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Age-related changes in S100β protein immunoreactivity in the periaqueductal gray (PAG) in rats

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Summary

The aim of this study was to investigate and compare S100β protein immunoreactivity in astrocytes of the periaqueductal gray in young and aged rats and to evaluate the morphology of these cells. Furthermore, the amount, astrocyte surface areas, and digital immunostaining intensity of the protein were morphometrically analysed.

The research was conducted on 100-day-old and 3-year-old male rats. Midbrain sections, containing PAG, were obtained from the animals. To detect S100β protein in astrocytes, the peroxydase-antiperoxydase immunohistochemical reaction with the S100β antibody was performed. Diaminobenzidine was used as a chromogen. For this method, specificity control was carried out. S100β-immunopositive astrocytes from the dorsal, dorsolateral, and ventrolateral parts of PAG were observed and photographed with a light microscope equipped with a digital camera. Morphometric analyses were performed.

In 3-year-old rats, astrocytes in all parts of PAG were characterised by similar S100β immunoreaction intensity as those in 100-day-old animals. Astrocyte nuclei were round or oval-shaped, and showed very weak, moderate or intensive immunostaining. Astrocytes in all parts of PAG were irregularly spread. Morphometric analyses confirmed the results of microscopic examination. There were no statistically significant differences in the number of glial cells between the three parts of PAG. The surface areas of astrocytes in all parts did not differ significantly. Digital immunostaining intensity analysis revealed slight differences in the dorsal and ventrolateral parts of PAG between the two age groups of rats. Our results and the available literature data indicate that S100β protein expression in young and aged animals may differ in various brain areas and depends on many factors.

Keywords: astrocytes, S100β, PAG, aging

Aging is a natural biological process characterised by irreversible appearance of intracellular disorders and a decrease in the organism's self-healing abilities. Astrocytes are an important subject of research on brain aging mechanisms. They are the most numerous cells in the central nervous system (CNS), and they participate in many functions regulating neuronal activities. It is shown that these cells lose their neuroprotective abilities during the neurodegenerative processes of aging (9, 17). S100β expression changes in different CNS areas in various animal species are well described in the literature (4, 7, 8, 12, 16, 17). However, the age-dependent S100β protein expression in the periaqueductal gray (PAG) in rats has not been examined until now.

S100β protein is an important astrocyte marker, which exhibits its immunoreactivity in all astrocytes except immature cells of neurogenic zones of the dentate gyrus (5, 13). It belongs to the calcium-binding protein family. It is also capable of binding zink ions (Zn^{2+}) and copper ions (Cu^{2+}). The ion binding by S100β is carried out with two distinct EF-hand motifs (2, 11). This protein is involved in many extracellular and intracellular functions. It extracellularly increases the activity of neurites, which leads to their extension and increases their survival rate. It affects neuronal and astrocitic apoptotic processes. It stimulates astrocyte proliferation, interleukin 6 (IL-6) neuronal secretion and nitric oxide (NO) secretion in astrocytes and microglial cells (2). Intracellularly, S100β is responsible for Ca^{2+} and cAMP signal transduction. It regulates the activity of enzymes affecting the energetic metabolism and cell cycle. It is thus responsible for cell growth regulation. The protein is involved in the inhibition of protein phosphorylation, including glial fibrillary acidic protein (GFAP). Thereby, it affects astrocitic

cytoskeletal elements via the inhibition of the assembly of tubulin into microtubules and via the inhibition of the construction of filament subunits (2, 14, 18). Moreover, S100β can also decrease the activity of numerous kinases inhibiting the phosphorylation of GFAP, thus affecting the balance between polymerised and free GFAP fractions (5). An overexpression of S100β protein was observed in neurodegenerative diseases, for example in Alzheimer's disease (10, 14).

The periaqueductal gray (PAG) is an area located around the cerebral aqueduct. It can be divided into three parts: dorsal, dorsolateral, ventrolateral (1). This area regulates the neurobiological functions of organisms by numerous afferent and efferent connections with many structures of the central nervous system. It receives projections from higher structures of the CNS, which, through PAG, modulate functions of lower areas. It affects neurotransmission and modulation of pain impulses from nociceptive neurons. It is responsible for descending analgesia. The dorsolateral part is responsible for non-opioid analgesia, and the ventrolateral part for opioid analgesia. PAG regulates processes associated with the feelings of fear and anxiety. The dorsolateral part is involved in active defensive reactions of animals, such as fight and flight, whereas the ventrolateral part is involved in passive defensive reactions, such as freezing and quiescence. It affects the autonomic nervous system by which it indirectly takes part in blood pressure and heart rhythm regulation associated witch emotions. The stimulation of the dorsolateral part, unlike that of the ventrolateral part, leads to increased blood pressure and tachycardia. Blood pressure is also increased by the stimulation of the dorsal part. Moreover, PAG plays a role in vocalisation, female sex behaviour, proper intestinal functioning and voiding (1, 6, 15). Differences in the expression of S100β protein may indicate a significant influence of the protein on PAG functioning, depending on the animal's age as a result of neurodegenerative alterations in the aging brain.

