

Morphological and quantitative analysis of cerebellar cortical cells in Alzheimer's disease

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Folia Neuropathol 2012; 50 (3): 250-260

DOI: 10.5114/fn.2012.30525

Abstract

Alzheimer's disease (AD) is a neurodegenerative amyloid disease, although a great deal of research has been done, its aetiology is still unknown. In Khachaturian's hypothesis on the involvement of calcium in the aetiology of Alzheimer's disease, particular attention is paid to the disorder of calcium metabolism. Ludo van Bogaert, describing AD, has drawn attention to the presence of a multi-system damage to the brain and described four forms of the disease, including two cerebellar types: cerebellar-pyramidal and cerebellar. The aim of our study was to analyze the expression of calcium-binding proteins (calbindin, calretinin, parvalbumin) in cortical neurons of the cerebellum in patients diagnosed with Alzheimer's disease (experimental group) and in a control group (patients without Alzheimer's disease). We performed the quantitative analysis of the density of Purkinje cells and Bergmann glial cells in the cerebellar cortex. We observed weak immunoreaction with a calretinin antibody in Lugaro cells, and with parvalbumin in Purkinje cells in the experimental group. A weaker expression of calcium-binding proteins in the experimental group may indicate the disturbance of the transport and buffering of intracellular Ca^{2+} levels. The quantitative analysis showed that the density of Bergmann glial cells was higher in the experimental group. Our study suggests the disturbance of calcium metabolism in Alzheimer's disease.

Key words: Alzheimer's disease, cortical neurons of cerebellum, calcium-binding proteins.

Introduction

Alzheimer's disease (AD) is a neurodegenerative amyloid disease, although a great deal of research has been done, its aetiology is still unknown. It is known that AD is associated with the impairment of certain metabolic pathways and its incidence increases with age. In Khachaturian's hypothesis on the involvement of calcium in the aetiology of Alzheimer's disease, particular attention is paid to the disorder of calcium metabolism [25]. Ca^{2+} , a secondary signal

molecule of a properly functioning cell located in the cytoplasm and the light of the endoplasmic reticulum (ER), is responsible for internal and external communication service. The concentration of Ca^{2+} in the endoplasmic reticulum is 1000 times higher than in the cytoplasm. During activation, Ca^{2+} is released from the endoplasmic reticulum to the cytoplasm through inositol 1,4,5-trisphosphate (IP_3) receptors and ryanodine receptors (RyR) involved in the internal induction of apoptosis [6]. There is a group of calcium-binding proteins (CaBP), strongly evolutionarily conserved.

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CaBPs are divided into two groups: proteins binding and buffering Ca^{2+} levels in the cell (parvalbumin, calretinin and calbindin) and CaBP for Ca^{2+} translocation to the cell, participating in the transduction signal (calmodulin). Calbindin-D28k appears first as early as at 4-5 hbd, but not later than the expression of parvalbumin occurs in the development process. CaBPs play a neuroprotective role and via Ca^{2+} binding inhibit caspase activity in the cell. It is also suggested that CaBPs are involved in the regulation of cell division and maturation of neurons in the cerebellum [3,29,31,55].

During the course of Alzheimer's disease the functions of the brain undergo different forms of gradual damage, causing an insidious and difficult to capture onset of the disease. Ludo van Bogaert, describing AD, has drawn attention to the presence of a multi-system damage to the brain and described four forms of the disease, including two cerebellar types: cerebellar-pyramidal and cerebellar [5]. Despite the features of the cerebellar cognitive-affective syndrome present in AD, other regions of the brain, including cerebellum, are collected optionally for neuropathological diagnosis.

The cerebellum is also linked with the structures associated with cognitive and behavioural functions: dorsal-lateral prefrontal cortex, medial frontal cortex, parietal lobe and the upper part of the temporal lobe, anterior cingulate, and the rear part of the hypothalamus [11]. Noradrenergic fibers, serotonergic and dopaminergic nuclei of the brainstem and cholinergic fibres reach the cerebellum. Noradrenaline facilitates learning processes occurring in the cerebellar cortex. The dorsal and ventral parts of the locus coeruleus are the source of noradrenergic fibres reaching all the layers of the cerebellar cortex [14,26]. Serotonin controls the response within the feedback loop involved in learning processes. Serotonergic fibres form a dense network of the cerebellar cortex, especially in Purkinje cells and granular layer [22,23,34]. Dopamine facilitates learning associated with intentional responses [47] through a cascade of cAMP and dopamine and cyclic AMP regulated phospho-protein-32 (DARPP-32), by modifying the sensitivity of Purkinje cells GABA [21]. Acetylcholine induces a strong and long-lasting response of Purkinje cells to glutamate, strengthening the synaptic transmission between Purkinje and granular cells [2,15].

