

## VOLUMETRIJSKA ANALIZA HIPOKAMPUSNE FORMACIJE I AMIGDALOIDNOG KOMPLEKSA U ANIMALNOM MODELU POSTTRAUMATSKOG STRESNOG POREMEĆAJA

## VOLUMETRIC ANALYSIS OF HIPPOCAMPUS AND AMYGDALA IN ANIMAL MODEL OF PTSD

Milica Ivanišević<sup>1</sup>, Milica Knežević<sup>1</sup>, Natalija Kojović<sup>1</sup>, Ana Starčević<sup>1,2</sup>

<sup>1</sup> Univerzitet u Beogradu, Medicinski fakultet, Beograd, Srbija

<sup>2</sup> Institut za anatomiju "Niko Miljanić", Medicinski fakultet, Beograd, Srbija

**Correspondence:** ivanisevicmilica4@gmail.com

### Abstract

**Introduction:** Posttraumatic stress disorder (PTSD) represents a mental disorder that occurs after life threatening situations. Animal models in psychiatry studies represent a base from which results and conclusions can be translated to human population. Amygdala and hippocampus are important neuroanatomical substrates possibly relevant to PTSD pathogenesis.

**Aim:** The aim of study was to investigate volumetric changes that occur in hippocampus and amygdala related to PTSD animal model.

**Material and methods:** Experiment was conducted on adult male Wistar rats. They were two groups, experimental and control. Experimental paradigm lasted for 31 days during which animals were exposed to acute and chronic stress. Acute stress was performed on the first day and ten days later. In between, animals were exposed to chronic social stress by pair rotations. Before second acute stress exposure, experimental group was divided in two subgroups from which one received dexamethasone dose. After the experiment ended, animals were sacrificed and the brain was extracted. Following the freezing process, brain tissue samples were cut and prepared for microscopy using. This was followed by volumetric analysis of hippocampus and amygdala. Measurements were performed bilaterally using Image Tool 3.0 Software.

**Results:** Results showed volumetric changes in these structures. Hippocampus had smaller volume in the experimental subgroup without dexamethasone ( $\bar{x} = 0.6144$ ) compared to the control group ( $\bar{x} = 0.9688$ ). Amygdala, as well, had smaller volumes in same subgroup compared to the control ( $\bar{x} = 10.0156$  compared to  $\bar{x} = 11.5041$ ).

**Conclusion:** Our study provided results in agreement with several previous studies on rodents and contributes to the assumption that hippocampus and amygdala have significance in PTSD etiology. Further goal is to expand our study which will help us to better understand the disorder itself.

### Keywords:

amygdala,  
animal model,  
hippocampus,  
volumetric analysis,  
posttraumatic stress  
disorder

## Sažetak

**Uvod:** Posttraumatski stresni poremećaj (PTSP) predstavlja mentalni poremećaj koji se javlja nakon životno ugrožavajućih situacija. Animalni modeli u psihijatrijskim studijama predstavljaju osnovu iz koje se dobijeni rezultati mogu preneti na humanu populaciju. Hipokampusna formacija i amigdaloidni kompleks su dva neuroanatomska supstrata, moguće ključna za patogenezu ovog poremećaja.

**Cilj:** Cilj ovog rada je izučavanje volumetrijskih promena vezanih za animalni model PTSP-a u hipokampusu i amigdali.

**Materijal i metode:** Eksperiment je vođen na odraslim Vistar pacovima. Životinje su bile podeljene u eksperimentalnu i kontrolnu grupu. Eksperimentalna paradigma je trajala 31 dan, tokom kojih su životinje izlagane akutnom i hroničnom stresu. Akutni stres je izveden prvog dana i deset dana kasnije. Tokom svih ostalih dana životinje su izlagane hroničnom socijalnom stresu izvođenjem rotacije. Pre drugog izlaganja akutnom stresu eksperimentalna grupa je bila podeljena na dve podgrupe, od kojih je jedna primila dozu deksametazona. Nakon završetka eksperimenta, životinje su žrtvovane i uzeti su mozgo-vi. Tkivo je zamrznuto i pripremljeni su iseći za mikroskopiju. Vršene su volumetrijske analize hipokampusu i amigdale. Merenja su bila bilateralna i korišćen je program *Image Tool 3.0. Software*. Obe eksperimentalne podgrupe su poređene sa kontrolnom grupom.

