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Effects of chronic stress on the brain – the evidence from morphological examinations of hippocampus in a chronic unpredictable stress (CUS) model in rats

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Abstract

Background. Chronic stress exposure deteriorates memory and increases the risk of psychiatric disorders, including depression.

Objectives. The objective of this study was to perform morphological studies in experimental model of neuropsychiatric disorder and to assess histologically the effect of chronic unpredictable stress procedure (CUS) influence on hippocampus.

Material and methods. Chronic unpredictable stress procedure (CUS) was applied for 8 weeks in rats by the modified method described by Katz et al. Experimental model of neuropsychiatric disorder was used based on morphological studies of hippocampal formation.

Results. Stress-induced alterations were observed in the hippocampus. Nerve cell changes included neuron shrinkage and dendritic remodeling. The most vulnerable hippocampal cells to chronic stress were CA3 and CA4 pyramidal neurons. In dentate gyrus chronic stress led to granule neuron shrinkage and slight exacerbation of apoptosis in the polygonal cell layer. CUS led to

statistically significant changes in quantitative characteristics of the CA3 and CA4 neuron size and nuclei diameter.

Conclusions. Chronic stress induces degeneration of hippocampal neurons. The observed neuronal changes indicate the damage of the neurons did not involve neither apoptosis nor necrosis Similarity between histological changes obtained in 8-week long CUS procedure applied in our research and morphological changes described in depressed patients confirms the usefulness of the applied stress procedure as the experimental model of depression.

Key words: stress, depression, hippocampus, chronic unpredictable stress procedure (CUS), animal model, morphology.

Introduction

The exposure to chronic stress deteriorates the memory and increases the risk of the occurrence of psychiatric disorders including depression, schizophrenia, autism spectrum disorders, anxiety, naming only few ((Bondi, Rodriguez, Gould, Frazer, & Morilak, 2008), (Harrison, 2002), (Corcoran et al., 2003)). Severe or prolonged stress also contributes to posttraumatic stress disorder ((Bremner, 2001), (Bremner et al. 2008)). The exposure to stressors is high in contemporary society and as a consequence mental health has deteriorated at the global scale. The WHO expects that mental disease, including stress-related disorders, will be the second leading cause of disabilities by 2020 (World Health Organization, 2004). This indicates that there is a need for systematic effort towards more effective prevention and treatment of stress related diseases. This can be achieved with the support of research that helps to understand how stress affects biological structures and functions of the organism and what are the psychological and behavioral consequences of those pathological changes. In this paper we present the effects of chronic stress on the brain, specifically showing the morphological changes of hippocampus, the key brain region involved in the memory, cognition and stress response.

In our study we used the chronic unpredictable stress procedure (CUS) which was applied for 8 weeks in rats by the modified method described by Katz et al (Katz et al., 1981). Although this method is widely used in neuropsychiatric studies, the evidence from existing research is not consistent and not unequivocal because of the variation is specific methodological conditions (ie. duration of the procedure) (Bondi et al., 2008; Harrison, 2002), (K Cieślik et al., 2006). (Yang et al., 2013), Therefore for most of the experimental models of neuropsychiatric disorders lack

comprehensive evidence regarding etiopathology, symptomatology complimented with pathomorphological examination of the brain structures. For instance, although 8 weeks of CUS has been shown to induce depressive-like behavior in animal models (Monteiro et al., 2015), it has not been confirmed what are the effects of this specific procedure on brain. Moreover, it is difficult to describe the complete and exact neuropathological consequences of stress exposure in the human brain as stress can occur in many different forms and depends on an individual's coping strategy and stress sensitivity. In order to treat neuropsychiatric disorders efficiently, knowledge of neurodegenerative phenotype of the condition together with the corresponding experimental models to search for neuroprotection strategies targeting accurately cell death cascade as well as regional and temporal changes underlying neuronal cell death (Yakovlev & Faden, 2004), (Stoica & Faden, 2010). Therefore research on the experimental stress models, which enable the development of new treatment strategies in stress-related disorders are of important interest.

In order to fill in this gap in our study we performed morphological examinations of hippocampus. Hippocampus is a key brain region involved in long term memory, cognition and the response to stress itself. For instance, in depressed patients structural alterations in the form of shrinkage of hippocampal structures have been reported (Frodl et al., 2014), (McEwen & Gianaros, 2010), (Kim & Diamond, 2002). The prior examinations carried out by the authors, has already showed that using chronic unpredictable stress stimuli one can cause in rats behavioral changes resembling ones observed in human suffering from depression (Ossowska, Danilczuk, Klenk-Majewska, Czajkowski, & Zebrowska-Lupina, 2004). It provided the foundations towards developing specific animal model of experimental depression. With the current contribution showing the effects of CUS on brain, we offer further evidence for future developments in the field of psychopathology and neuropharmacology.

Material and methods

The examinations were carried out on male Wistar rats (weighting initially 180–200 g). All animals were kept in one room. Animals were housed six per cage under a natural light/dark cycle. The ambient temperature was maintained at 21 ± 2°C°C. Food and water were provided *ad libitum*. All experimental procedures were carried out between 8 a.m. and 1 p.m. Rats were experimentally naive and tested once. All experimental procedures were conducted according to the NIH Animal Care and Use Committee Guidelines and were approved by the Ethics Committee of the Medical University of Lublin.