The cerebellar cortex consists of three layers: an outer molecular layer, a middle Purkinje cell layer (ganglionic layer) and an inner granular layer. In the molecular layer two groups of interneurons are distinguish-

ed: stellate and basket cells. A middle Purkinje cells layer consists of neurons about 70 μm in diameter. Purkinje cells are involved in many learning and memory processes, as well as in the process of neurons migration [47, 48]. Purkinje cells comprise all known types of inositol triphosphate (IP_3) receptors, which facilitate Ca^{2+} transportation, glutamatergic receptors, and glutamic acid decarboxylase (GAD) protein, an enzyme involved in the GABA metabolism, and cholinergic receptors implicated in the process of memory renewal [47]. In the ganglionic layer, besides Purkinje cells, there are Bergmann glial cells, a group of specialized astrocytes involved in synaptogenesis and signal transduction between Purkinje cells [7,33,36]. Of the four known subtypes of glutamate transporters, glutamate aspartate transporter (GLAST) and glutamate transporter-1 (GLT-1) are characteristic for astrocytes, and their expression occurs mainly in the cerebellum. Bergmann glial cells are engaged in the process of neurotransmitter amino group removal from the synaptic space [45,50].

In the granular layer, four types of interneurons are distinguished. Golgi small cells (granular cells) less than 6 μm in diameter, which form numerous synapses with Purkinje cell dendrites are thought to be the smallest human brain cells. Golgi large cells (size of 10-20 μm) are involved in the transmission of cortical feedback [27]. They form synapses with granular cells and molecular layer cells. Lugaro cells (size of 10-20 μm) occur in the upper part of the granular layer and form links with Purkinje cells, Golgi large cells, stellate and basket cells [30]. On account of their structure two types of Lugaro cells, two- and three-armed, are known [35]. Finally, there is one more type of cells, unipolar brush cells (UBC), a group of small interneurons (size of 8-14 μm), primarily regarded as a subclass of Golgi large cells characterized by the structure similar to a brush [10,13,20,36].

The aim of our study was to analyze the expression of CaBPs (calbindin, calretinin, parvalbumin) in cortical neurons of the cerebellum in patients diagnosed with Alzheimer's disease and to perform the quantitative analysis of the density of Purkinje cells and Bergmann glial cells in the cerebellar cortex.

Material and methods

The study material was examined with the use of light microscopy. The control group comprised cerebellar fragments derived from 15 patients (mean 75.67

years) who showed neither Alzheimer’s disease nor extensive neuropathological changes in the brain, especially in the cerebellum, and died from other diseases (e.g. cardio-pulmonary failure, heart attack, ischemic stroke). The experimental group consisted of cerebellar fragments derived from 13 patients (mean 76.77 years) diagnosed with Alzheimer’s disease lasting not longer than 10 years. After autopsy all cerebellar fragments were fixed in formalin, embedded in paraffin, and stained with haematoxylin-eosin, cresyl violet and Klüver-Barery to identify morphology.

The specimens of cerebellum were stained immunohistochemically with antibodies calbindin D-28k (CB) (Sigma, 1 : 2500), calretinin (CR) (DAKO, 1 : 75), parvalbumin (PV) (Sigma, 1 : 2000), and glial fibrillary acidic protein (GFAP) (Leica, 1 : 100).

The density of Purkinje cells and Bergmann glial cells, in the region of cortex declive surface of 14.6 mm², was analyzed in the experimental and control groups (Fig. 1). Preparations were analyzed in Zeiss Axiophot light microscope and then recorded by a set of scanning, including a table with unit labour Märzhäuser LUDL and KS RUN library. STATISTICA 9 was employed to analyze the results obtained. Spearman and Pear-

son’s correlation tests for the density of Purkinje and Bergmann glial cells, as well as analysis of variance ANOVA were performed.

Results

Molecular layer

In the material derived from patients not diagnosed with Alzheimer’s disease (control group) stellate cells staining in hematoxylin-eosin (H&E) were observed in the molecular layer at a height of roughly 2/3 of the Purkinje cell layer. They were characterized by the scant cytoplasm and dark nucleus. In staining with parvalbumin antibody only a weak immunohistochemical (IHC) reaction was observed (Table I) (Fig. 2A). In the patients with diagnosed Alzheimer’s disease (experimental group) stellate cells, observed in the molecular layer, did not show morphological differences. In IHC response a similar, equally IHC reaction with parvalbumin was noted (Fig. 2B). In light microscopy, H&E staining basket cells formed a group of cells with oval nuclei, located at a height of 1/3 width of the molecular layer above the Purkinje cells layer. In the control group, H&E staining basket cells showed a well pigment-

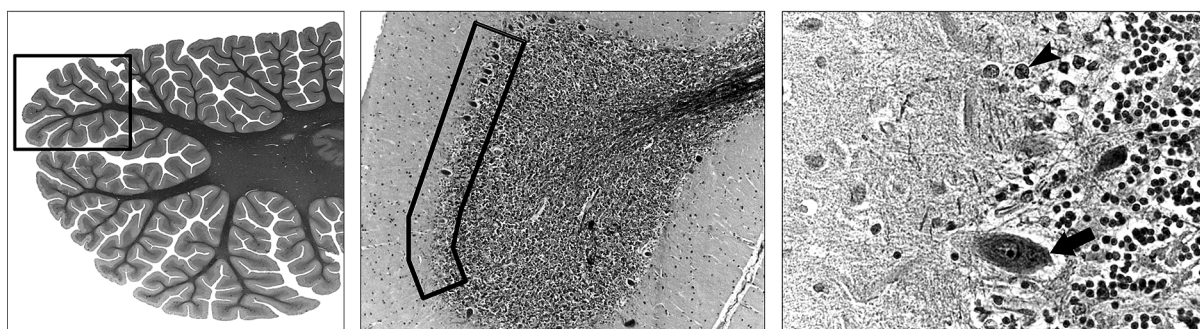


Fig. 1. Scheme of quantitative analysis of cerebellar cortex declive surface. Purkinje cells (arrow), Bergmann glial cells (head arrow).

