Neurochem Res
DOI 10.1007/s11064-015-1655-5

ORIGINAL PAPER

The Use of Agmatine Provides the New Insight in an Experimental Model of Multiple Sclerosis

Milica Ninkovic[1] · Ivana Stevanovic[1] · Ivana Stojanovic[2] · Srdjan Ljubisavljevic[3,4] · Jelena Basic[1] · Predrag Peric[5]

Received: 15 May 2015 / Revised: 26 June 2015 / Accepted: 28 June 2015

Abstract The aim of the study is to investigate the hypothesis that agmatine (AGM) enhances blood brain barrier (BBB) compounds properties in experimental autoimmune encephalomyelitis (EAE), which is an established animal model for studying multiple sclerosis (MS). Wild-type (WT) and knockout (KO) CBA/H iNOS−/− mice, 3 months old (15 ± 5 g) were used for EAE induction by myelin basic protein (MBP) dissolved in complete Freund's adjuvant (CFA). The animals were divided into control, CFA, EAE, EAE + AGM and AGM groups. After the development of full clinical remission, the animals were sacrificed and the immunohistochemical and biochemical examinations were performed in brain homogenates. We had noticed the increased expressions of occludin in WT and KO mice with EAE + AGM, compared to EAE groups in which these expressions were significantly decreased compared to the controls. The significant elevations of matrix metalloproteinases (MMPs)—MMP-3 and MMP-9 in WT and KO EAE animals were decreased during AGM treatment in both groups. AGM application post EAE in WT and KO mice caused decreased level of Iba-1 stain, compared to EAE groups. The obtained results suggest beneficial AGM effects in EAE on BBB components, which might be useful for novel therapeutic strategies in MS.

Keywords Experimental autoimmune encephalomyelitis · Agmatine · Occludin · Microglia · Matrix metalloproteinases

✉ Srdjan Ljubisavljevic
srljub@gmail.com

[1] Institute for Medical Research, Military Medical Academy, Belgrade, Serbia

[2] Faculty of Medicine, Institute for Biochemistry, University of Nis, Nis, Serbia

[3] Clinic for Neurology, Clinical Centre of Nis, Nis, Serbia

[4] Faculty of Medicine, Institute for Pathophysiology, University of Nis, Bul. Dr Zorana Djindjica 48, 18000 Nis, Serbia

[5] Clinic of Neurosurgery, Military Medical Academy, Belgrade, Serbia

Introduction

Experimental autoimmune encephalomyelitis (EAE) is a well-established cell-mediated autoimmune inflammatory disease of the central nervous system (CNS), which has been used as the model of the human multiple sclerosis (MS) [1, 2]. In MS/EAE, dysfunction of the blood brain barrier (BBB), followed by edema, leads to disrupted intercellular communication within the CNS [3, 4]. It is well documented that biphasic MS pathomechanism is based on demyelination [relapsing remitting (RR) phase] and neurodegeneration (progressive phase) [5].

The anatomical basis of BBB is represented by tight junctions between the cells surrounding the microvessels that perfuse the brain. Astrocytes play a critical role in the regulation of brain metabolism at BBB junctions. Their perivascular feet in processes surround the vascular barrier formed by brain vascular endothelial cells just outside the basal lamina, lining the abluminal membranes of endothelium. These processes also create direct connections to neurons. This unique arrangement allows astrocytes to act as gatekeepers regulating properties of BBB [6].

Axonal damage is believed to play a major role in the irreversible phase of MS [2]. Inflammation and BBB breakdown are the major hallmarks of disease in EAE [7].

Published online: 04 July 2015

The BBB is a crucial interface between the peripheral circulation and the CNS environment [8]. Occludin, an integral membrane protein of tight junctions (including those of the BBB) undergoes phosphorylation in association with altered tight junction permeability in cell culture systems [9].

Tight junctions are specialized cell–cell adhesion structures and critical components of the BBB that have previously been shown to be abnormally distributed in MS tissue [10]. Occludin junctional protein, localized in tight junctions in brain endothelial cells, showed strickingly increased migration in EAE. Occludin dephosphorylation coincided with the onset of inflammation, slightly preceding visible signs of disease, and was just prior to apparent changes in BBB permeability. These findings suggest occludin as a target for signaling processes in EAE, perhaps regulating the response of the BBB to the inflammatory environment, as it is seen in MS [7].

Over the last few decades it has been established that matrix metalloproteinases (MMPs) are the key enzymes in remodeling processes of extracellular matrix in physiological, as well as pathological states [11]. Also, these enzymes play an important role in the immunopathogenesis of MS, in part through the disruption of the BBB and the recruitment of inflammatory cells into the CNS. Moreover, MMPs can also enhance the cleavage of myelin basic protein (MBP) and the demyelination process, which, beside BBB breakdown, could be the crucial event in MS progression [12, 13]. The results of investigated occludin expression in human serum demonstrate its decreased level in endothelial cells [14].

Immunopathogenesis of MS means remodeling of extracellular matrix which involves enzymes of MMP family. As factors which are released from activated microglia, MMPs contribute to neuron damage by initiation of BBB disruption and direct neurotoxicity [15]. In vitro investigation has been shown that both MMP-3 and MMP-9, are released from microglia [16]. When microglial cell become activated, they undergo dramatic changes in cell shape, proliferation, migration and phagocytosis, which are all closely related to rearrangements of the actin cytoskeleton [17]. Ionized calcium-binding adaptor molecule 1 (Iba1) is protein with actin-binding properties and it represents a microglial marker in animal tissues [18].

