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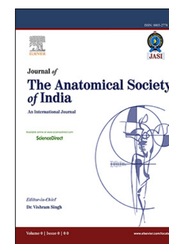

Original Article

# Morphometric analysis of the age-related changes of synaptophysin immunoreactivity in the human parahippocampal region

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ABSTRACT

*Introduction:* The aim of our research was to quantify the synaptic density in the parahippocampal region of the brain with densitometric measurements of synaptophysin immunoreactivity in cases of different ages.

*Methods:* The study was performed on post mortem obtained right parahippocampal region (PR) samples of 14 cadavers (10 males, 4 females) whose ages ranged from 35 to 80 years. The samples were classified into three age groups (I: 30–49; II: 50–69; III: 70 and older). The tissue was immunohistochemically processed and stained with an anti-human synaptophysin antibody. The densitometric analysis included measurement of the mean and median optical density (OD) of the PR gray and white matter (WM) digital images. This was performed with ImageJ.

*Results:* The histological analysis showed that the neuropil of the PR's layers showed two types of synaptophysin positive reactions. The first type was characterized by the presence of a granular pattern of synaptophysin immunoreactivity, and this was less intense in the second type. The synaptophysin immunopositivity of the PR gray matter neuropil decreased with age, especially in its superficial layers. The results of the densitometric analysis confirmed the significant decrease of the gray matter mean and median OD during aging. A One Way ANOVA showed that only the mean and median optical densities of the third age group were significantly lower than the values for the first age group.

*Discussion:* The synaptic density decreases with age in the PR, but this decrease is significant only in cases older than 70 years.

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## 1. Introduction

The parahippocampal gyrus is a gross anatomical term for the portion of the brain located at the inferomedial surface of the temporal lobe in primates and humans. In comparative framework, it is frequently named the parahippocampal region (PR), in which individual areas differ in their structural and connectional organization. It can be divided into the following regions: perirhinal, postrhinal, and entorhinal in rodents[1] or temporopolar, perirhinal, posterior parahippocampal, and entorhinal in humans.[2,3] In humans, the anterior tip of the PR is formed by the temporopolar cortex. Regions posterior to temporopolar are the entorhinal medially and the perirhinal cortices laterally. At the caudal end of the PR is the posterior parahippocampal cortex.[3] Some authors include the parasubiculum and presubiculum as the medial most parts of the PR (ref. 4). Histologically, the entorhinal, medial perirhinal cortices, parasubiculum, and presubiculum represent the periallocortex. The temporopolar cortex comprises the primary olfactory cortex, lateral perirhinal and posterior parahippocampal cortices are of isocortical type.[4]

The cortical areas such as the limbic lobe, visual, auditory, somatosensory, and multimodal association areas project either to the perirhinal or postrhinal area, which are further connected to the lateral and medial entorhinal cortices, respectively.[1,5] Axons of the entorhinal cortex neurons form the largest associational pathway of the temporal lobe, the perforant pathway. The perforant pathway shows a topographical organization with layer II component projecting to the dentate gyrus and hippocampal CA3 field, and layer III component terminating in the CA1 hippocampal area and subiculum.[5] To a lesser extent, the entorhinal cortex and hippocampal formation receive direct input from the latter mentioned associational systems. The deep layers of the entorhinal cortex mediate feedback projections of the hippocampal formation to the limbic lobe, orbitofrontal, medial frontal, anterior temporal and posterior temporal association cortices. From the above cited facts, it can be concluded that the PR controls the majority of the cortical input to the hippocampus.[1,6]

The cortical areas of the PR, together with the amygdala and hippocampus, form the system known as the medial temporal lobe. It is generally accepted that this system as a whole is somehow involved in memory processing. The fact that its components are seriously damaged in Alzheimer's disease (AD), a neurological disorder that represents a form of pathological brain aging accompanied by progressive loss of memory function, indirectly confirms this assumption.[2] Normal brain aging is associated with a gradual decline of cognitive function. Declarative memory mediated by the hippocampus as a part of medial temporal lobe and working memory mediated by the dorsolateral prefrontal cortex are the components of such decline that are the most vulnerable to aging.[7] To explain memory impairment in healthy aged individuals, numerous studies regarding the medial temporal lobe age-related changes were performed. The hippocampus was the most extensively analyzed region. The fact that the majority of the cortical input to the hippocampus is funneled through the PRs suggests that the PRs likely contribute to memory processes. Their different structures and connections indicate that they do not simply conduct but also process information for the hippocampus.[1] That is why aging changes of these cortical mantle areas have become the subject of numerous recent studies.

