on the floor of each cage.

Duration of disease is presented as a mean value of the days the animal had an EAE symptom. Duration of paralysis was generated by calculating the average number of days that the rats had a score ≥ 3. Mean maximum severity score – the mean of the maximum clinical score obtained by each rat in a group during the course of the experiment. The cumulative disease index or total disease load is the sum of the daily clinical values ≥ 3 over time for each female and male animal observed between day 8 and day 28.

The performed experimental procedures and animal care were under European ethical norms (Directive 2010/63/EU) on the protection of animals used for the experiments and other scientific purposes and under national regulations (given by the Animal Welfare Law of the Republic of Serbia (Official Gazette of the Republic of Serbia No. 41/2009)). The Ministry of Agriculture, Forestry, and Water Economy of the Republic of Serbia approved the experimental protocols (permit no. 323-07-05970/2020-05). The results presented here follow the ARRIVE guidelines. The experimental animals were kept in plastic cages with wood shavings, at a constant temperature, with 12 h dark/light cycle, including unlimited access to laboratory chow and water *ad libitum*.

Real-time PCR

The animals were perfused with cold saline. For gene expression studies, the spinal cords from both sexes (C, n = 5/group; Eo, n = 5/group; Ep, n = 6/group; Ee, n = 6/group) were rapidly dissected on ice and placed in sterile RNase/DNase-free microcentrifuge tubes with RNAlater RNA Stabilization Solution (Ambion, Applied Biosystems by Thermo Fisher Scientific, Waltham, MA) and kept at –80°C until RNA extraction. RNeasy Mini Kit (QIAGEN, Germany) was used for RNA extraction. RNA concentration was measured on a Nanophotometer N60 (IMPLEN, Munich, Germany) at 260 nm. The quality of the samples was estimated by OD260/OD280 and OD260/OD230 ratios. Reverse transcription was completed with a High Capacity cDNA Reverse Transcription Kit, and quantitative real time-PCR (qRT-PCR) analysis was performed using the QuantStudio 3 Real-Time PCR System (all from Applied Biosystems by Thermo Fisher Scientific). TaqMan PCR reactions were performed with Assay-on-Demand Gene Expression Products (Applied Biosystems, Carlsbad, CA, USA) (Table I). The samples were run in triplicate, and the mean value of each Ct triplicate was used for further calculation. In every analysis, an endogenous control was included to correct the differences in inter-assay amplification efficiency. The expression of the investigated genes was normalized to Gapdh expression. The results were analyzed using RQ Study Add ON software for the 7000 v 1.1 SDS instrument (ABI Prism Sequence Detection System) with a confidence level of 95% ($p < 0.05$).

Statistical analysis

Statistical analyses were performed using GraphPad Prism 8 software. The mean values between multiple groups were compared with two-way ANOVA followed by Fisher's least significant difference (LSD) test. Data are presented as mean ± SEM and were considered statistically significant for $p < 0.05$.

Table I. List of TaqMan probes

Target gene	Assay ID
Srebf1	Rn01495769_m1
Abca1	Rn00710172_m1
Lrp1	Rn01503901_m1
Ldlr	Rn00598442_m1
Soat1	Rn00579605_m1
Npc1	Rn01531821_m1

Results

In this study, we found that all female and male rats developed acute monophasic disease (Fig. 1). The onset of disease was similar in the female and male rats (11.55 ±0.27 and 11.47 ±0.28, respectively). All rats recovered completely 28 days after immunization. The parameters of the disease showed that there was no difference in the severity of the disease except for the duration of paralysis, which was longer in male rats. We also found that the disease duration was shorter in male rats, but the cumulative disease index was higher than in female rats (Table II).

The spinal cords from these experiments were used for the present study. We investigated whether sex and induction of EAE affect gene expression of proteins involved in cholesterol turnover. The gene expression patterns of these molecules were examined by real-time PCR (Fig. 2). We found a statistically significant difference in the average expression of three genes – *Srebf1*, *Ldlr1*, and *Soat1* – which were influenced by both sex and disease, with a significant interaction between these two factors (Table III). In contrast, the expression levels of *Abca1* and *Lrp1* were significantly affected only by sex, whereas the effects of EAE and the interaction between sex and EAE were significant for *Npc1* expression (Table III).

