

Evaluation of immunoreactive for glial fibrillary acidic protein astrocytes in the periaqueductal gray matter of rats treated with monosodium glutamate

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Summary

The aim of this study was to evaluate the glial fibrillary acidic protein immunoreactive (GFAP-IR) astrocytes in the periaqueductal gray matter (PAG) of rats treated with monosodium glutamate (MSG). This study was performed on PAG sections of rats treated subcutaneously with MSG (2 g/kg b.w. (group I) and 4 g/kg b.w. (group II)) and saline solution (group C). Immunohistochemical reactions were performed by peroxidase-antiperoxidase method using an antibody against GFAP protein. In animals receiving MSG in dorsomedial (dm) and dorsolateral (dl) parts of PAG the majority of astrocytes had GFAP-IR bodies and few thin, poorly branched processes. In lateral (l) and ventrolateral (vl) parts of PAG cells with numerous, branched processes with GFAP expression were observed. Some astrocytes in vl PAG of group II rats were characterized by strongly stained cytoplasmic bodies and thick, multiple branched processes. Statistically significant differences in the number of GFAP-IR astrocytes were shown between control animals and group II in dm PAG and dl PAG. All MSG-treated animals revealed statistically significantly fewer immunonegative structures for GFAP as well as all cells in the PAG. In the PAG of rats receiving MSG, especially at high dose, there is an increase in GFAP immunoreactivity in astrocytes. The hypertrophy of GFAP-IR cells and the probable lack of their proliferation may indicate isomorphic astroglia in response to increased extracellular glutamate concentrations. However, the decrease in their number in dm PAG and dl PAG may be related to MSG cytotoxicity.

Keywords: periaqueductal gray matter, GFAP, MSG, astrocytes

Glutamate (Glu)-induced excitotoxicity contributes largely to the damage and death of central nervous system structures. This phenomenon is described in the course of many acute as well as chronic neurological diseases. The neurotoxic effect of Glu occurs when its extracellular concentration exceeds regular values. Under physiological conditions, the appropriate level of this neurotransmitter in the perineuronal space ranges from 0.5 to 5 $\mu\text{M/L}$ (2, 21). The phenomenon of excitotoxicity is related to strong or prolonged stimulation of neuronal and glial Glu-specific receptors. Their activation results in a sudden increase in the intracellular calcium ion levels. As a result, a cascade of reactions is triggered, which may lead to damage or even death of neurons and glial cells (21, 23). The development of Glu-induced excitotoxicity also occurs in individuals treated with monosodium glutamate (MSG). This compound is a widely used

food additive. However, in addition to its palatability improvement, it also has toxic multisystemic effects on the body. Numerous studies proved that the MSG effect contributes to damage of structures in the central nervous system (CNS), retinal degeneration or hepatitis (4, 8). Moreover, this compound can result in the formation of reactive oxygen forms, activation of many enzymes and induction of apoptosis in cells found in rodents (6). The MSG is repeatedly used in animal models of excitotoxicity because its administration increases the extracellular concentration of Glu in the CNS (1, 8,9). These changes are accompanied by the phenomenon of reactive astroglia, as demonstrated in many diseases including epilepsy, stroke, traumatic brain injury, Alzheimer's disease, and multiple sclerosis (10, 14, 22).

Astroglia represents the most abundant population of glial cells in the CNS. Astrocytes are of stellate shape

and possess numerous branching processes extending radially from their bodies. These cells are organized into polygonal, three-dimensional and nonoverlapping domains. Astroglia is indispensable for the proper function of neurons and their survival rate. Astrocytes control the neuronal microenvironment, maintaining ion-metabolic homeostasis and proper concentrations of neurotransmitters, including Glu (10, 14, 28). In the course of reactive gliosis, astrocytes undergo numerous morphological and functional changes, which is most often expressed in the hypertrophy of their bodies and processes, enhanced synthesis of various proteins and molecules (5, 14, 16, 19, 22, 25, 30). One of the markers of reactive astroglia behaviour is an increase in intracellular expression of glial fibrillary acidic protein (GFAP). This compound is an essential structural component of type III intermediate filaments that form the cytoskeleton of cells. It is specific to mature astrocytes in the CNS. GFAP affects cell shape, structural stability and mechanical strength. This protein also controls astrocyte mobility and thus synaptic activity. The GFAP plays an important role in the stiffness of astrocytic processes as well as in the permeability and maintenance of the blood-brain barrier. The GFAP interacts with many other intracellular adhesion proteins and molecules, including specific transporters for glutamate. Thus, it participates in maintaining homeostasis of this neurotransmitter (5, 10, 16, 19).

