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Reactivity of astrocytes in the periaqueductal gray matter of rats treated with monosodium glutamate

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Abstract

Introduction. The astrocytic S100β calcium-binding protein performs numerous intra- and extracellular functions, promoting the survival of central nervous system (CNS) structures. Its increased synthesis and release are a manifestation of reactive glial behavior, crucial for the maintenance of proper neuronal function, particularly under the pathological conditions. The periaqueductal gray matter (PAG) is a mindbrain area composed of four parts dorsomedial (dm), dorsolateral (dl), lateral (l) and ventrolateral (vl)) which are involved in pain sensing and defensive reactions of the body. The aim of this study was to evaluate the S100β protein immunoreactive (S100β-IR) astrocytes in adult rats after administration of monosodium glutamate (MSG).

Material and methods. The animals were administered the saline solution (group C), 2 g/kg b.w. MSG (group I) and 4 g/kg b.w. MSG (group II). The study was carried out on the brain sections stained by immunohistochemical peroxidase-antiperoxidase method with a primary mouse antibody against the S100β protein.

Results. The analyses showed the presence of the S100β-immunoreactive cells in dm, dl, l, vl PAG of all animals. In the C and I group animals, the PAG astrocytes were characterized mainly by the presence of the studied protein in the nucleus and cytoplasm of the cell body. In the group II rats in all parts of PAG, the S100β-IR cells with numerous, thicker and branched processes were observed. A decrease in the number of the S100β-IR cells was found in dm, dl and l PAG in the MSG-treated animals, particularly with the larger dose. The number of cells with the S100β expression was comparable in vl PAG in all rats.

Conclusions. MSG administered parenterally to the higher dose to adult rats affects the immunoreactivity of S100β protein in PAG. Phenotypic changes of the studied cells may indicate reactivity of glial cells and increased expression of the studied protein whereas a decrease in their number may result from the increased protein secretion into the extracellular space or cytotoxic death of glial cells. *(Folia Histochemica et Cytobiologica 2020, Vol. 58, No. 2, 147–155)*

Key words: rat; monosodium glutamate; periaqueductal gray matter; S100β; astrocytes

Introduction

Astrocytes are glial cells responsible for maintenance and control of neural homeostasis in the central nervous system (CNS). These cells provide a structural-metabolic support for neurons, protecting them against various types of damage. They also play an active role in the synaptic transmission, including the neurotransmitter metabolism, and are a source of many active gliotrophic and neurotrophic substances [1–3]. Astrocytes play a key role in the pathogenesis of numerous neurological and CNS disorders. Moreover, their dysfunction may affect neuronal survival [2, 4]. The activity of glial cells changes dynamically depending on the functional state of the neuronal microenvironment. In order to protect neurons, astrocytes become activated in many types of CNS disorders and damage. The reactivity of astrocytes to various CNS insults is recognized to be a pathological feature of structural changes in the nervous system. In the course of the so-called reactive astrogliosis, numerous changes at the molecular, cellular and functional levels are observed including hypertrophy of cell body and cell processes, increased expression and synthesis of various proteins as well as the increased

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amplitude, duration and frequency of intracellular calcium signals [2–7].

Calcium ions take an active part in the intracellular signaling and communication. The maintenance of their homeostasis is essential for proper cell functioning. A sudden increase in cellular Ca^{2+} concentration may lead to the activation of a number of enzymatic reactions and consequently, to the induction of apoptotic cell death [8]. A rapid increase in Ca^{+2} levels in astrocytes was observed, among others, in the pilocarpine model of epilepsy [9]. During the kainate-induced seizures in the murine hippocampus a similar glial reaction was shown [10]. Kainic acid, as a cyclic analog of L-glutamate (Glu), is a strong agonist of Glu-specific ionotropic receptors [11]. Glu is the main excitatory neurotransmitter in CNS. It is present in the extracellular space at a low concentration. However, in the pathological states an increase in the extracellular Glu level is observed. This phenomenon results in the excessive stimulation of Glu-specific ionotropic receptors. Such activity can lead to excitotoxicity death caused by a rapid influx of calcium ions into the cell [12, 13]. Excitotoxicity is of significant importance for the pathogenesis of many pathological CNS conditions, *e.g.* ischemia [14], stroke [15], trauma [16], epilepsy [17], as well as neurodegenerative diseases [18–20]. In such pathological states, reactivity of astrocytes manifested by hypertrophy of their bodies and processes was observed [6, 7, 21]. Glu is also the main component of monosodium glutamate (MSG) which is used worldwide as a flavor enhancer. MSG is often used in experimental studies due to its toxicity [22–24]. In animals treated with MSG some brain lesions were observed [25–28].

