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Original article

Immunohistochemical evaluation of hippocampal CA1 region astrocytes in 10-day-old rats after monosodium glutamate treatment

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Abstract

High concentration of glutamate (Glu) is excitotoxic for nervous system structures. This may lead to glial reactivity ie. increased expression of glial fibrillary acidic protein (GFAP) and S100β protein, and also to hypertrophy and proliferation of cells which are determined by the presence of Ki-67 antigen. The aim of the study was to analyse the immunoreactivity of the GFAP, S100β and Ki-67 proteins in astrocytes of hippocampal CA1 region in young rats after administration of monosodium glutamate (MSG) at two doses: 2 g/kg b.w. (I group) and 4 g/kg b.w. (II group). In rats from I and II group morphologically altered astrocytes with the GFAP expression were observed in the SLM of the hippocampal CA1 region. The cells had eccentrically located nuclei and on the opposite site of the nuclei there were single or double, long and weakly branched processes. Moreover, in the SLM the increase of the number of GFAP and S100β immunopositive astrocytes and nuclei with Ki-67 expression, in contrary to control individuals, was observed. These results suggest the increased expression of the proteins in early reactions or hyperplasia which, together with cell hypertrophy, indicate late reactivity of astroglia in response to glutamate noxious effect.

Key words: astrocytes, MSG, glial reactivity, rats, hippocampus

Introduction

Hippocampus as a part of a limbic system is responsible for, inter alia, memory, cognitive processes and addictions. This area consists of CA1-CA4 regions of the hippocampus proper and the dentate gyrus with the hilus. It belongs to the rhinencephalon of the cortical temporal lobe . The regions of the hippocampus proper contain: stratum oriens (SO), stratum pyramidale (SP), stratum radiatum (SR) and stratum lacunosum-moleculare (SLM) (El-Falougy et al. 2006). Hippocampal dysfunction may be related to many central nervous system (CNS) diseases, for example to: diseases which lead to stupor (Alzheimer's disease), diseases like amnesia, epilepsy (Schwartzkroin 1994, Kesner et al. 2010, Marlatt et al. 2010). Glutamate (Glu) as a main excitatory neurotransmitter is involved in proper neuronal develop-

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ment and activity of the hippocampus (Meldrum 2000, Platt 2007). Glu takes part in a long term potentiation formation and synaptic inhibition. Moreover, it plays a role in neuronal plasticity and synaptogenesis. In developing brain, Glu influences migration, differentiation, neuronal survival and death (Meldrum 2000, Lujàn et al. 2005, Platt 2007). For the proper neuronal transmission in mammalian CNS, Glu appears in a minimal amount from 0.5 to 2 μM in the extracellular space (Hawkins 2009). In the state of high concentration of Glu, it is an excitotoxin which leads to cell damage and even death. The excitotoxicity phenomenon is crucial in the pathogenesis of epilepsy, brain stroke, Parkinson and Alzheimer's diseases (Castillo et al. 1996, Mattson 2003, Platt 2007, Yuan et al. 2007, López-Pérez et al. 2010). In this state astrocytic reactivity is observed. There are three glia reaction phases. In the first phase (direct phase) of reactivity the cells tend to compensation of extracellular environment alterations for example by regulation of ions and neurotransmitters levels. After a couple of hours, a second phase of early reactions develops. It is manifested by reactive synthesis and increased expression of various proteins like glial fibrillary acidic protein (GFAP) and S100β protein. The GFAP is a cytoplasmic protein which forms the intermediate glial filaments. It influences the structural stability, mobility, and cellular mitotic activity of astrocytic processes. The S100β is a nucleo-cytoplasmic, calcium-binding protein which regulates many calcium-dependent intra- and extracellular processes. Subsequently, the third phase (late phase) of reactivity develops in which the hypertrophy of cell bodies and processes is observed. There is also an increased proliferation (hyperplasia) and migration of glia (Montgomery 1994, Norenberg 1994, Eng et al. 2000, Rothermundt et al. 2003, Donato et al. 2009). The measure of the activity of cell divisions is a nuclear Ki-67 antigen which may be detected during interphase of the cell cycle (G1, S, G2) and during mitosis (Scholzen et al. 2000). It is possible to specify the level of astrocytes reactivity state by the GFAP, S100β and Ki-67 proteins immunohistochemical assessment as a response to damage of nervous system structures.

