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Nevena R. Todorović

EFEKTI FLUOKSETINA I KLOZAPINA NA ANTIOKSIDATIVNI SISTEM I PARAMETRE INFLAMACIJE U MOZGU I JETRI PACOVA U USLOVIMA HRONIČNE IZOLACIJE

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Nevena R. Todorović

THE EFFECTS OF FLUOXETINE AND CLOZAPINE ON ANTIOXIDATIVE SYSTEM AND INFLAMMATION PARAMETERS IN THE BRAIN AND LIVER OF CHRONICALLY ISOLATED RATS

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Mentori:

dr Dragana Filipović, naučni savetnik,
Univerzitet u Beogradu – Institut za nuklearne nauke „Vinča”

dr Jelena Đorđević, redovni profesor,
Univerzitet u Beogradu – Biološki fakultet

Članovi komisije:

dr Dragana Filipović, naučni savetnik,
Univerzitet u Beogradu – Institut za nuklearne nauke „Vinča”

dr Jelena Đorđević, redovni profesor,
Univerzitet u Beogradu – Biološki fakultet

dr Snežana B. Pajović, naučni savetnik,
Univerzitet u Beogradu – Institut za nuklearne nauke „Vinča”
Redovni profesor, Univerzitet u Nišu – Medicinski fakultet

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Efekti fluoksetina i klozapina na antioksidativni sistem i parametre inflamacije u mozgu i jetri pacova u uslovima hronične izolacije

SAŽETAK

Depresija je visokoprevalentno, multifaktorijalno i heterogeno psihijatrijsko oboljenje kompleksne neurobiološke osnove. S obzirom da veliki broj pacijenata (30–40%) ne postiže remisiju bolesti uprkos antidepresivnoj terapiji, patofiziologija depresije i mehanizmi delovanja antidepresiva i danas su aktuelne problematike iako se intenzivno istražuju decenijama unazad. Brojne studije ukazuju da hronični psihosocijalni stres, vodeći sredinski faktor rizika za razvoj depresije, izaziva oksidativni stres i inflamaciju u mozgu, procese koji imaju važnu ulogu u etiopatofiziologiji ovog oboljenja. Pored toga, sve je više podataka koji pokazuju da je disregulacija γ-aminobuterna kiselina (GABA) sistema u mozgu povezana sa depresijom.

U radu su analizirani efekti antidepresiva fluoksetina i atipičnog antipsihotika klozapina na glutation (GSH)-zavisni antioksidativni sistem i parametre inflamacije u hipokampusu i prečeonoj zoni kore cerebruma, moždanim regionima posebno osetljivim na stres. Takođe, ispitan je uticaj ovih lekova na GABA signalizaciju u medijalnoj prečeonoj zoni kore cerebruma, kao i njihova hepatotoksičnost. Ovi efekti su istraživani na životinjskom modelu depresije koji se zasniva na hroničnom izlaganju psihosocijalnom stresoru. Odrasli mužjaci pacova Wistar soja su podvrgavani 21-dnevnoj izolaciji, a fluoksetin i klozapin su davani stresiranim pacovima tokom izolacije, kao i nestresiranim (kontrolnim) pacovima u periodu od 21 dana.

Hronična izolacija je dovela do promena u ponašanju koje nalikuju depresivnom i anksioznom, kompromitovala antioksidativnu odbranu posredovanu glutation peroksidazom i izazvala porast nivoa faktora nekroze tumora α (TNF-α) u hipokampusu. Takođe, izolacija je uzrokovala smanjenje koncentracije GSH, aktivaciju nuklearnog faktora-κB i porast nivoa medijatora inflamacije ciklooksigenaze-2 (COX-2), interleukina-1β i TNF-α u prečeonoj zoni kore cerebruma. Fluoksetin i klozapin su onemogućili razvoj promena u ponašanju i proinflamatorne procese u obe ispitivane moždane strukture kod izolovanih pacova. Što se tiče GSH-zavisnog sistema, fluoksetin je pokazao protektivni efekat ali samo u prečeonoj zoni kore cerebruma pacova. Ovi

rezultati pokazuju ulogu proinflamatornih molekularnih promena u razvoju simptoma depresije, kao i antiinflamatornih efekata fluoksetina i klozapina u sprečavanju istih.

Uticaj izolacije, kao i potencijalni protektivni efekti fluoksetina i klozapina na broj parvalbumin pozitivnih (PV+) ćelija ispitani su u medijalnoj prečeonoj zoni kore cerebruma, moždanom regionu za koji je pokazano da je veoma značajan sa aspekta razvoja simptoma depresije. Utvrđeno je da je izolacija dovela do značajnog smanjenja broja PV+ ćelija u cingulatnoj kori 1, prelimbičkom (PrL) i infralimbičkom (IL) podregionu, kao i dorzalnoj pedunkularnoj kori medijalne prečeone zone kore cerebruma. Fluoksetin i klozapin su onemogućili izolacijom izazvano smanjenje u PrL i IL, što ukazuje na značaj koji GABA signalizacija u ovim podregionima ima za promene ponašanja u uslovima hronične izolacije.

Hronični tretmani fluoksetinom i klozapinom su doveli do oksidativnih oštećenja proteina i lipida u jetri, kako izolovanih, tako i nestresiranih pacova. Oksidativna oštećenja su uočena i u jetri izolovanih pacova koji nisu tretirani lekom. U jetri ovih pacova je uočena povećana proizvodnja azot-monoksida i COX-2, koja je onemogućena hroničnim tretmanom fluoksetinom. Fluoksetin nije značajno uticao na strukturu tkiva jetre za razliku od klozapina koji je izazvao brojne makrovezikularne masne promene i fokalne nekroze, naročito kod izolovanih pacova.

Studija opisuje molekularne promene uključene u patofiziologiju stanja nalik depresiji hronično izolovanih pacova. Te promene obuhvataju kompromitovanje GSH-zavisnog sistema, povećanje ekspresije medijatora inflamacije u hipokampusu i prečeonoj zoni kore cerebruma, kao i smanjenje broja PV+ ćelija u medijalnoj prečeonoj zoni kore cerebruma. Takođe, studija proširuje znanja o mehanizmima delovanja fluoksetina i klozapina izvan okvira serotoninske i dopaminske signalizacije. Antidepresivni i anksiolitički efekti ovih lekova praćeni su inhibicijom proinflamatornih promena u obe moždane strukture, kao i prevencijom smanjenja broja PV+ ćelija u PrL i IL podregionima prečeone zone kore cerebruma pacova u uslovima hronične izolacije. Rezultati studije jasno ukazuju i da klozapin ispoljava više štetnih efekata na jetru od fluoksetina.

KLJUČNE REČI: Fluoksetin, klozapin, hronična izolacija, hipokampus, prečeona zona kore cerebruma, jetra, antioksidativni sistem, parametri inflamacije, parvalbumin.

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The effects of fluoxetine and clozapine on antioxidative system and inflammation parameters in the brain and liver of chronically isolated rats

ABSTRACT

Depression is a highly prevalent, multifactorial and heterogeneous disorder with complex neurobiology. Large numbers of patients (30–40%) do not achieve disease remission despite receiving antidepressive therapy. Thus, despite the decades of research, the pathophysiology of depression and mechanisms of antidepressants' actions are ongoing and widespread issues. A growing body of evidence indicates that psychosocial stress, the leading environmental risk factor, causes oxidative stress and inflammation in the brain and that these processes have important role in the pathophysiology of depression. In addition, dysregulation of γ-aminobutyric acid (GABA)-ergic system is becoming increasingly associated with this disorder.

In this study, we analyzed the effects of antidepressant fluoxetine and atypical antipsychotic clozapine on the glutathione (GSH)-dependent antioxidative system and parameters of inflammation in hippocampus and prefrontal cortex, brain regions particularly sensitive to stress. Besides, we examined the effects of drugs on the GABA-ergic signalization, as well as their hepatotoxicity. These effects were investigated in an animal model of depression based on chronic psychosocial stress. Adult male Wistar rats were isolated for 21 days and fluoxetine and clozapine were applied to the stressed rats during isolation, as well as to non-stressed rats for 21 days.

Chronic isolation caused depressive- i anxiety-like behaviors, compromised glutathione peroxidase-mediated antioxidative defense and caused increase in tumor necrosis factor α (TNF-α) levels in hippocampus. Besides, isolation decreased GSH levels, activated nuclear factor-κB and elevated cyclooxygenase-2 (COX-2), interleukin-1β i TNF-α levels in prefrontal cortex. Fluoxetine and clozapine prevented behavioral changes and proinflammatory events in both examined brain structures in isolated rats. Regarding GSH-dependent defense, fluoxetine showed protective effects, which were restricted to the prefrontal cortex. These results indicate the significant role of proinflammatory molecular changes in the development of depressive symptoms, as well as the importance of the anti-inflammatory properties of fluoxetine and clozapine in their prevention.

The effects of isolation, as well as potential protective effects of fluoxetine and clozapine on the number of parvalbumin positive (PV+) cells were examined in medial prefrontal cortex, a brain region highly implicated in the development of depressive symptoms. Decreased number of PV+ cells in cingulate cortex area 1, prelimbic area (PrL), infralimbic area (IL) and dorsal peduncular cortex of medial prefrontal cortex were found in isolated rats. Fluoxetine and clozapine prevented this effect in PrL and IL subregions, which indicates the importance of intact GABA-ergic signalization in these areas for resistance against isolation-induced behavioral changes.

Chronic fluoxetine and clozapine treatment caused oxidative damages of proteins and lipids in the rat liver in both non-stressed and isolated rats. The chronic isolation itself also provoked oxidative damage. Besides, isolation increased nitrogen monoxide and COX-2 production in the liver, which were prevented by fluoxetine treatment. Fluoxetine did not significantly affect the hepatic architecture while clozapine caused numerous macrovesicular fatty changes and focal necrosis, particularly in isolated rats.

This study describes molecular changes involved in the pathophysiology of depressive-like state in chronically isolated rats. These changes include compromised GSH-dependent defense, elevated expression of proinflammatory mediators in hippocampus and prefrontal cortex, as well as decreased number of PV+ cells in medial prefrontal cortex. Furthermore, this study expands the knowledge about the fluoxetine and clozapine action beyond serotonergic and dopaminergic signaling. The antidepressive and anxiolytic effects of these drugs paralleled the inhibition of hippocampal and prefrontal cortical inflammation, as well as the prevention of decrease in PV+ cells number in PrL and IL. Finally, results of this study reveal that clozapine is more harmful for the liver, compared to fluoxetine.

KEY WORDS: Fluoxetine, clozapine, chronic isolation, hippocampus, prefrontal cortex, liver, antioxidative system, inflammation parameters, parvalbumin.

SCIENTIFIC FIELD: Biology

SPECIFIC TOPIC: Neurobiology

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SKRAĆENICE

ACTH – adrenokotrikotropni hormon

ALT – alanin transaminaza

AST – aspartat transaminaza

ATP – adenozin-5´ trifosfat

α_1 i α_2 – adrenalinski receptori

BDNF – moždani neurotrofički faktor (engl. *brain derived neurotrophic factor*)

BSA – goveđi albumin iz seruma (engl. *bovine serum albumin*)

CB – kalbindin

CBP – kalcijum-vezujući protein (engl. *calcium binding protein*)

Cg1 – cingulatna kora 1

COX-2 – ciklooksigenaza-2

CR – kalretinin

CRH – kortikotropin-oslobađajući hormon (engl. *corticotropin-releasing hormone*)

CYP – citohrom P450 enzimi

D_2 – dopaminski receptori

DILI – oštećenja jetre izazvana lekom (engl. *drug-induced liver injury*)

DNK – dezoksiribonukleinska kiselina

DP – dorzalna pedunkularna kora

DSM-V – dijagnostičko-statistički priručnik za duševne poremećaje V (engl. *Diagnostic i Statistical Manual of Mental Disorders*)

FLK – fluoksetin hidrohlorid

FR – fiziološki rastvor

GABA – γ-aminobuterna kiselina

GLR – glutation reduktaza

GPx – glutation peroksidaza

GR – glukokortikoidni receptor

GSH – glutation

GS-SG – glutation-disulfid

GST – glutation-S transferaza

HHA – hipotalamo-hipofizno-adrenokortikalni sistem

5-$HT_{1A,}$ 5-HT_{2A} – serotoninski receptori

ICD-11 – Međunarodna statistička klasifikacija bolesti i srodnih zdravstvenih problema 11 (engl. *International Statistical Classification of Diseases i Related Health Problems*)

IL – infralimbički podregion medijalne prečeone zone kore cerebruma

IL-1β – interleukin-1β

IL-6 – interleukin-6

IκB – inhibitorna subjedinica-κB

IZ – izolacija

KLZ – klozapin

KORT – kortizol / kortikosteron

LC-MS – tečna hromatografija kuplovana sa masenom spektrometrijom

LC-MS-MS – tečna hromatografija kuplovana sa tiemskom masenom spektrometrijom

LPS – lipopolisaharid

MARTA – antipsihotici koji se vezuju za veliki broj receptora (engl. *multi acting receptor targeted antipsychotics*)

MDA – malondialdehid

MDD – veliki depresivni poremećaj (engl. *major depressive disorder*)

MR – mineralokortikoidni receptor

NF-κB – nuklearni faktor-κB

NK – ćelije prirodne ubice (engl. *natural killer*)

NKT – prirodne T ćelije ubice

NMDA-R – N-metil-D-aspartat receptori

NOS – azot-monoksid sintaza

NOX – NADPH oksidaza

NS – nestresirani pacovi

OD – optička gustina (engl. *optical density*)

PFA – paraformaldehid

PrL – prelimbički podregion medijalne prečeone zone kore cerebruma

PV – parvalbumin

PVN – paraventrikularno jedro hipotalamusa (engl. *paraventricular nucleus*)

RNS – reaktivne vrste azota (engl. *reactive nitrogen species*)

ROS – reaktivne vrste kiseonika (engl. *reactive oxygen species*)

SAM – simpato-adrenomedularni sistem

SNRIs – inhibitori preuzimanja serotonina i noradrenalina (engl. *serotonin i norepinephrine reuptake inhibitors*)

SOD – superoksid dismutaza

SSRIs – selektivni inhibitori preuzimanja serotonina (engl. *selective serotonin reuptake inhibitors*)

TNF-α – faktor nekroze tumora α

SADRŽAJ

1 UVOD

Hronična izloženost psihosocijalnim stresorima predstavlja faktor rizika za psihijatrijska oboljenja, uključujući depresiju. Depresija je kompleksno, multifaktorijalno psihijatrijsko oboljenje od kog boluju milioni ljudi širom sveta. Predstavlja veliki teret savremenog društva kako u kliničkom, tako i u socijalnom i ekonomskom pogledu. Smatra se da će tzv. veliki depresivni poremećaj (engl. *major depressive disorder*, MDD) do 2020. godine biti drugi po redu uzročnik invaliditeta, odmah iza ishemijske bolesti srca (World Health Organization, 2001). Uzroci koji dovode do ovog poremećaja nisu još precizno definisani, ali jasno je da su za njegovu pojavu odgovorne kompleksne interakcije genetičkih i sredinskih faktora. Depresiju karakteriše veoma složena neurobiološka osnova koja uključuje promene na nivou neurotransmitera i njihovih receptora, neuroendokrinih sistema, mehanizama neuroplastičnosti i neuroinflamatornih procesa (Hasler, 2010). Farmakološki agensi koji su trenutno u upotrebi ne odgovaraju na najbolji način na izazove koje nameće heterogenost i kompleksnost ovog psihijatrijskog oboljenja jer samo 60−70% pacijenata ispoljava željeni odgovor na terapiju (Al-Harbi, 2012). Stoga je unapređivanje antidepresivne terapije jedan od globalnih zdravstvenih prioriteta, pa je velika pažnja usmerena ka detaljnom ispitivanju mehanizama delovanja postojećih medikamenata, kao i daljem istraživanju patofiziologije depresije radi identifikacije novih meta farmakoterapije.

1.1 STRES

Danas se u naučnoj i široj javnosti stres najčešće pominje kao jedan od najrasprostranjenijih psiholoških problema savremenog društva i faktor rizika za veliki broj zdravstvenih problema, uključujući i psihijatrijske poremećaje. Međutim, posmatrano u širem kontekstu, odgovor na stres je kompleksna, sofisticirana i pažljivo regulisana adaptacija koja je oblikovana prirodnom selekcijom jer povećava sposobnost organizma da se izbori sa situacijama koje zahtevaju akciju ili odbranu. S obzirom da svaka korist ima svoju cenu, odgovor na stres može imati ozbiljne, negativne posledice ukoliko je prenaglašen ili predugo traje (Nesse i Young, 2000).

Sredinom 19. veka Klod Bernar je primetio da održavanje života zavisi od očuvanja sklada i konstantnosti unutrašnje sredine uprkos promenama u spoljašnjoj sredini (Bernard, 1859). Kanon je 1929. uveo termin "homeostaza" koji označava upravo ono što je Bernard opisao decenijama unazad (Cannon, 1929). Termin stres prvi je upotrebio Hans Selije i definisao ga kao nespecifičan odgovor organizma na bilo šta što ugrožava homeostazu, dok je sam ugrožavajući faktor nazvao stresor (Selye, 1936, 1956). Decenijama nakon Kanonovog definisanja homeostaze, koja po njemu predstavlja set prihvatljivih opsega vrednosti za parametre unutrašnje sredine, definisan je pojam alostaza koji podrazumeva sposobnost održavanja stabilnosti unutrašnje sredine kroz stalne promene i prilagođavanja organizma (Sterling i Eyer, 1988; McEwen i Stellar, 1993). Usled delovanja hroničnog stresora, u organizmu se pokreću fiziološke, psihološke i ponašajne promene koje omogućavaju alostazu. Ukoliko je ovaj odgovor prenaglašen ili predugo traje može doći do tzv. alostatskog opterećenja (engl. *allostatic load*) i oštećenja tkiva i organa (Goldstein i McEwen, 2002).

U svojim radovima Selije je, kao stresore, koristio krvarenje, sepsu, injektovanje otrovnih supstanci i druge opasne, fizičke pretnje po organizam. On nije uzimao u obzir doprinos koji sama percepcija i interpretacija stresora može imati na odgovor na stres. Teorije koje su usledile pretpostavljaju da upravo percepcija individue, na koju utiču prethodno iskustvo, genetika i ponašanje, u velikoj meri oblikuje taj odgovor. Čak i stimulus koji ne predstavlja realnu opasnost po homeostazu može dovesti do odgovora na stres. Ovakvi stresori suptilnije prirode, poput psihosocijalnih stresora, naročito su česti u savremenom društvu (Goldstein, 2001). Dakle, savremeni koncept stresa uzima

u obzir realne pretnje, ali i stimuluse koji se doživljavaju kao pretnje po homeostazu a sam odgovor na stres se posmatra kao reakcija koja poseduje izvesne specifičnosti u zavisnosti od prirode stresora, načina percepcije stresora i sposobnosti organizma da na njega odgovori (Goldstein i Kopin, 2007).

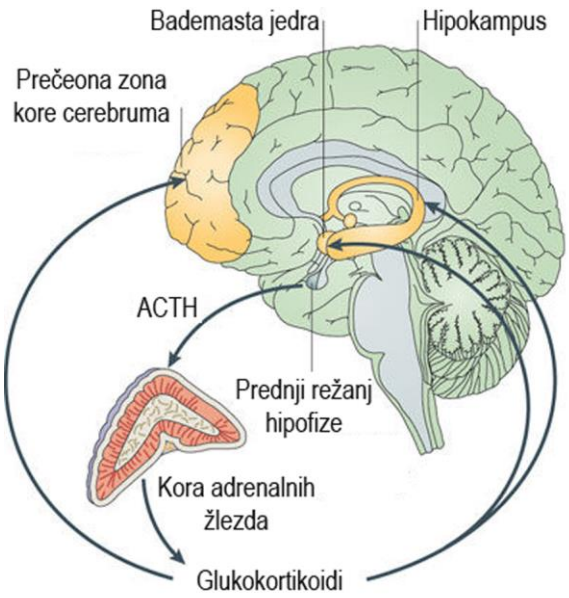
Stresori se na osnovu svoje prirode mogu podeliti na fizičke (hladnoća, toplota, radijacija, buka, različite hemijske supstance, itd.), psihološke (psihičko maltretiranje, nasilje, trauma itd.), socijalne (ljudi – gubitak posla, razvod, usamljenost itd.; životinje – izolacija, prisustvo dominantne jedinke) i stresore koji predstavljaju pretnju kardiovaskularnoj i metaboličkoj homeostazi (hipoglikemija, hemoragija, itd.) (Pacak i Palkovits, 2001). Neke stresore je teško uvrstiti u samo jednu od ovih grupa. Tako je izolacija koja je korišćena u ovoj studiji blagi stresor psihosocijalne prirode, dok npr. imobilizacija ima karakteristike stresora svih navedenih kategorija. Stresori variraju i po intenzitetu i dužini trajanja, tako da razlikujemo blage i jake, odnosno akutne, ponavljane i hronične stresore.

Održavanje homeostaze u prisustvu stresora podrazumeva aktivaciju kompleksnog odgovora na stres koji uključuje endokrini, nervni i imunski sistem (Chrousos i Gold, 1992). Odgovor na stres najčešće uključuje neuroendokrine sisteme simpato-adrenomedularni (SAM) i hipotalamo-hipofizno-adrenokortikalni (HHA) sistem, i pojačano oslobađanje "hormona stresa" kateholamina i kortikosteroida. Aktiviranje SAM sistema podrazumeva aktivaciju noradrenalinskih neurona *locus coeruleus*-a i oslobađanje kateholamina koji omogućavaju "bori se ili beži" odgovor. Percepcija stresora od strane viših moždanih centara dovodi do oslobađanja kortikotropin-oslobađajućeg hormona (engl. *corticotropin-releasing hormone*, CRH) iz paraventrikularnog jedra (engl. *paraventricular nucleus*, PVN) hipotalamusa. Ovaj hormon indukuje oslobađanje adrenokotrikotropnog hormona (ACTH) iz hipofize, koji stimuliše koru adrenalnih (nadbubrežnih) žlezda da sintetiše i luči glukokortikoide (kortizol kod ljudi i kortikosteron kod glodara, KORT). Glukokortikoidi regulišu svoju sintezu mehanizmom negativne povratne sprege tako što suprimiraju aktivnost HHA sistema vezujući se za glukokortikoidne i mineralokortikoidne receptore (GR i MR) u hipofizi, hipotalamusu, hipokampusu i medijalnoj prečeonoj zoni kore cerebruma. Po prolasku opasnosti, odnosno po završetku delovanja stresora, nivoi kateholamina i kortikosteroida bi trebalo da se vrate na bazalne. Ukoliko to izostane, produženo

delovanje hormona stresa može dovesti do alostatskog opterećenja sa patofiziološkim posledicama (Joëls i Ronald de Kloet, 1994; McEwen, 1998; de Kloet, 2000).

1.1.1 Mozak i stres

Mozak je ključni organ odgovora na stres jer, sa jedne strane, utvrđuje šta je to potencijalna pretnja, odnosno stresor, a sa druge strane određuje fiziološke i ponašajne odgovore organizma. U odgovor na stres uključena je dvosmerna komunikacija između mozga i kardiovaskularnog, imunskog i drugih sistema koja je posredovana nervnim i endokrinim putevima. Mozak je veoma podložan negativnim uticajima stresora, pri čemu su neke moždane strukture osetljivije od drugih. Najosetljivije su one koje su najistaknutije mete glukokortikoida – hipokampus, prečeona zona kore cerebruma i bademasta jedra (Slika 1).



Slika 1. ***Moždani regioni – mete glukokortikoida***

Stresori aktiviraju hipotalamo-hipofizno-adrenokortikalni sistem dovodeći do pojačanog oslobađanja glukokortikoida. Ovi hormoni se vezuju za mineralokortikoidne i glukokortikoidne receptore koji su, u mozgu, u najvećoj meri prisutni u hipokampusu, prečeonoj zoni kore cerebruma i bademastim jedrima. ACTH – adrenokotrikotropni hormon. Modifikovano iz (Krugers i sar., 2010).

Stres dovodi do strukturnih i morfoloških promena poput remodelovanja dendrita, smanjenja broja dendritskih trnova, gubitka glijskih ćelija i drugih promena u

ovim moždanim strukturama (McEwen, 2007). Strukturne i patofiziološke promene u nabrojanim strukturama, naročito u hipokampusu i prečeonoj zoni kore cerebruma, povezane su sa psihijatrijskim poremećajima (Godsil i sar., 2013). U ovoj studiji korišćen je model hronične izolacije pacova kao životinjski model depresije, te su parametri od interesa ispitivani upravo u ove dve moždane strukture. Kratak pregled literature koja se odnosi na promene u hipokampusu i prečeonoj zoni kore, a koje su u vezi sa hroničnim stresom i psihijatrijskim oboljenjima, sa akcentom na depresiji, prikazan je u narednim poglavljima.

1.1.1.1 Hipokampus

Hipokampus je moždana struktura uključena u brojne kognitivne funkcije i regulaciju raspoloženja, emocija i odgovora na stres. Ima značajnu ulogu u regulaciji aktivnosti HHA sistema posredstvom negativne povratne sprege, pa je bogat receptorima adrenalnih steroidnih hormona (GR i MR) (Sapolsky i sar., 1985). Samim tim je izrazito osetljiv na štetne efekte stresora. Hipokampus karakteriše velika plastičnost, sposobnost da prilagodi svoju strukturu i funkciju signalima iz spoljašnje i unutrašnje sredine. Mehanizmi neuroplastičnosti uključuju modifikaciju dendrita, broja sinapsi i nastanak novih neurona što se sve odražava na organizaciju i aktivnost hipokampusnih mreža i konačno na samo ponašanje jedinke. Međutim, pored toga što ima značajnu ulogu u procesima poput učenja i pamćenja, visok stepen plastičnosti hipokampusa se može negativno ispoljiti u vidu naglašene ranjivosti na nepovoljne uslove kao što je hronični stres (McEwen, 1994; Bartsch i Wulff, 2015). Sve je veći broj studija koje pokazuju da hronični stres indukuje promene u hipokampusu, kako na molekularnom, tako i na strukturnom nivou (McEwen i sar., 2016). Pored toga, ova moždana struktura se već dugo dovodi u vezu sa psihijatrijskim poremećajima (Sapolsky, 2000). Kod pacijenata obolelih od depresije primećeno je smanjenje zapremine ove strukture koje je srazmerno broju depresivnih epizoda i trajanju bolesti (Sheline i sar., 1996, 2003; McKinnon i sar., 2009). Pored toga, *postmortem* analizama je uočena veća gustina pakovanja glijskih ćelija, piramidnih i granularnih neurona, kao i smanjene dimenzije tela piramidnih neurona u hipokampusu ovakvih pacijenata (Stockmeier i sar., 2004). Slične promene koje uključuju smanjeni volumen i atrofiju dendrita hipokampusa, uočene su i kod hronično stresiranih životinja (Galea i sar.,

1997; Krugers i sar., 2010b). Takođe, pokazano je da hronični stres umanjuje adultnu neurogenezu i plastičnost u hipokampusu eksperimentalnih životinja tako što utiče na ćelijsku proliferaciju, neuronsku diferencijaciju, preživljanje novih neurona (Xu i sar., 2007; Ferragud i sar., 2010; Yun i sar., 2010) i sazrevanje njihovih sinapsi (Chen i sar., 2015b). Sa druge strane, antidepresivi i elektrokonvulzivna terapija povećavaju neurogenezu kod odraslih pacova (Malberg i sar., 2000), i primata (Perera i sar., 2007, 2011). Zbog svega navedenog, hipokampus je predmet istraživanja većine studija koje izučavaju efekte hroničnog stresa i patofiziologiju depresije.

1.1.1.2 Prečeona zona kore cerebruma

Prečeona zona kore cerebruma je moždani region koji je veoma osetljiv na stres i sudeći po sve većem broju kliničkih, i studija sprovedenih na životinjskim modelima, ima važnu ulogu u patofiziologiji psihijatrijskih oboljenja, uključujući depresiju (Ménard i sar., 2016; Negrón-Oyarzo i sar., 2016). Prečeona zona kore upravlja kognitivnim i socio-emocionalnim funkcijama i moduliše odgovor na stres (Cerqueira i sar., 2008). Poput hipokampusa, učestvuje u kontroli HHA sistema posredstvom negativne povratne sprege i sadrži veliki broj GR receptora (Herman i sar., 2003; Smith i Vale, 2006).

Godinama unazad, mnogi simptomi kliničke depresije se pripisuju disfunkciji prečeone zone kore (George i sar., 1994). Kvantitativnom meta-analizom utvrđena je hiperaktivnost ventromedijalne prečeone zone kore cerebruma u stanju mirovanja kod pacijenata obolelih od MDD (Kühn i Gallinat, 2013). Takođe, kod MDD pacijenata je zabeležena smanjena debljina kore u nekoliko prečeonih regiona (Van Tol i sar., 2014). Pored toga, pokazano je da hronični stres dovodi do strukturnih i funkcionalnih promena poput remodelovanja dendrita, gubitka dendritskih trnova i izmenjene sinaptičke transmisije u prečeonoj zoni kore (Cook i Wellman, 2004; Radley i sar., 2004; Liston i sar., 2006; Goldwater i sar., 2009). Analiza transkriptoma ovog regiona kod hronično stresiranih miševa koji pokazuju depresivno ponašanje otkrila je promene u GABA i dopaminskim sinapsama kao i u procesima recikliranja sinaptičkih vezikula, signalizaciji neurotrofinima i neuroimunskim procesima (Ma i sar., 2016).

Vang i saradnici su pokazali da od prirode sinapsi u medijalnoj prečeonoj zoni može zavisiti osetljivost jedinke na stres. Pored toga, uočili su da veštački indukovano povećanje aktivnosti neurona ovog regiona dovodi do prevođenja rezistentog fenotipa u fenotip osetljiv na stres kod miševa (Wang i sar., 2014). Zanimljivo, pokazano je da dubinska stimulacija mozga (engl. *deep brain stimulation*) u nivou medijalne prečeone zone ublažava simptome depresije, kako u slučaju pacijenata, tako i kod životinjskih modela (Mayberg i sar., 2005; Lozano i sar., 2008; Hamani i sar., 2010). Navedeni podaci ukazuju na značaj koji promene u aktivnosti ovog regiona kore cerebruma imaju u ispoljavanju depresivnog ponašanja, odnosno antidepresivnog efekta.

1.1.2 Oksidativni stres

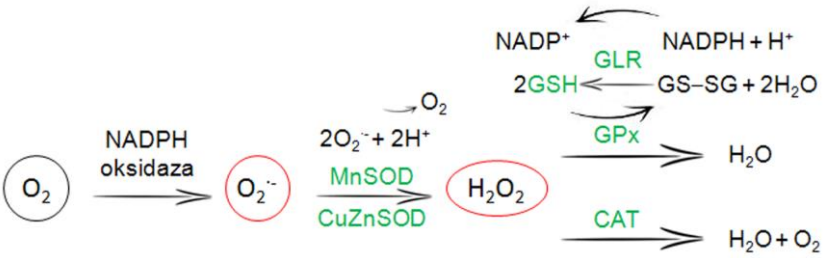
Pored toga što uzrokuje morfološke i neurohemijske promene, hronični stres izaziva oksidativni stres u mozgu. Oksidativni stres se definiše kao stanje narušene ravnoteže između nivoa prooksidanata i antioksidanata, u pravcu povećanja nivoa prooksidanata.

1.1.2.1 Prooksidanti

Najzastupljeniji prooksidanti u organizmu su reaktivne vrste kiseonika (engl. *reactive oxygen species*, ROS) i to superoksidni anjon radikal ($O_2^{\bullet -}$), vodonik-peroksid (H_2O_2) i hidroksilni radikal ($OH^{\bullet}$), kao i reaktivne vrste azota (engl. *reactive nitrogen species*, RNS) poput azot-monoksida (NO) i peroksinitritnog anjona ($ONOO^-$). Značajan izvor ROS predstavlja proces oksidativne fosforilacije u mitohondrijama, te su ćelije koje su metabolički aktivnije i koje sadrže veći broj ovih organela podložnije oksidativnom stresu. Ova jedinjenja nastaju strogo regulisanom aktivnošću enzima poput NADPH oksidaze (NOX) i NO sintaze (NOS) i imaju fiziološke funkcije – uništavaju patogene štiteći ćeliju od infekcija, imaju ulogu sekundarnih glasnika, učestvuju u regulaciji koncentracije kalcijuma, fosforilacije i defosforilacije proteina (Halliwell, 2006; Valko i sar., 2007). Remećenje redoks ravnoteže usled povećane produkcije oksidanata ili smanjenog antioksidativnog kapaciteta, može dovesti do oksidacije lipida, proteina i DNK, i time do oštećenja strukture i funkcije ćelije koje za posledicu mogu imati apoptozu ili nekrozu (Kohen i Nyska, 2002).

1.1.2.2 Antioksidativni sistem

Antioksidativni sistem je odbrambeni sistem sačinjen od neenzimskih i enzimskih komponenti uz pomoć kojih se ćelije suprotstavljaju toksičnim efektima koje imaju ROS i RNS. Glutation (GSH) je najzastupljeniji tiol u ćelijama i najvažniji neenzimski antioksidant, koji ima centralnu ulogu u održavanju fiziološkog redoks statusa. GSH je tripeptid koji održava visok nivo tiola u ćeliji i može direktno da reaguje sa ROS/RNS i onemogući ih da oštete proteine i druge biomolekule. Takođe, učestvuje u detoksifikaciji peroksida, degradaciji ksenobiotika, održavanju trodimenzionalne strukture mnogih proteina i regulaciji aktivnosti enzima putem S-glutationilacije, post-translacione modifikacije koja podrazumeva formiranje disulfidnih mostova između GSH i cisteinskih ostataka proteina. GSH je redukujući agens kojim se, pomoću enzima glutation peroksidaze (GPx), redukuju H_2O_2 i različiti organski peroksidi do vode, odnosno odgovarajućih alkohola. Tom prilikom GSH biva oksidovan do glutation-disulfida (GS-SG). GPx je homotetramerni enzim čije subjedinice sadrže atom selena u aktivnom mestu (Chambers i sar., 1986). Smanjena ekspresija i aktivnost ovog enzima narušavaju antioksidativnu zaštitu i povećavaju podložnost oksidativnom stresu koji je posredovan peroksidima. Redukcija GS-SG koji nastaje aktivnošću GPx vrši se pomoću NADPH, u reakciji koju katalizuje enzim glutation reduktaza (GLR) (Slika 2). U uslovima povećanog stvaranja peroksida, povećana aktivnost GPx dovodi do smanjenja zaliha GSH, koje se obnavljaju aktivnošću enzima GLR. Ukoliko povećana potrošnja GSH nije praćenja povećanom aktivnošću GLR dolazi do narušavanja GSH/GSSG ravnoteže u smeru prooksidativnog stanja. GSH je takođe kofaktor glutation-S transferaze (GST), enzima koji konjuguje ksenobiotike ili elektrofilne metabolite sa GSH (Sastre i sar. 2005).



Slika 2. ***Mehanizmi antioksidativne zaštite***

(objašnjenja u tekstu)

Dakle, u uslovima oksidativnog stresa dolazi do oksidacije GSH i smanjenja redukujućeg kapaciteta u ćelijama. Oksidacija GSH, odnosno smanjivanje rezervi redukovanog glutationa u mitohondrijama, za posledicu može imati glutationilaciju kompleksa I elektron-transportnog lanca i povećanu produkciju $O_2^{\bullet-}$, koji doprinosi prooksidativnim uslovima (Taylor i sar., 2003). GSH zajedno sa gorepomenutim enzimima predstavlja tzv. GSH-zavisni antioksidativni sistem. Pored ovog sistema, u odbrani od prooksidativnih jedinjenja učestvuju i superoksid dismutaze (SOD), enzimi koji katalizuju dismutaciju $O_2^{\bullet-}$ u H_2O_2 (McCord i Fridovich, 1988), kao i katalaza koja katalizuje degradaciju H_2O_2 do vode i molekulskog kiseonika (Chelikani i sar., 2004).

