DE GRUYTER OPEN

Bull Vet Inst Pulawy 58, 105-109, 2014
DOI: 10.2478/bvip-2014-0017

Immunoreactivity of S100β protein in the hippocampus of chinchilla

Aleksandra Krawczyk, Karol Rycerz, Radosław Szalak, Jadwiga Jaworska-Adamu

Department of Animal Anatomy and Histology, Faculty of Veterinary Medicine, University of Life Sciences, 20-950 Lublin, Poland
aleksandra.krawczyk@up.lublin.pl

Received: June 24, 2013 *Accepted: October 17, 2013*

Abstract

The aim of the study was to investigate S100β protein in astrocytes of CA1 and CA3 areas of the hippocampus proper and the dentate gyrus with the hilus yet undefined in mature males of chinchilla. The presence of S100β was determined using indirect immunohistochemical peroxidase-antiperoxidase method with specific monoclonal antibody against this protein. Most of the S100β-positive cells were detected in the subgranular zone of the dentate gyrus and in the middle part of the hilus. In CA3 area, it was found that the most numerous cells with S100β are in stratum radiatum. In CA1 area, there were single astrocytes expressing this protein. This data demonstrates species differences and a large quantity of S100β immunoreactive cells in the subgranular zone of the dentate gyrus of chinchilla, which may be associated with structural reorganisation of the hippocampus and with neurogenesis, learning, and memorising process dependent on the hippocampus.

Key words: chinchilla, S100β protein, immunoreactivity, astrocytes, hippocampus.

Introduction

The hippocampus is a part of archicortex classified to the rhinencephalon located in the medial temporal lobe of the brain. It is one of the elements of hippocampal formation, next to the subiculum, which connects it with neocortex through parahippocampal gyrus. The hippocampus consists of the hippocampus proper (*cornu Ammonis*) divided into four areas: CA1, CA2, CA3, CA4, and the dentate gyrus (DG) with the hilus (H). The hippocampus has a laminar structure. Areas of chinchilla's hippocampus proper are made up of four layers: stratum oriens (SO), stratum pyramidale (SP), stratum radiatum (SR), stratum lacunosum-moleculare (SLM). In the structure of the dentate gyrus there are distinguished stratum moleculare (SM) and stratum granulosum (SG). Stratum polymorphicum (SPM) constitutes a hilus of the dentate gyrus divided into two parts: a part rich in nerve fibres adjacent to stratum granulosum of the dentate gyrus and deeply located part with densely arranged nerve cells weakly myelinated (28). Between the hilus and stratum granulosum of the dentate gyrus there is a region about 25 μm wide called subgranular zone (SGZ) in which active neurogenesis takes place in the mature brain. This process is complex, dynamic and depends on the interaction of growth factors and neurotrophins (2, 18). The hippocampus is involved in the processes of learning, formation, and maintenance of declarative and spatial memory, which are based on structural and functional changes of neuronal networks (20). Many different substances modulate the activity of neurons and synaptic junctions, including S100β protein actively secreted by astrocytes in the central nervous system (CNS). It is one of twenty S100 calcium-binding proteins belonging to "EF-hand" family. In mammalian CNS, it is present as a homodimer composed of noncovalently linked two β subunits. (7, 10, 13, 24). S100β protein is localised in the cell nucleus and cytoplasm in a free form, as well as bound with intracellular membranes and cytoskeletal elements, such as type III intermediate filaments, cytoplasmic microtubules, centrioles, and mitotic spindles. It plays many functions in cell through interactions with effector proteins, which additionally increase S100β protein affinity to calcium ions. It is a sensory protein reacting to the changes of calcium ion level in the cytoplasm, as well as a protein that can reduce their number as a buffer. In addition, this protein affects the organisation of cell cytoskeleton,

blocking synthesis of microtubules or stimulating breakdown of type III intermediate filaments *e.g.* astroglial filaments composed of glial fibrillary acidic protein (GFAP). Moreover, it reduces the activity and metabolism of many enzymes, phosphorylation of different proteins, and gene transcription. It is involved in the processes of cell proliferation and differentiation. S100β is actively released into the extracellular space where it acts autocrinely and paracrinely on neurons and other glial cells (1, 7, 13). The effects of this protein activity depend on the level in which it occurs. In nanomolar concentrations, it has a trophic effect on the structure of CNS and in micromolar concentrations it is toxic and leads to the apoptosis of nerve and glial cells. Overexpression of this protein can be a cause of symptoms occurring in Alzheimer's disease or Down's syndrome, which is caused by trisomy of the 21st chromosome with a gene encoding S100β protein (7, 11, 24, 26).