**Rezultati:** Na osnovu merenja pronađene su statistički značajne volumetrijske promene u hipokampusu i amigdali. Hipokampus je imao manji volumen u eksperimentalnoj podgrupi koja nije primila deksametazon ( $\bar{x} = 0,6144$ ) u poređenju sa kontrolnom grupom ( $\bar{x} = 0,9688$ ). Amigdala je takođe imala manji volumen u ovoj podgrupi u poređenju sa kontrolom ( $\bar{x} = 10,0156$ ,  $\bar{x} = 11,5041$ ).

**Zaključak:** Rezultati istraživanja ukazuju na važnost hipokampusne formacije i amigdaloidnog kompleksa za etiologiju ovog poremećaja. Dalje proširenje studije bi nam moglo pomoći da bolje razumemo uzroke i promene koje nastaju u sklopu PTSP-a.

### Ključne reči:

amigdala,  
animalni model,  
volumetrijska  
analiza,  
posttraumatski  
stresni poremećaj,  
hipokampus

## Introduction

Stress is a prevailing problem of modern life. Apart from everyday stress-packed situations people face, some social groups suffer from trauma caused issues. People that suffered from traumatic event could develop physiological response to fear which includes activation of hypothalamic – pituitary – adrenal (HPA) axis that induces “fight or flight reaction”. Hypothalamic – pituitary – adrenal feedback mechanism activation shows that endocrine axis and glucocorticoid levels have a significant role in overcoming stressful events (1,2). Consequences of such feedback are usually present short-term, during maximum three months. Some people that survived traumatic events could have chronic effects that include symptoms of posttraumatic stress disorder (PTSD) (3). In order to establish PTSD diagnosis, it is critical to determine specific symptoms of this mental health issue. According to International Classification of Diseases (ICD 11), criteria for PTSD are initial exposure to trauma, intrusive flashbacks of event, avoidance of circumstances resembling or associated with initial event and significant changes in behavior like hyper-vigilance, outbursts of anger etc. For now, molecular, biochemical and morphological reasons for development of PTSD are not clear and completely understood and they continue to be in the spotlight of today researches (4). There is an assumption that hippocampus and amygdala are two neuroanatomical substrates with potentially major role in PTSD development. Hippocampus is a central structure in declarative

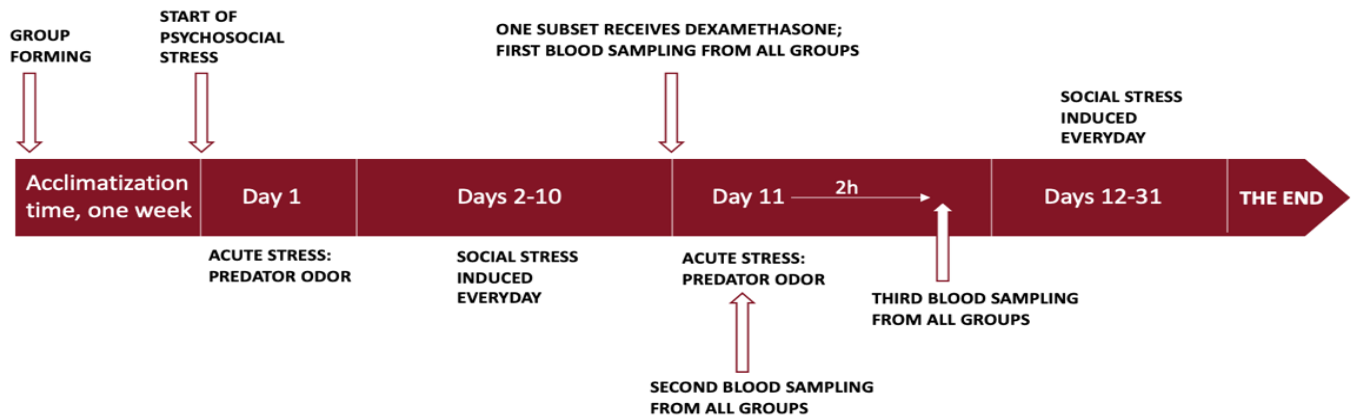
memory circuits. Symptoms, that appear to be crucial for PTSD, are connected to previously formed memories and stress related events. Since hippocampus is included in forming these memories, changes that appear in this structure could be a source of problems that PTSD patients face. Moreover, hippocampus is one of the structures with most glucocorticoid receptors and high levels of these hormones undoubtedly affect hippocampal cells (5). Amygdala, as another part of limbic system is connected to emotional memories, mostly fear, which is in connection with PTSD.

