NASTAVNO-NAUČNOM VEĆU FARMACEUTSKOG FAKULTETA
UNIVERZITETA U BEOGRADU
TO THE ACADEMIC COUNCIL OF THE FACULTY OF PHARMACY
UNIVERSITY OF BELGRADE

KOMISIJI ZA POSLEDIPLOMSKE STUDIJE
TO THE COMMITTEE FOR POSTGRADUATE STUDIES

Na sednici Nastavno-naučnog veća Farmaceutskog fakulteta, Univerziteta u Beogradu, održanoj 6.4.2017. godine imenovani su članovi Komisije za ocenu i odbranu završene doktorske disertacije kandidata diplomiranog farmaceuta Dušanke Stanić, pod naslovom:

The Academic Council of the Faculty of Pharmacy, University of Belgrade has nominated the Committee for evaluation and defense of doctoral dissertation of Dušanka Stanić at the meeting held on April 6, 2017, bachelor of pharmacy, entitled:

Uticaj oksitocina na aktivnost osovine hipotalamus-hipofiza-nadbubreg i ponašanje pacova

Effects of oxytocin on the Hypothalamic-Pituitary-Adrenal axis activity and behavior of rats

Komisija u sastavu/ *Committee consisting of:*

1. Dr Vesna Pešić, mentor rada, vanredni profesor, Univerzitet u Beogradu-Farmaceutski fakultet/ *supervisor, associate professor, University of Belgrade-Faculty of Pharmacy*
2. Dr Bosiljka Plećaš-Solarović, redovni profesor, Univerzitet u Beogradu-Farmaceutski fakultet/ *full professor, University of Belgrade-Faculty of Pharmacy*
3. Dr David Gurwitz, redovni profesor, Sakler Medicinski fakultet, Univerzitet u Tel Avivu, Izrael/ *full professor, Sackler Faculty of Medicine, Tel-Aviv University, Israel*

pregledala je priloženu disertaciju i podnosi Nastavno-naučnom veću Farmaceutskog fakulteta, univerziteta u Beogradu sledeći/

Based on detailed review of the submitted dissertation, presents to the Academic Council of the Faculty of Pharmacy-University of Belgrade the following

IZVEŠTAJ/*REPORT*

A. PRIKAZ SADRŽAJA DOKTORSKE DISERTACIJE/ *THE CONTENT OF THE DOCTORAL DISSERTATION*

Doktorska disertacija kandidata Dušanke Stanić pod naslovom **"Uticaj oksitocina na aktivnost osovine hipotalamus-hipofiza-nadbubreg i ponašanje pacova"** napisana je jasnim i preglednim stilom, sadrži 8 tabela, 48 slika i 309 literaturnih navoda. Disertacija je napisana na 180 strana i sadrži sledeća poglavlja: Uvod, Ciljeve rada, Materijal i metode, Rezultate, Diskusiju, Zaključak i Literaturu. Na početku disertacije dat je Sažetak rada na srpskom i engleskom jeziku, a na kraju Biografija autora.

*Doctoral dissertation of the candidate Dušanka Stanić entitled **"Effects of oxytocin on the Hypothalamic-Pituitary-Adrenal axis activity and behavior of rats"** is written in a clear and concise style and contains 8 tables, 48 figures and 309 references Dissertation is written on 180 pages and comprises of listed chapters: Introduction, The aims of research, Materials and methods, Results, Discussion, Conclusion and References. At the beginning of the dissertation, a short abstract is given on both Serbian and English, while dissertation ends with the Author biography.*

U **Uvodnom delu** date su teorijske osnove značajne za predmet proučavanja doktorske disertacije. Uvodni deo je podeljen na 8 većih odeljaka. U prvom odeljku uvodnog dela izneta su dosadašnja saznanja o oksitocinu, njegovoj strukturi, distribuciji u organizmu, efektima, kao i osnovni mehanizmi dejstva ovog hormona na ciljna tkiva preko oksitocinergičkih receptora

(OxtR). Zatim, u posebnim odeljcima unutar prvog odeljka uvodnog dela, navedeni su, do sada poznati, efekti oksitocina na CNS, društveno ponašanje, kao i rasprostranjenost oksitocina i OxtR u različitim regijama mozga. Nakon toga, ukazano je na značajan uticaj polimorfizma OxtR i verovatnoće oboljevanja od različitih psihijatrijskih poremećaja, kao što su anksioznost, depresivni poremećaji i šizofrenija. Na kraju prvog odeljka, opisani su, do sada pokazani, efekti oksitocina na osovinu hipotalamus-hipofiza-nadbubrežna žlezda (HPA), kao tzv. "perifernog modulatora". U drugom odeljku poglavlja Uvod, data je definicija depresivnih poremećaja, kao i podaci Svetske zdravstvene organizacije o njihovoj prevalenci na globalnom nivou. Takođe, navedeni su i najznačajniji faktori rizika oboljevanja od ovog poremećaja, sa akcentom na hroničnu izloženost stresu, kao vodećem epigenetskom faktoru. Zatim, opisani su mehanizmi uključeni u fiziološki odgovor organizma na stres, kao i činjenica da ovi mehanizmi mogu biti poremećeni ukoliko je jedinka izložena delovanju stresnih stimulusa tokom dužeg vremenskog perioda. U okviru trećeg i četvrtog odeljka uvodnog dela opisani su različiti eksperimentalni modeli hroničnog stresa i depresivnog ponašanja, koji su razvijeni u cilju proučavanja patofiziologije depresivnih poremećaja. Dat je pregled različitih karakterističnih promena koje se uočavaju kod eksperimentalnih životinja, a mogu se povezati sa depresivnom simptomatologijom, pre svega promene u ponašanju i disfunkcija HPA osovine. Ukazano je na probleme koji se mogu javiti kod različitih primenjenih modela a koji podrazumevaju aplikaciju stresnih stimulusa od strane eksperimentatora i kako se ovi problemi mogu prevazići upotrebom standardne doze egzogenog kortikosterona. Zatim je detaljno opisan animalni model hroničnog stresa korišćen u izradi ove disertacije, koji podrazumeva administraciju kortikosterona glodarima tokom tri nedelje. Takođe, dat je pregled rezultata dosadašnjih studija o karakterističnim promenama koje se javljaju kod eksperimentalnih životinja nakon hronične administracije kortikosterona, a koje se mogu povezati sa manifestacijom depresivnih poremećaja kod ljudi. U petom odeljku opisane su promene u morfologiji i aktivnosti nadbubrežne žlezde, glavnog ciljnog organa HPA osovine, tokom hronične izloženosti stresu i u depresivnim poremećajima. Nakon toga, u šestom poglavlju, ukazano je da su štetni efekti hronično povišenog nivoa glukokortikoida, tokom aktivacije HPA osovine u uslovima hroničnog stresa, u značajnoj meri posledica i povećane produkcije slobodnih radikala i sledstveno povećanog oksidativnog stresa. U sedmom poglavlju, sagledan je uticaj hroničnog stresa na

specifične moždane strukture, pre svega hipokampus. Poznato je da kod depresivnih pacijenata, a i u animalnim modelima depresije dolazi do atrofije hipokamusa, što može biti posledica negativnog uticaja glukokortikoida na ćelijsku proliferaciju. Zatim, dat je osvrt na pojam "adultne neurogeneze", proces koji je izuzetno izmenjen u uslovima hiperaktivnosti HPA osovine, a za koji se smatra da ima značajnu ulogu u odgovoru organizma na hronični stres. Poslednje, osmo poglavlje, bavi se problematikom savremene terapije depresivnih poremećaja, pre svega antidepresivima iz grupe selektivnih inhibitora preuzimanja serotonina (SSRI), a koja je, na žalost, u oko 50% slučajeva neuspešna. Iz tog razloga, novija farmakogenomska istraživanja se koriste u svrhu identifikacije potencijalnih biomarkera povoljnog odgovora na terapiju antidepresivima iz grupe SSRI, koja bi značajno olakšala izbor adekvatnog leka za svakog pacijenta.

*The **Introduction** contains theoretical bases relevant for the subject of doctoral dissertation. The introduction is organized in eight sections. In section one of the Introduction, previous findings on oxytocin, it's structure, distribution within the organism, effects, as well as the basic mechanisms of effect of this hormone on the target tissues via oxytocinergic receptors (OxtR) are presented. Subsequently, in next parts within the first section of the introductory chapter, the known effects of oxytocin on CNS, social behaviour, and the expression of oxytocin and OxtR in the different brain regions are listed. Thereafter, it is focused on the significant influence of OxtR polymorphism and its effect on the probability for developing various psychiatric disorders such as anxiety, depressive disorders and schizophrenia. At the end of the section one, up-to-date published effects of oxytocin on the hypothalamic-pituitary-adrenal (HPA) axis activity as „peripheral modulator“are described. The section two defines the depressive disorders as well as the data of The World Health Organization on their prevalence on global level. Also, the most significant risk factors for development of above-mentioned disorders are indicated, with the emphasis on the chronic exposure to stress as main epigenetic factor. In addition, the mechanisms involved in the physiological response to stress are described, along with explanation how these mechanisms could be disrupted in case that the individual is exposed to the stressful stimulants during long periods of time. Within the third and fourth sections of the Introduction, various experimental models of chronic stress, developed to study the pathophysiology of depression are described. An overview of the different characteristic changes*