There are many mechanisms that lead to a decrease in intracellular of Ca^{2+} levels. One of them is the presence of calcium-binding proteins that belong to the S100 family representing 25 relatively small proteins. Engaged in numerous intracellular and extracellular functions, they take part in the regulation of proliferation, differentiation, cell migration, apoptosis, energy metabolism and protein phosphorylation [29, 30]. Astrocytes exhibit the presence of S100β protein, which is an acid homodimer composed of two β subunits. It leads to the intracellular changes in the calcium ion levels and reduces their concentration acting as a buffer [31, 32]. Moreover, in the course of numerous acute and chronic disorders of the nervous system, presence of this protein in the extracellular space is demonstrated [33, 34].

The elevated level of S100β in the body fluids (peripheral blood, cerebrospinal fluid, urine, saliva) is observed during the traumatic brain injury [35, 36], Alzheimer's disease [37], Parkinson's disease [38], Down syndrome [39], schizophrenia [40] and mood disorders [41, 42]. Therefore, S100β is considered to be a biomarker of pathological states in the nervous system [32–34].

The periaqueductal gray matter (PAG) is a midbrain area. In rat it consists of four parts: dorsomedial (dm), dorsolateral (dl), lateral (l) and ventrolateral (vl). Through numerous descending and ascending pathways, PAG is connected with other CNS areas, participating in pain sensing and defensive reactions of the organism with characteristic autonomic symptoms in the respiratory and cardiovascular systems. Activity of this area depends on the appropriate level of neurotransmitters including Glu [43–46]. The proper concentration of this amino acid is controlled by astrocytes [2, 3]. Taking into account important functions and numerous PAG connections in CNS, it seems to be essential to estimate the astroglial activity under the conditions of the elevated Glu level in the extracellular space.

The aim of this study was to evaluate the effects of monosodium glutamate (MSG) on the morphology, distribution and density of immunoreactive S100β astrocytes in the periaqueductal gray matter of adult rats.

Material and methods

Animals and material collection. The experiments were approved by the 2nd Local Ethical Commission for Animal Experiments in Lublin (No. 7/2011). The study was performed on fifteen 60-day-old male Wistar rats. They were kept in metal cages at the temperature of 20–22°C, 60% air humidity, in a 12h:12h day/night cycle with continuous access to food and water. The rats were randomly divided into three groups of 5 animals each control (C), I and II. Rats of the C group were administered saline solution, whereas animals in groups I and II received monosodium glutamate (MSG) (Sigma-Aldrich, St. Louis, MO, USA) in the doses of 2 g/kg b.w. and 4 g/kg b.w., respectively, subcutaneously for 3 consecutive days. All animals were euthanized 24 h after the last injection of MSG or saline solution. Thereafter brains were immediately collected for examination. The material was fixed in the buffered 10% formalin and embedded in the paraffin blocks by the routine histological technique. Next, the frontal, 4 μm-thick sections were obtained and placed on the SuperFrost Plus slides.