1.1.2.3 Psihosocijalni stres – oksidativni stres – psihijatrijski poremećaji

Sve je veći broj podataka koji ukazuju da oksidativni stres u mozgu, uzrokovan hroničnim delovanjem psihosocijalnih stresora, doprinosi razvoju psihijatrijskih oboljenja poput depresije, anksioznosti, shizofrenije i bipolarnog poremećaja (Van Winkel i sar., 2008; Maes i sar., 2011; Schiavone i sar., 2012; Salim, 2014). Značajna uloga oksidativnog stresa u etiologiji velikog broja bolesti poput kancera, kardiovaskularnih i neurodegenerativnih bolesti, odavno je prepoznata (Aruoma, 1998). O značaju mehanizama oksidativnog stresa za zdravlje, kako fizičko, tako i emocionalno i kognitivno, možda najbolje govori Gingričeva kovanica "oksidativni stres je novi stres" (Gingrich, 2005).

Mozak je veoma podložan oksidativnom stresu, pre svega zbog velike potrošnje kiseonika i visokog sadržaja polinezasićenih masnih kiselina čije dvostruke veze predstavljaju lake mete slobodnih radikala. Stoga je održavanje redoks ravnoteže u ćelijama mozga veliki izazov koji zahteva efikasnu antioksidativnu zaštitu (Noseworthy i Bray, 1998). Brojne studije su pokazale da su mehanizmi antioksidativne zaštite u nekim moždanim regionima, pre svega hipokampusu i prečeonoj kori, oslabljeni kod hronično stresiranih pacova (Eren i sar., 2007; Ahmad i sar., 2010; Che i sar., 2015). Rezultati komparativne analize proteoma nestresiranih i hronično stresiranih pacova ističu metabolizam GSH kao jedan od bioloških puteva koji trpi najviše promena u prečeonoj zoni kore cerebruma, u uslovima stresa (Yang i sar., 2013).

Meta-analiza koja je obuhvatila 23 studije i gotovo 5000 učesnika pokazala je da je depresija povezana sa povećanim oksidativnim stresom i oslabljenim antioksidativnim sistemom (Palta i sar., 2014). Povišeni nivo markera oksidativnog stresa i smanjeni nivo antioksidanata i antioksidativnih enzima, kao i njihove aktivnosti, su u više navrata detektovani u krvi pacijenata koji boluju od depresije (Lopresti i sar., 2014; Maes i sar., 2012). *Postmortem* analizama je pokazano značajno smanjenje ekspresije nekih antioksidativnih enzima, uključujući GPx i glutation sintazu u hipokampusu pacijenata koji su bolovali od bipolarnog poremećaja (Benes i sar., 2006), kao i smanjen nivo GSH i GPx enzima u prečeonoj zoni kore pacijenata koji su bolovali od MDD, shizofrenije i bipolarnog poremećaja (Gawryluk i sar., 2011). Dakle, koncept da su psihijatrijska oboljenja spregnuta sa oksidativnim stresom ima znatnu podršku u eksperimentalnim podacima. U prilog tome govore i podaci da antidepresivi mogu redukovati oksidativni stres, što sugeriše da jačanje antioksidativne zaštite može biti jedan od mehanizama koji se nalazi u osnovi neuroprotektivnog dejstva ovih lekova (Behr i sar., 2012).

1.2 DEPRESIJA

Depresija je multifaktorijalno psihijatrijsko oboljenje složene neurobiološke osnove. Prevalencija depresije, po podacima Svetske zdravstvene organizacije iz 2015. godine je 4,4% globalne populacije (~322 miliona obolelih) (World Health Organization, 2017). Faktori rizika za nastanak ove veoma heterogene bolesti su brojni, i mogu biti genetički i sredinski. Studije sprovedene na blizancima svedoče o postojanju genetičke predispozicije za razvoj ovog oboljenja. Meta-analiza podataka dobijenih istraživanjem genetičke epidemiologije MDD je pokazala da je u 30–40% slučajeva uzrok razvoja ove bolesti genetičke prirode (Sullivan i sar., 2000). Međutim, još nisu sa sigurnošću utvrđene specifične genske varijante odgovorne za povećan rizik od nastanka ove bolesti. Što se tiče sredinskih faktora, hronično delovanje stresora, pre svega onih koji imaju psihosocijalnu prirodu, predstavlja najveće faktore rizika.

Dva najpoznatija i najčešće upotrebljavana priručnika koja se koriste prilikom uspostavljanja dijagnoze nekog depresivnog poremećaja su Dijagnostičko statistički priručnik za duševne poremećaje V (engl. *Diagnostic i Statistical Manual of Mental*

Disorders, DSM-V) izdat 2013. godine od strane Udruženja psihijatara Amerike, i Međunarodna statistička klasifikacija bolesti i srodnih zdravstvenih problema, revizija 11 (engl. *International Statistical Classification of Diseases i Related Health Problems,* ICD-11) koji je objavila Svetska zdravstvena organizacija.

Klasifikacija depresivnih poremećaja prema DSM-V (American Psychiatric Association, 2013):

1. Disruptivni poremećaj raspoloženja (engl. *disruptive mood dysregulation disored*);
2. Veliki depresivni poremećaj (engl. *major depressive disorder*, MDD);
3. Hronični depresivni poremećaj (distimija);
4. Predmenstrualni disforični poremećaj;
5. Depresivni poremećaj izazvan supstancom ili lekom;
6. Depresivni poremećaj uzrokovan drugim zdravstvenim problemom;
7. Drugi specifični depresivni poremećaj;
8. Nespecifični depresivni poremećaj.

Dijagnostički kriterijumi za MDD prema DSM-V (American Psychiatric Association, 2013):

1. Osoba ima pet ili više simptoma tokom najmanje dve nedelje, uz obavezno prisustvo jednog od prva dva. Simptomi su:

   - Depresivno raspoloženje tokom većeg dela dana;
   - Upadljivo smanjenje interesovanja ili zadovoljstava u svim ili većini aktivnosti;
   - Značajne promene telesne mase (više od 5% za mesec dana) i smanjenje ili povećanje apetita;
   - Insomnia ili hipersomnia;
   - Psihomotorna agitacija ili retardacija;
   - Umor ili gubitak energije;
   - Osećanje manje vrednosti, krivice i bespomoćnosti;
   - Smanjena sposobnost razmišljanja, usredsređivanja ili donošenja odluka;
   - Učestale misli o smrti, samoubistvu.

* simptomi su prisutni gotovo svakodnevno sa izuzetkom promena u telesnoj masi i suicidalnih misli

2. Simptomi uzrokuju klinički značajan distres ili narušavaju socijalno i društveno funkcionisanje;
3. Simptomi nisu posledica nekog drugog zdavstvenog problema ili uzimanja neke supstance;
4. Odsustvo manijačne ili hipomanijačne epizode.

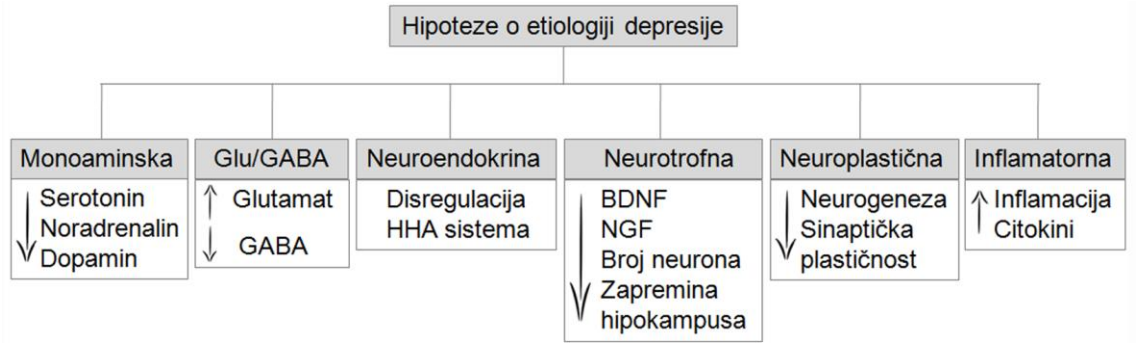
Treba istaći da bipolarni poremećaj, prema klasifikaciji priručnika DSM-V, nije ubrojan u depresivne poremećaje, već je opisan u posebnom poglavlju. U pitanju je kompleksni poremećaj koji karakterišu depresivne i manijačne/hipomanijačne epizode. Atipična depresija, koja takođe ne postoji u pomenutoj klasifikaciji kao kategorija u okviru depresivnih poremećaja, je fenomen prvi put opisan šezdesetih godina prošlog veka. Karakteriše je poboljšanje raspoloženja kao odgovor na pozitivne događaje, kao i dva ili više od navedenih simptoma:

1. Značajno povećanje telesne mase ili apetita;
2. Hipersomnia;
3. Osećaj težine u ekstremitetima (engl. *leaden paralysis*);
4. Povećana osetljivost na odbacivanje, što dovodi do problema u socijalnim odnosima.

Kod pacijenata se javljaju različiti kompleksi simptoma pa je klinička slika depresije veoma varijabilna. Pored kliničke slike, varijabilnost je u velikoj meri prisutna i u etiologiji i neurobiologiji ove bolesti (Strakowski, 2012). Potrebno je, upkos velikoj heterogenosti i varijabilnosti, pronaći univerzalne patofiziološke procese ovog oboljenja radi razvoja terapije kroz otkrivanje novih targeta farmakoloških agenasa.

1.2.1 Hipoteze o etiologiji depresije

Klinička i etiološka heterogenost depresivnih poremećaja otežava rasvetljavanje njihove patofiziologije. Postoji nekoliko istaknutih hipoteza o etiologiji depresije (Slika 3) od kojih svaka nalazi uporište u određenoj grupi pacijenata ili prekliničkih studija ali ne u svim, te je koncept jedinstvene hipoteze uveliko napušten.



Slika 3. ***Hipoteze o etiologiji depresije*** *(objašnjenja u tekstu)*

Glu – glutamat; GABA – γ-aminobuterna kiselina; HHA – hipotalamo-hipofizno-adrenokortikalni sistem; BDNF – neurotrofni faktor mozga; NGF – faktor rasta neurona

U daljem tekstu dat je kratak pregled najzapaženijih hipoteza o etiologiji depresije sa fokusom na inflamatornu i GABA hipotezu. Nijedna hipoteza pojedinačno ne može u potpunosti objasniti uzroke nastanka ovog heterogenog oboljenja ali zajedno pružaju uvid u njegovu kompleksnu neurobiologiju.

1.2.1.1 Monoaminska hipoteza depresije

Monoaminska hipoteza dominira već više od pola veka u istraživanjima vezanim za ispitivanje patofiziologije depresije. To se najbolje ogleda u činjenici da je u osnovi terapijskog efekta antidepresiva predominantna modulacija monoaminske transmisije. Prema ovoj hipotezi patologija depresije je uzrokovana smanjenom koncentracijom monoaminskih neurotransmitera, naročito serotonina i noradrenalina (Bunney i Davis, 1965; Schildkraut, 1965; Coppen, 1967). Ona je definisana početkom pedesetih godina prošlog veka kada je tokom kliničkih proba antituberkuloznog agensa iproniazida slučajno uočeno da isti pozitivno utiče na raspoloženje (Delay i sar., 1952; Selikoff i sar., 1952; Crane, 1957). Ubrzo je otkriven i triciklični antidepresiv imipramin, koji kao i iproniazid, povećava monoaminsku neurotransmisiju (Kuhn, 1958). To je dovelo do postavljanja ove hipoteze, ali šire gledano, otvorilo je vrata ispitivanju patofiziologije depresije uopšte i razvijanju odgovarajućih farmakoloških terapeutika. Međutim, ideja da je monoaminska deficijencija jedina odgovorna za razvoj depresije poljuljana je činjenicom da do ublažavanja simptoma bolesti dolazi tek nekoliko nedelja nakon porasta koncentracije monoamina u mozgu (Baldessarini, 1989), kao i otkrićem da tretmani koji ne uključuju monoamine, poput elektrokonvulzivne terapije, mogu imati

isti terapijski efekat (Pagnin i sar., 2004). Kako ni nakon višedecenijskog istraživanja nije pronađena direktna uzročno-posledična veza između promena u monoaminskoj transmisiji i depresije (Nestler, 1998), postulirane su druge hipoteze o etiologiji i neurobiologiji ovog oboljenja.

1.2.1.2 Glutamatska i GABA hipoteza depresije

Složene neuronske mreže u mozgu sačinjene su od projekcionih neurona i interneurona koji kao neurotransmitere najčešće koriste glutamat, odnosno GABA. Prema glutamatskoj hipotezi depresije, imbalans između glavnog ekscitatornog i glavnog inhibitornog sistema dovodi do razvoja simptoma depresije. Naime, disbalans između eksitatorne glutamatske i inhibitorne GABA neurotransmisije može dovesti do ekscitotoksičnosti za koju je pokazano da ima važnu ulogu u patofiziologiji depresivnih i anksioznih poremećaja (Sanacora i sar., 2012). Ova teorija postavljena je početkom devedesetih godina prošlog veka kada je pokazano da antagonisti jonotropnog N-metil-D-aspartat receptora za glutamat (NMDA-R) imaju antidepresivni efekat (Trullas i Skolnick, 1990). To je potvrđeno i drugim prekliničkim istraživanjima koja su pokazala da kompetitivni i nekompetitivni NMDA-R antagonisti smanjuju imobilnost glodara, koja se smatra indikatorom stanja nalik depresivnom u testu prinudnog plivanja (Skolnick, 2002). U prilog ovoj teoriji govore i rezultati *postmortem* studije Hašimota i saradnika, koji su pokazali povećan nivo glutamata u prečeonoj zoni kore cerebruma žrtava samoubistva koje su bolovale od MDD ili bipolarnog poremećaja (Hashimoto i sar., 2007). Zanimljivo je i to da ketamin, nekompetitivni NMDA-R antagonist, predstavlja jedini lek koji ispoljava brz antidepresivan efekat, i to nakon samo jedne doze, čak i u slučaju depresije rezistentne na ostale terapeutike (Hasselmann, 2014).

Inhibitorni GABA sistem ima značajnu ulogu u regulaciji glutamatske transmisije i smatra se da je upravo inhibicija ključna komponenta sinhronizovane korteksne aktivnosti (Isaacson i Scanziani, 2011). GABA hipoteza depresije pretpostavlja da je poremećeno funkcionisanje GABA sistema u mozgu uzročno povezano sa ovim poremećajem (Brambilla i sar., 2003; Luscher i sar., 2011). U prilog ovoj hipotezi govori sve veći broj prekliničkih i kliničkih studija koje pokazuju da deficit neurotransmitera GABA predstavlja jednu od patofizioloških promena kod

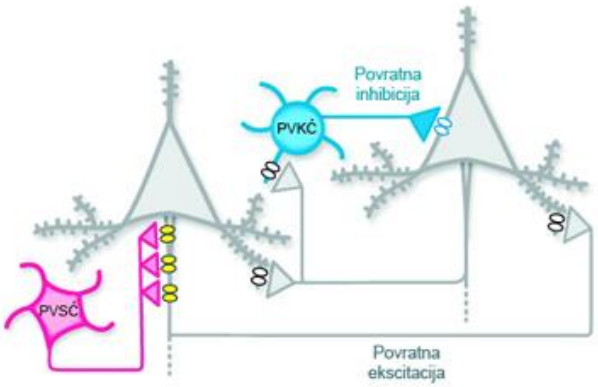
depresivnih i anksioznih poremećaja (Möhler, 2012). Transkranijalnom magnetnom stimulacijom je utvrđeno da pacijenti koji boluju od MDD imaju smanjenu korteksnu GABA neurotransmisiju. Osim toga, pokazano je da pacijenti sa ozbiljnijim simptomima bolesti i rezistencijom na terapiju imaju izraženiji deficit u GABA transmisiji u odnosu na pacijente sa blažim simptomima koji odgovaraju na terapiju (Levinson i sar., 2010). Takođe, kod osoba koje boluju od depresije, uočena je smanjena sinteza GABA (Thompson i sar., 2009), kao i gubitak GABA interneurona u određenim regionima kore cerebruma (Rajkowska i sar., 2007; Maciag i sar., 2010).

Inhibitorni GABA interneuroni predstavljaju veoma heterogenu grupu neurona unutar koje se, između ostalog, može izvršiti klasifikacija na osnovu Ca^{2+}-vezujućih proteina (engl. *calcium binding proteins*; CBP) koje neuroni eksprimiraju (Kelsom i Lu, 2013). Tako postoje parvalbumin (PV)- , kalbindin (CB)- i kalretinin (CR)- pozitivni interneuroni. CBP predstavljaju familiju malih kiselih proteina koji vezuju Ca^{2+} jone i regulišu njihovu unutarćelijsku koncentraciju, i na taj način modulišu prostorno-vremenske aspekte unutarćelijske signalizacije posredovane ovim jonima (Schwaller, 2010). Joni Ca^{2+} igraju značajnu ulogu u transmembranskom i unutarćelijskom prenosu signala, od oslobađanja neurotransmitera iz presinaptičkih završetaka do regulacije genske ekspresije. Smatra se da su gotovo sve reakcije u mozgu direktno ili indirektno regulisane ovim jonima (Augustine i sar., 2003) tako da je precizna regulacija njihove koncentracije unutar neurona veoma značajna. Pored toga, Ca^{2+} može direkto da aktivira enzime poput proteaza, nukleaza, fosfolipaza, pa nekontrolisano povećanje njegove koncentracije može biti neurotoksično (Berliocchi i sar., 2005).

PV+ interneuroni su inhibitorni neuroni koji privlače posebnu pažnju zbog fundamentalnog značaja koji imaju u organizaciji aktivnosti neurona kore cerebruma (Sohal i sar., 2009). PV je citosolni CBP relativne molekulske mase ~12 kDa, koji poseduje dva Ca^{2+} vezujuća domena (Cates i sar., 2002). Pored toga što visokim afinitetom vezuje Ca^{2+}, PV može srednjim afinitetom da vezuje i Mg^{2+}. PV brzo smanjuje količinu slobodnih Ca^{2+} jona u presinaptičkim ćelijama sprečavajući tako njihove štetne efekte i održavajući membranski potencijal u sinaptičkom regionu sličnim potencijalu mirovanja. Smanjena ekspresija PV može imati ozbiljne posledice jer dovodi do povećanog, asinhronog oslobađanja GABA iz PV+ interneurona što

negativno utiče na integraciju signala u piramidnim neuronima i samim tim narušava njihovo pravilno funkcionisanje (Manseau i sar., 2010).

PV+ interneuroni čine ~40% ukupnih GABA interneurona kore cerebruma glodara (Rudy i sar., 2011). Sa elektrofiziološkog aspekta, oni se karakterišu kao brzookidajući (engl. *fast-spiking*) neuroni zato što generišu repetititivne, kratkotrajne akcione potencijale, sa relativno kratkim periodima hiperpolarizacije, i brzim povratkom na potencijal mirovanja (Benes i Berretta, 2001). Mogu se izdvojiti najmanje dva morfološki različita podtipa ovih interneurona: korpasti interneuroni kore koji inervišu somu, dendrite i dendritske trnove piramidnih neurona i drugih PV+ korpastih ćelija, kao i malobrojniji svećnjasti interneuroni koji inervišu inicijalne segmente aksona piramidnih neurona kore (Markram i sar., 2004). Piramidni neuroni primaju signale od drugih piramidnih neurona i PV+ interneurona, integrišu ih i emituju na udaljene neurone lokalizovane u istoj ili drugoj nervnoj formaciji. Lokalne, povratne aksonske kolaterale piramidnih neurona pobuđuju obližnje projekcione neurone, ali i okolne korpaste PV+ interneurone, koji emituju brzu i jaku povratnu inhibiciju (Lewis i sar., 2012) (Slika 4). Zanimljivo je to što od svih GABA neurona kore, PV+ interneuroni primaju najjaču ekscitaciju glutamatom (Gulyás i sar., 1999). Dakle, recipročna povezanost lokalnim aksonskim kolateralama obezbeđuje povratnu ekcitaciju piramidnih neurona, dok ekscitatorni signal sa piramidnih neurona na korpaste PV+ interneurone dovodi do inhibicije piramidnih neurona.



Slika 4. ***Povezanost piramidnih neurona (sivo) i parvalbumin-pozitivnih korpastih (PVKĆ, plavo) i svećnjastih (PVSĆ, ljubičasto) interneurona u prečeonoj zoni kore cerebruma.***
Modifikovano iz Lewis i sar. 2012 (objašnjenja u tekstu).

Ove veze su značajne za održavanja balansa između ekscitacije i inhibicije, kao i za formiranje tzv. visokofrekventnih (30–100 Hz) gama oscilacija, koje su pak značajne za organizaciju aktivnosti neurona kore i sinhronizaciju korteksnih oscilacija (Bartos i sar., 2007; Sohal i sar., 2009). Brzookidajući inhibitorni interneuroni međusobno su povezani električnim sinapsama što omogućava sinhronizaciju velikog broja interneurona i sinhronizovane korteksne oscilacije (Gibson i sar., 1999). Svaki PV+ interneuron ostvaruje sinapse sa velikim brojem piramidnih neurona kore, te disfunkcija i malog broja ovih interneurona može da dovede do desinhronizacije značajnih delova kore cerebruma. Sve veći broj podataka govori u prilog tome da je poremećeno funkcionisanje PV+ interneurona kore u vezi sa različitim psihijatrijskim oboljenjima (Marín, 2012).

Treba istaći da brzookidajuća priroda zahteva visoku metaboličku aktivnost, pa ovi interneuroni imaju veću koncentraciju citohrom c oksidaze i veći broj mitohondrija od piramidnih ćelija (Gulyás i sar., 2006). To rezultuje povećanom proizvodnjom ATP, ali i ROS. Zbog toga su ovi interneuroni posebno podložni oksidativnom stresu.

1.2.1.3 Neuroendokrina hipoteza depresije

Funkcionalni i strukturni poremećaji u okviru HHA sistema su česti kod depresivnih pacijenata. Kod njih je uočena hiperkortizolemija, desenzitizacija GR, povećan nivo CRH u likvoru, kao i uvećanje hipofize i nadbubrežnih žlezda (Nemeroff i sar., 1984; Krishnan i sar., 1991; Rubin i sar., 1995; Holsboer, 2001). U prilog tome da je povećanje koncentracije KORT uključeno u etiologiju MDD govori i česta pojava depresivnih simptoma kod pacijenata sa Kušingovim sindromom koje odlikuje hiperkortizolemija (McEwen, 2007). Međutim, smanjena aktivnost HHA sistema je takođe primećena u različitim poremećajima povezanim sa stresom, uključujući i atipičnu depresiju (Fries i sar., 2005).

1.2.1.4 Neurotrofna hipoteza depresije

Sve veći broj studija sugeriše da je neurotrofni faktor mozga (engl. *brain derived neurotrophic factor*, BDNF) uključen u patofiziologiju psihijatrijskih poremećaja, što je dovelo i do formulisanja tzv. neurotrofne hipoteze depresije. Uočeno je da depresivni

pacijenti imaju niži nivo BDNF u serumu (Shimizu i sar., 2003), kao i da povećanje koncentracije ovog neurotrofina koincidira sa ublažavanjem simptoma depresije nakon tretmana antidepresivima (Azdemir i sar., 2005). Slični fenomeni su zapaženi i u životinjskim modelima. Pokazano je čak da intrakranijalna infuzija BDNF ima antidepresivan efekat u dva životinjska modela koja se koriste za ispitivanje depresije (Shirayama i sar., 2002). U prilog ovoj hipotezi govore i podaci da je depresija povezana sa redukovanim brojem neurona i glijskih ćelija, i smanjenjem zapremine hipokampusa i prečeone zone kore cerebruma (Drevets i sar., 2008).

1.2.1.5 Neuroplastična hipoteza depresije

Neuroplastična hipoteza spaja različita polja istraživanja depresije integrišući saznanja dobijena ispitivanjem mehanizama ekspresije neurotrofina, procesa neurogeneze, sinaptičke plastičnosti i remodelovanja neurona i neuronskih mreža. Neuroplastičnost predstavlja skup procesa od nastanka, preživljavanja, migracije i integracije novih neurona, do rasta neurita, sinaptogeneze i modulacije sinapsi. To je osnovni mehanizam neuronske adaptacije koji je, sudeći po sve većem broju podataka, narušen kako kod depresivnih pacijenata, tako i životinja kojima je stresom indukovano ponašanje nalik depresiji (Wainwright i Galea, 2013). Naime, patološki stres i depresija su povezani sa atrofijom dendrita, gubitkom dendritskih trnova i sinapsi, kao i smanjenim brojem glijskih ćelija u određenim moždanim regionima, o čemu je bilo reči u poglavlju 1.1.1. Tretman antidepresivima utiče na neuroplastičnost, podstičući neurogenezu, gliogenezu, formiranje sinapsi i preživljavanje ćelija u hipokampusu i prečeonoj zoni kore cerebruma, što takođe govori u prilog ovoj hipotezi (Serafini, 2012).

1.2.1.6 Inflamatorna hipoteza depresije

Inflamatorna ili citokinska hipoteza depresije pretpostavlja da prekomerna aktivacija imunskog sistema može doprineti patogenezi ovog oboljenja. Predložena je pre nešto više od dve decenije (Smith, 1991; Maes, 1995) i do danas je podržana brojnim studijama. Ova hipoteza je pružila objašnjenje za par uočenih fenomena koji povezuju proinflamatorne citokine i promene u ponašanju. Prvi fenomen je da tzv.

“sickness” ponašanje, koje je posledica aktivacije inflamatornog odgovora, ima zajedničke simptome sa depresijom – umor, anhedonija, letargija, smanjen unos hrane, usporena psihomotorika i kognitivni poremećaji (Dantzer, 2001). Drugi fenomen je da administracija egzogenog interferona u sklopu lečenja hepatitisa C ili malignih melanoma izaziva simptome depresije, uključujući i suicidalne misli (Loftis i Hauser, 2004).

Povišeni nivo proinflamatornih citokina interleukina-1β (IL-1β), interleukina-6 (IL-6) i faktora nekroze tumora α (TNF-α) detektovani su u krvi i likvoru depresivnih pacijenata. U krvi ovih pacijenata detektovan je i povišen nivo drugih biomarkera inflamacije poput proteina akutne faze, hemokina, adhezivnih molekula i prostaglandina (Miller i sar., 2009; Raison i sar., 2006). Takođe, povišeni nivo IL-1β, TNF-α i drugih proteina urođenog imuniteta pronađen je *postmortem* u mozgu žrtava samoubistva koje su bolovale od depresije (Miller i Raison, 2016).

Promene u nivou cirkulišućih proinflamatornih citokina uočene su i kod životinja koje su podvrgavane stresorima u cilju izazivanja depresivnog ponašanja (Himmerich i sar., 2013; Yazir i sar., 2015). Pokazano je da administracija lipopolisaharida (LPS) koji indukuje ekspresiju proinflamatornih citokina, provocira ispoljavanje ponašanja koje nalikuje depresivnom kod miševa (Frenois i sar., 2007), dok pretretman antidepresivnim supstancama sprečava povećanu ekspresiju citokina u mozgu i promene u ponašanju (Gawali i sar., 2016; Huang i sar., 2016). Ovi i mnogi drugi podaci iz literature govore u prilog tome da su medijatori inflamacije, indukovani u mozgu usled stresa, uključeni u patofiziologiju psihijatrijskih oboljenja i predstavljaju targete farmakološke terapije (Munhoz i sar., 2008; Calcia i sar., 2016).

IL-1β, IL-6 i TNF-α su glavni proinflamatorni citokini koji su konstitutivno eksprimirani u nervnim i glijskim ćelijama mozga zdravog, odraslog čoveka i predstavljaju neuromodulatore uključene u regulaciju funkcija neurona tokom na primer sna (Vitkovic i sar., 2000). Međutim, povećana ekspresija ovih citokina u mozgu povezana je sa psihijatrijskim poremećajima, uključujući depresiju (McNamara i Lotrich, 2012). Inflamatorni, kao i pro- i antioksidativni procesi regulisani su i koordinisani brojnim transkripcionim faktorima, između ostalih i jedarnim faktorom-κB (NF-κB) (Bakunina i sar., 2015). NF-κB proteini predstavljaju familiju transkripcionih

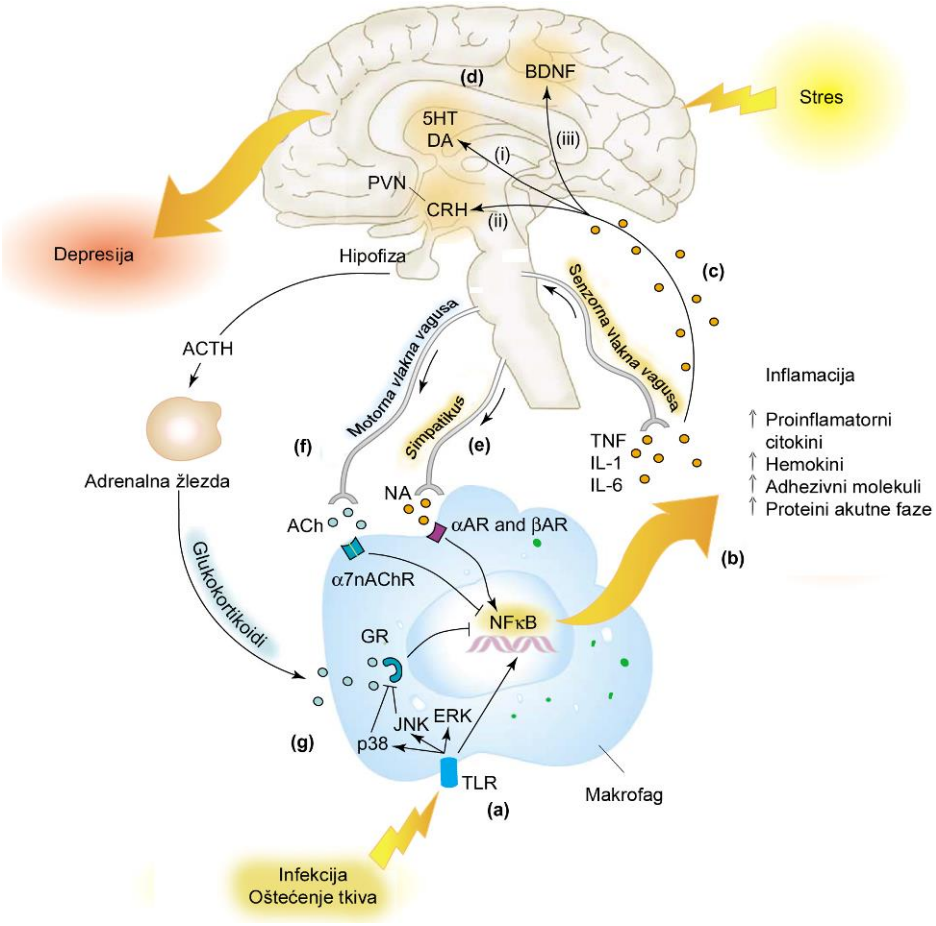
faktora koja reguliše veliki broj fizioloških procesa poput ćelijske proliferacije, diferencijacije i preživljavanja. Međutim, najpoznatija je uloga pozitivnog regulatora inflamatornog odgovora. NF-κB indukuje ekspresiju proinflamatornih citokina, uključujući IL-1β i TNF-α, kao i ciklooksigenaze-2 (COX-2), enzima koji katalizuje produkciju prostaglandina, važnih medijatora inflamacije (Inoue i Tanabe, 1998; Grilli i Memo, 1999; Jin i sar., 2008). Pored toga utiče na količinu ROS i RNS u ćeliji, tako što reguliše ekspresiju enzima koji dovode do njihovog formiranja (NOX-2, iNOS, COX-2, citohrom P450 enzimi itd.), ali i onih koji učestvuju u njihovom eliminisanju (antioksidativni enzimi poput GPx i SOD) (Morgan i Liu, 2011). Aktivnost ovog transkripcionog faktora je kontrolisana inhibitornom subjedinicom IκB (engl. *inhibitor kappa B*) koja se vezuje za NF-κB i zadržava ga u citoplazmi. Različiti stimulusi dovode do fosforilacije i posledične degradacije IκB što rezultuje translokacijom NF-κB iz citoplazme u jedro. U jedru, p50/p65 heterodimer NF-κB se vezuje za odgovarajuće DNK sekvence i reguliše ekspresiju brojnih ciljnih gena (Auphan i sar., 1995; Baldwin, 1996). Pokazano je da stres fizičkog sputavanja (engl. *restraint stress*) stimuliše translokaciju NF-κB u jedro ćelija kore cerebruma pacova već nakon 4 sata (Madrigal i sar., 2001).

NF-κB može biti aktiviran preko tzv. *Toll-like* receptora koji pokreću urođeni imunski odgovor, uključujući oslobađanje proinflamatornih citokina (Kawai i Akira, 2007) (Slika 5). Takođe, aktivaciju ovog transkripcionog faktora mogu izazvati stresori aktiviranjem simpatičkog nervnog sistema i povećanim oslobađanjem noradrenalina (Bierhaus i sar., 2003). Pored toga, poznato je da stresori smanjuju oslobađanje acetilholina i na taj način promovišu aktivaciju NF-κB (Pavlov i Tracey, 2005). Naime, vezivanje acetilholina za α7 subjedinicu nikotinskog acetilholinskog receptora (α7nAChR) ćelija imunskog sistema inhibira urođeni imunski odgovor. Dakle, aktivacija NF-κB je nishodni efektor neuroendokrinog odgovora na stresor. Osim toga, proteinske kinaze aktivirane mitogenima poput p38 i Jun amino-terminalne kinaze, mogu inhibirati GR i time osloboditi NF-κB inhibitornog uticaja ovog receptora (Raison i sar., 2006). Zanimljivo je da do aktivacije NF-κB mogu dovesti ROS, kao i medijatori inflamacije (TNF, IL-1, LPS) (Li i Karin, 1999; Kabe i sar., 2005). S obzirom na sprektar target gena sa jedne, i stimulusa koji ga aktiviraju sa druge strane, ovaj transkripcioni faktor se može smatrati posrednikom između oksidativnog stresa i

inflamacije (Fischer i Maier, 2015), dva veoma važna procesa u patofiziologiji depresije.

Poznato je da signali o perifernoj inflamaciji mogu dospeti do centralnog nervnog sistema (Slika 5). Signali o inflamatornim procesima u abdominalnoj šupljini mogu se preneti aferentnim vlaknima vagusa do mozga. Zatim, citokini i drugi medijatori inflamacije iz krvi mogu dospeti do mozga putem cirkumventrikularnih organa u kojima ne postoji krvno-moždana barijera. Takođe, citokini mogu interagovati direktno sa endotelnim ćelijama krvno-moždane barijere i indukovati oslobađanje inflamatornih molekula poput prostaglandina u parenim mozga (Perry i sar., 2007; Tracey, 2009).

Proinflamatorni citokini utiču na metabolizam neurotransmitera, neuroendokrine funkcije, sinaptičku plastičnost i ponašanje (Raison i sar., 2006) (Slika 5). Poznato je da utiču na sintezu serotonina, dopamina i glutamata, kao i na njihovo oslobađanje i preuzimanje iz sinaptičke pukotine. Tako, na primer, citokini preusmeravaju metabolizam triptofana na kinureinski put, umesto ka sintezi serotonina (Miller i sar., 2013). Zatim, citokini aktiviraju HHA sistem time što aktiviraju neurone PVN hipotalamusa, što dovodi do oslobađanja CRH, i posledično ACTH i KORT (Silverman i sar., 2005).