Agmatine, (4-aminobutyl)guanidine—AGM, is an amine that is formed by decarboxylation of L-arginine by the enzyme arginine decarboxylase (ADC) and hydrolyzed by the enzyme agmatinase to putrescine [19, 20]. AGM is a putative neurotransmitter and an intermediate in polyamine biosynthesis [21]. Also, AGM inhibits NO synthase (NOS), which is involved in the synthesis of NO and is located in neurons (nNOS) and endothelial cells (eNOS) in physiological conditions, or in mitochondria and microglia during inflammatory processes (iNOS) [22, 23]. It is known that the treatment with exogenous AGM exerts neuroprotective effects in animal models of ischemia and neurotrauma [24].

In order to determine the influence of iNOS in remodeling of extracellular matrix processes during EAE development, we examined the MMP-3 and MMP-9 concentrations in brain tissue, as well as the microglial activation and tight junction protein expression, after the treatment with AGM, as a continuity of our previous research [25–29].

Materials and Methods

Animals

The experimental animals were treated according to Guidelines for Animal Study, No. 282-12/2002 (Ethics Committee of the Military Medical Academy, Belgrade, Serbia and Montenegro).

The experiments were performed on CBA/H wild-type (WT) and knockout iNOS−/− (KO) mice females, weighing between 15 and 20 g, randomly divided into one control group (n = 8) and four experimental groups (n = 9, each). Animals were housed (five mice per cage) under standardized housing conditions (ambient temperature of 23 ± 2 °C, relative humidity of 55 ± 3 % and a light/dark cycle of 13/11 h) and had free access to standard laboratory pellet food and tap water. All the experiments were performed after 7 days period of adaptation to laboratory conditions, and were carried out between 9 a.m. and 1 p.m. The experimental protocols were approved by the Local Animal Care Committee and conformed to the recommendations given in "Guide for the Care and Use of laboratory Animals" (National Academy Press, Washington, DC, 1996).

Induction of EAE

Experimental autoimmune encephalomyelitis was induced by the subcutaneous injection of myelin basic protein, bovine type (100 μg dissolved in 0.1 ml complete Freund's adjuvant—CFA), in the hind foot pad of the animals and two intraperitoneal injections of Pertussis toxin (150 ng dissolved in 0.1 ml phosphate buffered saline—PBS) were given on days 0 and 1.

Each of 68 animals were randomly assigned to one of five groups: control (CG) (treated by PBS—0.1 ml/i.p./daily), EAE (PBS—0.1 ml/i.p./daily after EAE induction), CFA (CFA—0.1 ml in the hind foot pad of the animals was given on day 0 and PBS—0.1 ml/i.p./daily), EAE and AGM (AGM—75 mg/kg body weight/daily after EAE induction), AGM (AGM—75 mg/kg body weight/daily). The animals were treated every day during the course of

the experiment. Twenty-four days after EAE induction (24 days post immunization—24 dpi), the animals from each group were sacrificed and the tissues were collected for biochemistry and immunohistochemistry.

Clinical Evaluation of EAE

All animals were scored for the clinical signs of EAE daily. EAE clinical expression was assessed as 1 = healthy; 2 = loss of tail tone; 3 = hindlimb weakness; 4 = hindlimb paralysis; 5 = hindlimb paralysis plus forelimb weakness; 6 = moribund or dead. Animals were sacrificed 24 days after EAE induction under Ketalar anesthesia with decapitation.

The Tissue Preparation

The brains from two animals from each of the experimental groups were dissected, removed from the skull, fixed in 4 % paraformaldehyde (TAAB Laboratory Equipment, Aldermaston, UK) for at least 24 h and cryoprotected in graded sucrose at 4 °C. The brains were frozen in methylbutane and stored at −70 °C until cryosectioning (CRIOCUT-E Reichert-Yung). From the remaining animals, the brains were removed, washed in PBS and 10 % homogenates of the whole encephalitic mass—WEM (consisting of the removed brain tissue without the cerebellum and brainstem) were made in PBS on ice with Teflon® pounder and stored at −20 °C for later biochemical analysis.

Immunohistochemistry

Frozen, 8 □m thick sections were deposited on Poly-L Lysine coated slides and allowed to air dry. DakoCytomation EnVision + System-HRP kit was used for two-step IHC staining technique. Criostat sections were fixed in acetone and endogenous peroxidase activity was blocked by peroxidase block (0.03 % hydrogen peroxide containing sodium azide) (Dako Cytomation) for 15 min. Slides were incubated with appropriate dilutions of rabbit polyclonal antibody—anti-Occludin antibody 1:25 (Abcam, Cambridge, UK) and mouse monoclonal antibody—anti-Iba1 antibody 1:50 (Abcam, Cambridge, UK), for 60 min. Afterwards, slides were incubated with the labelled polymer (DakoCytomation) conjugated to goat anti-rabbit immunoglobulins in Tris–HCl buffer, containing stabilizing protein and an anti-microbial agent with addition of 5 % normal rat serum for 30 min. Staining has been completed by a 5–10 min incubation with 3,3′-diaminobenzidine (DAB) + substrate-chromogen (DakoCytomation), which resulted in a brown-colored precipitate at the antigen site. In negative controls, slides were incubated with PBS, in the absence of primary antibody. Finally, slides were counterstained with hematoxylin and mounted with Kaiser gel.

Biochemical Analyses

Whole encephalitic mass—WEM was dissected from each frozen brain and was transferred separately into saline solution (0.9 % w/v). Aliquots (1 ml) were placed into a glass tube homogeniser (Tehnica Zelezniki Manufacturing, Slovenia). Homogenization was performed twice with a Teflon pestle at 800 rpm (1000 g) for 15 min at 4 °C. The supernatant was centrifuged at 2500*g* for 30 min at 4 °C. The resulting precipitate was suspended in 1.5 ml of deionised water [30].

Solubilisation of subcellular membranes in hypotonic solution was performed by constant mixing for 1 h using a Pasteur pipette. Thereafter, homogenates were centrifuged at 2000*g* for 15 min at 4 °C and the resulting supernatant was used for analysis. Total protein concentration was estimated with bovine serum albumin as a standard [31].

