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Theta burst stimulation promotes nestin expression in experimental autoimmune encephalomyelitis

Stimulacija teta praskovima pojačava ekspresiju nestina kod eksperimentalnog autoimunskog encefalomijelitisa

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Abstract

Background/Aim. Multiple sclerosis (MS) is an immune-mediated disease of the nervous system in which the myelin sheath is destroyed during the process of neurodegeneration. Experimental autoimmune encephalomyelitis (EAE) is an animal model of MS in which preservation of myelin and remyelination of axons can improve neuron survival. The aim of the study was to evaluate the activation capacity of neuronal tissue by autoimmune inflammation and treatment with intermittent (i) theta burst stimulation (TBS)-iTBS or continuous TBS (cTBS) based on the expression profiles of nestin in astrocytes, oligodendrocytes, and neurons. **Methods.** Two forms of TBS – iTBS and cTBS – were used to extend the period during which axons can be remyelinated. It was investigated how iTBS or cTBS protocols affect the expression profiles of nestin with glial fibrillary acidic protein, myelin basic protein (MBP), and neuronal nuclear protein in rat spinal cord. Changes at the molecular level were monitored using the immunofluorescence method. **Results.** The obtained results showed that both protocols (iTBS and cTBS) increased the expression of nestin and MBP and reduced astrogliosis in the spinal cord of EAE rats. **Conclusion.** The therapeutic potential of TBS in EAE contributes to the improvement of the intrinsic ability to recover from spinal cord injury.

Key words:
encephalomyelitis, autoimmune, experimental; multiple sclerosis; nerve regeneration; nestin; rats; spinal cord; transcranial magnetic stimulation.

Apstrakt

Uvod/Cilj. Multipla skleroza (MS) je bolest nervnog sistema posredovana imunskim mehanizmima u kojoj dolazi do oštećenja mijelinskog omotača u toku procesa neurodegeneracije. Eksperimentalni autoimunski encefalomielitis (EAE) je animalni model MS, u kome obnavljanje mijelina i remijelinizacija aksona mogu poboljšati preživljavanje neurona. Cilj rada bio je da se proceni aktivacioni kapacitet neuronskog tkiva pod uticajem autoimunskog zapaljenja i intermitentne (i) stimulacije teta praskovima (*theta burst stimulation* – TBS) ili kontinuirane (*continuous* – c) TBS (cTBS), na osnovu profila ekspresije nestina u astrocitima, oligodendrocitima i neuronima. **Metode.** Dva oblika TBS – iTBS i cTBS su korišćena za produženje perioda tokom koga aksoni mogu biti remijelinizovani. Ispitivan je uticaj iTBS i cTBS protokola na ekspresiju nestina sa glijalnim fibrilarnim kiselim proteinom, mijelin baznim proteinom (MBP) i neuronalnim nuklearnim proteinom u kičmenoj moždini pacova. Promene na molekulskom nivou su praćene primenom imunofluorescentne metode. **Rezultati.** Dobijeni rezultati su pokazali da oba protokola (iTBS i cTBS) povećavaju ekspresiju nestina i MBP i redukuju astrogliozu u kičmenoj moždini EAE pacova. **Zaključak.** Terapijski potencijal TBS u EAE doprinosi poboljšanju intrinzične sposobnosti za oporavak od povrede kičmene moždine.

Ključne reči:
encefalomijelitis, autoimunski, eksperimentalni; multipla skleroza; živac, regeneracija; nestin; pacovi; kičmena moždina; stimulacija, magnetna, transkranijalna.

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Introduction

Multiple sclerosis (MS) is a demyelinating disease of the central nervous system (CNS) characterized by repeated loss and regeneration of myelin sheaths. Pathological accumulation of degradation products during inflammation, neurodegeneration, and demyelination is thought to contribute to the onset and progression of the disease [1].

Experimental autoimmune encephalomyelitis (EAE) is a neurological disease characterized by disruption of blood-brain barrier, perivascular neuroinflammation, and neuronal damage due to progressive axon sheath destruction [2]. It is one of the most commonly used models of MS. Disruptions in the glial-neuronal network between astrocytes, oligodendrocytes, and neurons lead to metabolic deficits in all cell types, resulting in cellular dysfunction and death. Remyelination of CNS axons is essential for functional recovery after demyelinating injury. The reduction of astrogliosis has a positive effect on the remyelination process [1].

Various inhibitory signals in inflammatory demyelination prevent the required differentiation of oligodendrocytes and suppress the expression of myelin basic protein (MBP), which is required for the onset of healing [3]. The intense activity of oligodendrocytes is supported by a high metabolic rate, considering the high turnover in the formation of myelin sheaths [4]. Loss of integrative relationships between neurons, astrocytes, and oligodendrocytes leads to metabolic disturbances in these cells, permanent damage, and even death [5]. Stimulation of remyelination of axons in the CNS is crucial as it promotes the return of axons to a competent state suitable for myelination, regeneration, and thus, restoration of function and reduction of astroglia contribute to this [6].