Neuroradiological studies most frequently show significant atrophy of the entorhinal cortex in patients suffering from mild cognitive impairment and at different stages of AD in relation to healthy age-matched individuals.[8–10] The results of the longitudinal studies, performed to evaluate the dynamics of the medial temporal lobe component volumetric changes of the same individuals during the aging process, varied from the detection of minimal changes to an increased annual percentage of atrophy after the age of 50 (ref. 11–14).

Histological studies, performed on post mortem tissue from AD patients, have shown that the extensive presence of neurofibrillary pathology, neuronal cell loss, and the loss of synapses are observed first in the PR and later in other brain regions.[2] Researchers assumed that neuronal death in the medial temporal lobe of healthy aged individuals might alter the input in the hippocampus and consequently alter hippocampal learning.[15] Stereological studies that were focused on the quantification of neuronal numbers showed that besides a limited neuron loss within some hippocampal regions (the hilus and subiculum), there is no evidence for more extensive neuronal loss in this brain area.[15–18]

Current techniques use several proteins, which are integral synaptic components for the quantification of synapses. Syntaxin 1 and SNAP-25 are localized in the presynaptic plasma membrane. Synaptophysin, synaptobrevin, synaptotagmin, GAP-43, Rab3A, SV2, synapsin I, and EP10 represent synaptic vesicle proteins. Finally, neurogranin and synaptopodin are the components of the postsynaptic membrane.[19,20] Immunohistochemical method quantifies synaptic density by labeling presynaptic or synaptic vesicles proteins. Opposite to the immunoblotting, it preserves structural integrity of the tissue, maintaining the ability to measure immunoreactivity within different cortical layers. However, fixation of the tissue, which precedes immunohistochemical staining prevents precise determination of an absolute concentration of the analyzed protein in a given fragment of the brain tissue.[20]

Synaptophysin is the most abundant synaptic vesicle protein and is therefore often measured in attempts to quantify synapses. It is described as one of the most ubiquitous synaptic components of the nervous system, and is considered to be a direct measure of the presence of mature synapses.[20,21] Synaptophysin is a member of the physin family, which consists of synaptophysin, synaptoporin, pantophysin, mitsugumin, and synaptogyrin. Little is known about a definitive role of physins in the synaptic vesicle cycle. According to Rizzoli[22] synaptophysin interacts with cholesterol forming a core structure of the vesicle. Also, there are evidences, which indicate that it is a component of the vesicle trafficking mechanism important for fine tuning of neurotransmitter release, which effects memory and learning phenomenon such as long-term potentiation.[23]

All of these data and the fact that some newer studies in humans and animals suggested that age-related cognitive decline is linked with synaptic alterations rather than with

**Table 1 – Mean and median OD of the GM and WM of the PR in analyzed cases.**

| Case | Age | Age group | Gender | Cause of death | Postmortem interval (h) | Gray matter | | White matter | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Mean OD | Median OD | Mean OD | Median OD |
| 1 | 35 | I | Male | Asphyxia | 18 | 1.286 | 1.293 | 0.922 | 0.914 |
| 2 | 37 | I | Male | Asphyxia | 48 | 1.582 | 1.616 | 1.021 | 1.016 |
| 3 | 42 | I | Male | Drowning | 37 | 1.725 | 1.782 | 1.121 | 1.111 |
| 4 | 42 | I | Female | Corrosive poisoning | 41 | 1.620 | 1.644 | 1.032 | 1.024 |
| 5 | 43 | I | Male | Heart failure | 24 | 1.715 | 1.796 | 1.227 | 1.228 |
| 6 | 55 | II | Male | Poisoning | 9 | 1.377 | 1.377 | 1.047 | 1.046 |
| 7 | 56 | II | Male | Poisoning | 14 | 1.486 | 1.507 | 1.040 | 1.026 |
| 8 | 57 | II | Female | Cardiac arrest | 35 | 1.471 | 1.480 | 1.031 | 1.025 |
| 9 | 61 | II | Male | Traumatic cardiac rupture | 21 | 1.407 | 1.421 | 0.997 | 0.995 |
| 10 | 61 | II | Male | Lung cancer | 24 | 1.462 | 1.471 | 1.016 | 1.013 |
| 11 | 77 | III | Female | Heart failure | 48 | 1.316 | 1.319 | 1.017 | 1.009 |
| 12 | 78 | III | Male | Traumatic shock | 17 | 1.292 | 1.285 | 1.018 | 1.007 |
| 13 | 78 | III | Male | Spinal cord contusion | 22 | 1.458 | 1.469 | 1.015 | 1.014 |
| 14 | 80 | III | Female | Poisoning | 12 | 1.307 | 1.299 | 1.037 | 1.022 |

neuronal loss[7] led us to the assumption that the loss of synapses in the PR during aging might be important for the disruption of the cortico-entorhinal-hippocampal neuronal circuits that are important for normal memory function. The aim of our research was to assess the dynamics of synaptic vesicles density in the PR during the aging process with the quantification of its synaptophysin immunoreactivity.