Slika 5. ***Interakcije na relaciji stres – imunski odgovor – depresija***

(a) *Aktivacija NF-kB preko tzv. Toll-like receptora (TLR) usled nekog imunskog izazova dovodi do inflamatornog odgovora koji uključuje* ***(b)*** *oslobađanje proinflamatornih citokina TNF-α, IL-1 i IL-6.* ***(c)*** *Ovi citokini ulaze u mozak putem cirkumventrikularnih organa ili aferentnim nervnim vlaknima (npr. senzorna vlakna vagusa).* ***(d)*** *U mozgu, citokini doprinose razvoju depresije (narižasto) tako što:* ***(i)*** *dovode do promena u metabolizmu neurotransmitera serotonina (5-HT) i dopamina (DA);* ***(ii)*** *aktiviraju hipotalamo-hipofizno-adrenokortikalni (HHA) sistem i* ***(iii)*** *narušavaju sinaptičku plastičnost tako što utiču na neurotrofne faktore kao što je neurotrofni faktor mozga, BDNF. Stresori iz spoljašnje sredine promovišu aktivaciju NF-kB tako što:* ***(e)*** *povećavaju aktivnost simpatičkog nervnog sistema – povećano oslobađanje noradrenalina (NA) koji se vezuje za α (αAR) i β (βAR) receptore (žuto);* ***(f)*** *umanjuju inhibiciju posredovanu motornim vlaknima vagusa – smanjeno oslobađanje acetilholina (ACh), koji se vezuje za α7 subjedinicu nikotinskog acetilholinskog receptora (α7nAChR)] (plavo).* ***(g)*** *Aktivacija proteinski kinaza aktiviranih mitogenima, uključujući p38 i Jun amino-terminalnu kinazu (JNK) dovodi do oslobađanja NF-kB od inhibitornog uticaja glukokortikoidnog receptora (GR) (plavo). Modifikovano iz Raison i sar., 2006.*

1.3 ŽIVOTINJSKI MODELI DEPRESIJE

Istraživanja patofiziologije depresije koja se vrše na pacijentima susreću se sa brojnim ograničenjima. Pre svega, svedena su na ispitivanja uzoraka periferne krvi i *postmortem* izolovanog tkiva mozga. Pored toga, veliki broj promenljivih kao što su istorija terapije medikamentima, starost i pol pacijenata, i uslovi života mogu da utiču na dobijene rezultate. Studije na životinjskim modelima omogućavaju ispitivanje ćelijskih i molekularnih promena u kontrolisanim uslovima, kao i korišćenje invazivnih metoda poput manipulacije farmakološkim agensima i editovanja gena (Wang i sar., 2017).

Životinjski modeli depresije pokušavaju da proizvedu merljive promene koje koreliraju sa simptomima depresije. Simptomi i promene koje se uočavaju kod pacijenata koji boluju od depresije, a koje je moguće modelovati na pacovima i miševima prikazani su u Tabeli 1.

Tabela 1. ***Simptomi i osobine povezane sa depresijom koje je moguće modelovati na pacovima***

Ponašanje koje nalikuje anhedoniji
Ponašanje koje nalikuje anksioznom
Beznadežno ponašanje, očaj (engl. *behavioral despair*)
Promene u apetitu i telesnoj masi
Poremećaji cirkadijalnog ritma spavanja
Neuroendokrine promene – promene u HHA sistemu
Neuroanatomske promene – atrofija dendrita i smanjena zapremina hipokampusa

Validnost određenog životinjskog modela depresije najčešće se procenjuje na osnovu zadovoljavanja kriterijuma koje je opisao Vilner (Willner, 1984). Naime, da bi model bio zadavoljavajuć, pored toga što treba da daje ponovljive rezultate, neophodno je da ispunjava kriterijume validnosti sličnosti (fenomenološka sličnost sa simptomatologijom oboljenja), konstrukcijske validnosti (sličnost sa oboljenjem u strukturnom i neurohemijskom pogledu) i predikcione validnosti (zadovoljavajući odgovor na terapiju koja se koristi u lečenju pacijenata).

Ekspanzija različitih modela depresije počela je osamdesetih godina prošlog veka nakon otkrića da razvoj depresije, koja je do tada smatrana endogenim poremećajem, u velikoj meri zavisi od spoljašnjih faktora (Brown i Harris, 1978; Lloyd, 1980). To je pokrenulo veliki broj istraživanja koja su za cilj imala da utvrde vezu između stresa i depresije. Brojni modeli koji se danas koriste, od kojih su neki prikazani u Tabeli 2 zasnivaju se upravo na hroničnom izlaganju laboratorijskih životinja različitim stresorima.

Tabela 2. ***Modeli depresije koji se primenjuju na pacovima i miševima***

Tipovi modela	Modeli	Reference
Stres u adultnom periodu	Hronični (nepredvidivi) blagi stres	(Katz, 1982; Willner i sar., 1987)
	Hronična izolacija	(Wiberg i Grice, 1963)
	Naučena bespomoćnost	(Seligman i sar., 1975; Maier, 1984)
Stres u ranim fazama života	Odvajanje mladunaca od majke	(Matthews i Robbins, 2003)
Lezije	Bilateralna olfaktorna bulbektomija	(Cairncross i sar., 1979) (Jancsár i Leonard, 1984)
Farmakološki modeli	Reserpin*	(Stein i Ray, 1960)
Genetički modeli	Genetičke manipulacije: 5-HT transporter "*knockout*"	(Lira i sar., 2003) (Garcia-Garcia i sar., 2009)
	Selektivno ukrštanje: Wistar – Kyoto pacovi hipersenzitivni na stres	(Paré, 1989) (Solberg i sar., 2001)

*Reserpin neselektivno smanjuje nivo monoaminskih neurotransmitera serotonina, dopamina, noradrenalina u sinapsama tako što inhibira njihov transport u sinaptičke vezikule

1.3.1 Hronična izolacija

Dugotrajna izolacija je psihosocijalni stresor koji kod juvenilnih i adultnih pacova i miševa dovodi do tzv. sindroma izolacije koga karakterišu promene na nivou ponašanja, fiziologije i morfologije. Sindrom izolacije prepoznat je još šezdesetih godina prošlog veka kada je uočeno da izolovani pacovi ispoljavaju veću agresivnost i anksioznost prilikom kontakta sa eksperimentatorom, u poređenju sa pacovima koji su gajeni u kavezu sa drugim jedinkama (Wiberg i Grice, 1963). Tada je još primećeno da

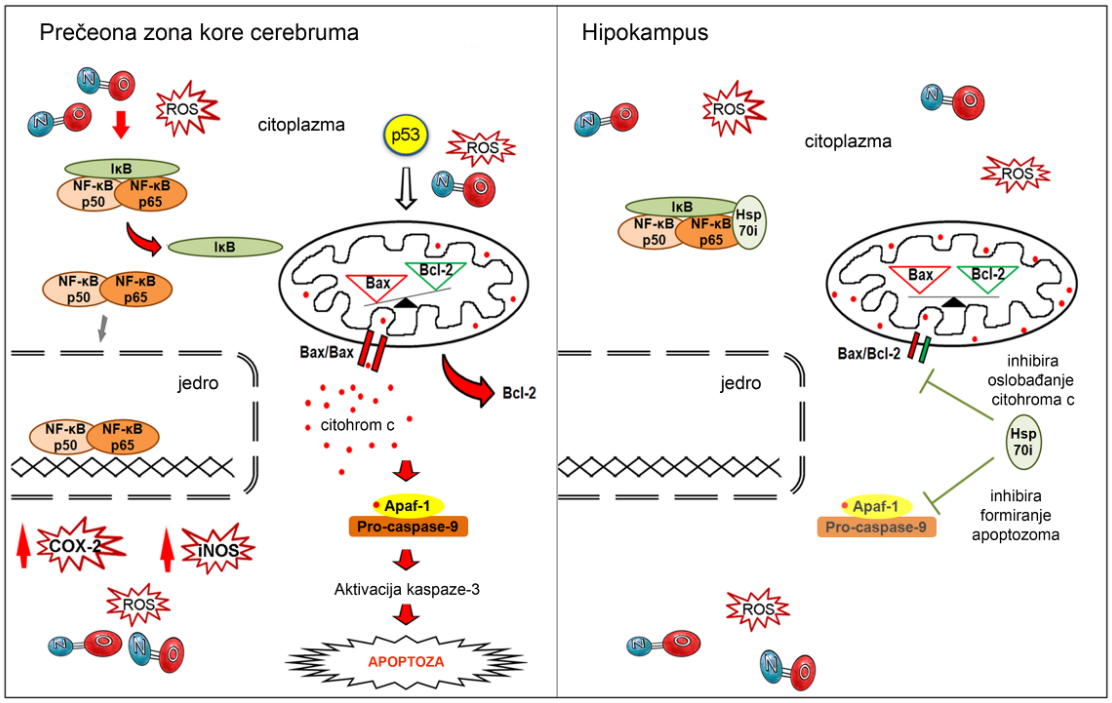
pacovi nakon 13 nedelja izolacije imaju uvećane adrenalne žlezde i tireoideu, kao i umanjenu slezinu i timus. Izolacija je blag stresor koji zbog svoje socijalne prirode, za pacove kao socijalne životinje, predstavlja prirodniji stresor od procedura koje uključuju fizičke stimuluse poput imobilizacije, elektrošokova i buke (Heinrichs i Koob, 2006). Hronična izolacija pacova se koristi kao životinjski model za proučavanje patofiziologije depresije i ispunjava sva tri Vilnerova kriterijuma validnosti (Abelaira i sar., 2013). Dodatno, ovaj model ispunjava i proširene uslove (Tabela 3) koje su definisali Belzung i Lemoina (2011).

Tabela 3. ***Validnost modela izolacije u odnosu na kriterijume koje su opisali Belzung i Lemoina***

Kriterijumi validnosti	Zahtevi za ispunjavanje kriterijuma	Validnost izolacije u odnosu na postavljene kriterijume
Validnost homologije	Životinjska vrsta koja se koristi u modelu mora biti odgovarajuća	Pacovi su socijalne životinje, izolacija prekida socijalne interakcije (Niesink i Van Ree, 1982a; Heinrichs i Koob, 2006)
Validnost patologije	Mora postojati sličnost u procesima koji dovode do razvoja simptoma kod pacijenata i modela	Izolacija ima psihosocijalan karakter kao i mnogi faktori koji povećavaju rizik za razvoj depresije kod ljudi (Joiner i Coyne, 1999; Moran i sar., 2008)
Validnost mehanizama	Biološki mehanizmi u osnovi poremećaja su isti kod pacijenata i modela	Disfunkcija HHA sistema (Sànchez i sar., 1998; Dronjak i sar., 2004; Filipović i sar., 2005)
Validnost sličnosti	Etološka i biološka validnost	Izolovani pacovi ispoljavaju anhedoniju i anksioznost (Carrier i Kabbaj, 2012) i kognitivne poremećaje (Einon, 1980; Ren i sar., 2015)
Predikciona validnost	Pozitivan odgovor na terapiju koja se koristi u lečenju pacijenata	Normalizacija ponašanja nakon tretmana antidepresivima (Niesink i Van Ree, 1982b; Djordjevic i sar., 2012a)

Izolacija, kod adultnih mužjaka pacova, izaziva ponašanje slično depresivnom i anksioznom već nakon dve nedelje (Carrier i Kabbaj, 2012). Takođe, pokazano je da 21-dnevna izolacija remeti regulaciju HHA aktivnosti tako što narušava efikasnost negativne povratne sprege u višim centrima u mozgu (Filipović i sar., 2005). U ovom modelu detektovane su i molekularne promene u ćelijama hipokampusa i prečeone zone kore cerebruma poput aktivacije puteva koji vode ka oksidativnom i nitrozativnom stresu. Intenzivnije promene su uočene u ćelijama prečeone zone u kojoj je detektovana

i proapoptotska signalizacija nakon primene dodatnog akutnog stresora (Filipović i sar., 2016) (Slika 6, detalji u legendi).



Slika 6. ***Šematski prikaz efekata 21-dnevne izolacije adultnih mužjaka Wistar pacova na ćelije prečeone zone kore cerebruma i hipokampusa***

U ćelijama prečeone zone kore cerebruma (levo), izolacija dovodi do aktivacije i translokacije NF-κB u jedro i povećane ekspresije enzima COX-2 i iNOS što rezultuje povećanom produkcijom ROS/RNS. Pored toga, delovanje akutnog stresora (2h imobilizacija ili hladnoća) kod izolovanih pacova izaziva proapoptotski odgovor koji podrazumeva: translokaciju p53 praćenu translokacijom proapoptotskog Bax proteina (engl. Bcl-2 associated X protein) u mitohondrije, i antiapoptotskog Bcl-2 (eng. B-cell lymphoma 2) u citoplazmu; oslobađanje citohroma c iz mitohondrija u citoplazmu; aktivaciju kaspaze-3.

U ćelijama hipokampusa (desno) izolacija u znatno manjoj meri povećava produkciju ROS/RNS,ali povećava nivo inducibilnog proteina toplotnog šoka 70 (engl. inducible heat shock protein 70, HSP70i) koji inhibira aktivaciju NF-κB i štitu od oksidativnog/nitrozativnog stresa, formiranja apoptozoma i apoptoze. Modifikovano iz (Filipović i sar., 2016).

Zanimljivo, u hipokampusu adultnih izolovanih pacova uočen je smanjen broj PV+ neurona (Filipović i sar., 2013, 2018). Promene u okviru GABA interneurona, naročito onih koji eksprimiraju PV, u kori i hipokampusu predstavljaju karakteristiku patofiziologije shizofrenije (Gonzalez-Burgos i sar., 2015). Inače, hronična izolacija pacova odmah po završetku perioda sisanja koristi se kao neurorazvojni model

shizofrenije (Möller i sar., 2013). Međutim, kao što je istaknuto ranije u tekstu (Poglavlje 1.2.1.2) promene u GABA sistemu su karakteristične i za depresivne poremećaje.

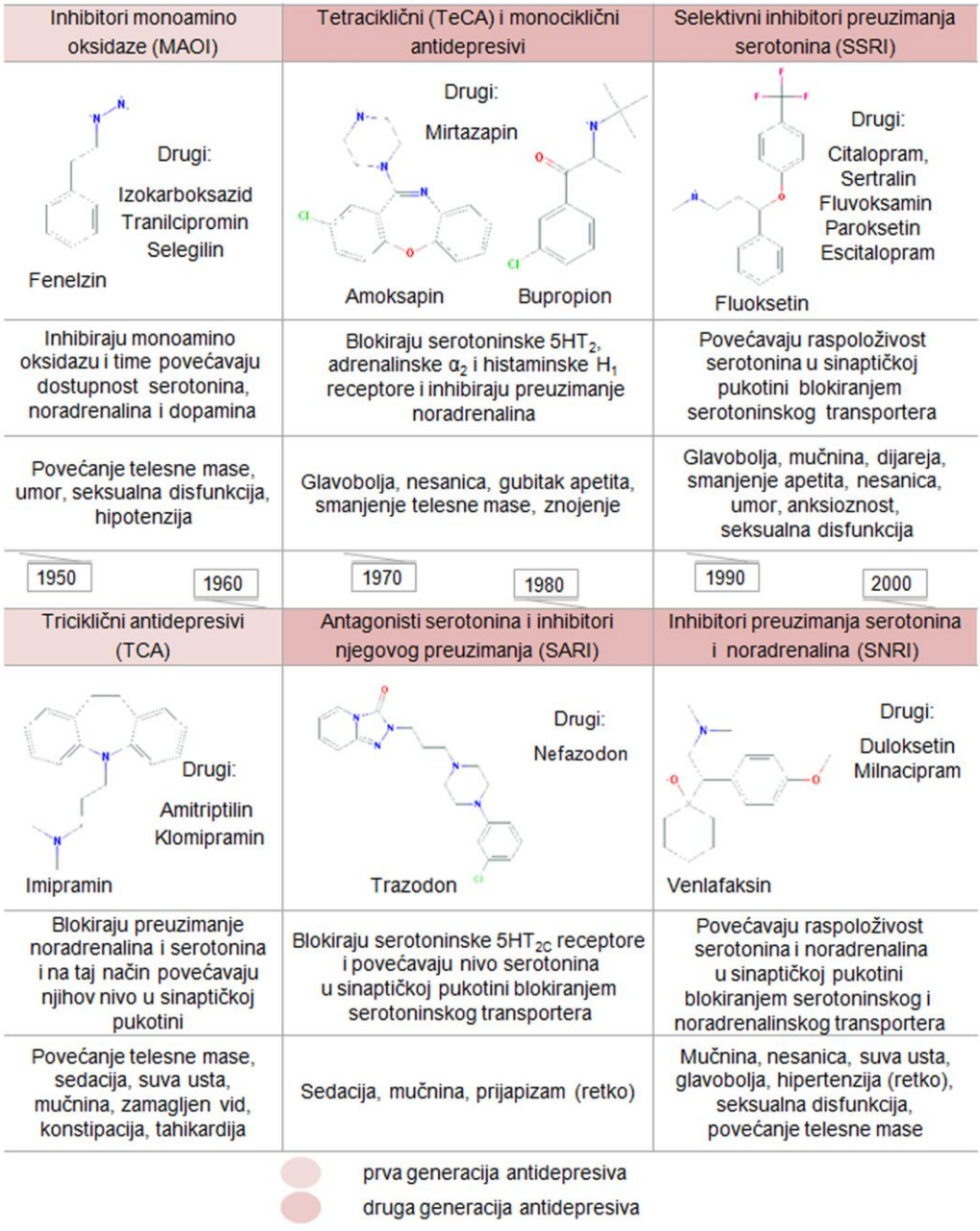
1.4 PSIHOTROPNI LEKOVI

Psihotropni lekovi (psihofarmaceutski ili psihoaktivni lekovi) su hemijske supstance koje menjaju percepciju, razmišljanje, raspoloženje i ponašanje tako što izazivaju promene u mozgu, pre svega delujući na neurotransmitersku signalizaciju. U njih se, između ostalih, ubrajaju lekovi poput antidepresiva, antipsihotika i anksiolitika koji se koriste u lečenju psihijatrijskih poremećaja. Lečenje psihijatrijskih oboljenja suočava se sa velikim izazovima. Jedan od vodećih je zabrinjavajuće niska efikasnost postojećih lekova. U slučaju depresije samo 60–70% pacijenata ima adekvatan odgovor na psihotropnu terapiju. Takođe, određivanje odgovarajuće doze je često veoma težak i dugotrajan proces, a veliki problem predstavljaju i neželjeni efekti i štetne posledice lekova (Al-Harbi, 2012).

1.4.1 Antidepresivi

Osnovni cilj tretmana antidepresivima je uklanjanje uzroka nastanka i ublažavanje simptoma depresije poput osećanja velike tuge, isrpljenosti, anksioznosti, problema sa spavanjem i pojave samoubilačkih misli, kao i sprečavanje njihovog ponovnog javljanja. Antidepresivi su prvi put uvedeni u kliničku upotrebu pedesetih godina prošlog veka (Tabela 4). Primena iproniazida–inhibitora monoamino oksidaze i imipramina–prvog predstavnika tricikličnih antidepresiva, predstavlja revoluciju u razvoju psihijatrije. Monoamino oksidaze katalizuju oksidaciju noradrenalina, serotonina i dopamina, pa njihovi inhibitori povećavaju dostupnost ovih neurotransmitera, dok triciklični antidepresivi blokiraju preuzimanje noradrenalina i serotonina iz sinaptičke pukotine. Antidepresivna svojstva iproniazida zapravo su otkrivena slučajno tokom kliničke probe ovog agensa koji je bio namenjen lečenju tuberkuloze. Njegov stimulišući efekat na nervni sistem koji je tom prilikom uočen, u prvom trenutku je tretiran kao sporedni efekat. Međutim, ubrzo je postao prvi specifični antidepresiv plasiran na tržište.

Tabela 4. ***Glavne grupe antidepresiva***

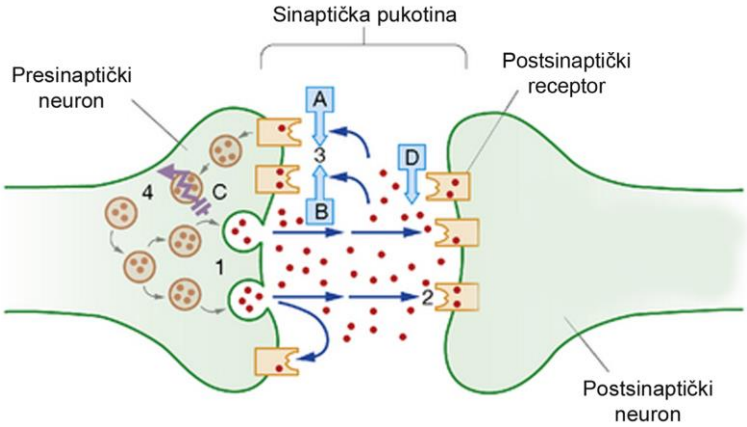


Predstavnci, mehanizmi delovanja i neželjeni efekti glavnih grupa antidepresiva, kao i vreme kada je odobrena njihova upotreba u kliničkoj praksi

Pripadnici prve generacije antidepresiva, tzv. klasični antidepresivi, danas su u ograničenoj upotrebi zbog ozbiljnih neželjenih efekata i prepisuju se tek ukoliko druge opcije ne dovedu do remisije. Uvođenje tzv. atipičnih antidepresiva (mirtazapin, trazodon i bupropion) sedamdesetih godina nije dovelo do velikog progresa u smislu povećanja efikasnosti i smanjenja neželjenih efekata. Ogroman korak unapred u tom

smislu napravljen je otkrićem selektivnih inhibitora preuzimanja serotonina (engl. *selective serotonin reuptake inhibitors*, SSRIs). Time su otvorena vrata razvoju drugih grupa tzv. savremenih antidepresiva poput inhibitora preuzimanja serotonina i noradrenalina (engl. *serotonin i norepinephrine reuptake inhibitors*, SNRIs). SSRIs blokiraju serotoninski transporter i na taj način sprečavaju preuzimanje serotonina iz sinaptičke pukotine u presinaptiči neuron. SNRIs blokiraju serotoninski, kao i noradrenalinski transporter, i na taj način povećavaju raspoloživost kako serotonina, tako i noradrenalina u sinaptičkoj pukotini (Lopez-Munoz i Alamo, 2009).

Iako predstavnici različitih grupa imaju specifične mehanizme kojima ostvaruju svoje terapijske efekte, u osnovi delovanja svih antidepresiva koji se danas koriste u kliničkoj praksi je modulacija monoaminske transmisije na nivou sinapse (Slika 7, detalji u legendi).



Slika 7. ***Mehanizmi delovanja antidepresiva u sinapsi***

Fiziološki procesi u sinapsi: 1. *Oslobađanje monoaminskog neurotransimtera iz presinaptičkog neurona u sinaptičku pukotinu;* ***2.*** *Vezivanje monoamina za receptore na postsinaptičkoj membrani;* ***3.*** *Vraćanje monoamina iz sinaptičke pukotine u presinaptički neuron;* ***4.*** *Razgradnja monoamina monoamino oksidazom.*

Efekti antidepresiva*:* ***A.*** *Triciklični antidepresivi sprečavaju preuzimanje noradrenalina i serotonina od strane presinaptičkog neurona;* ***B.*** *Selektivni inhibitori preuzimanja serotonina predominantno sprečavaju preuzimanje serotonina;* ***C.*** *Inhibitori monoamino oksidaze smanjuju aktivnost ovog enzima i na taj način povećavaju količinu monoamina dostupnog za oslobađanje u sinaptičku pukotinu;* ***D.*** *Neki antidepresivi (npr. nefazodon) direktno blokiraju postsinaptičke receptore. Preuzeto i modifikovano sa https://clinicalgate.com/psychotropic-drugs/.*

1.4.1.1Fluoksetin

Uvođenje fluoksetina (Prozak) u kliničku praksu krajem osamdesetih godina prošlog veka predstavlja drugu revoluciju u terapiji depresije. Fluoksetin je derivat fenilpropilamina i pripada grupi SSRIs antidepresiva koji predstavljaju lekove prvog izbora u lečenju simptoma depresije jer su veoma efikasni i relativno bezbedni u odnosu na druge antidepresive (Wilde i Benfield, 1998). SSRIs blokiraju serotoninske transportere, sprečavaju transport serotonina nazad u presinaptički neuron i time povećavaju njegovu koncentraciju u sinaptičkoj pukotini i produžavaju izloženost receptora na postsinaptičkoj membrani ovom neurotransmiteru. Uprkos tome što je ovaj efekat brz, do poboljšanja stanja pacijenta dolazi tek nakon nekoliko nedelja, što ukazuje da je akutno povećanje koncentracije serotonina potrebno, ali ne i dovoljno za suzbijanje simptoma depresije. Akutne promene u koncentraciji serotonina mogu da dovedu do dugotrajnih promena koje uključuju smanjenje ekspresije ili desenzitizaciju određenih receptora, promene u aktivnosti enzima uključenih u metabolizam serotonina, povećanje ekspresije neurotrofnih faktora i druge. S obzirom da akutni porast koncentracije serotonina može biti toksičan za okolne ćelije, navedene promene se mogu posmatrati kao protektivne adaptacije. Dakle, povećana koncentracija monoamina pokreće nishodne molekularne mehanizme koji dovode do ublažavanja simtoma depresije. Za te procese je potrebno 4–6 nedelja što odgovara latentnom periodu delovanja ovih antidepresiva (Santarsieri i Schwartz, 2015). Pored toga, pokazano je da fluoksetin indukuje neurogenezu (Malberg i sar., 2000; Santarelli, 2003). Pomenuti adaptivni procesi, kao i povećanje neurogeneze i plastičnosti sinapsi, mogu povratiti normalno funkcionisanje moždanih regiona koji su bili oštećeni i na taj način dovesti do remisije.

Fluoksetin i norfluoksetin, glavni metabolit fluoksetina koji takođe ima terapijsku aktivnost, imaju visok afinitet za serotoninski transporter, selektivno se vezuju za njega, blokiraju ga i na taj način dovode do povećanja koncentracije ovog neurotransmitera u sinaptičkoj pukotini i do sedam puta (Wong i sar., 1995).

1.4.2 Antipsihotici

Antipsihotici se koriste za lečenje psihoza, ozbiljnih psihičkih poremećaja čiju suštinu predstavlja izmenjen odnos obolele osobe prema stvarnosti. Najčešće se koriste za psihoze koje se javljaju u obliku shizofrenije, a ponekad i u lečenju bipolarnog poremećaja (manija i depresija) i teške depresije.

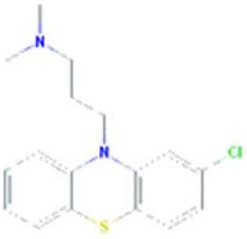
Prva generacija antipsihotika (neuroleptici) predstavlja snažne antagoniste dopaminskih D_2 receptora, koji deluju na deluzije i halucinacije (Miyamoto i sar., 2005). Ti tzv. tipični antipsihotici su povezani sa velikim rizikom za pojavu neuroloških simptoma kao što su tardivna diskinezija, distonija, akatizija, sekundarni parkinsonizam, koji su poznati pod imenom ekstrapiramidni neželjeni efekti (Poznić Jesić i sar., 2012) (Tabela 5).

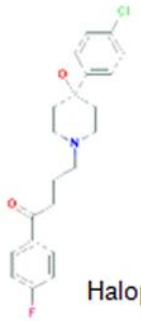
Antipsihotici druge generacije takođe blokiraju D_2 receptore ali manjim afinitetom, i razlikuju se od prethodnih po tome što blokiraju i serotoninske 5-HT_{2A} receptore, zbog čega su i dobili ime atipični antipsihotici. Atipičnost ovih antipsihotika se ogleda i u tome što efikasno deluju i na apatiju, anhedoniju, oskudan govor i kognitivne simptome, i što su delotvorni kod pacijenata rezistentnih na terapiju neurolepticima. Ovi lekovi se drugačije nazivaju serotonin-dopaminski antagonisti i zapravo imaju veći afinitet za 5-HT_{2A} nego D_2 receptore (Meltzer i Huang, 2008). Popularnost su stekli pre svega zbog značajno manjeg rizika od pojave ekstrapiramidnih simptoma. Oni pak mogu izazvati metaboličke neželjene efekte poput hiperglikemije, dislipidemije i gojaznosti. Atipični antipsihotici se koriste i u lečenju depresivnih poremećaja, sa ili bez psihotičnih simptoma, kako pojedinačno, tako i u kombinaciji sa antidepresivima (Wang i Si, 2013).

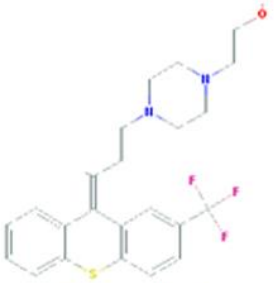
Parcijalni agonisti D_2 i 5-HT_{1A} receptora koji čine tzv. treću generaciju antipsihotika koriste se u lečenju shizofrenije, MDD, bipolarnog poremećaja, kao i Parkinsonove bolesti. Aripiprazol, glavni predstavnik ove grupe, ispoljava visoki afinitet i parcijalni agonizam prema D_2 receptorima, što mu omogućava da deluje kao svojevrstan modulator. Naime, smatra se da je hiperaktivnost dopaminske transmisije u mezolimbičkom putu (od ventralnog tegmentuma do *nukleus akumbensa*) uzrok pojave deluzija i halucinacija kod shizofrenih pacijenata. Aripiprazol zahvaljujući visokom afinitetu i parcijalnom agonizmu smanjuje dopaminsku transmisiju i ublažava pomenute

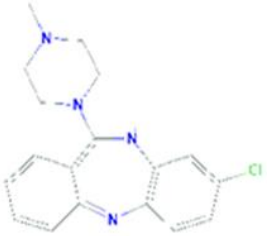
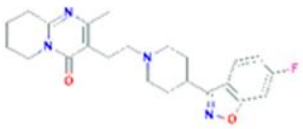
simptome. Sa druge strane, u mezokortikalnom putu (od ventralnog tegmentuma do prečeone zone kore cerebruma) obolelih osoba prisutna je hipofunkcija dopaminske transmisije, a aripriprazol je povećava i dovodi do poboljšanja kognitivnih simptoma, apatije i anhedonije (Etievant i sar., 2010).

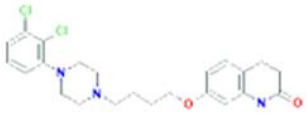
Tabela 5. ***Glavne grupe antipsihotika***

<table>
<tr><td colspan="3">Tipični antipsihotici (prva generacija)</td></tr>
<tr><td>Fenotiazini</td><td>Butirofenoni</td><td>Tioksanteni</td></tr>
<tr><td> 
Hlorpromazin

Drugi:
Flufenazin, tioridazin, promazin, perfenazin</td><td> 
Haloperidol

Drugi:
Benperidol, droperidol</td><td> 
Flupentiksol

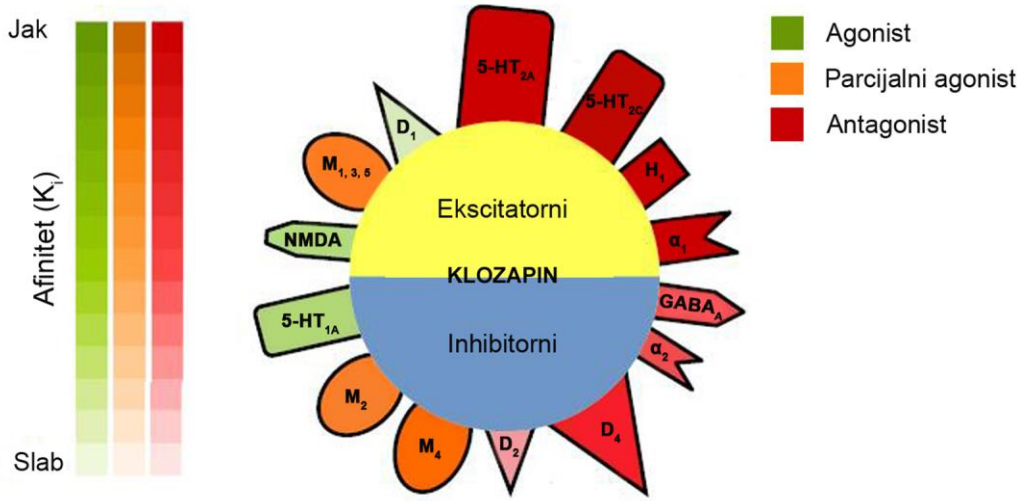
Drugi:
Zuklopentiksol, klopentiksol</td></tr>
<tr><td colspan="3">Antagonisti dopaminskih D_2 receptora
Efikasni protiv deluzija i halucinacija
Veliki rizik za pojavu ekstrapiramidnih neželjenih efekata</td></tr>
<tr><td colspan="3">Atipični antipsihotici</td></tr>
<tr><td colspan="2">Druga generacija</td><td>Treća generacija</td></tr>
<tr><td colspan="2">  
Klozapin Risperidon

Drugi:
Olanzapin, kvetiapin, ziprasidon, paliperidon, sulpirid, sertindol,</td><td> 
Aripiprazol</td></tr>
<tr><td colspan="2">Antagonisti serotoninskih i dopaminskih receptora ($5\text{-}HT_{2A}$, D_4, D_2)
Efikasni protiv deluzija, halucinacija, kao i apatije, anhedonije i kognitivnih simptoma
Manji rizik za pojavu ekstrapiramidnih neželjenih efekata u odnosu na tipične antipsihotike</td><td>Parcijalni agonist D_2 i $5\text{-}HT_{1A}$ receptora i antagonist $5\text{-}HT_{2A}$ receptora</td></tr>
</table>

Predstavnici i mehanizmi delovanja glavnih grupa antipsihotika

1.4.2.1Klozapin

Klozapin, derivat dibenzodiazepina, je prvi lek iz druge generacije antipsihotika i otkriven je 1958. godine (Hippius, 1999). Vezuje se za veliki broj receptora različitih neurotransmitera pa se ubraja u tzv. MARTA (engl. *multi acting receptor targeted antipsychotics*) grupu antipsihotika (Slika 8).



Slika 8. ***Pojednostavljeni šematski prikaz afiniteta klozapina kao agonista (zeleno), parcijalnog agonista (narižasto) i antagonista (crveno) ekscitatornih (žuto) i inhibitornih (plavo) receptora.*** *Različite nijanse (svetlo – tamno) zelene, narižaste i crvene boje odražavaju jačinu afiniteta (slab – jak) klozapina za svaki receptor. D_1, D_2, D_4 – dopaminski; 5-HT_{1A}, 5-HT_{2A}, 5-HT_{2C} – serotoninski; M_{1-5} – muskarinski; α_1 i α_2 – adrenalinski; H_1 – histaminski; NMDA – glutamatni; $GABA_A$ – jonotropni receptor za GABA. Modifikovano iz (O'Connor i O'Shea, 2015).*

Klozapin se pre svega primenjuje u lečenju shizofrenije i efikasan je u suzbijanju kako deluzija i halucinacija, tako i kognitivnih deficita, apatije i anhedonije. Ublažava anksioznst, depresiju, razdražljivost i suzbija suicidalne misli (O'Connor i O'Shea, 2015). Ispoljava snažan antipsihotički efekat uprkos niskom afinitetu za D_2 receptore, što ukazuje na postojanje drugih receptora i mehanizama odgovornih za njegov terapijski efekat (Lally i MacCabe, 2015). Iako je otkriven pre skoro 60 godina, klozapin i danas predstavlja zlatni stiard u lečenju pacijenata koji boluju od shizofrenije, rezistentne na tretman bar dva antipsihotika (Taylor, 2017). Pokazano je da je ovaj antipsihotik efikasan i u lečenju depresije koja je rezistentna na standardnu

antidepresivnu terapiju, bilo da se primenjuje u vidu monoterapije ili u kombinaciji sa antidepresivima (Rogóż, 2013; Li i sar., 2015b). Antidepresivni efekat atipičnih antipsihotika, između ostalih i klozapina, verovatno je posredovan 5-HT_{1A}, 5-HT_{2A} i α_2 receptorima (Rogóż, 2013).

Komparativna meta-analiza koja je poredila efikasnost i tolerabilnost 15 antipsihotika, pokazala je da klozapin u manjoj meri izaziva ekstrapiramidne neželjene efekte od drugih ispitivanih lekova, čak ne značajno više od samog placeba (Leucht i sar., 2013). Međutim, klozapin može imati druge veoma štetne neželjene efekte kao što su agranulocitoza, dijabetes i povećanje telesne mase. Jedno vreme je bio povučen iz kliničke upotrebe usled visokog rizika od agranulocitoze ali je vraćen zbog nezamenljivo velike efikasnosti u slučajevima tretman-rezistente shizofrenije (Naheed i Green, 2001; Mitchell i sar., 2013).