MMP-3 and MMP-9 were measured with standard ELISA kit (quantitative sandwich enzyme-immunoassay Quantikine ELISA Mouse Total MMP-3 and Quantikine ELISA Mouse Total MMP-9, R&D Systems, USA), at 450 nm. Data are expressed as nanograms per mg of proteins.

Reagents

All chemicals used in this study were of analytical grade and from the following sources. Saline solution (0.9 % w/v) was purchased by the Hospital Pharmacy (Military Medical Academy, Belgrade, Serbia). All drugs solutions were prepared on the day of the experiment.

Statistical Analysis

After verifying a normal distribution in all groups, using Kolmogorov–Smirnov test, the data presented were mean ± SD. Imunohistohemical, biochemical and data from EAE score were analyzed statistically by One Way ANOVA using Dunnett's C test. The linear regression analysis was performed to determine the relation between immunohistochemical and biochemical parameters, using the statistical program SPSS version 13. Statistical significance was defined as $p < 0.05$.

Results

Post active immunization, the expression of occludin positive cells (Fig. 1) in mice forebrain samples of EAE group significantly decreased in wild-type (WT-w) (e), as well as

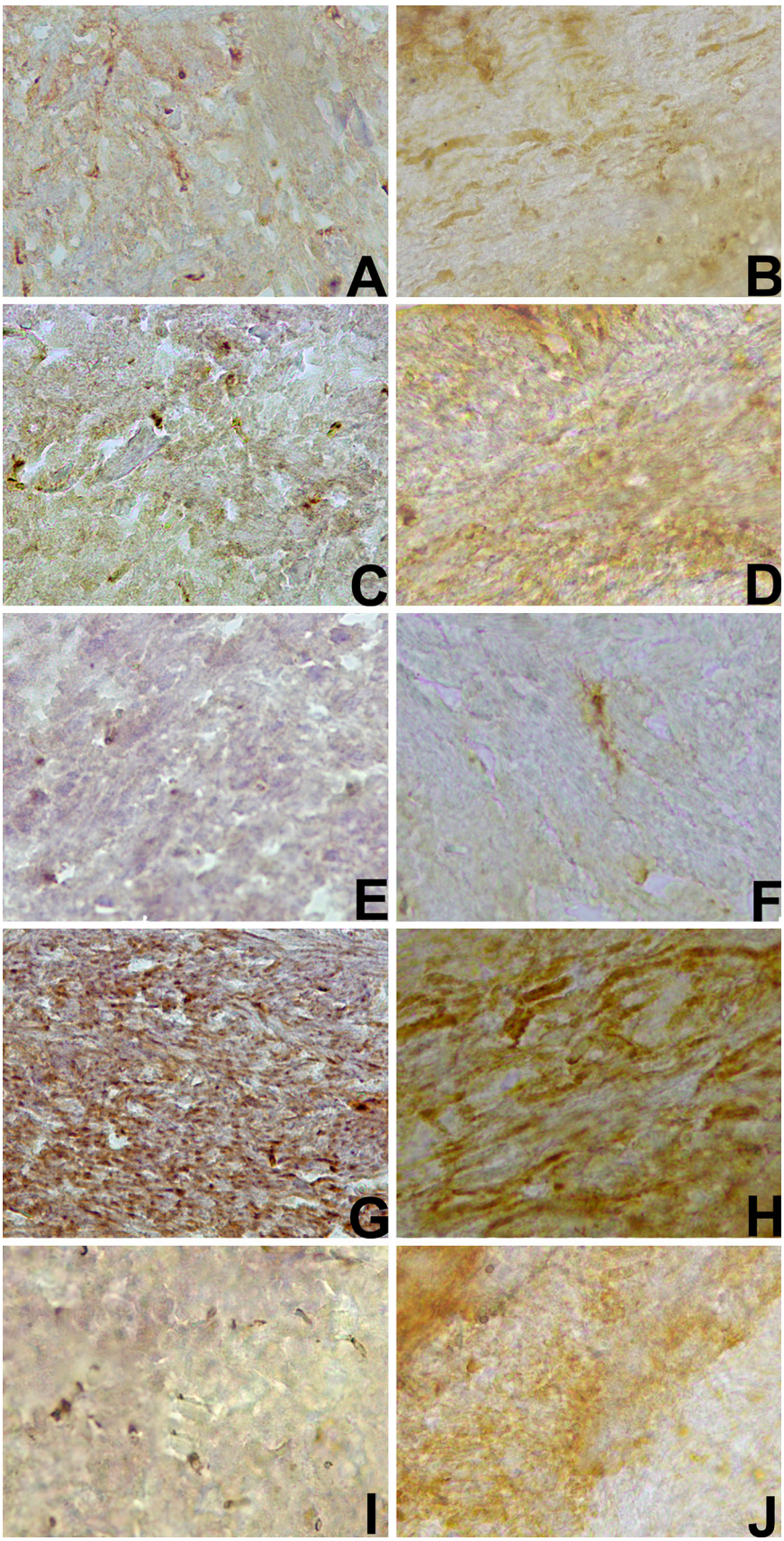

Fig. 1 The representative immunohistochemical staining of occludin in wild-type (**a**, **c**, **e**, **g**, **i**) and iNOS−/− knockout animals (**b**, **d**, **f**, **h**, **j**). **a** Control wild (CGw), **b** control knockout iNOS−/− (CGk), **c** complete Freund's adjuvant wild (CFAw), **d** complete Freund's adjuvant knockout iNOS−/− (CFAk), **e** EAE wild (EAEw), **f** EAE knockout iNOS−/− (EAEk), **g** EAE + Agmatine wild (EAE + AGMw), **h** EAE + Agmatine knockout iNOS−/− (EAE + AGMk), **i** Agmatine wild (AGMw), **j** Agmatine knockout iNOS−/− (AGMk). Immunostaining for occludin showed the strongest signal in EAE + AGMw (**g**), as well as EAE + AGMk (**h**) group of animals, at the end of the disease (24 dpi). However, occludin labelling demonstrated mild reaction in EAEw (**e**) and EAEk (**f**) group of animals (original magnification ×400)