Due to the complexity of disorder, biases and inconsistency, researchers' attention is shifted towards animal models of PTSD. Variety of animal models have been developed throughout years of establishing and improving this approach. The aim of this study was to investigate possible morphological changes connected to PTSD in particular brain parts, hippocampus and amygdala. So as to achieve it, an animal model of this disorder was conducted using experimental paradigm previously described by Zohar and Zoladz (6,7).

## Materials and methods

### Experimental procedure

In this experiment adult male albino Wistar rats were used, body mass 220-350g, 12 animals in total. They were raised in Galenika a.d. vivarium and held in properly marked macrolon cages with steel cover in pairs, under



**Figure 1.** Experiment timeline

conditions of alternating 12-hour light and dark intervals. Room temperature where animals stayed was 18-22°C, along with air humidity between 55% and 65% - with all the relevant records kept. They were fed with a full feed mixture that contained 20% of raw proteins (The Veterinary Institute Subotica). Water was from Belgrade Waterworks. Food and water were available *ad libitum*.

There were 12 animals included in this experiment in total. Prior to the beginning of experiment, all animals had acclimatization time of seven days. After this period, the rats were divided in two groups. Experimental group contained 8 rats that were included in experiment paradigm described by Zohar and Zoladz (6,7). This model involves acute and chronic stress in order to induce an animal model of PTSD during the time of experiment. Control group contained 4 rats and they were not exposed to any kind of stress. After acclimatization time which lasted one week, the experimental group was exposed to stress. Acute stress consisted of rat immobilization in plastic tubes for 20 minutes and exposure to predator odor. It was planned to implement acute stress two times during the experiment. First time it was performed on the first day of the experiment, after acclimatization period. Second time was 10 days later, on 11<sup>th</sup> day of the experiment. First acute stressing was conducted during daylight, between 9 and 11 AM, and second during night, between 7 and 9 PM. Every day in between two acute stress exposures we were inducing social stress in rodents. Social stress was provided by changing pairs of rats that shared a single cage. Care was taken not to repeat the same pair formation in animal rotation within 48 hours. Before second exposure to acute stress on 11<sup>th</sup> day, the experimental group was divided into two subsets. One subset received dexamethasone (Dexasone) treatment (50 mg/kg b.m.) via subcutaneous injection (Figure 1).

After applying second acute stress on rodents, social stress was induced every day for next 20 days. On 31<sup>st</sup> day experiment was finished.

### Volumetric analysis

After the 31<sup>st</sup> day, animals were put under anesthesia by intraperitoneal application of chloral hydrate. Following this procedure, 4% paraformaldehyde perfusion

