# Continuous versus intermittent theta burst stimulation in the treatment of experimental autoimmune encephalomyelitis: a glutamate transporters study

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# Abstract

Background: Synaptic overload with glutamate aggravates neurotransmission and worsen the progression of the neurodegenerative disease, such as multiple sclerosis (MS). The experimentally induced autoimmune encephalomyelitis (EAE) in rats is a well-established animal model to study MS. Glutamate reuptake occurs by glial glutamate transporter (GLT-1), and glutamate-aspartate transporter (GLAST) localized predominantly in astrocytes terminals. The focus of the study addressing the expression of these transporters in EAE rats and those subjected to theta burst stimulation (TBS), that promotes long-lasting modulation of neuronal activity in rats/humans. Leading by the reported outcomes of TBS, we examined if TBS underlying mechanisms refer to astroglial glutamate transporters status.

Methods : We studied changes in the expression of glial glutamate transporter GLT-1 and glutamate-aspartate transporter (GLAST), and glial fibrillary acidic protein (GFAP), in the spinal cord of EAE rats, subjected to intermittent (iTBS) and continuous (cTBS) theta burst stimulation. We quantified the expression of GLAST, GLT-1, and GFAP by immunofluorescence in control and experimental groups of Dark Agouti rats.

Results: EAE elevated expression of GFAP, GLAST, and GLT-1. Both TBSs reduced the expression of GFAP. Continual TBS did not interfere with glutamate transporters in EAE rats, while iTBS decreased GLT-1, and increased GLAST.

Conclusion: Continual TBS reduced astrogliosis more efficiently than iTBS, in EAE rats. Besides, it did not mitigate the glutamate transporters' expression; thus, glutamate reuptake remained upraised in cTBS exposed EAE rats. Accordingly, we concluded that cTBS might advance the remyelination of damaged neuronal cells in EAE rats. The future clinical trials on the treatment of MS may consider the data of this pre-clinical animal study.


# 1. Background

The experimentally induced autoimmune encephalomyelitis (EAE) in rats or mice is a well-established model to study multiple sclerosis (MS) [1]. Progressive neuronal deterioration mediated by cytokine and or chemokine associated formation of sclerotic plaques and inflammatory demyelination, as well as blood/brain barrier and axonal myelin sheaths damage (a cause of slowing or stopping nerve impulses transmission), characterize MS [2, 3]. Excessive excitatory signals or the absence of neuronal activity interfere with the differentiation of oligodendrocyte progenitor cells into oligodendrocytes during inflammatory demyelination. Oligodendrocyte progenitors' differentiation into oligodendrocytes depends on neuronal activity [4]. Myelin regeneration, i.e., remyelination, occurs spontaneously by oligodendrocytes in MS. Astrocytes are the glial cells that retrieve peripheral immune cells access to the central nervous system (CNS) [5, 6].

The neuronal activity involves both excitatory and inhibitory neuronal signal transmission. The consequence of the excitatory neuronal activity is the excessive glutamate release into synapsis.

Excitatory amino acid transporters, glial glutamate transporter GLT-1 and glutamate-aspartate transporter GLAST, uptake glutamate from synapses into astrocytes. The principle glutamate transporter is GLT-1, mainly distributed in astrocytes, but also neurons and other glial cells. The GLAST is primarily localized in astrocytes, but in considerably less amount, then GLT-1, and less in neurons. Its contribution to glutamate removal is markedly less then GLT-1 [1, 7].

Metabolic turnover of glutamate into glutamine occurs in astrocytes, by glutamine synthetase, and glutamine into gamma-aminobutyric acid in the presynaptic neuron, by glutamate decarboxylase. Astroglial and presynaptic terminal glutamine transporters participate in glutamine pass on. The described glutamate pathway (transport and metabolic turnover) results in modified excitatory neuronal activity [8].

The theta burst stimulation (TBS) is a form of repetitive transcranial magnetic stimulation. Depending on the pattern of short bursts of highly frequent low-intensity pulses emission/release, we considered two protocols, intermittent TBS (iTBS) and continually (cTBS). Pointed to the central area of the head (bregma), TBS promotes long-lasting modulation of neuronal activity in rats/humans. Intermittent TBS induces cortical facilitation, while cTBS causes depression of cortical activity. Both TBS protocols sustain neuronal activity, enabling oligodendrocyte progenitor cell differentiation into oligodendrocytes, and remyelination, eventually [4, 9, 10].

Glial fibrillary acidic protein (GFAP) is an intermediate filament protein in astrocytes, which coordinates CNS cells' responses to a variety of stressors, such as CNS diseases, trauma, or ischemia [11]. Thus, we tested GFAP as a sensitive marker of astrocyte responses to the applied treatments by direct immunohistochemical staining antibody-based method [12].

We used the EAE model to test our hypothesis that TBS reduces astrogliosis and sustains glutamate reuptake, which was recognized as crucial to slow MS progress. Accordingly, we study the expression of glutamate transporters GLT-1 and GLAST of astroglial or other origins, in EAE rats exposed to TBS. We used multicolor confocal immunofluorescence microscopy to access the expression of GLT-1, GLAST, and GFAP in the spinal cord of rats [13].