in knockout iNOS−/− (KO-k) (f) mice, compared with CG animals (CGw-a and CGk-b). Also, immunohistochemical data suggest that occludin positive neurons with mild reaction (+) were present in the EAE group (EAEw, EAEk), enhanced in CG (CGw, CGk) and AGM-treated animals (AGMw, AGMk), with maximum occludin expression (+++) in EAE + AGM groups: EAE + AGMw (Fig. 1g; $p < 0.05$) and EAE + AGMk (Fig. 1h; $p < 0.05$), Fig. 1.

The results of biochemical analysis showed that MMP-3 concentration in EAE animals were elevated in both WT and KO mice, compared to controls ($p < 0.05$). AGM application post EAE induced decrease of MMP-3 concentration compared to EAE groups in both WT and KO animals. In CFA group of KO animals MMP-3 concentration was increased compared to controls (Fig. 2).

Results of MMP-9 were similar to MMP-3: increased concentrations in EAE group compared to controls, decreased values in EAE + AGM compared to EAE and increased concentration in CFA KO animals compared to controls, ($p < 0.05$) (Fig. 3).

In EAE groups of both WT and KO animals, Iba1 showed higher positivity areas compared to controls, with strong staining in larger number of cells (+++) in EAE group of both WT and KO animals (Fig. 4e, f; $p < 0.05$). Mice brain samples from both WT and KO animals EAE + AGM (++) showed mild immunoreactivity compared to controls, but significantly decreased compared to EAE groups (Fig. 4g, h; $p < 0.05$). The number and density of Iba1 (Fig. 4i, j) positive cells were markedly

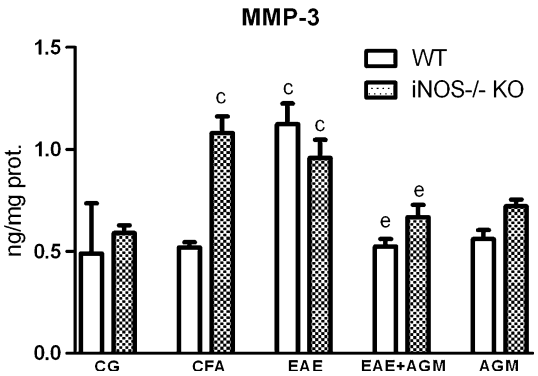

Fig. 2 MMP-3 concentration (ng per mg of proteins) in the wild type and knockout mice brain. *CG* control group animals treated with PBS the dose of 0.1 ml/i.p./daily, *CFA* animals treated with complete Freund's adjuvant the dose of 0.1 ml in the hind foot pad of the animals, *EAE* experimental autoimmune encephalomyelitis (for EAE induction protocol see "Animals" section), EAE + AGM—EAE rats treated with AGM the dose of 75 mg/kg body weight/daily, AGM—treated with AGM the dose of 75 mg/kg body weight/daily. *Bars* in the graph represent mean ± SD from 8 to 9 animals for each group. Labels of statistical significance: *c* compared to control group; *e* compared to EAE group. Statistical significance was considered at: $p < 0.05$, One Way ANOVA, Dunnett's C test

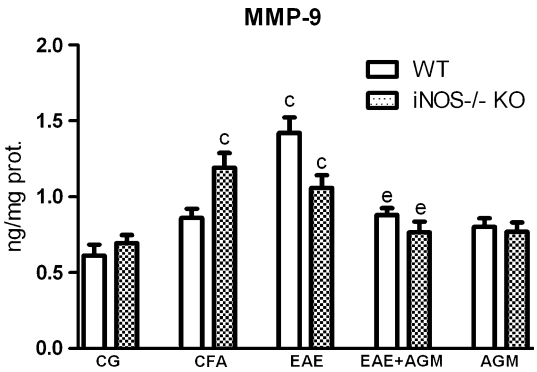

Fig. 3 MMP-9 concentration (ng per mg of proteins) in the wild type and knockout mice brain. *CG* control group animals treated with PBS the dose of 0.1 ml/i.p./daily, *CFA* animals treated with complete Freund's adjuvant the dose of 0.1 ml in the hind foot pad of the animals, *EAE* experimental autoimmune encephalomyelitis (for EAE induction protocol see Section Animals and methods), EAE + AGM—EAE rats treated with AGM the dose of 75 mg/kg body weight/daily, AGM—treated with AGM the dose of 75 mg/kg body weight/daily. *Bars* in the graph represent mean ± SD from 8 to 9 animals for each group. Labels of statistical significance: *c* compared to control group; *e* compared to EAE group. Statistical significance was considered at: $p < 0.05$, One Way ANOVA, Dunnett's C test

decreased in AGM group (+), than in other samples ($p < 0.05$). While for Iba 1 staining there was no difference between WT control and WT AGM group, staining for Iba1 in iNOS KO animals resulted in the increased signal in AGM group (+) (Fig. 4j; $p < 0.05$), compared to controls (−) ($p < 0.05$), Fig. 4.

During the disease development, the clinical manifestation of EAE is significantly decreased in the EAE groups treated with AGM compared to WT and KO EAE groups at 15, 20 and 24 dpi, Fig. 5 [29].

In Table 1 there are summary data of immunohistochemistry for occludin, Iba-1 and GFAP (data from previous published results).