Interestingly, expression of *Srebf1*, *Ldlr1*, and *Soat1* (Fig. 2A) was higher in females than in males, in contrast to *Abca1*, *Lrp1*, and *Npc1* (Fig. 2B). Post-hoc analysis revealed that the expression of *Srebf1* was significantly lower in male rats than in female rats under control conditions and at the end of the disease (Fig. 2A). In addition, significant pairwise differences were observed between *Srebf1* levels in female rats in groups Eo and Ep compared with the corresponding group C, whereas in male rats, Srebf1 expression was significantly upregulated in group Ee compared with the corresponding group C.

Sex differences were observed in *Ldlr1* and *Soat1* levels, with female rats showing significantly higher expression than males in all experimental groups (Fig. 2A). In view of this, changes in the expression levels of these genes during EAE were detected only in females and resulted in lower *Ldlr1* and increased *Soat1* expression in Ep and Ee groups compared with group C. *Lrp1* expression was significantly higher in all male groups than in the corresponding female groups, whereas *Abca1* expression was higher only in the Ep group when males were compared with females (Fig. 2B). Moreover, both gene expressions were also sensitive to EAE at the peak of the disease, with slightly higher expression in males compared with the corresponding control group. Sex differences were also partially observed in *Npc1* levels, with female rats showing significantly different expression than males under control conditions and in the most severe stage of EAE (Fig. 2B). Moreover, *Npc1* expression was significantly suppressed in male animals at all stages of EAE compared to the corresponding control.

Discussion

Cholesterol represents a significant constituent of mammalian cell membranes, and it is required to maintain structural integrity, fluidity, and lipid rafts [49,62]. Cholesterol maintains the morphology and normal function of the CNS. The disturbances in cholesterol homeostasis in the adult brain are related to the onset and progression of neurodegenerative diseases [16], including MS. Excess cellular cholesterol is toxic, and therefore the cholesterol-biosynthetic pathway must be tightly regulated and coupled to pathways that enable the removal of cholesterol. Previously, we observed that gene and protein expression of the cholesterol homeostasis regulators HMGCR1, ApoE, and CYP46A1 changes during clinical signs of EAE [31]. This study extends the aforementioned findings and provides new insights

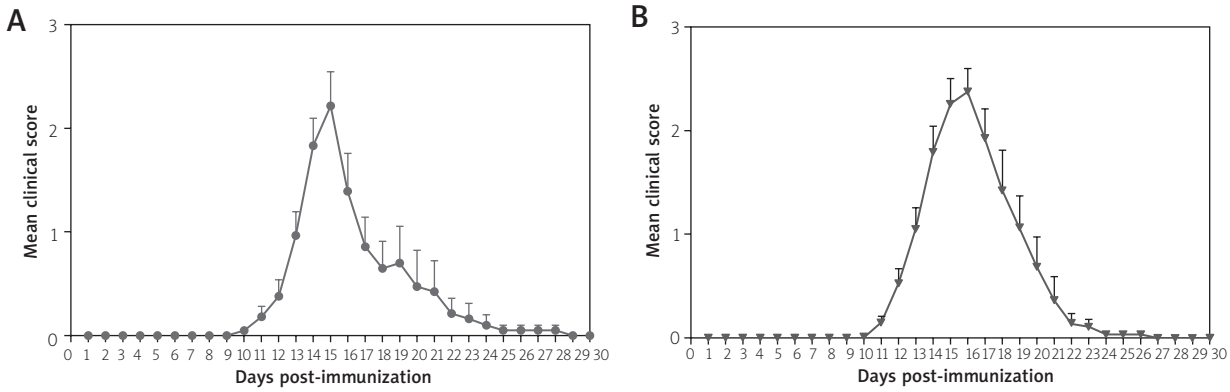

Fig. 1. Temporal change in symptoms of experimental autoimmune encephalomyelitis (EAE) in female (**A**) and male (**B**) rats. Data are expressed as mean ± SEM of daily measurements from individual animals.