observed in experimental animals that could therefore be linked to depressive symptomatology are overviewed, including the list of all changes related to behavior and dysfunction of the HPA axis. The potential problems during the application of various models involving the application of stressful stimuli by the experimenter are highlighted, and overcoming these problems by using the standard dose of exogenous corticosterone is described. Subsequently, the animal model of chronic stress applied in this thesis is described in detail. The above mentioned model includes the application of the corticosterone to rodents during the period of three weeks. An overview of the results of the previous studies describing characteristic changes, linking to the human depressive symptoms, that occur in experimental animals after chronic administration of corticosterone is also provided. Section five describes the changes in the morphology and activity of the adrenal gland, which is the main target organ for the HPA axis, during chronic exposure to stress and in depressive disorders. Thereafter, in section six, it is explained how adverse effects of chronically elevated glucocorticoids during activation of the HPA axis in the chronic stress conditions, could in large part be the consequence of increased production of free radicals and consequently, increased oxidative stress. Section seven provides description of the impact of chronic stress on specific brain structures, primarily hippocampus. Atrophy of the hippocampus was demonstrated in depressed patients and in animal models of depression, suggesting that it couldy be the consequence of the negative influence of glucocorticoids on cell proliferation. Thereafter, the concept of adult neurogenesis is described as the process which is hindered in the conditions of HPA axis hyperactivity, and which is also considered to have an important role in response of the organism to the chronic stress. The last, eight, section is dealing with the issues of contemporary therapy of depressive disorders, primarily the selective serotonin reuptake inhibitor (SSRI) antidepressants, which is unsuccessful in around 50% of the treated patients. For this reason, recent pharmacogenomic research has been used to identify potential biomarker(s) of favorable response to treatment with the SSRI antidepressants with the aim to assist in the selection of adequate therapy for each patient.

Ciljevi rada su definisani kao ispitivanje uticaja hormona oksitocina na ponašanje i parametre aktivnosti HPA osovine u modelu hroničnog stresa/depresije indukovane dugotrajnom primenom

kortikosterona kod pacova Wistar soja. Dalje, cilj je bio i da se kod eksperimentalnih životinja ispita efekat kombinovanog davanja oksitocina i citaloprama, antidepresiva iz grupe selektivnih inhibitora preuzimanja serotonina, lekova prvog izbora u terapiji pacijenata koji boluju od nekog od depresivnih poremećaja. Navedeni ciljevi su jasno definisani i na osnovu postavljenih ciljeva istraživanje je podeljeno u tri faze.

The aims of the research *are defined as evaluation of the influence of the hormone oxytocin on the behavior and parameters of HPA axis activity, in the model of the long-term corticosterone administration-induced depression-like symptoms in adult male Wistar rats. Furthermore, the aim was to examine the potential benefitial effect of administering oxytocin alongside citalopram. Citalopram is an antidepressant from the selective serotonin reuptake inhibitors (SSRI) group, the first line therapy in the treatment of patients suffering from depressive disorders. These aims are clearly defined and, based on the defined aims, the whole research was divided into three phases.*

U poglavlju **Materijal i metode** precizno su navedeni korišćeni materijali i reagensi, i detaljno opisana metodologija primenjena u istraživanju. Eksperimenti koji čine doktorsku disertaciju realizovani su u okviru tri faze.

In the ***Materials and methods*** *chapter, all used materials and reagents are clearly defined, as well as the methodology used in the research. Experimental work from this doctoral dissertation has been conducted in three phases.*

U **prvoj fazi** istraživanja ispitivan je uticaj akutnog i subhroničnog tretmana oksitocinom na ponašanje eksperimentalnih životinja i nivo biogenih amina u plazmi. Ispitivani su efekti dve doze oksitocina, 0.25 IU/400 μL i 10 IU/400 μL. Rezultati ove faze su bili potrebni za određivanje adekvatne doze i dužine trajanja tretmana oksitocinom koja je korišćena u narednim fazama istraživanja. Životinje su bile podeljene u eksperimentalne grupe koje su bile izložene sledećim tretmanima: oksitocin akutno (0.25 IU/400 μL i 10 IU/400 μL, *s.c.*), oksitocin subhronično (0.25 IU/400 μL i 10 IU/400 μL, *s.c*. tokom 14 dana) i kontrolne grupe koje su primale fiziološki rastvor akutno (400 μL, *s.c.*) i subhronično (400 μL, *s.c.*, 14 dana).

*In the **first phase** of research, the effects of acute and subchronic treatment, with two different doses (0.25 IU/400 μL and 10 IU/400 μL), of oxytocin on behavior and plasma levels of biogenic amines in experimental animals were evaluated. The results obtained in the first experimental phase of the dissertation were used to determine the appropriate oxytocin dosage and treatment duration that was applied in next phases of the research. Animals were randomly divided in the following experimental groups and treated with: acute oxytocin (0.25 IU/400 μL and 10 IU/400 μL, s.c.), subchronic oxytocin (0.25 IU/400 μL and 10 IU/400 μL, s.c. for 14 days) and respective control groups treated with saline acutely (400 μL, s.c.) or subchronicaly (400 μL, s.c. for 14 days).*

U **drugoj fazi** ispitivan je efekat subhroničnog tretmana oksitocinom (10 IU/400 μL) na ponašanje eksperimentalnih životinja i parametre aktivnosti HPA osovine u uzorcima plazme (nivo kortikosterona, adrenalina i noradrenalina). Pored toga, analiziran je odgovor HPA osovine na akutni stres, kvantifikovani su parametri oksidativnog statusa u plazmi i stepen oštećenja DNK limfocita periferme krvi, kao i parametri koji podržavaju adultnu neurogenezu u hipokampusu. Svi ovi parametri određivani su u animalnom modelu hroničnog stresa/depresije indukovane hroničnom primenom kortikosterona. Životinje su bile podeljene u sledeće eksperimentalne grupe na osnovu primenjenih tretmana: kortikosteron (CORT, 100 mg/L, 21 dan, *per os*), oksitocin (OXY, 10 IU/400 μL, *s.c*. 14 dana), kortikosteron i oksitocin (CORT+OXY, oksitocin tokom poslednjih 14 dana) i kontrolna grupa tretirana rastvaračem *per os* i fiziološkim rastvorom *s.c.* tokom 14 dana.

*In the **second experimental phase**, the effect of subchronic treatment with oxytocin (10 IU/400 μL) on behavior and HPA axis activity (plasma levels of corticosterone, adrenaline and noradrenaline) in experimental animals were determined. Furthermore, the HPA axis response to acute stress challenge, oxidative stress parameters in plasma, levels of DNA damage in peripheral blood lymphocytes, and parameters promoting adult hippocampal neurogenesis were investigated. All these parameters were examined in animal model of chronic stress/depression induced by chronic corticosterone administration. Adult, male rats were randomly divided into four experimental groups according to treatment: corticosterone (CORT, 100 mg/L, 21 days, per os), oxytocin (OXY, 10 IU/400 μL, s.c., daily for 14 days), corticosterone and oxytocin*

(CORT+OXY, oxytocin during last 14 days of experiment) and control group of animals treated with solvent per os and saline s.c daily for 14 days.

U **trećoj fazi** istraživanja, u navedenom animalnom modelu depresije, ispitivan je dodatni efekat dvonedeljnog tretmana oksitocinom uz tretman citalopramom, antidepresivom iz grupe SSRI. Ispitivani su efekti kombinovanog tretmana na ponašanje životinja, a u uzorcima prefronatlanog korteksa ispitivana je ekspresija gena za Itgb3 i Chl1, proteine koji se smatraju potencijalnim biomarkerima povoljnog odgovora na terapiju antidepresivima iz grupe SSRI. Životinje su bile podeljene u sledeće eksperimentalne grupe: kortikosteron (CORT, 100 mg/L, 21 dan, *per os*), oksitocin (OXY, 10 IU/400 µL, *s.c*. tokom poslednjih 14 dana), citalopram (CIT, 10 mg/ kg TM *s.c*. tokom poslednjih 14 dana), kortikosteron i oksitocin (CORT+OXY), kortikosteron i citalopram (CORT+CIT), kortikosteron, oksitocin i citalopram (CORT+OXY+CIT) i kontrolne grupe. U sva istraživanja obuvaćena ovom doktorskom tezom uključeno je ukupno 291 mužjaka pacova *Wistar* soja, straih dva meseca na početku eksperimenta.