The results suggest a significant positive correlation between GFAP expression in EAE KO animals and EAE WT animals (Table 2, r = 0.6889, $p < 0.05$).

Discussion

During the disease development, agmatine expressed strong protective effect on EAE clinical symptoms, reducing significantly the clinical score in rats with EAE compared to EAE group in both WT and KO animals. Our results show that, during the entire time of the disease, EAE neurological signs in WT EAE animals were significantly more severe when compared to KO mice, suggesting the importance of NO metabolism in EAE pathogenesis, which was also supported by the results of other authors [32]. It has to be mentioned that iNOS KO

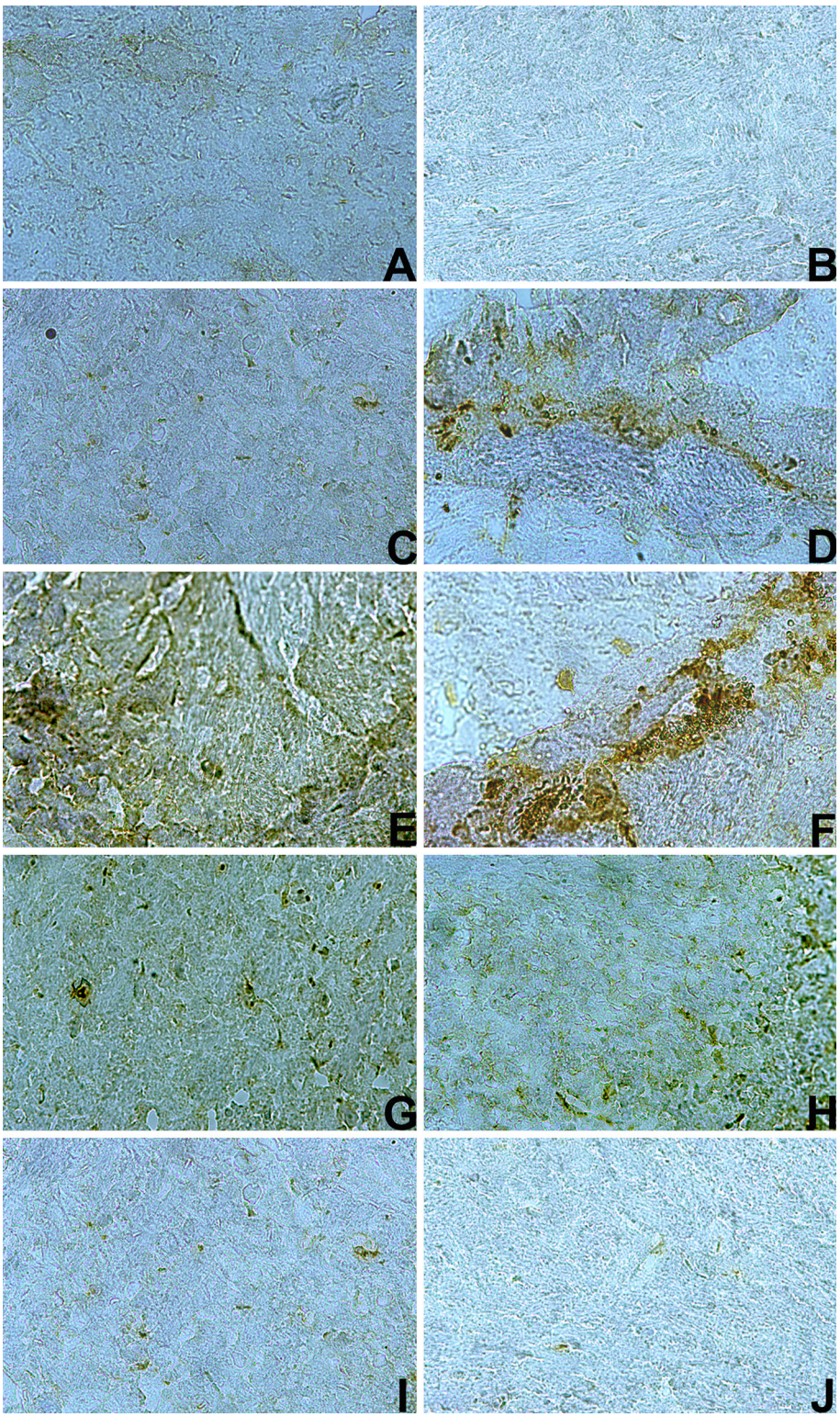

Fig. 4 The representative immunohistochemical staining of Iba-1 in wild-type (**a**, **c**, **e**, **g**, **i**) and iNOS−/− knockout animals (**b**, **d**, **f**, **h**, **j**). **a** Control wild (CGw), **b** control knockout iNOS−/− (CGk), **c** complete Freund's adjuvant wild (CFAw), **d** complete Freund's adjuvant knockout iNOS−/− (CFAk), **e** EAE wild (EAEw), **f** EAE knockout iNOS−/− (EAEk), **g** EAE + Agmatine wild (EAE + AGMw), **h** EAE + Agmatine knockout iNOS−/− (EAE + AGMk), **i** Agmatine wild (AGMw), **j** Agmatine knockout iNOS−/− (AGMk). Immunostaining for Iba-1 showed the strongest signal in EAEw (**e**), as well as EAEk (**f**) group of animals, at the end of the disease (24 dpi). However, Iba-1 labelling demonstrated mild reaction in EAE + AGMw (**g**) and EAE + AGMk (**h**) group of animals, and the predominant low staining in AGMw (**i**) and AGMk (**j**) groups. (original magnification ×400)

animals also had the increased NO levels, but significantly lower that those in WT animals (our unpublished data), indicating that not only iNOS was the source of NO in these conditions. Literature data show that agmatine irreversibly inhibits nNOS and induces down regulation of iNOS [33]. However, the significantly better clinical score in KO mice compared to WT animals, during agmatine treatment, suggests that agmatine suppresses proinflamatory pathways in EAE independently of iNOS activity, which is in accordance with the findings of others [34].