on temperature of 4°C was conducted. The animals were sacrificed and brain samples were placed in 4% paraformaldehyde solution for another 24 hours. The experiment was conducted in compliance with the current national (Animal Welfare Law) and European (Directive 2010/63/EU; European convention for the protection of vertebrate animals used for experimental and other scientific purposes) regulations. After 24 hours, brain samples were transferred to sucrose solutions of growing concentrations (10%, 20%, 30%) in 0.1M phosphate buffer with pH=7.4. Brains were promptly frozen on -80°C by immersing in 2 methyl butane. Frozen brains were cut using cryocut (Leica Instruments, Nussloch, Germany) on 20°C below zero, with 25 µm thickness of each section. Sections were placed on super frost microscope slides (Menzel, Braunschweig, Germany) and kept on -20°C. They were stained by Nissl staining using methylene blue in order to mark brain structures for quantification in stereological analysis. All examined structures of rodent telencephalon were determined by Paxinos and Watson atlas. (8) We measured volumes of hippocampus and amygdala. Volumetric analysis was conducted using Stereo Investigator System (Micro Bright Field, Inc. Colchester, VT). Personal computer with hardware-software system was connected to BH 2 microscope (Olympus America Inc, Melville, NY) which was used as visualisation instrument with high resolution video camera. From complete series of sections, from all brain samples, every fourth section was used to perform volumetric analysis on random starting point. Borders of each structure were constructed by ImageJ software, magnified on monitor 140 times using 2.5x objective. Final volumetric calculations were made following the Cavalieri's principle, using data gained from encircled structures of each section in combination with original section thickness. All measures were made bilaterally. Whole neuromorphological substrates were taken in consideration during volume evaluation, using Image Tool 3.0. Software.

### Results

Results of comparing hippocampal volumes of rodents' brain samples show statistical relevance. Experimental group that did not receive dexamethasone

treatment had decreased arithmetic mean in hippocampal volume, compared to the control group ( $\bar{x}_1 = 0.6144\text{mm}^3$ ;  $\bar{x}_2 = 0.9688\text{mm}^3$ ;  $p < 0.05$ ) (Table 1). Result of comparing amygdala volume in experimental group without dexamethasone and control group also showed high statistical relevance ( $\bar{x}_1 = 10.015\text{mm}^3$ ;  $\bar{x}_2 = 11.5041\text{mm}^3$ ;  $p < 0.05$ ) (Table 2). Graphical representations of these hippocampal volume difference (Figure 2) and amygdala volume difference (Figure 3) show that changes in volumes were more drastic in case of hippocampus.

## Discussion

Post-traumatic stress disorder persists as a frequent problem in psychiatry. People that have undergone a certain type of trauma develop symptoms that disturb their everyday life due to the phenomenon called “re-experiencing” events that led to trauma. In order to find specific and effective treatment, psychiatrists and scientists are continually trying to improve both psychotherapy and pharmacotherapy for the sake of improving patient’s life quality. Understanding the changes that occur in this disorder is crucial for development of novel therapy approaches. Studies on the effective animal model of PTSD, examination of physiological functions and neuroanatomical structures provide new research possibilities (9).

There are different stressors that are used in animal model studies with the goal to induce PTSD, such as electrical foot shock, underwater trauma, housing instability and predator exposure (7,10–12). Since people suffering

from PTSD have different types of trauma that led to this syndrome, different kinds of acute stressors in animal models are convenient as long as they are ethical and previously proved to be successful (13). Our group considered predator odor exposure as the most fitting type of stressor for the research, along with immobilization in plastic tubes in order to enhance feeling of helplessness in life-threatening situation. Rats were exposed twice to such stress during the experiment. Besides exposing rodents to obvious threat of predator, it is critical to provide symptoms as analogous as possible to those connected with PTSD in patients. Therefore, induced continuous social stress supposed to enhance PTSD symptoms in rats.

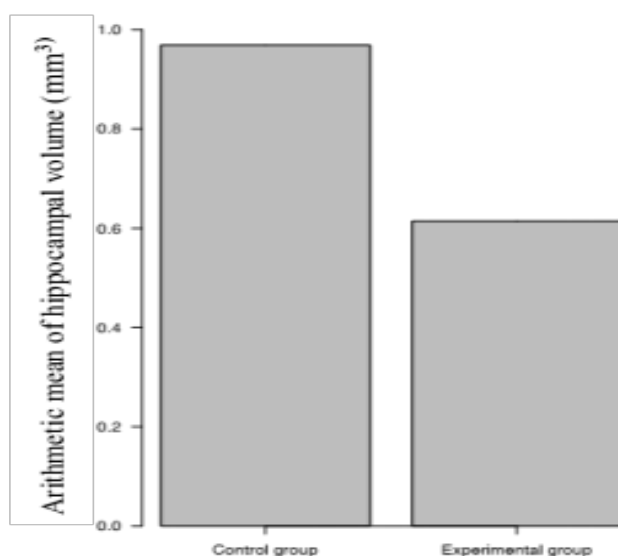
The experiment showed that hippocampal volume had noteworthy differences between the control and experimental group without dexamethasone treatment. The experimental group had total hippocampal volume reduction compared to control group. These results are in correlation with previous studies conducted on animal model of PTSD that showed hippocampal deficit (14,15) and other studies conducted on patients had same results, as well (16). Nevertheless, several studies on patients have shown contradictory results of hippocampal volume increase (17,18). An interesting fact is that numerous individuals from control groups, in particular studies conducted on patients, had similar, smaller hippocampal structure (19). This opened a question whether decreased hippocampal volume is a risk factor for developing PTSD at some moment in life or it is a consequence of a particular traumatic event (20).