The aim of the study was to investigate S100β protein immunoreactivity in astrocytes of the periaqueductal gray in young and old rats. In this study the distribution and staining intensity of S100β-positive cells were determined and compared in the dorsal, dorsolateral, and ventrolateral parts of PAG in different age groups of rats. Morphometric analyses were performed, and the results obtained were compared with results for other areas of the brain of different animal species.

Material and methods

The studies were conducted on 10 white male Wistar rats in two age groups. One group consisted of five 100-day-old rats, and the other comprised five 3-year-old rats. The studies on animals were approved by the Second Local Ethical Committee (7/2011). After the animals had been killed with an intra muscular injection of 10% Ketamine, the brain was immediately dissected. The midbrain, containing the periaqueductal gray, was sampled from the material. Small tissue blocks were fixed in 200 ml of fresh buffered (pH = 7.0) 10% formalin for 12 hours at 4°C, and then paraffin blocks were made by the routine histological technique. Frontal 6 μm-thick sections containing PAG were obtained, and then deparaffinized and hydrated in a graded series of alcohols. In order to eliminate endogenous peroxidase activity, the sections were incubated with 0.4% H_2O_2 with phosphate buffer for 30 min. at room temperature. After triple rinsing in fresh buffer, the sections were treated with normal goat serum (Sigma) for 20 min. at room temperature to eliminate background coloration. In order to reveal S100β protein in astrocytes and its intracellular location, the immunohistochemical peroxidase-antiperoxidase reaction was performed on sections derived from each animal. A set of antibodies was used for immunostaining, diluted according to the producer's recommendations. The sections were incubated with the primary astrocyte-specific anti-S100β protein antibody for 24 h at 4°C temperature. Then, they were incubated with anti-IgG goat antibody, and with the peroxidase-antiperoxidase (PAP) complex. After triple rinsing in buffer, diaminobenzidine (DAB) was used as a chromogen. Incubation with DAB was carried out for 30 min. at room temperature. Water-insoluble brown reaction products of different intensity were found. Subsequently, the sections were rinsed in distilled H_2O, dehydrated, cleared in xylene and mounted in DPX (distyrene plasticizer xylene). For the immunohistochemical technique applied, a specificity control was performed, in which the primary antibody was omitted or replaced with normal goat serum. S100β-immunopositive astrocytes from the dorsal, dorsolateral and ventrolateral parts of the periaqueductal gray were analysed and photographed with an Olympus BX51 light microscope equipped with a Color View III digital camera. Photomicrographs were archived, and then morphometric analyses of S100β-immunoreactive astrocytes were performed by the Cell^D program. The average quantity of S100β-positive astrocytes in 1×10^{-2} mm^2 and their areas were determined. The digital immunostaining intensity of cell nuclei was measured as optical units per μm in cells with positive immunohistochemical reaction. The results obtained were analysed by one-way ANOVA with a significance factor of 0.05. Statistical analyses were carried out by the R 3.0.2 program.

Results and discussion

In 100-day-old Wistar rats, astrocytes from the dorsal, dorsolateral and ventrolateral parts of the periaqueductal gray were morphologically analysed. In the dorsal part of the area, as in the other parts, astrocytes were distributed unevenly. These glial cells exhibited different intensity of S100β immunostaining. However, most of them (ca. 80%) were characterised by intensive immunoreactivity, especially in cell nuclei. Glial processes were bright. Among astrocytes there were cells (ca. 20%) with moderate brown staining, and their cell nuclei exhibited various intensity of immunohistochemical reaction (Fig. 1). In the dorsolateral part of PAG, there were astrocytes (ca. 90%) with intensive