1.4.3 Delovanje fluoksetina i klozapina izvan okvira serotoninske i dopaminske signalizacije

Molekularni mehanizmi terapijskog efekta fluoksetina i klozapina, izvan serotoninske i dopaminske neurotransmisije, su u velikoj meri nepoznati. Od posebnog značaja su antioksidativni i imunomodulatorni efekti ovih lekova; interes za ova svojstva psihotropnih lekova je sve veći, jer je sve više dokaza da oksidativni stres i inflamacija doprinose patofiziologiji depresije (Maes i sar., 2009; Bakunina i sar., 2015).

Antioksidativni efekti antidepresiva, uključujući fluoksetin, intenzivno su ispitivani u prekliničkim i kliničkim studijama. U prekliničkim studijama (*in vitro* i životinjski modeli) uočeni su antioksidativni, ali i prooksidativni efekti fluoksetina, naročito u ćelijama jetre pacova pri velikim dozama. Kliničke studije su, u više navrata, pokazale da fluoksetin i drugi antidepresivi mogu smanjiti oksidativni stres koji se uočava kod MDD pacijenata (Behr i sar., 2012). Antioksidativna svojstva klozapina su u znatno manjoj meri ispitivana. Nedavno je jedna *in vitro* studija pokazala antioksidativne efekte klozapina, koji bi mogli doprineti njegovoj terapijskoj efikasnosti (Sadowska-Bartosz i sar., 2016).

Što se tiče imunomodulatornih efekata fluoksetina i klozapina, literaturni podaci su nedosledni. Te nedoslednosti su verovatno jednim delom uzrokovane razlikama u metodologiji, odnosno razlikama u dužini tretmana i primenjenim dozama (Baumeister i sar., 2015). Najveća neslaganja se uočavaju prilikom poređenja rezultata *in vitro* i *in vivo* studija, mada je mali broj *in vivo* studija koje se bave ovom tematikom. Ideja da neuroprotektivni efekat atipičnih antipsihotika, makar delom, proizilazi iz njihove sposobnosti da regulišu stresom izazvane promene u inflamatornom sistemu, podržana je nedavnom studijom na paliperidonu (MacDowell i sar., 2015). Rezultati te studije su pokazali da paliperidon sprečava neuroinflamaciju u modelu akutnog i hroničnog fizičkog sputavanja. Podaci koji se odnose na fluoksetin, sumirani u nedavno objavljenom radu, ukazuju da je, uprkos nekim suprotstavljenim eksperimentalnim rezultatima, fluoksetin sposoban da moduliše imunski odgovor (Di Rosso i sar., 2015).

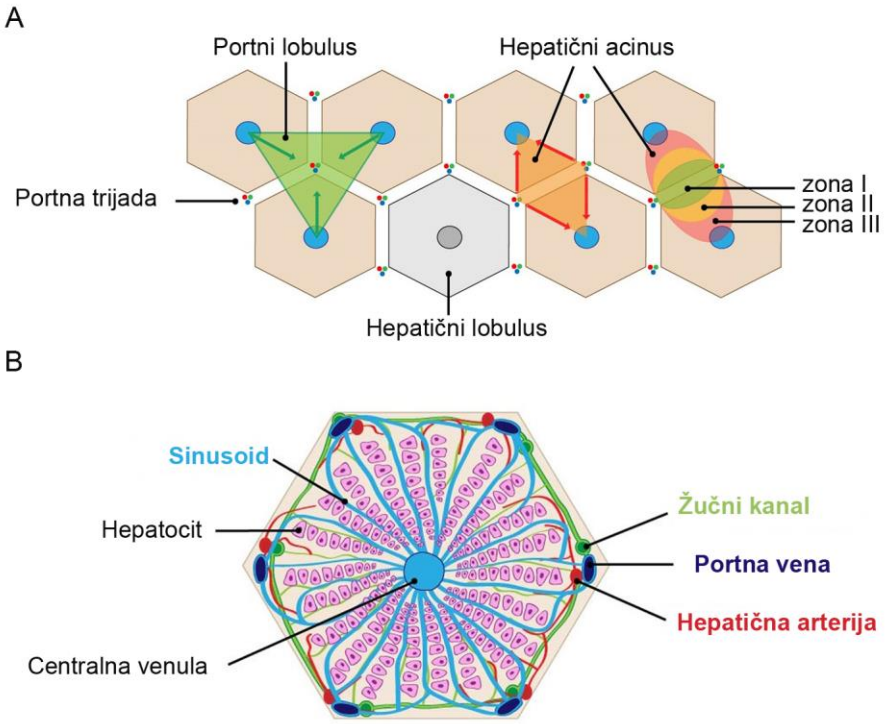
Neki podaci ukazuju da fluoksetin svoj antidepresivni efekat delom ispoljava delujući na GABA signalizaciju. Interesantno je da serotoninski receptori generalno imaju ekscitatorni efekat na GABA neurone, pa je moguće očekivati da povećan nivo serotonina usled delovanja fluoksetina dovodi do povećanja GABA neurotransmisije (Pehrson i Sanchez, 2015). Slično je pokazano i u slučaju klozapina. Naime, uočeno je da je terapijski efekat ovog antipsihotika u vezi sa pojačanom inhibitornom neurotransmisijom posredovanom $GABA_B$ metabotropnim receptorima spregnutim sa proteinom G (Kaster i sar., 2015).

1.5 HISTOLOŠKE KARAKTERISTIKE JETRE

Jetra je organ u kom se odvija veliki broj metaboličkih procesa pa se često naziva biohemijskom laboratorijom organizma. U njoj se odvija metabolizam proteina, masti i ugljenih hidrata, steroida i drugih hormona, detoksifikacija toksina, sinteza holesterola, albumina i faktora koagulacije krvi, skladištenje glikogena i vitamina, formiranje žuči. Između ostalog, ovaj organ predstavlja primarno mesto bioaktivacije i detoksifikacije lekova.

Parenhim jetre je izgrađen od tzv. hepatičnih lobulusa – poligonalnih struktura u čijem središtu se nalazi centralna venula (Slika 9, A). Hepatični lobulusi su okruženi krvnim sudovima i žučnim kanalima koji su organizovani u portne trijade. Portna trijada

se sastoji od ogranaka hepatične arterije, portne vene i žučnog kanala (Slika 9, B) (McCuskey, 2012).



Slika 9. ***Funkcijska organizacija parenhima jetre (A) i građa hepatičnog lobulusa (B)***

Preuzeto i modifikovano sa http://fblt.cz/en/skripta/ix-travici-soustava/5-jatra-a-biotransformace-xenobiotik/

Jetra ima dva aferentna (hepatična arterija i portna vena) i jedan eferentni krvni sud (hepatična vena). Oksigenisana krv se doprema hepatičnom arterijom, a dezoksigenisana krv bogata hranljivim materijama portnom venom. Krv se sinusoidima razvodi po hepatičnom lobulusu, a zatim se drenira u centralnu venulu lobulusa. Centralne venule se spajaju u hepatične vene kojima se krv odvodi u donju šuplju venu. Što se tiče ćelijske populacije, hepatociti čine 80% ćelija jetre, a od neparenhimskih ćelija najzastupljenije su endotelne ćelije, limfociti i Kupferove ćelije, rezidentne makrofage koje odstranjuju umrle ćelije i patogene agense krvi. Ove ćelije imaju ulogu i u prezentaciji antigena, sekreciji proinflamatornih medijatora (IL, TNF, prostaglandini), deponovanju metala i lipida. Rezidentne ćelije jetre su i ćelije prirodne ubice (engl. *natural killer*, NK), prirodne T ćelije ubice (NKT) i antigen-prezentujuće dentritske ćelije. U perisinusoidnom prostoru se nalaze stelatne ćelije koje predstavljaju depo vitamina A i masti (Dong i sar., 2007; McCuskey, 2012).

1.5.1 Patologija jetre

Bolesti jetre imaju visoku prevalenciju u svetu. Dugotrajna izloženost virusima (hepatitis B i C), alkoholu ili nekim metabolitima može dovesti do oštećenja jetre. Hronična oštećenja mogu uzrokovati niz patoloških stanja poput steatoze (masna jetra), steatohepatitisa (steatoza sa inflamacijom), fibroze (nagomilavanje komponenti vanćelijskog matriksa), ciroze (formiranje fibroznih septuma i narušavanje normalne arhitekture jetre) i hepatocelularnog karcinoma. Brojni podaci ukazuju da su oksidativni stres i inflamacija najvažniji uzročnici različitih bolesti ovog organa, bez obzira na njihovu etiologiju (Reyes-Gordillo i sar., 2017). Njihova zajednička osnova je povećana produkcija ROS, a glavni izvori ovih reaktivnih vrsta u jetri su mitohondrije i citohrom P450 enzimi u hepatocitima, kao i Kupferove ćelije i neutrofili. ROS i RNS mogu biti neutralisani antioksidantima u jetri poput GSH, vitamina C, A i E, kao i antioksidativnim enzimima SOD, katalazom i GPx (Mari i sar., 2010). Ukoliko proizvodnja prooksidanata prevazilazi kapacitete antioksidativnog sistema dolazi do oštećenja ćelija jetre (Zhu i sar., 2012). U odgovoru na apoptotična tela poreklom od oštećenih hepatocita regrutuju se i aktiviraju Kupferove ćelije koje produkuju citokine i druge medijatore inflamacije. Kritična komponenta inflamacije je infiltracija imunskih ćelija poput neutrofila, monocita i limfocita, koje takođe oslobađaju medijatore inflamacije (citokini, hemokini, NO) i intenziviraju oksidativni stres produkujući ROS i RNS. Ova kaskada procesa se takoreći pretvara u začarani krug u kom oštećenja nastala usled oksidativnog stresa i inflamacije promovišu patogenezu bolesti jetre (de Irade i sar., 2015).

1.5.2 Jetra i stres

Uprkos tome što je poznato da psihosocijalni stres negativno utiče na fizičko zdravlje individue, njegova uloga u nastanku i toku bolesti vezanih za jetru je dobila malo naučne pažnje. Međutim, postoje podaci koji ukazuju da psihosocijalni stres utiče na razvoj, progres i ishod bolesti jetre. Tako je nedavno sprovedena meta-analiza ukazala na asocijaciju psihosocijalnog stresa i oboljenja jetre (Russ i sar., 2015). Analizom proteoma jetre pokazano je da hronični stres fizičkog sputavanja, životinjski model depresije, dovodi do promena u ekspresiji čak 98 proteina, mahom onih koji su uključeni u transportne, metaboličke i imunološke procese (Li i sar., 2017). Smatra se

da stres posredstvom glukokortikoida i kateholamina utiče na inflamatorni odgovor u jetri i na taj način promoviše patološke promene (Vere, 2009).

1.5.3 Jetra i psihotropni lekovi

Imajući u vidu da se metabolizam lekova primarno dešava u jetri, važno je poznavati efekte određenog leka i njegovih metabolita na strukturni integritet ovog organa. Cilj metaboličke obrade jeste prevođenje leka u oblik koji je pogodan za ekstrakciju iz organizma, što najčešće podrazumeva njegovo prevođenje u polarniji metabolit. Lekovi se metabolišu i na drugim mestima u organizmu poput pluća, zida creva, bubrega i plazme. Međutim, u najvećoj meri se metabolišu u jetri, pre svega jer je jetra metabolički najaktivnije tkivo po jedinici mase, a pored toga, jetra je veliki organ i entero-hepatičnom cirkulacijom kroz nju prolazi krv u kojoj se nalazi lek koji je apsorbovan u crevu (Njoku, 2014).

Hepatotoksičnost leka je jedan od uzroka akutne insuficijencije jetre i čest je razlog za povlačenje nekog farmakološkog agensa sa tržišta. Akutna insuficijencija jetre ima visoku stopu smrtnosti, i u oko 50% slučajeva uzrokovana je štetnim delovanjem leka (engl. *drug-induced liver injury* - DILI), a može biti i posledica virusnog hepatitisa ili idiopatskog autoimunskog hepatitisa. Klinička manifestacija DILI dosta varira, od asimptomatskog povećanja nivoa transaminaza u krvi, do iznenadne i brze insuficijencije jetre (Barnes i sar., 2013). Za razliku od virusnog ili autoimunskog hepatitisa koji se dijagnostifikuju serološkim testiranjima na hepatitis A–E virusne antigene, odnosno na osnovu autoantitela i hiperglobulinemije, DILI se teško otkiva zbog odsustva specifičnog, pouzdanog biomarkera. Dijagnoza se pre svega zasniva na isključivanju drugih potencijalnih uzroka i oslanja se na informacije o upotrebi agenasa, između ostalih i lekova, za koje je pokazano da izazivaju oštećenja jetre (Foureau i sar., 2015). Informacije vezane za hepapotoksičnost lekova je potrebno imati i da bi bilo moguće odrediti optimalnu dozu, kao i da bi se redovnim analizama pratilo stanje kod pacijenata koji primaju lekove koji sa sobom nose rizik od oštećenja jetre.

Centralnu ulogu u razvoju hepatotoksičnosti nekog leka igra sam metabolizam tog leka. Lek ili njegov reaktivni metabolit mogu direktno uzrokovati hepatotoksičnost vezivanjem za makromolekule, ili indirektno promovisanjem inflamacije. Neki reaktivni

metaboliti se mogu kovalentno vezati za nativne proteine, sprečiti samoprepoznavanje od strane imunskog sistema i na taj način promovisati hepatitis posredovan autoimunskim odgovorom (Njoku, 2014). Decenijama unazad se smatra da je DILI posredovan kako inflamacijom, tako i oksidativnim stresom. Povećana produkcija slobodnih radikala, pre svega ROS i RNS molekula, se čak smatra ranim indikatorom hepatotoksičnog potencijala nekog leka. Pokazano je da mnogi lekovi mogu da indukuju oksidativni stres u jetri time što smanjuju nivo antioksidanata, povećavaju nivo prooksidanata i izazivaju lipidnu peroksidaciju (Li i sar., 2015).

Fluoksetin i klozapin se intenzivno metabolišu u jetri izoenzimima iz najveće familije enzima odgovornih za metabolizam lekova – citohrom P450 (CYP) enzimima. To su monooksidaze koje sadrže hem i učestvuju u metabolizmu ksenobiotika, ali i steroida, masnih kiselina i vitamina (Furge i Guengerich, 2006). Fluoksetin podleže biotransformaciji u aktivni metabolit norfluoksetin (N-desmetilfluoksetin) i mnoge druge metabolite, prevashodno delovanjem CYP2D6 enzima (Hiemke i Hartter, 2000). Glavni metabolit klozapina je norklozapin (N-desmetilklozapin), koji se formira aktivnošću CYP1A2, enzima koji je odgovoran za 70% metabolizma klozapina, kao i enzima CYP3A4 u jetri (Prior i sar., 1999).

Antidepresivi mogu uzrokovati DILI. Što se tiče fluoksetina, asimptomatsko povećanje nivoa transaminaza u krvi, koje upućuje na hepatocelularna oštećenja, primećeno je kod 0,5% pacijenata koji su pod dugoročnom terapijom (Voican i sar., 2014). Takođe, zabeleženo je nekoliko slučajeva akutnog hepatitisa (Cai i sar., 1999). Što se animalnih modela tiče, Souza i sar. (1994) su pokazali da fluoksetin i norfluoksetin utiču na energetski metabolizam mitohondrija jetre pacova i da mogu biti toksični ukoliko se primenjuju u visokim dozama. Povećani nivo indikatora oksidativnih oštećenja lipida i proteina u jetri, kao i povećana aktivnost alanin transaminaze (ALT) i aspartat transaminaze (AST) u serumu, detektovani su kod pacova koji su mesec dana tretirani fluoksetinom (Inkielewicz-Stêpniak, 2011).

Veći je broj podataka u literaturi koji ukazuju na hepatotoksičnost klozapina, u poređenju sa fluoksetinom. Iako je veoma efikasan antipsihotik, klozapin se, zbog ozbiljnih neželjenih efekata, koristi kao lek drugog izbora u lečenju uporne shizofrenije i depresije. Od svih antipsihotika koji se nalaze u kliničkoj upotrebi, klozapin je u

najvećoj meri povezan sa metaboličkim sindromom, gojaznošću, dijabetesom i dislipidemijom. Međutim, njegova upotreba je ograničena u prvom redu zbog visokog rizika od fatalne agranulocitoze, zbog čega su obavezne redovne kontrole leukocita kod pacijenata koji konzumiraju ovaj lek (pregled u Klementila, 2016). Hepatotoksični efekti klozapina su privlačili manje pažnje istraživača od navedenih kardiometaboličkih i hematoloških negativnih efekata. Najčešća asimptomatska abnormalnost kod pacijenata tretiranih klozapinom je povećanje nivoa aminotransferaza u krvi. Povišeni nivo ALT detektovan je u 53%, a AST u 39% pacijenata, na uzorku od 164 pacijenta (Macfarlane i sar., 1997). U literaturi je opisano nekoliko kliničkih slučajeva hepatitisa koji je indukovan klozapinom (Macfarlane i sar., 1997; Fong i sar., 2005; Wu Chou i sar., 2014). Etiologija nije poznata ali se smatra da je hepatitis indukovan klozapinom posledica metaboličke ili imunoalergijske idiosinkratske reakcije organizma na lek (Hummer i sar., 1996).

2 CILJEVI

Poznato je da hronični psihosocijalni stres izaziva oksidativni stres i inflamaciju u mozgu i da ovi procesi imaju važnu ulogu u etiologiji i patofiziologiji psihijatrijskih oboljenja. Takođe je poznato da su psihijatrijska oboljenja povezana sa poremećenom GABA signalizacijom, što uključuje i promene u funkcionisanju PV+ interneurona kore cerebruma, koji su zbog svoje brzookidajuće prirode posebno osetljivi na oksidativni stres. Molekularni mehanizmi delovanja antidepresiva fluoksetina i antipsihotika klozapina, izvan domena serotoninske i/ili dopaminske signalizacije, još su nedovoljno razjašnjeni. Nedovoljno su istraženi i efekti ovih psihotropnih lekova na jetru, organ u kome je njihov metabolizam najintenzivniji. Imajući u vidu navedeno, cilj doktorske disertacije bio je da se ispita efekat hronične izolacije na ponašanje, parametre antioksidativne odbrane i inflamacije u hipokampusu i prečeonoj zoni kore cerebruma, kao i na broj PV+ interneurona u medijalnoj prečeonoj zoni kore mozga pacova. Takođe, zadatak je bio da se analizira uticaj hronične primene fluoksetina i klozapina na pobrojane parametre u pomenutim moždanim strukturama, kao i efekti ovih lekova na jetru, sa ciljem da se ispita njihovo antioksidativno, antiinflamatorno i hepatotoksično dejstvo u životinjskom modelu depresije.

Primarni ciljevi doktorske disertacije bili su da se ispita uticaj hronične izolacije pacova, kao i hroničnog tretmana antidepresivom fluoksetinom i antipsihotikom klozapinom na:

1. Ponašanje pacova, i to parametre koji ukazuju na anhedoniju i anksioznost;
2. Antioksidativni sistem i parametre inflamacije u hipokampusu i prečeonoj zoni kore cerebruma;
3. GABA signalizaciju, odnosno broj PV+ ćelija u medijalnoj prečeonoj zoni kore cerebruma;
4. Parametre oksidativnih oštećenja, antioksidativne odbrane i histološke promene u jetri.

3 MATERIJAL I METODE

3.1 ŽIVOTINJE

U eksperimentima su korišćeni odrasli mužjaci pacova soja Wistar, starosti ~2,5 meseca, mase 300–350 g. Pacovi su odgajani u vivarijumu Instituta za nuklearne nauke „Vinča“, Laboratorije za molekularnu biologiju i endokrinologiju, u standardnim laboratorijskim uslovima (temperatura 22 ± 2 °C; vlažnost vazduha 55%; svetlosni režim 12h svetlost/12h mrak) u kavezima (dužina 59,5 cm; širina 38 cm i visina 20 cm) sa hranom i vodom dostupnim *ad libitum*. Eksperimenti su odobreni od strane Etičkog komiteta za upotrebu laboratorijskih životinja Instituta za nuklearne nauke „Vinča“ (broj dozvole 02/11), i izvedeni su u skladu sa propisima Srpskog udruženja za upotrebu životinja u istraživanju i obrazovanju. Broj korišćenih životinja i njihova patnja tokom eksperimenata svedeni su na neophodan minimum.

3.2 PRIPREMA RASTVORA LEKOVA

U radu su korišćene Flunisan tablete (Hemofarm, Srbija) koje sadrže 20 mg fluoksetin-hidrohlorida, i Leponeks tablete (Novartis Pharmaceuticals, UK) koje sadrže 25 mg klozapina. Flunisan tablete su mrvljene u avanu i rastvarane u destilovanoj, sterilnoj vodi. Leponeks tablete su mrvljene i rastvarane u 1 N HCl, uz zagrevanje. Dobijeni rastvor je razblaživan destilovanom vodom i neutralisan do pH 5,1 dodavanjem 1 N NaOH (Halim i sar., 2004). Rastvori oba leka su mešana na magnetnoj mešalici (1 sat), zatim su prebacivani u ultrazvučno kupatilo (3 × 10 min) i na kraju filtrirani Whatman No. 42 filter papirom. Koncentracije odgovarajućih lekova su određivane tečnom hromatografijom ultravisokih performansi (Kovacevic i sar., 2006). Na osnovu tih koncentracija i mase pacova, koja je merena jednom nedeljno, određivana je zapremina rastvora leka koju je bilo potrebno aplicirati da bi se postigla željena doza.

3.3 TRETMAN ŽIVOTINJA

Životinje su podeljene u šest eksperimentalnih grupa (Tabela 6). Nestresirane (NS) životinje su smeštane po 3 ili 4 u jedan kavez, dok su pacovi u izolaciji (IZ) bili sami u kavezima tokom 21 dana.

Tabela 6. ***Pregled eksperimentalnih grupa i tretmana***

Eksperimentalna grupa	Stresor	Lek
NS+FR	/	Fiziološki rastvor (0,9% NaCl)
NS+FLK	/	Fluoksetin-hidrohlorid (15 mg/kg/dan)
NS+KLZ	/	Klozapin (20 mg/kg/dan)
IZ+FR	21d izolacija	Fiziološki rastvor (0,9% NaCl)
IZ+FLK	21d izolacija	Fluoksetin-hidrohlorid (15 mg/kg/dan)
IZ+KLZ	21d izolacija	Klozapin (20 mg/kg/dan)

NS – nestresirani pacovi, FR – fiziološki rastvor, FLK – fluoksetin, KLZ – klozapin, IZ – izolovani pacovi

Između kaveza izolovanih pacova postavljane su neprozirne, kartonske pregrade kako bi se životinje lišile vizuelnog kontakta (Slika 10). Izolovane životinje su doživljavale prisustvo drugih jedinki jedino pomoću čula sluha i mirisa. U okviru nestresiranih i izolovanih pacova, pacovi su podeljeni na one tretirane fluoksetin-hidrohloridom, odnosno klozapinom (NS+FLK/NS+KLZ, odnosno IZ+FLK/IZ+KLZ).

Slika 10. ***Izolacija pacova***

Uzimajući u obzir stres koji izaziva samo apliciranje leka, u cilju pravilnog tumačenja dobijenih rezultata kontrolne grupe nestresiranih i stresiranih životinja tretirane su fiziološkim rastvorom (0,9% NaCl) (NS+FR/IZ+FR). Lekovi su ubrizgavani intraperitonealno (i.p.) svakog dana u periodu od 21 dana. Izolovanim pacovima lekovi su aplicirani uporedo sa izlaganjem stresoru. Doze fluoksetina od 15 mg/kg/dan i

klozapina od 20 mg/kg/dan izabrane su na osnovu podataka iz literature koji su pokazali da se ovakvom primenom dostiže nivo ovih lekova u serumu koji odgovaraju terapijskim nivoima izmerenim kod pacijenata (Halim i sar., 2004; Czeh i sar., 2005). Opravdanost korišćenja ovih doza je potvrđenja merenjem koncentracije fluoksetina, odnosno klozapina u serumu tretiranih pacova (Tabela 7). Koncentracije fluoksetina merene su tečnom hromatografijom kuplovanom sa masenom spektrometrijom (LC-MS) (Djordjevic i sar., 2005), dok su koncentracije klozapina određivane tečnom hromatografijom kuplovanom sa tandemskom masenom spektrometrijom (LC-MS-MS) (Song i sar., 2009).

Tabela 7. ***Koncentracije fluoksetina i klozapina izmerene u serumu tretiranih pacova***

Lek	Doza (mg/kg/dan)	Koncentracije leka u serumu (ng/mL)		Terapijski nivoi kod pacijenata (ng/mL)
Fluoksetin	15	NS+FLK	280 ± 50	100–700 (Dulawa i sar., 2004)
		IZ+FLK	230 ± 28	
Klozapin	20	NS+KLZ	103 ± 18	100–700 (Sadock i Sadock 2008)
		IZ+KLZ	123 ± 18	

NS – nestresirani pacovi, FLK – fluoksetin, IZ – izolovani pacovi, KLZ – klozapin

3.4 TESTOVI PONAŠANJA

Nestresirani i izolovani pacovi su podvrgavani testu prinudnog plivanja, testu zainteresovanosti za zaslađeni rastvor i testu zakopavanja klikera kako bi se ispitao efekat izolacije na ponašanje, odnosno da bi se potvrdila etološka validnost 21-dnevne izolacije kao modela za ispitivanje depresije. Kod izolovanih pacova detektovani su očaj, anhedonija i anksioznost. Nakon validacije modela započet je eksperiment koji je imao za cilj da utvrdi efekte fluoksetina, odnosno klozapina, kako na ponašanje, tako i na druge parametre predstavljene dalje u tekstu. Životinje svih šest eksperimentalnih grupa iz Tabele 6 su podvrgavane testu zainteresovanosti za zaslađeni rastvor i testu zakopavanja klikera s obzirom da njihova neinvazivnost omogućava korišćenje tkiva od interesa u daljim analizama. Za razliku od njih, test prinudnog plivanja sam po sebi izaziva stres koji bi uticao na rezultate dobijene biohemijskim, imunoblot, imunofluorescentnim i histopatološkim metodama.

3.4.1 Test prinudnog plivanja

Test prinudnog plivanja je test kojim se ispituje prisustvo očaja i tzv. beznadežnog ponašanja, simptoma koji su karakteristični za ponašanje nalik depresivnom kod glodara (Porsolt i sar., 1977). Testu su podvrgavani nestresirani i izolovani pacovi tretirani fiziološkim rastvorom da bi se ispitalo da li 21 dan izolacije izaziva ove simptome. Test je izvođen u providnom cilindru prečnika 20 cm i visine 50 cm, koji je napunjen vodom sa česme (24 ± 1 °C) do visine od 30 cm (Slika 11). Jedan dan pre samog testiranja pacovi su, u sklopu faze navikavanja, provodili po 15 min u vodi. Voda u cilindru je menjana posle svakog pacova. Na dan testiranja, pacovi su provodili po 5 min u vodi i njihovo ponašanje je snimano video kamerom. Na snimcima su praćeni i kvantifikovani sledeći parametri:

- Plutanje – minimalni pokreti neophodni da bi se njuška održala iznad vode;
- Penjanje/bežanje – energični, ka gore usmereni, pokreti prednjih šapa duž zida cilindra;
- Plivanje – aktivno pokretanje prednjih šapa u centru ili uz zid cilinda uključujući i ronjenje.

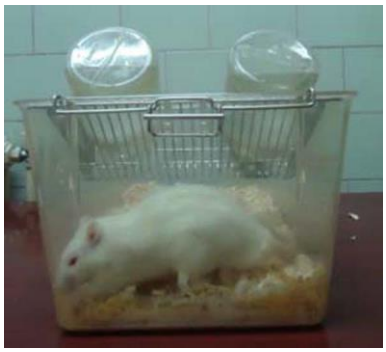
Slika 11. ***Test prinudnog plivanja***

Kvantifikaciju ispitivanih parametara su obavljala dva eksperimentatora nesvesna tretmana kom je ispitivan pacov podvrgavan. Rezultati, predstavljeni kao srednja vrednost dva merenja, izraženi su u sekundama i predstavljaju vreme tokom kog su pacovi ispoljavali nabrojane vidove ponašanja.

3.4.2 Test zainteresovanosti za zaslađeni rastvor

Test zainteresovanosti za zaslađeni rastvor meri apetit pacova za ukusnim, slatkim napitkom i često se, u modelima depresije, koristi za merenje anhedonije,

smanjenja ili odsustva uživanja (Willner i sar., 1987). Svi pacovi su testirani pre početka (0d) i na kraju (21d) odgovarajućeg tretmana. Tokom testiranja, u svakom kavezu se nalazila po jedna životinja. Dve flaše, od kojih je jedna sadržala vodu sa česme, a druga 1% rastvor saharoze, merene su i postavljane na svaki kavez (Slika 12).



Slika 12. Test zainteresovanosti za zaslađeni rastvor

Nakon sat vremena, boce su sklanjane i ponovo merene. Količina popijene tečnosti je računata kao razlika u težinama flašica (g) pre i nakon eksperimenta. Zainteresovanost za zaslađeni rastvor, kao mera hedonije kod pacova, izražena je kao procenat popijene 1% saharoze u odnosu na ukupnu popijenu tečnost.

3.4.3 Test zakopavanja klikera

Zakopavanje objekata je odbrambena reakcija glodara na neprijatan stimulus koju suzbija primena anksiolitika, pa se često koristi kao pokazatelj anksioznosti (Ho i sar., 2002). Test je sprovođen na svim pacovima pre početka (0d) i na kraju (21d) odgovarajućeg tretmana. Testirani pacovi su stavljani po jedan u odvojene kaveze sa čistom piljevinom. Po šest staklenih klikera prečnika 2,5 cm su pravilno raspoređivana po površini piljevine (Slika 13). Nakon 30 min, pacovi su sklanjani iz kaveza i brojani su zakopani klikeri, odnosno klikeri kojima su bar dve trećine prekrivene piljevinom. Eksperimentator u trenutku brojanja nije imao uvid u tretman kom je ispitivan pacov podvrgavan. Rezultati su prikazani kao srednja vrednost broja zakopanih klikera.

Slika 13. Test zakopavanja klikera

3.5 ŽRTVOVANJE ŽIVOTINJA I IZOLOVANJE TKIVA

Životinje su žrtvovane 24 sata nakon poslednje primljene doze leka. Pacovi su anestezirani kombinacijom ketamina i ksilazina (100/5 mg/kg i.p.). Krv je izolovana iz leve komore srca, ostavljana u polipropilenskim epruvetama na sobnoj temperaturi 30 min da koaguliše, nakon čega je centrifugirana 10 min na 1500 × g, na 4 °C (Sorvall GLC-3). Dobijeni serumi su čuvani na -20 °C. Po izolaciji krvi, pacovi su *in situ* perfundovani fiziološkim rastvorom. Nakon perfuzije, izolovano je tkivo jetre. Tkivo jetre koje je korišćeno u biohemijskim i imunoblot analizama zamrzavano je u tečnom azotu i čuvano na -80 °C, dok je tkivo namenjeno histopatološkim ispitivanjima fiksirano 48h u 10% formalinu. Nakon izolovanja jetre, pacovi su dekapitovani giljotinom (Harvard Apparatus, Holliston, MA, SAD) i izolovan je ceo mozak. Prečeona zona kore cerebruma i hipokampus izolovani su od ostatka mozga na ledeno hladnoj podlozi. Odmah po izolovanju, tkiva su zamrzavana u tečnom azotu i čuvana su na -80 °C do analiziranja. U slučaju uzoraka koje su namenjeni za imunofluorescentnu analizu, u celosti izolovani mozgovi su inkubirani 24h na 4 °C u 4% paraformaldehidu (PFA), pH 7,4. Nakon toga mozgovi su prebacivani u 0,4% PFA, sečeni na vibratomu, i dobijeni preseci su čuvani na -20 °C do analiziranja.

3.6 PRIPREMA CITOSOLNIH I JEDARNIH FRAKCIJA

3.6.1 Hipokampus i prečeona zona kore cerebruma

Hipokampus i prečeona zona kore cerebruma su homogenizovani u staklenom Potter-Elvehjem homogenizeru teflonskim tučkom (40 pokreta) u dve zapremine hladnog (4 ºC) pufera za homogenizaciju [15 mM Tris-HCl pH 7,9; 0,25 M saharoza; 16 mM KCl; 15 mM NaCl; 5 mM etilen-diamin tetrasirćetna kiselina (EDTA); 1 mM etilenglikol tetrasirćetna kiselina (EGTA); 1 mM ditiotreitol (DTT); 0,15 mM spermin i 0,15 mM spermidin sa dodatkom koktela proteaznih inhibitora (cOmplete™, Mini Protease Inhibitor Cocktail, Roche, Mannheim, Nemačka)]. Uzorci su centrifugirani 10 min na 2000 × *g*, 4 °C (SS-34 Sorvall centrifuga). Dobijen supernatant (S1) je korišćen za dobijanje citosolne, a talog (T1) za dobijanje jedarne frakcije.

Supernatant S1 je centrifugiran 20 min na 15000 × g, 4 °C (ultracentrifuga Beckman L8-M, Ti50i). Dobijeni supernatant (S2) je dalje centrifugiran 60 min na 100000 × g, 4 °C (ultracentrifuga Beckman L8-M, Ti50i), i dobijen supernatant je korišćen kao citosolna frakcija.

Talozi (T1) su dva puta ispirani pažljivom resuspenzijom u 4 zapremine hladnog (4 ºC) pufera za ispiranje (10 mM HEPES, pH 7,9; 1,5 mM $MgCl_2$; 10 mM KCl i koktel proteaznih inhibitora) i centrifugiranjem na 4000 × g, 4 ºC u trajanju od 10 min (Eppendorf 5417R mikrocentrifuga). Dobijeni talozi (T2) su zatim resuspendovani u jednoj zapremini pufera za lizu jedara (10 mM HEPES, pH 7,9; 0,75 mM $MgCl_2$; 0,5 M KCl; 0,5 mM EDTA; 12,5% glicerol i koktel proteaznih inhibitora), homogenizovani u staklenom homogenizeru staklenim tučkom i inkubirani na ledu 30 min na mešalici sa povremenim vorteksovanjem. Supernatant, koji predstavlja jedarni ekstrakt, dobijen je finalnim centrifugiranjem (30 min, 14000 × *g*, 4 °C, Eppendorf 5417R mikrocentrifuga).

3.6.2 Jetra

Tkivo jetre je homogenizovano u staklenom Potter-Elvehjem homogenizeru teflonskim tučkom (20 pokreta) u 4 zapremine hladnog (4 ºC) pufera za homogenizaciju jetre (50 mM Tris–HCl pH 7,4; 250 mM saharoza; 100 mM NaCl; 5 mM $MgCl_2$; 1 mM

Na_2EDTA; 1 mM EGTA; 1 mM DTT; 0,15 mM spermidin; 0,1 mM PMSF). Homogenat je zatim centrifugiran (10 min, 2000 × g, 4 ºC, SS-34 Sorvall), talog (T1) je korišćen za dobijanje jedarnih frakcija a supernatant je dalje centrifugiran 60 min na 100000 × g, 4 ºC (Beckman L8-M, Ti50) čime je dobijana citosolna frakcija. Talozi T1 su isprani tri puta u tri zapremine pufera za homogenizaciju (10 min, 2000 × g, Eppendorf 5417R mikrocentrifuga). Finalno, talozi su resuspendovani u jednoj zapremini hladnog (4 ºC) pufera za lizu jedara (10 mM Tris-HCl, pH 7,4; 10% glicerol; 0,3 M NaCl; 1,5 mM EDTA; 1 mM DTT; 0,5 mM PMSF; 0,15 mM spermin; 0,15 mM spermidin), homogenizovani u staklenom homogenizeru staklenim tučkom (1 min, 4 °C) i inkubirani na ledu 30 min na mešalici sa povremenim vorteksovanjem. Jedarna frakcija je dobijena uzimanjem supernatanta nakon finalnog centrifugiranja (30 min, 14000 × *g*, 4 °C, Eppendorf 5417R mikrocentrifuga).