Table II. Parameters of clinical severity during experimental autoimmune encephalomyelitis (EAE) in female and male rats

Gender	Parameters			
	Duration of disease	Duration of paralysis	Maximal clinical score	Cumulative disease index
Female	8.3 ±2.3	1.8 ±0.8	2.8 ±0.4	10.7 ±1.4
Male	7.3 ±1.6	2.5 ±1.2	3.0 ±0.2	11.6 ±3.1

Table III. Effects of sex and experimental autoimmune encephalomyelitis (EAE) on the expression of genes involved in cholesterol turnover

ANOVA table			
Dependent variable	Independent variables		
Gene expression	Sex	EAE	Interaction Sex*EAE
Srebf1	$F(1, 32) = 16.53$ $p = 0.0003$*	$F(3, 32) = 11.52$ $p < 0.0001$*	$F(3, 32) = 3.622$ $p = 0.0234$*
Ldlr1	$F(1, 34) = 493.9$ $p < 0.0001$*	$F(3, 34) = 10.21$ $p < 0.0001$*	$F(3, 34) = 10.12$ $p < 0.0001$*
Soat1	$F(1, 32) = 77.13$ $p < 0.0001$*	$F(3, 32) = 8.716$ $p = 0.0002$*	$F(3, 32) = 4.713$ $p = 0.0078$*
Abca1	$F(1, 33) = 11.54$ $p = 0.0018$*	$F(3, 33) = 2.680$ $p = 0.0629$	$F(3, 33) = 0.8809$ $p = 0.4610$
Lrp1	$F(1, 34) = 255.5$ $p < 0.0001$*	$F(3, 34) = 1.181$ $p = 0.3314$	$F(3, 34) = 1.450$ $p = 0.2454$
Npc1	$F(1, 33) = 0.1437$ $p = 0.7071$	$F(3, 33) = 18.32$ $p < 0.0001$*	$F(3, 33) = 10.13$ $p < 0.0001$*