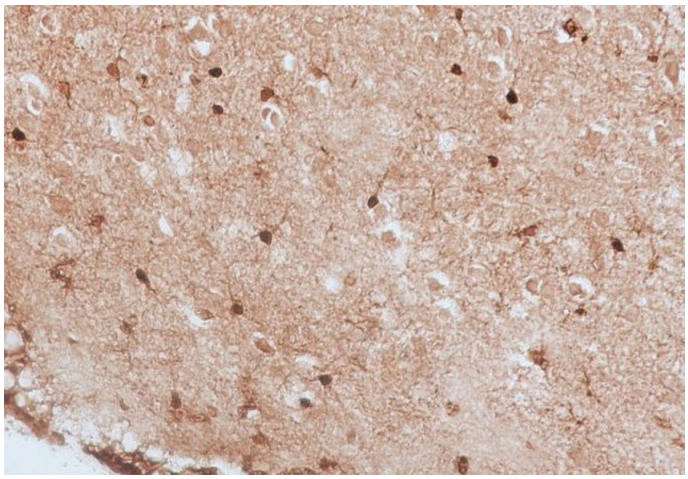

Fig. 1. S100β-immunoreactive astrocytes – the dorsal part of PAG in a 100-day-old rat

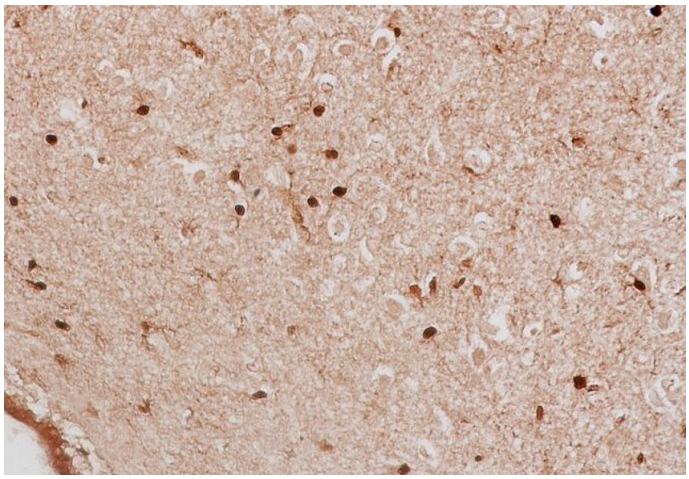

Fig. 2. S100β-immunoreactive astrocytes – the dorsolateral part of PAG in a 100-day-old rat

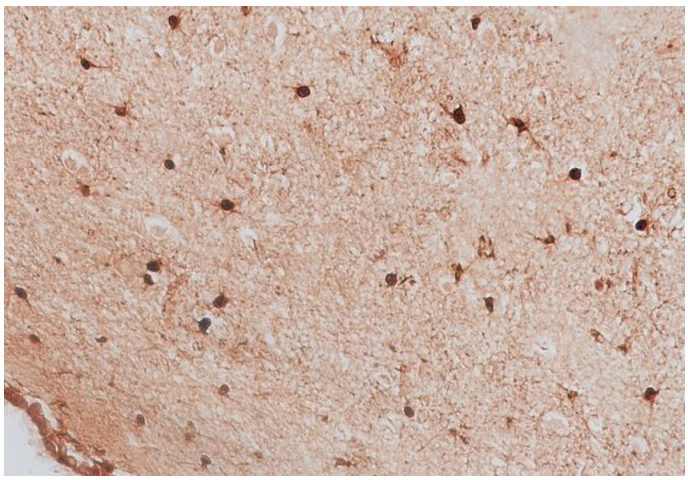

Fig. 3. S100β-immunoreactive astrocytes – the ventrolateral part of PAG in a 100-day-old rat

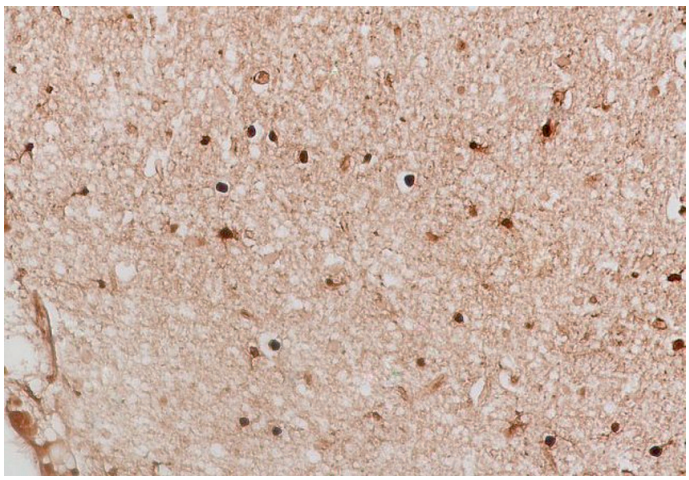

Fig. 4. S100β-immunoreactive astrocytes – the dorsal part of PAG in a 3-year-old rat