# 2. Methods

# 2.1. Animals and experimental procedure

The Ethical Community of the Military Medical Academy (Belgrade, Serbia) approved the animal experiment, within which a part relates to the protocol used in this paper (license no. 323-07-00622/2017-05) following the principles of the governmental policy of Official Gazette Republic of Serbia (No. 14/2009) and Directive 2010/63/EU [14].

The pre-clinical animal study design included female Dark Agouti rats (n = 27) weighed 150–200 g and were 10–14 weeks old were used in this study [15]. The animals were randomly housing in polyethylene

cages (three rats per pen, which corresponds to experimental or control groups). They habituated to the husbandry conditions (temperature of 23 ± 2 ºC, relative humidity of 55 ± 3%, a light/ dark set of 13/11 h). The animals had free access to chow and water and were accommodated to the laboratory environment and staff five days before the experiment. A wire lid to hold food and water were bottoms placed in cages were EAE rats were allocated, for the animal welfare (more convenient access to food and water).

To induce EAE in rats, they were firstly anesthetized intraperitoneally with pentobarbital 45 mg/kg, (Trittay, Germany) and then immunized subcutaneously with 0.1 mL mixture of Dark Agouti rats` spinal cord tissue homogenate and Complete Freund's Adjuvant (1:2, v/v), containing 1 mg/mL Mycobacterium tuberculosis (CFA, Sigma, St. Louis, MO, USA), into the right hind footpad [16].

Two arms, control and experimental, of Dark Agouti rats were included in this study [17], [18]. Control arm included four groups ( n = 3/group) referring to the Control group - not treated rats, CFA group – subcutaneously treated with Complete Freund's adjuvant (the solvent for Mycobacterium tuberculosis, used to induce EAE) and iTBS and cTBS groups – rats subjected to iTBS and cTBS. The five experimental groups (n = 3/group) were EAE group – with induced EAE (with Mycobacterium tuberculosis suspended in Complete Freund's adjuvant), EAE + iTBS and EAE + cTBS groups – EAE rats exposed to iTBS or cTBS, respectively, and EAE + iTBSsh and EAE + cTBSsh groups – sham stimulated EAE rats exposed to the sound artifacts of iTBS and cTBS. Exposure of rats to iTBS or cTBS or their sound artifacts started from 14th dpi for the next ten days, until the 24th dpi. Rats were anesthetized deeply intraperitoneally with sodium pentobarbital and perfused intracardially with 20 mL of the cold saline solution immediately before sacrificing by decapitation.

Blood removal from minimizes interferences for immunostaining of microscopic preparations [19].

The TBS protocols were applied with a MagStim Rapid2 with a 25 mm shape-of-eight coil (MagStim Company, Whitland, Dyfed, UK), consistent with Huang et al. (2007). The pattern of iTBS was composed of 20 trains of 10 bursts, with three pulses at a frequency of 50 Hz, repeated at 5 Hz (duration 192 s with 10 s pause) [10]. The cTBS pattern encompassed a serial of bursts (single 40 s repeated at 5 Hz with the block of 600 pulses). The intensity of the TBS stimulus was 30% of maximal stimulator output (merely under a motor threshold, anticipated as an observed upper limb contraction). The TBS was manually applied to rats, and animals were not posed to any additional stress. The coil was positioned over bregma in direct physical contact with the animal head [9].

The assessed experimental outcomes referred to iTBS and cTBS impact on the astroglial glutamate transporters capacity in EAE rats model.

Decapitation was performed 24 hours after the last TBS treatment. Prior decapitation, all animals were intraperitoneally anesthetized with sodium pentobarbital [19].

No adverse events occurred in any of the experimental groups.

# 2.2. Immunofluorescence

The crowding up of spinal cords was performed by pumping with a high volume cold saline solution. The lumbar part of the spinal cord (the ventral horn region) was rapidly cut up and fixed in 4% paraformaldehyde (0.1 M PBS, pH 7.4, 12 h at 4 °C), transferred into the sucrose medium (10–30% in 0.1 M PBS, pH 7.4), then frozen in 2-methyl butane and kept at -80 °C, until slicing on a cryotome (Leica CM 1850, Germany) [20]. The serial od 25 µm thick sections were placed onto glass microscope plates, dried for two hours at room temperature, and stored at -20 °C until immunochemistry procedures. Ahead of immunostaining the microscopy preparations were rinsed in PBS, wrapped up in 0.25% Triton X-100 in PBS for 15 min, and three-time washed with PBS. Unspecific binding was suppressed using 5% BSA in PBS (Sigma-Aldrich, Germany) for one hour at room temperature. Herein, we applied the technique of double immunostaining [21, 22].

To ensure the specificity of the staining, we performed three "negative controls." Briefly, we applied BSA to the microscopic preparations, and no fluorescence was developed after the addition of the secondary antibodies, suggesting the absence of non-specific bindings.