The animals were divided into two groups one control and one experimental (twelve animals in each group). Animals from the experimental group were subjected to long-term stress procedure -

chronic unpredictable stress procedure (CUS) - for a period of 8 weeks. The CUS procedure was a variant of the method of Katz et al. ((Katz et al., 1981), (Monteiro et al., 2015)) and consisted of several stressors which were performed every day for 8 weeks. The rats from the experimental group were subjected to the following kinds of unpredictable stressors (one stressor per day): 20 s exposure to electric footshock (3 mA, 0.2 s duration every 2 s), 2 h periods of immobilization at 20°C or at 4°C, 5 min exposure to an electric bell, 3 min periods of swimming in cold water (12°C) and 5 min periods of illumination (80 ± klx) and 1-day long periods of food deprivation (with water *ad libitum*) (Table.1).

The rats were sacrificed by decapitation twenty-four hrs after the end of the CUS procedure. Following decapitation brains were rapidly removed from the cranial cavity, fixed in 4% formalin at 4^0C for at least 24h. Specimens were dehydrated in graded ethanol solutions and embedded in paraffin. Seven-μm thick paraffin slices were serially cut in the frontal plane and subjected to the following examinations:

- histological (cresyl violet staining),
- immunocytochemical (using monoclonal anty-MAP2 antibodies),
- examinations for the detection of apoptotic cells with TUNEL method using in Situ Cell Death Detection Kit AP (Roche) and
- morphometric examinations using planimeter and a computer analyser of histological pictures (Lobophot 2, Nicon).

For all immunocytochemistry and TUNEL method, sections from both stress and control groups were processed simultaneously to avoid any possible artefacts as a consequence of the labeling procedure.

Histological study

For histological analysis selected paraffin-embedded tissue slices were stained with cresyl violet and assessed using a light microscope. We examined morphology of neurons in the dorsal hippocampus of both hemispheres.

Immunocytochemical study

For immunocytochemical analysis, paraffin-embedded tissue sections were processed on glass slides covered with Vectabond reagent (Vector Laboratories). Sections were repeatedly rinsed in phosphate buffered saline (PBS), then pretreated with 1%H_2O_2 to block any possible endogenous peroxidase. After incubation in 5% normal serum, sections were put into 0,2%Triton X-100. The sections were rinsed in PBS and incubated in a 1:500 solution of monoclonal antibody anti-MAP2, at 4^0C for 72h. Then, they were incubated in a solution of biotinylated secondary antibody for 24 h at 4^0C and in a solution of avidin-biotin peroxidase complexes for 1 h at room temperature. The bound peroxidase was detected by incubating the sections in a medium containing 0,05% 3,3'-diaminobenzidine (DAB, Sigma) and 0,01%H_2O_2 for 10 min at room temperature. Rinsing the sections

in H_2O stopped the reaction. Sections were finally dehydrated in graded alcohols and cleared in xylene.

TUNEL method

TUNEL method using in Situ Cell Death Detection Kit AP (Roche) was processed on paraffin-embedded tissue sections. Sections were repeatedly rinsed in phosphate buffered saline (PBS), then were put into Triton X-100 (Sigma) solution for 8 minutes. The sections were rinsed in PBS and incubated in a mixture of Enzyme solution and Label solution (Roche) at 37^0C for 1h. Then, they were incubated in 3% solution of BSA in PBS (sigma) for 1 h at 37^0C , following by AP converter for 12 h at 37^0C and TRIS (Sigma). Finally sections were treated with a medium containing Fast Red (Roche) for 30 minutes at 37^0C.

Quantitative analysis and statistics

Quantitative analysis of morphological changes was carried out by counting the number of normal and shrunken neurons and measuring the neuron size and neuron nuclei diameter using a planimeter and a computer analyser of histological pictures (Lobophot 2, Nicon) in cresyl violet stained slides. We examined neurons in the dorsal hippocampus of both hemispheres. Cells with round nuclei and visible nucleoli were considered normal, while cells with dark shrunken nuclei were considered shrunken. Cell counts were made within 40x microscopic fields in the pyramidal cell layer (the CA1-CA4) and dentate gyrus. The point directly ventral to the most lateral extension of the upper limb of the dentate granule cell layer was established as boundary between the CA3 and CA4 neurons. The percentage number of normal and shrunken neurons in the CA1-CA4 and dentate gyrus was counted in all groups. Statistical analysis of animal mortality was assessed using Chi-square test. For statistical processing of the body weight changes and neuron number results we used Student's T test criteria. The difference was considered statistically significant at $p<0,05$.

Results

The influence of stress on animal mortality in the examined groups. Stress procedure used in the present paper did not significantly increase animal mortality (Fig. 1).

The influence of stress on the body weight changes of animals in examined groups during the experiment. Stressed animals were characterized by smaller body gain in comparison with the control group (Table 2, Fig. 2).