## 2. Materials and methods

The material included the right PR samples of 14 cadavers (10 males and 4 females) that ranged in age from 35 to 80 years. The samples were dissected during routine autopsies performed at the Institute of Forensic Medicine of the Medical faculty in Niš, Serbia. The post mortem period of the obtained samples was not longer than 48 h (Table 1). The tissue samples were taken from the rostral part of the parahippocampal gyrus, which lies between the collateral and uncal sulcus. According to Insausti et al.[2], this area of the PR gross-anatomically corresponds to the entorhinal cortex, but the presence of the adjoining perirhinal and posterior parahippocampal cortices in the obtained samples cannot be excluded. The samples size was 10 mm × 10 mm. The samples were fixed by immersion in 10% buffered formalin for the next 24 h. They were then embedded in paraffin, cut into sections 5 μm thick, and mounted on FLEX IHC Microscope Slides (Dako, Glostrup, Denmark). The accompanied medical documentation did not have data regarding previously diagnosed neurological or psychiatric disorders of the cadavers included in this study (Table 1). The antemortem cognitive status of the donors was unknown. In all cases, the neuropathological evaluation was normal. The tissue samples used in this study were obtained according to the rules of the faculties Internal Ethical Committee.

The obtained tissue sections were further immunohistochemically processed and stained with a monoclonal mouse anti-human synaptophysin antibody (Dako, Code IS776, Clone SY38, Ready – t- Use, EnVision FLEX System). To confirm the specificity of the immunoreactions, omission of the primary antibody was performed. No positive structures were found in these control sections. The immunohistochemical reaction of the PR white matter (WM) served as an internal negative control. The stained sections were histologically analyzed with a light microscope under 5×, 10×, and 40× lens magnification.

The immunohistochemically stained sections were further morphometrically analyzed. Twenty fields of vision selected by an unbiased method from all layers of the PRs gray matter (GM) and five fields of vision of the WM selected by the same method were captured by digital camera (1.3 megapixel resolution) under 40× lens magnification in each of the 14 evaluated cases. The images were captured under the same light and optical conditions, and they contained only PR tissue without blank spaces or artifacts due to the accuracy of the following densitometric measurements.

According to Masliah et al.[24], a densitometric analysis was used for the quantification of the synaptophysin immunoreactivity. This was performed using ImageJ. The system was spatially calibrated with an object micrometer (1:100). The optical density (OD) calibration was performed with a Kodak No. 3 calibrated step tablet according to the manual provided on the software's website (http://rsb.info.nih.gov/ij/docs/examples/calibration/). To perform the densitometric analysis, the obtained 24 bit RGB images were split into three 8-bit channels (red, green and blue). The blue channel, on which the synaptophysin positive areas appeared as different shades of gray and the negative areas were light, was used for the analysis. This allowed for the influence of neuronal perikarya, glial cells and blood vessels on the results of measurement to be minimized. The mean and median OD of the entire image area were measured for all of the captured fields of vision in each case, and the obtained values were averaged for the gray and WM.

The statistical analysis of the obtained densitometric parameters was performed by SPSS (version 16). The correlations between the mean and median OD values and the age of the evaluated cases were established by linear regression analysis. To more thoroughly evaluate the dynamics of latter

cited parameters with age, the analyzed cases were classified into three age groups. The first age group included the cases with ages ranging from 30 to 49 years. The second group included the cases with ages ranging from 50 to 69 years, and the third group included the cases aged 70 years and older. The significance of differences of the latter cited age groups' morphometric parameters was established by One Way ANOVA and by Tukey's post hoc test. The gender differences were analyzed by Student's *t*-test for two independent samples, while the differences between gray and WM mean and median OD were analyzed by Student's *t*-test for two dependent samples. Confounding of the post mortem interval on the mean and median OD values was analyzed by bivariate correlation analysis.

## 3. Results

### 3.1. Histological analysis

The PR cortex cytoarchitectonic features such as thickness, lamination, neuronal size, and shape were well preserved in all of the cases. In younger cases, immunoreactivity of the PR superficial layers, which contained numerous small to medium sized neurons, was significantly stronger in relation to the deep layers (Fig. 1A). In older cases, such differences of the synaptophysin immunoreactivity between the layers of the PR were not observed (Fig. 1B). The neuronal perikarya, glial cells, and blood vessels were synaptophysin negative (Fig. 1C–F).