Thus, after the application of primary monoclonal mouse anti-GFAP antibody (1:500; Abcam, Germany) and overnight incubation at 4 °C, we applied the secondary donkey anti-mouse, Alexa Fluor 555 (1:250; Invitrogen, Carlsbad, USA) for the next two hours of incubation in the dark, at room temperature. Then we performed the second round of immunostaining incubated by applying the primary rabbit polyclonal anti-GLAST (1:200; Abcam, Germany) and rabbit polyclonal anti-GLT-1 (1:1000; Abcam, Germany). After overnight incubation, at 4 °C, we added secondary goat anti-rabbit Alexa Fluor 488 (1:500; Invitrogen, Carlsbad, USA), and the slides were incubated for two hours in the dark, at room temperature. Finally, the slides were treated with the Mowiol medium (Sigma Aldrich) and prepared for confocal microscopy (Zeiss Axiovert 200M, LSM 510 laser module).

Three sections of the ventral horn region per rat were considered for the fluorescence measurement by using ImageJ software, Fijy version. Five photomicrographs (section area of interest), randomly chosen from each section, were captured for the magnification (40x objective) and analyzed by two channels (green for the transporters, GLAST and GLT-1) and red (for the GFAP) and analyzed by Coloc2 program processor of the ImageJ software [23].

# 2.3. Statistical analysis

By using Pearson's correlation coefficient was estimated the extent of overlapped fluorescence from the glutamate transporters and GFAP based on the shapes, not the intensity of fluorescence (by the Coloc2 program processor of the ImageJ software, Fiji version).

Mander's colocalization coefficients (the average value for five photomicrographs measurements per section, presented by grey-scale range) was used to estimate the contribution of the individual intensity of green or red fluorescences to the overlapped fluorescence.

The values of Pearson's and Mander's analyses are graphically presented as means ± SD. The $p < 0.05$ was considered as significant. One-way ANOVA and Tukey's post hoc multiple tests were used (software

GraphPad Prism, version 5.03) for statistical data analysis.

# 3. Results

By comparing immunofluorescence images of GLAST, GLT-1, and GFAP from the spinal cord of rats (Figs. 1, 3 - GFAP-positive cells in the spinal cord from rats with EAE were uniformly spaced apart in accomplished astrogliosis) we quantified the signal intensities (Figs. 2ab, 4ab). Pearson's correlation coefficients between the targeted glutamate transporters and GFAP were presented graphically (Figs. 2c, 4c) and by the images (the 3rd column in Figs. 1 and 3). The contribution extent of the single fluorescence spectra of the examined transporters and GFAP to the overall fluorescence was presented by the Manders` colocalization coefficients (M1 refers to glutamate transporters and M2 to GFAP) (Figs. 2d, 4d). The ratios of GLAST to GLT-1 for the examined groups of rats were presented in Fig. 5. Green fluorescence immunoreactivity refers to GLAST and GLT-1, while red to GFAP.

GFAP positive cells in the spinal cord from rats with EAE were uniformly spaced apart in accomplished astrogliosis (Figs. 1, 3)

The increase of GLAST ($p < 0.05$) and GLT-1 ($p < 0.01$) and GFAP ($p < 0.05$), when measured with GLAST, and $p < 0.01$, when measured with GLT-1) was achieved in the EAE group, compared to the controls (Fig. 1, 2ab, 3, 4ab).

Intermittent TBS increased astroglial and neuronal GLAST expression significantly (from $*p < 0.05$ to $**p < 0.01$; GFAP remained elevated), and reduced astroglial GLT-1 expression significantly (from $**p < 0.01$ to the control values) and GFAP (from $**p < 0.01$ to the control values), in exposed EAE rats. Continual TBS did not affect GLAST ($p < 0.05$) or GLT-1 ($p < 0.05$), but reduced GFAP (from $**p < 0.01$ to the control values) (Figs. 2 cd, 4 cd).

The ratio GLAST/GLT-1 was the highest in the EAE group compared to the controls (Fig. 5).

# 4. Discussion

Astrogliosis restricts neuronal tissue damage and is a crucial defending mechanism against different insults to CNS [1]. According to the literature, TBS-induced neuronal activity could be associated with OPCs proliferation, differentiation, and remyelination of axons. We were tempted to examine if and to what extent glutamate transporters in the area of the spinal cord of EAE rats respond to iTBS and cTBS.

The applied experimental method and performed negative controls ensured us about the specificity of the staining and minimizing the risk, though we cannot rule out that some staining may be due to non-specific binding to blood vessels or other cells that may express these proteins.

Stressors such as neurodegenerative diseases, brain medical or physical treatments, etc. may favor the expression of a specific type of cells or molecules/transporters placed on/within neuronal or glial

terminals cells [24, 25]. We ascertained that EAE promotes astrogliosis, based on the increased fluorescence of GFAP ($p < 0,05$, $p < 0,01$, by two ways of measurements, in average for ~ 28%), GLT-1 ($p < 0,01$) for 54% (mostly present in astrocytes, but also other glial and neuronal cells), and GLAST ($p < 0,05$) for 67% (to a radically lesser extent it is present in astrocytes and neurons), compared to the controls. Because glutamate transporters are mostly located in astrocyte terminals within the CNS, an increased number of astrocytes (documented in astrogliosis) may be found as a predominant reason for enhanced expression of glutamate transporters in EAE rats.