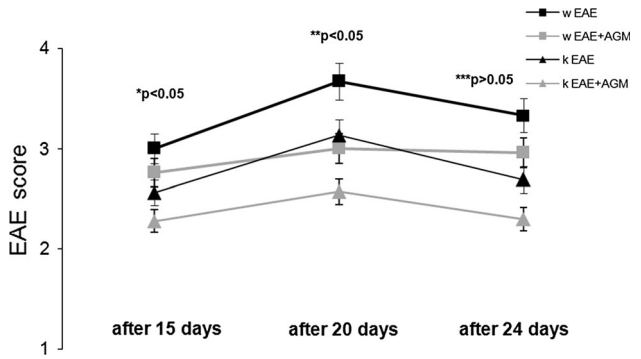

Fig. 5 The EAE score after 15, 20 and 24 days of EAE induction (15, 20, 24 day post immunization—15 dpi, 20 dpi, 24 dpi). 1—no clinical signs, 2—loss of tail tone, 3—hindlimb weakness—ataxia, 4—one to two limbs plegic, 5—three to four limbs plegic, 6—moribund or dead. The EAE score was higher in both examined groups (wild-type and knockout) than in animals from the same groups which have been treated with AGM. Generally, wild-type animals have had worse EAE score compared to the iNOS knockout animals. $*p < 0.05$, (EAEw vs EAEk—after 15 dpi, $**p < 0.05$, (EAEw vs EAEk, EAEw vs EAEw + AGM, EAEk vs EAEk + AGM—after 20 dpi), $***p > 0.05$, (EAEw vs EAEk—after 24 dpi), One Way ANOVA, Dunnett's C test

Table 1 Summary of immunohistochemical examinations of mice sagittal sections following EAE lesions

Group	Antibody		
	Occludin	GFAP	Iba1
CGw	++	+	+
CGk	++	+	−
CFAw	++	+	+
CFAk	++	+	++
EAEw	+	+++	+++
EAEk	+	+++	+++
EAE + AGMw	+++	++	++
EAE + AGMk	+++	+	++
AGMw	++	+	+
AGMk	++	+	+

CGw control wild, *CGk* control knockout iNOS−/−, *CFAw* complete Freund's adjuvant wild, *CFAk* complete Freund's adjuvant knockout iNOS−/−, *EAEw* EAE wild, *EAEk* EAE knockout iNOS−/−, *EAE + AGMw* EAE + Agmatine wild, *EAE + AGMk* EAE + Agmatine knockout iNOS−/−, *AGMw* Agmatine wild, *AGMk* Agmatine knockout iNOS−/−

(+), mild reaction; (++), strong reaction; (+++), very strong reaction

Post active immunization, decreassed expression of occludin positive cells (Fig. 1) in mice forebrain samples of WT, as well as KO animals, directs to the importance of occludin signaling pathways, which may provide novel insights into EAE and MS pathology and offer new therapeutic possibilities. Nitta and colleagues speculate that occludin's role in tight junction physiology may be of a regulatory and signaling nature rather than structural [35]. Studies indicated that occludin, highly expressed in the endothelial cells of the BBB, seemed to undergo dephosphorylation in the BBB of EAE animals [7, 13]. Occludin phospholylation changes may signal alterations in permeability of tight junctions of the BBB. During the course of EAE, occludin dephosphorylation could be involved in BBB breakdown and/or trafficking of immune cells into the CNS.

Decrease of occludin along with an increase of MMP-3 and MMP-9 in both WT and KO mice shows the potential for direct disruption of BBB in EAE. MMPs have been implicated in BBB disruption in different neuroinflammatory diseases [34, 36]. MMP-9 belong to gelatinase B group and digest the denatured collagens, gelatins Beside the digesting of extracellular matrix components, MMP-3 activates a number of proMMPs, such as proMMP-1 [37]. The results of these investigations prove that in the model of EAE in both WT and KO mice, activated microglia releases MMP-3 and MMP-9. The more prominent increases of both MMP-3 and MMP-9 in WT animals are in accordance with the more expressed clinical signs in these groups. The results of Swanson and coleaques show that it happens under the influence of TNFα which also needs the activation of poli(ADP)polymerase (PARP) [38]. Decreased neuron mortality induced by microglia activation could be related to farmacological inhibition of PARP and MMP-9 [39]. Increased MMP-9 and Iba-1 expressions in our investigation are consistent to these data. In addition, GFAP staining in EAE group indicates the astrocytes activation which was equally expressed in both WT and KO groups compared to controls [29]. The number and distribution of Iba1 positive cells show similar trend of GFAP expression changes in all experimental groups (Table 1), which may mean the time dependent consistence of astrocyte and microglia activation. Upon inflammation, astrocytes and microglia are activated, and can act as immune competent cells [40]. A significant positive correlation between GFAP expression in EAE KO animals and EAE WT animals (Table 2, $r = 0.6889$, $p < 0.05$) suggest that astrocyte activation influences the course of EAE progression. Deteriorated clinical expression in WT compared to KO mice in EAE group published in our previous reference, indicates the essential role of iNOS in EAE [24]. This is in accordance with other literature data [32].