Intermediate filaments influence cell type and developmental stage. Nestin is a class VI intermediate filament protein transiently expressed in adult neural stem cells (NSCs) and immature neural progenitor cells and disappears when the cells enter differentiation. It is commonly used as a marker for NSCs in both the embryo and the adult brain. Nestin is the main marker for immature astroglial cells and multipotent progenitor cells while maturing astrocytes contain glial fibrillary acidic protein (GFAP) [7]. Nestin expression has been shown to be reinitiated in reactive astrocytes [8]. Nestin-expressing cells indicate an active stage of embryonic progenitor cells and are involved in the repair of damaged CNS tissue during the course of EAE [9].

Nestin is mainly distributed in the cytoplasm, and its expression is an indicator of cell growth and proliferation. The level of nestin messenger ribonucleic acid is more pronounced in damaged tissue than in intact tissue [10]. Various factors regulate the expression of nestin. It was originally thought to be exclusively associated with NSCs [11].

The neuron-specific nuclear protein (NeuN) is a small soluble protein mainly localized in the nucleus [12]. It is considered a relevant marker for *post*-mitotic neurons [13]. NSCs compete for a central role in the recovery process after tissue injury [14]. Consequently, the changes in NSCs in EAE might be reflected in the expression of nestin. NeuN, a specific marker for mature neurons, is present in most neurons of adult neurogenesis [15].

Repetitive transcranial magnetic stimulation (TMS) enhances neuronal activity through synaptic potentiation induced by high stimulation frequency and subsequent suppression of EAE-induced tissue damage [16]. Our studies have shown that two different TMS stimulation protocols, intermittent theta burst stimulation (TBS) – iTBS and continuous TBS (cTBS), are structured types of repetitive TMS that induce changes in redox homeostasis in both healthy and EAE animals [17, 18]. While iTBS induces cortical facilitation similar to long-term potentiation (LTP), cTBS causes depression of cortical activity reminiscent of long-term depression (LTD) [19]. Our previous report indicated that neuroplasticity induced by LTP and LTD could lead to local changes in the brain at the molecular level, such as increased availability of brain-derived neurotrophic factor, which can reduce inflammation and support myelin repair [20].

The aim of this study was to evaluate the activation capacity of neuronal tissue by autoimmune inflammation and treatment with iTBS or cTBS using the expression profiles of nestin in astrocytes, oligodendrocytes, and neurons.

Methods

Animals

The experimental animals were handled in accordance with the ethical guidelines for the use of animals in research. The experimental procedures were approved by the Ethics Committee from the Ministry of Agriculture and Environmental Protection – the Veterinary Directorate of the Republic of Serbia No. 323-07-00622/2017-05.

Female Dark Agouti rats aged 10–14 weeks weighing 150–200 g were used for the experiments. The animals were held in cages under constant environments (light-dark cycle of 13/11 hrs, temperature of 23 °C ± 2 °C, and humidity of 55% ± 3%). Food and water were provided *ad libitum*, while water was offered manually in cases of severe paralysis.

EAE induction and TMS treatment

The rats were anesthetized intraperitoneally (i.p.) with ketamine (50 mg/kg) and xylazine (10 mg/kg) before EAE induction by subcutaneous (s.c.) injection of 0.1 mL suspension of rat spinal cord tissue homogenate (50% weight/volume – w/v in saline) dissolved in Complete Freund's Adjuvant (CFA) containing 1 mg/mL *Mycobacterium tuberculosis* (Sigma, St. Louis, MO, USA) into the right posterior footpad [21].

TMS was performed with a specific coil (25 mm figure-of-eight) using a MagStim Rapid [2] device (The MagStim Company, Whitland, Dyfed, UK). The center of the coil was located directly above the bregma. The iTBS pattern (iTBS group) consisted of 20 trains of ten bursts of three pulses at a frequency of 50 Hz repeated at 5 Hz (duration 192 s with 10 s

pauses between series) [17]. The cTBS pattern (cTBS group) consisted of a single 40 s train of bursts repeated at 5 Hz. Each pattern consisted of 600 pulses. The intensity of the stimulus was 30% of the maximal stimulator output, which was currently below the motor threshold (expressed as apparent upper limb contraction in all TBS-treated rats).

Experimental procedure

The experimental animals were randomly divided into seven groups: Control group (C), n = 3; CFA treated group (CFA), n = 3; EAE-immunized animals group (EAE), n = 3; two groups of EAE-immunized animals treated with iTBS (EAE+iTBS), n = 3, or cTBS (EAE+cTBS), n = 3, and two groups of healthy animals stimulated with iTBS (iTBS), n = 3 or cTBS (cTBS), n = 3. Healthy animals were treated with iTBS or cTBS for ten days, while both treatment protocols were applied to EAE animals for ten days from day 14 post-immunization (p.i.).

Clinical evaluation of EAE was performed daily as a part of a double-blind study up to day 24 p.i. [22]. All animals were anesthetized i.p. with sodium/pentobarbital 45 mg/kg body weight and decapitated 24 hrs after the last TBS protocol.