We showed that GLAST fluorescence originates from astrocytes (GLAST and GFAP coefficients convergent to one point), unlikely to GLT-1 (divergent of GLT-1 and GFAP coefficients), which fluorescence originates from other glial or neuronal cells, in addition to astrocytes (Figs. 2d, 4d) [13]. The ratio GLAST/GLT-1 was the highest in the EAE group in comparison to different experimental groups (Fig. 5). From that standpoint, it seems that glutamate transporters responded differently to immunization for EAE [26]. Our results are in line with the study of Sulkowski et al. (2014) reporting that elevated GLT-1 and GLAST messenger RNAs in the forebrain of rats that are associated with EAE [13]. Astrogliosis is a feedback response to toxic levels of glutamate in the CNS during inflammation and neurodegeneration. [27]. It protects the functional integrity of neurons (through increased glutamate uptake), supports oligodendrocytes myelin production within lesion areas, but can lead to harmful effects under specific circumstances or be primary causes of CNS disorders, such as astrocytopathy [28].

Intermittent TBS and cTBS protocols are prototypes for long-term potentiation or depression, respectively [29]. Nevertheless, neuronal activity is excitatory or inhibitory; it supports neurogenesis through different pathways, such as glutamate uptake and metabolic turnover, oligodendrogenesis, and corticospinal tract remyelination. Besides, TBS associated long-term potentiation or depression widespread through different parts of the brain [9]. Huang YZ et al. (2007) showed that human TBS treatment resulted in synaptic plasticity and variations in the activity of cortical inhibitory and excitatory pathways [10, 30].

The causes of the lower ratio GLAST/GLT-1 in iTBS or cTBS treated EAE rats comparing to EAE rats may refer to reduced or suppressed astrogliosis, exclusion or inclusion of the non-astroglial glutamate transporters responds, or glutamate transporters intrinsic susceptibility to specific stressors.

The changes in expression of glutamate transporters and GFAP in health rats exposed to cTBS or iTBS are in the range of control values (Figs. 2 and 4). Continual TBS withdrawn astrogliosis in EAE rats (reduced GFAP to the control values), while astroglial GLAST (unchanged, $p < 0.01$) and GLT-1 (dropped from $p < 0.01$ to $p < 0.05$) remained elevated. Excessive glutamate transporters` activity implies sufficient glutamate removal from synapses, i.e., prevention of excitation and its metabolic turnover into glutamine, within astrocytes [31].

Intermittent TBS decreased astrogliosis, and GLT-1 originated from other neuronal or glial cells, in addition to astrocytes, in EAE rats (according to Manders' colocalization coefficients). As GLT-1 is the most abundant glutamate transporters (mainly localized in astrocytes), it has been considered essential for glutamate removal from synapses. The glutamate transporter GLAST that originates from other glial

or neuronal cells, in addition to astrocytes, is barely present in CNS and spinal cord. Thus, iTBS may result in inadequate glutamate removal from synapses [25].

Our results confirmed that GFAP responded to the applied stressors, such as immunization for EAE, and TBSs [32]. We showed that glutamate transporters react differently to stressors depending on their origin. Thus, astroglial GLAST and not exclusively astroglial GLT-1 rose in EAE, iTBS affected glutamate transporters of non-astroglial origin predominantly, while cTBS did not affect glutamate transporters.

# 5. Conclusions

We demonstrated that astrogliosis developed in rats with induced EAE, based on the elevated expression of GFAP, and glutamate transporters. We showed that cTBS reduced astrogliosis more efficiently than iTBS in EAE rats. Concerning glutamate transporters, iTBS reduced GLT-1 and increased GLAST expression, contrary to cTBS, which did not interfere. Hence GLT-1 is the most abundant glutamate transporter, and that its activity was not affected by cTBS, we concluded that glutamate reuptake remained upraised in cTBS exposed EAE rats. Accordingly, we found that cTBS might advance the remyelination of damaged neuronal cells in EAE rats. The cTBS treatment might be of therapeutic relevance in addition to conventional pharmacologic therapy of MS. The clinical trials of new MS treatment may consider the data of this pre-clinical animal study.

# Abbreviations

CFA - Complete Freund's Adjuvant

CNS - central nervous system

cTBS –continuous theta burst stimulation

cTBSsh – sham stimulated EAE rats, exposed to the sound artifacts of cTBS

dpi –postimmunization days

EAE - experimental autoimmune encephalomyelitis

GFAP - glial fibrillary acidic protein

GLAST - glutamate-aspartate transporter

GLT-1 - glial glutamate transporter

iTBS – intermittent theta burst stimulation

iTBSsh – sham stimulated EAE rats, exposed to the sound artifacts of iTBS

MS – multiple sclerosis

TBS - theta burst stimulation

# Declarations

**Ethics approval:** The Ethical Community of the Military Medical Academy (Belgrade, Serbia): license no. 323-07-00622/2017-05, following the principles of the governmental policy of Official Gazette Republic of Serbia (No. 14/2009) and Directive 2010/63/EU.