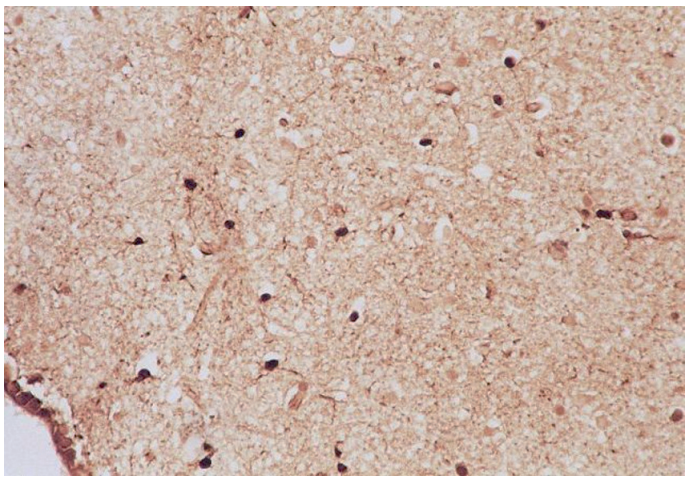

Fig. 5. S100β-immunoreactive astrocytes – the dorsolateral part of PAG in a 3-year-old rat

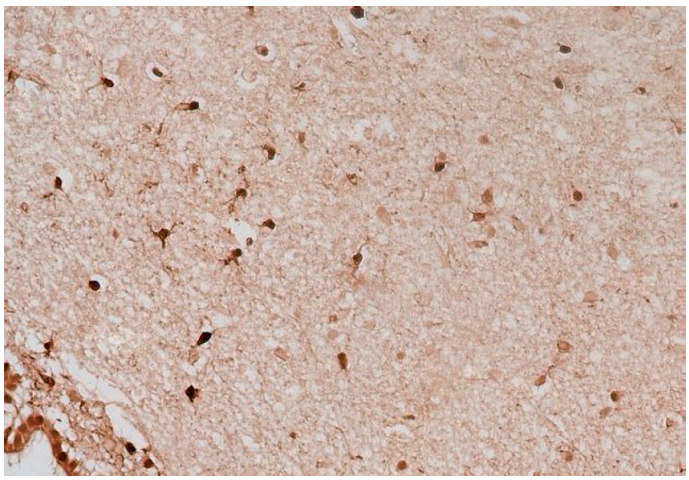

Fig. 6. S100β-immunoreactive astrocytes – the ventrolateral part of PAG in a 3-year-old rat

immunoreactivity of S100β protein. Very few glial cells (ca. 15%) had processes, which were stained bright in comparison with oval and round nuclei (Fig. 2). In the ventrolateral part of PAG, numerous, unevenly distributed intensively S100β-immunoreactive astrocytes (ca. 80%) were observed, as in the dorsolateral part. Most of the glial cells (ca. 65%) had no processes, and their cell bodies contained round or oval-shaped, dark brown cell nuclei. Less numerous astrocytes (ca. 35%) scattered in this part of PAG were characterised by the presence of glial processes, which were extending from star-shaped cells. These processes were less S100β immunostained than their cell nuclei (Fig. 3). In 3-year-old animals, as in 100-day-olds, astrocytes were characterised by dif-

ferent intensity of S100β immunostaining, but they had less glial processes. Astrocyte nuclei were round and oval. Some (ca. 20%) exhibited weak reaction (Fig. 4). The dorsolateral part of PAG in 3-year-old rats, as well as in 100-day-olds, was characterised by the presence of glial cells with intensive S100β immunoreactivity. Some of these astrocytes (ca. 10%) had short, single processes (Fig. 5). In the ventrolateral part of PAG in 3-year-old rats, astrocytes with different S100β immunostaining intensity were observed. Some glial cells (ca. 50%) showed intensive immunoreaction, and some had processes extending from their cell bodies. Equally numerous astrocytes (ca. 50%) were present with moderate immunostaining intensity, and a few of them had glial processes (Fig. 6).