The periaqueductal gray matter (PAG) surrounds the Sylvius (cerebral) aqueduct along the length of the midbrain in the form of longitudinally arranged columns. They constitute the dorsomedial (dm PAG), dorsolateral (dl PAG), lateral (l PAG), and ventrolateral (vl PAG) parts in rats. Each of them forms selective connections with the prefrontal cortex, amygdala, thalamus, hypothalamus, and spinal cord, being an important component of the nociceptive pathway. The PAG is responsible for the integration and coordination of specific patterns of autonomic, motor, and behavioral system responses to various stressors of internal (e.g., pain) and external (e.g., threat) origins. This area is also involved in mechanisms controlling vocalization, micturition, and thermoregulation. Proper functioning of the PAG depends on correct neurotransmission involving, among other things, Glu (3, 18, 26).

So far the expression of GFAP in astrocytes in individual parts of PAG under the conditions of increased Glu concentration has not been determined.

The aim of this study was to evaluate the glial fibrillary acidic protein immunoreactive (GFAP-IR) astrocytes in the PAG of rats that received parenterally MSG.

Material and methods

Adult male Wistar rats (15 animals) were used for the experiment based on the approval No. 7/2011 II of the Local Ethical Committee for Animal Experiments in Lublin. The

animals were kept in cages (12 h day/12 h night, ambient temperature 20-22°C, 60% air humidity) with permanent access to the feed and water throughout the experiment. After reaching the age of 60 days and the average body weight (BW) of 250 g the individuals were randomly divided into three groups: i.e., control (C), I and II, each including 5 animals. Then the rats were injected subcutaneously (s.c.) three times at 24 h intervals with the saline solution (group C) and MSG (Sigma-Aldrich, St. Louis, MO, USA, 49621) at doses of 2 g/kg BW (group I) and 4 g/kg BW (group II). On the fourth day of the experiment, all animals were euthanized. After slaughtering the rats with an overdose of 10% ketamine administered intramuscularly, their brains were collected immediately. The material was fixed in the buffered 10% formalin (pH = 7.0, 4°C, 12 h). Then the brains were secured in paraffin blocks from which frontal 4 µm-thick PAG sections were obtained in the next step.

Immunohistochemistry. The indirect peroxidase-antiperoxidase method and Sigma-Aldrich antibodies were used to prove the presence of GFAP in the PAG astrocytes. The reagents were diluted according to the manufacturer's recommendations in 0.5 M Tris buffered saline (pH = 7.6), which was also used to rinse the sections according to the staining procedure. In the first reaction step, all dewaxed and rehydrated PAG sections from each animal were incubated at room temperature (RT) with 3% H₂O₂ for 30 min and then with goat serum (G9023, 1 : 10) for 20 min. Subsequently, the primary rabbit anti-GFAP antibody (G 9269, 1 : 80) was applied for 16 h at 4°C. After this time the sections were rinsed and further incubated at RT for 1 h with a secondary goat anti-rabbit IgG antibody conjugated to a peroxidase-antiperoxidase complex (A9169, 1 : 400). Then the chromogen (3,3'-diaminobenzidine tetrachloride) was used. Following the above, the sections were rinsed in distilled water and subjected to a routine histological staining procedure using the Mayer's hematoxylin. A negative control for the primary antibody bypass reaction was performed simultaneously. In our earlier studies the specificity of the primary antibody was confirmed using the rat brain sections (24).

Morphological and morphometric analyses. All obtained stained slides containing dm PAG, dl PAG, l PAG, and vl PAG were observed for the GFAP-IR evaluation of astrocytes and photographed using an Olympus BX 40 light microscope coupled to an Olympus Color View III digital camera (Olympus, Tokyo, Japan).