**Table 1.** Results of hippocampal volume ( $\text{mm}^3$ ) comparison

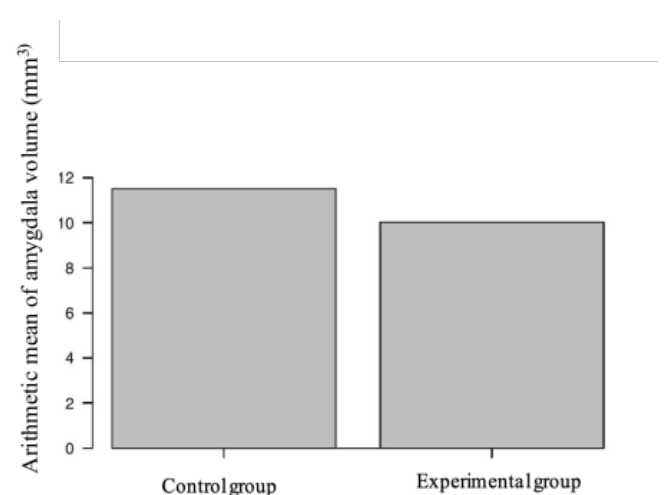
	Group	N	$\bar{x}$	SE	p
Hippocampus	Experimental	25	0.6144	0.02093	< 0.01
	Control	25	0.9688	0.05967	

**Table 2.** Results of amygdala volume ( $\text{mm}^3$ ) comparison

	Group	N	$\bar{x}$	SE	p
Amygdala	Experimental	25	10.0156	0.11661	< 0.01
	Control	25	11.5041	0.13026	



**Figure 2.** Graphical representation of changes in volume of hippocampus



**Figure 3.** Graphical representation of changes in volume of amygdala

Amygdala, another neuroanatomical substrate with potentially crucial role in PTSD, has a major part in keeping and evoking memories of traumatic events (21) which is the reason why it is a central structure in current examinations when it comes to this mental disorder. Results of studies that had neuroimaging as a method of analysis were, in this case as well, uncertain. Several studies have showed unilateral volume changes with either smaller (22) or larger (23) left amygdala. Some results surprisingly do not show any differences in amygdala volume in patients compared to control group (24). Our measures of amygdala volume showed reduction of this structure in rodents that were exposed to stress without dexamethasone compared to the control group. In previous animal model studies researchers used a single – prolonged stress model in order to induce PTSD and their results show enhanced apoptosis in amygdala neurons (25) which correlates with lower total volume.

Volumetric analysis shows that changes in central nervous tissue are significant when rats do not receive any glucocorticoid treatment. Potential explanation of this event could lay in promotive effect of glucocorticoids on stress adaptation and therefore protection of brain structures important for behavior in stressful situations (26).

For further research, we tend to expand the study since this study had limited number of experimental animals included. Bigger groups should improve our insight and provide new information related to this disorder.

## Conclusion

To conclude, the study showed that in an animal model of PTSD, significant changes in structure appear in amygdala and hippocampus, both having a reduction of total volume in animals that did not receive any treatment. These results contribute to the assumption that hippocampus and amygdala are involved in PTSD development. Volumetric and other types of analysis on animal models could contribute to understanding pathophysiology of PTSD, as well as other mental health issues, and therefore reveal new therapeutic approaches.

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