Immunohistochemistry. The brain sections containing PAG from each animal were stained with indirect peroxidase-antiperoxidase (PAP) method using the antibodies and reagents from Sigma-Aldrich. The reaction was performed with dilutions in 0.5M TBS (Tris-buffered saline) at pH 7.6 according to the manufacturer's recommendations. In the

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first stage, all sections were dewaxed and rehydrated. Next, they were treated with 3% H_2O_2 at room temperature (RT) for 30 min and then with the goat serum (G9023, 1:10) for 20 min. Then incubation with the primary mouse antibody against the S100*β* protein (S2532, 1:2000) was performed at 4°C for 16 hours. Later the secondary goat antibody against the mouse IgG conjugated with the peroxidase–antiperoxidase complex (A9917, 1:150) was used at room temperature for 1 hour. To visualize the reaction, the DAB chromogen (RT, 30 min.) was applied. At the final stage, all sections were stained with Mayer's hematoxylin, and slip-covered using DPX (Fluka, Buchs, Switzerland). At the same time, negative control of the immunohistochemical reactions by omission of the primary antibody was performed. The specificity of the primary antibody was confirmed on the rat brain sections in our earlier study [47]. The stained sections were analyzed and microphotographs were obtained in Olympus BX40 microscope connected to the digital Olympus Color View III camera (Olympus, Tokyo, Japan).

Microscopic and morphometric analyses. The morphology and distribution of immunoreactive cells for S100*β* (S100*β*-IR) were evaluated on the brain sections of each animal. The measurements of 50 sections from each group of rats (10 sections/animal) were made based on the microscopic observations. The number of S100*β*-IR astrocytes identified by the presence of brown reaction product was measured in the individual parts of PAG (dm, dl, l and vl) of each animal from all groups (C, I, II). The measurements were conducted in 100 squares (2.0×10^{-2} mm^2) using the grid. The analyses were made in 20 randomly selected fields per animal (2 fields/section) using the Cell^D program. The obtained results from the dm, dl, l and vl PAG of rats of each group were presented as the average number of S100*β*-IR cells (N) in the area of 2.0×10^{-2} mm^2.

Statistical analysis. The data were subjected to the statistical analyses using the Statistica software (version 12.5, StatSoft, Krakow, Poland). The normal distribution of data was verified using the Shapiro-Wilk test. The Friedman test was applied to compare the results between the individual parts of PAG within one group. The data obtained from the groups were compared using the Kruskal-Wallis test. The post-hoc Dunn test was applied in both cases. A value of $p < 0.05$ was considered as statistically significant.

Results

The microscopic analyses showed that in all animals in the studied groups S100*β*-IR cells were present in PAG. In all parts of the studied area of the control animals and in dorsomedial, dorsolateral and lateral part of PAG of the rats treated with the lower dose of MSG a brown reaction product was observed in the nucleus and cytoplasm of astrocytes. However, in ventrolateral part of PAG of animals from group I astrocytes containing brown reactivity product in the initial parts of processes were more frequent. These immunoreactive processes were usually less intensely stained, thin and did not branched. In all parts of PAG of rats treated with the higher dose of MSG, the S100*β*-IR cells with the brown nucleus, cell body cytoplasm and numerous thick and branched processes were present. The remaining S100*β*-IR cells showed a similar location of studied protein to that in the control (Figs. 1, 2).

The morphometric analyses proved the largest density of S100*β*-IR cells in dorsomedial part of PAG in the control group of animals. In rats which received MSG at both doses, astrocytes with the brown product of reaction were the most numerous in ventrolateral part of PAG. The average number of cells with the expression of the S100*β* protein was comparable in this part of PAG in both control and MSG-treated rats (Fig. 3). In dorsomedial part of PAG of groups I and II, a statistically significant decrease in the S100*β*-IR density of astrocytes was observed in comparison to the control group. In dorsolateral and lateral part of PAG, the mean number of S100*β*-IR cells in groups C and I animals was comparable and statistically significantly higher than in the group II (Fig. 4).