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All authors approved the submitted manuscript.

All authors have agreed both to be personally accountable for the author's own contributions and to ensure that questions related to the accuracy or integrity of any part of the work, even ones in which the author was not personally involved, are appropriately investigated, resolved, and the resolution documented in the literature.

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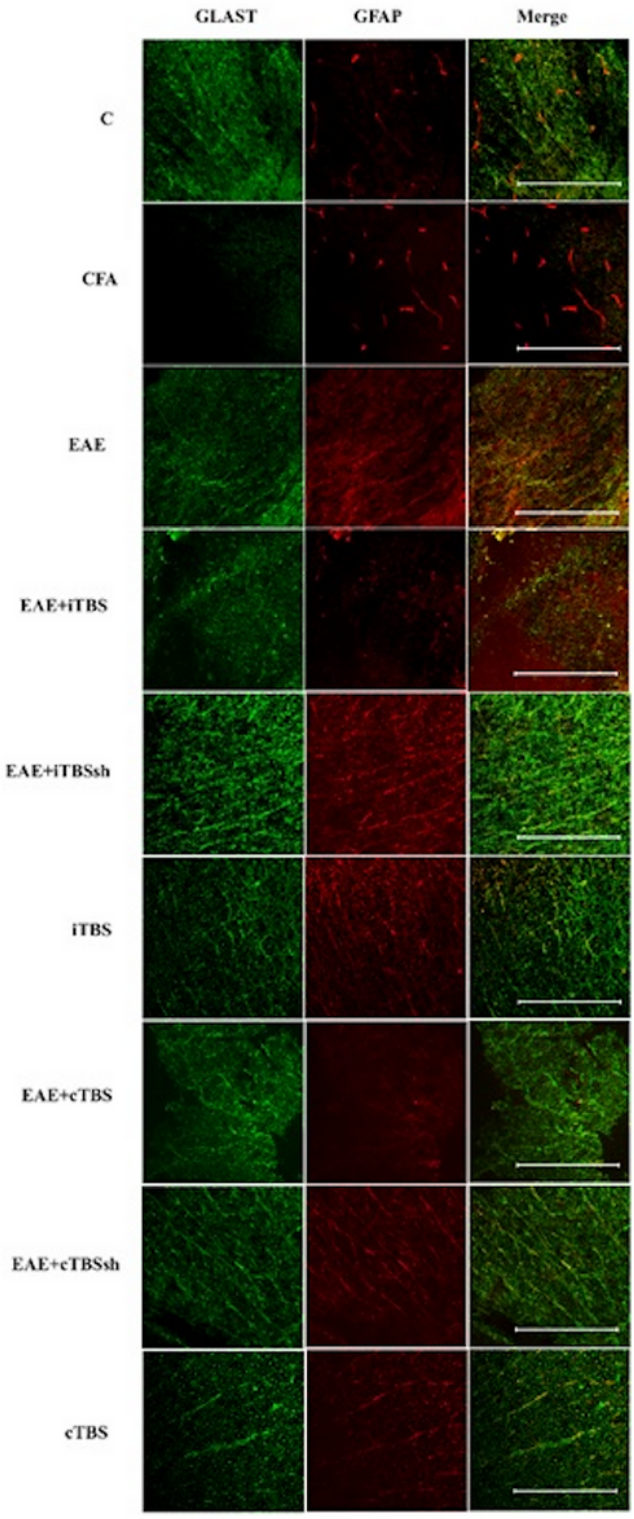
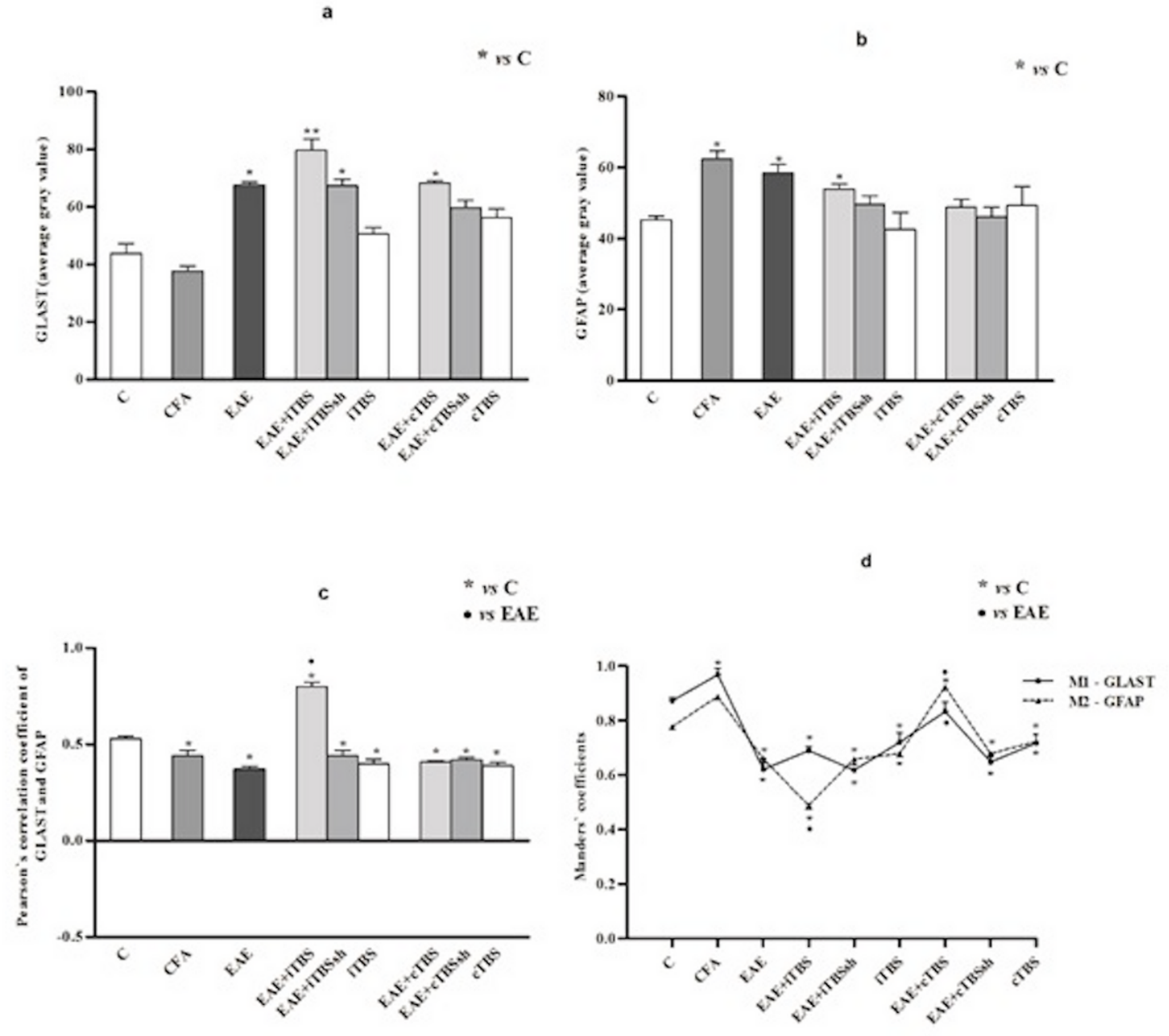
# Figures