*In the **third phase** of the study, in above-mentioned animal model of depression, the effect of additional two-week treatment with along with citalopram, antidepressant, was evaluated. The effects of the combined treatment on behavior of experimental animals were investigated. In the samples of prefrontal cortices, the expression of potential biomarkers of good response to SSRI therapy, mRNA for Itgb3 and Chl1, were analyzed by real-time PCR. Animals were randomly assigned to one of the following experimental groups: corticosterone (CORT, 100 mg/L, 21 days, per os), oxytocin (OXY, 10 IU/400 µL, s.c., last 14 days of experiment), citalopram (CIT, 10 mg/kg BW, s.c., last 14 days of experiment), corticosterone and oxytocin (CORT+OXY, corticosterone and citalopram (CORT+CIT) corticosteone, oxytocin and citalopram (CORT+OXY+CIT) and respective control group. A total of 291 animals, male Wistar rats, two months old at the beginning of the experiment, were used for the entire experimental work included in this dissertation.*

Za ispitivanje ponašanja eksperimentalnih životinja korišćeni su sledeći testovi: test otvorenog polja (eng. Open Field Test, OFT), uzdignuti plus lavirint (eng. Elevated Plus Maze, EPM) i test forsiranog plivanja (eng. Forced Swim Test, FST). Načini izvođenja testova, kao i analizirani parametri detaljno su opisani u ovom delu disertacije. U tkivu srži nadbubrežne žlezde

određivana je količina dva najznačajnija transportera za kateholamine, noradrenalinskog transportera (NAT) i vezikularnog transportera za monoamine 2 (VMAT2), primenom Western blot metode. Način čuvanja žlezde nakon žrtvovanja, izolacija tkiva medule, metoda izolovanja proteina, elektroforetsko razdvajanje, kao i detaljne karakteristike antitela korišćenih u Western blot analizi date su u ovom odeljku. Lančana reakcija polimerizacije u realnom vremenu (RT-PCR) korišćena je za analizu ekspresije gena Slc6a2 i Slc18a2 koji kodiraju proteine NAT i VMAT2 u srži nadbubrežne žlezde, kao i ekspresiju gena Itgb3 i Chl1 u prefrontalnom korteksu pacova. Postupak izolacije iRNK, kao i njena ekstrakcija, sinteza komplementarne DNK i karakteristike početnih sekvenci korišćenih za RT-PCR analizu jasno su navedeni. Zatim, detaljno je opisan postupak sprovođenja, kalupljenja u parafinu i sečenja tkiva nadbubrežne žlezde koje je korišćeno za histološku analizu. Takođe, objašnjeno je na koji način je izvođena morfometrijska analiza tkiva i navedeno korišćeno uveličanje. Koncentracija biogenih amina u plazmi određivana je primenom reverzno-fazne tečne hromatografije pod visokim pritiskom sa elektrohemijskom detekcijom (RP-HPLC-ED), dok je koncentracija kortikosterona određivana sistemom tečna hromatografija-elektrosprej jonizacija-tandem masena spektrometrija (LC-ESI-MS/MS). Karakteristike ovih metoda, kao i način pripreme i čuvanja uzoraka plazme detaljno su opisani. Za procenu promena u integritetu DNK i stepena njenog oštećenja korišćen je comet test (gel elektroforeza pojedinačnih ćelija) prema tačno definisanom protokolu. Dalje, određivana je koncentracija lipidnih hidroperoksida, malondialdehida i aktivost enzima superoksid dizmutaze u plazmi eksperimentalnih životinja, u cilju procene oksidativnog statusa/antioksidativne zaštite životinja po jasno i detaljno opisanoj metodologiji. Kako bi se ispitao proces adultne neurogeneze u hipokampusu, primenjena su imunohistohemijska bojenja preseka moždanog tkiva i određivanje gustine BDNF i Ki-67 imuno-pozitivnih ćelija. Svi detalji pripreme uzoraka, karakteristike i razblaženja antitela korišćenih za ova bojenja, kao i način kvantifikacije detaljno su navedeni. Za statističku obradu, u zavisnosti od analiziranih rezultata korišćeni su Studentov t-test, kao i jednofaktorska i dvofaktorska ANOVA sa odgovarajućom *post-hoc* analizom. Svi relevantni podaci vezani za statističku obradu rezultata detaljno su navedeni u posebnom odeljku ove celine.

For evaluation of the effects of applied treatments on behavior of animals, the following tests were used: open field test (OFT), elevated plus maze (EPM) and forced swim test (FST).

Deatiled procedure of tests, as well as all parameters that were analyzed, are described in detail in this section of dissertation. The amounts of the two main catecholamines transporters, noradrenaline transporter (NAT) and vesicular monoamine transporter 2 (VMAT2), in the adrenal medulla of Wistar rats were determined by using the Western blot analysis. In this section are listed: conditions for storage of adrenal gland tissue, methods for adrenal medulla and proteins isolation, electrophoretic separation of proteins, as well as all relevant characteristics of antibodies used for the Western blot analysis. Real time polymerase chain reaction (RT-PCR) was used for quantifying of the gene expression levels of Slc6a2 and Slc18a2 genes in the adrenal medulla, as well as Itgb3 and Chl1 mRNA in the prefrontal cortex. Methodology for mRNA isolation and extraction, synthesis of complementary DNA and caracteristics of primers used are clearly described. Furthermore, detailed procedure for preparation of paraffin embedded sections of adrenal gland tissues used for histological analysis was described. In addition, morphometric analyses and magnification used in microscopy were explained. Plasma levels of biogenic amines were determined using Reversed Phase High Pressure Liquid Chromatography with an Electrochemical Detector (RP-HPLC-ECD), while corticosterone levels were evaluated with Liquid Chromatography-Electrospray Ionization-Tandem mass spectrometry (LC-ESI-MS/MS) system. All relevant properties of the applied methods, storage and preparation of plasma samples are described in detail. For evaluation of the DNA damage levels in peripheral lymphocytes, precisely defined protocol for comet test (single gel electrophoresis) was used. Next, in order to evaluate oxidative status/antioxidative protection mechanism in the experimental animals, plasma concentrations of lipid hydroperoxde (LOOH), malondialdehyde (MDA) and superoxide dismutase activity (SOD) were evaluated and the methodology was explained in detail. Immunohistochemical staining of brain tissues was used to investigate the hippocampal adult neurogenesis and density of the BDNF and Ki-67 immunopositive cells in this brain region. All details about the sample preparation procedure, dilution and characteristics of antibodies used for the staining, as well as the methodology for quantification of positive cells are presented in this section. Depending on the results, for statistical analysis Student t-test, one-way and two-way ANOVA with appropriate post hoc test were used. All relevant data about statistical analysis of the results are fully explained in the corresponding section of Materials and Methods chapter.

B. OPIS POSTIGNUTIH REZULTATA/DESCRIPTION OF THE OBTAINED RESULTS

U poglavlju **Rezultati** dat je pregled rezultata dobijenih na osnovu sprovedenih istraživanja. Rezultati su prikazani u vidu grafikona ili tabela. Prikaz rezultata je organizovan u skladu sa postavljenim ciljevima istraživanja. Rezultati su grupisani u 3 veće i 9 manjih celina. U prvoj većoj celini, prikazani su rezultati prve eksperimentalne faze disertacije. Kako je pokazano da tretman manjom korišćenom dozom oksitocina (0.25 IU/400 μL), bez obzira na dužinu trajanja, nije imao uticaja na ispitivane parametre, grafički su jasno prikazani efekti veće korišćene doze (akutno i subhronično) na ponašanje životinja i koncentracije biogenih amina u plazmi. Rezultati su prikazani za svaki ispitivani test ponašanja, kao i za svaki analizirani hormon ponaosob. Dobijeni rezultati ukazuju da subhronični tretman oksitocinom (10 IU/400 μL) pokazuje određeni anksiolitički i antidepresivni potencijal i dovodi do značajnih promena u ponašanju životinja u svim izvođenim testovima. Takođe, tretman oksitocinom bez obzira na dužinu trajanja, doveo je do značajnog povećanja koncentracija adrenalina i serotonina u plazmi pacova.
U drugom poglavlju Rezultata, u vidu pet manjih celina, prikazani su rezultati druge eksperimentalne faze disertacije. U prvom odeljku opisani su i grafički predstavljeni rezultati uticaja eksperimentalnih tretmana na porast telesne mase, kao i unos hrane i vode životinja. Pokazano je da životinje hronično tretirane kortikosteronom, imaju značajno manji prinos telesne mase, što je bila potvrda dobro poznatog efekta hronično povišenih vrednosti glukokortikoida (Conrad i sar., 2007; Gourley i Taylor, 2009). Sa druge strane, tretman ositocinom je ublažio ove negativne efekte kortikosterona u CORT+OXY grupi životinja. U drugom manjem odeljku, predstavljeni su uticaji tretmana na ponašanje životinja u testovima otvorenog polja, uzdignutog plus lavirinta i forsiranog plivanja. Dobijeni rezultati ukazuju na pojačanu anksioznost životinja CORT grupe u testu otvorenog polja, kao i na promene u ponašanju koje se mogu povezati sa depresivnom simptomatologijom u testu forsiranog plivanja. Sa druge strane, koadminitracija oksitocina tokom poslednje dve nedelje tretmana kortikosteronom je u ovim testovima (test otvorenog polja i test forsiranog plivanja) pokazala jasan anksiolitički i antidepresivni potencijal i dovela do značajnih promena u ponašanju životinja u poređenju sa životinjama tretiranim samo