In the literature data there is no information about hippocampal astrocytes activity in young rats under the influence of monosodium glutamate (MSG) based on the GFAP, S100β and Ki-67 parallel immunoreactivity assessment. The aim of the study was the microscopic and morphometric analysis of the GFAP, S100β and Ki-67 immunoreactivity in the hippocampal CA1 region in 10-day-old rats after parenteral MSG injections.

Materials and Methods

For the experiments fifteen 7-day-old (P7) Wistar rats were used. The animals were randomly divided into 3 groups: two experimental groups (I,II) and control group (C). Experimental rats were treated with the MSG (L-Glutamic acid monosodium salt monohydrate, Sigma-Aldrich) subcutaneously for three consecutive days in two doses: 2 g/kg b.w. (I group) and 4 g/kg b.w. (II group). In C group the animals received the appropriate amount of saline (0.9% NaCl).

Animals were kept in optimal housing conditions (12x12 h light-dark cycle, temperature 20-22°C) with their dams and received mother's milk. The dams were kept in separated cages with free access to food and water. Experiments were performed according to the permission of Second Local Ethical Committee (7/2011).

24 hours after the last MSG and 0.9% NaCl injection 10-day-old animals (P10) were euthanized. For the studies brains were dissected and fixed in fresh buffered 10% formalin and embedded in paraffin blocks using a routine histological technique. Next, 4 μm-thick frontal sections containing hippocampus were obtained from the material. The sections were placed on superfrost plus slides.

Immunohistochemistry

Immunohistochemical reactions by Sternberger's indirect peroxidase-antiperoxidase method were conducted on a deparaffinised and hydrated sections. Every third section was heated in microwave in 0.01M citrate buffer (ph=6.0) in 3 cycles for 5 min to retrieve the antigen. To carry out further steps of the reaction, the antibodies and reagents were used according to the producer's recommendations (Sigma-Aldrich, St. Louis, Missouri, USA). The antibodies and reagents were diluted in 0.5M Tris buffer (TBS, pH=7.6). First, all the sections were incubated in 0.4% H_2O_2 (room temperature, 30 min) to inhibit the endogenous peroxidase activity. Subsequently, the sections were treated with normal goat serum (room temperature, 20 min) to remove the non-specific background staining. Every third section was incubated with primary antibodies (temp. 4°C, 16h): anti-glial fibrillary acidic protein (GFAP) produced in rabbit, anti S100β protein and anti-Ki-67 antigen produced in mouse. The antigen was retrieved before. The sections were treated with appropriate secondary antibody (room temperature, 1h) consecutively and then with peroxidase-antiperoxidase complex (room temperature, 1h). The sections were rinsed in buffer

after every step of the reaction (3x15 min). In the next step a chromogen, 3,3'diaminobenzidine tetrachloride (DAB), (room temperature, 30 min) was used. After that the sections were rinsed in distilled H_2O, counterstained with Mayer's haematoxylin, dehydrated, cleared in xylene and mounted in DPX (Fluka, Buchs, Switzerland). For the immunohistochemical reactions the specificity control was conducted by omitting the primary antibody and replacing it with normal goat serum. The obtained sections were qualitatively and quantitatively analysed under Olympus BX51 (Olympus, Tokyo, Japan) light microscope with digital camera (Olympus Color View III). Colour microphotographs with 400x magnification were archived.

Microscopic analysis

For the microscopic assessment and photography the sections (10 per animal) were chosen randomly from all collected sections from each animal. On the basis of the GFAP, S100β and Ki-67 immunoreactivity a distribution and density of glial cells with a positive reaction in the CA1 hippocampal region were analysed according to a semiquantitive scale: (-) lack of cells, (-/+) few cells, (+) average number of cells, (++) numerous cells, (+++) very numerous cells. The morphology of the GFAP and S100β immunostained astrocytes in the studied brain area was also analysed.

Morphometric analysis

The morphometric analyses were performed with the Cell^D programme (Olympus) on one photomicrograph per section. A grid of squares of $2.5x10^{-3}mm^2$ was imposed on randomly chosen photomicrographs from the CA1 region. The size of the squares and the test area was the same for all layers of the studied region. Only the squares which entirely covered the studied layer of the hippocampal CA1 region, were chosen to be counted, but not more than 2 squares per photo in one layer. Immunopositive and immunonegative cells for the GFAP, S100β and Ki-67 were counted in 20 squares in each layer per animal, which resulted in the measurements of 100 squares in the control rats and MSG-treated rats per each studied layer.