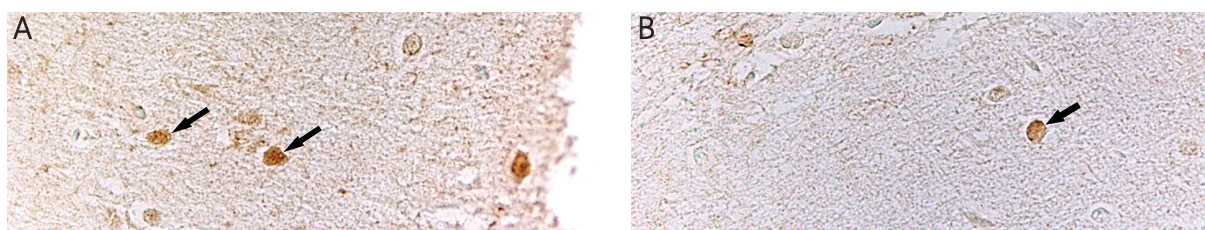


Fig. 2. A) Control group. Stellate cells of the cerebellar cortex (arrows). Parvalbumin. Magnification × 400. B) Alzheimer’s disease group. An IHC reaction in stellate cells (arrow). Parvalbumin. Magnification × 400.

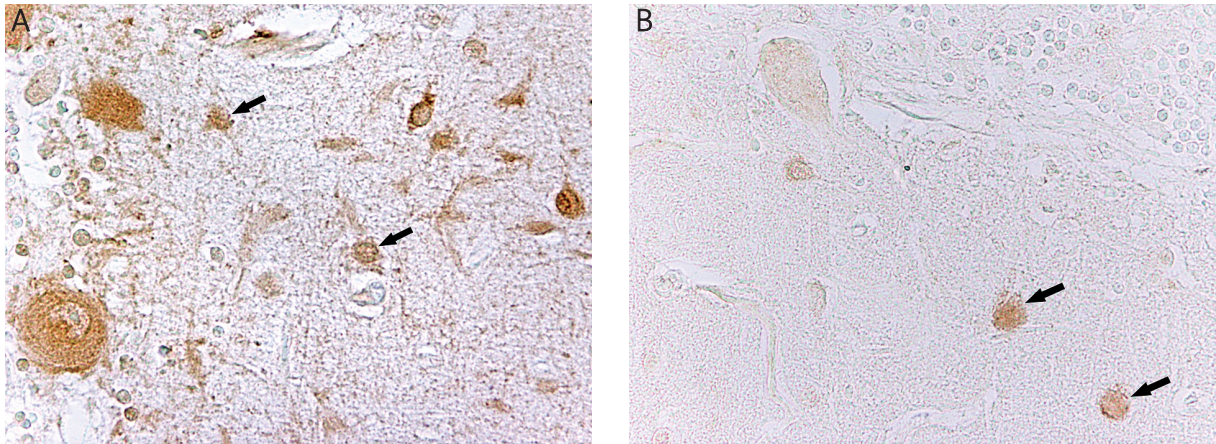


Fig. 3. **A)** Control group. Parvalbumin: a slight IHC reaction in basket cells of the cerebellar cortex (arrows). Magnification $\times 400$. **B)** Alzheimer's disease group. Parvalbumin: an IHC reaction in basket cells (arrows). Magnification $\times 400$.

ed, sparse, light pink cytoplasm. In the experimental group, a picture of the basket cell morphology did not differ from that of caged cells in the control group. In the control group, basket cells showed a very clearly marked reaction only with parvalbumin antibody (Fig. 3A). The basket cells showed neither reaction with calbindin-D28k nor with calretinin antibodies (Table I). In the experimental group, a very weak IHC reaction with parvalbumin was observed only in single basket cells (Fig. 3B).

Ganglionic layer/Purkinje cells layer

In the control group, Purkinje cells in H&E staining showed a well preserved shape and uniformly stained bright pink cytoplasm. A fragment of axon and single dendrites could be sometimes seen. In the control group, uniformly and strongly labelled perikaryon and the Purkinje cell axon were observed in calbindin-D28k labelling (Fig. 4A). In the experimental group, the IHC reaction with calbindin-D28k labelling was as strong as that in the control group. The reaction

Table I. Immunohistochemical reaction in cerebellar cortical cells in control and experimental groups (AD).

Cerebellar cortical cells	Antibody							
	Calbindin D-28k (CB)		Calretinin (CR)		Parvalbumin (PV)		GFAP	
	Control	AD	Control	AD	Control	AD	Control	AD
Stellate cells	N	N	N	N	P	P	N	N
Basket cells	N	N	N	N	P	S-L	N	N
Purkinje cells	P	P	N	N	S-P	S-L	N	N
Lugaro cells	N	N	P	L	N	N	N	N
Golgi small cells	N	N	N	N	N	N	N	N
Golgi large cells	N	N	P	P	N	N	N	N
Unipolar brush cells	N	N	P	P	N	N	N	N
Bergmann glial cells	N	N	N	N	N	N	S-P	S-P