3.7 SPEKTROFOTOMETRIJSKE ANALIZE

3.7.1 Određivanje koncentracije proteina

Koncentracija proteina u citosolnim i jedarnim frakcijama hipokampusa, prečeone zone kore cerebruma i jetre određivana je po Lorijevoj metodi (Lowry i sar., 1951), modifikovanoj od strane Markvela i saradnika (Markwell i sar., 1978). Goveđi albumin iz seruma (engl. bovine serum albumin, BSA) je korišćen za pravljenje standardne krive raspona koncentracija 1–10 mg/mL.

Tabela 8. Reagensi korišćeni za određivanje koncentracije proteina

Reagens A	2 % Na_2CO_3 + 0,16 % Na-K-tartarat + 1 % SDS
Reagens B	4% $CuSO_4$
Reagens C	Reagens A: Reagens B = 99 : 1
Reagens D	Folin & Ciocalteu's phenol reagens : dH_2O = 1 :1

Po 10 µL rastvora BSA/uzorka je razblaživano sa dH_2O do 900 mL nakon čega je dodavano po 100 µL 1M NaOH, mešano i inkubirano 10 min. Ceo eksperiment je izvođen na sobnoj temperaturi. Nakon toga je dodavano po 2 mL rastvora C (Tabela 8), mešano i ostavljano da stoji 15 min. Na kraju je dodavano po 0,3 mL reagensa D, mešano i inkubirano 45 min. Optička gustina (engl. *optical density*, OD) je merena na

talasnoj dužini od 750 nm (spektrofotometar S-30 Boeco, Nemačka). Koncentracije proteina u uzorcima su računate na osnovu jednačine koja opisuje standardnu pravu

$$OD_{750nm} = nagib\ prave \times koncentracija\ proteina + odsečak\ na\ apscisi$$

3.7.2 Određivanje nivoa GSH

Nivo GSH u citosolu hipokampusa, prečeone zone kore cerebruma i jetre određivan je Elmanovom metodom (Ellman, 1959), modifikovanom od strane Hisina i Hilfa (Hissin i Hilf, 1976). Esej se zasniva na redukciji 5,5-ditio-bis-nitrobenzoeve kiseline (DTNB) pomoću GSH, i formiranju 2-nitro-5-merkaptobenzoeve kiseline jake žute boje. U reakcionu smešu (10 mM Tris-HCl pH 8,2; 1 mM EDTA, 50 mM DTNB, 25% metanol) je dodavano po 20 µL dH_2O (blank), standarda ili uzorka. Po dodavanju, uzorci su centrifugirani na 3000 × g (Eppendorf 5417R mikrocentrifuga), 5 min, na sobnoj temperaturi. OD supernatanta je merena na 405 nm (spektrofotometar S-30 Boeco, Nemačka). Koncentracija GSH u uzorcima je računata na osnovu jednačine standardne krive koja je dobijana pomoću serije rastvora poznatih koncentracija GSH u rasponu 0,25–2 mM. Finalna koncentracija GSH izražena je kao nmol/mg ukupnih proteina.

3.7.3 Određivanje aktivnosti enzima GPx i GLR

Aktivnosti enzima GPx i GLR su merene u citosolu hipokampusa i prečeone zone kore cerebruma. GPx aktivnost merena je Ransel kitom (kat. br. RS504, Riox Laboratories, Crumlin, UK), po uputstvima proizvođača. Protokol je baziran na metodi Palje i Valentina (Paglia i Valentine, 1967) po kojoj se meri pad OD na 340 nm. Naime, GPx aktivnost se meri posredno, preko GLR enzima koji katalizuje redukciju GS-SG, trošeći pritom NADPH što uzrokuje pad OD_{340nm}. Što je veća GPx aktivnost, to je viši nivo GS-SG pa samim tim i pad OD koji se meri. GLR aktivnost je određivana metodom po Halivelu i Fojeru (Halliwell i Foyer, 1978), merenjem opadanja OD_{340nm}, uz male izmene radi dobijanja linearne zavisnosti OD od vremena. Reakciona smeša sadržala je: 100 µL 0,5 M fosfatnog pufera koji sadrži 1 mM EDTA (pH 7,8); 25 µL uzorka; 10 µL 10 mM NADPH u 50 mM fosfatnom puferu koji sadrži 1 mM EDTA (pH 7,8); 20 µL 10 mM GS-SG u 10 mM fosfatnom puferu i dH_2O do zapremine od 1

mL. GS-SG je dodavan na kraju i to 2 min nakon ostalih komponenti da bi se nespecifična oksidacija NADPH odvila pre početka snimanja. Nakon dodavanja GS-SG, OD je očitavana svakih 30s tokom 5 min (spektrofotometar S-30 Boeco, Nemačka). Aktivnosti oba enzima su računate na osnovu Lamber-Berovog zakona koristeći sledeću jednačinu:

$$\text{Aktivnost enzima}\left(\frac{\text{U}}{\text{l}}\right) = \frac{(\Delta A_{\text{uzorak}} - \Delta A_{\text{blank}})}{\text{minuti}} \times \frac{V_{ukupno}}{V_{uzorak} \times 6{,}22}$$

A – apsorbancija; V – zapremina; blank – umesto uzorka dodata dH_2O; 6,22 predstavlja molarni ekstinkcioni koeficijent NADPH izražen u $mM^{-1}cm^{-1}$

Izračunate vrednosti su deljene koncentracijama proteina tako da su na kraju aktivnosti enzima izražene kao mU/mg ukupnih proteina. Inače, jedan U predstavlja količinu enzima koja katalizuje oksidaciju 1 µmol NADPH u minutu.

3.7.4 Određivanje aktivnosti enzima GST

GST aktivnost je merena u citosolnoj frakciji jetre na osnovu sposobnosti ovog enzima da katalizuje reakciju konjugacije GSH sa 1-hloro-2,4-dinitrobenzenom (CDNB) čiji je produkt konjugat GS-DNB (Habig i sar., 1974). Produkcija GS-DNB dovodi do povećanja OD_{340nm} koje je direktno proporcionalno aktivnosti enzima GST. Reakciona smeša je sadržala 490 µL fosfatnog pufera pH 6,5; 5 µL 100 mM CDNB i 5 µL 100 mM GSH. Po 450 µL ove smeše je inkubirano u kiveti 5 min na 25 °C. Nakon toga je dodavano po 50 µL uzorka (fosfatni pufer u slučaju blanka) i merena je OD_{340nm} na svakih 30s u roku od 5 min. Aktivnost enzima je računata na osnovu formule prikazane u prethodnom odeljku uz korišćenje molarnog ekstinkcionog koeficijenta GS-DNB (9,6 $mM^{-1}cm^{-1}$) i izražena je u µmol/min/mg ukupnih proteina.

3.7.5 Određivanje nivoa NO metabolita (NO_x^-)

NO je veoma reaktivan molekul pa se njegov nivo najčešće prati indirektno, merenjem nivoa njegovih metabolita nitrata (NO_3^-) i nitrita (NO_2^-). Nivo NO_x^- metabolita mereni su u citosolu jetre, pri čemu su prvo NO_3^- prevođeni u NO_2^- u prisustvu kadmijuma (Cortas i Wakid, 1990). Kako bi se uspostavila odgovarajuća katalitička površina, granule kadmijuma su inkubirane 3 min, uz mešanje u rastvoru za

aktivaciju (5 mM $CuSO_4$; 200 mM Na-glicin pufer, pH 9,7). Nivo NO_2^- je određivan kolorimetrijskim esejem, uz pomoć Grisovog reagensa [1% sulfanilamid; 2,5% H_3PO_4; 0,1% N-(1-naftil) etilendiamin dihidrohlorid (NED)], u čijem prisustvu dolazi do formiranja azo jedinjenja koja rastvoru daju ljubičastu boju (Navarro-Gonzálvez i sar., 1998). Nakon 15 min inkubiranja sa kadmijumom, uzorci su mešani sa reagensom A (0,1% NED u dejonizovanoj vodi) i reagensom B (1% sulfanilamid u 2,5% H_3PO_4] u odnosu 2:1:1. Reakciona smeša je inkubirana 15 min uz mešanje, u mraku, a potom je merena OD na 550 nm na ELISA čitaču (WALLAC 1420 - Victor^2 Multilabel Counter, LKB, UK). Koncentracije NO_2^- u uzorcima su preračunavane na osnovu jednačine standardne krive koja je konstruisana merenjem OD_{550} serije rastvora $NaNO_2$ poznatih koncentracija opsega 0,5–10 μM. Finalna koncentarcija nitrita izražena je kao nmol/mg ukupnih proteina.

3.7.6 Merenje oksidativnih oštećenja proteina

Stepen oksidativnih oštećenja proteina meren je u jetri određivanjem količine karbonilnih grupa (Levine i sar., 1994). Metoda se zasniva na reakciji karbonilnih grupa i 2,4-dinitrofenilhidrazina (DNPH) i formiranja odgovarajućeg hidrazona koji može biti analiziran spektrofotometrijski. Uzorci su razblaživani do iste koncentracije proteina (5 mg/mL). Zatim je uzorak, u odnosu 1:1, mešan sa 10 mM DNPH, rastvorenim u 2 M HCl i inkubiran je 1h u mraku, na sobnoj temperaturi uz vorteksovanje na svakih 10 min. Za svaki uzorak je pravljen blank koji se sastojao od uzorka i 2 M HCl. Nakon inkubiranja dodavana je trihlorsirćetna kiselina (TCA), do finalne koncentracije 10% i reakciona smeša je inkubirana 10 min na 4 °C. Smeša je zatim centrifugirana 15 min na 11000 × g, 4 °C. Talog dobijen centrifugiranjem je ispiran 3 puta sa 5 mL smeše etanol/etilacetat (1:1) da bi se uklonio slobodan DNPH (15 min na 11000 × g, 4 °C). Nakon finalnog ispiranja talozi sa proteinima su rastvarani u po 0,6 mL 6 M guanidin hidrohlorida i 20 mM KH_2PO_4, pH 2,3 i inkubirani su 60 min na 37 °C. Smeša je potom centrifugirana (3 min na 3000 × g) i merena je OD_{380nm} supernatanta. Količina karbonilnih grupa proteina je računata na osnovu formule:

$$\text{Količina karbonilnih grupa proteina } \left(\frac{nmol}{mg}\right) = \frac{(A_{uzorak} - A_{blank}) \times V_{ukupno}}{V_{uzorak} \times 22}$$

A – apsorbancija; V – zapremina; 22 predstavlja molarni ekstinkcioni koeficijent odgovarajućeg hidrazona, izražen u $mM^{-1}cm^{-1}$

3.7.7 Merenje oksidativnih oštećenja lipida

Malondialdehid (MDA) je proizvod peroksidacije lipida i predstavlja najčešće korišćen biohemijski marker oksidativnih oštećenja ovih makromolekula. Nivo MDA je određivan u ukupnom ćelijskom ekstraktu jetre pomoću reakcije sa tiobarbiturnom kiselinom (TBA) (Albro i sar., 1986). Po 50 µL uzorka, pomešanih sa 950 µL 50 mM Tris-HCl, pH 7,4 i 2 mL TBA reagensa (15% TCA; 0,375% TBA; 0,25 M HCl) inkubirano je u vodenom kupatilu 60 min na 95 °C. Nakon hlađenja, uzorci su centrifugirani 10 min na 3000 × g. Merena je OD_{535nm} supernatanta i nivo MDA je određivan na osnovu standardne krive koja je konstruisana pomoću rastvora poznate koncentracije MDA (Sigma Aldrich). Rezultati su predstavljeni kao nmol/mg ukupnih proteina.

3.8 ELEKTROFORETSKO RAZDVAJANJE PROTEINA I WESTERN BLOT ANALIZA

Proteini citosolne i jedarne frakcije hipokampusa, prečeone zone kore cerebruma i jetre su razdvajani po molekulskoj masi SDS-poliakrilamidnom gel elektroforezom (SDS-PAGE). Nakon razdvajanja, proteini su prebacivani na membranu na kojoj je vršena imunološka detekcija proteina od interesa i njihova relativna kvantifikacija.

3.8.1 SDS-PAGE

Uzorci su prvo razblaživani do iste koncentracije proteina, a zatim su, u odnosu 4:1, mešani sa puferom za pripremu uzoraka proteina za SDS-PAGE (5 × *Laemmli Sample Buffer*, LSB: 200 mM Tris-HCl, pH 6,8; 42,5% glicerol; 25% β-merkaptoetanol; 10% SDS; 0,05% bromfenol plavo). Pre nanošenja na SDS-poliakrilamidni gel uzorci su inkubirani 5 min na 90 ˚C nakog čega su ostavljani da se ohlade do sobne temperature. Proteini su koncentrovani na 5%, a razdvajani na 10% ili 12% poliakrilamidnom denaturišućem gelu (Tabela 9), pomoću *Mini-Protean II*

Electrophoresis Cell sistema (Bio-Rad, SAD) napunjenog puferom za elektroforezu (25 mM Tris; 192 mM glicin; 0,1% SDS, pH~8.3).

Tabela 9. ***Sastav gelova za koncentrovanje i razdvajanje proteina***

Gel za koncentrovanje	Gel za razdvajanje
125 mM Tris pH 6,8 5% Akrilamid/bisakrilamid (30:1) 0,1% SDS 0,1% APS 0,1% TEMED	375 mM Tris pH 8,8 10 ili 12% Akrilamid/bisakrilamid (30:1) 0,1% SDS 0,1% APS 0,06% TEMED

SDS - natrijum dodecil-sulfat, APS - amonijum-persulfat, TEMED - tetrametiletilendiamin

Na gel je nanošena ista količina ukupnih proteina za svaki uzorak (30-60 µg). Na svaki gel nanošen je i standard za molekulske mase proteina (*Pierce Prestained protein Molecular Weight Marker*, Thermo Scientific, SAD). Proteini su razdvajani elektroforezom pri konstantnom naponu od 120 V u trajanju od 90 min, na sobnoj temperaturi.

3.8.2 Prenos proteina sa poliakrilamidnog gela na membranu

Nakon završene elektroforeze, vršen je prenos razdvojenih proteina sa gela na poliviniliden difluorid (PVDF) membranu (BioRad, Corporation, SAD). PVDF membrana je aktivirana u metanolu u trajanju od 1 min, a zatim je prebacivana u pufer za transfer (25 mM Tris; 192 mM glicin; 20% metanol pH~8,3). Formiran je sendvič koji se sastojao, redom, od sunđera, filter papira (Mini Trans-Blot®, Bio Rad), gela, membrane, filter papira i sunđera. Sendvič je postavljan u uređaj za vlažni transfer (Trans-Blott Cell, Bio Rad) i prelivan puferom za transfer. Prenos proteina sa gela na membranu odvijan je pri konstantnoj struji od 130 mA, na 4 °C u trajanju od 2 sata.

3.8.3 Western blot analiza

Nakon prenosa proteina, membrane su inkubirane 1h u puferu za blokiranje (5% nemasno mleko u prahu rastvoreno u TBS-T puferu: 20 mM Tris-HCl pH 7,4; 140 mM NaCl; 0,1% Tween-20), na sobnoj temperaturi uz blago mešanje. Membrana je zatim, na osnovu standarda, poprečno sečena na određenim molekulskim masama i isečci

membrana su inkubirani preko noći, na 4 °C sa odgovarajućim primarnim antitelima (Tabela 10).

Tabela 10. ***Primarna antitela korišćena u imunoblot analizi***

Antitelo	Kataloški broj	Proizvođač	Razblaženje
anti-GPx	sc-30147	Santa Cruz Biotechnology, SAD	1:2000
anti-GLR	sc-32886	Santa Cruz Biotechnology, SAD	1:2000
anti-NF-κB-p65	sc-372	Santa Cruz Biotechnology, SAD	1:1000
anti-COX-2	sc-1747	Santa Cruz Biotechnology, SAD	1:1000
anti-IL-1β	AB1832P	Merck Millipore, Nemačka	1:500
anti-TNF-α	AB1837P	Merck Millipore, Nemačka	1:500
anti-CuZnSOD	SOD-100	Stressgene Biotechnologies, Kanada	1:4000
anti-β-aktin	sc-1616-R	Santa Cruz Biotechnology, SAD	1:1000

Sva navedena antitela su zečja, poliklonska antitela

Membrane su zatim ispirane TBS-T puferom (3 puta po 15 min) a potom inkubirane 2h na sobnoj temperaturi, uz mešanje sa sekundarnim anti-zečijim antitelom konjugovanim sa peroksidazom rena (engl. *horseradish peroxidase*, HRP) (sc-2004, Santa Cruz Biotechnology, SAD). Membrane su posle toga opet ispirane u TBS-T puferu (3 puta po 15 min). Kao kontrola ujednačenosti prilikom nalivanja uzoraka u bunariće gela korišćen je β-aktin. Proteini od interesa detektovani su metodom hemiluminiscence primenom luminola i rastvora H_2O_2 u odnosu 1:1 (engl. *Immobilon Western Chemiluminescent HRP substrate,* Merc Millipore, SAD). U prisustvu HRP enzima i H_2O_2 dolazi do oksidacije luminola i oslobađanja svetlosne energije koja je detektovana na aparatu Chemidoc-MP System (Bio-Rad) ili pomoću filma (ORTHO CP-GU X-ray film, AGFA, Belgija), pri čemu je dužina ekspozicije varirala od 1 do 10 min u zavisnosti od proteina. Signali snimljeni na Chemidoc-MP aparatu su analizirani u kompjuterskom programu Image Lab 5.0 (BioRad), dok su signali detektovani na filmu kvantifikovani u programu ImageJ. Bez obzira na način detekcije, za svaki

ispitivani protein je urađena normalizacija u odnosu na odgovarajući intenzitet trake dobijene za β-aktin. Količina proteina je izražena kao % od količine detektovane u grupi nestresiranih pacova tretiranih fiziološkim rastvorom (NS+FR, kontrola).

U nekim slučajevima, nakon detekcije signala, membrane su stripovane da bi se detektovao drugi protein sa iste membrane. Stripovanje je vršeno inkubiranjem membrane 30 min na 50 °C u puferu za stripovanje (62,5 mM Tris-HCl pH 6,8; 2% SDS i 100 mM β-merkaptoetanol). Nakon toga membrane su ispirane TBS-T puferom, tri puta po 10 min, blokirane i dalje tretirane prema opisanom protokolu.

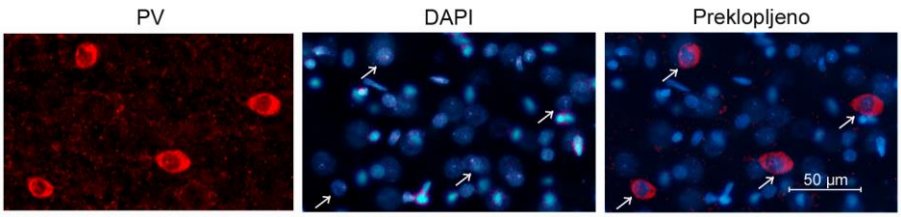
3.9 IMUNOFLUORESCENTNA ANALIZA

Broj PV+ ćelija u podregionima medijalne prečeone zone kore cerebruma analiziran je indirektnom imunofluorescencijom. Mozgovi, fiksirani u 4% PFA (poglavlje 3.5), su isečeni na koronalne preseke debljine 40 μm upotrebom vibratoma (VT 100 S; Leica, Nemačka). Preseci su čuvani na -20 °C u krioprotektivnom rastvoru (0,05 M PBS, pH 7,4; 15% glukoza; 30% etilen glikol; 0,04% natrijum-azid) do dalje upotrebe.

3.9.1 Imunofluorescentno obeležavanje PV+ ćelija

PV+ ćelije su vizualizovane na presecima mozga pacova u nivou medijalne prečeone zone kore cerebruma (udaljenost od bregme 3–3,7 mm). Slobodno plutajući (engl. *free-floating*) preseci su ispirani tri puta TBS-T puferom (20 mM Tris-HCl pH 7,4; 0,05% Triton X-100), a zatim inkubirani 1h u rastvoru za blokiranje (3% kozji serum, Vector Laboratories, Burlingame, CA, SAD, ratvoren u 20 mM Tris-HCl pH 7,4; 0,3% Triton X-100), na sobnoj temperaturi, da bi se sprečilo nespecifično obeležavanje. Nakon toga preseci su, preko noći na 4 °C, inkubirani sa poliklonskim, zečjim primarnim anti-PV antitelima (SWANT, Švajcarska, razblaženje 1:1000), pripremljenim u rastvoru za blokiranje. Preseci su zatim ispirani tri puta u TBS-T puferu i inkubirani 2h u mraku na sobnoj temperaturi sa anti-zečjim sekundarnim antitelima konjugovanim sa Alexa-Fluor 555 (razblaženje 1:1000) (Molecular Probes, Invitrogen, Eugene, SAD) u TBS sa dodatkom 3% normalnog kozjeg seruma. Nakon tri ispiranja u TBS puferu preseci su montirani na Superfrost Plus mikroskopske pločice (Thermo

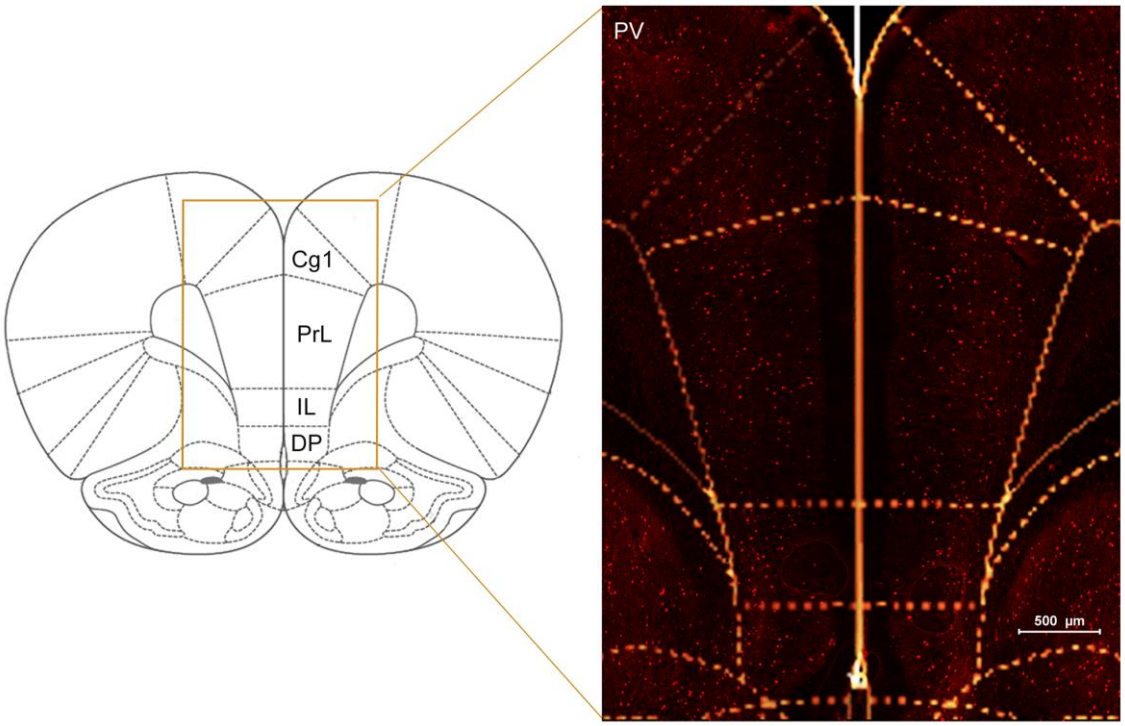
Scientific, SAD), ostavljeni da se osuše a zatim su zalivani medijumom za montiranje fluorescentnih preparata (Dako, Agilent Technologies, Danska) koji je sadržao reagens za obeležavanje jedara (4',6-diamidino-2-fenilindol, DAPI, 10 μL/mL). Bojenje jedara je potvrdilo ćelijsku prirodu signala koji je dobijen obeležavanjem PV (Slika 14). Negativna kontrola obeležavanja je tretirana po istom principu, sa izuzetkom inkubiranja u rastvoru primarnih antitela, i potvrdila je specifičnost signala dobijenih na uzorcima. Imunofluorescentno obeleženi preparati snimani su na fluorescentnom mikroskopu Leica DMI6000B, digitalnom kamerom DFC, korišćenjem programskog paketa LAS AF-TCS SP8 (Leica Microsystems). Prilikom snimanja je korišćen objektiv HC PL FLUOTAR 10x0.30 DRY.



Slika 14. ***Potvrda ćelijske prirode signala koji je dobijen nakon obeležavanja PV*** *(PV, parvalbumin – crveno, DAPI – plavo). Na preklopljenoj slici strelice označavaju PV+ ćelije sa obeleženim jedrima. Kalibraciona oznaka iznosi 50 µm.*

3.9.2 Kvantifikacija obeleženih ćelija

Za svaki moždani presek je snimljeno oko 40 serijskih mikrografija duž X i Y ose. Ove mikrografije su spojene u jednu pomoću kompjuterskog programa Image Composite Editor (Microsoft Resarch, SAD). Podregioni medijalne prečeone zone kore cerebruma su razgraničeni prema anatomskom atlasu mozga pacova (Paxinos i Watson, 2005) u kompjuterskom programu Adobe Photoshop (version CS3) (Adobe, SAD) (Slika 15).



Slika 15. ***Koronalni presek mozga pacova u nivou medijalne prečeone zone kore cerebruma.*** *Levo: šema preseka, +3,72 mm od bregme (modifikovano iz Paxinos i Watson, 2005). Cg1 – cingulatna kora 1, PrL – prelimbički podregion, IL- infralimbički podregion, DP – dorzalna pedunkularna kora. Desno: reprezentativna slika koronalnog preseka medijalne prečeone zone kore cerebruma (približno +3,72 mm od bregme) nestresiranog pacova tretiranog fiziološkim rastvorom, sa obeleženim parvalbumin pozitivnim ćelijama. Slika je dobijena spajanjem pojedinačnih snimaka napravljenih uz upotrebu objektiva 10x i podeljena je na podregione na osnovu levo prikazane šeme. Kalibraciona oznaka iznosi 500 µm.*

Ukupan broj jasno obeleženih PV+ ćelija, u svakom ispitivanom podregionu pojedinačno, je brojan u kompjuterskom programu Fiji's (ImageJ) pomoću alata za brojanje ćelija (engl. *cell counter*). Brojanje PV+ ćelija je rađeno u obe hemisfere, u duplikatu, bez uvida u tretman kome je ispitivan pacov podvrgavan. Prilikom statističke analize podataka, korišćene su srednje vrednosti dva brojanja sa obe hemisfere.

3.10 HISTOLOŠKA ANALIZA JETRE

3.10.1 Priprema tkiva i rastvora za bojenje hematoksilinom i eozinom

Isečci jetre ukalupljeni u parafin sečeni su na preseke debljine 5 μm upotrebom mikrotoma. Sastav Majerovog hematoksilina koji je korišćen za bojenje jedara prikazan je u Tabeli 11. Rastvor je kuvan 5 min na 100 °C, ohlađen i pre upotrebe profiltriran kroz Whatman papir #1. Stok rastvor alkoholnog eozina (Tabela 11) je pre upotrebe 4 × razblaživan 80% etanolom da bi se dobio radni rastvor korišćen za bojenje citoplazme.

Tabela 11. ***Sastav rastvora hematoksilina i eozina***

Majerov hematoksilin	Alkoholni eozin (stok)
1 g hematoksilina 1000 mL dH_2O 0,2 g $NaIO_3$ 50 g $AlK(SO_4)_2 \times 12\ H_2O$ ostavljeno preko noći 50 g hloral hidrata 1 g kristala limunske kiseline	1 g eozina B 20 mL dH_2O 80 mL 96% alkohola

3.10.2 Procedura bojenja

Preseci su prvo deparafinizovani ksilenom i rehidratisani potapanjem u opadajuće koncentracije etanola (100%, 96%, 70%), zatim su potapani u hematoksilin, nakon čega su ispirani nekoliko puta mlakom vodom sa česme dok jedra nisu poplavela. Nakon toga preseci su potapani u eozin. Višak eozina je uklanjan i preseci su dehidratisani potapanjem u rastuće koncentracije etanola (70%, 96%, 100%). Nakon sušenja, preseci su zalivani Eukitt (Sigma Aldrich, SAD) medijumom za montiranje. Obojeni tkivni preseci su analizirani svetlosnim mikroskopom Olympus BX50F4 i snimani digitalnom kamerom Olympus DP70. Procedura bojenja kao i analiza mikrografija u odnosu na histopatološke promene poput portne inflamacije, fokalne nekroze hepatocita i mikrovezikularnih i makrovezikularnih masnih promena, je urađena od strane patologa (Prof. dr Nada Tomanović, Institut za patologiju, Medicinski fakultet Univerziteta u Beogradu).

3.11 STATISTIČKA ANALIZA PODATAKA

Za statističku obradu podataka korišćen je kompjuterski programski paket STATISTICA Release 7. Rezultati dobijeni poređenjem ponašanja izolovanih i nestresiranih pacova analizirani su jednofaktorskom analizom varijanse (ANOVA) za ponovljena merenja [faktor stresor (nivoi: nestresirani i izolovani), ponovljeni faktor vreme (nivoi: 0d i 21d)]. Rezultati testova ponašanja rađeni na nestresiranim i izolovanim pacovima koji su tretirani lekovima su analizirani dvofaktorskim ANOVA testom za ponovljena merenja [faktori: lek (nivoi: fiziološki rastvor, fluoksetin, klozapin), stresor (nivoi: nestresirani i izolovani); ponovljeni faktor vreme (nivoi: 0d i 21d)]. Rezultati biohemijskih i imunoblot analiza, kao i rezultati imunofluorescentne analize, analizirani su dvofaktorskim ANOVA testom [faktori: lek (nivoi: fiziološki rastvor, fluoksetin, klozapin), stresor (nivoi: nestresirani i izolovani)]. Razlike između eksperimentalnih grupa analizirane su pomoću *Duncan post-hoc* testa i smatrane su statistički značajnim za vrednosti $p < 0,05$. Rezultati su predstavljeni kao srednja vrednost ± standardna greška za 5 – 6 životinja po grupi.

4 REZULTATI

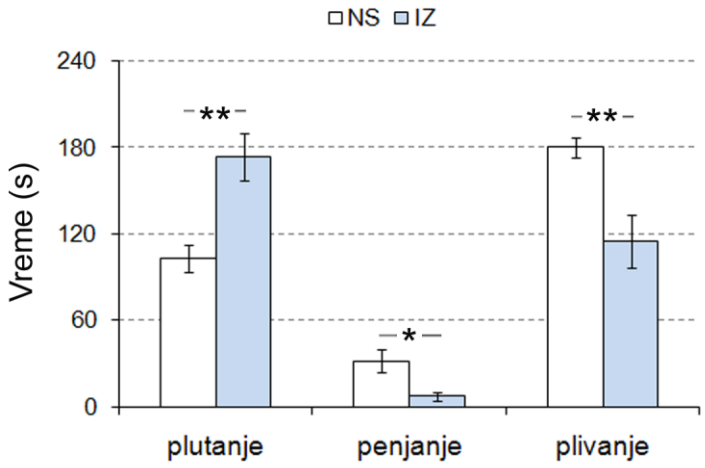
U okviru disertacije ispitivani su antioksidativni i antiinflamatorni efekti antidepresiva fluoksetina i antipshihotika klozapina na životinjskom modelu hroničnog psihosocijalnog stresa izolacije koji se koristi za ispitivanje ponašanja nalik depresivnom. Praćen je uticaj 21-dnevne izolacije na ponašanje odraslih mužjaka pacova Wistar soja, kao i GSH-zavisni antioksidativni sistem i medijatore inflamacije u hipokampusu i prečeonoj zoni kore cerebruma, moždanim strukturama posebno osetljivim na stres. Analiziran je i uticaj izolacije na broj PV+ ćelija u medijalnoj prečeonoj zoni kore cerebruma. Takođe, ispitivano je da li fluoksetin i klozapin, davani tokom hronične izolacije, ispoljavaju protektivne efekte u okviru pomenutih parametara. Budući da se biotransformacija lekova primarno odvija u jetri, ispitivana je i hepatotoksičnost primenjenih lekova praćenjem parametara oksidativnih oštećanja, antioksidativne zaštite, kao i histoloških karakteristika jetre.

4.1 REZULTATI TESTOVA PONAŠANJA

4.1.1 Efekat izolacije na ponašanje pacova

Izolovani i nestresirani pacovi su podvrgavani testovima ponašanja pre početka i na kraju eksperimentalnog protokola kako bi se ispitala mogućnost primenjenog stresora da izazove simptome ponašanja nalik depresivnom i anksioznom.

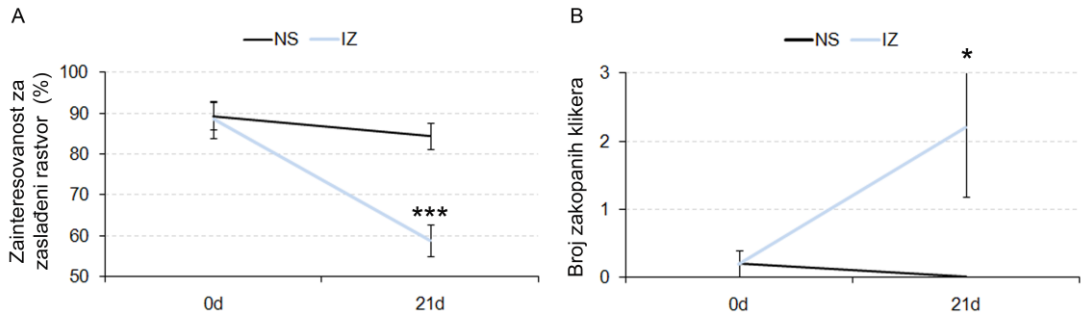
Testom prinudnog plivanja ispitivano je da li izolacija dovodi do tzv. beznadežnog ponašanja i očaja. Duži periodi plutanja, odnosno kraći periodi penjanja i plivanja, odslikavaju nevoljnost i stanje nalik očaju. Jednofaktorskim ANOVA testom pokazan je značajan efekat izolacije na sve praćenje parametre: plutanje ($F_{1.8}$ = 13,81; $p < 0,01$), penjanje ($F_{1.8}$ = 8,49; $p < 0,05$) i plivanje ($F_{1.8}$ = 10,59; $p < 0,01$). *Post-hoc* testom je pokazano da su periodi plutanja značajno duži ($p < 0,01$), dok su periodi penjanja ($p < 0,05$) i plivanja ($p < 0,01$) značajno kraći kod izolovanih u poređenju sa nestresiranim pacovima (Slika 16).



Slika 16. ***Test prinudnog plivanja:*** *ukupno trajanje perioda plutanja, penjanja i plivanja nestresiranih (NS) i izolovanih (IZ) pacova. Simboli ukazuju na značajne razlike:* $^{*}p < 0,05$ *i* $^{**}p < 0,01$*, izolovani vs. nestresirani pacovi.*

Test zainteresovanosti za zaslađeni rastvor i test zakopavanja klikera su primenjivani kako bi se ispitalo da li izolacija dovodi do ponašanja koje nalikuje anhedoniji, odnosno anksioznosti. Jednofaktorskim ANOVA testom za ponovljena merenja pokazani su značajni efekti izolacije ($F_{1.8}$ = 13,48; $p < 0,01$), vremena ($F_{1.8}$ = 18,55; $p < 0,01$), i interakcije vremena i izolacije ($F_{1.8}$ = 9,41; $p < 0,05$) na

zainteresovanost za zaslađeni rastvor. *Post-hoc* testom je pokazano da je zainteresovanost u grupi stresiranih pacova značajno manja posle 21 dana izolacije nego pre početka izolacije ($p < 0,001$) (Slika 17, A), što ukazuje na anhedoniju. U grupi nestresiranih pacova nisu uočene značajne razlike u zainteresovanosti između ove dve vremenske tačke ($p > 0,05$). Što se tiče zakopavanja klikera, pokazan je značajan efekat interakcije vremena i izolacije ($F_{1.8} = 5,76$; $p < 0,05$), kao i značajno povećanje u broju zakopanih klikera u grupi izolovanih pacova nakon 21 dana, u odnosu na početne vrednosti (0d) ($p < 0,05$), (Slika 17, B). Zakopavanje predstavlja odbrambenu reakciju pacova na strani objekat u kavezu i povećan broj zakopanih klikera u odnosu na kontrolne vrednosti ukazuje na ponašanje nalik anksioznom. Kao i u slučaju zainteresovanosti za zaslađeni rastvor, u okviru grupe nestresiranih pacova nisu primećene značajne razlike.