Discussion

Our studies showed that PAGs of all animals had nuclear-cytoplasmic immunoreactive cells for S100*β* protein. Additionally, in the MSG administered animals, a phenotypically different S100*β*-IR type of astrocyte was observed. The cells presented S100*β* immunoreactivity in nucleus, cell body cytoplasm and cellular processes. Such morphology may indicate the increased protein synthesis being a sign of reactive astrogliosis. This phenomenon is most likely to occur in response to the intracellular calcium ion levels increase due to the enhanced activation of glutamate specific ionotropic receptors [48]. In the cell S100*β* protein controls calcium-dependent processes and regulates enzymatic activity. Its interactions with other molecules influence, among others, the growth, differentiation and proliferation of astrocytes. This protein modulates cytoskeleton remodeling, which allows for dynamic changes in cell morphology [32]. In group II rats, the phenotypically modified cells occurred in all parts of PAG while in group I they were present only in vl PAG. This may be related to the increased loss of calretinine (CR) immunoreactive neurons being affected by MSG in this part of PAG as has been shown by us recently [49]. The CR-positive cells are

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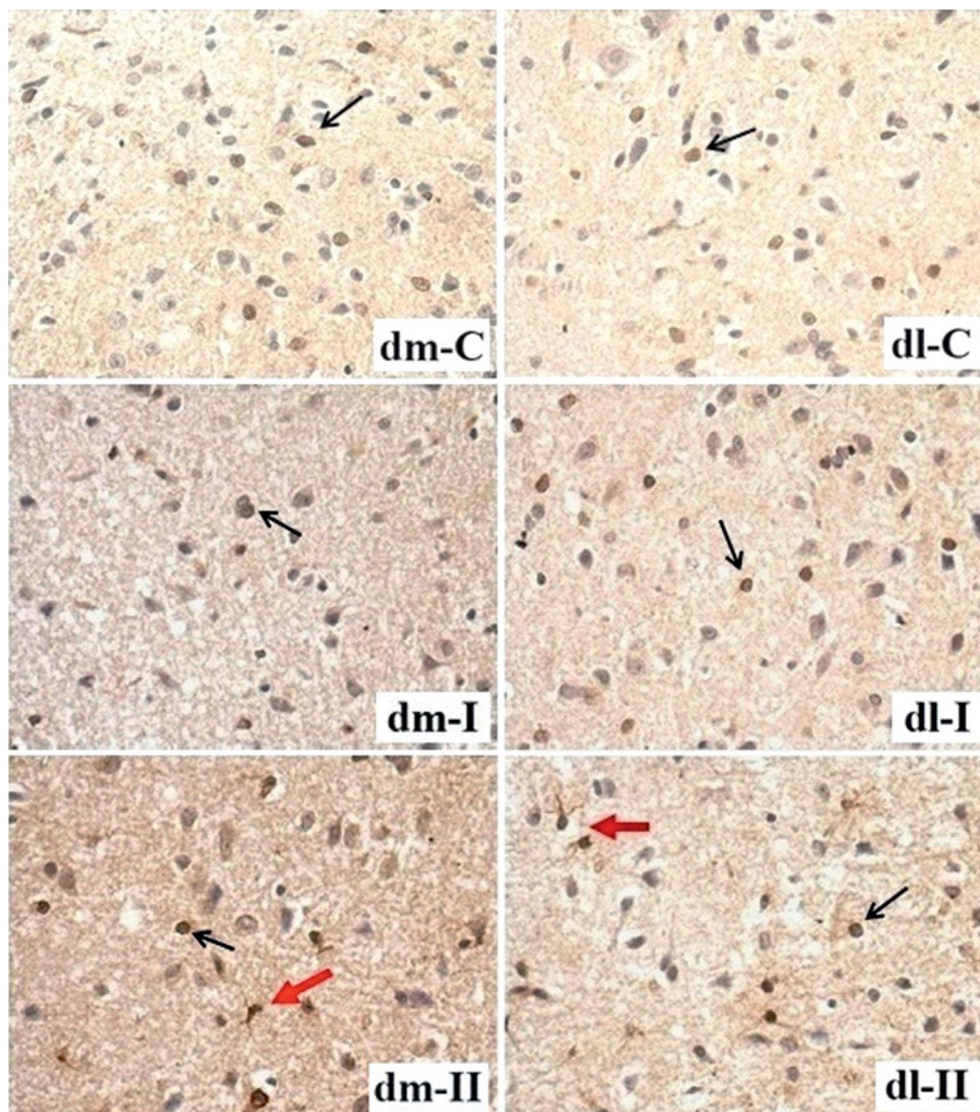