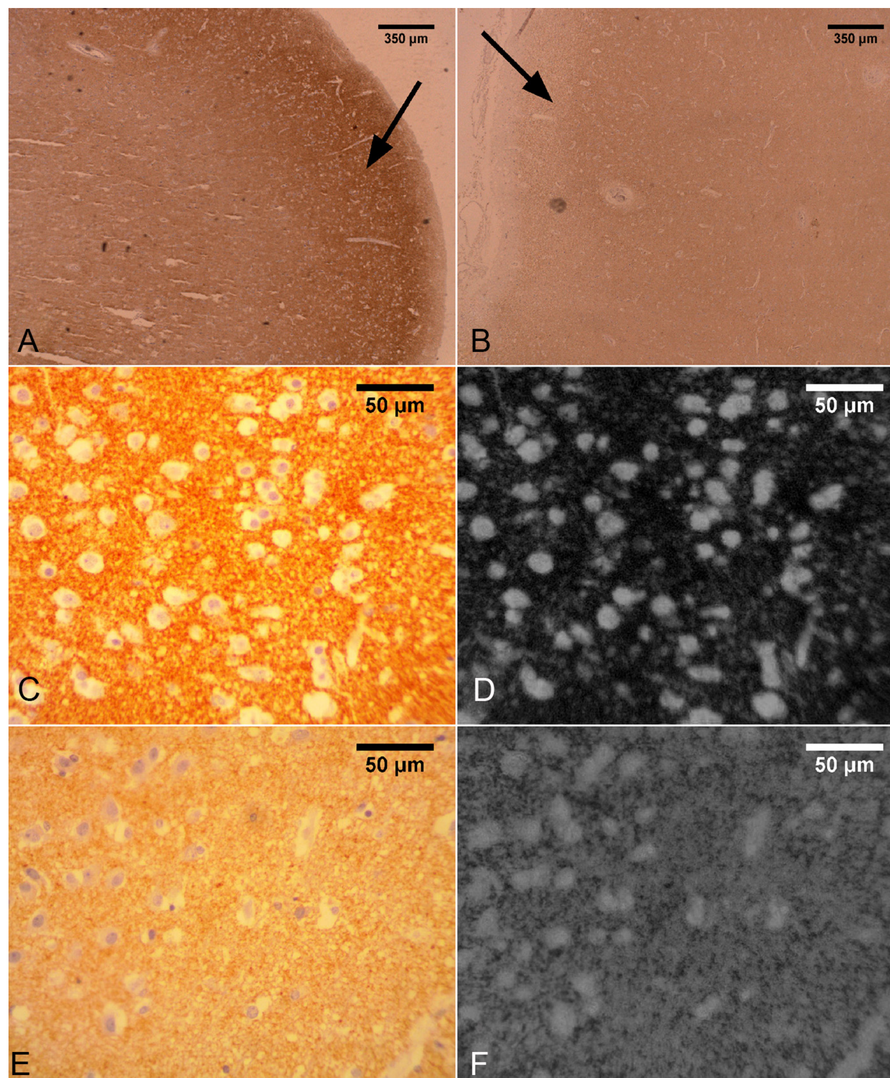


**Fig. 1 – Parahippocampal region (PR) immunoreactivity on the anti-synaptophysin antibody. (A) The PR superficial layers synaptophysin immunoreactivity (arrow) in a case of a 42-year-old male; (B) The PR superficial layers synaptophysin immunoreactivity (arrow) in a case of a 78-year-old male; 5× lens magnification; SY38 anti–human synaptophysin antibody; EnVision FLEX System; (C) Neuropil of the PR with predominant type I synaptophysin immunoreactivity in a case of a 42-year-old male; (D) Eight bit blue channel of image C; (E) Neuropil of the PR with predominant type II synaptophysin immunoreactivity in a case of a 78-year-old male; (F) Eight bit blue channel of image E; 40× lens magnification; SY38 anti–human synaptophysin antibody; EnVision FLEX system.**

The neuropil of PR layers showed a granular pattern of synaptophysin immunoreactivity (Fig. 1C–F). The immunohistochemical reactions of PR layers were characterized by the presence of two different types of positive reactions. The first was characterized by the presence of larger and dark brown stained synaptophysin immunopositive grains. The second type included areas of neuropil that were light brown and with significantly less intense granular immunostaining pattern (Fig. 1C–F). Generally, these types of positive reaction were approximately equally distributed in majority of the layers of the PR. However, the first type predominated in relation to the second one only in the superficial layers of the PR of the younger cases (Fig. 1C and D). In older cases, the frequency of the type I synaptophysin immunoreactivity in the superficial layers of the PR significantly decreased (Fig. 1E and F). This probably caused the differences in the intensity of the synaptophysin staining between the superficial and deep layers of the PR to disappear in the latter cases (Fig. 1B). The PR WM neuropil was negative and showed a focal type I positive reaction. The intensity of the positive reaction of the deep layers of the PR, as well as its WM insignificantly varied with age.