Slika 17. ***Zainteresovanost za zaslađeni rastvor (A) i broj zakopanih klikera (B) kod nestresiranih (NS) i izolovanih (IZ) pacova.*** *Rezultati su izraženi kao procenat popijene 1% saharoze u odnosu na ukupnu popijenu tečnost, odnosno kao broj zakopanih klikera. Simboli ukazuju na značajne razlike:* $^{***}p < 0,001$ *i* $^{*}p < 0,05$ *vrednosti za zainteresovanost za zaslađeni rastvor, odnosno broj zakopanih klikera nakon 21 dana izolacije (21d) vs. vrednosti pre početka izolacije (0d).*

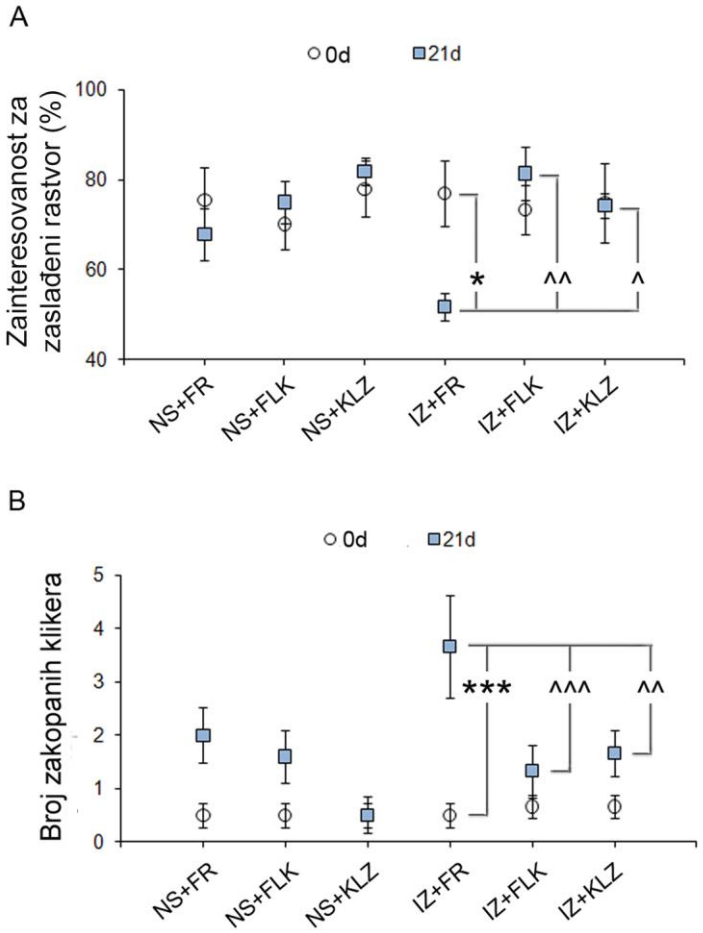
Rezultati testova ponašanja pokazuju da 21-dnevna izolacija dovodi do promena u ponašanju odraslih mužjaka pacova. Naime, izolacija izaziva simptome koji nalikuju očaju, anhedoniji i anksioznosti koji su uobičajeni za ponašanje slično depresivnom kod glodara.

4.1.2 Uticaj fluoksetina i klozapina na razvoj ponašanja nalik anhedoničnom i anksioznom izazvanih izolacijom

Efikasnost primenjenih doza lekova je, na nivou ponašanja, ispitana pomoću testa zainteresovanosti za zaslađeni rastvor i testa zakopavanja klikera. Test prinudnog plivanja sam po sebi izaziva stres koji bi uticao na rezultate drugih analiza te nije sprovođen u ovom eksperimentu. Podaci dobijeni ovim testovima su analizirani dvofaktorskim ANOVA testom za ponovljena merenja. Rezultati *post-hoc* testa su pokazali da su izolovani pacovi koji su primali fiziološki rastvor imali značajno manju zainteresovanost za zaslađeni rastvor nakon (21d), nego pre (0d) izolacije ($^{*}p < 0,05$) (Slika 18, A), što odgovara rezultatima prethodnog eksperimenta.

Kod drugih eksperimentalnih grupa nije primećena značajna razlika u zainteresovanosti između ove dve vremenske tačke. Poređenjem vrednosti izmerenih na kraju eksperimenta (21d), izolovani pacovi koji su tretirani fluoksetinom ili klozapinom pokazali su značajno veću zainteresovanost za zaslađeni rastvor u poređenju sa izolovanim pacovima koji su primali fiziološki rastvor ($^{\wedge\wedge}p = 0,01$, $^{\wedge}p < 0,05$), što ukazuje da i fluoksetin i klozapin imaju uticaj na ponašanje koje nalikuje anhedoniji.

U slučaju testa zakopavanja klikera, rezultati dvofaktorskog ANOVA testa za ponovljena merenja su pokazali značajne efekte izolacije ($F_{1.30} = 5,05$; $p < 0,05$) i lekova ($F_{2.30} = 5,49$; $p < 0,01$), kao i značajne efekte vremena ($F_{1.30} = 19,56$; $p < 0,001$) i interakcije vremena i lekova ($F_{2.30} = 3,97$; $p < 0,05$). Poređenjem vrednosti dobijenih na kraju (21d) i na početku (0d) eksperimenta u okviru svake grupe, uočena je promena samo kod izolovanih pacova tretiranih fiziološkim rastvorom, i to značajno povećanje broja zakopanih klikera nakon 21-dnevnog stresa ($^{***}p < 0,001$) (Slika 18, B). U drugim eksperimentalnim grupama nije bilo značajnih razlika u vrednostima koje su dobijene na kraju, u odnosu na one sa početka eksperimenta. Na kraju eksperimenta (21d), značajno manji broj zakopanih klikera uočen je kod izolovanih pacova koji su tretirani fluoksetinom ili klozapinom nego kod izolovanih pacova koji su primali fiziološki rastvor ($^{\wedge\wedge\wedge}p < 0,001$, $^{\wedge\wedge}p < 0,01$), što ukazuje da i fluoksetin i klozapin sprečavaju razvoj ponašanja nalik anksiznom uzrokovano izolacijom.



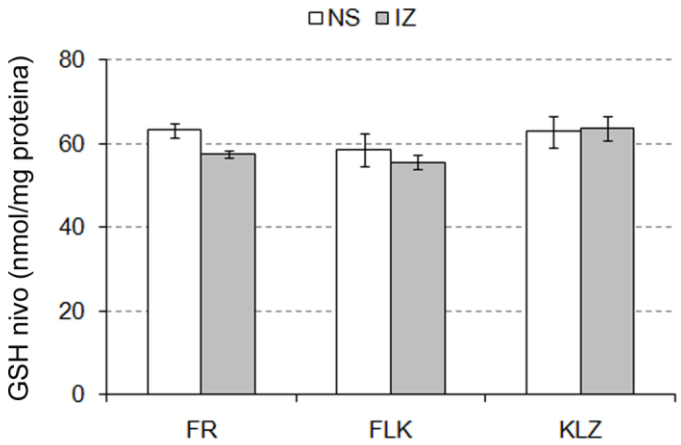
Slika 18. ***Zainteresovanost za zaslađeni rastvor (A) i broj zakopanih klikera (B) kod nestresiranih (NS) i izolovanih (IZ) pacova tretiranih fiziološkim rastvorom (FR), fluoksetinom (FLK, 15 mg/kg/dan) ili klozapinom (KLZ, 20 mg/kg/dan) na početku (0d) i na kraju (21d) eksperimenta.*** *Rezultati su izraženi kao procenat popijene 1% saharoze u odnosu na ukupnu popijenu tečnost, odnosno kao broj zakopanih klikera. Simboli ukazuju na značajne razlike:* $^{*}p < 0,05$ *i* $^{***}p < 0,001$ *IZ+FR (21d) vs. IZ+FR (0d);* $^{\wedge\wedge}p = 0,01$ *i* $^{\wedge\wedge\wedge}p < 0,001$ *IZ+FLK (21d) vs. IZ+FR (21d);* $^{\wedge}p < 0,05$ *i* $^{\wedge\wedge}p < 0,01$ *IZ+KLZ (21d) vs. IZ+FR (21d).*

Dakle, tretmani fluoksetinom (15 mg/kg/dan), odnosno klozapinom (20 mg/kg/dan), tokom 21-dnevne izolacije, sprečili su pojavu ponašanja koja nalikuju anhedoniji i anksioznosti kod odraslih mužjaka pacova.

4.2 REZULTATI BIOHEMIJSKIH I IMUNOBLOT ANALIZA HIPOKAMPUSA

4.2.1 Uticaj fluoksetina i klozapina na GSH-zavisni sistem u hipokampusu nestresiranih i izolovanih pacova

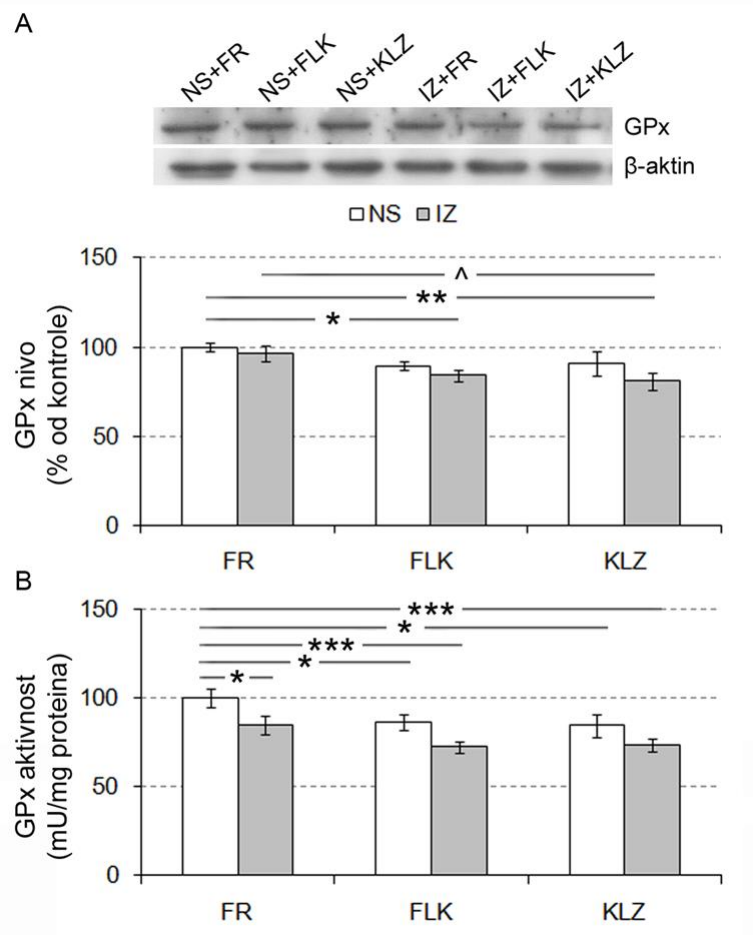
Kako bi se ispitali efekti izolacije i primenjenih lekova na GSH-zavisni sistem u hipokampusu, praćen je nivo GSH, GPx, GLR, kao i aktivnost GPx i GLR. Rezultati dvofaktorskog ANOVA testa nisu pokazali značajne razlike u nivou GSH među eksperimentalnim grupama (Slika 19).



Slika 19. ***Nivo GSH u citosolu hipokampusa nestresiranih (NS) i izolovanih (IZ) pacova tretiranih fiziološkim rastvorom (FR), fluoksetinom (FLK, 15 mg/kg/dan) ili klozapinom (KLZ, 20 mg/kg/dan).***

Rezultati dvofaktorskog ANOVA testa su pokazali značajan efekat lekova ($F_{2.29}$ = 5,66; $p < 0,01$) na nivo proteina GPx u citosolu hipokampusa. *Post-hoc* testom je utvrđeno da izolovani pacovi tretirani fluoksetinom ili klozapinom imaju značajno niži nivo ovog enzima u hipokampusu u poređenju sa nestresiranim pacovima koji su primali fiziološki rastvor ($^{*}p < 0,05$; $^{**}p < 0,01$) (Slika 20, A). Međutim, samo je kod pacova tretiranih klozapinom uočen niži nivo ovog enzima kod tretiranih izolovanih u odnosu na samo izolovane pacove ($^{\wedge}p < 0,05$). Rezultati ispitivanja GPx aktivnosti dvofaktorskim ANOVA testom su pokazali značajne efekte izolacije ($F_{1.24}$ = 13,07; p = 0,001) i lekova ($F_{2.24}$ = 5,72; $p < 0,01$). *Post-hoc* testom je pokazano da je aktivnost ovog enzima značajno niža u hipokampusu svih eksperimentalnih grupa u odnosu na

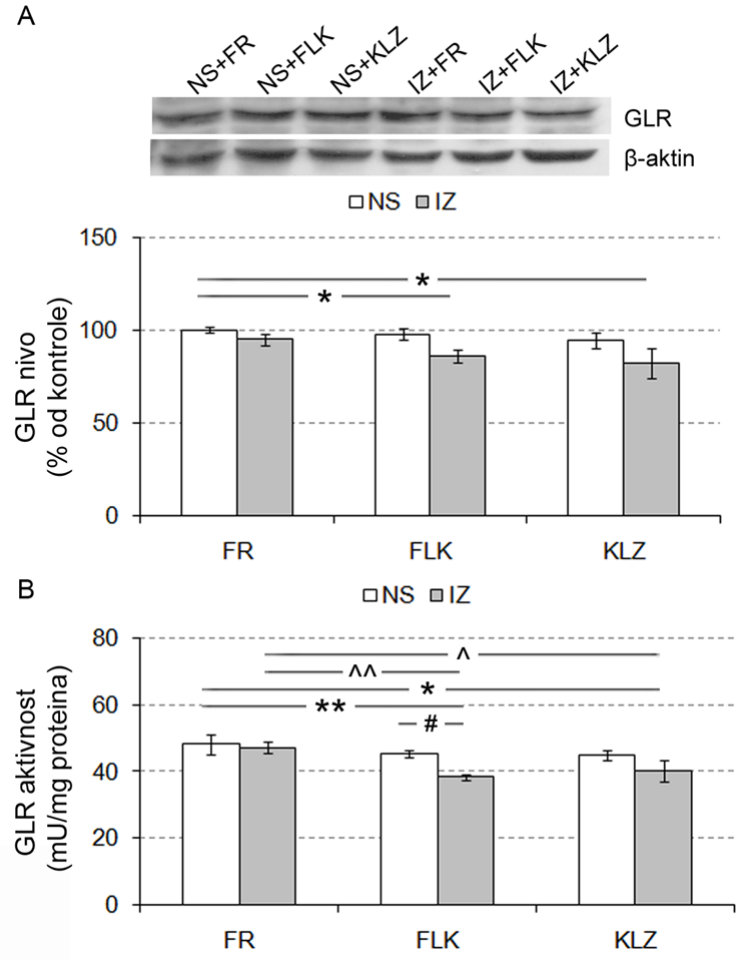
kontrolnu grupu (nestresirani pacovi tretirani fiziološkim rastvorom) ($^{*}p < 0,05$; $^{***}p < 0,001$) (Slika 20, B).



Slika 20. ***Nivo (A) i aktivnost (B) GPx enzima u citosolu hipokampusa nestresiranih (NS) i izolovanih (IZ) pacova tretiranih fiziološkim rastvorom (FR), fluoksetinom (FLK, 15 mg/kg/dan) ili klozapinom (KLZ, 20 mg/kg/dan).*** *Simboli ukazuju na značajne razlike: $^{*}p < 0,05$, $^{**}p < 0,01$ i $^{***}p < 0,001$ u odnosu na NS+FR (kontrola); $^{\wedge}p < 0,05$ u odnosu na IZ+FR.*

Rezultati dvofaktorskog ANOVA testa su pokazali značajan efekat izolacije ($F_{1.30} = 7,40$; $p < 0,05$) na nivo enzima GLR u citosolu hipokampusa. *Post-hoc* testom je pokazano da je nivo ovog enzima u hipokampusu izolovanih pacova tretiranih fluoksetinom ili klozapinom značajno niži u odnosu na nestresirane pacove ($^{*}p < 0,05$), ali ne i u odnosu na izolovane pacove tretirane fiziološkim rastvorom (Slika 21, A). Što se tiče GLR aktivnosti, uočeni su značajni efekti izolacije ($F_{1.24} = 6,93$; $p < 0,05$) i lekova ($F_{2.24} = 5,01$; $p < 0,05$). Rezultati *post-hoc* testa su pokazali da je aktivnost ovog enzima u hipokampusu izolovanih pacova tretiranih fluoksetinom ili klozapinom

značajno niža nego kod nestresiranih pacova tretiranih fiziološkim rastvorom ($^{**}p < 0,01$; $^{*}p < 0,05$) (Slika 21, B). Pored toga, značajno su niže aktivnosti u hipokampusu izolovanih pacova tretiranih fluoksetinom ili klozapinom u poređenju sa aktivnostima izmerenim u hipokampusu izolovanih jedinki tretiranih fiziološkim rastvorom ($^{\wedge\wedge}p < 0,01$; $^{\wedge}p < 0,05$), kao i kod izolovanih tretiranih fluoksetinom u poređenju sa nestresiranim pacovima tretiranim istim lekom ($^{\#}p < 0,05$).



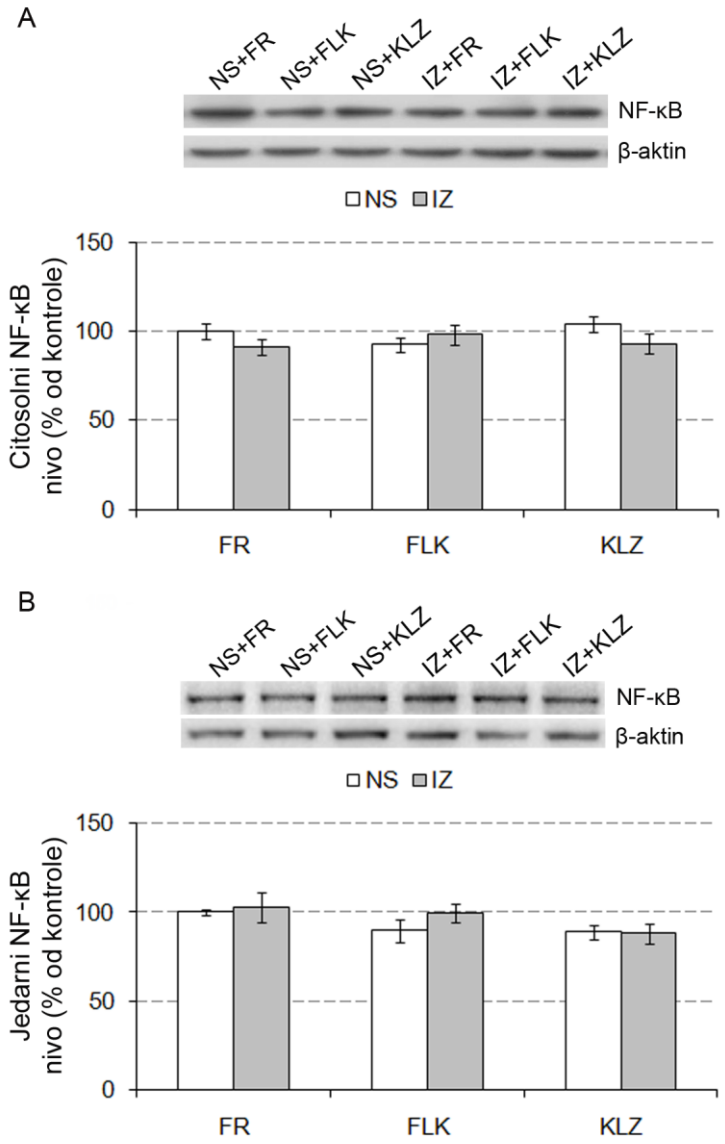
Slika 21. ***Nivo (A) i aktivnost (B) GLR enzima u citosolu hipokampusa nestresiranih (NS) i izolovanih (IZ) pacova tretiranih fiziološkim rastvorom (FR), fluoksetinom (FLK, 15 mg/kg/dan) ili klozapinom (KLZ, 20 mg/kg/dan).*** *Simboli ukazuju na značajne razlike: $^{*}p < 0,05$ i $^{**}p < 0,01$ u odnosu na NS+FR (kontrola); $^{\wedge}p < 0,05$ i $^{\wedge\wedge}p < 0,01$ u odnosu na IZ+FR; $^{\#}p < 0,05$ IZ+FLK vs. NS+FLK .*

Dakle, izolacija je značajno smanjila samo aktivnost GPx enzima u hipokampusu, a zajedničko delovanje izolacije i lekova je kompromitovalo

funkcionisanje GSH-zavisnog antioksidativnog sistema tako što je smanjilo količinu i aktivnosti enzima GPx i GLR.

4.2.2 Uticaj fluoksetina i klozapina na distribuciju NF-κB-p65 subjedinice u hipokampusu nestresiranih i izolovanih pacova

Rezultati dvofaktorskog ANOVA testa nisu pokazali značajne razlike u nivou NF-κB-p65 subjedinice ni u citosolnoj (Slika 22, A), ni u jedarnoj (Slika 22, B) frakciji hipokampusa.

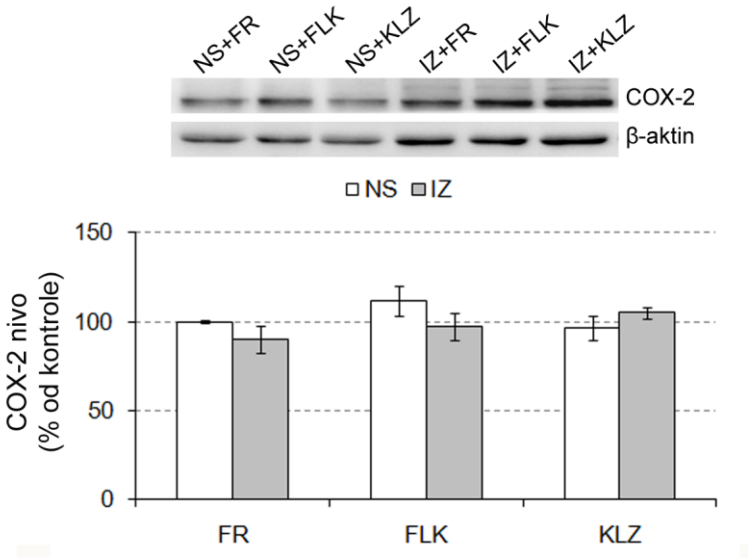


Slika 22. ***Nivo NF-κB-p65 u citosolnoj (A) i jedarnoj (B) frakciji hipokampusa nestresiranih (NS) i izolovanih (IZ) pacova tretiranih fiziološkim rastvorom (FR), fluoksetinom (FLK, 15 mg/kg/dan) ili klozapinom (KLZ, 20 mg/kg/dan); NS+FR, kontrola.***

Može se zaključiti da izolacija nije dovela do translokacije NF-κB-p65 subjedinice iz citoplazme u jedro. Takođe, tretmani ispitivanim lekovima nisu menjali nivo p65 subjedinice NF-κB kako u hipokampusu nestresiranih, tako i izolovanih pacova.

4.2.3 Uticaj fluoksetina i klozapina na medijatore inflamacije u hipokampusu nestresiranih i izolovanih pacova

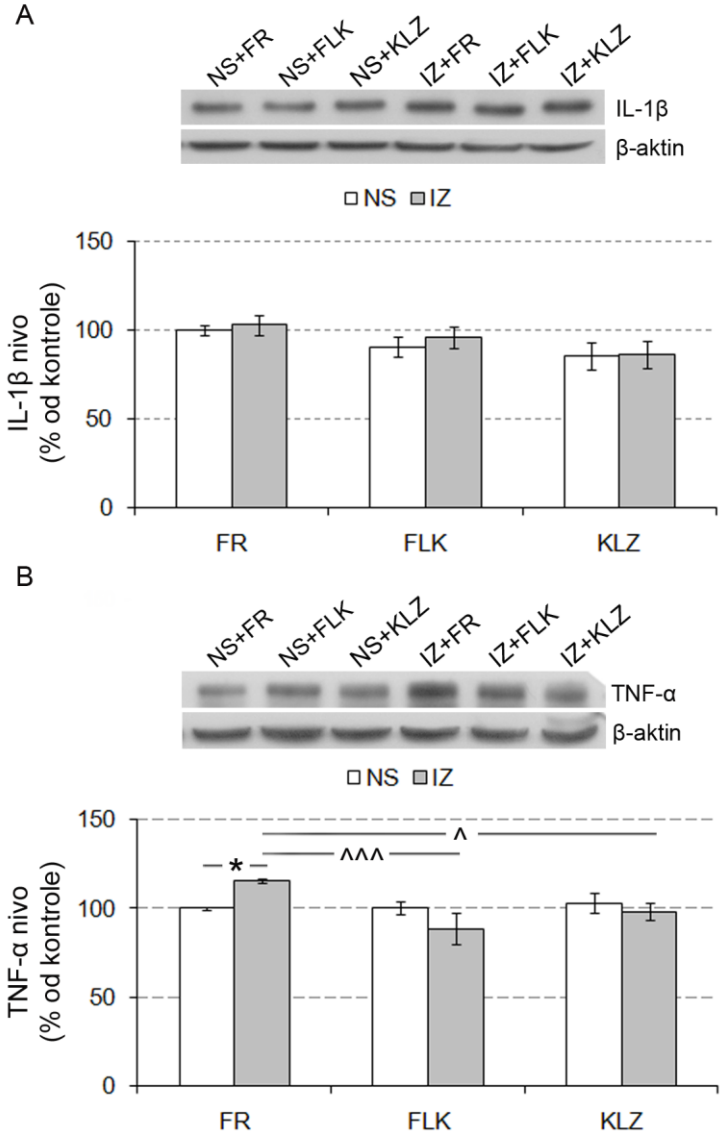
Kako bi se ispitali imunomodulatorni efekti fluoksetina i klozapina, praćen je nivo enzima COX-2 i proinflamatornih citokina IL-1β i TNF-α u citosolu hipokampusa nestresiranih i izolovanih pacova tretiranih ovim lekovima. Rezultati dvofaktorskog ANOVA testa nisu pokazali značajne razlike u nivou COX-2 (Slika 23) i IL-1β (Slika 24, A) među eksperimentalnim grupama.



Slika 23. ***Nivo COX-2 u citosolu hipokampusa nestresiranih (NS) i izolovanih (IZ) pacova tretiranih fiziološkim rastvorom (FR), fluoksetinom (FLK, 15 mg/kg/dan) ili klozapinom (KLZ, 20 mg/kg/dan); NS+FR, kontrola.***

Međutim, uočeni su značajni efekti lekova ($F_{2.24} = 3,71$; $p < 0,05$) i interakcije izolacije i lekova ($F_{2.24} = 4,51$; $p < 0,05$) na nivo TNF-α. *Post-hoc* testom je pokazano da je nivo ovog citokina značajno viši u hipokampusu izolovanih nego nestresiranih pacova ($^{*}p < 0,05$), ali da se značajno smanjuje kod izolovanih pacova tretiranih fluoksetinom ili klozapinom, u odnosu na izolovane pacove koji su primali fiziološki rastvor ($^{\wedge\wedge\wedge}p = 0,001$; $^{\wedge}p < 0,05$) (Slika 24, B). Nisu uočene značajne promene u nivoima

ispitivanih medijatora inflamacije u hipokampusu nestresiranih pacova tretiranih lekovima.



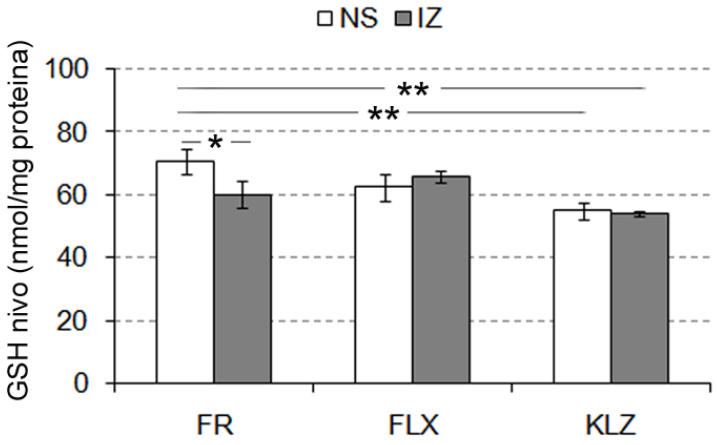
Slika 24. ***Nivo IL-1β (A) i TNF-α (B) u citosolu hipokampusa nestresiranih (NS) i izolovanih (IZ) pacova tretiranih fiziološkim rastvorom (FR), fluoksetinom (FLK, 15 mg/kg/dan) ili klozapinom (KLZ, 20 mg/kg/dan).*** *Simboli ukazuju na značajne razlike: $^{*}p < 0,05$ u odnosu na NS+FR (kontrola); $^{\wedge}p < 0,05$ i $^{\wedge\wedge\wedge}p < 0,001$ u odnosu na IZ+FR.*

Dakle, izolacija dovodi do povećanja nivoa TNF-α, ali ne i IL-1β i COX-2 u hipokampusu. Tretmani fluoksetinom i klozapinom održavaju nivo ovog citokina na kontrolnom kod izolovanih pacova.

4.3 REZULTATI BIOHEMIJSKIH I IMUNOBLOT ANALIZA PREČEONE ZONE KORE CEREBRUMA

4.3.1 Uticaj fluoksetina i klozapina na GSH-zavisni sistem u prečeonoj zoni kore cerebruma nestresiranih i izolovanih pacova

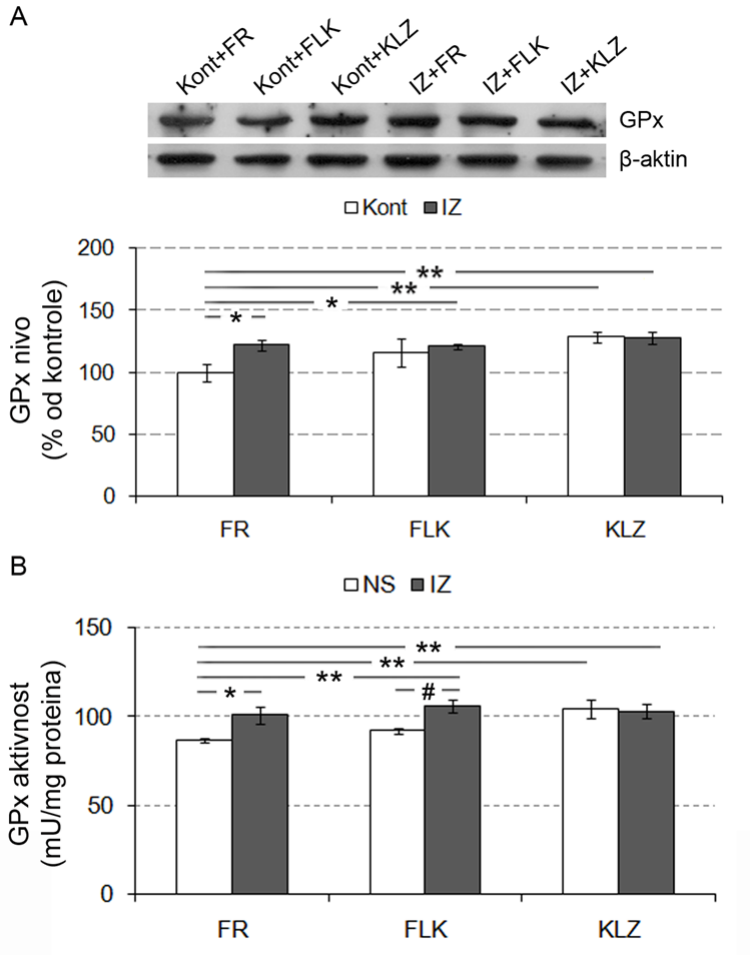
Uticaj hronične izolacije na GSH-zavisni sistem prečeone zone kore cerebruma, kao i potencijalni protektivni efekti fluoksetina, odnosno klozapina, praćeni su merenjem nivoa GSH, kao i nivoa i aktivnosti enzima GPx i GLR u citosolnoj frakciji ovog moždanog regiona. Rezultati dvofaktorskog ANOVA testa su pokazali značajan efekat lekova na nivo GSH ($F_{2.24} = 6,90$; $p < 0,01$). *Post-hoc* testom je pokazano da je nivo GSH u prečeonoj zoni kore izolovanih pacova tretiranih fiziološkim rastvorom značajno niži u odnosu na nestresirane pacove tretirane ovim rastvorom ($^{*}p < 0,05$) (Slika 25). Takođe, značajno niži nivo uočen je u prečeonoj zoni nestresiranih i izolovanih pacova tretiranih klozapinom u poređenju sa nestresiranim životinjama koje su primale fiziološki rastvor ($^{**}p < 0,01$).



Slika 25. ***Nivo GSH u citosolu prečeone zone kore cerebruma nestresiranih (NS) i izolovanih (IZ) pacova tretiranih fiziološkim rastvorom (FR), fluoksetinom (FLK, 15 mg/kg/dan) ili klozapinom (KLZ, 20 mg/kg/dan).*** *Simboli ukazuju na značajne razlike: $^{*}p < 0,05$ i $^{**}p < 0,01$ u odnosu na NS+FR (kontrola).*

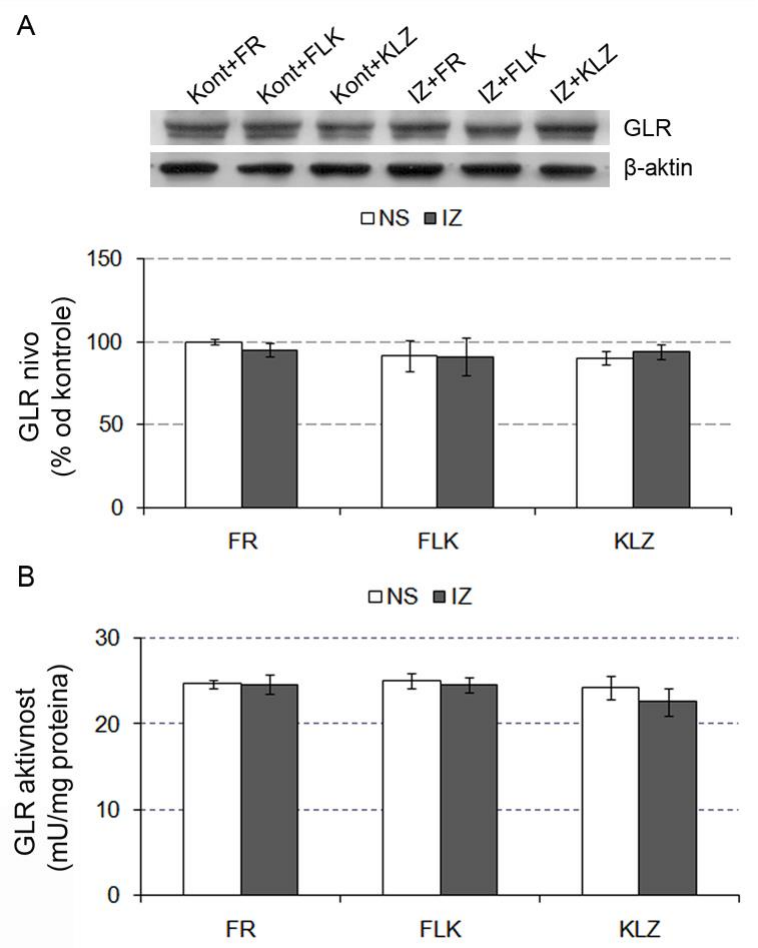
Rezultati dvofaktorskog ANOVA testa su pokazali značajan efekat lekova ($F_{2.25} = 4,78$; $p < 0,05$) na nivo GPx u citosolu prečeone zone kore cerebruma. *Post-hoc* testom je pokazano da izolacija povećava nivo GPx ($^{*}p < 0,05$), kao i da samo klozapin povećava nivo ovog enzima u citosolu prečeone zone nestresiranih pacova ($^{**}p < 0,05$)

(Slika 26, A). Takođe, u prečeonoj zoni izolovanih pacova tretiranih fluoksetinom ili klozapinom uočeno je značajno povećanje nivoa GPx u odnosu na nestresirane životinje koje su primale fiziološki rastvor ($^{*}p < 0,05$; $^{**}p \leq 0,01$), ali ne i u odnosu na samo izolovane pacove. Dvofaktorskim ANOVA testom pokazani su značajni efekti izolacije ($F_{1.27} = 8,63$; $p < 0,01$) i lekova ($F_{2.27} = 3,64$; $p < 0,05$) na aktivnost GPx. Značajno povećane aktivnosti ovog enzima, u odnosu na nestresirane pacove koje su primale fiziološki rastvor, uočeno je u prečeonoj zoni kore sve tri grupe izolovanih pacova ($^{*}p < 0,05$; $^{**}p < 0,01$), kao i nestresiranih pacova tretiranih klozapinom ($^{**}p < 0,01$). Takođe, značajne razlike su primećene između izolovanih i nestresiranih pacova tretiranih fluoksetinom ($^{\#}p < 0,05$).