N – negative, *P* – positive, *L* – low sensitivity, *S* – some of cells

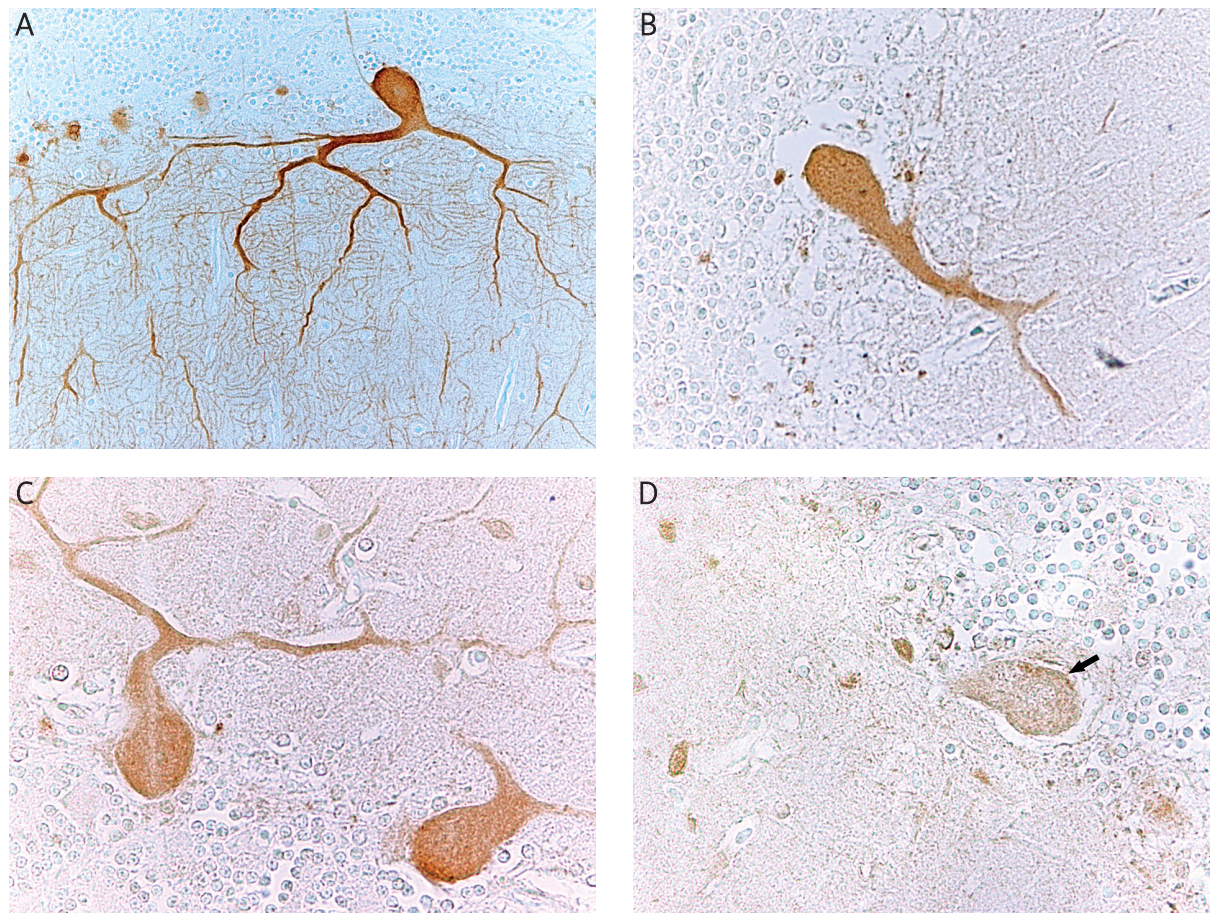


Fig. 4. **A)** Control group. Calbindin: a strong IHC reaction in Purkinje cells and their processes. Magnification $\times 400$. **B)** Alzheimer's disease group. Calbindin: a strong reaction in Purkinje cells and a weak reaction in their processes. Magnification $\times 400$. **C)** Control group. Parvalbumin: a strong reaction in Purkinje cells and a weaker reaction in their axon. Magnification $\times 400$. **D)** Alzheimer's disease group. Parvalbumin: a weaker IHC reaction in Purkinje cells (arrow). Magnification $\times 400$.

occurred in perikaryon and Purkinje cell axons. An IHC reaction with calbindin-D28k was observed in all Purkinje cells (Fig. 4B). In calretinin labelling no IHC reaction in Purkinje cells was observed in any of the groups under study. In parvalbumin labelling a stronger immunohistochemical reaction in perikaryon and a weaker reaction in the Purkinje cells axon were found (Fig. 4C). In the experimental group, H&E staining, showed in some cases blurred boundaries of Purkinje cells and the cytoplasm stained lighter compared with the control group. In parvalbumin labelling a weak IHC reaction in axon and perikaryon was revealed (Table I). Relatively small amounts of Purkinje cells were found in both study groups (Fig. 4D).

In the control group, Bergmann glial cells placed near the Purkinje neurons were observed in both H&E

staining and in the reaction with GFAP antibody. In H&E staining Bergmann glial cells were visible as a dispersed chromatin nuclei sometimes with a narrow seam of cytoplasm with clearly visible nucleoli without visualization of the processes. In the reaction with GFAP antibody, Bergmann glial cells became visible as a bright nucleus and a few thick insets, laying parallel to the layers of web press but also running perpendicular to the surface of the cerebellar cortex. Not all cells with Bergmann glial morphology, showed a positive reaction with GFAP antibody (Fig. 5A). The cellular nuclei were slightly enlarged and brighter. In the reaction with GFAP antibody only single cells showed a positive reaction (Table I). Gliofilaments, showing a positive reaction with GFAP antibody were mostly in short, thickened glial processes (Fig. 5B).