### 3.2. *Morphometric analysis*

The results of the densitometric analysis of the evaluated fields of vision of the analyzed cases' PR are presented in Table 1.

A linear regression analysis was conducted to evaluate the relationship between the age as a predictor and the mean and median OD as the outcome variables, respectively. This analysis showed that there was a significant decrease of the GM mean ($F(1,12) = 6.62$, $P = 0.024$) and median OD ($F(1,12) = 7.003$, $P = 0.021$) of the analyzed cases with age (Fig. 2A). These relationships can be identified by the two following models: mean OD = 1.78–0.006 $\times$ age and, median OD = 1.85–0.006 $\times$ age. The first model shows that the age explained 30% (adjusted $R$ square = 0.30) and the second model shows that the age explained 32% (adjusted $R$ square = 0.32) of the mean and median OD variance. These represented large effect sizes (for the mean OD $d = 1.48$ and for the median OD $d = 1.53$). The mean and median OD of the WM did not significantly change with age ($P > 0.05$).

To more thoroughly establish the dynamics of the evaluated densitometric parameters with age, the analyzed cases were classified into three age groups (Table 2). The ages were significantly different ($F(2,11) = 206.638$, $P < 0.001$, $d = 6.45$). Tukey's post hoc test showed that the mean ages of the first (39.80 $\pm$ 3.56 years) and second (58 $\pm$ 2.83 years) and third (78.25 $\pm$ 1.26 years) age groups were mutually significantly different ($P < 0.05$). The results of the one way ANOVA test showed that GMs mean ($F(2,11) = 4.84$, $P = 0.031$) and median OD ($F(2,11) = 5.20$, $P = 0.026$) of the evaluated groups were significantly different. These differences were characterized by large effect sizes (mean OD $d = 1.22$, median OD $d = 1.30$). Tukey's post hoc test showed that mean OD of the first group was insignificantly ($P > 0.05$) higher than that of the second group, and its value was significantly higher than the mean OD of the third age group ($P < 0.05$). The mean OD of the second age group was insignificantly lower than the first age group and insignificantly higher than the value of the third age group ($P > 0.05$) (Table 2, Fig. 2B). The same trend was observed for the median OD (Table 2, Fig. 2B). As in the linear regression analysis, the WMs densitometric parameters were not significantly different between the evaluated age groups ($P > 0.05$) (Table 2).

Significant gender differences were not observed in terms of the age, mean OD, or median OD ($P > 0.05$). Nevertheless, the number of the evaluated female cases was too small for the accurate statistical analysis. The mean ($t = 14.44$, $df = 13$, $P < 0.001$, $d = 10.75$) and median OD ($t = 13.15$, $df = 13$, $P < 0.001$, $d = 9.31$) of the GM were significantly higher than the same parameters of the WM. These differences indirectly additionally confirmed the positive reaction.

Mean and median OD of the PR GM (Mean: $r = 0.072$, $n = 14$, $P = 0.81$; Median: $r = 0.075$, $n = 14$, $P = 0.80$), as well as WM (Mean: $r = 0.427$, $n = 14$, $P = 0.13$; Median: $r = 0.418$, $n = 14$, $P = 0.14$) did not correlate significantly with post mortem interval of the evaluated PR samples.

From the above described results of the histological and densitometric analyses, it can be concluded that synaptophysin immunoreactivity decreases in the PR with age. This decrease was significant only in the third age group or in the individuals older than the age of 70, probably due to reduced number of the synapses in the superficial layers of this brain region.

## 4. Discussion

Though significant neuron loss was not observed in the cerebral cortex, some authors consider that the neurobiological basis of the age-related hypofunction of the brain represents synaptic changes. Researchers measured two major parameters to support these assumptions. These parameters were the numerical density of synaptic contacts and the packing density of the synaptic vesicles in presynapses.[25] Studies that attempted to reveal underpinnings of the age-related cognitive decline assumed that such changes of synapses in the hippocampus and prefrontal cortex might be responsible for its occurrence.[7] Synaptic aging in the prefrontal cortex is associated predominantly with the loss of glutamatergic axospinous synapses and thin dendritic spines of the neurons in layer III, whose axons project to the temporal cortex. Latter cited findings correlate positively with the level of age-related cognitive impairment. In contradiction to the previously mentioned findings, the hippocampus is characterized by less intensive and region specific synaptic aging alterations associated with the impairment of memory. Such changes include an age-related reduction in the size of postsynaptic densities of perforated synapses in the CA1 region, immunohistochemical and electron microscopic detection of large and complex axospinous synapse loss in the lacunosum-moleculare layer of the hippocampal CA3 region and molecular layer of the dentate gyrus.[7] Nakamura et al.[25] stated that the synaptic density gradually decreases with aging. In the human frontal cortex, the synaptic density reaches its maximum in the fifth year of life. It then decreases for the next 10 years to the adult level. Active use of the synapses with learning and environmental complexity contributes to synaptic plasticity and maintenance of the synaptic density and function throughout adulthood.[26] A sharp