The aim of this study was to investigate the S100β protein immunoreactivity in astrocytes of the hippocampus in adult chinchilla males, as well as to determine, compare, and evaluate morphometrically the S100β positive cells in the layers of CA1 and CA3 areas of the hippocampus proper and the dentate gyrus with the hilus. The obtained results were analysed in relation to the similar results obtained in other animal species.

Material and Methods

The examinations were carried out on brains of five (18-month-old) chinchilla males. The brains were collected immediately after the slaughter. After 12 h fixation in 10% buffered formalin (pH 7, at 4°C), the material was embedded in paraffin blocks by routine histological technique. Afterwards, 6 µm-thick frontal sections of the hippocampus were placed on glass slides and held for 30 min at 56°C, deparaffinised in xylene, and hydrated in alcohols of decreasing concentrations. In order to trace the distribution and localisation of S100β protein in astrocytes of the hippocampus, immunohistochemical peroxidase-antiperoxidase (PAP) method was applied according to Stenberger (27). For immunostaining, a set of antibodies and reagents from Sigma-Aldrich (USA) was used. They were diluted, according to the manufacturer's instructions, in 0.5 M Tris buffered saline (TBS, pH 7.6).

In the first stage, activity of endogenous peroxidase was inhibited by treating hydrated slices with 0.4% H_2O_2 for 30 min at room temperature. Then, the slices were rinsed in 0.5 M TBS. Subsequently, incubation with normal goat serum (Sigma G9023) was carried out at room temperature for 20 min in order to remove the background non-specific staining.

Using specific mouse monoclonal S100β protein antibody (Sigma S2532), PAP reaction was carried out for 24 h at 4°C. Afterwards, the slices were incubated for one hour with goat monoclonal antibody against mouse immunoglobulins (Sigma A9917), and then with mouse monoclonal peroxidase-antiperoxidase complex antibody (Sigma P3039) also for one hour. To visualise the reaction, the slices were treated for 30 min at room temperature with 3,3-diaminobenzidine tetrachloride (Fluka 32750), which was used as a chromogen giving insoluble reaction product of varying intensity of brown staining. Subsequently, sections were rinsed in distilled water, dehydrated, cleared in xylene, and closed in DPX. Specificity control for the used immunohistochemical technique was performed, in which the primary antibody against the antigen was omitted or replaced with normal goat serum.

S100β protein immunopositive astrocytes were photographed under a light microscope (Olympus BX 40) connected with a digital camera (Olympus Color View IIIu). The obtained microphotographs were archived and next quantitative analyses were made in cell^D programme. S100β-positive cells were counted at magnification about 400x in two areas (each of about 0.005 mm^2) from each of seven sections per animal. Mean, minimum (MIN), and maximum (MAX) values and standard deviations were determined from the obtained results. The differences between the means were verified by one-way ANOVA test. Statistically significant differences were assumed at $P < 0.05$.

Results

Immunopositive astrocytes with cytoplasmo-nuclear expression of S100β protein were demonstrated in all layers of CA1 and CA3 areas of the hippocampus proper, dentate gyrus, and hilus of the dentate gyrus. Most of them were observed in SGZ of the dentate gyrus (++++) and in less myelinated part of the hilus (+++). Single (+) S100β protein immunopositive cells were demonstrated in the part of the hilus adhering to SG of the dentate gyrus, as well as in SM of the dentate gyrus (Fig. 1), and in all layers of CA1 area scattered (Fig. 2).