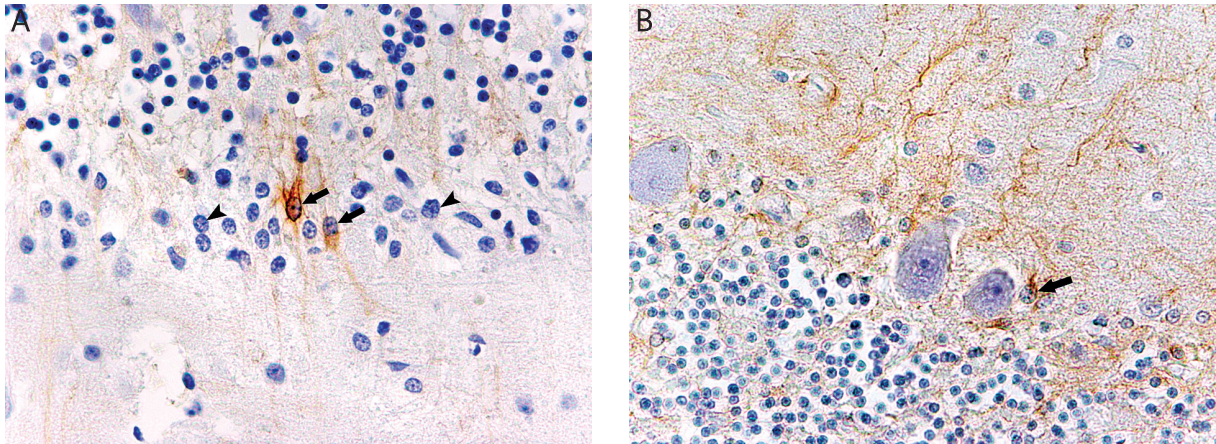


Fig. 5. **A)** Control group. Bergmann glial cells of the cerebellar cortex with an IHC reaction (arrows) and without immunoreaction (head arrows). GFAP. Magnification $\times 400$. **B)** Alzheimer's disease group. A GFAP positive reaction in Bergmann glial cells with thickened glial processes (arrow). Magnification $\times 400$.

Granular layer

In the control group, H&E stained large Golgi cells did not exhibit characteristic features that allow to distinguish them from UBCs. In the control group, calretinin-labelled large Golgi cells showed a positive reaction in perikaryon (Fig. 6A). In the experimental group, the IHC reaction intensity was comparable to the response observed in the control group (Fig. 6B). In the control group, small Golgi cells in the granular layer formed a well-distinguished group of rather evenly distributed neurons. In the H&E staining, this group was characterized by a dark round nucleus and almost invisible cytoplasm. We also observed decreased

density of small Golgi cells. No positive IHC reactions with calbindin-D28k, calretinin or parvalbumin antibodies were observed. In the control group in H&E, cresyl violet and Klüver-Barrera staining Lugaro cells were characterized by a horizontal arrangement of the granular layer of cerebellar cortex under the Purkinje cells layer. In the control group, in the IHC reaction with calretinin, Lugaro cells showed a calretinin-positive reaction, visible in the cytoplasm of neurons and in the processes of both cellular type (Fig. 7A). In the experimental group, an expression of calretinin was significantly weaker than that in the control group (Fig. 7B). In the control group, H&E stained UBCs did not exhibit characteristic features that allow to distinguish

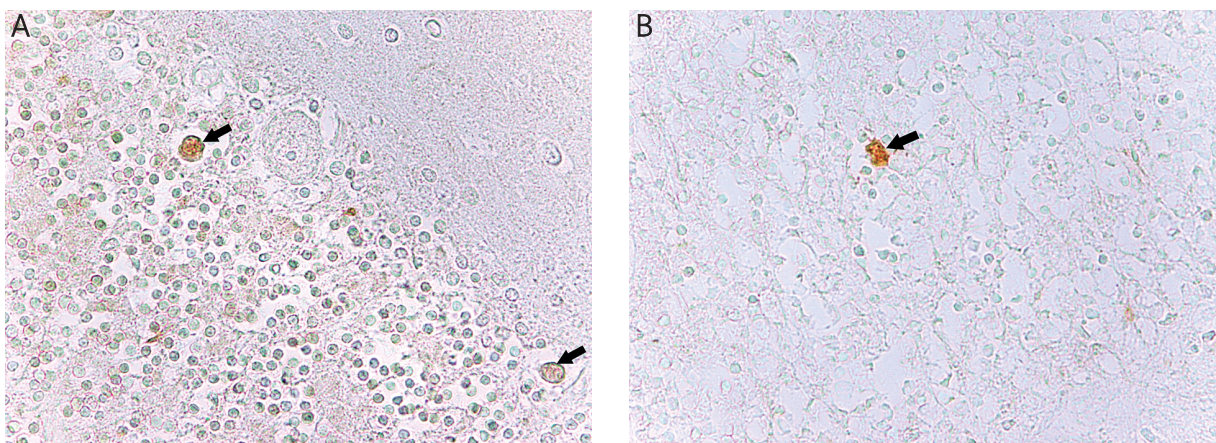


Fig. 6. **A)** Control group. Calretinin: a positive reaction in Golgi cells of the cerebellar cortex (arrows). Magnification $\times 400$. **B)** Alzheimer's disease group. Calretinin: a reaction in Golgi cells (arrow). Magnification $\times 400$.