Figure 1. S100β-immunoreactive cells in the dorsomedial (dm) and dorsolateral (dl) parts of periaqueductal gray matter (PAG) in rats from the control group (C) and rats treated *sc* with monosodium glutamate (MSG) in the dose of 2 g/kg b.w. (group I) and 4 g/kg b.w. (group II). Black arrows show non-reactive astrocytes. Red arrows show reactive astrocytes. S100β-immunoreactivity was detected as described in Material and methods. Objective magnification: 40×.

probably a subpopulation of inhibitory GABA-ergic interneurons. Their reduced number may lead to the increased activity of glutamatergic neurons due to a lack of GABA-dependent inhibition. This process increases further the glutamate (Glu) amount in the extracellular space which enhances the glial response [50].

Numerous studies have proved harmful influence of MSG on the nervous system [25–28, 51, 52]. The intraperitoneal administration of MSG at the dose of 3.5 mg/g b.w. caused a decrease in number of Purkinje cells in rat cerebellum [27]. Neurotoxic effect of MSG leads to degenerative changes and death of Purkinje cells in the cerebellar cortex of the rats which were administered 3 g/kg b.w. of MSG orally for 14 days [26]. Astrocytes are less sensitive to the Glu-induced damage than neurons [53]. However, some authors suggest that the excess of this neurotransmitter in the extracellular space may also lead to the death of these glial cells [1, 26]. This process has been observed in both *in vitro* and *in vivo* studies. The cortical cultured astrocytes treated with glutamate for 24 hours (50–100 mM) showed changes in nucleus morphology, DNA fragmentation and activation of the caspase pathway, indicating apoptosis induction [1]. Death of astroglial cells, probably due to the oxidative stress, was also found in the cerebellar cortex of the rats which were administered 3 g/kg b.w. of MAG orally for 14 days