The results of histological examinations. Animals exposed to stress procedure showed changes both within the hippocampus proper and dentate gyrus. Within hippocampus proper the most sensitive to destructive action of chronic stress were pyramidal neurons in the CA3 and CA4 regions. One observed shrinkage of nerve cell bodies in the CA3 and CA4 regions and stratum radiatum width decrease. Within the dentate gyrus stress led to shrinkage of granular layer neurons (Fig. 3).

Immunocytochemical examination results. The immunocytochemical examinations showed stress-induced decrease of immunoreactivity in nerve cell bodies and the change in the character of reaction from diffuse to granular within dendrites (Fig. 4).

TUNEL method results. TUNEL method examinations showed the presence in stressed animals single TUNEL positive neurons (apoptotic nerve cells) in dentate gyrus (Fig. 5).

Morphometric analysis. In morphometric analysis there were no statistically significant neuron number decrease in stressed animals. The stressed group was characterized by higher average percentage number of shrunken neurons in the pyramidal layer of hippocampus and statistically significant smaller values of average neuron size (Fig. 6, 7, 8).

Discussion

The carried out examinations showed that that 8 week chronic unpredictable stress procedure in animals resulted in neuronal degeneration. The observed neuronal changes (the shrinkage of perikaryons, neuronal cytoplasm condensation, negative results in TUNEL method in pyramidal neurons, a lack of inflammatory infiltration) indicate the damage of the neurons did not involve neither apoptosis nor necrosis (Martin et al., 1998). The remaining morphological changes observed in stressed rats were cytoskeleton remodeling, atrophy of dendrites (decreased stratum radiatum width) and slight intensification of apoptosis in dentate gyrus. Results of the present research indicate that the most vulnerable to the damaging effect of chronic stress hippocampal nerve cells were CA3 and CA4 pyramidal neurons. It may be caused by the presence of mossy fibers endings on their surface from which the glutamate activating the NMDA receptors is released (Ayarapetyanz et al., 2007).

Shrinkage of hippocampal structures (10-20%) is the best demonstrated change observed in depressed patients (Frodl et al., 2014) . One of the most important reasons of hippocampal volume decrease in depression is dendritic regression due to decreased accessibility of neurotrophic factors e.g. BDNF protein (brain-derived neurotrophic factor) (Dwivedi et al., 2003). It was shown that chronic unpredictable stress procedure induces reduction in the hippocampal BDNF gene expression (Katarzyna Cieślik et al., 2011).

Immunocytochemical examinations with the use of monoclonal anti-MAP2 (microtubule-associated protein 2) antibodies performed in the present study revealed stress-induced changes in the distribution of MAP2 within dendrites. MAP2 (microtubule-associated protein 2) is considered to be the dendritic hallmark and it is thought that the intensity of MAP2-immunoreaction correlates with dendritic integrity and plasticity (Di Stefano et al., 2001). Earlier research in depressed patients demonstrated the changes of dendritic tree within the hippocampus in the form of smaller arborization of apical dendrites in patients with mood disorders, the reversibility of dendritic remodeling and its disappearance after successful antidepressant therapy (Harrison, 2002). Dendritic remodeling and several other stress-induced brain changes are plastic and reversible in nature. Therefore for this

phenomena the term “neuroplasticity” is used, which refers to the general capacity of the brain to adapt functionally or structurally to a change in demands. But when stress is severe or prolonged it can cause permanent morphological alterations in the brain. Some scientists indicated the regression of dendrites as one of the most important causes of hippocampal volume decrease in depression (Czéh & Lucassen, 2007). Hippocampal volume reductions in depression are by now one of the best-replicated findings in biological psychiatry, but whether it is cause or consequence of the disorder remains unclear. Predictors of lower hippocampal volumes in depressed patients were: a more extensive depressive episode duration and recurrence, the size of their integrated cortisol responses and a history of early life stress (Lucassen et al., 2014).

The unique feature of neurons in the dentate gyrus in adults is their capacity to the formation of new nerve cells. In the present research, single apoptotic (TUNEL-positive) neurons in the dentate gyrus were observed. In depressed patients apoptotic nerve cells were revealed in very small number, and interestingly they were not present in the area of the most severe risk of damage, like the CA3 and CA4 regions, but they were found mainly in the dentate gyrus. There was shown that stress inhibits the physiological production of granule neurons in the dentate gyrus. Such decrease in the production of new neurons may lead to the functional alterations in hippocampus and related brain structures (Lucassen et al., 2006), (Sahay, Drew, & Hen, 2007).

Conclusions

In this study we revealed degeneration of hippocampal neurons induced by 8-week long CUS procedure. Similarity of histological changes obtained in chronic stress procedure applied in our research to morphological changes described in depressed patients suggests the usefulness of the applied stress procedure as the experimental model of depression, regarding etiopathology, symptomatology as well as pathomorphological changes in the brain which should be confirmed in further studies.

Table 1. Scheme of the chronic unpredictable stress procedure (CUS) for one week.

Stressed animals were subjected to CUS procedure for 8 weeks.