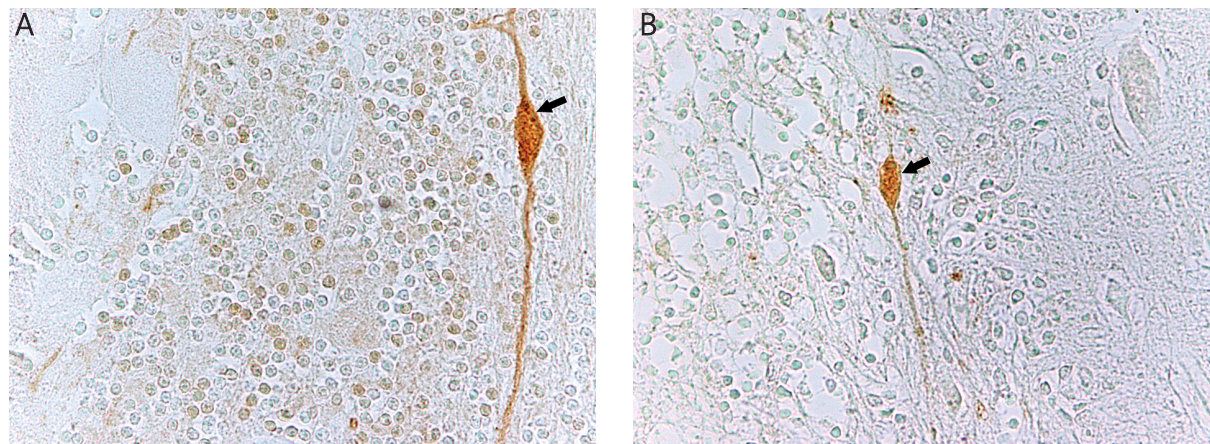


Fig. 7. **A)** Control group. Calretinin: an intense reaction observed in Lugaro cells (arrow). Magnification $\times 400$. **B)** Alzheimer's disease group. Calretinin: a weak reaction IHC in Lugaro cells of the cerebellar cortex (arrow). Magnification $\times 400$.

them from Golgi large cells (Table I). In both study groups, UBCs showed the same reaction with calretinin antibody in the neuron cytoplasm and axon initial segments (Figs. 8A and B).

Quantitative analysis

The density of Purkinje cells and Bergmann glial cells in the region of cortex declive surface of 14.6 mm^2 were analyzed in both study groups. Due to aging changes, the experimental and control groups were divided into two age groups, under 80 (< 80) and over 80 (≥ 80) years of age and in the experimental group, the disease duration was less than 10 years (< 10 years). Aver-

age density of Purkinje cells in cerebellar cortex was 9.33 ± 2.42 and 9.11 ± 2.07 in the experimental group and control group, respectively. Average density of Purkinje cells in the control subgroup was 9.37 ± 1.94 (< 80) and 8.73 ± 2.39 (≥ 80) and in experimental subgroups, it was 9.75 ± 2.12 (< 80) (Fig. 9) and 8.85 ± 2.86 (≥ 80) (Fig. 10).

Average density of Bergmann glial cells in the cerebellar cortex was 72.10 ± 32.11 and 58.49 ± 14.09 in the experimental group and the control group, respectively. Average density of Bergmann glial cells in the experimental subgroups was 81.27 ± 39.22 (< 80) and 61.39 ± 19.33 (≥ 80) and in the control subgroups it was 59.02 ± 16.93 (< 80) and 57.69 ± 9.78 (≥ 80).

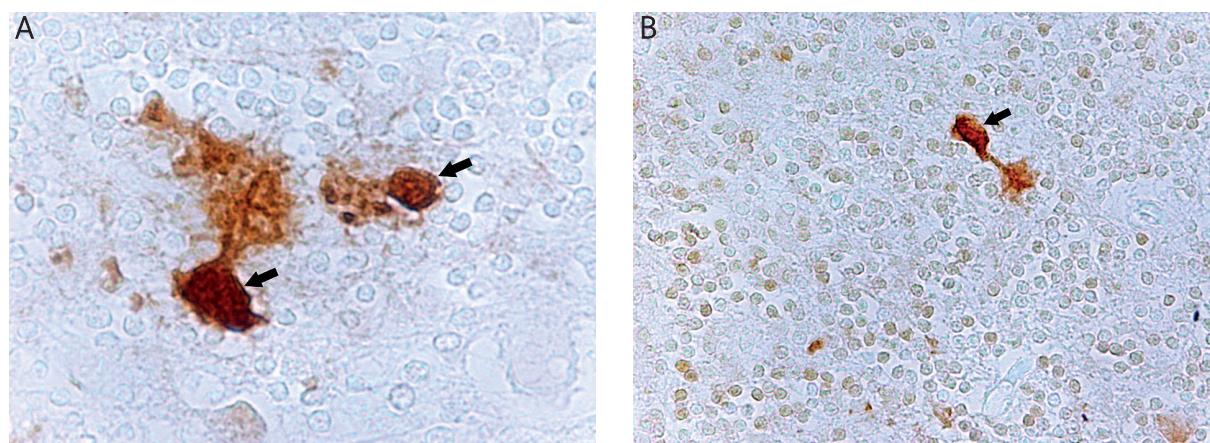


Fig. 8. **A)** Control group. A very intense calretinin reaction observed in unipolar brush cells and their processes (arrows). Magnification $\times 630$. **B)** Alzheimer's disease group. A comparable calretinin IHC reaction in unipolar brush cells to the control group (arrow). Magnification $\times 400$.