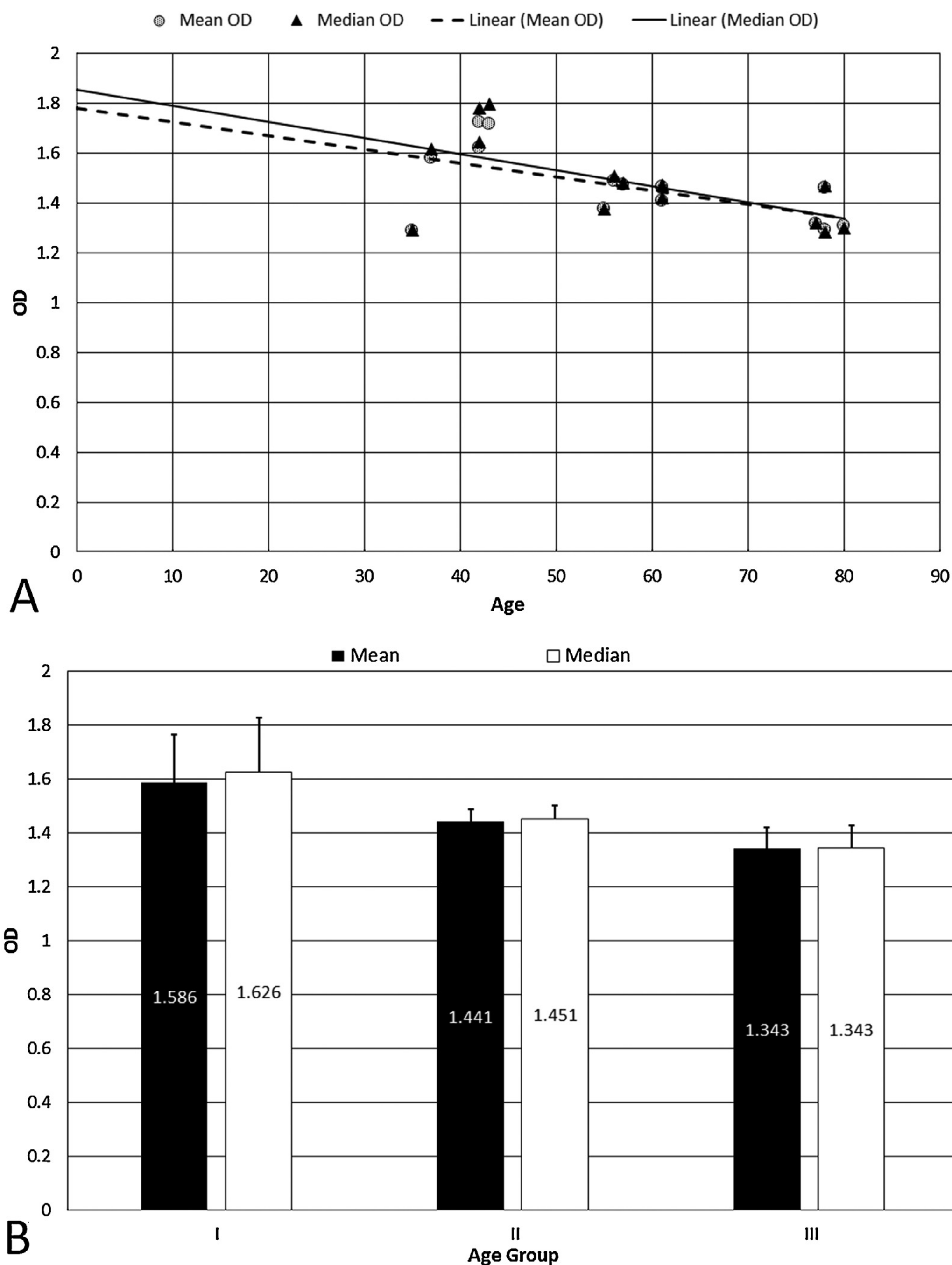


**Fig. 2 – Graphical display of the results of the densitometric analysis. (A) Correlation between the mean and median OD of the PRs GM and the age of the evaluated cases; (B) mean and median OD of the PRs GM of the analyzed age groups.**