## Figure 1

Double fluorescence staining showing immunoreactivity for GLAST and GFAP in rats' spinal cord. Light grey (green in the color photograph) represents GLAST positivity, and darker grey (red in color photograph) represents GFAP positivity. Three rats per group were used. Groups were as follows: C - control group of rats; EAE - rats with experimentally induced autoimmune encephalomyelitis, decapitated at 24 dpi; CFA - rats treated with Complete Freund`s Adjuvant; EAE+iTBS - EAE rats exposed to iTBS );

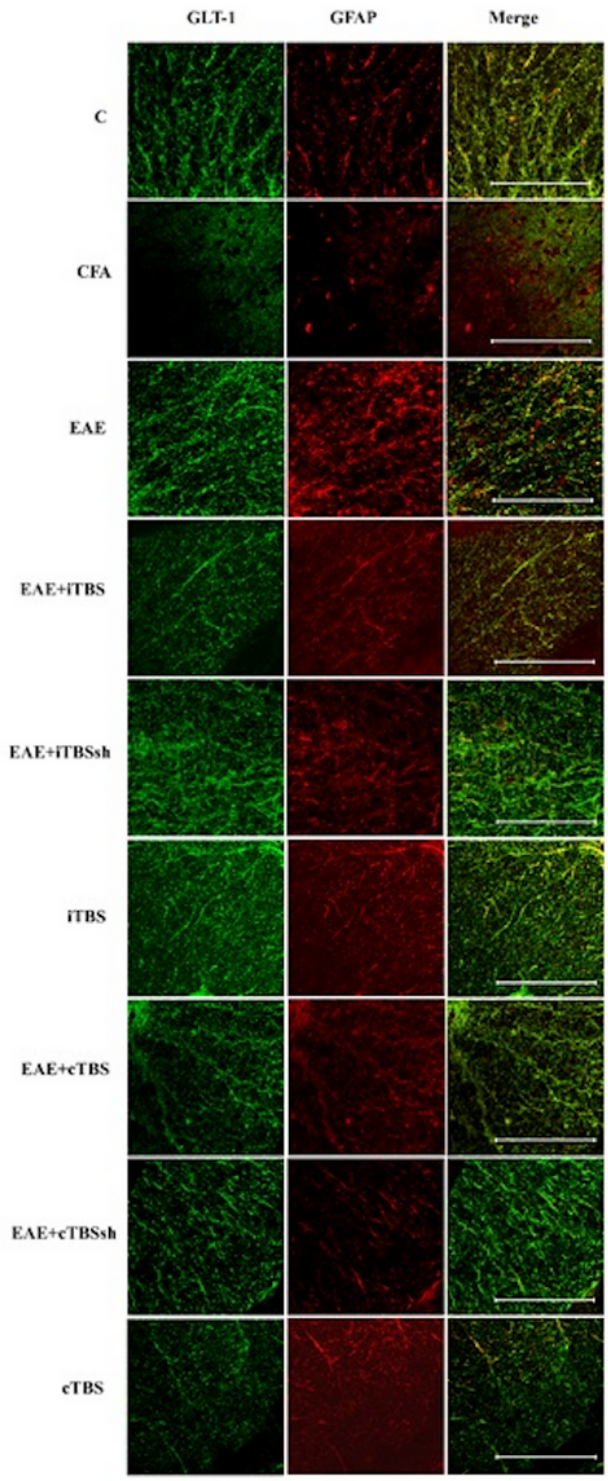
EAE+iTBSsh - EAE rats exposed to artifact noise of iTBS; iTBS - health rats exposed to iTBS; EAE+cTBS - EAE rats exposed to cTBS); EAE+cTBSsh - EAE rats exposed to artifact noise of cTBS; cTBS - health rats exposed to cTBS The photomicrographs were taken at magnification x 400. Scale bar corresponds to 200 μm.