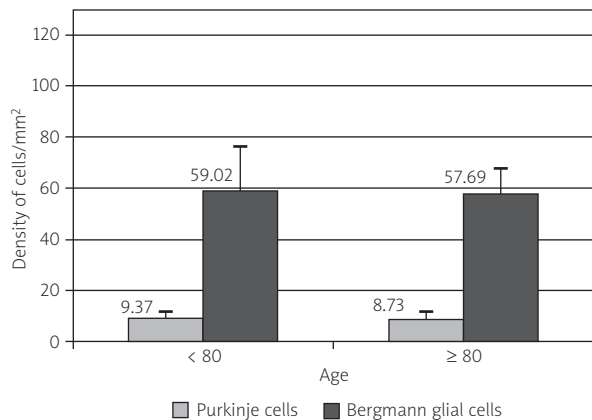


Fig. 9. Average value density of Purkinje cells and Bergmann glial cells in the cerebellar cortex in the control group below 80 years (< 80) and over 80 years (≥ 80) ($p = 0.05$).

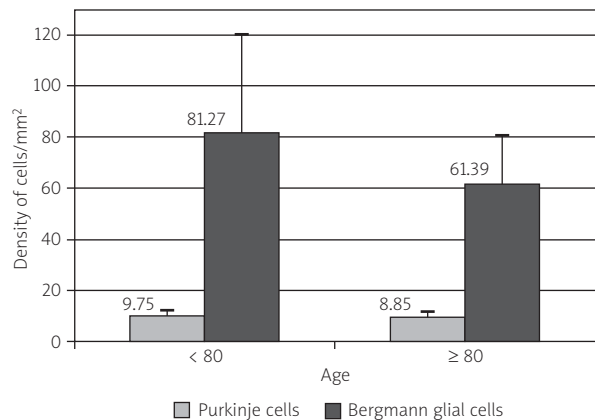


Fig. 10. Average value density of Purkinje cells and Bergmann glial cells in the cerebellar cortex in the experimental group below 80 years (< 80) and over 80 years (≥ 80) ($p = 0.05$).

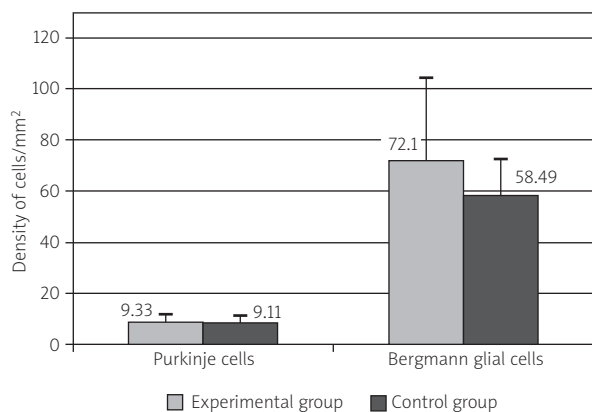


Fig. 11. Average value density of Purkinje cells and Bergmann glial cells in the cerebellar cortex in the control and experimental group ($p = 0.05$).

The analysis of the density of Purkinje and Bergmann glial cells showed slightly higher values in the experimental group; on average there were 6.4 Bergmann glial cells per one Purkinje cell in the control group and 7.7 cells in the experimental group. The results of the quantitative analysis showed slight differences in the density of Purkinje cells and Bergmann glial cells in the cortex of the cerebellum between the study groups. The relationship between the density of Purkinje cells, Bergmann glial cells and age (Spearman and Pearson correlation tests) showed no statistically significant differences ($p = 0.05$) between the groups and within them. Testing the hypothesis of homogeneity of variance (Levene's test) indicated that the assumption of homogeneity of

variance was satisfied only for Bergmann glial cells (0.918). However, the analysis of variance (ANOVA) for univariate classification did not reveal any statistically significant differences (0.841) in the Bergmann glial cell density between the groups and within them.

Discussion

The changes in the Purkinje cells observed in the experimental group occur in the blurred boundaries of neurons and brighter, homogeneous staining of the cytoplasm. This type of morphological changes, observed in light microscopy, has been described as a disease of non-specific mixing degeneration of neurons [12]. This type of neuronal degeneration results most frequently from ischemic/hypoxic neurons and concerns Purkinje cells usually accompanied by Bergmann glial cells proliferation and microglia. The quantitative analysis showed that the ratio of Purkinje cells to Bergmann glial cells was higher in the experimental group than in control group and amounted to 7.7 and 6.4, respectively. Similar observations were described by other authors [16,39].