**Table 2 – Mean and median OD of the PR GM and WM in the obtained age groups.**

| Age group | Gray matter | | | | White matter | | | |
|---|---|---|---|---|---|---|---|---|
| | Mean OD | | Median OD | | Mean OD | | Median OD | |
| | Mean | SD | Mean | SD | Mean | SD | Mean | SD |
| I ($n = 5$) | 1.586 | 0.178 | 1.626 | 0.203 | 1.064 | 0.115 | 1.059 | 0.118 |
| II ($n = 5$) | 1.441 | 0.046 | 1.451 | 0.052 | 1.026 | 0.02 | 1.021 | 0.019 |
| III ($n = 4$) | 1.343 | 0.077 | 1.343 | 0.085 | 1.022 | 0.01 | 1.013 | 0.007 |

decrease occurs after the age of 60 or 70 years, probably due to the shrinkage of the large neurons, perikarya, which then cannot maintain their normally huge synaptic contacts.[26–28] These results are in agreement with our finding of significantly decreased synaptophysin immunoreactivity in the PR of the cases older than 70 years.

According to Masliah et al.[24], the density of synapses can be measured by quantification with the electron microscope, then with the immunochemical quantification of synapse-associated proteins in brain homogenates and, by counting the dendritic spines in Golgi-stained sections. All three of these methods have limitations. The electron microscopic method requires well-preserved tissue, which in the case of human tissue usually requires a brain biopsy, and the size of the examined area is very small. A major restriction of the immunochemical quantification of synapse-associated proteins in brain homogenates is that this method cannot identify the distribution of the synapses in the cortical laminae. The quantification of the spines in the Golgi stained sections represents a very partial count of synapses.[24] According to Masliah et al.[24], measuring of the OD of the synaptophysin immunoreactivity, as we did in our study, presents a rapid, practical and reliable method for quantification of the density of the synaptic vesicles in all of the cortical laminae.

According to Honer et al.[27], specific presynaptic proteins such as synaptic vesicle protein synaptophysin are very important for cognitive reserve in the healthy elderly, and the loss of this protein correlates with antemortem cognitive dysfunction. Researchers analyzed the immunoreactivity of this protein in different cortical regions. Masliah et al.[28] showed a 23% decrease of the synaptic contacts in the superior frontal cortex of the cases older than 60 years compared with the cases 60 years old and younger with the computer aided quantification of the anti-synaptophysin immunolabeled neuropil. Keleshian et al.[29] measured synaptophysin in the frontal cortex homogenate with the western blot method and reported a significant decrease in the protein and mRNA levels in the aged compared with the middle-aged group.

Synaptophysin immunoreactivity of the medial temporal lobe during aging was also studied, with the hippocampal regions being the most frequently analyzed. Hof and Morrison[30] cited that expression of the presynaptic protein synaptophysin declines in the hippocampal regions that receive input from the entorhinal cortex as a component of the PR via the perforant path. Smith et al.[31] found that in a majority of the examined hippocampal regions, the average synaptophysin intensity values were insignificantly lower for the aged group than the younger cohort. Ojo et al.[32] used an optical segmentation procedure to quantify the immunoreactivity of different hippocampal subfields on three synaptic markers: synaptophysin, PSD-95, and CD200. The presence of synaptophysin was reduced in the aged compared to the young animals in all of the hippocampal regions, with significantly lower levels measured in the CA1 area and dentate gyrus.