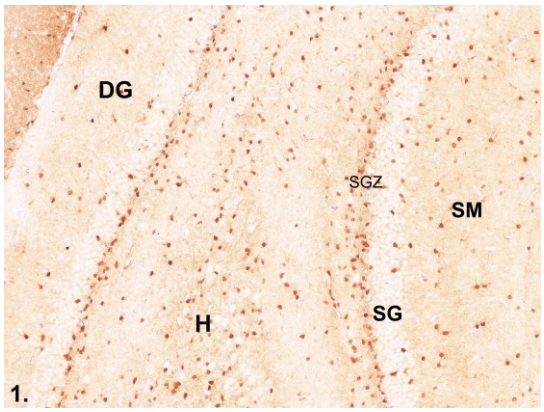


Fig. 1. S100β immunopositive astrocytes localised mostly in SGZ and middle part of the hilus. About 100x

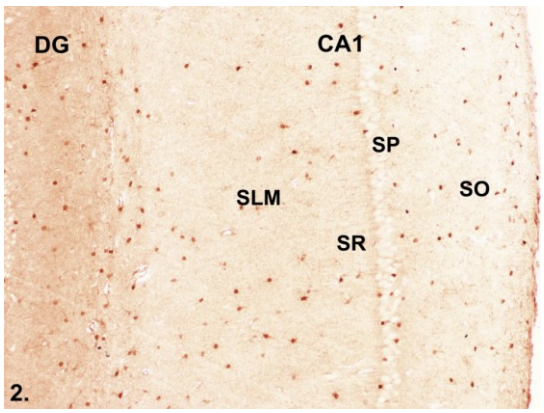


Fig. 2. Single astrocytes with the expression of S100β protein distributed in all CA1 layers of the hippocampus proper. About 100x

In CA3 area of the hippocampus proper, distribution of cells with the expression of the protein was laminar with their concentration especially in SR (+++) and in SP (++). Single cells (+) with the expression of S100β protein were shown in SO and SLM (Fig. 3).

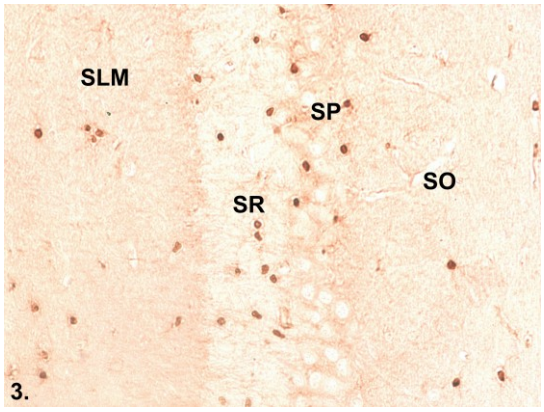


Fig. 3. S100β immunopositive astrocytes localised mainly in SR and SP of CA3 area of the hippocampus proper. About 400x

Observations under a light microscope corresponded to the results of morphometric analysis (Table 1).

Discussion

The distribution of astrocytes, immunoreactive for S100β protein in chinchilla's hippocampus, only partly corresponds to that defined in rat or mouse. Most S100β-positive cells were shown in the dentate gyrus of the examined brain area in rat, as well as in chinchilla (25). The results of our examinations point to a slightly different distribution of these structures, because in chinchilla most of them were observed in SGZ of the dentate gyrus. On the other hand, in rat they were mostly scattered in SM and the hilus of the dentate gyrus. Not much of this glia was demonstrated in SG of the dentate gyrus. Moreover, in CA3 area of rat's hippocampus proper, the greatest immunoreactivity for S100β was demonstrated in SO and SR. In astrocytes of SP, a weak expression of the examined protein was observed (3). In CA3 area of chinchilla's hippocampus proper, most S100β –positive structures occurred in SR and SP. The fewest examined cells were present in SO. In mouse, as compared to rat, scarce and single astrocytes with the expression of the examined protein in SP of CA1 and CA3 areas of the hippocampus proper and SG of the dentate gyrus were shown. Most immunostained structures were demonstrated in SLM of CA1 and CA3 area and in the hilus of the dentate gyrus (21).

The most important layers in the hippocampus proper and dentate gyrus are SP and SG, in which neuronal bodies are located. They form numerous junctions with other areas of the CNS (8, 9, 14). Excitatory nervous impulses from enthorinal cortex reach the hippocampus through perforant pathway. Neurons axons of enthorinal cortex reach dendrites of granular neurons of the dentate gyrus, which transfer information through mossy fibres mainly to pyramidal neurons of CA3 area.