## Figure 2

Effects of iTBS and cTBS on GLAST (a) and GFAP (b) expression in the spinal cord. Results were expressed as average grey value ± SD (n=3). Pearson's correlation coefficients (c) and Manders' colocalization coefficients, M1 (GLAST) and M2 (GFAP) (d): Values are presented as means ± SD.

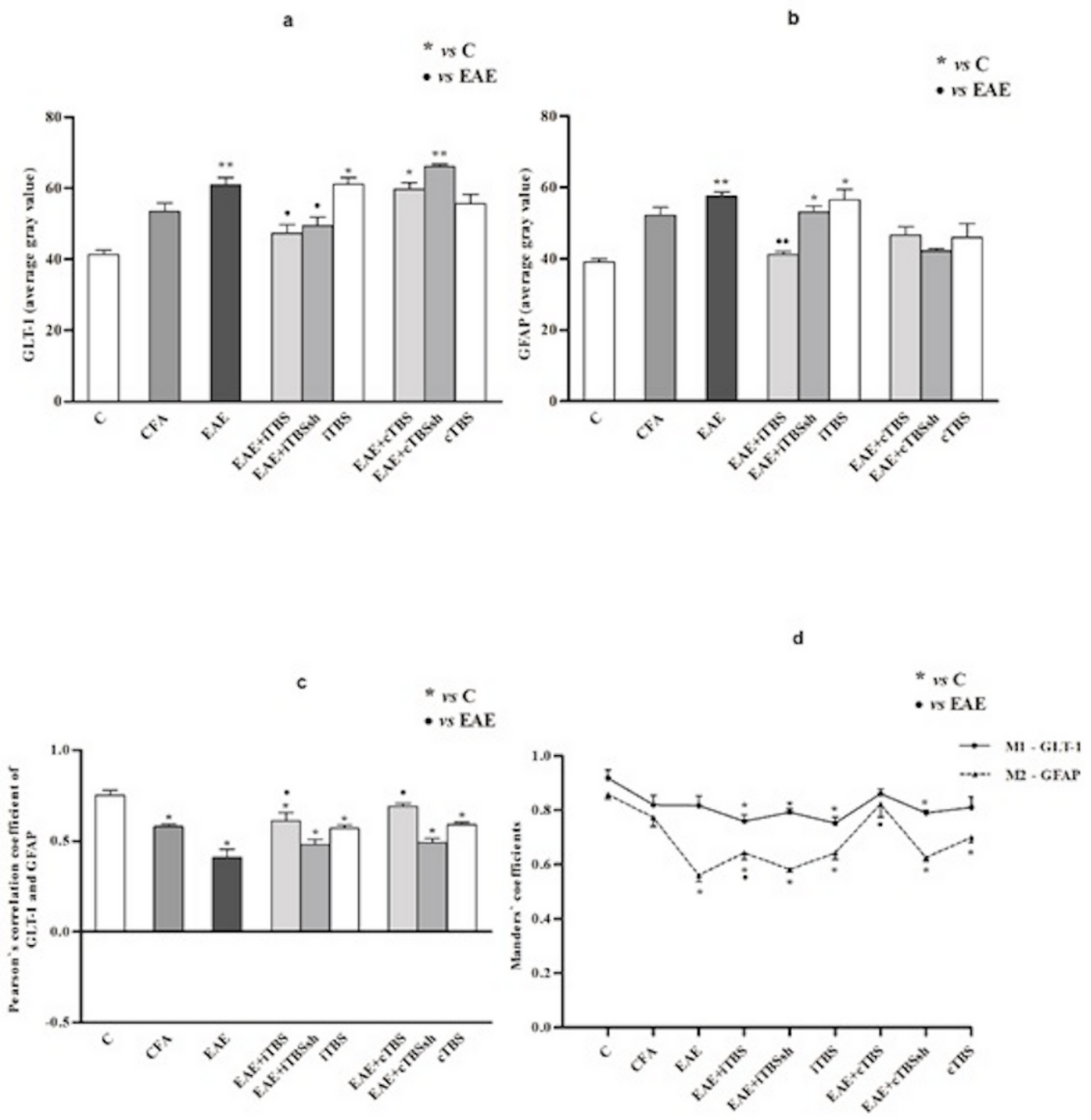
Labeling of differences considered statistically significant: *p<0.05, **p<0.01 - compared to control, and ●p<0.05, ●●p<0.01 - compared to EAE.