The number of immunopositive and immunonegative for GFAP cells in 100 areas of 2.0×10^{-2} mm² was analyzed in the PAG sections of rats from groups C, I, and II using the Cell[^]D software. Analyses were performed using a grid in randomly selected 20 areas per animal. The results were presented as the mean number of immunopositive and immunonegative cells for GFAP and all cells present in dm PAG, dl PAG, l PAG, and vl PAG along with the standard deviation.

Statistical methods. Statistical analysis were performed using Statistica software (version 13.3, StatSoft, Poland). The Shapiro-Wilk test was used to assess the compliance of the distribution of the examined variables with the normal

distribution. The one-way ANOVA (with Tukey post-hoc test) or the Kruskal-Wallis test (with Dunn post-hoc test) were used to compare the amount of the cells between groups. A value of $p < 0.05$ was considered as statistically significant.

Results and discussion

Microscopic analyses showed the GFAP-IR cells in the PAG of all tested rats (groups C, I, II). In the control group in all parts of the PAG astrocytes revealed a moderate expression of the tested protein mainly in the cytoplasm surrounding round or oval nucleus and at the point of separation from the bodies of processes. Some of the GFAP-IR cells were adendritic. Moreover, astrocytes with less GFAP immunoreactive, sparse, thin, little-branched processes were also present in I PAG and vl PAG. Astrocytes with longer and divided into smaller branches processes with stronger expression of the tested protein were only in vl PAG (Fig. 1, 2). The animals receiving MSG in dm PAG and dl PAG mainly showed cells with moderate immunoreactivity for GFAP in the cytoplasm of bodies surrounding the round or oval nuclei and in sparse, thin, poorly branched processes. In addition, astrocytes with more numerous and distinctly thicker and stained processes were also present in dl PAG in the group II animals (Fig. 1).

In I PAG and vl PAG of MSG-treated males, astrocytes showing moderate GFAP expression in the cytoplasm of bodies and in numerous processes – some of which were longer with secondary branches – were frequently observed. In addition, the group II animals in vl PAG also had astrocytes with a strong expression of the tested protein in a significant amount of cytoplasm surrounding eccentrically located round or oval nuclei and at the centres of their bodies, at the point of separation of thick and repeatedly branched processes (Fig. 2).

The morphometric analyses in animals treated with MSG with 4 g/kg BW showed a statistically significant decrease in the number of cells with the GFAP expression in dm PAG and dl PAG compared to the control group. In the other parts of the PAG, there were no changes in the density of astrocytes immunopositive for the tested protein. At the same time the analyses in the I and II groups rats showed a statistically significant decrease in the number of structures immunonegative for GFAP, as well as in all cells present in the differ-