Table 1. Mean, minimum (MIN), and maximum (MAX) values and standard deviations (SD) for the number of S100β immunopositive cells in individual layers of CA1, CA3, the dentate gyrus, and the hilus (area ~ 0.005 mm^2, *P < 0.05)

	CA1				CA3				DG		H	
	SO	SP	SR	SLM	SO	SP	SR	SLM	SM	SG	Internal part	External part
Mean	1.1	1.1	0.9	1.0	0.9	1.7*	2.6*	1.2	1.1	5.3*	3.2*	0.9
SD	1.1	0.8	0.9	0.9	1.0	1.1	1.5	0.9	0.8	2.0	2.7	1.3
MIN/MAX	0/4	0/4	0/3	0/4	0/4	0/5	0/7	0/5	0/3	1/10	0/10	0/5
Total cells in layer	77	80	67	71	68	121	185	85	78	373	229	67
Total cells in region	295				459				451		296	

SO - stratum oriens, SP - stratum pyramidale, SR - stratum radiatum, SLM - stratum lacunosum-moleculare, DG - dentate gyrus, SM - stratum moleculare, SG - stratum granulosum, H - hilus

Afterwards, nerve impulse through Schaffer's collaterals reaches neurons of CA1 area, where it is directed through subiculum to enthorinal cortex, and by feedback collaterals to the same and adjacent cells of CA3 area. Fibres of perforant pathway and pyramidal and granular neurons are glutamatergic. Junctions of the hippocampus play an important role in the processes of development and maintenance of memory. The ability of learning and memorising depends on the neuron plasticity, for example, structural and functional changes involving an increase in the number and density of dendritic branching, creation of new synapses, as well as quality changes of already existing junctions (2, 20). Synaptic efficiency can be increased (long-term potentiation – LTP) or decreased (long-term depression LTD). It has been shown that electrical induction of LTP in the dentate gyrus of rat provides cellular and molecular conditions favourable for proliferation and survival of newly generated neurons in this area in adult animals (4, 20).

Generation of new neurons takes place in SGZ located between the hilus and SG of the dentate gyrus. They differentiate locally from precursor cells migrating towards SG (6, 16), where they transform into stimulating glutamatergic neurons or GABA-ergic inhibitory interneurons (29) connected functionally with CA3 area of the hippocampus proper. Numerous experiments have shown that in the processes of learning and memory, neurogenesis plays a crucial role. An increase in a number of new neurons in the dentate gyrus of rats was proved by hippocampus-dependent memory tests. In addition, a reduced ability to learn and properly perform these tasks in animals, in which exchange of nerve cells in the hippocampus was inhibited for example as a result of irradiation, was observed (2).

The neuronal plasticity, which is necessary for the formation of memory, is affected by many substances including S100β protein secreted by astrocytes into the extracellular space. An increase in this protein synthesis in the brain was observed during learning. Intracerebral injection of S100β antibodies inhibits memory consolidation, causes amnesia, and blocks the formation of LTP (17, 24). The examinations carried out on transgenic mice revealed that the effect of the examined protein depends on the concentration in which it is formed. In animals with its overexpression impaired ability of solving spatial tasks was observed and it was confirmed by numerous behavioural tests (12, 17). S100β in micromolar concentrations acts neurotoxically leading to overproduction of reactive oxygen species and activation of caspase cascade inducing cell apoptosis (7, 13, 24). Moreover, *in vitro* studies revealed an increase in the level of mRNA of inducible nitric oxide synthase, and activity of this enzyme leading to the death of cells in mixed cultures of neurons and glia (13). S100β protein can be compared to cytokine, which through multiligand receptor RAGE (receptor for advanced glycation endproducts) in high concentrations acts toxically, while in low concentrations trophically on the structures of CNS. S100β in nanomolar concentrations stimulates the growth of axons, increases the survival of neurons during development, and in early stages of brain damage. It also prevents the degeneration of motor neurons in new-born rats after cutting the sciatic nerve, and regenerates damaged peripheral nerves. In addition, S100β has a positive effect indirectly inducing the proliferation of astrocytes, stimulating uptake of neurotoxic glutamate by these cells, as well as reducing cytotoxicity of neurons and astrocytes in hypothermia. The protective effect of S100β is manifested by increased expression of anti-apoptotic BCL-2 protein in the cells and activation of many proexperiential signalling pathways (7, 13). During brain injury, intracerebral injection of this protein induces generation of new cells in the hippocampus (18, 19). Acute brain injury is one of the factors causing an increase in S100β secretion from astrocytes into the extracellular space. This glia is activated by stimulation of metabotrophic glutamate receptor 3 and serotonin receptor 5HT1A, which is regarded as a regulator of S100β protein in the brain. There was a stimulating effect of 5HT1A receptor antagonists on S100β release from a culture of primary astrocytes (24). Moreover, an increase in this protein's level under the influence of antidepressant (fluoxetine), raising the amount of serotonin, was shown. It is suggested that antidepressants stimulate the expression of S100β protein in astrocytes and also a number of RAGE receptors in proliferating neuroblasts. In this way they become sensitive to its effect. Therefore, it is assumed that antidepressants contribute to the proliferation of neuroblasts through increased release of S100β, and its interaction with neuronal RAGE receptors (7). In addition, it has been demonstrated that blocking of 5HT1A receptor causes a 30% decrease in the number of newly created cells in the dentate gyrus in rat (23). It has been observed that trophic effect of this protein on serotonergic neurons, and its too low level can even inhibit the development of these nerve cells (22).