Slika 26. ***Nivo (A) i aktivnost (B) GPx enzima u citosolu prečeone zone kore cerebruma nestresiranih (NS) i izolovanih (IZ) pacova tretiranih fiziološkim rastvorom (FR), fluoksetinom (FLK, 15 mg/kg/dan) ili klozapinom (KLZ, 20 mg/kg/dan).*** *Simboli ukazuju na značajne razlike: $^{*}p < 0,05$ i $^{**}p < 0,01$ u odnosu na NS+FR (kontrola); $^{\#}p < 0,05$ IZ+FLK vs. NS+FLK.*

Međutim, rezultati dvofaktorskog ANOVA testa nisu pokazali značajne razlike u nivou i aktivnosti GLR kod svih ispitivanih eksperimentalnih grupa (Slika 27, A i B).

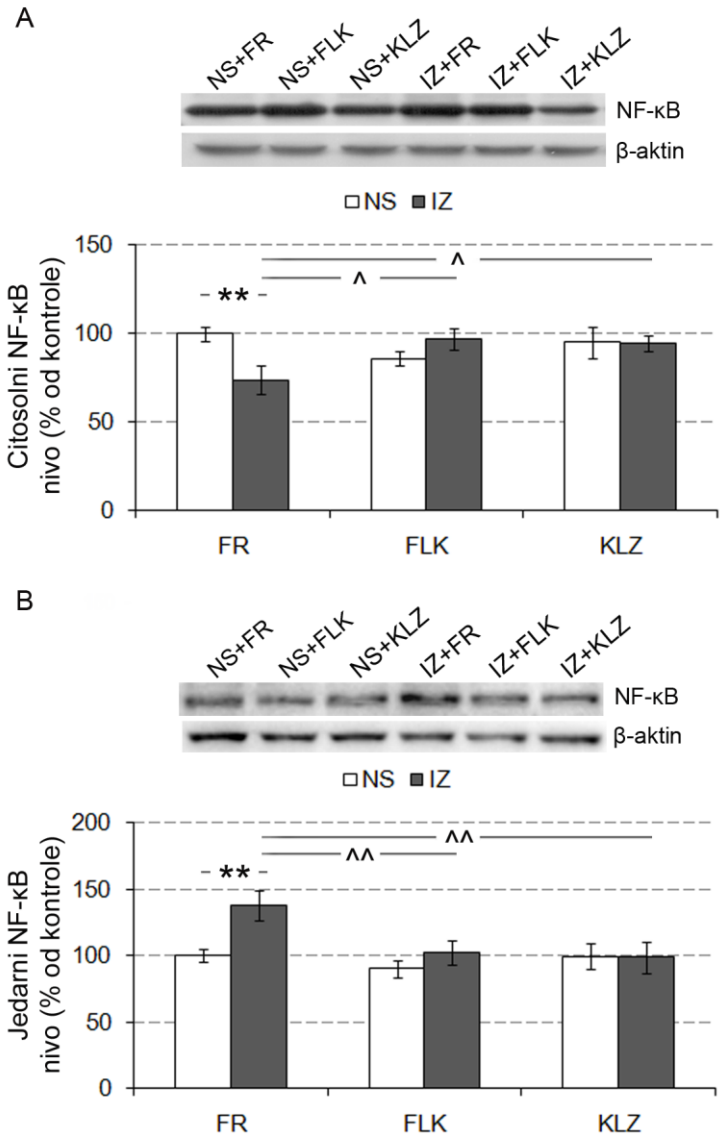


Slika 27. ***Nivo (A) i aktivnost (B) GLR enzima u citosolu prečeone zone kore cerebruma nestresiranih (NS) i izolovanih (IZ) pacova tretiranih fiziološkim rastvorom (FR), fluoksetinom (FLK, 15 mg/kg/dan) ili klozapinom (KLZ, 20 mg/kg/dan).***

Može se zaključiti da je hronična izolacija povećala antioksidativnu aktivnost posredovanu GPx enzimom i kompromitovala GSH-zavisni antioksidativni sistem u prečeonoj zoni kore cerebruma s obzirom na to da je dovela do smanjenja nivoa GSH. Tretman fluoksetinom je, kod izolovanih pacova, održao ovaj parametar na nivou zabeleženom kod kontrolnih životinja, dok klozapin nije ispoljio protektivno dejstvo, budući da se GSH nivo još više smanjio.

4.3.2 Uticaj fluoksetina i klozapina na distribuciju NF-κB-p65 subjedinice u prečeonoj zoni kore cerebruma nestresiranih i izolovanih pacova

Kako bi se ispitali efekti izolacije, odnosno fluoksetina i klozapina na aktivaciju proinflamatornog transkripcionog faktora NF-κB, praćen je nivo NF-κB-p65 subjedinice u citosolnoj i jedarnoj frakciji prečeone zone kore cerebruma. Rezultati dvofaktorskog ANOVA testa su pokazali značajan efekat interakcije izolacije i lekova na citosolni nivo NF-κB-p65 ($F_{2.29} = 4,69$; $p < 0,05$), kao i značajne efekte izolacije ($F_{1.26} = 4.71$, $p < 0,05$) i lekova ($F_{2.26} = 3.84$, $p < 0,05$) na nivo NF-κB-p65 u jedru. *Post-hoc* testom je utvrđeno da je citosolni nivo ove subjedinice značajno niži ($^{**}p = 0,01$) (Slika 28, A), a jedarni značajno viši ($^{**}p = 0,01$) (Slika 28, B) u prečeonoj zoni izolovanih u poređenju sa nestresiranim pacovima koji su primali fiziološki rastvor. Fluoksetin i klozapin su omogućili održavanje nivoa NF-κB-p65 u prečeonoj zoni kore cerebruma izolovanih pacova na nivou zabeženom kod kontrolnih pacova kako u citosolnoj ($^{\wedge}p < 0,05$), tako i u jedarnoj frakciji ($^{\wedge\wedge}p = 0,01$). U slučaju nestresiranih pacova, nisu uočene značajne promene nakon tretmana ispitivanim lekovima.

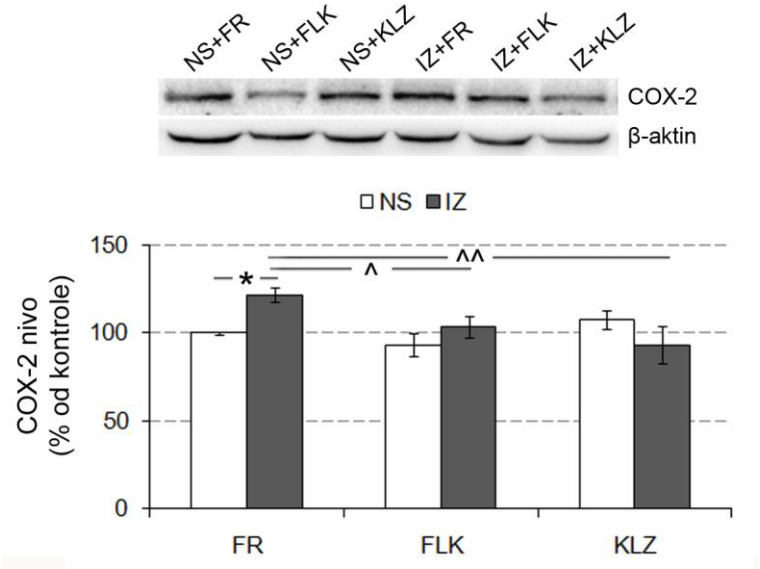


Slika 28. ***Nivo NF-κB-p65 subjedinice u citosolnoj (A) i jedarnoj (B) frakciji prečeone zone kore cerebruma nestresiranih (NS) i izolovanih (IZ) pacova tretiranih fiziološkim rastvorom (FR), fluoksetinom (FLK, 15 mg/kg/dan) ili klozapinom (KLZ, 20 mg/kg/dan).*** *Simboli ukazuju na značajne razlike:* $^{**}p < 0,01$ *u odnosu na NS+FR (kontrola);* $^{\wedge}p < 0,05$ *i* $^{\wedge\wedge}p < 0,01$ *u odnosu na IZ+FR.*

Dakle, 21-dnevna izolacija dovodi do translokacije NF-κB-p65 iz citosola u jedro, u ćelijama prečeone zone kore cerebruma. Fluoksetin i klozapin međutim onemogućavaju ovu translokaciju u prečeonoj zoni kore cerebruma izolovanih pacova.

4.3.3 Uticaj fluoksetina i klozapina na medijatore inflamacije u prečeonoj zoni kore cerebruma nestresiranih i izolovanih pacova

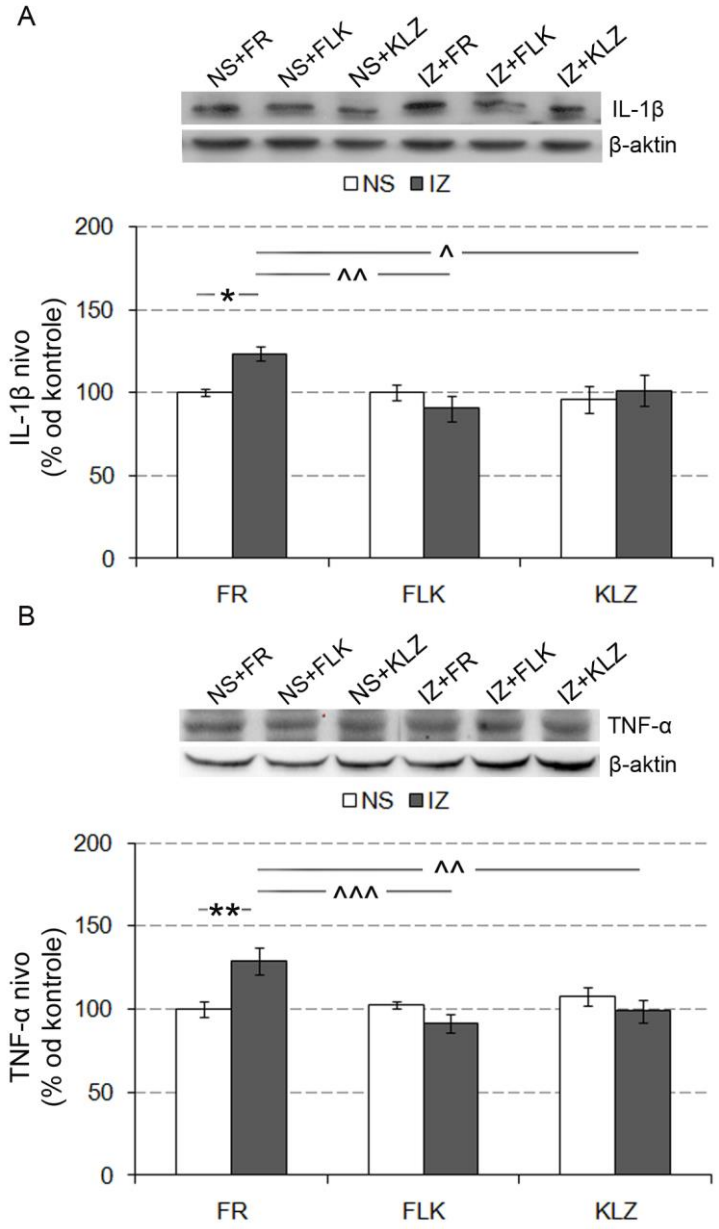
Imunomodulatorni efekti fluoksetina i klozapina ispitani su praćenjem ekspresije enzima COX-2 i citokina IL-1β i TNF-α u citosolu ćelija prečeone zone kore cerebruma nestresiranih i izolovanih pacova. Rezultati dvofaktorskog ANOVA testa su pokazali značajan efekat interakcije izolacije i lekova ($F_{2.25}$ = 4,63; $p < 0,05$) na nivo COX-2 proteina. *Post-hoc* testom je pokazano da je nivo ovog proteina značajno viši u citosolu ćelija prečeone zone kore cerebruma izolovanih u poređenju sa nestresiranim pacovima ($^{*}p < 0,05$) (Slika 29), i da tretman izolovanih pacova fluoksetinom ili klozapinom značajno smanjuje ovu vrednost u poređenju sa izolovanim pacovima koji su primali fiziološki rastvor ($^{\wedge}p < 0,05$; $^{\wedge\wedge}p<0,01$).



Slika 29. ***Nivo COX-2 u citosolu prečeone zone kore cerebruma nestresiranih (NS) i izolovanih (IZ) pacova tretiranih fiziološkim rastvorom (FR), fluoksetinom (FLK, 15 mg/kg/dan) ili klozapinom (KLZ, 20 mg/kg/dan).*** *Simboli ukazuju na značajne razlike: $^{*}p < 0,05$ u odnosu na NS+FR (kontrola); $^{\wedge}p < 0,05$ i $^{\wedge\wedge}p < 0,01$ u odnosu na IZ+FR.*

Rezultati dvofaktorskog ANOVA testa su pokazali značajan efekat lekova ($F_{2.30}$ = 3,52; $p < 0,05$) na nivo IL-1β, kao i značajne efekte lekova ($F_{2.30}$ = 4,70; $p < 0,05$) i interakcije izolacije i lekova ($F_{2.30}$ = 7,80; $p < 0,01$) na nivo TNF-α. Značajno viši nivo ovih citokina u prečeonoj zoni kore cerebruma izolovanih, u poređenju sa nestresiranim pacovima, pokazan je *post-hoc* testom ($^{*}p < 0,05$, Slika 30, A; $^{**}p < 0,01$, Slika 30, B).

Fluoksetin i klozapin značajno su snizili nivo IL-1β i TNF-α u prečeonoj zoni kore izolovanih pacova ($^{\wedge}p < 0,05$; $^{\wedge\wedge}p < 0,01$; $^{\wedge\wedge\wedge}p < 0,001$). Što se tiče nestresiranih pacova, nisu uočene značajne razlike u medijatorima inflamacije nakon tretmana ispitivanim lekovima.

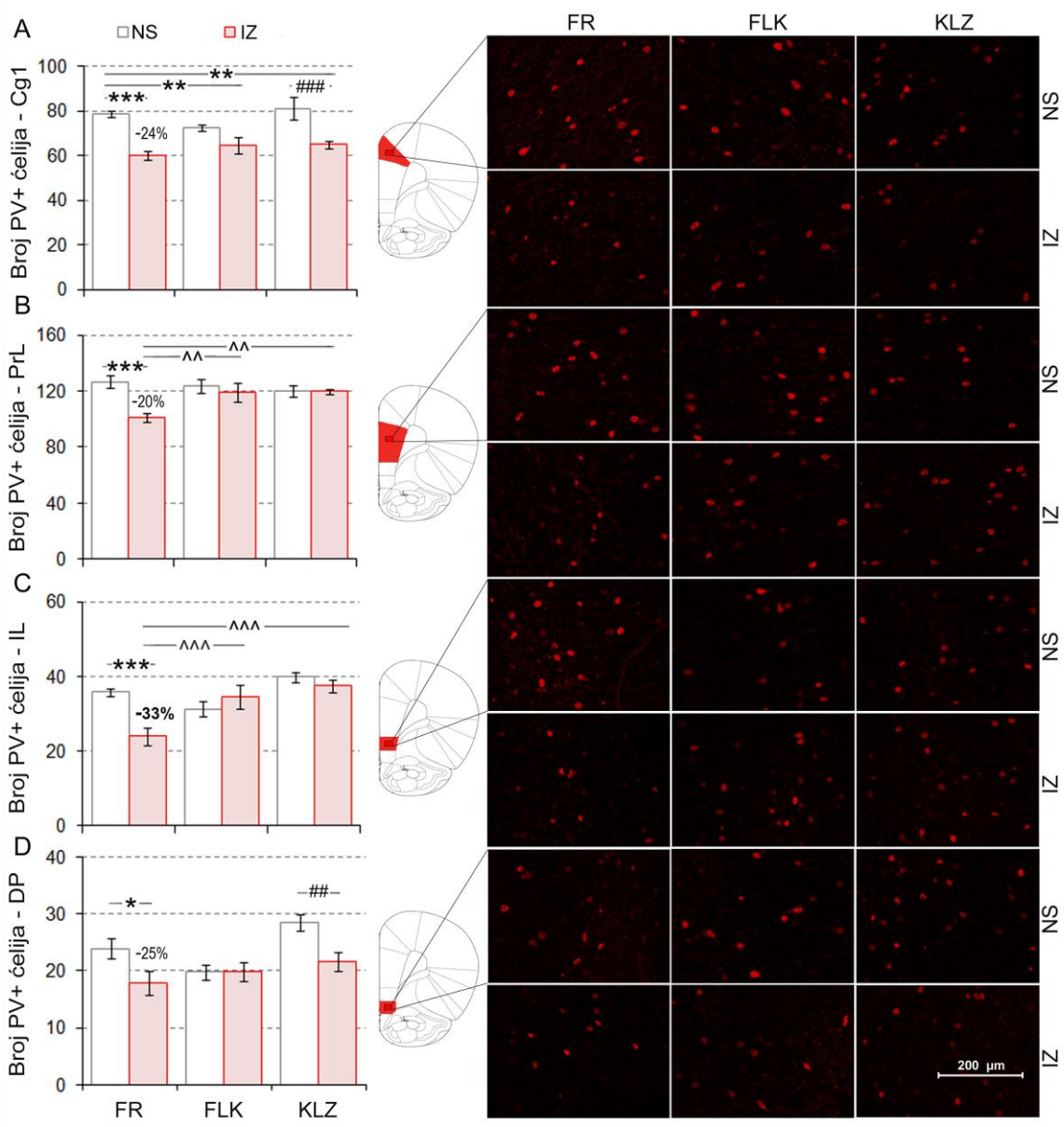


Slika 30. ***Nivo IL-1β (A) i TNF-α (B) u citosolu prečeone zone kore cerebruma nestresiranih (NS) i izolovanih (IZ) pacova tretiranih fiziološkim rastvorom (FR), fluoksetinom (FLK, 15 mg/kg/dan) ili klozapinom (KLZ, 20 mg/kg/dan).*** *Simboli ukazuju na značajne razlike: $^{*}p < 0,05$ i $^{**}p < 0,01$ u odnosu na NS+FR (kontrola); $^{\wedge}p < 0,05$; $^{\wedge\wedge}p < 0,01$ i $^{\wedge\wedge\wedge}p < 0,001$ u odnosu na IZ+FR.*

Dakle, 21-dnevna izolacija povećava nivo COX-2, IL-1β i TNF-α u prečeonoj zoni kore cerebruma, a fluoksetin, kao i klozapin, tokom stresa ispoljavaju imunomodulatorno dejstvo budući da onemogućavaju izolacijom indukovano povećanje.

4.4 UTICAJ FLUOKSETINA I KLOZAPINA NA BROJ PV+ ĆELIJA U MEDIJALNOJ PREČEONOJ ZONI KORE CEREBRUMA

Praćen je uticaj izolacije, kao i potencijalni protektivni efekti fluoksetina i klozapina, na broj PV+ ćelija u cingulatnoj kori 1 (Cg1), prelimbičkom (PrL) i infralimbičkom (IL) podregionu, kao i dorzalnoj pedunkularnoj kori (DP) prečeone zone kore cerebruma (Slika 31). Dvofaktorskim ANOVA testom utvrđen je značajan efekat izolacije na broj PV+ ćelija u Cg1 ($F_{1.27} = 35,36$; $p < 0,001$), PrL ($F_{1.29} = 8,07$; $p < 0,01$), IL ($F_{1.27} = 5,02$; $p < 0,05$) i DP podregionu ($F_{1.26} = 9,53$; $p < 0,01$). Takođe, pokazan je značajan efekat lekova na broj ovih ćelija u IL ($F_{2.27} = 10,52$; $p < 0,001$) i DP ($F_{2.26} = 5,59$; $p < 0,01$), kao i interakcije izolacije i lekova u PrL ($F_{2.29} = 4,75$; $p < 0,05$) i IL podregionu($F_{2.27} = 7,17$; $p < 0,01$). Hronična izolacija je smanjila broj PV+ ćelija u svim ispitivanim podregionima (Cg1, PrL i IL, $^{***}p < 0,001$; DP, $^{*}p < 0,05$) (Slika 31 A–D). Fluoksetin i klozapin su održali broj ćelija na nivou zabeleženom kod nestresiranih u PrL i IL podregionu kore izolovanih pacova ($^{\wedge\wedge}p < 0,01$; $^{\wedge\wedge\wedge}p \leq 0,001$), dok je u Cg1 ostao snižen ($^{**}p < 0,01$). Pokazano je i da je broj ovih ćelija značajno manji u Cg1 i DP podregionu izolovanih pacova tretiranih klozapinom u odnosu na nestresirane pacove koji su primali ovaj lek ($^{\#\#\#}p = 0,001$; $^{\#\#}p = 0,01$). Tretmani ispitivanim lekovima nisu doveli do značajnih promena u broju PV+ ćelija nestresiranih pacova.



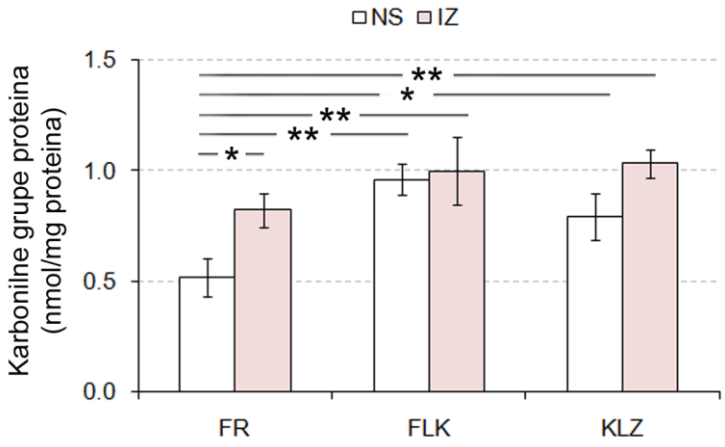
Slika 31. ***PV+ ćelije u Cg1 (A), PrL (B), IL (C) i DP (D) podregionima medijalne prečeone zone kore cerebruma nestresiranih (NS) i izolovanih (IZ) pacova tretiranih fiziološkim rastvorom (FR), fluoksetinom (FLK, 15 mg/kg/dan) ili klozapinom (KLZ, 20 mg/kg/dan).*** *Broj PV+ ćelija u ispitivanim podregionima (levo). Dijagrami sa označenim podregionima od interesa (sredina). Reprezentativne slike PV+ ćelija u odgovarajućim podregionima (desno). Simboli ukazuju na značajne razlike: $^{*}p < 0,05$; $^{**}p < 0,01$; $^{***}p < 0,001$ u odnosu na NS+FR (kontrola); $^{\wedge\wedge}p < 0,01$; $^{\wedge\wedge\wedge}p < 0,001$ u odnosu na IZ+FR; $^{\#\#}p = 0,01$; $^{\#\#\#}p = 0,001$ IZ+KLZ vs. NS+KLZ. Brojevi koji se nalaze iznad stubića IZ+FR predstavljaju procenat smanjenja broja PV+ ćelija u odnosu na kontrolu. Kalibraciona oznaka iznosi 200 µm. PV – parvalbumin, Cg1 – cingulatna kora 1, PrL – prelimbički podregion, IL- infralimbički podregion, DP – dorzalna pedunkularna kora.*

Dobijeni rezultati pokazuju da 21-dnevna izolacija dovodi do smanjenja broja PV+ ćelija u svim podregionima medijalne prečeone zone kore cerebruma. Fluoksetin i klozapin ispoljavaju protektivni efekat u PrL i IL podregionima.

4.5 REZULTATI BIOHEMIJSKIH I IMUNOBLOT ANALIZA JETRE

4.5.1 Efekat fluoksetina i klozapina na oksidativna oštećenja proteina i lipida u jetri nestresiranih i izolovanih pacova

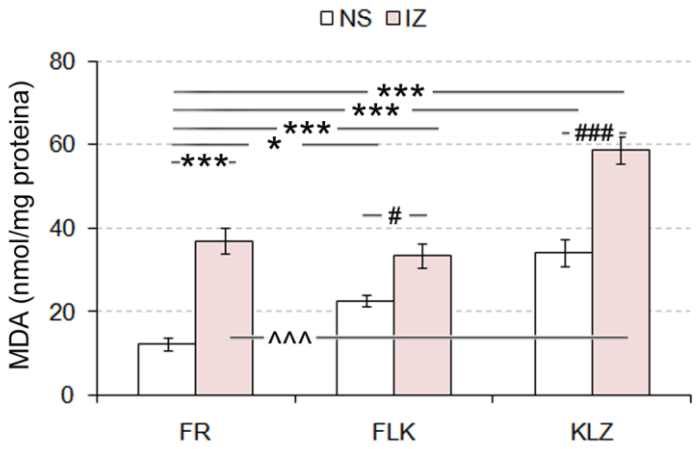
Nivo karbonilnih grupa proteina i MDA praćeni su da bi se ispitalo da li izolacija i tretmani ispitivanim lekovima dovode do oksidativnih oštećenja u jetri. Rezultati dvofaktorskog ANOVA testa su pokazali značajne efekte izolacije ($F_{1.26}$ = 6,09; $p < 0,05$) i lekova ($F_{2.26}$ = 6,06; $p < 0,01$) na nivo karbonilnih grupa proteina, kao i značajne efekte izolacije ($F_{1.30}$ = 77,41; $p < 0,001$), lekova ($F_{2.30}$ = 33,87; $p < 0,001$) i interakcije izolacije i lekova ($F_{2.30}$ = 4,33; $p < 0,05$) na nivo MDA.



Slika 32. ***Nivo karbonilnih grupa proteina u ćelijskom ekstraktu jetre nestresiranih (NS) i izolovanih (IZ) pacova tretiranih fiziološkim rastvorom (FR), fluoksetinom (FLK, 15 mg/kg/dan) ili klozapinom (KLZ, 20 mg/kg/dan).*** *Simboli ukazuju na značajne razlike: $^{*}p < 0,05$ i $^{**}p < 0,01$ u odnosu na NS+FR (kontrola).*

Izolacija je povećala kako nivo karbonilnih grupa proteina ($^{*}p < 0,05$, Slika 32), tako i MDA u jetri ($^{***}p < 0,001$, Slika 33). Fluoksetin i klozapin su povećali oba markera oksidativnih oštećenja u jetri i to kako kod nestresiranih ($^{*}p < 0,05$; $^{**}p < 0,01$; $^{***}p < 0,001$), tako i izolovanih pacova ($^{**}p < 0,01$, $^{***}p < 0,001$). Pored toga, *post-hoc*

testom je pokazano da je MDA nivo značajno viši u jetri izolovanih pacova koji su tretirani klozapinom u odnosu na one koji su primali fiziološki rastvor ($^{\wedge\wedge\wedge}p < 0,001$). Takođe, značajne razlike u MDA nivoima su uočene između izolovanih pacova tretiranih lekovima sa jedne, i odgovarajućih nestresiranih pacova tretiranih lekovima, sa druge strane ($^{\#}p < 0,05$; $^{\#\#\#}p < 0,001$).



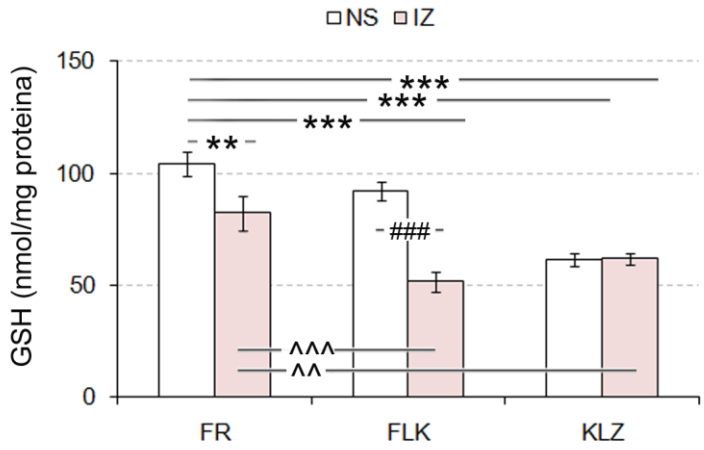
Slika 33. ***Nivo malondialdehida (MDA) u ćelijskom ekstraktu jetre nestresiranih (NS) i izolovanih (IZ) pacova tretiranih fiziološkim rastvorom (FR), fluoksetinom (FLK, 15 mg/kg/dan) ili klozapinom (KLZ, 20 mg/kg/dan).*** *Simboli ukazuju na značajne razlike:* $^{*}p < 0,05$ *i* $^{***}p < 0,001$ *u odnosu na NS+FR (kontrola);* $^{\wedge\wedge\wedge}p < 0,001$ *u odnosu na IZ+FR;* $^{\#}p < 0,05$ *IZ+FLK vs. NS+FLK,* $^{\#\#\#}p < 0,001$ *IZ+KLZ vs. NS+KLZ.*

Dakle, 21-dnevni tretmani fluoksetinom i klozapinom izazivaju oksidativna oštećenja proteina i lipida kako u jetri izolovanih, tako i nestresiranih pacova. Sama hronična izolacija takođe dovodi do ovih oštećenja.

4.5.2 Uticaj fluoksetina i klozapina na antioksidativni kapacitet u jetri nestresiranih i izolovanih pacova

Da bi se ispitao efekat lekova, samog stresora, kao i kombinovani efekat lekova i stresora, na antioksidativni sistem u jetri, praćen je nivo GSH, CuZnSOD, kao i aktivnost GST. Rezultati dvofaktorskog ANOVA testa su pokazali značajne efekte izolacije ($F_{1.30} = 29,93$; $p < 0,001$), lekova ($F_{2.30} = 23,66$; $p < 0,001$), kao i interakcije izolacije i lekova ($F_{2.30} = 10,22$; $p < 0,001$) na nivo GSH. *Post-hoc* testom je pokazano da je nivo GSH značajno niži od kontrolnog u jetri pacova svih eksperimentalnih grupa,

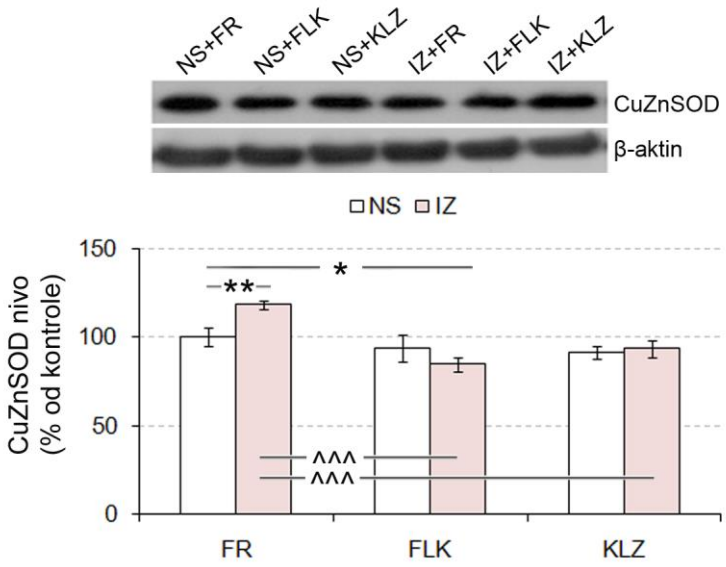
sa izuzetkom nestresiranih pacova tretiranih fluoksetinom ($^{**}p < 0,01$; $^{***}p < 0,001$) (Slika 34).



Slika 34. ***Nivo GSH u ćelijskom ekstraktu jetre nestresiranih (NS) i izolovanih (IZ) pacova tretiranih fiziološkim rastvorom (FR), fluoksetinom (FLK, 15 mg/kg/dan) ili klozapinom (KLZ, 20 mg/kg/dan).*** *Simboli ukazuju na značajne razlike: $^{**}p < 0,01$ i $^{***}p < 0,001$ u odnosu na NS+FR (kontrola); $^{\wedge\wedge}p < 0,01$ i $^{\wedge\wedge\wedge}p < 0,001$ u odnosu na IZ+FR; $^{\#\#\#}p < 0,001$ IZ+FLK vs. NS+FLK.*

Takođe, značajno niži nivo GSH je uočen u jetri izolovanih pacova tretiranih fluoksetinom ili klozapinom u odnosu na samo izolovane pacove ($^{\wedge\wedge}p < 0,01$; $^{\wedge\wedge\wedge}p < 0,001$), kao i kod izolovanih pacova tretiranih fluoksetinom u odnosu na nestresirane pacove tretirane ovim lekom ($^{\#\#\#}p < 0,001$).

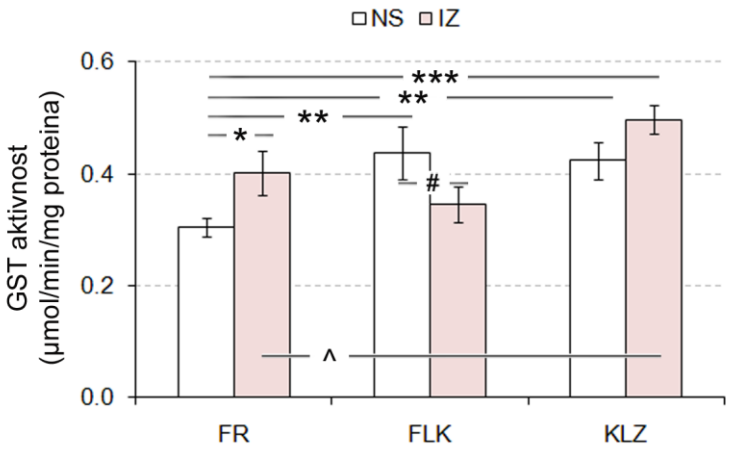
Rezultati dvofaktorskog ANOVA testa pokazali su značajne efekte lekova ($F_{2.30} = 12,59$; $p < 0,001$), i interakcije izolacije i lekova ($F_{2.30} = 5,17$; $p < 0,05$) na nivo CuZnSOD u citosolu jetre. Značajno povećanje nivoa ovog enzima uočeno je u jetri izolovanih u odnosu na nestresirane pacove ($^{**}p < 0,01$) (Slika 35).