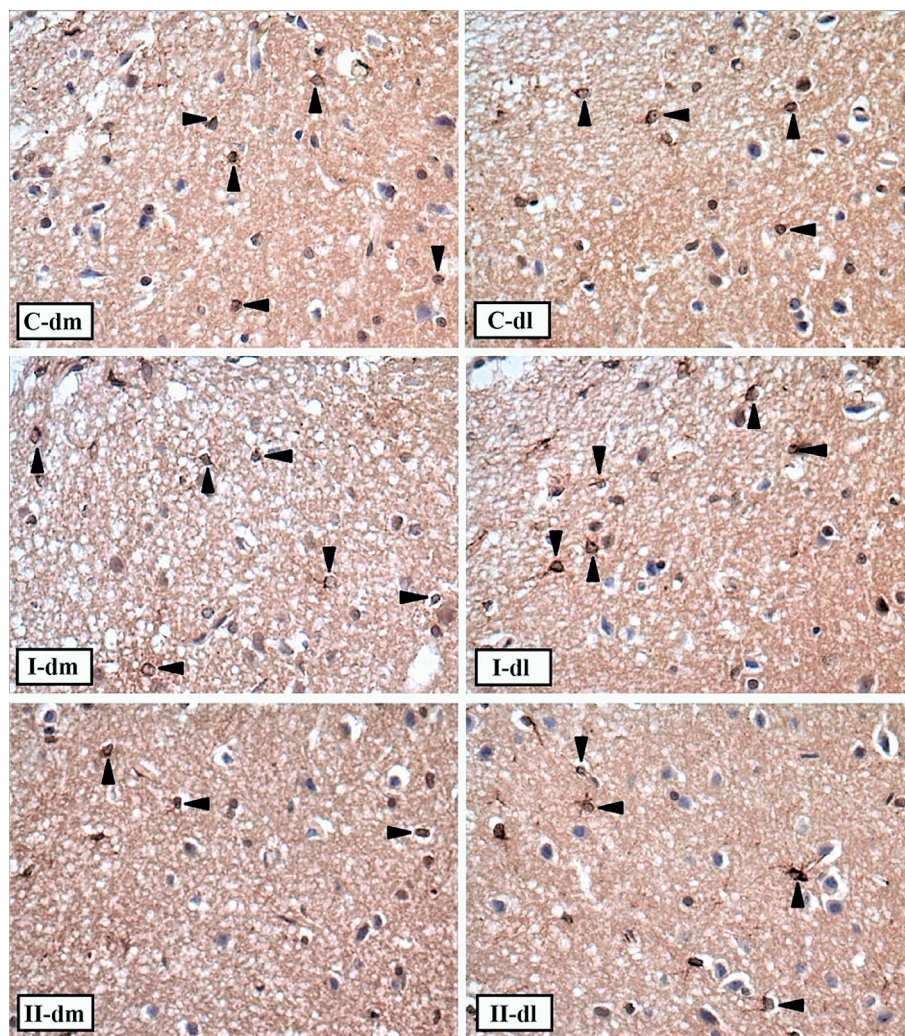


Fig. 1. GFAP-immunoreactive cells (arrowhead) in the dorsomedial (dm) and dorsolateral (dl) parts of PAG in the control (C) and MSG-treated (I, II) rats. GFAP-immunoreactivity was detected as described in Material and methods. Objective magnification: 40 ×

ent parts of PAG in comparison with the control group (Fig. 3).

Our study revealed that the parenteral administration of MSG to rats affects the GFAP expression in PAG astrocytes. An increased glial immunoreactivity for the tested protein was observed in these animals compared to the control groups. This phenomenon was manifested by hypertrophy of astrocyte bodies and processes. The degree of such changes varied depending on the PAG part and the dose of MSG administered to the rats. The most severe glial response was demonstrated in vl PAG of animals receiving 4 g/kg BW MSG (group II). These results indicate a state of astrocyte reactivity occurring most probably secondarily to an increase in the Glu concentration in the extracellular space. Astrocyte hypertrophy and an increase in the GFAP levels were observed in other brain areas of animals with MSG administered both parenterally and orally (1, 8, 9). In the excitotoxicity states, astrocytes tend to decrease extracellular Glu levels. This process is mediated by membrane transporters specific for this neurotransmitter present in the astrocytic processes,