Immunofluorescence

For fluorescence staining, spinal cords were quickly isolated and fixed for 12 hrs at 4 °C in 4% paraformaldehyde. For cryoprotection, the tissues were placed in a graded concentration of sucrose (10%, 20%, and 30% sucrose, pH 7.4). The spinal cord was frozen in 2-methyl butane and stored at -80 °C before sectioning with the cryotome (Leica CM 1850, Germany). The 25 µm thick tissue sections were mounted on glass slides, dried at room temperature for 2 hrs, and stored at -20 °C before staining. The primary mouse monoclonal anti-Nestin antibody (1:100; Abcam, Germany) was applied overnight at 4 °C. The secondary donkey anti-mouse antibody Alexa Fluor 555 (1:250; Invitrogen, Carlsbad, CA, USA) was used for 2 hrs in the dark (at room temperature). The slides were then washed several times before being incubated overnight at 4 °C with a new primary rabbit polyclonal antibody: anti-GFAP (1:1,000; Abcam, Germany), anti-MBP (1:1,000; Abcam, Germany), or anti-NeuN (1:1,000; Abcam, Germany). To complete the staining, the slides were incubated with the secondary goat anti-rabbit Alexa Fluor 488 antibody (1:500; Invitrogen, Carlsbad, CA, USA) for 2 hrs in the dark (at room temperature). All antibodies were diluted in phosphate-buffered saline-PBS with 1% bovine serum albumin-BSA. Slides were mounted on microscope slides with Mowiol medium (Sigma Aldrich) and analyzed under a confocal microscope (Zeiss Axiovert 200M, LSM 510 laser module).

Immunofluorescence quantification

Three sections of the ventral horn *per* animal were used to define fluorescence intensity using the Fiji version of ImageJ software. Five random photomicrographs of the section of interest were taken at one magnification (×40) and analyzed using the Coloc2 program processor of the ImageJ software [23].

Image processing and calculation of a combined colocalization coefficient

Quantitative colocalization analysis is an advanced digital imaging tool for the observation of antigens in immunofluorescence images obtained by confocal microscopy. It uses specialized algorithms that calculate many coefficients from which colocalization can be quantitatively estimated [24].

Colocalization is presented in the form of a plate with three images consisting of fluorescence images for red and green channels and a third merged image in which the channels are combined (overlapping pixels turn yellow). The analysis is done using computer software based on the evaluation of the color of the selected pair of channels.

The images were imported into the Fiji version of the free image processing software – ImageJ. Fiji contains several pre-installed plugins, including a colocalization analysis method called Coloc 2, which calculates several colocalization parameters, such as Pearson's and Manders' coefficient, based on correlation measurements of pixel intensity. In addition to the numerical correlation parameters, a 2D intensity histogram is also generated to visualize the correlation between the two channels. For more information, see Stevanovic et al. [20].

Statistical analysis

A one-way ANOVA and Tukey's *post hoc* multiple tests (GraphPad Prism software, version 6.0) were used for statistical data analysis. Values are presented as mean ± standard deviation. The correlation coefficients were determined using the Spearman test, whereby the differences were considered statistically significant if $p < 0.05$.

Results

The microscopic images of dual fluorescent immunoreactivity of nestin with GFAP (Figure 1), nestin with MBP (Figure 2), or nestin with NeuN (Figure 3) in spinal cord tissue were used for quantitative colocalization analysis (Figures 4 and 5).

In the EAE group, the expression of nestin and GFAP increased (Figure 1), while MBP decreased (Figure 2). Compared to the EAE group, the application of both the iTBS and cTBS protocols resulted in increased nestin expression with a simultaneous decrease in GFAP and increased MBP. The expression of NeuN showed no difference between the groups, independent of the increased nestin expression (Figure 3).

The increase in nestin immunoreactivity was measured in the EAE group compared to control values (Figure 4A, $p < 0.05$). Increased nestin expression was also measured after