kortikosteronom. Treću manju celinu čini prikaz rezultata o uticaju eksperimentalnih tretmana na strukturu i funkciju nadbubrežne žlezde. Ovaj segment podeljen je na manje celine u okviru kojih su prikazani uticaji tretmana na apsolutnu masu i strukturu žlezde, ekspresiju gena Slc6a2 i Slc18a2 i količinu proteina NAT i VMAT2 u srži, bazalnu i stresom indukovanu aktivnost nadbubrežne žlezde, odnosno koncentracije hormona kortikosterona, adrenalina i noradrenalina u plazmi pre i nakon testa forsiranog plivanja. Dobijeni rezultati ukazali su na primetnu atrofiju nadbubrežne žlezde kod životinja hronično tretiranih kortikosteronom, što se ogledalo u smanjenju površine ekvatorijalnih preseka korteksa i medule. Najintenzivniji efekat unutar korteksa zapažen je u zoni fascikulati, čija je površina bila značajno manja kod CORT u poređenju sa životinjama kontrolne grupe. Koadministracija oksitocina pokazala je pozitivan efekat na strukturu nadbubrežne žlezde, što se ogledalo u povećanju površine korteksa, a što se odrazilo i na apsolutnu masu žlezde. Dodatno, jasan efekat tretmana oksitocinom bila je i vazodilatacija krvnih sudova srži nadbubrežne žlezde, koja je bila najintenzivnija u OXY grupi životinja. Takođe, tretman oksitocinom delovao je stimulatorno na ekspresiju gena Slc6a2 i Slc18a2 i/ili količinu proteina transportera za kateholamine NAT i VMAT2 u meduli, na taj način povećavajući kapacitet žlezde za skladištenje kateholamina. Na kraju ovog odeljka, prikazan je uticaj eksperimentalnih tretmana na bazalne i stresom indukovane nivoe kortikosterona i kateholamina u plazmi eksperimentalnih životinja. Tronedeljni tretman kortikosteronom doveo je do malog, ali statistički značajnog smanjenja bazalne koncentracije ovog hormona u plazmi, kao i do smanjenja odgovora žlezde na akutni stres, tako da nije primećen uobičajen porast koncentracije ovog hormona u plazmi životinja CORT i CORT+OXY grupe nakon testa forsiranog plivanja u poređenju sa bazalnim vrednostima. Svi eksperimentalni tretmani doveli su do porasta bazalnih koncentracija adrenalina u plazmi u poređenju sa kontrolnom grupom. Interesantan je nalaz da su kod životinja tretitanih okistocinom (OXY i CORT+OXY grupe) koncentracije adrenalina i/ili noradrenalina u plazmi nakon akutnog stresa bile značajno niže u poređenju sa odgovarajućim bazalnim vrednostima. Četvrti manji odeljak druge sekcije poglavlja Rezultati čine rezultati o uticaju eksperimentalnih tretmana druge faze na parametre oksidativnog stresa u krvi Wistar pacova. U okviru ove celine, prvo su prikazani rezultati komet testa koji pokazuju oštećenja DNK limfocita periferne krvi bez i nakon *in vitro* inkubacije sa vodonik-peroksidom. Rezultati su prikazani kao ukupan broj ćelija sa oštećenjem,

broj ćelija sa niskim stepenom oštećenja DNK, broj ćelija sa visokim stepenom oštećenja DNK i odgovarajući skor kometa. Rezultati ukazuju da tretman kortikosteronom povećava osetljivost ćelija na oštećenja indukovana *in vitro* tretmanom vodonik-peroksidom, dok je koadministracija oksitocina, dovela do održavanja oštećenja DNK na niskom nivou. Ovaj nalaz je značajan u svetlu činjenice da je pokazano da se nizak nivo oštećenja DNK u ćelijama može uspešno reparirati. Nakon ovih rezultata, prikazan je uticaj tretmana na parametre oksidativnog stresa i antioksidativne zaštite u uzorcima plazme eksperimentalnih životinja. Iako tretmani nisu imali uticaja na koncentraciju malondialdehida u plazmi, tretman oksitocinom poslednje dve nedelje administracije kortikosterona značajno je smanjio koncentraciju lipidnih hidroperoksida, koji se smatraju ranim produktima oksidacije ćelijskih lipida, u plazmi Wistar pacova. Takođe, tretman kortikosteronom doveo je do značajnog smanjenja aktivnosti enzima antioksidativne zaštite, superoksid dizmutaze, dok je dodatni tretman oksitocinom uz kortikosteron značajno povećao njegovu aktivnost i na taj način pokazao antioksidativni zaštitni potencijal. U narednom odeljku prikazani su rezultati uticaja eksperimentalnih tretmana druge faze na ekspresiju pozitivnog modulatora adultne neurogeneze - neurotrofičnog faktora poreklom iz mozga (BDNF) kao i nuklearnog proteina Ki-67, u hipokamusu Wistar pacova, kao i količinu proteina BDNF određenu Westren blot analizom. Dobijeni rezultati ukazali su na negativan uticaj tretmana kortikosteronom na proces neurogeneze, koji se ogledao u značajnom smanjenju gustine BDNF+ ćelija u dentatnom girusu i CA2+CA3 regiji hipokampusa, kao i gustine Ki-67+ ćelija u dentatnom girusu u poređenju sa životinjama kontrolne grupe. Tretman oksitocinom poslednje dve nedelje administracije kortikosterona pokazao je regionalno specifičan, pozitivan uticaj na proces hipokampalne neurogeneze, prvenstveno povećavajući ekspresiju BDNF u dentatnom girusu i CA2+CA3 regionima, kao i ukupnu količinu ovog proteina. U okviru treće velike celine poglavlja Rezultati, prikazani su dobijeni rezultati treće eksperimentalne faze, o uticaju dodatnog tretmana oksitocinom uz tretman citalopramom na ponašanje i ekspresiju gena Itgb3 i Chl1 koji kodiraju protieine ITGB3 i CHL1 u prefrontalnom korteksu pacova, u kortikosteronom-indukovanom modelu hroničnog stresa i depresije. Ovo poglavlje je podeljeno na manje odeljke u kojima su pojedinačno opisani uticaji tretmana na svaki izvođeni test ponašanja, kao i na ekspresiju iRNK Itgb3 i Chl1. Kod kortikosteronom-tretirane grupe životinja, u testu forsiranog plivanja, pokazano je ponašanje koje se može povezati sa depresivnom simptomatologijom,

potvrđen je antidepresivni efekat citaloprama, a isti efekat je pokazan i kombinacijom tretmana oksitocina i citaloprama. U poslednjem odeljku grafički su prikazani efekti korišćenih tretmana na ekspresiju gena za protein ITGB3 i CHL1, koji se smatraju potencijalnim biomarkerima povoljnog odgovora na terapiju antidepresivima iz grupe SSRI, u tkivu prefrontalnog korteksa. Pokazano je da tretman kortikosteronom značajno smanjuje ekspresiju iRNK za Itgb3 u prefrontalnom korteksu, dok koadministracija oksitocina (CORT+OXY), kao i kombinacije oksitocina i citaloprama (CORT+OXY+CIT) dovodi do povećanja ekspresije ovog biomarkera u poređenju sa tretmanom samim kortikosteronom. Sa druge strane, nije utvrđeno postojanje razlika u ekspresiji iRNK za Chl1 između svih eksperimentalnih grupa treće eksperimentalne faze ove disertacije.

*The **Results** chapter contains all the results obtained in the experimental work of the dissertation. Results are shown as figures and tables and presented with regard to the previously set objectives. The results are arranged in three major sections and nine sub-sections. In the first section, results of the first experimental phase of dissertation are displayed. As it was shown that treatment with lower dose of oxytocin (0.25 IU/ 400 μL), regardless of the treatment duration, did not affect the analyzed parameters, the effects of higher oxytocin dose used (applied acutely and subchronically) on behavior and biogenic amines plasma levels of adult male Wistar rats were clearly presented. Results are shown for each behavioral test and analyzed hormone, respectively. The obtained results suggested that subchronic treatment with oxytocin (10 IU/400 μL) showed anxiolytic- and antidepressive-like potential in all behavioral paradigms used. Also, regardless of the duration, oxytocin administration significantly increased plasma levels of adrenaline and serotonine in plasma of adult male rats. The next section of Results chapter represents the results of the second experimental phase of dissertation. In the first sub-section, the effects of experimental treatments on body weight gain and water and food intake are described and graphically presented. It was shown that the animals chronically treated with corticosterone gained significantly less weight compared to the untreated animals, which is a very well known effect of chronicaly elevated glucocorticoid levels (Conrad et al., 2007; Gourley and Taylor, 2009). On the other hand, co-administration of oxytocin attenuated the negative effects of corticosterone on body weight gain in the CORT+OXY experimental group. In the second sub-section, the effects of applied treatments on behavior of animals in OFT, EPM and*

FST are presented. The results indicated that the animals belonging to the CORT experimental group displayed increased anxiety in OFT and depressive-like phenotype in FST. Co-administration of oxytocin for the last two weeks of corticosterone treatment showed an apparent anxiolytic and antidepressive potential in those tests (OFT and FST) compared to the group of animals treated with corticosterone only. The third sub-section covers the results about the effects of experimental treatments on adrenal gland structure and function. This section is divided into parts describing treatment effects on: glandular weight and morphometric parameters; gene expression of Slc6a2 and Slc18a2 and amounts of catecholamine transporters noradrenaline transporter (NAT) and vesicular monoamine transporter 2 (VMAT2) in adrenal medulla; basal and plasma levels of corticosterone, adrenaline and noradrenaline after acute stress challenge induced by 15 min. of forced swimming. The obtained results indicated noticeable adrenal gland atrophy and decreased mean cortical and medullary area in the animals treated chronically with corticosterone. The most prominent effect in the adrenal gland cortical zones was noticed in zona fasciculate; the area of this cortical zone was significantly decreased compared to control animals. Co-administration of oxytocin in the model of chronic stress induced by corticosterone showed positive effects on adrenal gland morphometry, primarily by increasing mean cortical area, which was reflected in adrenal weight as well. An additional effect of oxytocin treatment was a noticeable vasodilatation of medullary blood vessels, and this effect was the most prominent in the OXY group. Oxytocin treatment had a positive effect on the gene expression and/or amount of proteins NAT and VMAT2 in adrenal medulla, thus increasing the storage capacity for catecholamines in adrenal gland. At the end of this section, the effects of the treatments on basal and stress induced corticosterone and catecholamines plasma levels in experimental animals were shown. Three-weeks of corticosterone administration resulted in a slight, but significant reduction of the basal corticosterone plasma concentration and blunted glandular response to the acute stress. Therefore, the usual increase in corticosterone plasma concentration after forced swimming compared to basal levels in CORT and CORT+OXY group was not detected. All used experimental treatments increased basal adrenaline levels compared to the controls. In both experimental groups treated with oxytocin (OXY and CORT+OXY group), the response of chromaffine cells and thus catecholamines levels after acute stress challenge was somewhat