Next, the average number of cells with the studied protein expression was specified in relation to all cells in the studied layers of the hippocampal CA1 region.

Statistical analysis

One-way analysis of variance (ANOVA) with post hoc test Tukey and nonparametric Kruskal-Wallis test were used to compare the average number of cells. The statistically significant differences were recognised for $p<0.05$.

Results

GFAP immunoreactivity in the hippocampal CA1 region in P10 rats

In control and experimental (I,II) animals in the SO and SR the GFAP immunoreactive cells were average numerous (+). In the SP there were single cells (-/+). In the SLM an immunopositive glia was numerous in rats of C and I group. In animals from the II group the astrocytes with brown reaction product were very numerous (+++). Most of the GFAP immunoreactive cells of the hippocampal CA1 region had oval or round nuclei located centrally in the scant amount of dark brown cytoplasm. They were without glial processes. Only in the SLM layer in animals of the I and II group a different appearance of cells was found. Most of them had oval or round nuclei eccentrically located and from the opposite site of cells there were single or double, long and weakly branched processes (Fig. 1A, 1B, 1C).

Morphometric and statistical studies of the GFAP immunoreactive cells revealed statistically significant differences in the number of studied cells in the SLM of the hippocampal CA1 region between the three groups of animals (C,I,II) (ANOVA, $p<0.05$). Other layers of the hippocampal CA1 region were not significantly different (Table 1).

S100β immunoreactivity in the hippocampal CA1 region in P10 rats

In rats from C, I and II group in the SO an average number of the S100β immunoreactive astrocytes (+) was found. In the SP, the cells were single (-/+). In the SR and SLM there were average numerous S100β-positive cells in group C (+) and numerous cells (++) in groups I and II. In the CA1 region of the hippocampus in all individuals (C, I, II) the S100β protein was localised mainly in nuclei and cell bodies. Only few cells demonstrated the reaction product in the initial parts of their processes (Fig. 1D, 1E).

In the hippocampal CA1 region in individuals treated with MSG (I,II) a statistically significant similar increase of the S100β positive astrocytes was found