Morphometric analyses confirmed the morphological examination of S100β-immunoreactive astrocytes. There were no statistically significant differences between 100-day-old and 3-year-old rats in the number of glial cells in the dorsal, dorsolateral and ventrolateral parts of PAG (ANOVA $p > 0.05$) (Tab. 1). The analysis of mean astrocyte areas in the three parts of PAG did not show statistically significant differences between the two groups of animals (ANOVA $p > 0.05$) (Tab. 2). Digital immunostaining intensity analysis indicated slight differences between 100-day-old and 3-year-old rats in the dorsal and ventrolateral parts of PAG (ANOVA $p < 0.05$). In the ventrolateral part, however, there were no statistically significant differences between the two groups of animals (ANOVA $p > 0.05$) (Tab. 3).

Tab. 1. Comparison of the average number of astrocytes in PAG in 100-day-old and 3-year-old male rats

PAG areas	Number of 1×10^{-2} mm^2 squares	100-day-old rats	3-year-old rats
		Average ± Standard deviation	
Dorsal	100	2.31 ± 1.5	2.44 ± 1.8
Dorsolateral	100	2.48 ± 1.4	2.41 ± 1.5
Ventrolateral	100	2.45 ± 1.6	2.19 ± 1.5

Tab. 2. Comparison of the average surface areas in PAG in 100-day-old and 3-year-old male rats

PAG areas	Number of cells measured	100-day-old rats	3-year-old rats
		Average ± Standard deviation (μm^2)	
Dorsal	100	25.69 ± 3.7	29.79 ± 9.3
Dorsolateral	100	26.43 ± 5.2	25.47 ± 4.9
Ventrolateral	100	22.37 ± 4.7	23.74 ± 3.1

Tab. 3. Comparison of the average digital immunostaining intensity in PAG in 100-day-old and 3-year-old male rats

PAG areas	Number of cells measured	100-day-old rats	3-year-old rats
		Average ± Standard deviation (ou/μm^2)	
Dorsal	100	197.70 ± 7.7*	186.34 ± 10.3*
Dorsolateral	100	181.69 ± 15.2	178.08 ± 10.9
Ventrolateral	100	184.22 ± 5.1*	163.74 ± 4.2*

Explanation: * – differences between groups are significant at $p < 0.05$ (ANOVA)

In the available literature, there is a considerable variety in research results concerning age-related changes in S100β expression in different species. Some authors suggest that S100β expression in astrocytes increases with age. Even the amount of immunopositive glial cells is increased. An increase in this protein was shown, for example, in the brain cortex and hippocampus of the rat. Similar changes were found in the human brain (4, 12, 16). It is thought that the age-related increase in S100β expression and in the number of immunopositive cells is associated with the presence of characteristic neurodegenerative changes in old animals. In the case of S100β overexpression, the protein increases the expression of proinflamatory cytokins, which has a toxic effect and leads to the apoptosis of neurons and glial cells (10, 14). Moreover, the hippocampus is particularly sensitive to aging, which may result in disorders of spatial learning, while other brain areas, including PAG, may function properly. These disorders are probably caused by Ca^{2+}-dependent processes, which affect long-term potentiation (LTP) and long-term depression (LTD) (3). The presence of highly reactive astrocytes surrounding neuritic plaques was observed in the temporal lobe in patients with Alzheimer's disease. An increased reactivity of this protein was also present in patients with Down's syndrome (3, 5, 12). Age- and sex-related changes in S100β expression were observed in the brain cortex and hippocampus. In female rats, the highest expression was present at the beginning of the rest phase of the daily cycle, whereas in males at the beginning of the motor activity phase. Older animals exhibited increased protein expression in contrast to the young (8).

Studies of the hippocampal dentate gyrus in rats revealed a decrease in the number of S100β-positive astrocytes, which correlated with a decrease in the number of serotonergic fibers (7). The present study demonstrated that both the numbers and areas of S100β-immunoreactive astrocytes were similar in the periaqueductal gray of 100-day-old and 3-year-old rats. Only slight immunoreactivity differences between 100-day-old and 3-year-old animals were found in the dorsal and ventrolateral parts of PAG. Similar results were obtained by the examination of S100β reactivity in the mouse hippocampus. There were no significant differences between young and old mice in the expression of the protein in CA1, CA2, CA3 hippocampal regions, which indicates an increased role of GFAP in this process (17).

The diversity of the results for S100β immunostaining intensity and for the number of S100β-positive astrocytes indicates that the aging process in the CNS is not fully understood. Our studies of S100β immunoreactivity in astrocytes in both age groups suggest that the protein has no significant role in the aging of

the periaqueductal gray in rats. It is possible that other calcium-binding proteins are involved in changes in PAG during natural aging. Further studies of different areas of the brain are needed.

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