The fact that there were no differences in calbindin-28Dk expression in Purkinje cells in both groups does not confirm the reports on the neuroprotective role of calbindin-28Dk [17,19]. None of the study groups showed differences in the intensity of calretinin reaction in UBC, either in perikaryon neurons, initial segments of axon or large Golgi cells of the granular layer. In the experimental group, a significantly weaker IHC reaction in Lugaro cells than in the control group

was observed. Despite conflicting reports on the calretinin expression in the ganglionic layer, we observed no IHC reaction in Purkinje cells [41,46]. However, this situation can be explained by the fixation procedure and conduct of the material, which caused damage to the less stable calretinin domain in Purkinje cells (Table I) [40].

A positive reaction with the antibody against parvalbumin, without changed expression intensity in both study groups was observed in neurons of molecular layers in stellate cells, while the weakening of the reaction in the basket cells and Purkinje cells was found in the experimental group. In Purkinje cells there are all known types of IP₃ receptors [18]. Parvalbumin plays an important role in the transport and buffering of intracellular Ca²⁺ levels. Interacting with IP₃ and RyR receptors, it regulates the flow of Ca²⁺ from the endoplasmic reticulum. A weaker expression of parvalbumin may indicate the disturbance of this mechanism.

A GFAP expression was observed in some Bergmann glial cells. Due to a small amount of fibrillary acidic protein, not all Bergmann glial cells were labelled with GFAP. Bergmann glial cells are involved in signal transduction between Purkinje cells [32,33,36]. Astrocytes play an important role in the removal of amino residues of neurotransmitters from synaptic space. Of the four known subtypes of glutamate transporters, GLAST and GLT-1, are characteristic for astrocytes, and their expression occurs mainly in the cerebellum, in the Bergmann glial cells [45,50]. The GLAST transporter is involved in transformation of the dendritic tree from the multi- to single-pole changes Purkinje cells [52]. There are reports on the increased degeneration of granular cells and Purkinje cells correlated with the reduced expression of GLAST [9] so that the protective role of Bergmann cells and their involvement in the protection of neurons against glutamate toxic effects has increased [43,44,51]. Studies carried out on GLAST^{-/-} mice showed an impaired motor function and an increased susceptibility to cerebellar injury [53]. This can be explained by the occurrence of cerebellar ataxia in Alzheimer's patients which can be caused by the damage to GLAST transporter and its reduced expression. It is likely that the death of neurons, β-amyloid cytotoxicity and atrophy of astrocytes, make it more difficult to capture amino residues of alternating glutamate providing a complete picture of the pathomechanism of Alzheimer's disease [42].

In the study material, histological staining revealed a slight increase in Bergmann glial cell density and

a brighter nuclear chromatin. Thus, it appears that this pathomorphological change in Bergmann glial cells, in the pool of star-shaped glial cells probably indicates pathognomic changes of Alzheimer's disease accompanied by astroglia [37,42]. In Alzheimer's disease pathomorphological changes in astrocytes located around senile plaques in perivascular astroglia, as well as in other forms of neuroglia are described. It becomes even more clear that astroglia are actively involved in the pathogenesis of Alzheimer's disease. In astrocytes apolipoprotein E (ApoE) is synthesized, as well as pro-inflammatory proteins and a form of the enzyme induction of nitric oxide synthase (NOS) are produced. These compounds are implicated in the pathomechanism of AD development [56]. In the brains derived from the patients of the experimental group, a decreased density of Golgi small cells in the granular layer was also observed, however, it was not subjected to the quantitative analysis. The atrophy of the granular layer is usually described in paraneoplastic syndromes, alcohol encephalopathy, but it also occurs in the ischemic/hypoxic brain [12,28,38]. In Alzheimer's disease atrophy of the cerebellar vermis, atrophy of the Purkinje cells and gliosis in the molecular layer have also been reported [49]. In patients with AD advanced stages, some authors have noted the reduction of Purkinje cells up to 32% [54].

The results of the quantitative analysis show a higher density of Purkinje cells in the cerebellar cortex in patients below 80 years of age in both study groups (Figs. 9 and 10). This may indicate that in patients aged under 80 years, changes in the density of Purkinje cells did not produce the picture of progressive pathology in any of the study groups. The reduced Purkinje cell density in patients over 80 years of age in both groups may indicate progressive degenerative processes due to physiological aging and also in the mechanism of AD development. Analyzing the density of Purkinje and Bergmann glial cells, one should remember about cerebellar progressive atrophy in Alzheimer's disease, frequently reported in the literature, which can affect the density of both types of cells [1,4,54]. Purkinje cells are particularly sensitive, they may die in many pathologies and normal aging. It could be explain why we did not observe any changes in the density of Purkinje cells between the control and experimental groups. The analysis of Purkinje cell density in the experimental and control groups did not show statistically significant differences (Fig. 11). The increased density of Bergmann glial cells in the

patients below 80 years of age of both study groups can imply a protective role of protoplasmic glial cells. The correlation between the occurrence of fronto-temporal dementia and increased gliosis was also observed [8]. It was found that in early stages of fronto-temporal dementia, the amount of cortical astroglia may be four or even five times higher in the experimental group than in controls [24]. This may evidence the protective role of Bergmann glial cells involved in the process of removing toxic glutamate residues. In view of numerous clinical symptoms, indicating the signs of the cerebellar cognitive-affective syndrome, the results of these studies suggest that the diagnosis of Alzheimer's disease should be supplemented by the neuropathological analysis of the cerebellum.

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