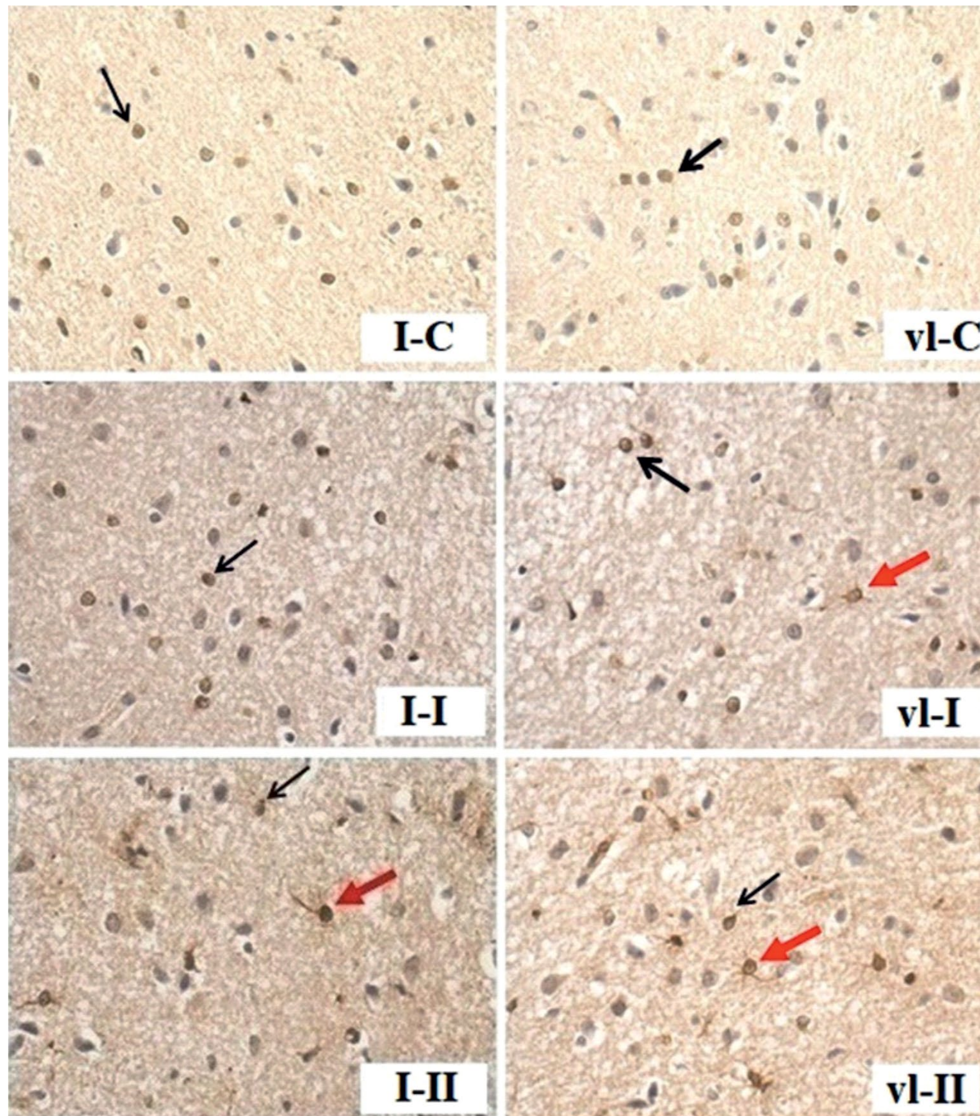

Figure 2. S100β-immunoreactive cells in the lateral (l) and ventrolateral (vl) parts of PAG in the control (C) and monosodium glutamate-treated (I, II) rats. Black arrows show non-reactive astrocytes. Red arrows show reactive astrocytes. S100β-immunoreactivity was detected as described in Material and methods. Objective magnification: 40×.

[26]. Cytotoxic glial damage may be also the cause of decrease in the S100β-IR density of astrocytes in PAG of the MSG treated animals as found in our study (group I and II). High concentration of Glu in the extracellular space leads to intracellular exhaustion of glutathione and accumulation of reactive oxygen species [54]. As a consequence, mitochondrial DNA damage and cell death may occur [1, 14, 23, 26, 55]. However, a decrease in the number of S100β-IR astrocytes may also be associated with the increased protein secretion into the extracellular space. This phenomenon is, among others, a result of the activation of metabotropic Glu receptors [48, 56]. The increased secretion of S100β protein into the extracellular space occurs also after stimulation of astrocytic serotonin receptors (5HT1A). In the dorsal part of PAG, there are numerous serotonergic endings of the dorsal raphe nucleus neurons. Their activity increases in response to the increased Glu concentration [32, 50, 57, 58]. This may account for the greatest reduction in S100β-IR in dm PAG cells of the MSG administered rats observed in our study. Moreover, some authors suggest that the S100β protein present in the extracellular space can intensify apoptotic changes in neurons and glial, as it is toxic at the micromolecular concentrations [29, 30, 32]. Therefore, further studies are necessary to determine whether the decrease in the number of S100β-IR cells observed in our study