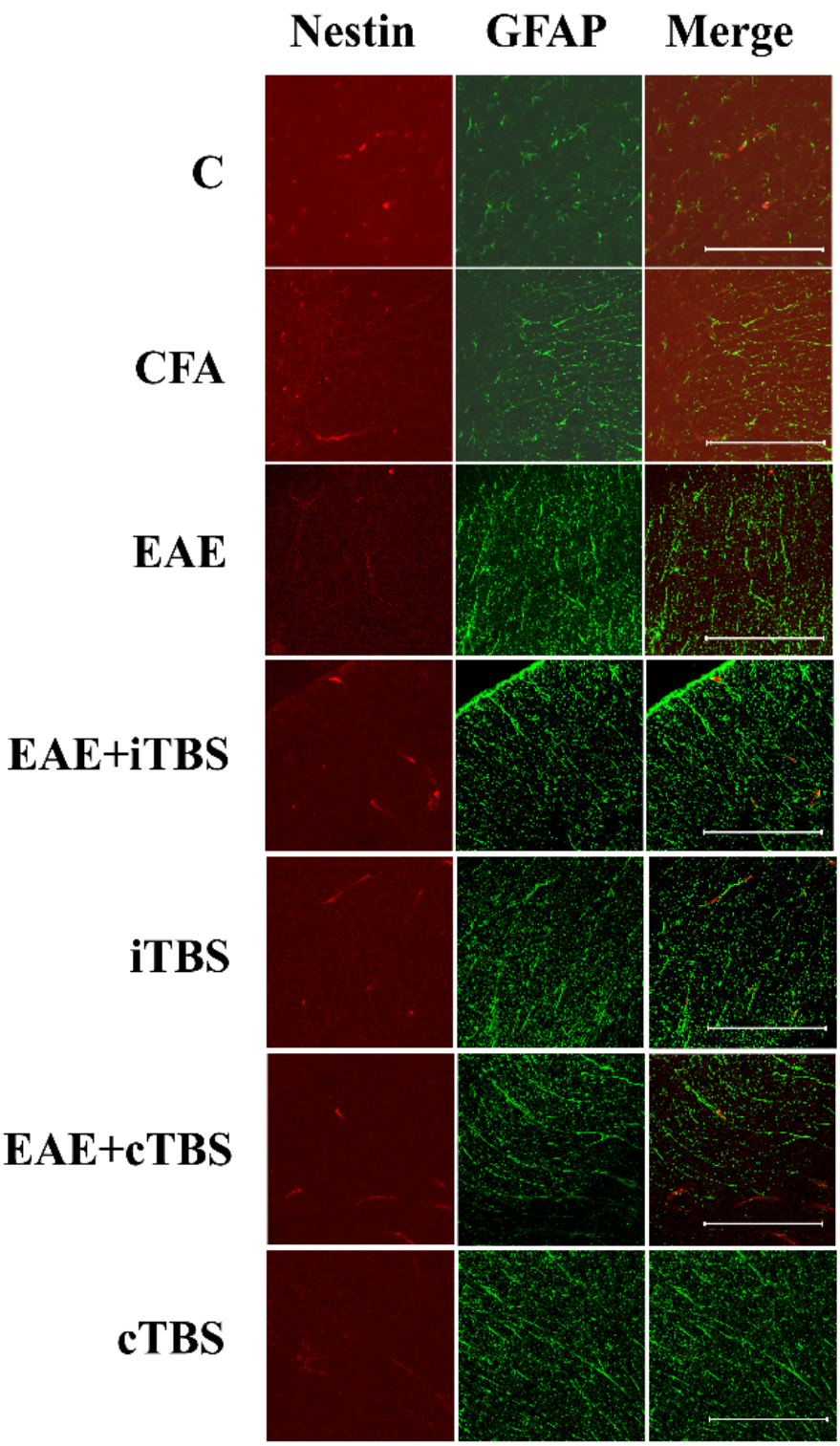

Fig. 1 – Double fluorescence staining showing immunoreactivity for nestin and GFAP in rat's spinal cord. Red represents nestin positivity, and green in the photograph represents GFAP positivity. C – control group; EAE – rats with EAE; CFA – rats treated with CFA; EAE+iTBS – iTBS treatment on EAE animals; iTBS – iTBS treatment on healthy animals; EAE+cTBS – cTBS treatment on EAE animals; cTBS – cTBS treatment on healthy animals. Photomicrographs of the stained spinal cord sections were taken at a magnification of ×40. The scale bar represents 200 μm. GFAP – glial fibrillary acidic protein; CFA – Complete Freund's Adjuvant; EAE – experimental autoimmune encephalomyelitis; iTBS – intermittent theta burst stimulation; cTBS – continuous theta burst stimulation.