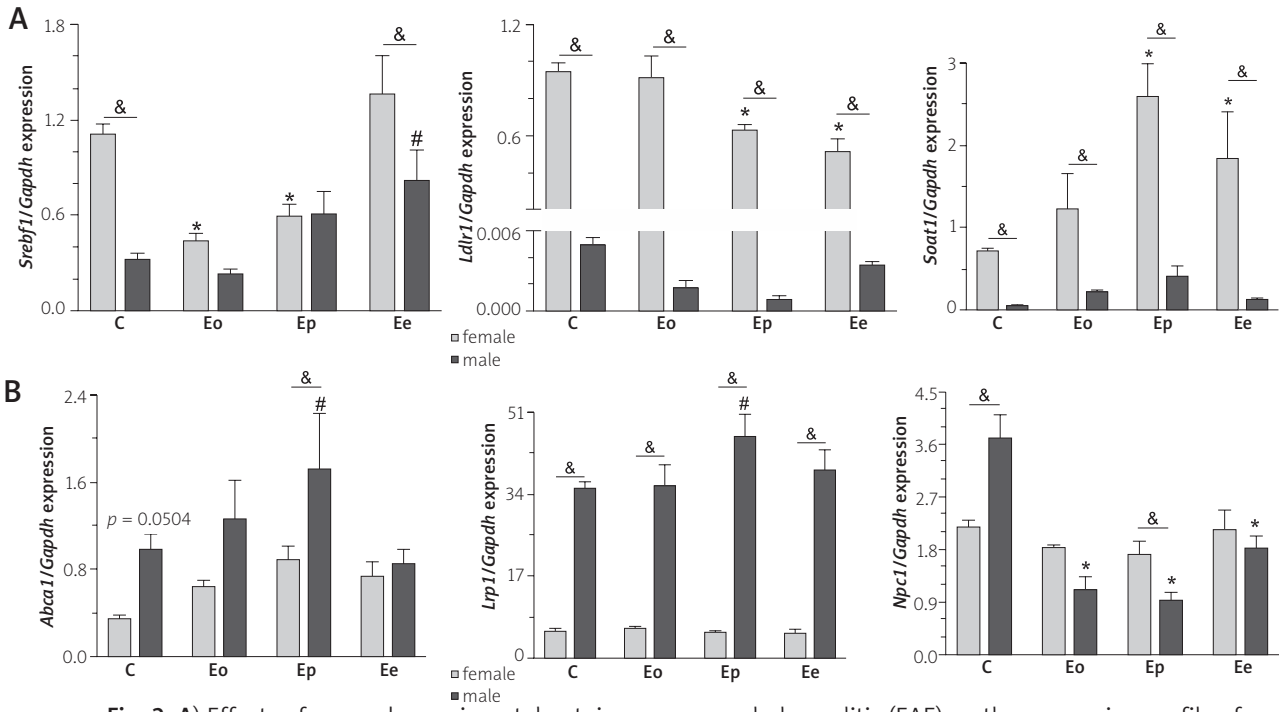

Fig. 2. A) Effects of sex and experimental autoimmune encephalomyelitis (EAE) on the expression profile of the genes *Srebf1*, *Ldlr1*, and *Soat1* in spinal cords of female and male rats. **B**) Effects of sex and EAE on the expression profile of the genes *Abca1*, *Lrp1*, and *Npc1* in spinal cords of female and male rats. RNA isolated from spinal cords of animals at different stages of disease and control animals ($n = 5$ to 6 animals/group) were subjected to qRT-PCR analysis. Levels of target genes are expressed relative to the expression of the *Gapdh* gene. The results were analyzed by factorial ANOVA followed by Fisher's least significant difference (LSD) test. Data are presented as mean ± SEM; $^{*}p < 0.05$ compared with the female control group, $^{\#}p < 0.05$ compared with the male control group, $^{\&}p < 0.05$ comparing corresponding female and male groups.

into the components of cholesterol and sex- and disease-related differences in gene expression in male and female rats during EAE.