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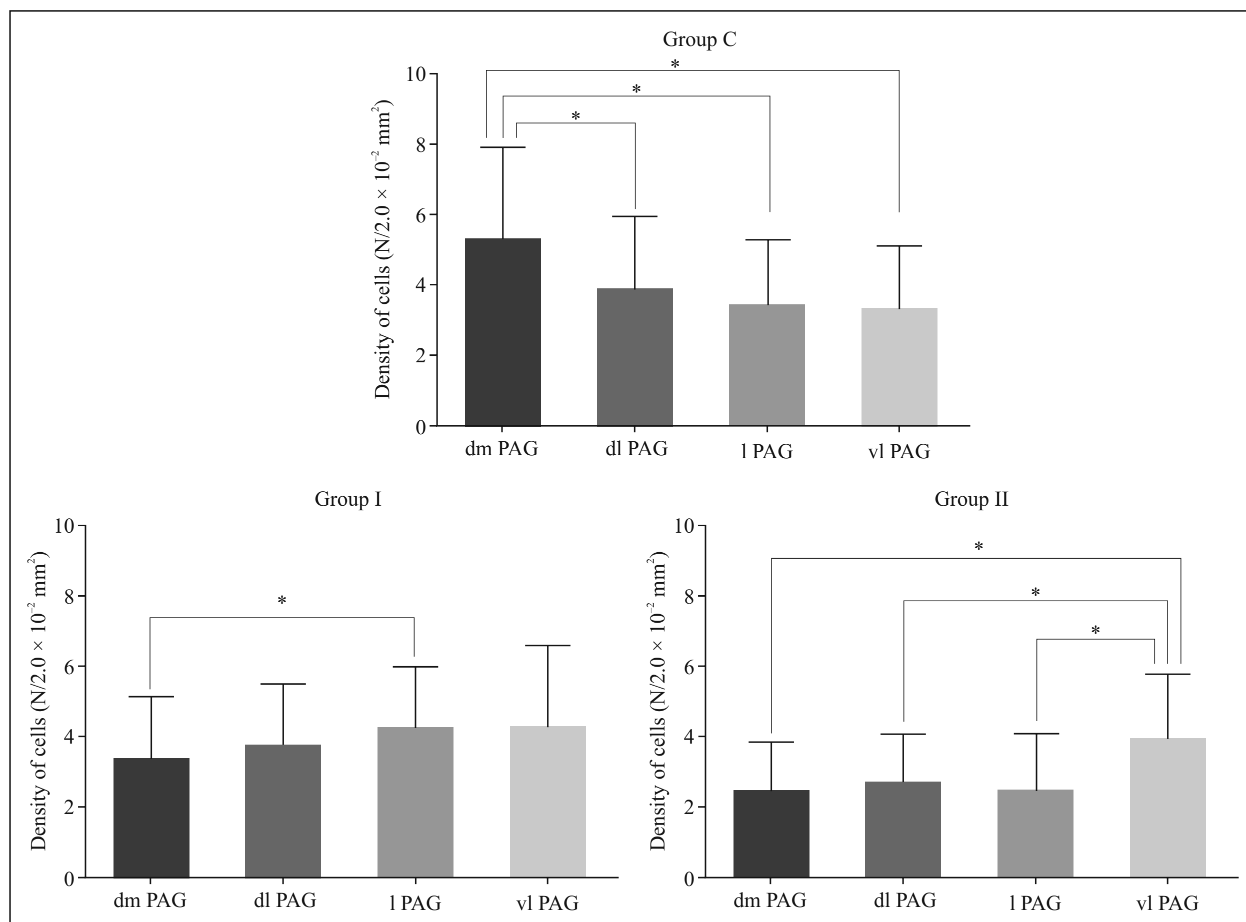

Figure 3. The density of S100β-immunoreactive (S100β-IR) cells in the dorsomedial (dm), dorsolateral (dl), lateral (l) and ventrolateral (vl) parts of PAG in the control (C) and MSG-treated rats (groups I and II). The bars show the mean density of S100β-IR cells in the area of 2.0×10^{-2} mm^2 and the whiskers represent standard deviation. *$p < 0.05$ — statistically significant differences between the parts of PAG related to the same group (Friedman test).

is the result of increased S100β protein secretion into the extracellular space and/or cell death.

In conclusion, the administration of MSG to the adult rats, particularly in large doses, affects the immunoreactivity of S100β protein in the PAG astrocytes. Phenotypic changes of these cells may indicate reactivity of glial cells and increased expression of the studied protein whereas a decrease in their number may result from the increased S100β secretion into the extracellular space and/or cytotoxic glial death.