surprising. Namely, contrary to the increase in adrenaline and noradrenaline levels after acute stress observed in control group, decrease of plasma catecholamine levels after acute stress was noted in both groups of rats treated with oxytocin. In the fourth sub-section, the effects of treatments on oxidative stress parameters in blood samples of Wistar rats are displayed. Within this section, the results of the comet test, which detects DNA damage in peripheral blood lymphocytes before and following in vitro treatment with hydrogen-peroxyde are presented. The results are shown as the total number of damaged cells, cells with moderately damaged DNA and cells with highly damaged DNA, as well as the appropriate comet scores. The obtained data indicated that the treatment with corticosterone reduced lymphocyte capacity in defense against an additional oxidative insult by hydrogen-peroxyde. Co-administration of oxytocin managed to keep DNA damage at moderate levels, and it is known that low levels of DNA damage is repairable. Next, the results about effects of experimental treatments on oxidative stress and antioxidative protection parameters in plasma of animals are shown. Although the applied treatments did not affect plasma malondialdehyde (MDA) levels, treatment with oxytocin for the last two weeks of corticosterone administration significantly decreased the plasma concentration of lipid hydroperoxides (LOOH), which are considered to be early products of cell lipid oxidation. Also, the treatment with corticosterone decreased plasma activity of antioxidative defense enzyme, superoxide dismutase, while additional treatment with oxytocin significantly increased its activity, thus showing certain antioxidative and protective potential. The next sub-section is composed of the results of immunohistochemical analysis, showing the effects of experimental treatments on brain-derived neurotrophic factor (BDNF) and nuclear protein Ki-67 expression in hippocampus of Wistar rats, which are considered to have a supportive role in the processes of hippocampal adult neurogenesis. Additionally, the amount of BDNF protein in the hippocampal tissue of rats was determined using Western blot analysis. The results indicated that the treatment with corticosterone had negative effects on neurogenesis, which was detected as significantly decreased density of BDNF+ neurons in the dentate gyrus (DG) and CA2+CA3 regions of the hippocampus, as well as density of Ki-67+ neurons in DG, compared to control animals. Treatment with oxytocin for the last two weeks of corticosterone administration displayed the regionally specific positive effect on hippocampal neurogenesis, primarily by increasing the expression of BDNF in the DG and CA2+CA3 regions, as well as the amount of

BDNF protein in hippocampal tissue. In the last section of the Results chapter, the effects of experimental treatments applied in the third experimental phase of the dissertation, describing combined oxytocin-citalopram treatment on behavior and expression of integrin beta-3 (Itgb3) and Chl1 mRNA in the prefrontal cortex in animal model of chronic stress/depression are shown. This section is divided into two parts in which the effects of treatments in each behavioral test performed (OFT, EPM and FST) and expression of Itgb3 and Chl1 mRNA are shown separately. Animals treated with corticosterone displayed depressive-like behavior in the FST, antidepressive effect of citalopram has been confirmed, and same effect was demonstrated with combination of oxytocin and citalopram. In the last section, the effects of treatments on expression of genes Itgb3 and Chl, encoding ITGB3 and CHL1 molecules, that are considered to be biomarkers of the positive response to therapy with SSRI group of antidepressants, in the prefrontal cortex tissues of rats is depicted. It is shown that the treatment with corticosterone significantly decreased expression of Itgb3 mRNA in prefrontal cortex, while co-administration of oxytocin (CORT+OXY group), as well as the combination of oxytocin and citalopram (CORT+OXY+CIT group) significantly increased expression of this biomarker compared to the CORT group. On the other hand, no differences in Chl1 mRNA expression levels were observed between groups of the third experimental phase of this dissertation.

C. UPOREDNA ANALIZA REZULTATA SA PODACIMA IZ LITERATURE/ *COMPARISON OF THE OBTAINED RESULTS WITH THE PUBLISHED DATA*

U poglavlju **Diskusija**, dobijeni rezultati su detaljno analizirani i sagledani u svetlu postojećih literaturnih podataka. Ovo poglavlje podeljeno je u tri veće celine, u skladu sa eksperimentalnim fazama disertacije. U okviru prve faze, sagledane su razlike u uticaju akutnog i subhroničnog tretmana oksitocinom na ponašanje i parametre aktivnosti HPA osovine kod Wistar pacova. Rezultati su razmotreni u svetlu publikovanih literaturnih podataka studija u kojima su ispitivani efekti oksitocina, kako na eksperimentalnim životinjama, tako i na humanoj populaciji. U drugoj celini poglavlja Diskusija, sagledani su efekti subhroničnog tretmana oksitocinom na ponašanje, parametre aktivnosti HPA osovine, oksidativni status i proces hipokampalne neurogeneze u animalnom modelu hroničnog stresa i depresije indukovane tronedeljnom primenom

kortikosterona, dok su u trećoj celini analizirani pokazani efekti kombinovanog tretmana oksitocinom i citalopramom na ponašanje i ekspresiju ITGB3 i CHL1 iRNK u prefrontalnom korteksu životinja, u navedenom modelu.

*In the **Discussion** chapter, the results are analyzed in detail and reviewed considering the current literature. This chapter is divided into three major sections, in line with experimental phases of the dissertation. In the first phase, the differences in effects of acute and subchronic treatments with oxytocin on behavior and HPA axis activity of Wistar rats were elaborated. The results are reviewed with regard to the published literature on oxytocin effects, both in human populations and in experimental models. In the second major section of the Discussion chapter, the effects of subchronic treatment with oxytocin on behavior, HPA axis activity, oxidative status and hippocampal neurogenesis are reviewed in the animal model of chronic stress-induced depression-like behavior by three-week corticosterone administration. Also, in the third section, the effects of combined oxytocin and citalopram treatment on behavior and expression of Itgb3 and Chl1 mRNA in prefrontal cortex of the animals, in stated model, are analyzed.*

Poslednjih decenija znatno se povećao broj studija u kojima je istraživan uticaj oksitocina na ponašanje. U ovim studijama, pokazano je da oksitocin menja reakciju organizma na stresne stimuluse (Svanidze i sar., 2012; Jovanović i sar., 2014), učestvuje u adaptaciji na hronični stres (Zheng i sar., 2009), ispoljava antinociceptivni efekat (Rash i Campbell 2014), poboljšava socijalno prepoznavanje (Lieberwirth i Wang, 2014) i reguliše društvene strahove (Guzman i sar., 2014). Takođe, pokazano je da ovaj neuropeptid ispoljava efekte i na nadbubrežnu žlezdu, glavni efektorni organ HPA osovine. Naime, oksitocin povećava broj hromafinih ćelija u srži nadbubrega pacova, dovodi do promena u broju ćelija i zapremini unutrašnjih zona kore i povećava količinu adrenalina, noradrenalina, dopamina i serotonina u žlezdi (Plećaš i sar., 1989). Centralna administracija oksitocina ženkama pacova uticala je na bazalne koncentracije adrenokortikotropnog hormona i kortikosterona u krvi i značajno umanjila odgovor ovih hormona nakon izlaganja životinja stresnoj situaciji (Windle i sar., 2004).

The number of studies analyzing effects of oxytocin on behavior has significantly increased over the last decades. In these studies, it has been shown that oxytocin modulates reaction of animals to stressful stimuli (Svanidze et al., 2012; Jovanović et al., 2014), mediates adaptation mechanism against chronic stress (Zheng et al., 2009), has antinociceptive effect (Rash and

Campbell, 2014), improves social bonding (Lieberwirth and Wang, 2014) and modulates social fear (Guzman et al., 2014). It has been shown that this neuropeptide also exhibits effects on the adrenal gland, which is the main effector organ of the HPA axis. Namely, oxytocin increases the number of chromaffin cells in the adrenal medulla, changes the number of cells and volume of cortical zones of the adrenal gland, and increases its content of adrenaline (A), noradrenaline (NA), dopamine and serotonin (Plećaš, 1989). Central oxytocin administration to female rats affects their basal adrenocorticotropic hormone (ACTH) and corticosterone levels and significantly decreases the response of this hormones to stress (Windle et al., 2004).

Poremećaji raspoloženja, uključujući i depresiju, su ozbiljni zdravstveni problemi koji, makar u jednoj manifestovanoj epizodi tokom života, pogađaju oko 20% svetske populacije. Prema istraživanjima Svetske zdravstvene organizacije, depresija postaje vodeći uzrok radnog onesposobljavanja širom sveta (Zhao i sar., 2008). Poznato je da pored naslednih faktora, u razvoju depresivnih epizoda veliki uticaj ima i hronična izloženost stresnim situacijama, kao i to da kod ovih pacijenata često dolazi do poremećaja u funkciji HPA osovine (Zhao i sar., 2008; Jovanović i sar., 2014). Budući da je pokazano da oksitocin utiče na nadbubrežnu žlezdu (Plećaš i sar., 1989) i da postoji povezanost koncentracije ovog hormona i određenih vrsta ponašanja, među kojima i depresivnog, može se pretpostaviti da bi ispitivanja uticaja oksitocina na aktivnost HPA osovine mogla biti od pomoći u sagledavanju etiologije i terapije depresije.