It has been suggested that the LXR pathway may be a potential therapeutic target for MS [27,34,59]. Previously, LXR agonists were shown to limit the release of proinflammatory cytokines and chemokines in stimulated microglia and reactive astrocytes [58,61]. Moreover, LXR activation decreases the inflammatory response in myeloid cells by reducing proinflammatory mediators (*Nos2*, *Cox2*, and *Il6*) [44]. Indeed, the marked activation of LXR has been shown to exert anti-inflammatory effects in phagocytes in active lesions during MS [34]. Studies also suggest that LXR deficiency exacerbates EAE, whereas its activation ameliorates EAE [15,44,56,57], in a T cell-dependent manner [8]. It has been postulated that activation of SREBP1a and SREBP1c underlies LXR activation and inhibits Th17 differentiation [15], which is involved in the pathogenesis of EAE [2]. Consistent with these studies, we found that *Srebf1* expression is decreased in female rats during EAE. Interestingly, we observed upregulation of *Srebf1* at the end of the disease in males. In our study, in males we observed an increase in *Abca1* expression at the peak of EAE, suggesting that cholesterol efflux is affected by disease progression. In female rats, there are no significant changes in *Abca1* expression during EAE. It has been postulated that *Ldlr* expression is regulated by intracellular cholesterol content in neurons and astrocytes [10,21]. During acute EAE disease, gene expression in neurons related to cholesterol metabolism, including *Ldlr*, was consistently downregulated [6]. Our results confirm this finding that *Ldlr* expression is downregulated in females during the peak and at the end of the disease. In our study, we observed strong sexual dimorphism in *Ldlr* expression, as it was barely detectable in males. Sexual dimorphism in *Ldlr* expression has been noted previously. Thus, compared with *Ldlr*-/- male mice, *Ldlr*-/- female mice suffered from less severe EAE [34]. These results suggest that immune modulation of macrophages is behind LDLR deficiency. Specifically, macrophages from female EAE mice show a decreased inflammatory response and reduced phagocytosis capacity, modifying EAE disease. Previously, astrocytes were found to have high *Ldlr* expression in control rats [6]. The observed downregulation may be due to decreased cholesterol transport to the plasma membrane, which is related to decreased expression of two transcription factors, LXR and *Srebf1*. Demyelination is a hallmark of MS disease, in which phagocytosis of damaged myelin by macrophages contributes to the exacerbation of neurological symptoms [24,26]. Our study showed that the expression of *Lrp1* did not change in female rats during the disease, whereas in males there was a modest increase of 30%. There is an association between *Lrp1* deficiency and a decreased ability to differentiate oligodendrocytes, so it remains to be determined whether this increase has relevance to EAE pathology given the marked *Lrp1* expression in male rats [33,42]. Excess cholesterol is removed *via* CYP46A1, and disruption of this pathway leads to the formation of cholesterol esters via SOAT1 to prevent accumulation. Cholesterol esters are sequestered in the form of lipid droplets, which are increased in several neurological diseases, including MS [45]. In particular, cholesterol ester concentrations have been found to be increased near white matter demyelination foci. In human MS tissue, no changes in the synthesis of cholesterol esters were detected, but the high levels were due to incomplete hydrolysis in MS [45]. Our results showed an increase in the mRNA level of SOAT1 at the peak and end of EAE in female rats, as previously detected in neurons in the acute phase of EAE [6]. This could lead to an increase in cholesterol ester levels in the spinal cord at the peak of the disease, which could be due to increased cholesterol synthesis. On the other hand, we observed decreased cholesterol synthesis [31]; therefore, we hypothesize that the excess cholesterol is due to demyelination. Finally, no change in *Npc1* expression was detected in female rats, whereas in male rats downregulation was detected from the onset to recovery of EAE. This gene is ubiquitously expressed throughout the brain [39], being mainly expressed in oligodendrocytes and microglia [6]. In MS patients, higher expression of *Npc1* was found in neurons, oligodendrocytes, and astrocytes in acute lesions. This suggested that the function of NPC1 is critical for proper maturation of oligodendrocyte progenitor cells and maintenance of existing myelin [52]. Previously massive microgliosis was associated with loss of NPC1 [5,38], and in our earlier studies we noted microgliosis in both male and female rats during EAE [28,30]. Here, we observed a steady state of *Npc1* expression in females and significant reduction in males, suggesting either sex differences in response to microgliosis or some other pathological event responsible for the downregulation of this gene in males during EAE. Furthermore, loss of NPC1 function suppresses SREBP-dependent gene expression and lack of activation of LXR signaling pathways [63], leading to intracellular cholesterol accumulation. Therefore, our study supports the involvement of *Npc1* in EAE pathology.

Conclusions

The present study extends current knowledge on differences in the expression of components involved in the maintenance of cholesterol homeostasis during EAE in the spinal cord of male and female rats. Overall,

genes that were more highly expressed in females were also affected by EAE in this sex in at least at two disease stages, whereas EAE had very little effect on genes that were more highly expressed in males, and only at the peak of the disease. Our study identified *Srebf1* and *Soat1* as targets that may be important for EAE pathology in females. It appears that male rats are less sensitive to changes in the expression of genes involved in cholesterol turnover during EAE, with the exception of *Npc1*, suggesting the need for further investigation of this protein. These results shed light on other disease-related networks and warrant further investigation, particularly with regard to sex differences in the course of EAE/MS.

Data availability

All reported data have been provided as part of the submitted article.

Funding

This work was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia, Contract No. 451-03-66/2024-03/200007.

Disclosures

The study was approved by the Bioethics Committee of the Ministry of Agriculture, Forestry and Water Management of the Republic of Serbia, Department for Animal Welfare (Approval No. 323-07-05970/2020-05).

The authors report no competing interests.

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