Praca oryginalna *Original paper*

Immunohistochemical study of glial fibrillary acidic protein in astrocytes of the periaqueductal gray matter in adult males of the chinchilla

JADWIGA JAWORSKA-ADAMU, ALEKSANDRA KRAWCZYK, KAROL RYCERZ, RADOSŁAW SZALAK, IZABELA KRAWCZYK-MARĆ, AGATA WAWRZYNIAK

Department of Animal Anatomy and Histology, Faculty of Veterinary Medicine, University of Life Sciences, Akademicka 12, 20-033 Lublin, Poland

Received 03.10.2014 *Accepted 10.12.2014*

Jaworska-Adamu J., Krawczyk A., Rycerz K., Szalak R., Krawczyk-Marć I., Wawrzyniak A.

Immunohistochemical study of glial fibrillary acidic protein in astrocytes of the periaqueductal gray matter in adult males of the chinchilla

Summary

Glial fibrillary acidic protein (GFAP) associated with intermediate filaments is a specific marker of astrocyte cells. Brain areas in adult animals of various species show a relatively constant level of differentiation of astrocytes, as well as of the density and expression of GFAP. The periaqueductal gray matter (PAG) of the midbrain integrates behavioural responses that include, among others, defence, pain and emotional reactions. Astrocytes of this region can play a key role in these processes. Under the influence of neurodegenerative diseases and many other factors, these glial cells undergo morphological and functional changes. Therefore, it seems essential to know the distribution and structure of astrocytes in the pathologically unchanged brain. The aim of this study was to determine the distribution and morphology of GFAP-immunoreactive astrocytes and to perform a morphometric evaluation of the PAG in adult males of the chinchilla. In the present study, peroxidase-antiperoxidase immunohistochemical reaction was carried out with a specific antibody against GFAP. GFAP-immunopositive astrocytes of the PAG were examined under a light microscope. A uniform distribution of many glial cells was demonstrated in the dorsal, dorsolateral and ventrolateral parts of the PAG. Morphometric analysis revealed the highest number of astrocytes in the dorsolateral PAG. Our research suggests that a significant accumulation of astrocytes in this part of the PAG in adult males of the chinchilla may reflect their involvement in active defensive mechanisms such as fight and flight. Our results regarding astrocyte GFAP in the PAG may provide a basis for further research on the impact of stressors and pathogens on the number, distribution and morphology of astrocytes and the expression of GFAP.

Keywords: astrocytes, GFAP, periaqueductal gray matter, chinchilla

The periaqueductal gray matter (PAG) of the midbrain is the site of anatomical interconnections between higher brain structures, i.e. the hypothalamus, corpus amygdaloideum, thalamus, cerebral cortex, raphe nuclei, and lower structures, i.e. the brain stem and spinal cord. The PAG integrates behavioural responses, including body functions, such as changes in pain (analgesia), defence (including aggression), and emotional responses, memory, lordosis, vasculo-cardiac control, vocalisation and urination. The area consists of dorsal, dorsolateral and ventrolateral parts. The richness in classical and atypical neurotransmitters and a variety of neuropeptides proves the complicated functions of the PAG. Many neurotransmitters and neuromodulators may be found within this area (1, 2, 6, 10, 11, 14). Although its structure and ultrastructure in mammals have long been well known, the PAG deserves special attention, and its functions are still being explained (3, 4, 7).

Astrocytes play an important role in the proper functioning of neurons in the central nervous system (CNS). These cells are a glial scaffold for neurons which form and maintain the blood-brain barrier, as well as regulate the blood flow, neurotransmitters and ionic homeostasis. They also produce trophic factors, phagocytise and serve as nutritional immunoregulators involved in synaptic plasticity (13). In adults, the number of astrocytes remains relatively constant, but it may also increase. Glial fibrillary acidic protein (GFAP) is an astrocyte-specific marker associated

with the glial intermediate filaments of the cytoskeleton. Immunohistochemical studies in rats revealed substantial differences between different regions of the brain in terms of the density and expression of glial fibrillary acidic protein (GFAP). A considerable density and intense immunoreactivity of GFAP can be observed in areas that come into contact with the surface of the brain during its ontogeny. One of such areas is the periaqueductal gray matter. Glia constitute a heterogeneous group of cells in terms of morphology, biochemistry and functions. The distribution of GFAP-immunoreactive astrocytes in the PAG of the rat has been described extensively, but without the analysis of particular parts of the PAG (8, 9, 12, 15).