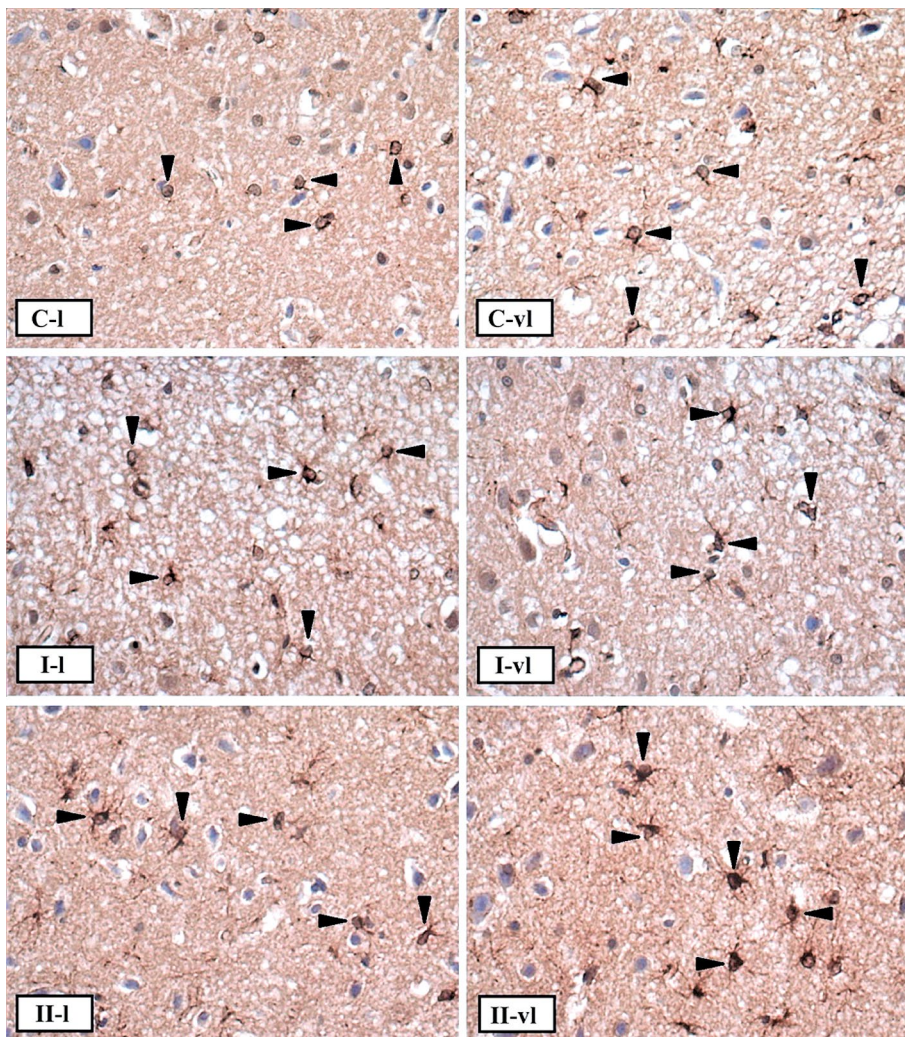


Fig. 2. GFAP-immunoreactive cells (arrowhead) in the lateral (l) and ventrolateral (vl) parts of PAG in the control (C) and MSG-treated (I, II) rats. GFAP-immunoreactivity was detected as described in Material and methods. Objective magnification: 40 ×

the activity of which increases in the states of neuronal hyperarousal. Owing to them astroglia captures about 80% of accumulated extracellular Glu (28). The *in vitro* studies proved that GFAP plays a key role in modulating the transport and function of glutamate-transporting proteins in astrocytes (11). In mice lacking the ability to synthesize GFAP and vimentin a significant reduction in the ability of astrocytic transporters to carry Glu was observed, which has harmful effects on other CNS structures (15).

Under the Glu-induced excitotoxicity conditions, it was shown that generally astroglial reactivity accompanies degenerative neuronal changes. The hippocampus of rats with MSG administered both orally (120 mg/kg BW for 6 weeks) and intraperitoneally (4 g/kg BW for 7 days) showed astrogliosis and neuronal loss in the CA1 and CA3 areas (1, 9). On the other hand, in the cerebellum of rats that received 3 g/kg BW of MSG with food for 14 days, reactive astrocytes were present with degenerated Purkinje cells (8). In our parallel study we proved that under the influence of MSG, the number of calretinin-immunoreactive neurons in the

PAG, especially in its ventrolateral part, decreases (12). This may account for the increased astrocyte reactivity and GFAP expression observed in this part of the PAG in the MSG-treated rats.

Reactive gliosis occurs in the states of any CNS injury. However, its nature and severity are varied. Astrogliosis can assume an isomorphic or anisomorphic form. The first type develops within hours of the damaging agent and is characterized by mild and reversible changes. This phenomenon is characterized by the presence of hypertrophic astrocytes with the preserved domain organization and increased GFAP expression. Such conditions promote regeneration of neuronal networks. Isomorphic astrogliosis occurs, among others, in the cases of axotomies or chemical-induced damage. Anisomorphic astrogliosis is characterized by astrocyte hypertrophy with the increased GFAP expression and astroglia proliferation, leading to disorganization of astrocytic domains and formation of permanent glial scars. These changes are irreversible. The glial scars aim mainly at confinement and isolation of the injury site from the properly functioning tissues of the CNS parts, but at the same time they are a mechanical obstacle to the regeneration of neuronal networks

(25, 27, 29). In our study, we did not observe an increase in the density of GFAP-positive reactive astrocytes in the MSG-treated rats. With a decrease in the total cell number and immunonegative structures for the tested protein this may indicate a lack of glial proliferation and thus reversible isomorphic astrogliosis.