Day	Kind of stressor	Time
1	Electric footshock (3 mA, 0.2 s duration every 2 s)	20 s
2	Immobilization at 20°C	2 h
3	Electric bell	5 min
4	Immobilization at 4°C	2 h
5	Swimming in cold water (12°C)	3 min
6	Illumination (80 ± klx)	5 min
7	Food deprivation	24 h

Table 2. Body weight parameters at the beginning and after 8-week long stress procedure.

Body weight at the beginning (g)				**Body weight after 8 weeks (g)**			
Group	**n**	**$\bar{x}$**	**SD**	**Group**	**n**	**$\bar{x}$**	**SD**
Control	12	211,667	23,034	Control	12	350,833	23,614
Stress	12	206,667	17,480	Stress	10	260,000	25,298

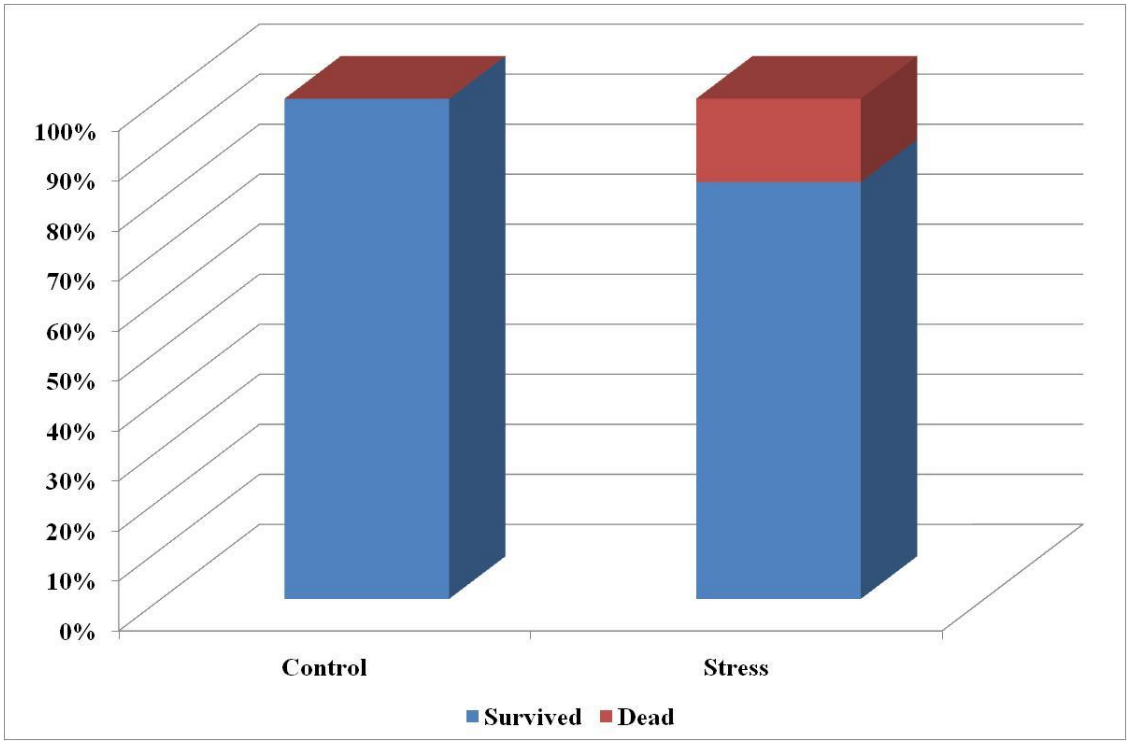


Fig. 1. The influence of stress on animal mortality in the examined groups, n=12 in the control group, n=12 in stressed group (Stress vs. Control: 16,67% vs. 0%).

