Use of ImageJ for Measuring Optical Density

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ABSTRACT: *In the current digital image processing work, we did the densitometric analysis using the tool ImageJ that helped to measure the optical density. This led to the labelling of the parahippocampal regions of the grey and white parts in the processed digital images. We have used the linear regression analysis to decrease the synaptophysin immune reactivity of the brain parts.*

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1. Introduction

The measurement of medical images often represents crucial method for routine diagnostics, as well as acquiring scientific data. However, with time, development of the more sophisticated medical image acquisition systems caused images to become larger and more complex. This, subsequently required implementation of advanced, time-consuming processing techniques to achieve precise quantification of the structures to be analyzed. Consequently, automation becomes necessity, so that medical specialists could perform simple tasks easily and focus on biomedical issues of the actual problem [1]. Level of the automation process to a large degree depends on the types of measurements that are required. Generally, analysis can include measurements of the entire scenes or individual features of the region of the interest, which should be well defined, either by edges or unique brightness, color, texture, or some combination of the latter cited factors [2, 3]. In the majority of the cases some human effort is needed at least to oversee the image processing operations which precede automatic measurement in order to verify that the features of interest are actually extracted from the more complex image. ImageJ represents key powerful tool to process and perform analysis to quantify medical images to aid in the detection of the features of interest [1].

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 [4]. Synaptophysin is the most abundant synaptic

vesicle protein and is therefore often measured in attempts to quantify synapses. It is considered to be a direct measure of the presence of mature synapses [5].

The cortical areas of the parahippocampal region (PR) of the temporal lobe of the brain, are generally accepted to form the system somehow involved in memory processing [6].

Taking into the consideration the fact that the biological underpinnings of the age - related cognitive decline are still unknown, all of these data led us to the assumption that the loss of synapses in the PR during the aging might be important for the disruption of normal memory function. The aim of our research was to, employing image processing and analysis, to assess the dynamics of synaptic density in the PR during the aging process with the quantification of synaptophysin immunoreactivity of the on the digital images of immunohistochemically stained histological slides of this brain area.

2. Material 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 obtained tissue was routinely histologically processed and further immunohistochemically stained with a monoclonal mouse anti-human synaptophysin antibody (Dako, Code IS776, Clone SY38, Ready – t- Use, EnVision FLEX System).In the immunohistochemically stained sections of each of the 14 evaluated cases, twenty fields of vision were selected by an unbiased method from all layers of the PRs gray matter (GM) and, five fields of vision of the white matter (WM) were selected by the same method and afterwards captured by digital camera (1.3 megapixel resolution) under x 40 lens magnification. 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 measurements.

According to Masliah et al. [7], a densitometric analysis was used for the quantification of the synaptophysin immunoreactivity. This analysis includes the evaluation of the brightness of the certain regions, or entire scene of the histological slides via the measurement of integrated optical density (IOD). Optical density is defined in Eq.1:

$$OD = -log_{10}\left(\frac{I}{I_0}\right) \tag{1}$$

where I/I_0 is the fraction of the incident light that penetrates through the sample without being absorbed or dispersed. Values of the OD range between 0 and 2.6. Integrated optical density represents the sum of the OD of all analyzed pixels[3]. Analysis was performed using ImageJ. The system was spatially calibrated with an object micrometer (1:100). The optical density 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/). In order 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 (Figures 1 and 2). This allowed for the influence of neuronal perikarya, glial cells and blood vessels on the results of measurement to be minimized. The IOD of the entire image area was measured for all captured fields of vision in each case, and the obtained values were averaged for the GM and WM, respectively.

The statistical analysis of the obtained densitometric parameter was performed by SPSS (version 16). The correlation between the IOD values and the age of the evaluated cases was established by linear regression analysis. The gender differences were analyzed by Student's *t*- test for two independent samples, while the differences between GM and WM IOD were analyzed by paired samples Student's *t*- test.