Using the method of double immunohistochemical staining of astrocytes in mouse's hippocampus, it was observed that S100β colocalised with astroglial marker *i.e.* glial fibrillary acidic protein (GFAP) only in 80% (21). GFAP-negative cells were particularly present in SP and SLM of CA3 area of the hippocampus in mice, and can represent a certain subpopulation of astrocytes. However, recent data also indicates colocalisation of S100β protein with NG2- chondroition sulphate proteoglycan (15). Among the glial cells showing positive reaction to NG2 inter alia precursor cells for oligodendrocytes were distinguished, as well as synantocytes considered by some as the fifth type of glia in CNS (5).

In conclusion, the basis of memory and learning depends on the exchange of nerve cells, activity of single neurons, increase in the number and density of

dendritic branching, creation of new synaptic junctions, or the efficiency changes of already existing synapses. These are very complex processes taking place in response to the information coming from the environment. Many substances affect neuronal plasticity including S100β protein actively released from astrocytes. Thus, a large number of cells immunoreactive for S100β in SGZ of the dentate gyrus in chinchilla can be associated with structural reorganisation of the hippocampus and neurogenesis, which is related to the process of learning and development of memory dependent on the hippocampus. Further examinations are necessary for accurate and complete identification of S100β positive cells in the hippocampus of chinchilla.