In recent years it has been shown that reactive astrocytes may have neuroprotective properties as well as neurotoxic effects. Based on the genomic studies (transcriptome analysis) two subtypes of reactive astrocytes were distinguished, i.e. A1 and A2, with different gene expression profiles. The first one is characterized by a negative influence on the surrounding structures in response to inflammation. These astrocytes were observed during the CNS injury induced by a single intraperitoneal injection of lipopolysaccharide. The inflammation thus induced activated microglia to produce cytokines. Under their influence, astrocytes produce and release compounds that induce neuronal and oligodendroglial death and inhibit axonal growth, synaptogenesis and impair phagocytosis (19, 30). Whereas the A2 subtype of astrocytes was found

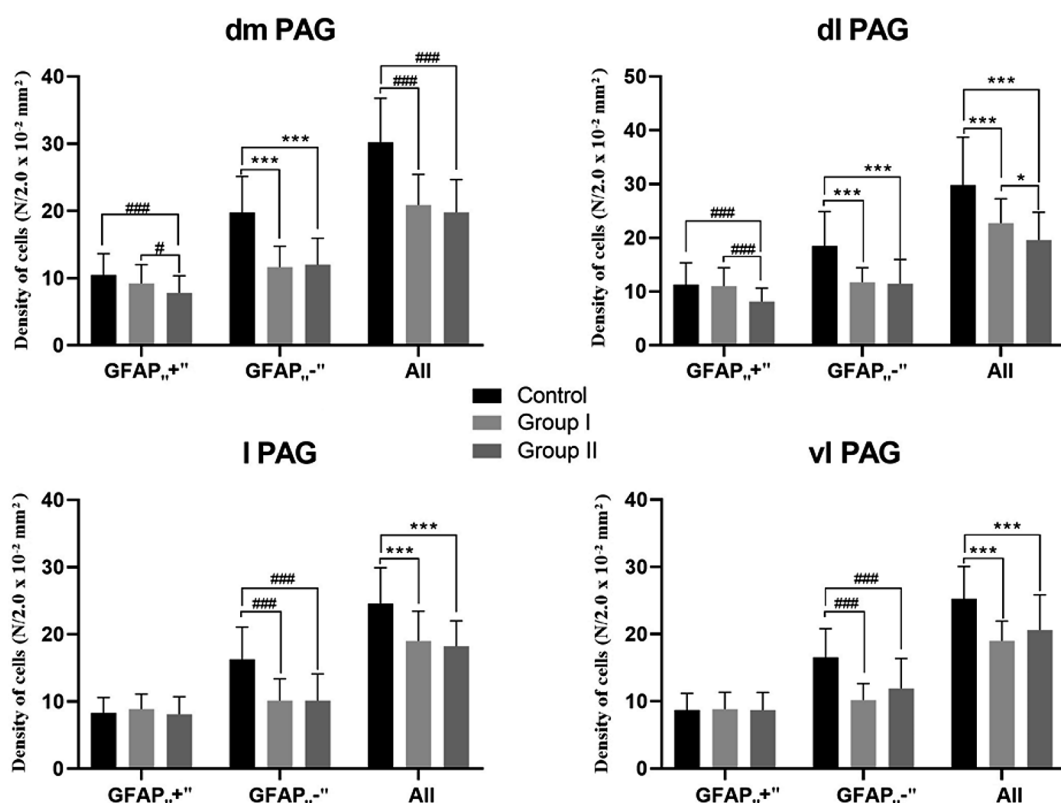


Fig. 3. The density of GFAP-immunopositive cells (GFAP_{+,+}), GFAP-immunonegative cells (GFAP_{+, -}) and all cells in the dorsomedial (dm), dorsolateral (dl), lateral (l) and ventrolateral (vl) parts of PAG in the control (C) and MSG-treated rats (groups I and II)