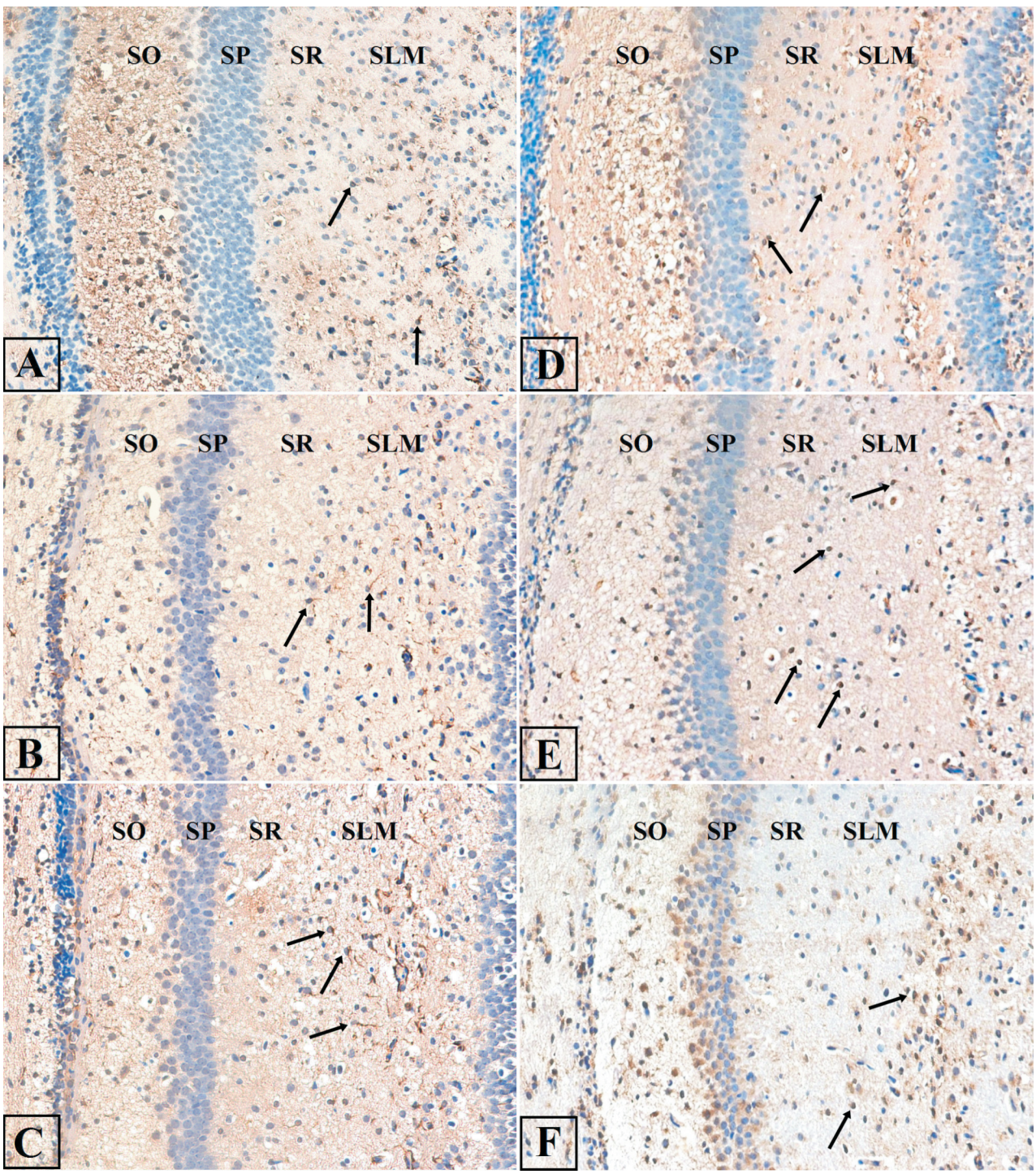

Fig. 1. Immunoreactivity of the GFAP (A, B, C), S100β (D, E) and Ki-67 (F) positive astrocytes in the hippocampal CA1 region of P10 rats in control group (A, D), group I (B) and group II (C, E, F). The immunopositive cells (→) are present in layers: stratum oriens (SO), stratum pyramidale (SP), stratum radiatum (SR), stratum lacunosum-moleculare (SLM). x400.

Table 1. The number of the GFAP, S100β and Ki-67 immunoreactive cells in the hippocampal CA1 region of control and MSG treated rats. The numeric data represent the mean with standard deviation in the area of 0.0025 mm^2.

P10 rats		CA1							
		SO		SP		SR		SLM	
GFAP	C	0.31 ± 0.25	–	0.01 ± 0.016	–	0.09 ± 0.081	–	0.21 ± 0.141[a]	–
	I	0.26 ± 0.216	–	0.02 ± 0.033	–	0.1 ± 0.147	–	0.37 ± 0.186[b]	↑
	II	0.24 ± 0.142	–	0.01 ± 0.033	–	0.11 ± 0.133	–	0.45 ± 0.194[c]	↑↑
S100β	C	0.26 ± 0.158	–	0.01 ± 0.006[a]	–	0.07 ± 0.096[a]	–	0.10 ± 0.095[a]	–
	I	0.29 ± 0.228	–	0.03 ± 0.032[b]	↑	0.33 ± 0.263[b]	↑	0.41 ± 0.22[b]	↑
	II	0.31 ± 0.237	–	0.03 ± 0.037[b]	↑	0.37 ± 0.234[b]	↑	0.42 ± 0.212[b]	↑
Ki-67	C	0.08 ± 0.084[a]	–	0.003 ± 0.008	–	0.01 ± 0.016[a]	–	0.03 ± 0.02[a]	–
	I	0.09 ± 0.051[a]	–	0.001 ± 0.006	–	0.015 ± 0.012[a]	–	0.12 ± 0.109[b]	↑
	II	0.14 ± 0.163[b]	↑	0.006 ± 0.002	–	0.08 ± 0.05[b]	↑	0.13 ± 0.125[b]	↑