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 analyzed cases. The neuropil of PR layers showed two different types of positive reaction. The first characterized by the presence of larger and dark brown stained synaptophysin immunopositive grains and the second which was observed in areas of neuropil that were light brown and with significantly less intense granular immunostaining pattern (Figures 1 and 2). In

younger cases, immunoreactivity of the PR superficial layers was significantly stronger in relation to the deep layers (Figure 1). In older cases, such differences of the synaptophysin immunoreactivity between the layers of the PR were not observed (Fig. 2). The neuronal perikarya, glial cells and blood vessels were synaptophysin negative (Figures 1 and 2). The first of the two observed types of the immunopositive reaction predominated in relation to the second one only in the superficial layers of the PR of the younger cases (Figure 1). In older cases, the frequency of the first type of synaptophysin immunoreactivity significantly decreased in the superficial layers of the PR (Figure 2). The parahippocampal region WM neuropil was negative and showed a focal presence of the first type of positive reaction. The intensity of the positive reaction of the PRWM 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 I. A linear regression analysis was conducted to evaluate the relationship between the age as a predictor and the IOD as the outcome variable. This analysis showed that there was a significant decrease of the GM IOD (F (1,12) = 6.62, p = 0.024) of the analyzed cases with age. Such relationship can be identified by the following model (Eq.2):

$$IOD = 12.03 \times 10^5 - 0.037 \times Age \quad (2)$$

which explained 36% (adjusted R square = 0.36) IOD variance and represented large effect size. The IOD of the WM did not significantly change with age ($p > 0.05$). Significant gender differences were not observed in terms of the age and IOD ($p > 0.05$). The IOD of the GM was significantly higher than the same parameters of the white matter ($t = 14.44$, $df = 13$, $p < 0.001$). These differences indirectly additionally confirmed the positivity of immunohistochemical reaction.

From the above described results of the histological and densitometric analysis, it can be concluded that synaptophysin immunoreactivity decreases in the PR with age.

Case	Age	Gender	IODGM (x 105)	IODWM (x 105)
1	35	Male	8.689	6.225
2	37	Male	10.687	6.895
3	42	Female	10.948	6.968
4	42	Male	11.656	7.573
5	43	Male	11.588	8.290
6	55	Male	9.304	7.072
7	56	Male	10.043	7.024
8	57	Female	9.938	6.963
9	61	Male	9.505	6.734
10	61	Male	9.879	6.861
11	77	Female	8.889	6.872
12	78	Male	8.731	6.860
13	78	Male	9.849	6.880
14	80	Female	8.829	7.003

Table 1. Results of the densitometric analysis of all 14 analyzed cases

4. Discussion

Though significant neuron loss was not observed in the cerebral cortex, some authors considered that the neurobiological basis of the age-related cognitive decline represent synaptic changes, predominantly in the hippocampus and prefrontal cortex [4, 8].

According to Honer et al. [9], 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. Synaptophysin immunoreactivity of the

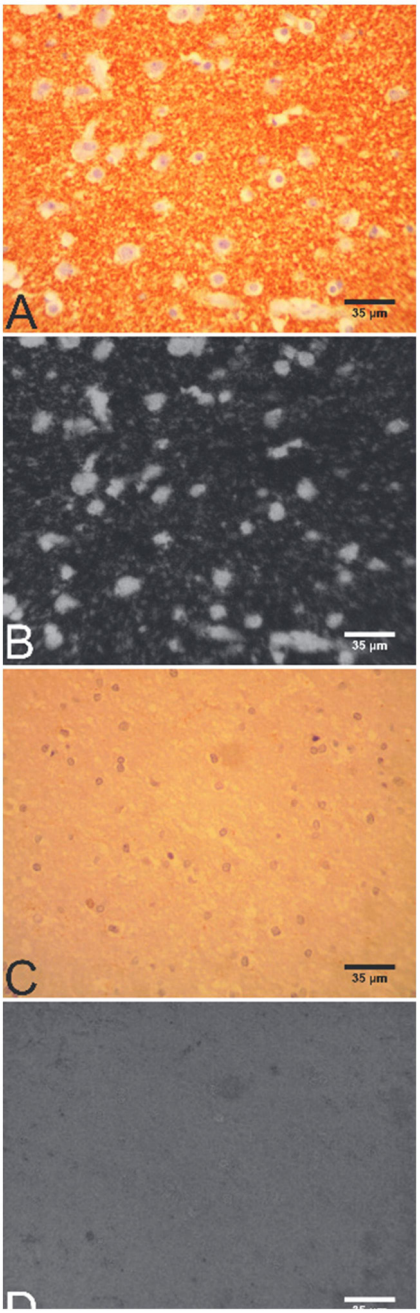

Figure 1. A – The PR GM synaptophysin immunoreactivity in a case of a 42 year old male; B – Eight bit blue channel of image A; C - The PR WM synaptophysin immunoreactivity of the latter male case; D - Eight bit blue channel of image C; x 40 lens magnification; SY38 anti – human synaptophysin antibody; EnVision FLEX System