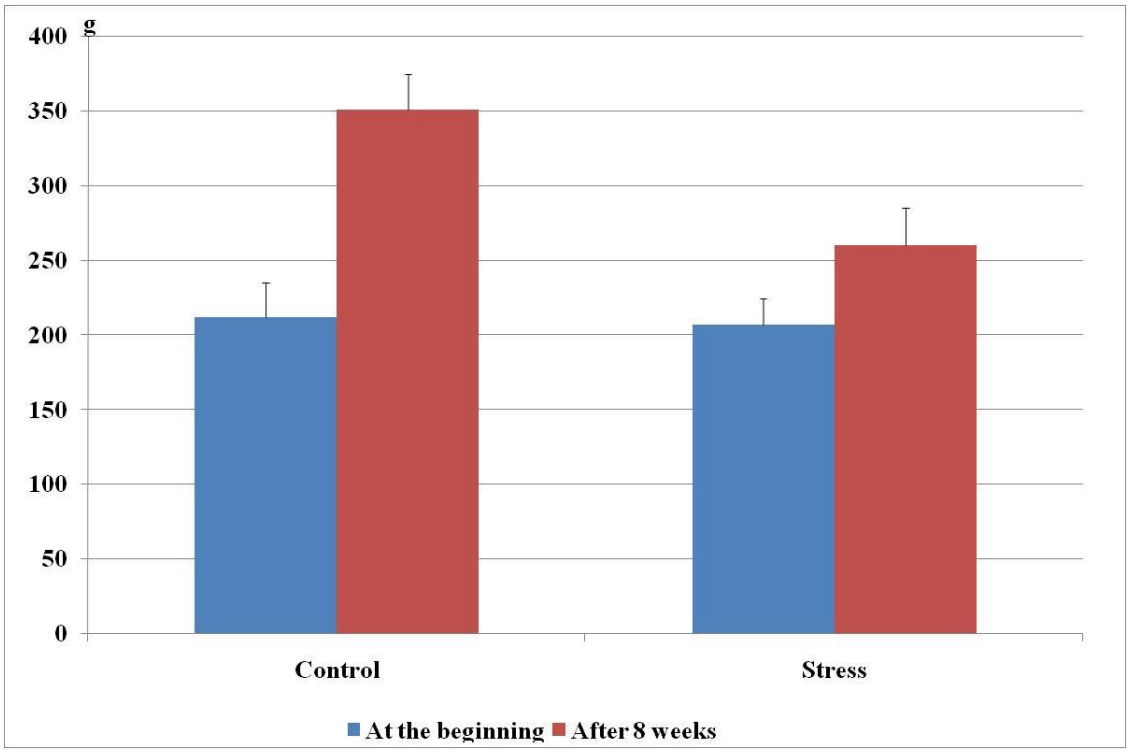
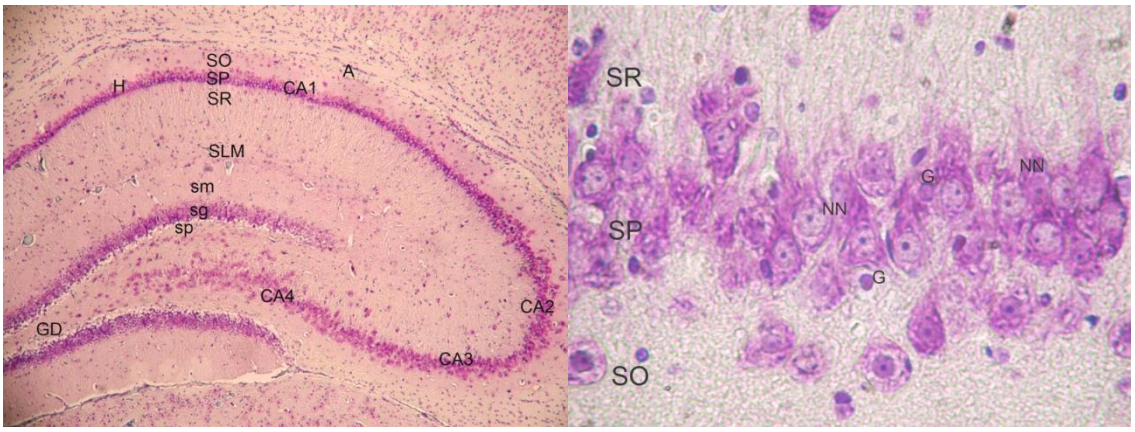


Fig. 2. Body weight parameters at the beginning and after 8-week long stress procedure. Stressed animals were characterized by smaller body gain in comparison with the control group. (Stress vs. Control: 260,00g vs. 350,83g, P<0,0001).

Control



Stress

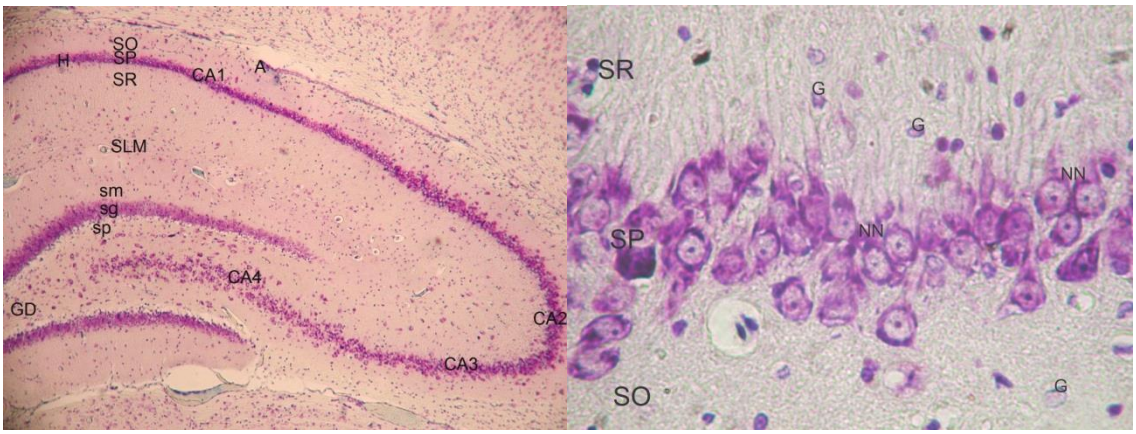


Fig. 3. Cresyl violet staining in the hippocampus of a control animal (top) and stressed animal (bottom). The left photomicrographs show small magnifications of the dorsal hippocampus in respective groups. Note stratum radiatum width decrease in stressed animal. Magnification, x 100. The right photomicrographs show high magnification of the CA3 region in respective groups. Note the difference between staining patterns of the groups in the hippocampal CA3 subarea, the shrinkage of nerve cell bodies in the CA3 region. Magnification, x 400. H – hippocampus proper (A – alveus, SO – stratum oriens, SP – stratum pyramidale: CA1-CA4 region, SR – stratum radiatum, SLM – stratum lacunosum-moleculare). GD – gyrus dentatus (sm – stratum moleculare, sg – stratum granulosum, sp – stratum pleomorphum).