Different superscript letters indicate statistically significant differences ($p<0.05$) in the studied area depending on the MSG dose; differences assessment: (–) lack of cells, (↑) increase of cells number, (↑↑) large increase of cells number; CA1 – hippocampal region, SO – stratum oriens, SP – stratum pyramidale, SR – stratum radiatum, SLM – stratum lacunosum–moleculare, C – control rats, I – rats treated with 2 g/kg b.w. of MSG, II– rats treated with 4 g/kg b.w. of MSG.

in the SP (Kruskal-Wallis, $p<0.05$), SR (ANOVA, $p<0.05$) and SLM (ANOVA, $p<0.05$) layers in relation to control rats. In the SO layer of the hippocampal CA1 region, there were no statistically significant differences in the number of astrocytes with the S100β expression between the studied groups of animals (C, I, II) (Table 1).

Ki-67 immunoreactivity in the hippocampal CA1 region in P10 rats

In the group C of animals in the hippocampal CA1 region a few cells (-/+) with the Ki-67 protein expression were found. In experimental animals (I, II) average numerous Ki-67 positive cells in the SO (+) and a few cells in the SP (+/-) were demonstrated. In the SR, the nuclei with the Ki-67 expression were single (-/+) in group I individuals and average numerous (+) in group II rats. In the SLM of I and II group of animals average numerous immunopositive for the studied protein cells were found (Fig. 1F).

Morphometric and statistical studies demonstrated statistically significant increase of the number of nuclei with the Ki-67 expression in the SO (Kruskal-Wallis, $p<0.05$) and SR (ANOVA, $p<0.05$) layers in rats of II group. In animals from groups C and I the SO and SR layers were not statistically different. There were no statistically significant differences in the SP layer between the individuals from all groups (C, I, II). In the SLM, the number of studied cells was increased similarly in both experimental groups in relation to control rats (ANOVA, $p<0.05$) (Table 1).

Discussion

Immunohistochemical assessment of the hippocampal CA1 region astrocytes showed glial reactions in P10 rats after subcutaneous MSG treatment. Under the light microscope a differential immunoreactivity for the GFAP, S100β protein and Ki-67 antigen was found in the studied cells. Morphometric analyses confirmed the microscopic results.

In numerous studies the excitotoxic destruction of nervous cells in animals after parenteral MSG treatment was demonstrated (Takasaki 1978). Many experimental studies indicate that high doses of MSG in neonatal rodents cause a loss of pyramidal cells in the hippocampal CA1 region (Kubo et al. 1993, Beas-Zárate et al. 2002). This phenomenon is related to long and strong excitation of Glu specific receptors, mainly N-methyl-D-aspartate receptors (NMDA). This leads to prolonged depolarisation of cell membranes and increased intracellular calcium ions influx. These alterations occur in just tens of minutes after cells activation. Subsequently, a calcium-dependent enzymatic reactions occur, which result in neuronal damage and even death (Mattson 2003, Platt 2007). Numerous NMDA receptors were shown in hippocampus where they play an important role in the process of learning and memory. For this reason, this area is highly sensitive to Glu-induced toxicity (Schmidt-Kastner et al. 1991, Meldrum 2000, Platt 2007). The excitotoxicity phenomenon is accompanied by astrocytes reactivity: hypertrophy of glial processes, increase of the GFAP immunoreactivity (astrogliosis) and proliferation (astrocytosis) (Montgomery 1994, Eng et al. 2000, Li et al. 2008).

In the presented own studies, reactive astroglia was shown mainly in the SLM layer of the hippocampal CA1 region using anti-GFAP, anti-S100β and anti-Ki-67 antibodies after subcutaneous MSG injections. In experimental individuals (I, II) the GFAP immunoreactive astrocytes were mainly characterized by eccentrically located nuclei and hypertrophy of one or two weakly branched processes. The structural alterations were related to the increase of the number of cells with the GFAP expression according to MSG dose. Moreover. in both experimental groups (I, II) in the SLM a similar increase of the number of the S100β and Ki-67 immunoreactive cells was demonstrated.