The PAG deserves special attention in morphological studies mainly because its importance in the role of astrocytes in the integration of different behavioural instances. Up till now, no other studies of the distribution and morphology of astrocytes immunoreactive for GFAP or morphometric studies of these cells in the periaqueductal gray matter of adult males of the chinchilla have been carried out. The inclusion of the functions of glial cells in the PAG is also discussed in the present study.

Material and methods

For this study, 5 sexually mature (about 1.5-year-old) males of the chinchilla from the RABA farm in Myślenice were used. Immediately after the animals had been slaughtered, their brains were sampled and the midbrain, containing the periaqueductal gray matter (PAG), was dissected. After fixation and embedding in paraffin blocks by a routine histological technique, 6 μm-thick frontal slices of the midbrain were obtained. They were then exposed to a 0.4% H_2O_2 solution for 30 minutes at room temperature to inhibit endogenous peroxidase activity. In order to remove the background coloration, the slices were treated with 10% goat serum (Sigma) for 20 minutes. Immunohistochemical detection of glial fibrillary astrocyte marker acidic protein (GFAP) was performed by the indirect peroxidase-antiperoxidase method. For immunostaining, an antibody kit (Sigma) and reagents diluted in 0.5 M tris buffer (TBS) at pH 7.0 were used. The sections were rinsed in the same buffer after the administration of each antibody. Incubation with primary rabbit monoclonal antibody against GFAP was performed overnight at 4°C. Following this, the secondary monoclonal anti-rabbit IgG antibody and peroxidase-anti-peroxidase complex were used for 1 h at room temperature. Antibody dilutions were made in accordance with the manufacturer's instructions. Diaminobenzidine (DAB) was used as chromogen. After immunostaining, the sections were stained with Mayer's haematoxylin. The specificity control reaction was performed without the primary antibody or by replacing it with normal goat serum. Brown GFAP-immunopositive astrocytes from the dorsal (dPAG), dorsolateral (dlPAG) and ventrolateral parts (dvPAG) were analysed and photographed under a light microscope (Olympus BX 40) connected to a digital camera (Olympus Color View IIIu).

Stellate shaped cells with large oval or round nuclei which showed cytoplasmic brown immunostaining both in the perinuclear cytoplasm and processes were categorised as astrocytes. For microscopic assessment and photography, 10 sections per each animal were obtained. The sections containing the PAG were random and not consecutive. Photomicrographs of the three parts of the PAG were analysed morphometrically. A grid of squares of 1×10^{-2} mm^2 was imposed on 50 randomly selected photomicrographs from the PAG. The total number of cells with nuclei coloured in blue (GFAP-negative) and the number of GFAP-immunoreactive astrocytes coloured in brown on the surface (GFAP-positive) were assessed in 100 squares of 1×10^{-2} mm^2 (2 squares per photomicrograph). The percentage ratio of GFAP-positive astrocytes to all cells (GFAP-positive + GFAP-negative) was determined in each part of the PAG. In order to compare the number of immunopositive cells from the dPAG, dlPAG and vlPAG, a nonparametric Kruskal-Wallis test was used. The ANOVA test with Tukey HSD and Scheffe's post hoc tests were used to compare the percentage ratios of GFAP-immunopositive astrocytes in different parts of the PAG. Differences were considered statistically significant for $p < 0.05$ (Kruskal-Wallis test) and $p < 0.01$ (ANOVA test). Statistical analyses were performed by the R 3.0.2 software.

Results and discussion

The examination of the brains of adult chinchilla males under a light microscope revealed a high density of GFAP-immunopositive astrocytes around the aqueduct in all the parts tested: dorsal (dPAG), dorsolateral (dlPAG) and ventrolateral (vlPAG).

Small stellate cells were evenly distributed in the PAG. Large, oval or round nuclei without immunostaining of GFAP were seen in the middle of the perikarions of astrocytes. Dark brown precipitate equally spaced within a small amount of perinuclear cytoplasm was observed.