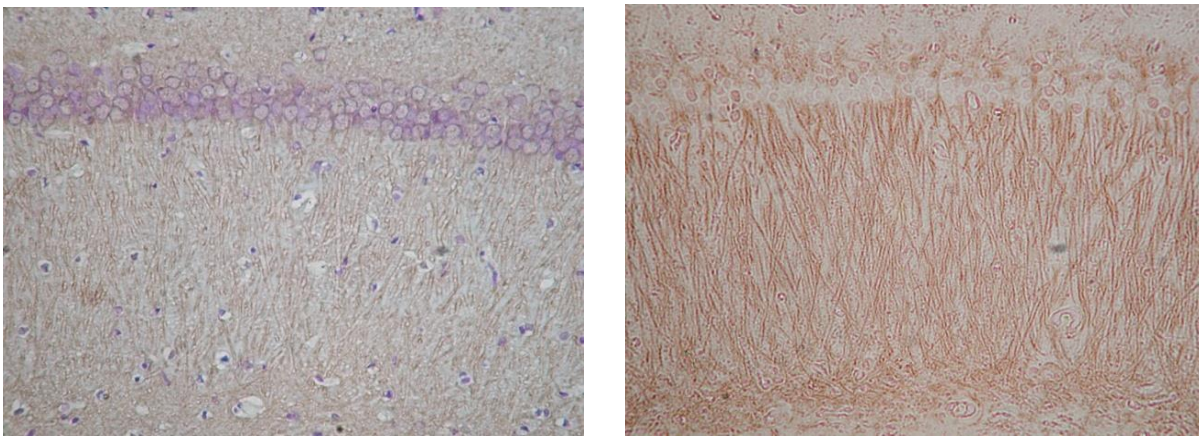


Fig. 4. Immunohistochemical staining for MAP2 in the hippocampus of control animal (left side) and stressed animal (right side). Stress-induced change in the distribution of MAP2 within dendrites in the form of the change in the character of reaction from diffuse to granular within dendrites. Magnification, x 200.