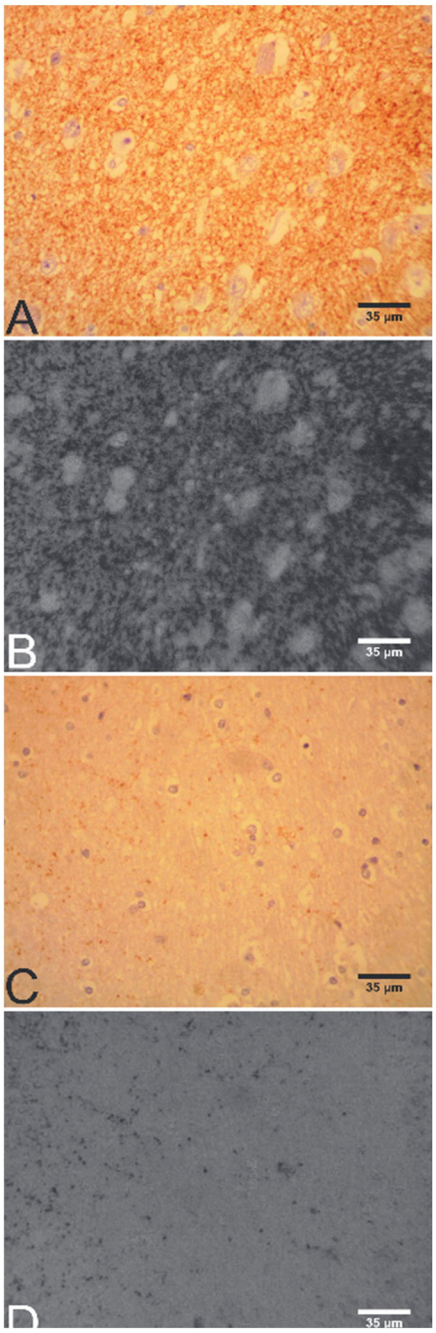

Figure 2. A – The PR GM synaptophysin immunoreactivity in a case of a 78 year old male; B – Eight bit blue channel of image A; C - The PR WM synaptophysin immunoreactivity of the latter male case; D - Eight bit blue channel of image C; x 40 lens magnification; SY38 anti – human synaptophysin antibody; EnVision FLEX System

temporal lobe during aging was also studied, with the hippocampal regions being the most frequently analyzed. The immunoreactivity of the PR on synaptophysin was less frequently studied. We found two studies in the literature that analyzed, among other markers, the synaptophysin immunoreactivity of the entorhinal cortexas a largest field of all of the PRs areas. Eastwood et al. [10] 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. According to them synaptophysin immunoreactivity of theelderly's group was insignificantly higher than the same of the adult group, which is in opposition to our results. The second study by Stranahan et al. [11] used fluorescence labeling for

synaptophysin and established its reduced immunoreactivity around the reelin labeled cells in layer II of the lateral entorhinal cortex of the cognitively impaired aged rats. Nevertheless, the latter cited changes were not detected in the aged cognitively unimpaired animals. However, according to Masliah et al. [7], opposite to our study, methods applied in the latter cited studies have limitations. A major restriction of the studies such as Eastwood et al. [10] which use 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 electron microscopic method, used in the study by Stranahan et al. [11], 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. Application of the image processing and subsequent densitometric analysis during our research enabled quick and thorough quantification of the synaptophysin immunoreactivity in all layers of the PR cortex. Opposite to the latter studies [10, 11], we detected gradual decrease of the synaptophysin immunoreactivity in this brain region, which could point to the conclusion that the density of the synaptic vesicles, and hence neuronal synapses decrease with advanced age in this region of the brain.

5. Conclusions

Finally, according to all above cited and the results of our study, it can be concluded that decreased synaptophysin immunoreactivity probably reflects the age-related loss of synapses in this region of the brain, which has potentially deleterious effects for normal memory processing in the elderly. Image processing and analysis of the immunohistochemically labeled PR histological slides enables measuring of the OD of the synaptophysin immunoreactivity and represents rapid, practical and reliable method for quantification of the density of the synaptic vesicles in all of the cortical laminae of this brain region.

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