Mood disorders, including depression, are severe health problems which, affect about 20% of world population during lifetime. According to The World Health Organization, depression is becoming the leading cause of disability worldwide (Zhao et al., 2008). It is known that, beside genetic, one of the most important predisposing factors in the development of depression is chronic stress, and that patients suffering from depressive disorders often display a dysregulation of HPA axis function (Zhao et al., 2008; Jovanović et al., 2014). Keeping in mind that oxytocin displays effects on the adrenal gland (Plećaš, 1989), and that correlation between oxytocin level and certain behavioral changes, including depressive-like behavior has been demonstrated, it could be assumed that analyzing effects of oxytocin on the HPA axis activity could help in explaining the ethiology and pathophysiology of depression.

Rezultati prve faze disertacije, ukazali su da kod Wistar pacova tretman oksitocinom u dozi 10 IU/400 μL 30 min. pre izvođenja testova ponašanja, kao i tokom 14 dana pokazuje određeni

anksiolitički i antidepresivni potencijal. Kako su intenzivniji efekti na ponašanje životinja i aktivnost HPA osovine registrovani tokom subhroničnog tretmana oksitocinom, u animalnom modelu depresije indukovane hroničnom primenom kortikosterona ispitivan je uticaj subhroničnog tretmana oksitocinom.

Results of the first phase of the dissertation have shown that the administration of oxytocin to Wistar rats in dose of 10 IU/400 μL, 30 minutes before the behavioral testing, and during 14 days, showed certain anxiolytic and antidepressive potential. Since more intensive effects on behavior and HPA axis activity have been observed during subchronic treatment with oxytocin, in animal model of depression induced by chronic corticosterone application, the effects of subchronic oxytocin treatment were further examined.

Rezultati druge faze disertacije potvrdili su dosadašnja saznanja o negativnom uticaju hroničnog tretmana kortikosteronom na porast telesne mase životinja (Donner i sar., 2012). Sa druge strane, tretman oksitocinom poslednjih 14 dana eksperimentalnog protokola ublažio je negativan efekat hiperkortizolizma na porast telesne mase, dok sam oksitocin nije imao uticaja na masu životinja, što je u skladu sa literaturnim podacima (Bjorkstrand u Uvnas-Moberg, 1996) koji ukazuju da *i.c.v.* i *s.c.* tretman oksitocinom nije doveo do promena telesne mase kod mužjaka pacova. U okviru ove faze disertacije, dobijeni rezultati testova ponašanja potvrđuju dosadašnja saznanja da hronična izloženost visokim koncentracijama glukokortikoida dovodi do promena u ponašanju pacova koja se mogu povezati sa povećanom anksioznošću i depresivnom simptiomatologijom (Hill i sar., 2003; Gregus i sar., 2005; Johnson i sar., 2006). Tačnije, životinje CORT grupe su u testu forsiranog plivanja značajno više vremena provodile u imobilnom položaju i pokazale kraće latentno vreme u poređenju sa životinjama kontrolne grupe, što upućuje na pojavu depresivnog fenotipa (Detke i sar., 1996; Cryan i sar., 2002; Johnson i sar., 2006). U testu otvorenog polja, tretman kortikosteronom doveo je do značajnog povećanja vremena imobilnosti i smanjenja vremena provedenog u centralnoj zoni arene, što jasno ukazuje na povećanu anksioznost ovih životinja. Koadministracija oksitocina poslednje dve nedelje tretmana kortikosteronom povećala je eksplorativnu aktivnost životinja u testu otvorenog polja i pokazala antidepresivni potencijal u testu forsiranog plivanja produžavajući latentno vreme. Ovi rezultati idu u prilog dosadašnjim podacima o anksiolitičkim i antidepresivnim efektima oksitocina, dobijenim u drugim modelima hroničnog stresa, ali takođe i na humanoj populaciji (Grppo i sar., 2009; Yan i sar., 2014;

Scantamburlo i sar., 2015; Ji i sar., 2016). Međutim, tačan mehanizam kojim oksitocin ostvaruje ovakve efekte na ponašanje još uvek je nepoznat.

The results obtained in the second phase of dissertation have confirmed previously collected findings about negative effects of chronic corticosterone treatment on body weight gain of experimental animals (Donner et al., 2012). On the other hand, the treatment with oxytocin lasting 14 days has diminished negative effect of the hypercortisolism on body weight gain, while oxytocin itself did not affect the animal's body weight. This is in alignment with the literature (Bjorkstrand and Uvnas-Moberg, 1996) pointing that i.c.v. and s.c. oxytocin administration did not affect body weight in male rats. Results of behavioral testing from this phase of the dissertation confirmed current knowledge that exposure of animals to chronically high glucocorticoids leads to behavioral changes, which can be linked to increased anxiety and depressive-like behavior (Hill et al., 2003; Gregus et al., 2005; Johnson et al., 2006). In particular, animals in CORT experimental group spent significantly more time in immobile position in forced swim test, and showed shorter latency compared to control animals, which suggests the appearance of depressive phenotype in this group of animals (CORT group) (Detke et al., 1996; Cryan et al., 2002; Johnson et al., 2006). In the open field test, corticosterone treatment significantly increased immobility time and decreased time animals spent in the central zone of arena, which indicates to increased anxiety in those animals. Co-administration of oxytocin during the last two weeks of corticosterone treatment increased explorative activity of animals in open field test, and showed antidepressive potential in the forced swim test by increasing latency to iommobility in the animals belonging to CORT+OXY treatment group. These results are in accordance with previous findings about anxiolytic and antidepressive properties of oxytocin, obtained in different models of chronic stress, as well as in humans (Grppo et al., 2009; Yan et al., 2014; Scantamburlo et al., 2015; Ji et al., 2016). However, the exact mechanism of the behavioral effects of oxytocin is still unknown.

Na dalje su diskutovani rezultati ove disertacije koji su doneli nova saznanja o uticaju oksitocina na aktivnost HPA osovine u eksperimentalnom modelu depresije indukovane hroničnom primenom kortikosterona a koja bi mogla doprineti razumevanju mehanizma pokazanih anksiolitičkih i antidepresivnih efekata ovog neuropeptida. Koadministracija oksitocina ublažila

je atrofiju nadbubrežne žlezde koja je bila posledica hroničnog tretmana kortikosteronom, povećala površinu korteksa žlezde i ublažila odgovor hormona na akutni stres. Takođe, putem povećanja ekspresije Slc6a2 i Slc18a2 gena i/ili količine proteina za kateholaminske transportere u srži žlezde NAT i VMAT2, oksitocin je doveo do povećanja kapaciteta nadbubrega za skladištenje kateholamina, što se smatra jednim od glavnih mehanizama adaptacije organizma na uslove hronične izloženosti stresu (Eisenhofer i sar., 2004).

Further, the results of dissertation which have brought new insight about effects of oxytocin on HPA axis activity in an animal model of depression induced by chronic corticosterone administration, and which could contribute to understanding the mechanisms of displayed anxiolytic and antidepressive effects of this neuropeptide, are discussed. Co-administration of oxytocin alleviated the adrenal gland atrophy induced by chronic corticosterone treatment, increased cortical area, and diminished hormonal response to acute stress challenge. Also, by increasing gene expression of Slc6a2 and Slc18a2 and/or amount of proteins of catecholamines transporters in the adrenal medulla, NAT and VMAT2, oxytocin treatment increased adrenal gland catecholamines storage capacity, which is considered to be one of the main adaptation mechanisms to chronic stress conditions (Eisenhofer et al., 2004).

Rezultati ove disertacije ukazali su da administracija oksitocina u animalnom modelu hroničnog stresa indukovanog *per os* primenom kortikosterona, ublažava oštećenja DNK limfocita periferne krvi nastala usled *in vitro* tretmana vodonik-peroksidom, jakim oksidativnim agensom. Ovakvi efekti oksitocina mogu se pripisati njegovom antioksidativnom potencijalu (Vargas-Martinez i sar., 2014; Gonzalez-Reyes i sar., 2015; Houshmand i sar., 2015). Mehanizam antioksidativnog dejstva oksitocina, makar delimično, može da uključuje supresiju lipidne peroksidacije i povećanje antioksidativnog kapaciteta, pre svega povećanje aktivnosti enzima superoksid dizmutaze u plazmi, na šta upućuju rezultati ove disertacije.

The results of this dissertation have shown that oxytocin administration in animal model of chronic stress induced by per os administration of corticosterone diminished DNA damage of peripheral blood lymphocytes induced by in vitro treatment with hydrogen peroxide, a potent oxidative agent. Observed effects of oxytocin treatment can be explained by its antioxidative potential (Vargas-Martinez et al., 2014; Gonzalez-Reyes et al.; 2015; Houshmand et al., 2015).

The mechanism of oxytocin's antioxidative effect could include, at least in part, the suppression of the lipid peroxidation and increase of antioxidative capacity, namely, by increasing activity of superoxide dismutase in plasma, which was observed in the results of this dissertation.