A hypertrophy of astrocytic processes and the increase of the number of GFAP immunoreactive cells were also found in fronto-parietal cortex in rats treated with 4 mg/g b.w. of MSG injected subcutaneously on the first, second, third, fifth and seventh day after birth (Martínez-Contreras et al. 2002). A hypertrophy of astrocytic processes may be a result of the necessity to capture the excessive amount of Glu from the extracellular space by specific glial transporters. Some results indicate the colocalisation of glial glutamate transporter (GLT-1) with gliofilaments and also a role of cytoskeleton in transporting GLT-1 along astrocytic processes (Zhou et al. 2004). At the same time, the astrocytic processes prolongation and increase of their branches may influence the stability of neuronal nets and modulation of synaptic activity (Middeldorp et al. 2011). In physiological conditions astrocytes surround about 60% of synapses in the hippocampus, in particular in the SLM layer of the hippocampal CA1 region. In the SLM there are the final dendritic branches of pyramidal neurons of the CA1 region which receive terminals of glutamatergic fibers of perforant tract and Schaffer's colaterals of pyramidal cells of the CA3 region (Korzhevskii et al. 2005). Such cytoarchitecture may explain morphological and quantitative alterations of the GFAP immunoreactive astrocytes limited to this location in the CA1 region, which was observed in own studies. Furthermore, astrocytic processes are highly mobile and they prolong in the direction of Glu releasing synapses. Astrocytes play an important role in a stabilization of newly formed synaptic connections, inter alia, by hypertrophy (Hawrylak et al. 1993, Barker et al. 2010) Synaptogenesis and hypertrophy of glial processes were observed in the hippocampal CA1 region in rats in, inter alia, kindling model of epilepsy (Hawrylak et al. 1993, Morimoto et al. 2004).

Elevated levels of Glu activate regulatory mechanisms in astrocytes which results in intensive the S100β synthesis. This protein increases the frequency of mitotic divisions via reconstruction of cells cytoskeleton and inhibition of gliofilaments (Yasuda et al. 2004). Interactions of this protein with the GFAP influence the cell cycle, growth and differentiation of astrocytes (Montgomery 1994, Norenberg 1994, Eng et al. 2000, Rothermundt et al. 2003, Donato et al. 2009). In addition, the S100β is released to extracellular space, for example via excitation of metabotropic glutamate group II (mGluR3) receptors. Such effect was demonstrated in mice hippocampus in epilepsy model (Sakatani et al. 2008). The increased secretion of this protein results in a decrease of the number of S100β immunoreactive astrocytes (Donato et al. 2009). Thus, it suggests that the 4 g/kg b.w. of MSG in P10 animals causes excessive the S100β release. It explains the similar number of astrocytes with the S100β expression observed in both experimental groups (I, II). The S100β in extracellular space modulates astrocytic Glu uptake and it stimulates glia to divisions, which protect neurons from excitotoxicity (Tramontina et al. 2006). Similar effect was suggested in the S100β knockout mice in epileptic model (Dyck et al. 2002). The S100β may be also involved in new dendritic spines formation and synaptic reorganization. The increased expression of this protein was demonstrated during reactive synaptogenesis in rat;s dentate gyrus (McAdory et al. 1998). Moreover, the increase of the number of astrocytes with the S100β expression was observed in human brain during epilepsy, which suggest its part in synaptic plasticity (Griffin et al. 1995).

The increased number of the GFAP and S100β immunoreactive astrocytes in the SLM layer of the hippocampal CA1 region was also related to the increase of nuclei number with the Ki-67 expression. This indicates the glia proliferation in P10 MSG treated animals. However, the GFAP is a marker of almost all reactive astrocytes but not non-reactive ones which contain the GFAP undetectable for immunohistochemical methods (Kimelberg 2004). During mild or moderate astrogliosis there is a considerably increased the GFAP expression and hypertrophy of almost all astrocytes. It may falsely indicate a glial proliferation (Norton et al. 1992, Sofroniew 2009).

In conclusion, the indicated increase of the number of the GFAP, S100β and Ki-67 immunopositive astrocytes in the hippocampal CA1 region of P10 individuals suggest an increased expression of these proteins during early reactions or hyperplasia. Astrocytes with hypertrophy indicate a late astroglia reactivity in response to harmful Glu influence. Through the immunoreactivity assessment of the three studied proteins in astrocytes the severity of reactive gliosis may be specified in response to Glu excess in extracellular space. The excitotoxicity phenomenon induced by Glu is involved in many pathomechanisms of acute and

chronic nervous system diseases. Hence, establishing the character and level of glia reactivity severity may allow to specify proper targeting of neurological disorders therapy in the future.

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