Explanations: the bars show the mean density of GFAP-IR cells in the area of $2.0 \times 10^{-2} \text{ mm}^2$ and the whiskers represent the standard deviation. A single asterisk (*) refers to the statistically significant difference between the control group and the MSG groups (ANOVA, $P < 0.05$). A triple asterisk (***) refers to the statistically significant difference between the control group and the MSG groups (ANOVA, $P < 0.001$). A single cross (#) refers to the statistically significant difference between the control group and the MSG groups (Kruskal-Wallis, $P < 0.05$). A triple cross (###) refers to the statistically significant difference between the control group and the MSG groups (Kruskal-Wallis, $P < 0.001$).

to be neuroprotective in ischemia. The cells secrete numerous neurotrophic compounds and cytokines (brain-derived neurotrophic factor, cardiotrophin-like cytokine factor, interleukin-6 and thrombospondins) that stimulate repair of damaged synapses. Both the first and the other subtypes of reactive astrocytes (A1 and A2) are characterized by the increased GFAP expression, which was observed as early as the first day after exposure to the damaging agent for at least a week (16, 17, 30). The decrease in the total cell number as well as in the immunonegative structures for GFAP revealed in our study may indicate indirectly the presence of A1 subtype of reactive astrocytes. Their neurotoxic properties may additionally aggravate destructive changes in PAG induced by the elevated extracellular Glu concentration.

At the same time, a decrease in the density of astrocytes with the GFAP expression was observed in dm PAG and dl PAG of rats receiving the large dose MSG (group II). This phenomenon may be a result of diminished synthesis of tested protein in the cells; it may also indicate damage to the astroglia. Inhibition of the GFAP synthesis under the increased Glu concentration condition may be related to the elevated

serotonin secretion. The dorsal part of the PAG (dm and dl) contains numerous serotonergic neuron endings, the bodies of which are located mainly in the dorsal raphe nucleus (20). The *in vitro* studies showed that the addition of serotonin to the astrocytic culture inhibited the GFAP synthesis in cells (13). However, damage to the serotonergic endings in the hypothalamus of adult rats resulted in an increase in the GFAP expression in astrocytes (7).

The decrease in the number of GFAP-IR astrocytes in dm PAG and dl PAG may also be associated with damage to this glia, confirmed indirectly by a decrease in all cells and immunonegative structures for GFAP. In many neuropathological conditions, astroglia undergoes atrophy and asthenia, resulting in impaired hemostatic and

neuroprotective properties. In the early stages of amyotrophic lateral sclerosis, astrodegeneration and reduced Glu uptake capacity of astrocytes were demonstrated which is crucial for the development of neurotoxicity. Atrophy and functional weakness of astroglia were also observed in neurodegenerative (Alzheimer's disease, Huntington's disease) and neuropsychiatric diseases (depression, schizophrenia) (29). Moreover, the astrocyte death was found in the cerebellar cortex of adult rats fed with MSG at a dose of 3 g/kg BW for 14 days. Glial damage is likely to occur as a result of oxidative stress induced by high concentrations of extracellular Glu. This mechanism of excitotoxicity was confirmed in numerous studies (2, 8, 9).

In conclusion, the GFAP immunoreactivity in astrocytes is increased in the PAG of rats receiving MSG, especially at a large dose. The degree of astroglia reactivity varies in different parts of the study area. The hypertrophy of astrocytes with the GFAP expression and the probable lack of astroglia proliferation may indicate isomorphic astrogliosis. On the other hand, the decrease in their number observed in dm PAG and dl PAG may be due to cytotoxic damage. It is worth undertaking further studies to determine the subtype

of reactive astrocytes and their positive or detrimental effects on the surrounding structures. Indeed, therapeutic support of the neuroprotective effects of astroglia could minimize the negative results of excitotoxicity in both the PAG and many other areas of the CNS. Understanding the role of reactive astroglial and learning how to regulate its function can contribute to the development of effective clinical strategies for the treatment of many acute and chronic CNS disorders.

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