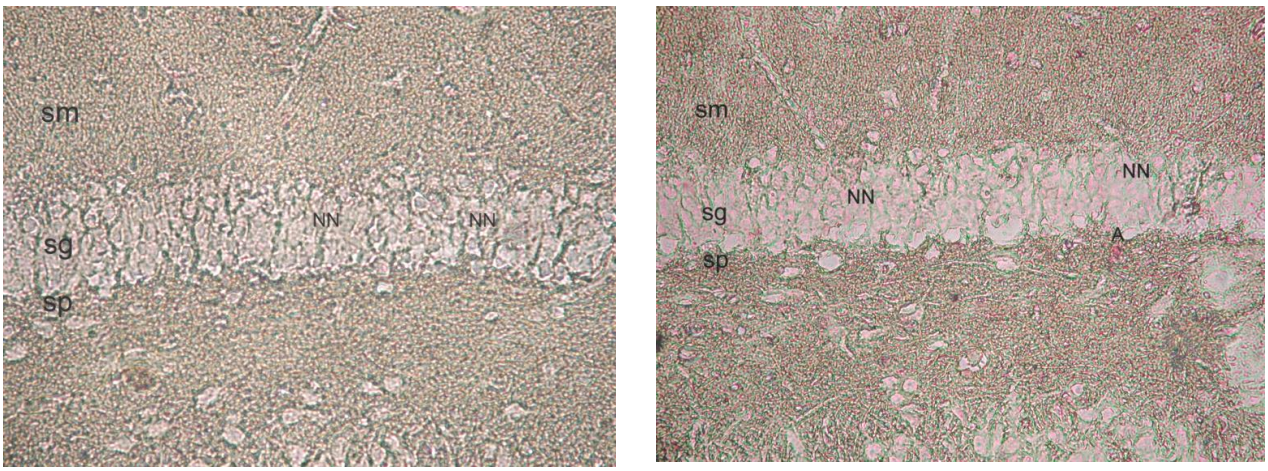
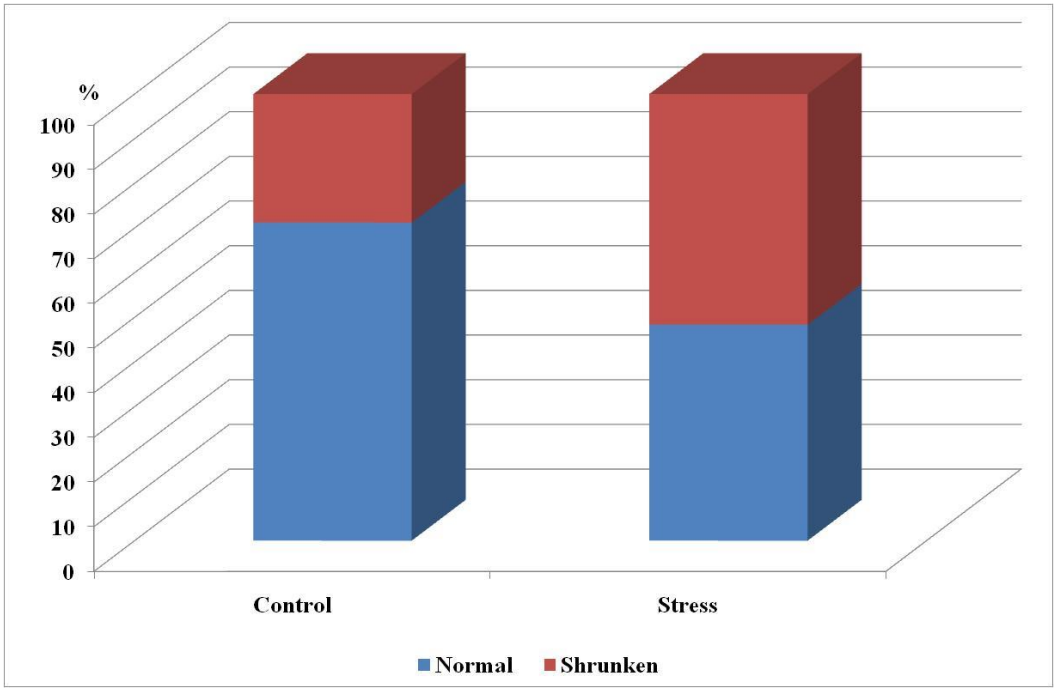
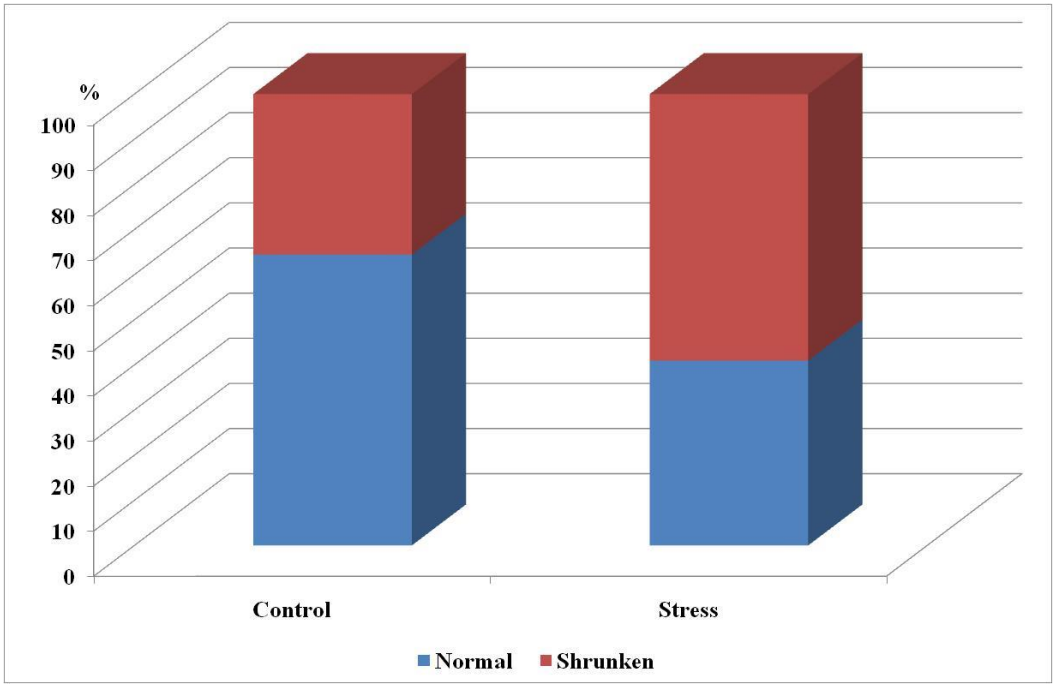


Fig. 5. TUNEL method using in Situ Cell Death Detection Kit AP results in dentate gyrus of control animal (left side) and stressed animal (right side). TUNEL method examinations showed the presence in stressed animals single TUNEL positive neurons (apoptotic nerve cells) in dentate gyrus. NN - neurons, A - apoptotic nerve cells, sm – stratum moleculare, sg – stratum granulosum, sp – stratum pleomorphum). Magnification, x 200.