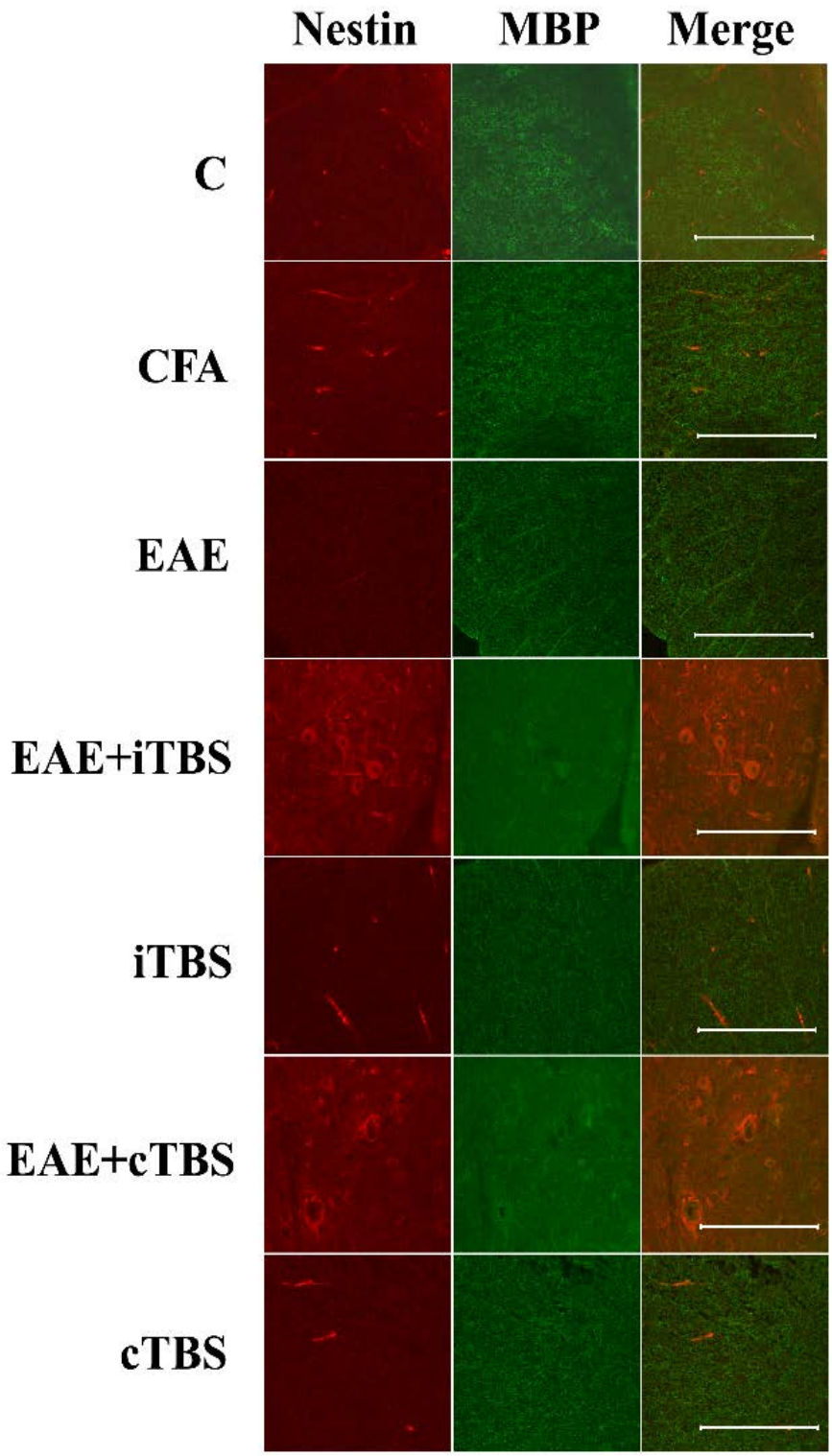

Fig. 2 – Double fluorescence staining showing immunoreactivity for nestin and MBP in rat's spinal cord. Red represents nestin positivity, and green represents MBP positivity. Photomicrographs of the stained spinal cord sections were taken at a magnification of ×40. The scale bar shows 200 μm.
MBP – myelin basic protein. For other abbreviations, see Figure 1.