The immunoreactivity of the other medial temporal lobe components on synaptophysin was less frequently studied. We found two studies in the literature that analyzed, among other markers, the synaptophysin immunoreactivity of the entorhinal cortex. As described above, entorhinal cortex presents the largest field of all of the PRs and is bidirectionally connected to the hippocampus and the associational cortices. Eastwood et al.[33] used immunoautoradiographic detection and in situ hybridization to measure the synaptophysin protein and synaptophysin mRNA levels in the post mortem obtained human hippocampus and the parahippocampal gyrus samples. Age of the cases analyzed in their study ranged from 5 weeks to 86 years. They classified evaluated cases into the following four age groups: infants, adolescent/young adults, adults and elderly. Age of their third (adults) and fourth (elderly) age groups approximately correspond to the age range of our cases. Generally, Eastwood and associates[33] found that synaptophysin immunoreactivity increases with age and additionally showed that the values of the synaptophysin signal in CA4 and CA3 hippocampal regions, subiculum and parahippocampal gyrus were significantly lower only in the infant group than in all subsequent age groups. According to them, synaptophysin immunoreactivity of the elderly's group was insignificantly higher than the same reactivity of the adult group, which is in opposition to our results. Latter cited authors detected opposite trend for synaptophysin mRNA, which significantly declined from infancy to adult group in the case of the parahippocampal gyrus. Further, comparison of synaptophysin mRNA between parahippocampal neurons in emulsion-dipped sections showed that the reduction seen in aging was present in superficial, but not in deep laminae. This was the case with synaptophysin protein immunoreactivity of the superficial layers of the PR in the oldest cases of our research. Finally, the same authors[33] performed correlation analysis in order to establish the association between the synaptophysin immunoreactivity and the age of the analyzed cases for each of the evaluated age groups separately. Its results showed that synaptophysin expression in the infants' group significantly positively correlated with age in CA4 and CA1 hippocampal fields and in the parahippocampal gyrus. In the same group, similar trend was observed for synaptophysin mRNA of the dentate gyrus and CA4 hippocampal region. Within the adolescent/young adult group, significant inverse correlations with age were not observed and Eastwood and associates[33] explained that with pruning related phenomenon. Nevertheless, across the six elderly subjects synaptophysin immunoreactivity significantly declined with age in the parahippocampal gyrus. Data about the trend of synaptophysin protein expression in their third (adult) age group, which corresponded to our first and second age groups, were not presented in their paper. Our linear regression analysis showed, opposite to the results of the Eastwood et al.[33], a continuous gradual decrease of synaptophysin expression through the whole adult period, and it becomes significant in the third age group, or in the cases older than the age of 70 years. Significant decrease of synaptophysin immunoreactivity in the elderly group of the Eastwood et al.[33] might partially correspond to the latter cited results of our study. The second study by Stranahan et al.[34] established reduced expression of reelin and its mRNA and, reduced synaptic marker immunoreactivity followed by an increase in tau phosphorylation in the lateral entorhinal cortex of the cognitively impaired aged rats. They used fluorescence labeling for synaptophysin and described a significant reduction in the positive reaction around the reelin

labeled cells in layer II of the lateral entorhinal cortex of these animals. Nevertheless, the latter cited authors did not detect such changes in the aged cognitively unimpaired animals. In our study, significant decrease of synaptophysin immunoreactivity was observed in the superficial areas of the PR in the cases older than 70 years. However, it should be emphasized that antemortem cognitive status of our cases was unknown which makes the results of our research incomparable with the results of the study by Stranahan et al.[34]

According to Clare et al.[20], decline of synaptic density is universal element in the pathologic changes associated with some types of dementia. Firstly, the correlation between the synaptic loss and AD was established. Afterwards, decline of synaptic density was observed in frontotemporal dementia (FTD), diffuse Lewy body disease (DLBD), ischemic vascular dementia (IVD), and spongiform encephalopathy. So far, results of the conducted studies show a region-specific reduction of the concentration of the synaptic proteins in different types of dementia. Thus, in AD synaptic loss was found predominantly in hippocampus and entorhinal cortex, as well as in frontal cortex. Synapse density is reduced in Creutzfeldt-Jakob disease as a form of the spongiform encephalopathy, too. IVD is quite heterogeneous from a neuropathologic perspective, and decrease of the synaptic proteins was found within frontal, hippocampal, and occipital regions in both patients with vascular dementia of the Binswanger type and Spatz-Lindenberg's disease. Significant decrease in synaptic density of the superficial layers of the prefrontal cortex was observed in cases with FTD, while in cases with DLBD detected loss of synapses was not significant. Medical documentation of our cases did not contain the data about any kind of neurological or psychiatric disorder during their life or immediately before death. Also, neuropathologic features characteristic for the latter cited types of dementias such as neuritic plaques, neurofibrillary tangles, Lewy bodies or Lewy neurites, macroscopic infarcts, microinfarcts, and microhemorrhages[35] were not observed in our material. So, it can be, with a reasonable certainty concluded that decline of synaptophysin immunoreactivity observed during our study does not represent the consequence of misdiagnosed dementia, but the result of the aging process. Nevertheless, a potential limitation of our study relates to the absence of the data about the cognitive status of the analyzed individuals during the period prior to death. Hence, the possibility that those cases, who were non-demented could potentially had mild cognitive impairment cannot be excluded. So, future researches should include the samples of the PR of the cases, in which antemortem cognitive status is known. Furthermore, in cases with normal cognitive status and post mortem observed neuropathologic changes characteristic for the above cited dementias more precise diagnostic neuropathologic criteria should be applied, too. In such way, more accurate correlation between the decline of the synaptophysin immunoreactivity in this region of the brain and memory impairment, which occurs with advancing age, would be achieved.

## 5. Conclusions

Finally, it can be concluded that synaptophysin immunoreactivity gradually decreases with age in the PR. This decrease becomes significant after the age of 70 years and affects predominantly superficial layers. Such immunohistochemical reactivity likely reflects the age-related loss of synaptic vesicles in this region of the brain, which has potentially deleterious effects on the integrity of the cortico-entorhinal-hippocampal neuronal circuits important for normal memory processing.

## Conflicts of interest

The authors have none to declare.

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