Imunohistohemijska analiza moždanog tkiva pokazala je i još jednom potvrdila postojanje veoma štetnog efekta glukokortikoida na proces adultne neurogeneze u hipokampusu, što se ogledalo u značajnom smanjenju gustine ćelija koje eksprimiraju BDNF i Ki-67 u dentatnom girusu i BDNF+ ćelija u CA2+CA3 regionu. Novija istraživanja ukazuju na činjenicu da kompleksni mehanizam dejstva antidepresiva podrazumeva i pozitivnu stimulaciju adultne neurogeneze u hipokampusu (Dwivedi i sar., 2003), procesa za koji se smatra da je od velikog značaja u ulozi hipokampusa u regulaciji odgovora organizma na stres (Egeland i sar., 2015). Rezultati imunohistohemijske analize koji su pokazali da tretman oksitocinom uz hroničnu administraciju kortikosterona značajno povećava ekspresiju ova dva parametra neurogeneze u tkivu hipokampusa, mogu doprineti objašnjenju pokazanog antidepresivnog i anksiolitičkog potencijala oksitocina.

Immunohistochemical analysis of the brain tissues has shown, and once again confirmed, the existence of detrimental effects of glucocorticoids on the process of adult neurogenesis in the hippocampus, which has been measured as significant decrease in density of cells expressing BDNF and Ki-67 in the dentate gyrus, and BDNF+ cells in CA2+CA3 region. Recent studies point out to the fact that complex mechanisms of antidepressants action also implies positive modulation of adult neuroegenesis in hippocampus, (Dwivedi et al., 2006), a process considered to be of great importance in stress response regulation by hippocampus (Egeland et al., 2015). The results of the immunohistochemical analysis have shown that the oxytocin treatment along with chronic corticosterone administration significantly increased the expression of these two parameters of neurogenesis in hippocampus, and can contribute toward understanding the exhibited antidepressive and anxiolytic potential of oxytocin.

U trećoj fazi disertacije još jednom su potvrđeni rezultati dobijeni tokom izrade druge faze disertacije o anksiolitičkom i antidepresivnom potencijalu oksitocina pokazanom u testovima ponašanja. Sa druge strane, do sada nepoznati, podaci o uticaju kombinovanog tretmana oksitocinom i citalopramom na ekspresiju iRNK Itgb3 i Chl1 u prefrontalnom korteksu

eksperimentalnih životinja, u skladu su sa prethodnim nalazima. Kod ljudi, ITGB3 i CHL1 se smatraju biomarkerima povoljnog odgovora na terapiju antidepresivima iz grupe SSRI (Oved i sar., 2013). Rezultati ove disertacije su ukazali da kod hronično povišenog nivoa glukokortikoida dolazi do redukcije ekspresije iRNK Itgb3 u prefrontalnom korteksu, dok ositocin ili citalopram dovode do normalizacije ekspresije ovog gena. Najizraženiji efekat povećanja ekspresije ovog biomarkera zapažen je u grupi životinja koja je bila tretirana i oksitocinom i citalopramom, uz hroničnu administraciju kortikosteronom. Ovaj podatak ukazuje na potencijalni sinergizam hormona oksitocina i antidepresiva iz SSRI grupe u modelu depresije koja je praćena povišenim nivoom glukokortikoida, što bi trebalo da bude predmet daljih istraživanja. Takođe, prikazani rezultati ukazuju na potencijalnu mogućnost da bi kod pacijenata tretman oksitocinom, uz standardnu terapiju antidepresivima iz grupe SSRI, mogao povećati verovatnoću povoljnog odgovora na osnovnu terapiju i na taj način smanjiti procenat pacijenata koji ne reaguju adekvatno na lekove prvog izbora. Mada bi trebalo napomenuti da i pored toga što je odobrena *i.v.* primena oksitocina, pokazani su i neželjeni efekti njegove upotrebe.(Dyer i sar., 2011). Sa druge strane, potrebno bi bilo sprovesti klinička ispitivanja u cilju istraživanja poboljšanja efekta antidepresivne terapije lekova SSRI grupe korišćenjem oksitocina kao dodatne terapije.

In the third phase of dissertation, the results related to the anxiolytic and antidepressive potential of oxytocin, obtained during the second phase of dissertation are once again confirmed. On the other hand, the novel findings on the effect of combined treatment of oxytocin and citalopram on the expression of Itgb3 and Chl1 mRNA in the prefrontal cortex of experimental animals were complementary to these findings. In humans, ITGB3 and CHL1 are considered to be biomarkers of the favorable response to therapy with antidepressants from SSRI group (Oved et al., 2013). These results have shown that, in terms of chronically elevated glucocorticoids, the reduction of Itgb3 expression in prefrontal cortex is observed, while co-administration of oxytocin or citalopram lead to normalization of Itgb3 expression. The most pronounced positive effect on this biomarker's expression is observed in the group of animals treated with both oxytocin and citalopram, along with chronic administration of corticosterone. This finding indicates to potential synergism of favorable effects of the oxytocin and

antidepressants from SSRI group in the animal model of depression accompanied with increased levels of glucocorticoids, and should be a topic of further research. Also, the obtained results indicate the possibility that, in depressive patients, treatment with oxytocin together with standard therapy with antidepressants from SSRI group, could increase the possibility of favorable response to the basic therapy, and in that way possibly decrease the percentage of non-responding patients to this first-line therapy. Although, it should be noted that, while oxytocin (i.v.) is approved for human use, it also has undesired side effects (Dyer et al., 2011). On the other hand, clinical trials are required for exloring the potential of augmenting the anti-depressant effects of SSRI drugs by oxytocin (i.v.) as add-on therapy.

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D. OBJAVLJENI REZULTATI KOJI ČINE DEO DISERTACIJE/ *RESULTS WHICH ARE PART OF THE DOCTORAL DISSERTATION*

Radovi objavljeni u naučnim časopisima međunarodnog značaja/ *Papers published in the international scientific journals*

1. Stanić D., Plećaš-Solarović B., Mirković D., Jovanović P., Petrović J., Dronjak, S., Marković, B., đorđević T., Ignjatović S., Pešić V. 2017. Oxytocin in corticosterone induced chronic stress model: Focus on adrenal gland function. Psychoneuroendocrin. 80, 137-146. M21a/2014, M21/2015; IF: 5.183/ 2015.

2. Stanić D., Plećaš-Solarović B., Petrović J., Bogavac-Stanojević N., Sopić M., Kotur-Stevuljević J., Ignjatović S., Pešić V. 2016. Hydrogen peroxide-induced oxidative damage in peripheral blood lymphocytes from rats chronically treated with corticosterone: The protective effect of oxytocin treatment. Chem. Bio. Int. 256, 134-141. M22/2015; IF 2.935/2015.

Usmena izlaganja po pozivu (M31)/ *Oral presentations*

1. Stanić, D. Oxytocin ameliorates behavioural and molecular phenotypes of stress response in rats, VI Kongres endokrinologa sa međunarodnim učešćem, Beograd. 9-12.12.2016.

Saopštenja sa međunarodnih skupova štampana u celini (M33)/*Abstracts at the international scientific meetings*

1. Pešić, V., Stanić, D., Petrović, J., Puškaš, N., Plećaš, B., Ignjatović, S., Oxytocin affects changes in behaviour, BDNF and Ki-67 expression in hippocampus caused by chronic corticosterone treatment. The 29th European College of Neuropsychopharmacology (ECNP) Congress, 17-20 September 2016, Vienna, Austria. Published in European Neuropsychopharmacology, Volume 26, Supplement 2, Page S289.

2. Stanić, D., Petrović, J., Mirković, D., Đorđević, T., Đuroć, V., Jovanović, P., Dronjak, S., Pešić, V. Oxytocin modulates HPA axis activity and hormone response to sress in rats chronically treated with corticosterone. The 29th European College of Neuropsychopharmacology (ECNP) Congress, 17-20 September 2016, Vienna, Austria. Published in European Neuropsychopharmacology, Volume 26, Supplement 2, Page S204.

3. Petrović, J., Stanić, D., Puškaš, N., Oved, K., Gurwitz, D., Mirković, D., Plećaš, B., Pešić, V., Oxytocin promotes neurotrophic growth, increases integrin subunit beta 3 (ITGB3) and ameliorates depressive- and anxiety-like behaviour in rats. ECNP Workshop for junior scientists in Europe, 9-12 March, 2017, Nice, France. Published in European Neuropsychopharmacology, Volume 27, Supplement 1, Page S34-S35.

4. Stanić, D., Petrović, J., Mirković, D., Batinić B., Plećaš, B., Pešić, V., Influence of oxytocin on behavior and serotonin plasma level after long-term corticosterone treatment of Wistar rats. II Kongres farmaceuta Crne Gore sa međunarodnim učešćem, Bečići, 2015.

D. ZAKLJUČAK – OBRAZLOŽENJE NAUČNOG DOPRINOSA DISERTACIJE/ *CONCLUSION – JUSTIFICATION OF SCIENTIFIC CONTRIBUTION OF THE DOCTORAL DISSERTATION*

Doktorska disertacija kandidata diplomiranog farmaceuta Dušanke Stanić bazirana je na ispitivanju uticaja hormona oksitocina na ponašanje i parametre aktivnosti HPA osovine pacova, sa fokusom na model hroničnog stresa i depresije indukovane tronedeljnom *per os* primenom kortikosterona, što predstavlja tematiku koja je veoma aktuelna i značajna u oblasti neuronauka. Detaljnom analizom priložene doktorske disertacije Komisija je konstatovala da je disertacija prikazana na jasan i pregledan način i da su svi postavljeni ciljevi doktorske disertacije u potpunosti realizovani. Eksperimenti su organizovani i sprovedeni u skladu sa savremenim standardima u proučavanoj naučnoj oblasti, što je omogućilo dobijanje rezultata kojima se ostvaruju prethodno postavljeni ciljevi. Na kraju doktorske disertacije prikazani su zaključci izvedeni na osnovu dobijenih rezultata i podataka dostupnih u literaturi. Podaci prezentovani u disertaciji daju originalan doprinos boljem razumevanju sagledavanog problema. Svemu navedenom u prilog ide činjenica da su rezultati ove doktorske disertacije do sada publikovani u okviru dva rada u međunarodnim časopisima kategorija M21 (M21a/2014) i M22, sa ukupnim IF=8.118, četiri saopštenja sa međunarodnih skupova, i jednim usmenim izlaganjem po pozivu na skupu sa međunarodnim učešćem.