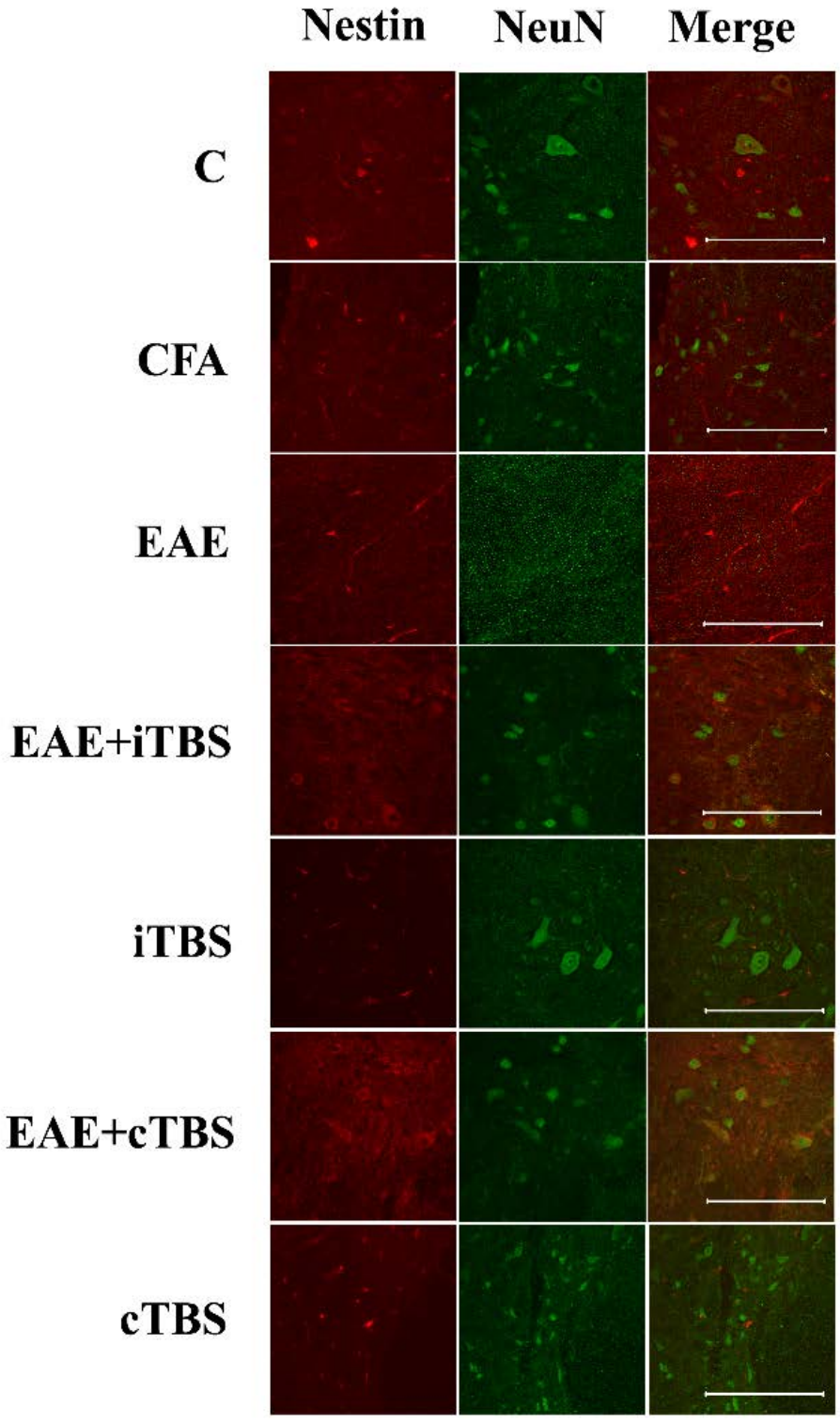

Fig. 3 – Double fluorescence staining showing immunoreactivity for nestin and NeuN in rat's spinal cord. Red represents nestin positivity, and green represents NeuN positivity. Photomicrographs of the stained spinal cord sections were taken at a magnification of ×40.
The scale bar shows 200 μm.
NeuN – neuronal nuclear protein. For other abbreviations, see Figure 1.

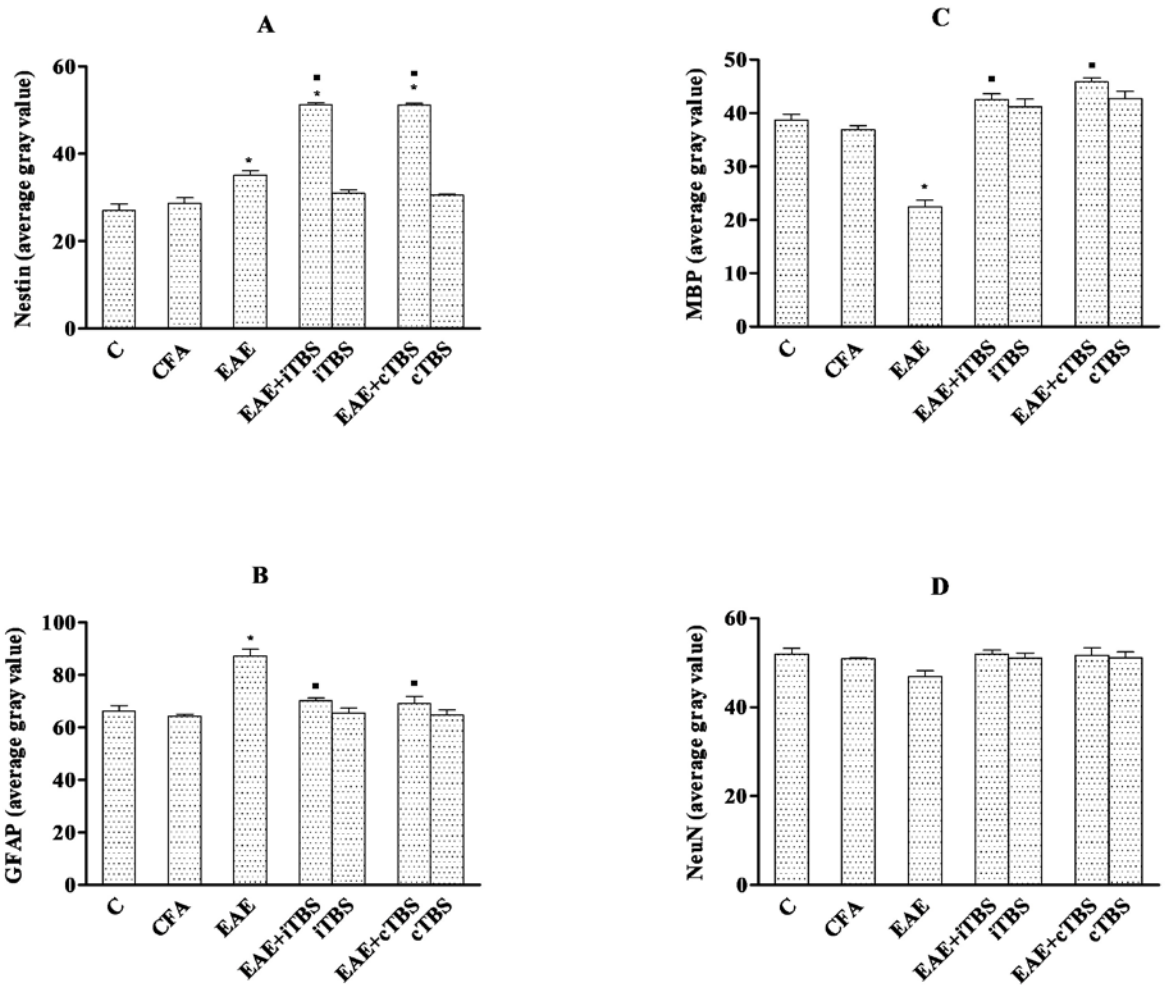

Fig. 4 – Effects of iTBS and cTBS on double staining of nestin (A), nestin with GFAP (B), nestin with MBP (C), or nestin with NeuN (D) in the rat spinal cord. Results were expressed as average grey value ± SD (n = 3). Analysis of variance (ANOVA) was performed followed by Tukey's multiple comparison test with GraphPad Prism 6.0.
MBP – myelin basic protein; NeuN – neuronal nuclear protein; SD – standard deviation. For other abbreviations, see Figure 1.
***$p < 0.05$ compared to the control group; ▪ $p < 0.05$ compared to the EAE group.**

both iTBS/cTBS treatments in the EAE animals compared to controls and compared to the EAE group (Figure 4A, $p < 0.05$). In contrast to nestin, GFAP expression increased in EAE animals compared to the controls (Figure 4B, $p < 0.05$). However, iTBS/cTBS treatment significantly decreased the expression of GFAP in the EAE animals (Figure 4B, $p < 0.05$).