Numerous thick primary glial processes branching into thinner secondary branches that expressed intense immunoreactivity for GFAP were seen protruding from cell bodies. Bright cells, i.e neurons and other types of glial cells with nuclei of different sizes, oval or round, tinted blue by Mayer's haematoxylin, were located between astrocytes. Glial processes of immunopositive astrocytes, similar to their perikarions, were often located on unstained nerve cell bodies and in capillary blood vessels (Figs. 1, 2, 3).

No immunohistochemical reaction product was seen in the control histological sections, in which the primary antibody against GFAP was omitted or replaced by a normal goat serum. Morphometric analysis revealed the highest percentage ratio of cells in the GFAP-positive dlPAG (43.49%), less in the vlPAG (36.23%) and the least in the dPAG (31.57%). The results of statistical analysis comparing the number of GFAP-immunopositive cells among the three parts of the PAG revealed significant differences between the

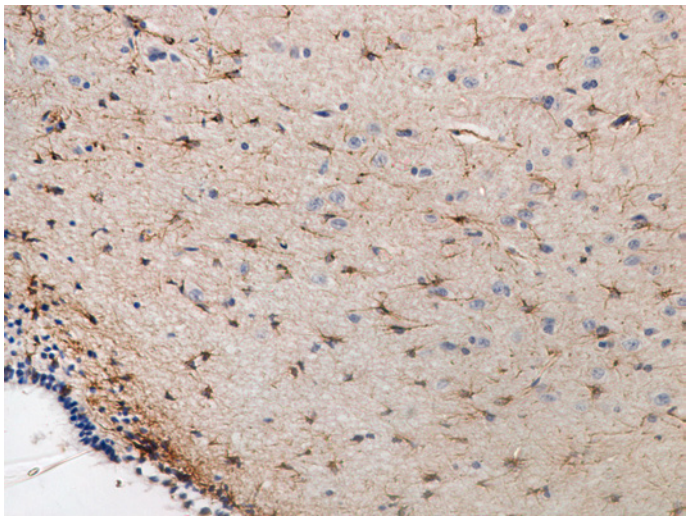

Fig. 1. GFAP-immunoreactive astrocytes located in the dorsal part of the PAG. Magn. 400 ×

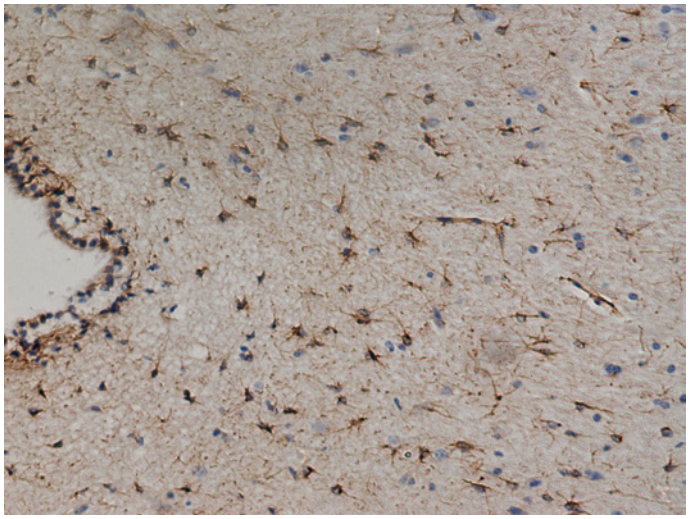

Fig. 2. GFAP-immunoreactive astrocytes located in the dorsolateral part of the PAG. Magn. 400 ×