Activated microglia release of proinflammatory and neurotoxic compounds, such as TNFα, ROS and NO. As a marker of activated microglia, increased Iba-1 expression indicates the rearrangement of the membrane cytoskeleton. Disruption of BBB in EAE could be the result of reduced occludin, activated microglia and astrocytes, as well as

Table 2 Pearson correlation between GFAP expression in iNOS−/− knockout animals and GFAP expression in WT animals

	GFAP-CGk	GFAP-EAEk	GFAP-EAE + AGMk	GFAP-AGMk
GFAP-CGw	0.1562	−0.3273	−0.2182	−0.2500
GFAP-EAEw	0.2030	0.6889	−0.2837	0.3714
GFAP-EAE + AGMw	−0.0471	−0.3948	0.0658	0.4523
GFAP-AGMw	−0.2906	0.1353	−0.4060	0.3101

CGw control wild, *CGk* control knockout iNOS−/−, *EAEw* EAE wild, *EAEk* EAE knockout iNOS−/−, *EAE + AGMw* EAE + Agmatine wild, *EAE + AGMk* EAE + Agmatine knockout iNOS−/−, *AGMw* Agmatine wild, *AGMk* Agmatine knockout iNOS−/−

elevated production of MMP-3 and MMP-9. Compromised BBB facilitates the influx of inflammatory cells which enhance neuroinflammation [41].

Agmatine application post EAE induces MMP-3 and MMP-9 depletion in both WT and KO animals (Figs. 2, 3). This could be in accordance with data that endogenous AGM inhibits MMP-9 expression through regulation of NO level [42]. Yang et al. [43] showed that AGM application inhibited MMP-9 and significantly decreased the level of cells mortality in CNS through NOS inhibition and depleted NO production. This is in accordance with our results—consequence of such AGM effect (decreased neuronal damage) in brain correlates with better clinical signs in WT and KO animals compared to EAE groups. The effects of applicated AGM are supported with less prominent expression of Iba-1 in both WT and KO EAE groups, while GFAP expression indicates the less degree of activated astrocytes (Table 1). GFAP expression in EAEk mice due to AGM application is on control level indicating intact astrocyte component of BBB. BBB integrity could be supported with elevated occludine expression post AGM application, compared to EAE animals (Fig. 1). Unactivated astrocytes in EAE post AGM application, which are found in KO mice (Table 1) support its protective effect upon BBB. This could be an explanation for better clinical score that we found in EAE + AGM KO animals [29], Fig. 5.

An interesting point of AGM application effect in control animals was its stimulative influence on Iba-1 expression in KO animals, which was not noticed in WT animals (Fig. 4). This was not followed with any further change of investigated parameters. A potential of AGM to inhibit constitutive NOS together with iNOS in KO animals could influence the grade of microglia activation [44].

Conclusions

The present study indicates benefit effects of AGM on BBB integrity in mice EAE model, which could be useful for the new insight into the mechanisms and potential therapeutic strategies in MS.

Acknowledgments This work was supported by a Grant from Scientific Project Number 41018 financed by the Ministry of Education and Science, Republic of Serbia.

Conflict of interest None.

References

1. Moscarello M, Mastronardi FG, Wood DD (2007) The role of citrullinated proteins suggests a novel mechanism in the pathogenesis of multiple sclerosis. Neurochem Res 32:251–256
2. Brück W, Gold R, Lund BT et al (2013) Therapeutic decisions in multiple sclerosis: moving beyond efficacy. JAMA Neurol 70(10):1315–1324
3. Baxter AG (2007) The origin and application of experimental autoimmune encephalomyelitis. Nat Rev Immunol 7:904–912
4. Wolburg-Buchholz K, Mack AF, Steiner E et al (2009) Loss of astrocyte polarity marks blood–brain barrier impairment during experimental autoimmune encephalomyelitis. Acta Neuropathol 118(2):219–233
5. Gonsette RE (2008) Neurodegeneration in multiple sclerosis: the role of oxidative stress and excititoxicity. J Neurol Sci 274:48–53
6. Haseloff RF, Dithmer S, Winkler L et al (2015) Transmembrane proteins of the tight junctions at the blood–brain barrier: structural and functional aspects. Semin Cell Dev Biol 38:16–25
7. Morgan L, Shah B, Rivers LE et al (2007) Inflammation and dephosphorylation of the tight junction protein occludin in an experimental model of multiple sclerosis. Neuroscience 147(3):664–673
8. Rubin LL, Staddon JM (1999) The cell biology of the blood–brain barrier. Ann Rev Neurosci 22:11–28
9. Harhaj NS, Antonetti DA (2004) Regulation of tight junctions and loss of barrier function in pathophysiology. Int J Biochem Cell Biol 36(7):1206–1237
10. Bennett J, Basivireddy J, Kollar A et al (2010) Blood–brain barrier disruption and enhanced vascular permeability in the multiple sclerosis model EAE. J Neuroimmunol 229(1–2):180–191
11. Le NT, Xue M, Castelnoble LA (2007) The dual personalities of matrix metalloproteinases in inflammation. Front Biosci 12:1475–1487
12. Furuse M, Sasaki H, Tsukita S (1999) Manner of interaction of heterogeneous claudin species within and between tight junction strands. J Cell Biol 147:891–903
13. Hirase T, Staddon JM, Saitou M et al (1997) Occludin as a possible determinant of tight junction permeability in endothelial cells. J Cell Sci 110(Pt 14):1603–1613
14. Minagar A, Ostanin D, Long AC et al (2003) Serum from patients with multiple sclerosis downregulates occludin and VE-cadherin expression in cultured endothelial cells. Mult Scler 9:235–238