A significant decrease in MBP immunoreactivity was observed in the EAE group compared to controls, and then both protocols (iTBS and cTBS) applied to EAE animals significantly increased MBP expression compared to the EAE group (Figure 4C, $p < 0.05$). There was no significant difference between nestin and NeuN in any of the experimental groups (Figure 4D).

We found that the nestin and GFAP molecules had high numerical colocalization values, according to the intensity-based coefficients (Pearson's and Manders' correlation parameters) (Figure 5A, 5D). High correlation values were found after labeling the same compartments with nestin and GFAP in the EAE, EAE+iTBS, and EAE+cTBS groups compared to controls. In contrast, Pearson's correlation was lower in the EAE+iTBS and EAE+cTBS groups compared to the EAE group. The M2 Manders' coefficient (GFAP) increased in EAE and EAE+iTBS compared to the controls, while it decreased in the EAE+cTBS group compared to the EAE animals (Figure 5D).

Pearson's correlation coefficient of colocalizations between nestin and MBP showed increased values in the EAE+cTBS group compared to the control and in both TBS protocols in the EAE animals compared to the EAE group (Figure 5B, $p < 0.05$). The Manders' coefficients of correlations and colocalizations between nestin and MBP were not significant (Figure 5E).

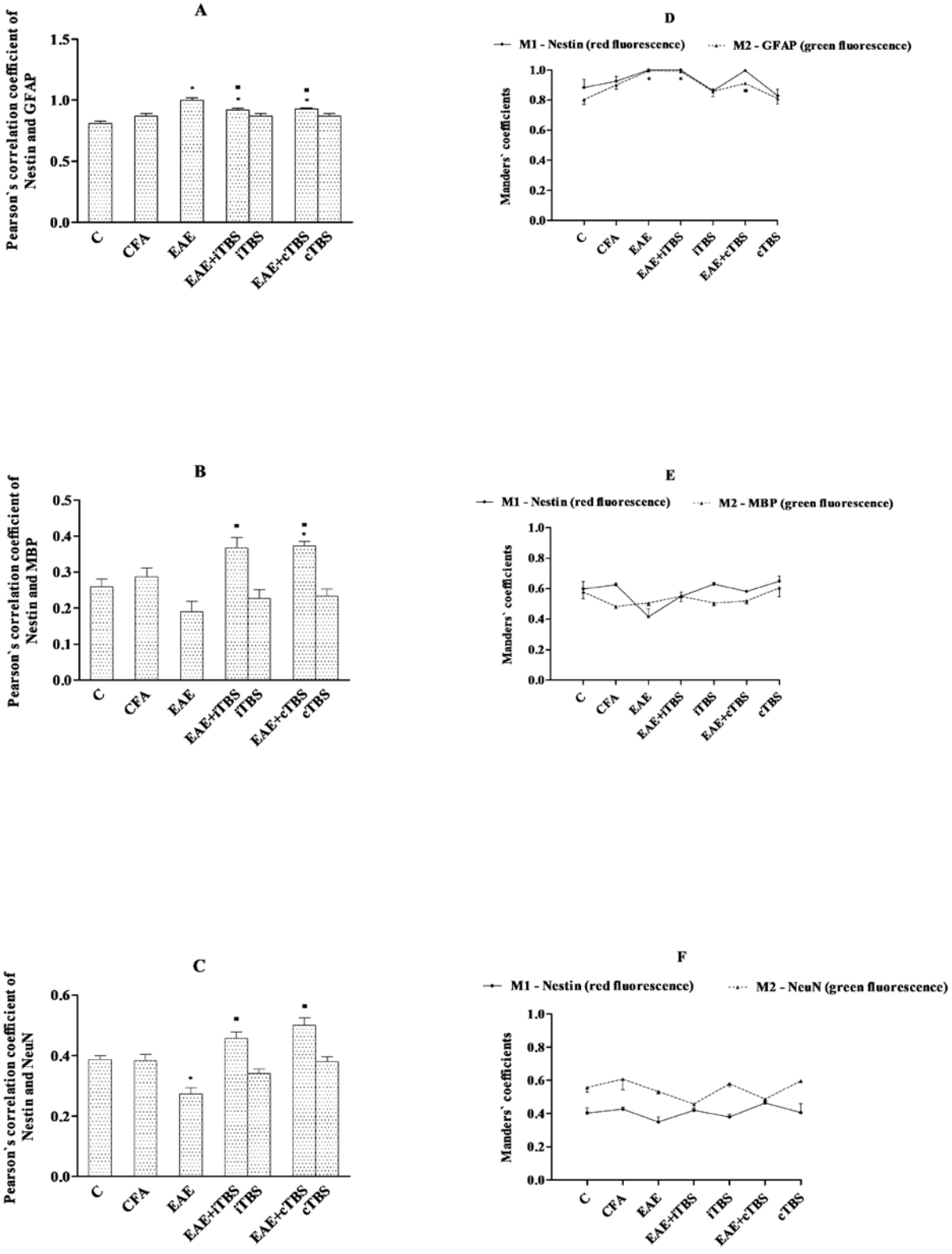

Fig. 5 – Colocalization parameters proposed as Pearson's coefficient (A, B, C) and Manders' coefficient (D, E, F): M1 (nestin) and M2 (GFAP, MBP, NeuN) after double labeling of nestin with GFAP (A, D), nestin with MBP (B, E), or nestin with NeuN (C, F) in the rat spinal cord. Results were expressed as average grey value ± SD (n = 3). Analysis of variance (ANOVA) was performed followed by Tukey's multiple comparison test with GraphPad Prism 6.0.

MBP – myelin basic protein; NeuN – neuronal nuclear protein; SD – standard deviation. For other abbreviations, see Figure 1.

***$p < 0.05$ compared to the control group; ▪ $p < 0.05$ compared to the EAE group.**

The Pearson's coefficients between nestin and NeuN showed increased values, albeit at a low level, in the EAE group compared to the controls (Figure 5C, $p < 0.05$), while both TBS treatments increased the same coefficient in the EAE animals compared to the EAE group (Figure 5C, $p < 0.05$). The Manders' coefficients between these two proteins were not significant (Figure 5F).

Discussion

Both applied TBS protocols in a model of actively induced EAE showed comparable effects of the expression of nestin, GFAP, and MBP in the spinal cord.

During recovery from EAE inflammation, nestin changed dynamically. Previous studies have shown weak nestin immunoreactivity in vascular endothelial cells but not in the neural parenchyma of the spinal cord of healthy rats [25]. EAE immunization can induce the appearance of progenitor cells/radial glia, which is supported by increased nestin expression. Neuronal damage is confirmed by the detected GFAP overexpression in and around the lesions [26].