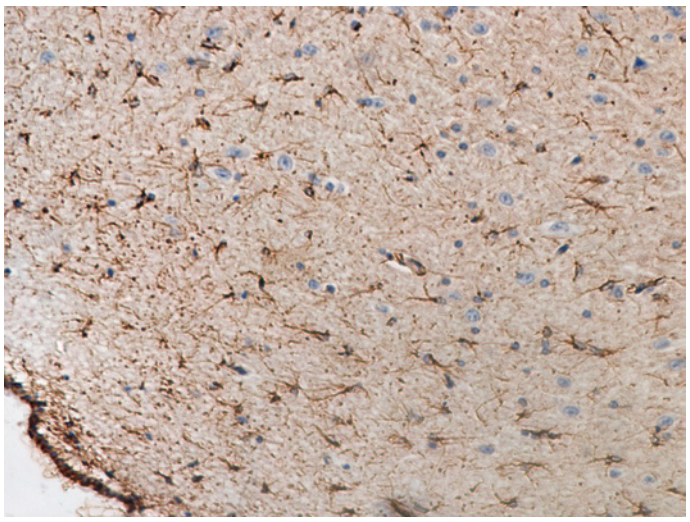

Fig. 3. GFAP-immunoreactive astrocytes located in ventrolateral part of the PAG. Magn. 400 ×

dPAG and vlPAG (Kruskal-Wallis $p < 0.05$) and between the dlPAG and vlPAG (Kruskal-Wallis $p < 0.05$). On the other hand, there were no significant differences between the dPAG and dlPAG (Kruskal-Wallis $p > 0.05$). Analyses of the percentage ratio of GFAP-positive astrocytes in relation to all cells tested in an area of 1×10^{-2} mm^2 showed statistically significant differences between the dPAG and dlPAG and between the dlPAG and vlPAG (ANOVA $p < 0.01$) (Tab. 1).

Tab. 1. The average number of GFAP-immunoreactive cells and their average percentage ratio to the total number of cells in 1×10^{-2} mm^2 in the PAG of chinchilla

PAG parts	GFAP-positive cells Average ± Standard deviation	The percentage ratio of cells
Dorsal	4.35 ± 1.84[a]	31.57%[1]
Dorsolateral	4.83 ± 1.74[a]	43.49%[2]
Ventrolateral	3.74 ± 1.69[b]	36.23%[1]

Explanations: Different letters and numbers in superscript indicate statistically significant differences between particular parts of the PAG (Kruskal-Wallis $p < 0.05$ for letters and ANOVA $p < 0.01$ for numbers).

Regional differences in the distribution of GFAP-positive astrocytes were described in a number of areas, among others in nuclei of the mesencephalon, rhombencephalon and spinal cord of adult rats. A significant accumulation of GFAP-immunoreactive cells was seen around the aqueductus of the brain, substantia nigra and the interpeduncular nucleus. However, in this species of mammals, the distribution of PAG astrocytes in particular parts of this area has not been studied (8). Regional differences in the content of these glial cells may be closely related to specific functions of the PAG. Our study revealed the least number of astrocytes in the vlPAG in adult males of the chinchilla. In the dPAG and dlPAG, a comparable number of GFAP-immunopositive astrocytes were found. However, the percentage ratio of these cells in the dlPAG was statistically higher, which may be due to its role in the formation of fear and anxiety (1).

These glia constitute a heterogeneous group of cells in terms of morphology, biochemistry and functions. Depending on the area of the brain where astrocytes are located, they transform and specialize as a result of the presence of neurotransmitters and neuromodulators.

In adult cats, neurotransmitters in the dPAG were shown to regulate and modulate defensive rage behaviour (3). In the PAG, astrocytes capture glutamate and GABA in synaptic spaces and convert them to glutamine with the participation of glutamate synthetase, which returns to neurons. This substance is used in nerve cells for the synthesis of these two neurotransmitters. The knowledge of the distribution and morphology of GFAP-immunopositive astrocytes in the PAG in adult males of the chinchilla is important because of the relation of the PAG to different types of behaviour.

In adult rats in a chronic restraint stress model, but not in subacute restraint stress, a significant decrease in the GFAP protein level and in the level of glial

membrane excitatory amino acid glutamate transporter (EAAT2) was demonstrated, especially in the vlPAG. Moreover, chronic restraint stress induced mechanical hypersensitivity and aggressive behaviour. The results of these studies suggest that chronic restraint stress induces a dysfunction of the PAG in the modulation of pain perception, which may be related to a decrease in the GFAP level (9). Other authors showed an increased activity of glial cells, manifested by a significant increase in immunoreactivity for GFAP in astrocytes of the vlPAG, in 2-month-old Sprague-Dawley rats, regardless of sex, after 60 minutes of morphine administration. These results suggest that these glial cells are possible modulators of morphine-induced analgesia. Opioids are ultimately acting on glial cells (5, 12).