15. Jourquin J, Tremblay E, Décanis N et al (2003) Neuronal activity-dependent increase of net matrix metalloproteinase activity is associated with MMP-9 neurotoxicity after kainate. Eur J Neurosci 18(6):1507–1517
16. Yang Y, Thompson JF, Taheri S et al (2013) Early inhibition of MMP activity in ischemic rat brain promotes expression of tight junction proteins and angiogenesis during recovery. J Cereb Blood Flow Metab 33(7):1104–1114
17. Mitchison TJ, Cramer LP (1996) Actin-based cell motility and cell locomotion. Cell 84:371–379
18. Ito D, Tanaka K, Suzuki S et al (2001) Enhanced expression of IBA1, ionized calcium-binding adapter molecule 1, after transient focal cerebral ischemia in rat brain. Stroke 32:1208–1215
19. Bhutada P, Mundhada Y, Humane V et al (2012) Agmatine, an endogenous ligand of imidazoline receptor protects against memory impairment and biochemical alterations in streptozotocin-induced diabetic rats. Prog Neuropsychopharmacol Biol Psychiatry 37:96–105
20. Piletz JE, Aricioglu F, Cheng JT et al (2013) Agmatine: clinical applications after 100 years in translation. Drug Discov Today 18:880–893
21. Rushaidhi M, Zhang H, Liu P (2013) Effects of prolonged agmatine treatment in aged male Sprague-Dawley rats. Neuroscience 234:116–124
22. Pautz A, Art J, Hahn S et al (2010) Regulation of the expression of inducible nitric oxide synthase. Nitric Oxide 23(2):75–93
23. Ibragic S, Sofic E, Suljic E et al (2012) Serum nitric oxide concentration in patients with multiple sclerosis and patients with epilepsy. J Neural Transm 119(1):7–11
24. Wilcox G, Fiska A, Haugan F et al (2004) Central sensitization. J Pain 5(3):S19
25. Ljubisavljevic S, Stojanovic I, Pavlovic D et al (2011) Aminoguanidine and *N*-acetyl-cysteine supress oxidative and nitrosative stress in EAE rat brains. Redox Rep 16(4): 166–172
26. Ljubisavljevic S, Stojanovic I, Pavlovic D et al (2012) Correlation of nitric oxide levels in the cerebellum and spinal cord of experimental autoimmune encephalomyelitis rats with clinical symptoms. Acta Neurobiol Exp 72(1):33–39
27. Ljubisavljevic S, Stojanovic I, Pavlovic R et al (2012) The reduced glutathione and S-nitrosothiols levels in acute phase of experimental demyelination—pathophysiological approach and possible clinical relevancy. Neuroscience 219:175–182
28. Ljubisavljevic S, Stojanovic I, Pavlovic D et al (2013) Suppression of the lipid peroxidation process in the CNS reduces neurological expression of experimentally induced autoimmune encephalomyelitis. Folia Neuropathol 51(1):51–57
29. Stevanovic I, Ninkovic M, Stojanovic I et al (2013) Beneficial effect of agmatine in the acute phase of experimental autoimmune encephalomyelitis in iNOS−/− knockout mice. Chem Biol Interact 206:309–318
30. Gurd JW, Jones LR, Mahler HR et al (1974) Isolation and partial characterization of rat brain synaptic membrane. J Neurochem 22:281–290
31. Lowry OH, Rosenbrongh NJ, Farr AL et al (1951) Protein measurement with the folin phenol reagent. J Biol Chem 193:265–275
32. de Lago E, Moreno-Martet M, Cabranes A et al (2012) Cannabinoids ameliorate disease progression in a model of multiple sclerosis in mice, acting preferentially through CB1 receptor-mediated anti-inflammatory effects. Neuropharmacology 62(7):2299–2308
33. Kim S, Moon C, Wie MB, Kim H, Tanuma N, Matsumoto Y, Shin T (2000) Enhanced expression of constitutive and inducible forms of nitric oxide synthase in autoimmune encephalomyelitis. J Vet Sci 1(1):11–17
34. Kim JH, Yenari MA, Giffard RG, Cho SW, Park KA, Lee JE (2004) Agmatine reduces infarct area in a mouse model of transient focal cerebral ischemia and protects cultured neurons from ischemia-like injury. Exp Neurol 189(1):122–130
35. Nitta M, Yogo K, Ohashi M et al (2010) Identification and expression analysis of connexin-45 and connexin-60 as major connexins in porcine oocytes. J Anim Sci 88(10):3269–3279
36. Kim YS, Joh TH (2012) Matrix metalloproteinases, new insights into the understanding of neurodegenerative disorders. Biomol Ther (Seoul) 20(2):133–143
37. Niu W, Qi Y (2012) Matrix metalloproteinase family gene polymorphisms and risk for coronary artery disease: systematic review and meta-analysis. Heart 98(20):1483–1491
38. Swanson RA, Ying W, Kauppinen TM (2004) Astrocyte influences on ischemic neuronal death. Curr Mol Med 4(2):193–205
39. Chase AJ, Bond M, Crook MF et al (2002) Role of nuclear factor-kappa B activation in metalloproteinase-1, -3, and -9 secretion by human macrophages in vitro and rabbit foam cells produced in vivo. Arterioscler Thromb Vasc Biol 22(5):765–771
40. Magliozzi R, Howell OW, Reeves C et al (2010) A Gradient of neuronal loss and meningeal inflammation in multiple sclerosis. Ann Neurol 68(4):477–493
41. D'Aversa TG, Eugenin EA, Lopez L et al (2013) Myelin basic protein induces inflammatory mediators from primary human endothelial cells and blood–brain barrier disruption: implications for the pathogenesis of multiple sclerosis. Neuropathol Appl Neurobiol 39:270–283
42. Jung HJ, Yang MZ, Kwon KH et al (2010) Endogenous agmatine inhibits cerebral vascular matrix metalloproteinases expression by regulating activating transcription factor 3 and endothelial nitric oxide synthesis. Curr Neurovasc Res 7(3):201–212
43. Yang MZ, Mun CH, Choi YJ et al (2007) Agmatine inhibits matrix metalloproteinase-9 via endothelial nitric oxide synthase in cerebral endothelial cells. Neurol Res 29(7):749–754
44. Mori M, Gotoh T (2004) Arginine metabolic enzymes, nitric oxide and infection. J Nutr 134(10 Suppl):2820S–2825S