Proper regulation of NSCs in injured neural tissue is a prerequisite for CNS remodeling. This study revealed increased reactivity of nestin throughout the spinal cord along with GFAP. In the EAE group, nestin immunoreactivity was detected in gray and white matter astrocytes on day 24 p.i. EAE underscores the accumulation of enlarged, multipolar GFAP immunoreactive astroglial cells within inflammatory demyelinating lesions. The accumulation of astroglia at the edges of lesions is followed by depletion of oligodendroglia, which may indicate that marked gliosis is not immediately followed by infiltrating oligodendrocytes near the lesions [27]. This is consistent with the decreased MBP immunoreactivity in the EAE group compared to controls.

In the mature CNS, it appears that glia have multipotent capabilities that can be triggered by a noxious attack such as neuroinflammation and can transform into neurons, oligodendroglia, or astrocytes [28]. The data on unaltered NeuN presentation in the spinal cord on day 24 p.i. suggest that nestin-positive multipotent cells in the spinal cord convert their phenotype into oligodendrocytes or astrocytes. This speculation is supported by the discovery of nestin-positive cells with long processes that resemble both preoligodendrocytes and mature oligodendrocytes [29].

Following EAE-immunization, several complexes of secondary injury cascades develop, offering great potential for therapeutic intervention. Nestin-positive NSCs could be induced by TBS treatments and help replace lost cells, raising the prospect of effective integration into neural circuits. Therefore, we hypothesize that TBS treatment contributes to functional recovery and pathophysiological changes by increasing the number of nestin-positive cells. Remarkably, the number of nestin/GFAP double-positive cells in the spinal cord sections decreased after iTBS or cTBS treatment in the diseased animals compared to the EAE group, indicating a beneficial effect of stimulation. Our study provides evidence that TMS promotes the formation of nestin-positive cells in the spinal cord of adult rats, suggesting that this may be related to the potential for functional recovery.

In contrast to the increase in the number of GFAP-positive cells in the EAE animals, which is indicative of astrogliosis, treatment with iTBS or cTBS reduced the proliferation of astrocytes in the EAE animals. Compared to the EAE animals, some of the cells with intense nestin labeling in the diseased animals treated with iTBS or cTBS gradually showed weak GFAP reactivity along with increased MBP expression, indicating the considerable therapeutic potential of TMS in functional recovery. These data complement our previous study, which confirmed that TMS treatment has a positive impact on astroglia and microglia, along with a better clinical outcome, including disease duration and exposed paralysis [30]. The therapeutic effect is likely to be a modulation of astrocyte activity in the zone of neural tissue damage [31].

Conclusion

The current study suggests that both theta burst stimulation protocols improve histologic recovery. These treatments appear to lead to an increase in nestin- and myelin basic protein-positive cells with decreasing astrogliosis in EAE animals, promising reconstructed neuronal differentiation. The therapeutic potential of theta burst stimulation is thus recommended in EAE as it helps improve the intrinsic ability to recover from spinal cord injury.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgement

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