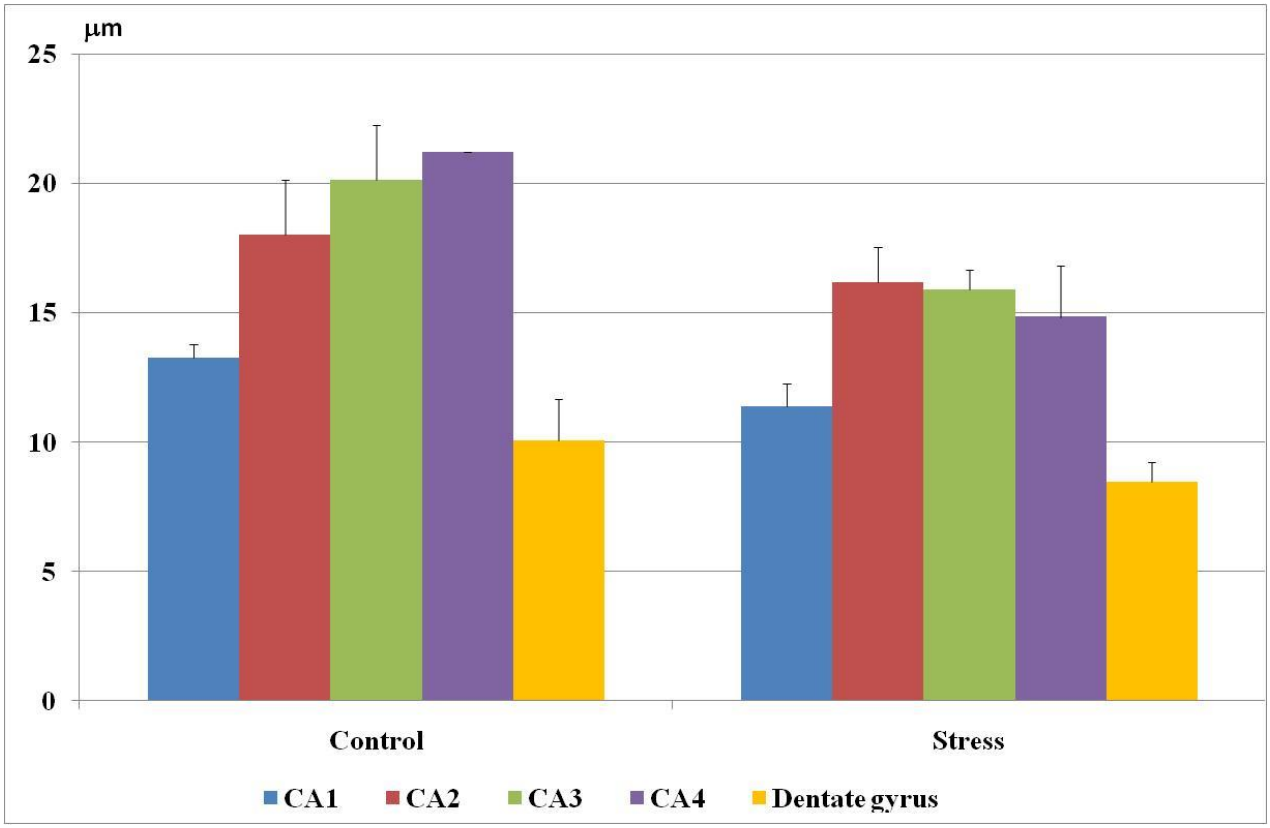
	CA3-normal			CA3-shrunken			CA3		
Group	**n**	**$\bar{x}$**	**SD**	**n**	**$\bar{x}$**	**SD**	**n**	**$\bar{x}$**	**SD**
Control	40	11,750	3,455	40	4,750	3,527	40	16,500	3,775
Stress	80	7,313	5,654	80	7,813	4,003	80	15,125	3,314

Fig. 6. Average percentage number of normal and shrunken neurons in the CA3 region in the examined groups. Number of slices per group: n=40 in the control group, n=80 in stressed group respectively. Stressed group was characterized by higher average percentage number of shrunken pyramidal hippocampal neurons in the CA3 region (51,653% vs 28,788%, P<0,001).



	CA4-normal			CA4-shrunken			CA4		
Group	**n**	**$\bar{x}$**	**SD**	**n**	**$\bar{x}$**	**SD**	**n**	**$\bar{x}$**	**SD**
Control	20	14,500	6,874	20	8,000	6,325	20	22,500	1,118
Stress	40	8,500	5,148	40	12,250	4,684	40	20,750	2,905

Fig. 7. Average percentage number of normal and shrunken neurons in the CA4 region in the examined groups. Number of slices per group: n=20 in the control group, n=40 in stressed group respectively. Stressed group was characterized by higher average percentage number of shrunken pyramidal hippocampal neurons in the CA4 region (59,036% vs 35,556%, P<0,001).



	CA1			CA2			CA3			CA4			Dentate gyrus		
Group	**n**	$\bar{x}$	**SD**	**n**	$\bar{x}$	**SD**	**n**	$\bar{x}$	**SD**	**n**	$\bar{x}$	**SD**	**n**	$\bar{x}$	**SD**
Control	20	12,500	0,500	20	17,000	2,000	20	19,000	2,000	20	20,000	0,000	20	9,500	1,500
Stress	20	10,750	0,829	20	15,250	1,299	20	15,000	0,707	20	14,000	1,871	20	8,000	0,707

Fig. 8. Average neuron diameter in the CA1-CA4 and dentate gyrus in the examined groups. Stressed group was characterized by statistically important smaller average neuron diameter in the CA3 and CA4 regions.

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