## Figure 3

Double fluorescence staining showing immunoreactivity for GLT-1 and GFAP in rats' spinal cord. Light grey (green in the color photograph) represents GLAST positivity, and darker grey (red in color

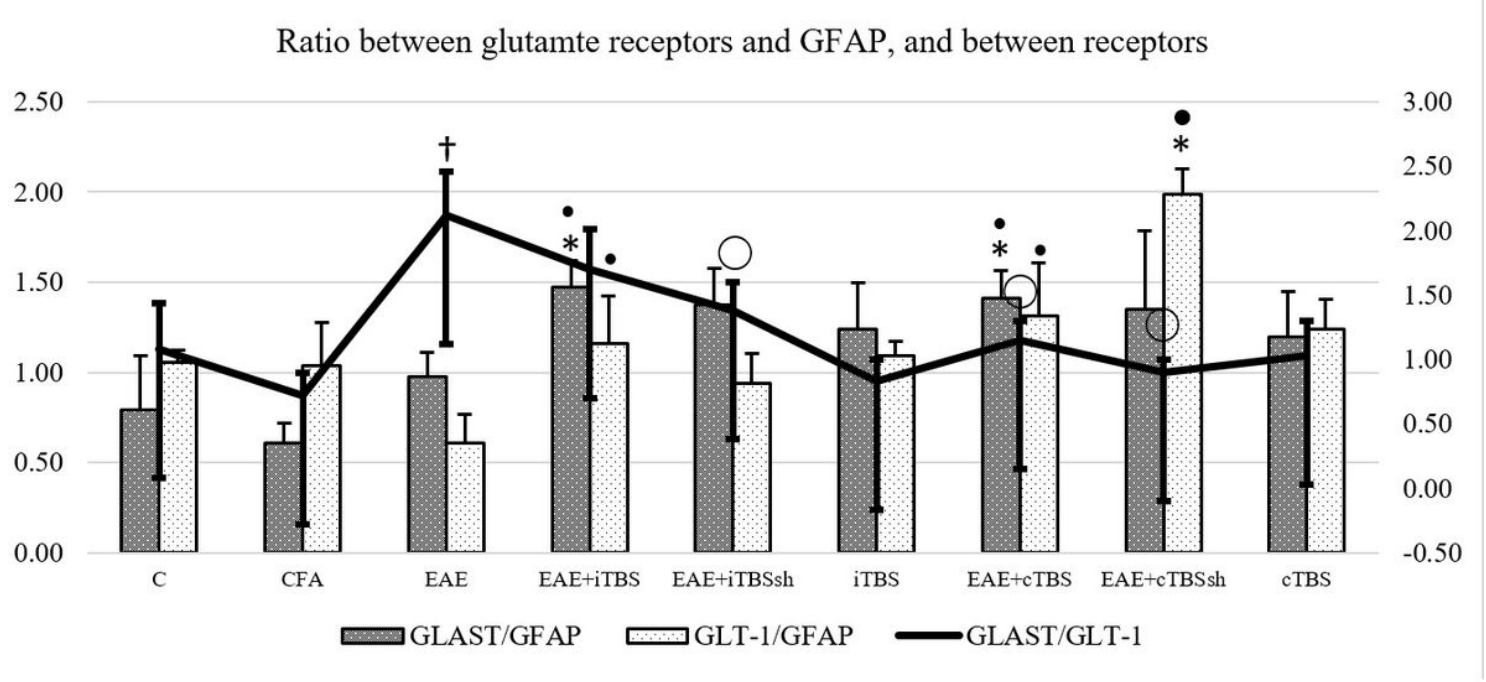
photograph) represents GFAP positivity. Three rats per group were used, and the groups are as described in Figure 1. The photomicrographs were taken at magnification x 400. Scale bar corresponds to 200 µm.




## Figure 4

Effects of iTBS and cTBS on GLT-1 (a) and GFAP (b) expression in spinal cord. Results were expressed as average grey value ± SD (n=3). Pearson's (c) and Manders' coefficients (d): M1 (GLT-1) and M2 (GFAP).

Values are presented as means ± SD. Differences, considered statistically significant: *p<0.05, **p<0.01 - compared to control and ●p<0.05, ●●p<0.01 - compared to EAE.




## Figure 5

The ratio between glutamate transporters and GFAP and between transporters in the spinal cord. Results were expressed as an average grey value ± SD (n=3). Values are presented as means ± SD. Bars represent ratios between the transporters and GFAP, and significant differences were labeled as *p<0.05 - compared to control and ●p <0.05 - compared to EAE. The thick line represents the ratio between the transporters (GLAST/GLT-1), and significance was labeled as †p<0.05 - compared to control and ⓘp <0.05 - compared to EAE.