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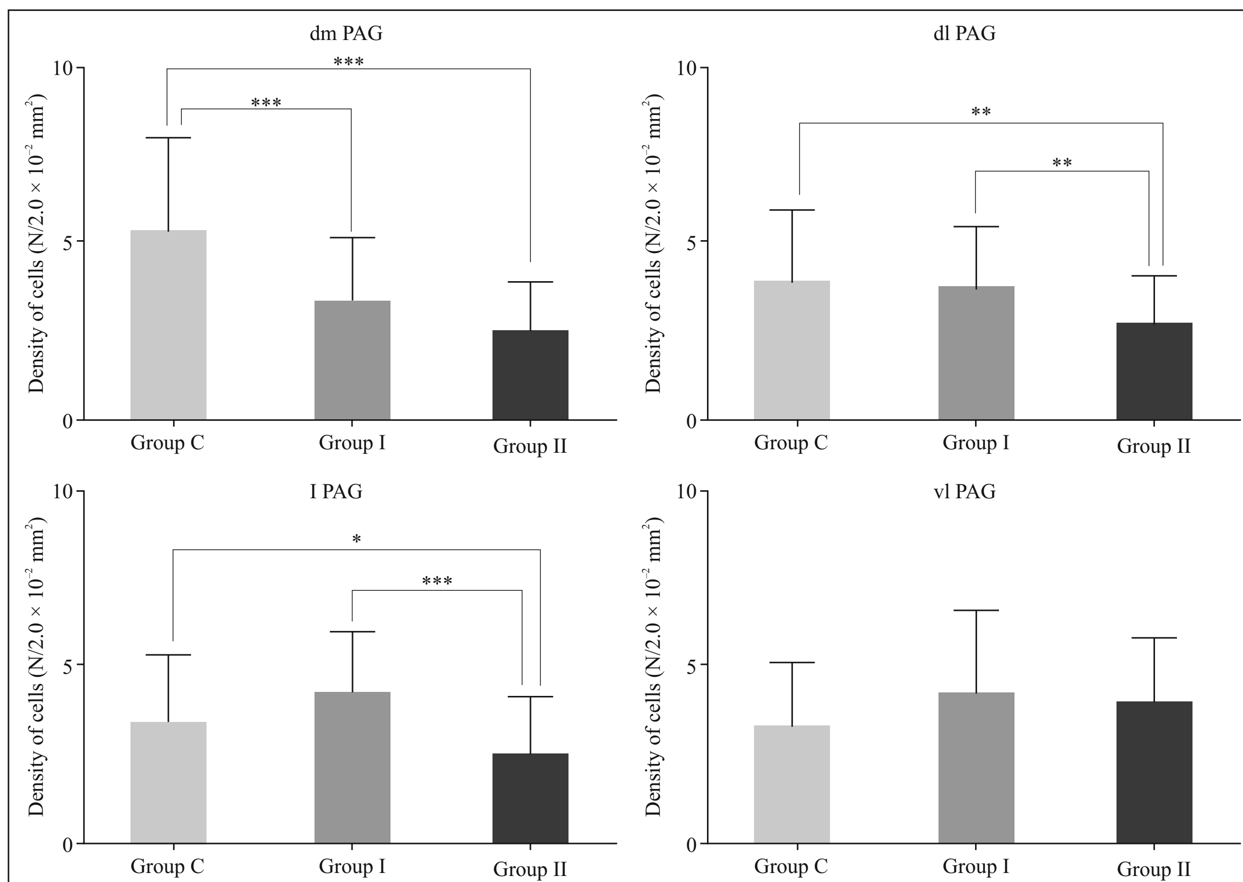

Figure 4. The density of S100β-immunoreactive cells in the dorsomedial (dm), dorsolateral (dl), lateral (l) and ventrolateral (vl) parts of PAG in control rats (group C) and MSG-treated rats (groups I and II). The bars show the mean density of S100β-IR cells in the area of 2.0 $\times 10^{-2}$ mm^2 and the whiskers represent standard deviation. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ — statistically significant differences between the control rats and the MSG-treated animals in relation to the same part of PAG (Kruskal-Wallis test).

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