In conclusion, a significant accumulation of astrocytes shown in the dorsal, dorsolateral and ventrolateral parts of the PAG in adult males of the chinchilla suggests that they may be involved in the proper functioning of neurons. Understanding the distribution of GFAP-immunoreactive astrocytes in the PAG in this species is necessary for further studies of the role of these glial cells in the modulation of emotional reactions, defence and pain, the process of feeling fear and anxiety, urination and sexual behaviour.

References

1. *Behbehani M. M.*: Functional characteristics of the midbrain periaqueductal gray. Prog. Neurobiol. 1995, 46, 575-605.
2. *Benarroch E. E.*: Periaqueductal gray. An interface for behavioral control. Neurology 2012, 78, 210-217.
3. *Bianchi R., Gioia M.*: Ultrastructural features of the synapses of the periaqueductal gray matter (PAG) of the cat. J. Hirnforsch. 1984, 25, 275-283.
4. *Buma P., Veenning J., Hafmans T., Joosten H., Nieuwenhuys R.*: Ultrastructure of the periaqueductal grey matter of the rat: an electron microscopical and horseradish peroxidase study. J. Comp. Neurol. 1992, 319, 519-535.
5. *Edison L. N., Murphy A. Z.*: Persistent peripheral inflammation attenuates morphine-induced periaqueductal gray glial cell activation and analgesic tolerance in male rat. J. Pain 2013, 14, 393-404.
6. *Fogaça M. V., Lisboa S. F., Aguiar D. C., Moreira F. A., Gomes F. V., Casarotto P. C., Guimareãs F. S.*: Fine-tuning of defensive behaviors in the dorsal periaqueductal gray by atypical neurotransmitters. Braz. J. Med. Biol. Res. 2012, 45, 357-365.
7. *Gioia M., Tredici G., Bianchi R.*: The structure of the human periaqueductal gray matter (PAG). Riv. It. Biol. Med. 1992, 12, 1-4.
8. *Hájos F., Kálmán M.*: Distribution of glial fibrillary acidic protein (GFAP)-immunoreactive astrocytes in the rat brain. II Mesencephalon, rhombencephalon and spinal cord. Exp. Brain Res. 1989, 78, 164-173.
9. *Imbe H., Kimura A., Donishi T., Kaneoke Y.*: Chronic restraint stress decreases glial fibrillary acidic protein and glutamate transporter in the periaqueductal gray matter. Neurosci. 2012, 223, 209-218.
10. *Jansen A. S. P., Farkas E., Sams M., Loewy A. D.*: Local connections between the columns of periaqueductal gray matter: a case for intrinsinc neuromodulation. Brain Res. 1998, 784, 329-336.
11. *Oka T., Yokota S., Tsumori T., Niu J.-G., Yasui Y.*: Glutamatergic neurons in the lateral periaqueductal gray innervate neurokinin-1 receptor-expressing neurons in the ventrolateral medulla of the rat. Neurosci Res. 2012, 74, 106-115.
12. *Sauzier J.-M. A.*: Glial cell activity within the ventrolateral periaqueductal gray of male and female rats. Biology Honors Theses 2012, 3, 1-11.
13. *Sofroniew M. V., Vinters H. V.*: Astrocytes: biology and pathology. Acta Neuropathol. 2010, 119, 7-35.
14. *Vianna D. M. L., Brandão M. L.*: Anatomical connections of the periaqueducal gray: specific neural substrates for deferent kinds of fear. Braz. J. Med. Biol. Res. 2003, 36, 557-566.
15. *Zilles K., Hájos F., Kálmán M., Schleicher A.*: Mapping of glial fibrillary acidic protein-immunoreactivity in the rat forebrain and mesencephalon by computerized image analysis. J. Comp. Neurol. 1991, 308, 340-355.

Corresponding author: prof. dr hab. Jadwiga Jaworska-Adamu, Akademicka 12, 20-950 Lublin; e-mail: jadwiga.jaworska@up.lublin.pl