Doctoral dissertation of candidate Dušanka Stanić, bachelor in pharmacy, is based on the examination of effects of oxytocin on behavior and HPA axis activity in rats, with a focus on the model of chronic stress/depression induced by three-week per os corticosterone administration, which is relevant and important subject in the field of neuroscience. After detailed review of submitted doctoral dissertation, Committee ascertains that dissertation is presented in clear and concise manner and that all defined aims of doctoral dissertation are fully accomplished.All experiments were organized and conducted in accordance with contemporary standards in the research field studied, which enabled obtaining results that sustainedachievementof predefined aims of the dissertation. At the end of the doctoral

dissertation, the conclusions were made according toobtained results, as well as, findings available in the literature. Findings presented in the dissertation provide a genuine contribution to research efforts in the explored scientific field. All above mentioned is supported by the fact that up to now the results of this doctoral dissertation are published in two papers in international journals category M21 (M21A/2014) and M22, with a total IF=8.118, four short papers presented on the international scientific meetings, and one invited oral presentation at the national conference with international participation.

Na osnovu iznetih rezultata i diskusije doktorske disertacije, doneti su sledeći zaključci:

- Dvonedeljni tretman oksitocinom u modelu hroničnog stresa i depresije pokazuje anksiolitički i antidepresivni potencijal što je procenjeno primenom odgovarajućih testova ponašanja (test otvorenog polja, uzdignuti plus lavirint i test forsiranog plivanja).
- Oksitocin pokazuje zaštitni efekat na strukturu i funkciju nadbubrežne žlezde u modelu kortikosteronom indukovanog hroničnog stresa i depresije. Ovaj efekat ogleda se u:
    - prevenciji atrofije žlezde indukovane hronično povišenim nivoom glukokortikoida. Oksitocin prvenstveno dovodi do povećanja površine ekvatorijalnog preseka korteksa žlezde, što se odrazilo i na apsolutnu masu organa,
    - upadljive vazodilatacije u srži nadbubrežne žlezde,
    - pozitivnoj modulaciji ekspresije gena Slc6a2 i Slc18a2 koji kodiraju dva najznačajnija transportera za kateholamine, noradrenalinskog transportera (NAT) i vezikularnog transportera za monoamine 2 (VMAT2). Na ovaj način, oksitocin, povećava kapacitet srži za skladištenje kateholamina, što se smatra jednim od glavnih mehanizama adaptacije i zaštite organizma u uslovima izloženosti hroničnom stresu.
    - dvosmernom uticaju na nivo hormona nadbubrežne žlezde u plazmi, pre i nakon akutnog stresa
- Oksitocin ispoljava antioksidativni potencijal i povoljno utiče na procese regulacije oksidativnog stresa/antioksidativne zaštite kod životinja hronično tretiranih kortikosteronom. Ovaj efekat se ogleda u:

- smanjenju stepena oštećenja DNK limfocita periferne krvi pacova nakon *in vitro* inkubacije sa vodonik-peroksidom, što najverovatnije omogućava adekvatno odvijanje procesa reparacije DNK.
- smanjenju koncentracije lipidnih hidroperoksida u plazmi, karakterisričnih markera oksidativnog oštećenja,
- povećanju aktivnosti enzima antioksidativne zaštite, superoksid dizmutaze.

- Tretman oksitocinom u značajnoj meri umanjuje negativan efekat kortikosterona na proces adultne neurogeneze u hipokampusu, a što se ogledalo u:
  - povećanoj gustini BDNF+ neurona u dentatnom girusu i CA2/CA3 regionu hipokampusa kao i količini BDNF proteina u tkivu hipokampusa.
  - povećanju gustine ekspresije Ki-67+ neurona u dentatnom girusu, mada ovaj efekat nije zapažen u modelu hroničnog stresa/depresije indukovane kortikosteronom, već kod pacova tretiranh samo oksitocinom.

- Dodatni tretman oksitocinom uz citalopram, antidepresiv iz grupe selektivnih inhibitora preuzimanja serotonina, u modelu kortikosteronom indukovanog hroničnog stresa i depresije, dovodi do povećanja ekspresije gena za ITGB3 u tkivu prefrontalnog korteksa. Kako se ITGB3 protein može smatrati biomarkerom povoljnog odgovora na terapiju antidepresivima iz grupe SSRI i neophodan je za funkcionisanje transportera za serotonin, može se pretpostaviti da bi tretman oksitocinom mogao povećati verovatnoću adekvatnog odgovora pacijenata na lekove prvog izbora u terapiji depress ivnih poremećaja.

Konačno, može se zaključiti da rezultati ove doktorske disertacije promovišu hormon oksitocin kao potencijalni dodatni tretman uz antidepresivnu terapiju kod pacijenata koji pate od nekog od depresivnih poremećaja a kod kojih je hronično povišen nivo glukokortikoida.

Based on the presented results, as well as the discussion of doctoral dissertation, the following conclusions have been made:

- *The two–week-long oxytocin treatment in the model of chronic stress/depression showed anxiolytic and antidepressant potential, which was evaluated using appropriate behavioral tests (open field test, elevated plus maze and forced swim test).*

- *Oxytocin exerts a protective effect on the adrenal gland function and structure in a model of corticosterone-induced chronic stress/depression. This effect was reflected as:*

    - *prevention of adrenal gland atrophy induced by chronically elevated level of glucocorticoids. Namely, oxytocin increased cortical area of equatorial adrenal sections, which affected absolute weight of the gland,*
    - *noticeable vasodilatation in the adrenal medulla,*
    - *positive modulation of gene expression of Slc6a2 and Slc18a2 encoding two main catecholamine transporters, noradrenaline transporter (NAT) and vesicular monoamine transporter 2 (VMAT2). In this way, oxytocin administration increased adrenal medulla storage capacity for catecholamines, which is considered to be one of the main adaptation mechanisms to the chronic stress condition,*
    - *bidirectional impact on adrenal gland hormone basal, and levels after acute stress challenge.*

- *Oxytocin exhibited antioxidative potential and positive influence on regulation of oxidative stress/antioxidative protection in animals chronically treated with corticosterone. This effect was reflected as:*

    - *decreased DNA damage of peripheral lymphocytes after in vitro incubation with hydrogen peroxide, which most probably enables adequate reparation of DNA,*
    - *decreased plasma concentration of lipid hydroperoxides, characteristic biomarkers of oxidative damage,*
    - *increased activity in plasma of antioxidative enzyme, superoxide dismutase.*

- *Treatment with oxytocin markedly attenuated negative effects of corticosterone on adult hippocampal neurogenesis, which was reflected as:*

- *increased density of BDNF+ neurons in dentate gyrus and CA2+CA3 hippocampal region, as well as increased amount of BDNF protein in hippocampal tissue,*
- *increased expression of Ki-67+ neurons in dentate gyrus, however this effect was not noticed in a model of chronic stress/depression induced by corticosterone, nonetheless in rats treated with oxytocin only.*

- *Additional treatment with oxytocin along with citalopram, antidepressant from selective serotonin reuptake inhibitors group, in an animal model of corticosterone-induced chronic stress/depression, exhibited increase in expression of gene Itgb3 encoding ITGB3 protein in prefrontal cortex tissue of rats. As ITGB3 protein could be considered as biomarker of favorable response to therapy with antidepressants from SSRI group, and is required for serotonin transporter activity, it could be concluded that, in patients, treatment with oxytocin could increase possibility for adequate response to first line drugs used in the therapy of depressive disorders.*

Finally, it could be concluded that results of this doctoral dissertation promote hormone oxytocin as potential additional treatment, along with therapy with antidepressants, in patients suffering from depressive disorders accompanied with chronically elevated glucocorticoid level.

Na osnovu svega izloženog, može se zaključiti da je kandidat ispunio postavljene ciljeve u doktorskoj disertaciji pod nazivom **"Uticaj oksitocina na aktivnost osovine hipotalamus-hipofiza-nadbubreg i ponašanje pacova"**, te predlažemo Nastavno-naučnom veću Farmaceutskog fakulteta da prihvati Izveštaj i omogući kandidatu dipl. farm. Dušanki Stanić odbranu doktorske disertacije.

*Based on the above consideration, it can be concluded that candidate fulfilled defined aims of the doctoral dissertation entitled: **"Effects of oxytocin on the Hypothalamic-Pituitary-Adrenal axis activity and behaviour of rats"**, and therefore we advise the Academic Council of the Faculty of Pharmacy – University of Belgrade to accept this Report and permits candidate, bachelor in pharmacy, Dušanka Stanić, defence of this doctoral dissertation.*

Beograd/ *Belgrade*

4.5.2017.

Članovi komisije/ *Committee members*

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