References

1. Adami C., Sorci G., Blasi E., Agneletti A.L., Bistoni F., Donato R.: S100B Expression in and effects on microglia. Glia 2001, 33, 131–142.
2. Bartkowska K.: Generacja komórek w mózgu dorosłych zwierząt. Kosmos 2004, 53, 167–181.
3. Bendotti C., Guglielmetti F., Tortarolo M., Samanin R., Hirst W.D.: Differential expression of S100B and glial fibrillary acidic protein in the hippocampus after kainic acid–induced lesions and mossy fiber sprouting in adult rat. Exp Neurol 2000, 161, 317–329.
4. Bruel–Jungerman E., Davis S., Rampon C., Laroche S.: Long–term potentiation enhances neurogenesis in the adult dentate gyrus. J Neurosci 2006, 26, 5888–5893.
5. Butt A.M., Hamilton N., Hubbard P., Pugh M., Ibrahim M.: Synantocytes: the fifth element. J Anat 2005, 207, 695–706.
6. Cameron H.A., McKay R.D.: Adult neurogenesis produces a large pool of new granule cells in the dentate gyrus. J Comp Neurol 2001, 435, 406–417.
7. Donato R., Sorci G., Riuzzi F., Arcuri C., Bianchi R., Brozzi F., Tubaro C., Giambanco I.: S100β's double life: intracellular regulator and extracellular signal. Biochim Biophys Acta 2009, 1793, 1008–1022.
8. Förster E., Zhao S., Frotscher M.: Laminating the hippocampus. Nat Rev Neurosci 2006, 7, 259–267.
9. Friedman D.P., Aggleton J.P., Saunders R.C.: Comparison of hippocampal, amygdala, and perirhinal projections to the nucleus accumbens: combined anterograde and retrograde tracing study in the Macaque brain. J Comp Neurol. 2002, 450, 345–365.
10. Garbuglia M., Verzini M., Sorci G., Bianchi R., Giambanco I., Agneletti A.L., Donato R.: The calcium–modulated proteins, S100A1 and S100B, as potential regulators of the dynamics of type III intermediate filaments. Braz J Med Biol Res 1999, 32, 1177–1185.
11. Gazzolo D., Bruschettini M., Corvino V., Lituania M., Sarli R., Bruschettini P., Michetti F.: Amniotic fluid levels of S100B protein in normal and trisomy–21 foetuses. Clin Chim Acta 2003, 330, 131–133.
12. Gerlai R., Wojtowicz J.M., Marks A., Roder J.: Overexpression of a calcium–binding protein, S100 beta, in astrocytes alters synaptic plasticity and impairs spatial learning in transgenic mice. Learn Mem 1995, 2, 26–39.
13. Jastrzębska B., Filipek A.: Function of calcium binding proteins of the S100 family. Post Biol Komórki 2003, 30, 383–397.
14. Jones B.F., Witter M.P.: Cingulate cortex projections to the parahippocampal region and hippocampal formation in the rat. Hippocampus 2007, 17, 957–976.
15. Karram K., Goebbels S., Schwab M., Jennissen K., Seifert G., Steinhauser C., Nave K.A., Trotter J.: NG2–expressing cells in the nervous system revealed by NG2–EYFP–knockin mouse. Genesis 2008, 46, 743–757.
16. Kempermann G., Gast D., Kronenberg G., Yamaguchi M., Gage F.H.: Early determination and long–term persistence of adult–generated new neurons in hippocampus of mice. Development 2003, 130, 391–399.
17. Kiełbiński M., Sołtys Z.: S100B protein, astrocytes and memory. Post Biol Komórki 2008, 35, 3–13.
18. Kleindienst A., McGinn M.J., Harvey H.B., Colello R.J., Hamm R.J., Bullock M.R.: Enhanced hippocampal neurogenesis by intraventricular S100B infusion is associated with improved cognitive recovery after traumatic brain injury. J Neurotrauma 2005, 22, 645–655.
19. Kleindienst A., Ross Bullock M.: A critical analysis of the role of the neurotrophic protein S100B in acute brain injury. J Neurotrauma 2006, 23, 1185–1200.
20. Longstaff A.: Neurobiology. Short lectures. edited by PWN, Warszawa, 2002, pp. 485–504.
21. Ogata K., Kosaka T.: Structural and quantitative analysis of astrocytes in the mouse hippocampus. Neuroscience 2002, 113, 221–233.
22. Pawluski J.L., Galea L.A., Brain U., Papsdorf M., Oberlander T.F.: Neonatal S100B protein levels after prenatal exposure to selective serotonin reuptake inhibitors. Pediatrics 2009, 124, 662–670.
23. Radley J.J., Jacobs B.L.: 5–HT1A receptor antagonist administration decreases cell proliferation in the dentate gyrus. Brain Res 2002, 955, 264–267.
24. Rothermundt M., Peters M., Prehn J.H., Arolt V.: S100B in brain damage and neurodegeneration. Microsc Res Tech 2003, 60, 614–632.
25. Savchenko V.L., McKanna J.A., Nikonenko I.R., Skibo G.G.: Microglia and astrocytes in the adult rat brain: comparative immunocytochemical analysis demonstrates the efficacy of lipocortin 1 immunoreactivity. Neuroscience 2000, 96,195–203.
26. Selinfreund R.H., Barger S.W., Pledger W.J., Van Eldik L.J.: Neurotrophic protein S100β stimulates glial cell proliferation. Proc Natl Acad Sci USA 1991, 88, 3554–3558.
27. Sternberger L.A.: Immunocytochemistry. John Wiley and Sons, New York, 1986.
28. Szalak R., Eustachiewicz R., Lonc G., Łuszczewska–Sierakowska I.: Morphology neurons and topography of formatio hippocampi in chinchillas (Chinchilla brevicaudata). Med Weter 2006, 62, 444–447.
29. Van Praag H., Schinder A.F., Christie B.R., Toni N., Palmer T.D., Gage F.H.: Functional neurogenesis in the adult hippocampus. Nature 2002, 415, 1030–1034.