Slika 35. ***Nivo CuZnSOD u citosolu jetre nestresiranih (NS) i izolovanih (IZ) pacova tretiranih fiziološkim rastvorom (FR), fluoksetinom (FLK, 15 mg/kg/dan) ili klozapinom (KLZ, 20 mg/kg/dan).*** *Simboli ukazuju na značajne razlike: $^{*}p < 0,05$ i $^{**}p < 0,01$ u odnosu na NS+FR (kontrola); $^{\wedge\wedge\wedge}p < 0,001$ u odnosu na IZ+FR.*

U jetri izolovanih pacova tretiranih fluoksetinom uočen je značajno niži nivo u poređenju sa nestresiranim ($^{*}p < 0,05$), i izolovanim pacovima ($^{\wedge\wedge\wedge}p < 0,001$) koji su primali fiziološki rastvor. Takođe, značajno niži nivo uočen je u jetri izolovanih pacova koji su tretirani klozapinom, u odnosu na samo izolovane pacove ($^{\wedge\wedge\wedge}p < 0,001$).

Značajni efekti lekova ($F_{2.30} = 9,50$; $p < 0,001$) i interakcije izolacije i lekova ($F_{2.30} = 7,79$; $p < 0,001$) na aktivnost GST pokazani su dvofaktorskim ANOVA testom. Izolacija je povećala aktivnost ovog enzima u citosolu jetre ($^{*}p < 0,05$). Takođe, oba leka su povećala aktivnost GST u jetri nestresiranih pacova ($^{**}p < 0,01$), međutim kod izolovanih je samo klozapin doveo do povećanja ($^{***}p < 0,001$) (Slika 36). U jetri izolovanih pacova tretiranih klozapinom, uočena je značajno viša aktivnost GST i u poređenju sa izolovanim pacovima koji su primali fiziološki rastvor ($^{\wedge}p < 0,05$). Pored toga, u jetri izolovanih pacova tretiranih fluoksetinom zabeležene su značajno niže aktivnosti ovog enzima u poređenju sa nestresiranim pacovima koji su tretirani istim lekom ($^{\#}p < 0,05$).

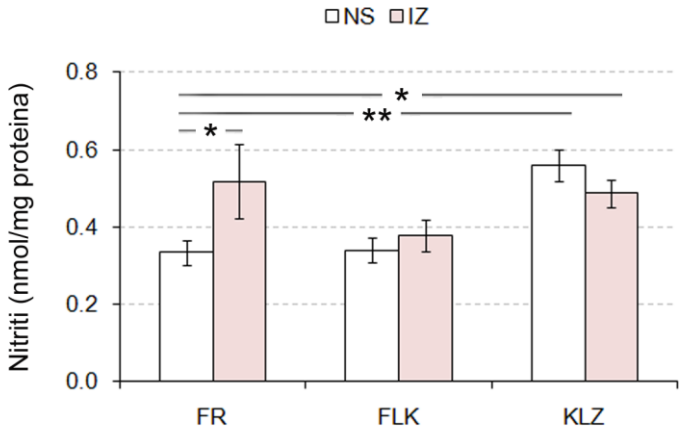


Slika 36. ***GST aktivnost u citosolu jetre nestresiranih (NS) i izolovanih (IZ) pacova tretiranih fiziološkim rastvorom (FR), fluoksetinom (FLK, 15 mg/kg/dan) ili klozapinom (KLZ, 20 mg/kg/dan).*** *Simboli ukazuju na značajne razlike: $^{*}p < 0,05$; $^{**}p < 0,01$ i $^{***}p < 0,001$ u odnosu na NS+FR (kontrola); $^{\wedge}p < 0,05$ u odnosu na IZ+FR; $^{\#}p < 0,05$ IZ+FLK vs. NS+FLK.*

Može se zaključiti da 21-dnevna izolacija i ispitivani lekovi, pre svega klozapin, pojačavaju antioksidativnu odbranu posredovanu enzimom GST i kompromituju GSH-zavisnu odbranu u jetri.

4.5.3 Uticaj fluoksetina i klozapina na nivo nitrita u jetri nestresiranih i izolovanih pacova

Nivo nitrita je meren kako bi se indirektno pratio nivo NO. Rezultati dvofaktorskog ANOVA testa su pokazali značajne efekte lekova ($F_{1.30} = 6,37$; $p < 0,01$) i interakcije izolacije i lekova ($F_{1.30} = 4,22$; $p < 0,05$) na nivo NO u citosolu jetre. Rezultati *post-hoc* testa su pokazali značajno povećanja nivoa NO nakon izolacije i tretmana klozapinom ($^{*}p < 0,05$; $^{**}p < 0,01$) (Slika 37).

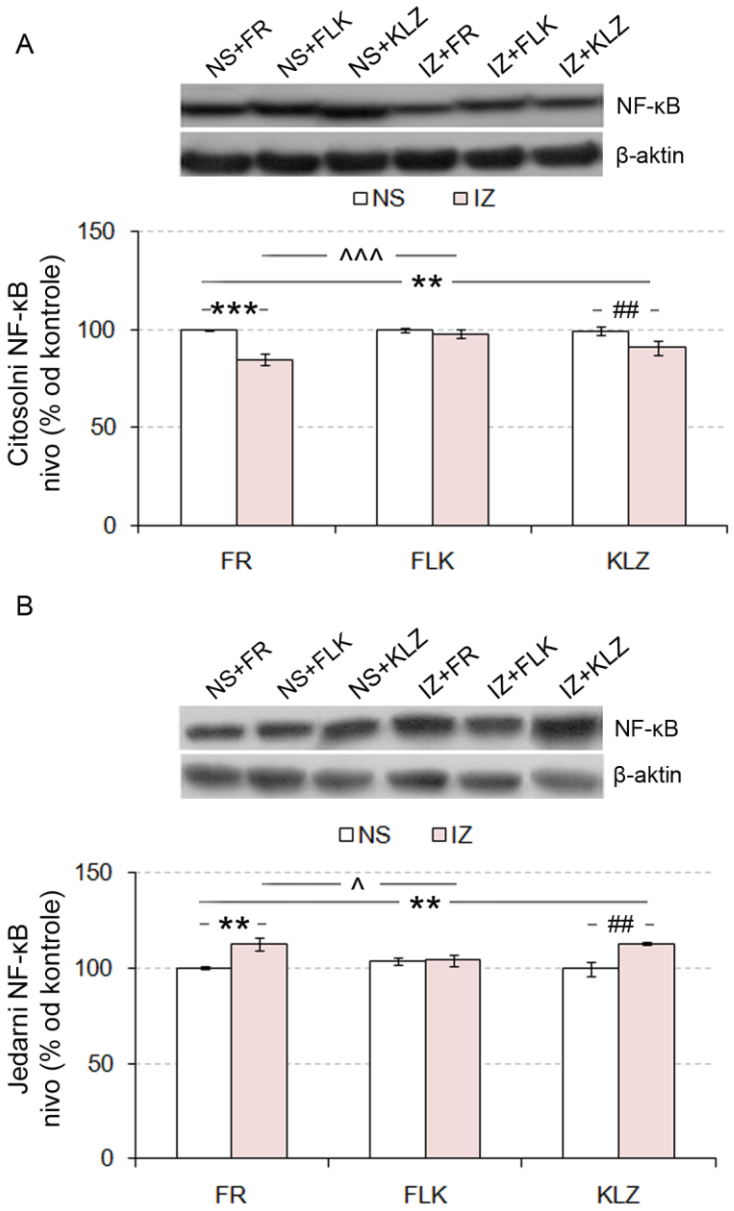


Slika 37. ***Nivo nitrita u citosolu jetre nestresiranih (NS) i izolovanih (IZ) pacova tretiranih fiziološkim rastvorom (FR), fluoksetinom (FLK, 15 mg/kg/dan) ili klozapinom (KLZ, 20 mg/kg/dan).*** *Simboli ukazuju na značajne razlike: $^{*}p < 0,05$ i $^{**}p < 0,01$ u odnosu na NS+FR (kontrola).*

Izolacija dovodi do povećane NO produkcije u jetri, sudeći po značajno višim nivoima nitrita u poređenju sa nestresiranim životinjama. Klozapin povećava nivo nitrita u jetri nestresiranih i izolovanih pacova, dok fluoksetin ne utiče na ovaj parametar kod obe grupe životinja.

4.5.4 Uticaj fluoksetina i klozapina na distribuciju NF-κB-p65 subjedinice u jetri nestresiranih i izolovanih pacova

Ispitivanjem nivoa NF-κB-p65, otkriveni su značajni uticaji izolacije ($F_{1.29}$ = 22,96; $p < 0,001$), lekova ($F_{2.29}$ = 4,72; $p < 0,05$), i interakcije izolacije i lekova ($F_{2.29}$ = 4,68; $p < 0,05$) na citosolni, kao i značajni efekti izolacije ($F_{1.24}$ = 17,21; $p < 0,001$) i interakcije izolacije i lekova ($F_{2.24}$ = 4,01; $p < 0,05$) na jedarni nivo ove subjedinice.



Slika 38. ***Nivo NF-κB-p65 subjedinice u citosolnoj (A) i jedarnoj (B) frakciji jetre nestresiranih (NS) i izolovanih (IZ) pacova tretiranih fiziološkim rastvorom (FR), fluoksetinom (FLK, 15 mg/kg/dan) ili klozapinom (KLZ, 20 mg/kg/dan).*** *Simboli ukazuju na značajne razlike:* $^{**}p < 0,01$ *i* $^{***}p < 0,001$ *u odnosu na NS+FR (kontrola);* $^{\wedge}p < 0,05$ *i* $^{\wedge\wedge\wedge}p < 0,001$ *u odnosu na IZ+FR;* $^{\#\#}p < 0,01$ *IZ+KLZ vs. NS+KLZ.*

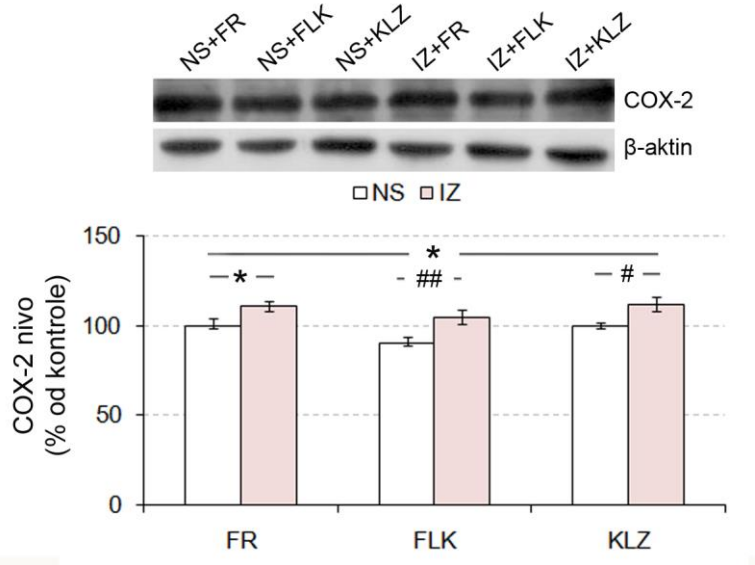
Izolacija je značajno smanjila nivo NF-κB-p65 subjedinice u citosolu ($^{***}p < 0,001$) (Slika 38, A), a povećala ga u jedru ($^{**}p < 0,01$) (Slika 38, B) ćelija jetre. Isti efekat je zabeležen u jetri izolovanih pacova tretiranih klozapinom ($^{**}p < 0,01$). U jetri izolovanih pacova tretiranih fluoksetinom uočen je značajno viši nivo NF-κB-p65 subjedinice u citosolu ($^{\wedge\wedge\wedge}p < 0,001$), i značajno niži u jedru ($^{\wedge}p < 0,05$), u poređenju sa

samo izolovanim pacovima. Takođe, u jetri izolovanih pacova tretiranih klozapinom uočen je značajno niži citosolni, i viši jedarni nivo NF-κB-p65, u odnosu na nestresirane pacove tretirane istim lekom ($^{\#\#}p < 0,01$).

Rezultati analize distribucije NF-κB-p65 subjedinice pokazuju da izolacija dovodi do njene translokacije u jedro ćelija jetre pacova. Fluoksetin, za razliku od klozapina, onemogućava translokaciju ovog transkripcionog faktora u jedro ćelija jetre izolovanih pacova.

4.5.5 Uticaj fluoksetina i klozapina na nivo COX-2 u jetri nestresiranih i izolovanih pacova

Rezultati dvofaktorskog ANOVA testa su pokazali značajan efekat izolacije ($F_{1.26} = 17,97$; $p < 0,001$) na nivo COX-2 u citosolu jetre. *Post-hoc* testom je pokazano da je nivo ovog proteina značajno viši od kontrolnog u jetri izolovanih pacova koji su primali fiziološki rastvor ili klozapin ($^{*}p < 0,05$) (Slika 39), kao i u jetri lekovima tretiranih izolovanih pacova u odnosu na odgovarajuće, lekovima tretirane nestresirane životinje ($^{\#\#}p < 0,01$; $^{\#}p < 0,05$).



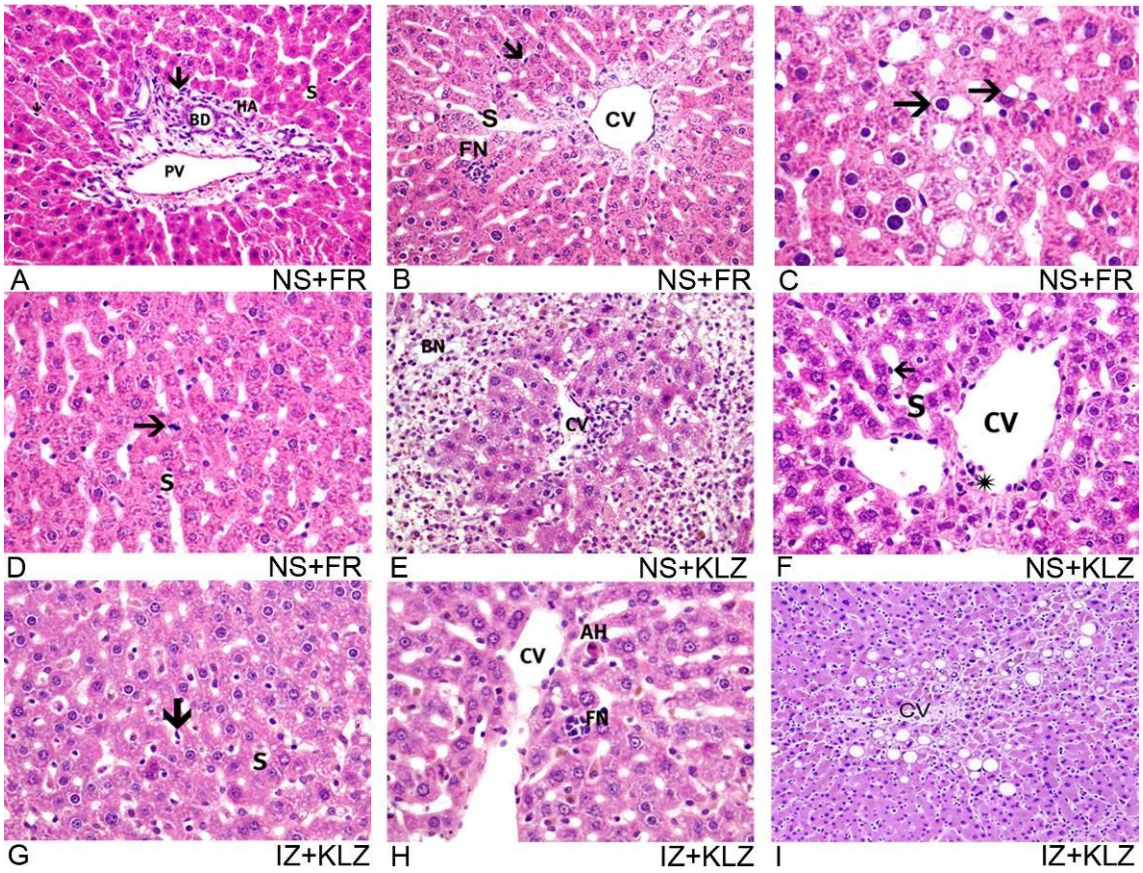
Slika 39. ***Nivo COX-2 u citosolu jetre nestresiranih (NS) i izolovanih (IZ) pacova tretiranih fiziološkim rastvorom (FR), fluoksetinom (FLK, 15 mg/kg/dan) ili klozapinom (KLZ, 20 mg/kg/dan).*** *Simboli ukazuju na značajne razlike: $^{*}p < 0,05$ u odnosu na NS+FR (kontrola); $^{\#}p < 0,05$ IZ+KLZ vs. NS+KLZ; $^{\#\#}p < 0,01$ IZ+FLK vs. NS+FLK.*

4.6 UTICAJ FLUOKSETINA I KLOZAPINA NA HISTOLOŠKE PARAMETRE JETRE NESTRESIRANIH I IZOLOVANIH PACOVA

Kako bi se ispitali efekti fluoksetina i klozapina, kao i same izolacije, na strukturu tkiva jetre, praćene su promene u okviru sledećih parametara: portna inflamacija, broj Kupferovih ćelija, nekroza hepatocita i masne promene jetre. Rezultati su prikazani na Slici 40 i u Tabeli 12.

Mali portni inflamatorni infiltrati, sačinjeni pre svega od limfocita, makrofaga i retko od neutrofila, uočeni su u jetri sve tri grupe nestresiranih životinja (Slika 40, A), ali u većoj meri kod pacova tretiranih fluoksetinom ili klozapinom. Zatim, Kupferove i inflamatorne ćelije primećene su u sinusoidnim prostorima jetre nestresiranih pacova tretiranih fiziološkim rastvorom ali je njihov broj povećan kod nestresiranih životinja koje su tretirane klozapinom (Tabela 12). Fokalna nekroza (nekroza pojedinačnih hepatocita) u jetri nestresiranih pacova detektovana je sporadično kod jedinki koje su primale fiziološki rastvor (Slika 40, B), za razliku od pacova koji su tretirani klozapinom ili fluoksetinom, kod kojih je detektovana u znatno većoj meri.

Što se tiče apoptotičnih hepatocita, oni su povremeno uočavani u jetri nestresiranih jedinki tretiranih fluoksetinom, dok su kod jedinki tretiranih klozapinom najčešće bili odsutni. Lekovi su imali efekat na jetru nestresiranih pacova i u pogledu masnih promena. Naime, makrovezikularne promene su primećene u svim eksperimentalnim grupama, uključujući i kontrolne pacove (Slika 40, C), s tim da kod njih promene nisu obuhvatale više od 10% hepatocita po uzorku. Kod pacova tretiranih fluoksetinom ili klozapinom, te promene su bile intenzivnije (Tabela 12).



Slika 40. ***Histopatološke promene u jetri pacova*** *(hematoksilin/eozin bojenje, uvećanje 400 ×). Nestresirane životinje tretirane fiziološkim rastvorom (NS+FR, kontrola):* ***(A)*** *Portna trijada sa ograncima hepatične arterije (HA), portne vene (PV) i žučnog kanala (BD), sa neupadljivim inflamatornim infiltratima sačinjenim od limfocita i makrofaga (velika strelica), normalna građa parenhima i Kupferove ćelije (mala strelica) u sinusoidima (S);* ***(B)*** *Fokalna nekroza hepatocita (FN) u blizini centralne venule (CV), Kupferove ćelije (strelica) u sinusoidima (S);* ***(C)*** *Fokalne makrovezikularne masne promene u hepatocitima (strelice);* ***(D)*** *Hepatocit u mitozi (strelica), sinusoidi (S). Nestresirane životinje tretirane klozapinom (NS+KLZ):* ***(E)*** *Spojna nekroza hapatocita (BN), centralna venula (CV), mikrovezikularne masne promene u hepatocitima;* ***(F)*** *Endotelitis centralne venule (CV) (zvezdica), sinusoidi (S) sa Kupferovim ćelijama (strelica). Izolovani pacovi tretirani klozapinom (IZ+KLZ):* ***(G)*** *Povećan broj Kupferovih ćelija (strelica) u sinusoidima (S);* ***(H)*** *Fokalna nekroza hepatocita (FN) i apoptotični hepatocit (AH) u blizini centralne venule (CV);* ***(I)*** *Makrovezikularne masne promene u hepatocitima oko centralne venule (CV).*

Tabela 12. ***Histopatološke promene u određenim strukturama tkiva jetre***

Histopatološki parametri	Tretman					
	NS			IZ		
	FR	FLK	KLZ	FR	FLK	KLZ
	Portna trijada					
Infiltrati neutrofila, limfocita i makrofaga	-	++	++	+/-	++	++
	Sinusoidi					
Kupferove i inflamatorne ćelije	+	+	++	+	+	++
Endotelitis centralne venule	-	-	-*	-	-	-
	Lobulusi					
Spojna/konfluentna nekroza	-	-	-*	-	-	-
Fokalna nekroza	+/-	++	++	+/-	+/-	++
Apoptotični hepatociti	-	+/-	-	-	-	+/-
Mikrovezikularne masne promene	-	-	+/-	-	-	-
Makrovezikularne masne promene	-	+	+	+/-	+/-	++
Hepatociti u mitozi	-	-	-	-	-	-

Značenje simbola u tabeli: ++ prisutno kod skoro svih životinja u grupi, + pretežno prisutno, +/- sporadično prisutno, - pretežno odsutno, - prisutno samo kod jedne životinje u grupi. Broj životinja po grupi je 6. NS – nestresirani pacovi, IZ – izolovani pacovi, FR – fiziološki rastvor, FLK – fluoksetin, KLZ – klozapin*

Što se tiče mikrovezikularnih masnih promena, one su, pre svega, uočavane nakon tretmana klozapinom, mada su neupadljive mikrovezikularne promene detektovane i u jetri nestresiranih pacova koji su primali fiziološki ratsvor ali nisu obuhvatale više od 3% hepatocita. U slučaju jedne nestresirane životinje tretirane fiziološkim rastvorom uočen je hepatocit u mitozi (Slika 40, D). Što se tiče lobulusnih promena, spojna (engl. *bridging*) i konfluentna nekroza uočene su u jetri jedne nestresirane životinje tretirane klozapinom (Slika 40, E). Kod iste životinje uočen je i endotelitis centralne venule (Slika 40, F).

Kod izolovanih pacova, histološka analiza portnih trijada otkrila je prisustvo inflamatornih infiltrata sačinjenih od limfocita, makrofaga i, u retkim slučajevima,

neutrofila. Ove promene su, u pogledu morfologije, slične u svim uzorcima ali se razlikuju po kvantitetu. U jetri izolovanih pacova tretiranih fiziološkim rastvorom, prisustvo inflamatornih infiltrata u portnim prostorima je sporadično, dok su isti kod izolovanih pacova tretiranih fluoksetinom ili klozapinom prisutni u većem broju. Što se tiče Kupferovih i inflamatornih ćelija u sinusoidima, njihovo prisustvo, naročito u blizini centralne venule, je bilo izraženije u jetri izolovanih životinja tretiranih klozapinom (Slika 40, G), u odnosu na samo izolovane ili izolovane pacove tretirane fluoksetinom. Fokalna nekroza je uočena u svim uzorcima ali najzastupljenija je u jetri jedinki tretiranih klozapinom, kod kojih su zapažane i po 3 fokalne nekroze u jednom lobulusu (Slika 40, H). Takođe, jedino kod ovih jedinki su primećeni retki apoptotični hepatociti (Slika 40, H). Male i neupadljive mikrovezikularne masne promene uočene su tek u ponekim uzorcima. Za razliku od njih, primećene su jasne i brojne makrovezikularne masne promene. One su, poput drugih promena, bile najizraženije u jetri pacova tretiranih klozapinom, kod kojih su obuhvatale i do 60% jetre (Slika 40, I).

Dakle, spektar histoloških karakteristika jetre izolovanih pacova koji su primali fiziološki rastvor sličan je onom koji je uočen kod kontrolnih pacova, s tim da su neke promene, poput makrovezikularnih masnih promena, naglašenije kod izolovanih. Hronični tretman fluoksetinom nije izazvao krupne promene na nivou strukture tkiva jetre kako nestresiranih, tako i izolovanih pacova. Za razliku od fluoksetina, klozapin je izazvao upadljive promene u jetri nestresiranih i izolovanih pacova.

5 DISKUSIJA

Patogeneza i patofiziologija depresije su predmeti intenzivnog istraživanja još od pedesetih godina prošlog veka, međutim i danas se ispituju nesmanjenim intenzitetom. Aktuelnost ove problematike je pre svega posledica visoke prevalencije ovog oboljenja, njegove heterogenosti i kompleksnosti, kao i potrebe za unapređivanjem antidepresivne terapije. Ta potreba proizilazi iz činjenice da je kod velikog broja pacijenata nemoguće postići remisiju bolesti primenom dostupnih medikamenata.

Životinjski modeli se u velikoj meri koriste u istraživanju neurobiologije depresije i ispitivanju mehanizama delovanja antidepresiva. Studije na životinjskim modelima depresije omogućavaju testiranje novih neurobioloških hipoteza, kao i ispitivanje mehanizama delovanja psihotropnih lekova i identifikovanje novih target molekula terapije. Studije na glodarima su pokazale da izlaganje stresorima povećava rizik za pojavu ponašanja koja nalikuju na depresiju. Istorijski, u tim studijama često su primenjivani hronični fizički stresori poput električnih šokova, fizičkog sputavanja, niske temperature itd. Iako su ta istraživanja ukazala na povezanost stresa i depresije, često su bila kritikovana zbog prirode korišćenih stresora, jer fizički stresori ne reprezentuju u najboljoj meri stresore koji deluju na čoveka, a koji su najčešće socijalne prirode (Hollis i Kabbaj, 2014). Stoga su se istraživači fokusirali na modele koji se baziraju na hroničnom izlaganju životinja socijalnom stresu, kao što je hronična izolacija koja je korišćena u ovom radu.

Rezultati studije govore u prilog validnosti hronične izolacije odraslih mužjaka pacova Wistar soja kao modela za ispitivanje depresije. Oni potvrđuju da ovaj model ispunjava kriterijume validnosti sličnosti, konstrukcijske validnosti, kao i predikcione validnosti. Takođe, studija proširuje prethodna saznanja o dejstvu fluoksetina i klozapina izvan okvira serotoninske i dopaminske signalizacije. Ona pruža jasniji uvid u efekte koje ovi lekovi imaju na antioksidativni sistem i parametre inflamacije u hipokampusu i prečeonoj zoni kore cerebruma, kao i na GABA signalizaciju u medijalnoj prečeonoj zoni kore. Dodatno, upotpunjuje znanja vezana za hepatotoksične efekte ovih lekova.

5.1 ANTIDEPRESIVNI I ANKSIOLITIČKI EFEKTI FLUOKSETINA I KLOZAPINA

Depresija je stanje definisano na osnovu simptoma opisanih kod ljudi, u koje se ubrajaju anhedonija, anksioznost, osećaj krivice, razmišljanja o smrti itd. Uprkos tome što većina tih simptoma zahteva samosvest i samorefleksiju, postoje određeni parametri koje je moguće meriti kod životinja i koji koreliraju sa nekim od pobrojanih simptoma. Neki od tih parametara su interesovanje za hranu ili piće, reagovanje na strani objekat u kavezu i vreme potrebno da odustanu i predaju se kada su izloženi stresnoj situaciji.

U ovom radu praćene su promene ponašanja odraslih mužjaka pacova izazvane 21-dnevnom izolacijom. Rezultati su pokazali da izolacija dovodi do promena tipičnih za ponašanje nalik depresivnom kod glodara i ujedno sličnih uobičajenim simptomima pacijenata koji boluju od depresije, poput očaja, anhedonije i anksioznosti. Duži periodi plutanja kod izolovanih, u odnosu na nestresirane pacove, u testu prinudnog plivanja ukazuju na beznadežnost i očajanje, što je u saglasnosti sa ranije sprovedenom studijom (Djordjevic i sar., 2012b). Naime, pacovi primorani na plivanje, u početku su vrlo aktivni jer plivaju i penju se uz zid suda, a potom ulaze u stanje slabe pokretljivosti (plutanje, imobilnost) koje podrazumeva pokrete neophodne i dovoljne za održavanje glave iznad vode. Smatra se da je plutanje u toku ovog testa posledica povinovanja pacova eksperimentalnim uslovima i da odslikava stanje očajanja koje je posledica bezizlaznosti situacije. Stoga, kraći periodi aktivnih pokušaja da se izbavi, a duži periodi pasivnog plutanja ukazuju na depresivno raspoloženje, odnosno na nevoljnost i stanje nalik očaju. U prilog tome da se plutanje tokom prinudnog plivanja može smatrati indikatorom ponašanja nalik depresiji govore i podaci da primena terapeutika sa antidepresivnim dejstvom skraćuje njegovo trajanje (Porsolt i sar., 1977).

Smanjena zainteresovanost za zaslađeni rastvor kod izolovanih u odnosu na nestresirane pacove ukazuje na anhnedoniju uzrokovanu primenjenim stresorom. Anhedonija predstavlja delimičan ili potpuni gubitak interesovanja za aktivnosti koje mogu da izazovu osećaj zadovoljstva i jedan je od glavnih simptoma depresije. Veliki broj studija je pokazao da različiti tipovi stresora smanjuju zainteresovanost za zaslađeni rastvor, a da primena antidepresiva vraća zainteresovanost na kontrolne vrednosti (Willner i sar., 1987; Rygula i sar., 2006; Salari i sar., 2016), pa se ovaj

parametar često koristi kao mera anhedonije. Pored toga, značajno veći broj zakopanih klikera kod izolovanih nego kod nestresiranih životinja ukazuje da izolacija izaziva i ponašanje nalik anksioznom. Međutim, treba napomenuti da postoji mišljenje da je zakopavanje klikera posledica konzistentnog, upornog kopanja, pre nego anksioznosti uzrokovane novim predmetom u kavezu pacova. Tako je studija na miševima pokazala da ponavljanje eksperimenta, čime se omogućava navikavanje životinja na klikere, ima mali uticaj na rezultate testa (Thomas i sar., 2009). Zaista, rezultati testa zakopavanja klikera nisu uvek u saglasnosti sa rezultatima drugih testova koji ispituju ponašanje nalik anksioznom. Međutim, indicija anksioznosti, koja je u ovoj studiji detektovana kroz povećan broj zakopanih klikera kod izolovanih pacova ima potporu u literaturnim podacima. Naime, prethodno je pokazano da izolovani pacovi provode manje vremena u svetlom odeljku u testu sa "svetlo-tamnom" kutijom (engl. *light-dark box test*) (Carrier i Kabbaj, 2012), kao i da beleže manji broj ulazaka, i manje provedenog vremena u otvorenoj ručici u testu izdignutog plus lavirinta (engl. *elevated plus maze test*) (Djordjevic i sar., 2015). Takođe, rezultati testa sa izdignutom platformom (engl. *elevated platform test*) (Spasojevic i sar., 2007) podržavaju zaključak da hronična izolacija dovodi do ponašanja nalik anksioznom kod adultnih mužjaka pacova. Pored toga, Spasojević i saradnici (2007) su uočili da izolovani pacovi manje vremena posvećuju održavanju higijene krzna što ukazuje na letargiju i apatiju.

Apliciranje fluoksetina (15 mg/kg/dan), odnosno klozapina (20 mg/kg/dan), sprečilo je razvoj ponašanja koja nalikuju anhedoniji i anksioznosti u uslovima hronične izolacije. Ovi rezultati su očekivani s obzirom da su prethodne studije pokazale da fluoksetin značajno povećava zainteresovanost za zaslađeni rastvor kod pacova izlaganih različitim oblicima hroničnog stresa (Rong i sar., 2010; Yang i sar., 2014; Chen i sar., 2015a). U literaturi ne postoji mnogo podataka o uticaju klozapina na anhedoniju, međutim pokazano je da ovaj antipsihotik vraća zainteresovanost za zaslađene rastvore u jednom modelu shizofrenije (Vardigan i sar., 2010). Što se tiče anksioznosti, oba leka su u ranijim studijama pokazala anksiolitička svojstva kako u testu zakopavanja klikera na miševima (Bruins Slot i sar., 2008), tako i u drugim testovima ponašanja (Zhang i sar., 2000; Mead i sar., 2008). U prilog tome da fluoksetin i klozapin sprečavaju nastanak simptoma karakteristčnih za depresiju, govore i rezultati ranijih studija koji su pokazali da oba leka skraćuju periode plutanja u testu prinudnog

plivanja (Weiner i sar., 2003; Ciulla i sar., 2007; Brenes i Fornaguera, 2009; Chatterjee i sar., 2012).

5.2 EFEKTI FLUOKSETINA I KLOZAPINA NA GSH-ZAVISNI SISTEM I PARAMETRE INFLAMACIJE

Kako je opisano u uvodu, oksidativni stres i inflamacija su uključeni u neuroprogresiju MDD koja uključuje neurodegeneraciju, ćelijsku smrt, smanjenu neurogenezu i plastičnost, kao i pojačan autoimunski odgovor (Bakunina i sar., 2015). Psihosocijalni stres, jedan od glavnih uzročnika psihijatrijskih oboljenja, dovodi do oksidativnog stresa i ćelijske signalizacije posredovane inflamatornim molekulima, uključujući transkripcioni faktor NF-κB (Miller i sar., 2009; Hayashi, 2015). U ovoj studiji je ispitano kako hronični tretmani fluoksetinom ili klozapinom utiču na komponente GSH-zavisnog antioksidativnog sistema i medijatore inflamacije u hipokampusu i prečeonoj zoni kore cerebruma pacova, u uslovima hronične izolacije. Razumevanje ovih efekata je značajno sa aspekta personalizovane i efikasnije upotrebe ovih lekova u terapiji pacijenata.

5.2.1 Efekti fluoksetina i klozapina na GSH-zavisni sistem i parametre inflamacije u hipokampusu

Hipokampus je moždana struktura čija je osetljivost na različite tipove stresora potvrđena u velikom broju studija (Krugers i sar., 2010b; Kim i sar., 2015; McEwen i sar., 2016). U ovom radu je uočeno da pacovi u uslovima hronične izolacije, koji ispoljavaju ponašanje nalik depresivnom, imaju smanjenu aktivnost GPx enzima i povećan nivo proinflamatornog citokina TNF-α u citosolu hipokampusa. Primena fluoksetina, odnosno klozapina, tokom izolacije sprečila je promene nivoa TNF-α, ali nije uticala na štetne efekte izolacije na antioksidativnu odbranu posredovanu GPx enzimom.

Značajno smanjenje nivoa GPx i GLR je uočeno nakon zajedničkog delovanja stresa i fluoksetina, odnosno klozapina. Poznato je da glukokortikoidi povećavaju kapacitet antioksidativne odbrane tako što aktiviraju gene koji kodiraju antioksidativne

enzime CuZnSOD, katalazu i GPx (Grier i Halliday, 2004). Ranije je pokazano da izolacija negativno utiče na translokaciju GR iz citoplazme u jedro (Filipović i sar., 2016). Pored toga, pokazano je da antidepresivi, između ostalih i fluoksetin, mogu inhibirati glukokortikoidima-indukovanu transkripciju gena (Budziszewska i sar., 2000). Uzimajući u obzir navedeno, može se pretpostaviti da remećenje signalizacije posredovane GR doprinosi slabljenju sistema odbrane u hipokampusu pacova izlaganih kombinovanom efektu izolacije i leka. Uprkos tome što je antioksidativno dejstvo fluoksetina poznato od ranije, malo se zna o njegovom uticaju na aktivnost antioksidativnih enzima (Caiaffo i sar., 2016). Naime, prethodno je pokazano da ovaj antidepresiv obnavlja antioksidativni kapacitet u mozgu (Bilici i sar., 2001; Novío i sar., 2011), međutim Adžić i saradnici (2011) su otkrili da on negativno utiče na antioksidativni sistem tako što smanjuje aktivnost GLR enzima u eritrocitima. Što se tiče klozapina, pokazano je da direktna antioksidativna aktivnost doprinosi njegovoj terapijskoj efikasnosti (Sadowska-Bartosz i sar., 2016). Međutim studije antioksidativnih enzima, između ostalih i GPx i GLR, nisu pokazale protektivan efekat ovog antipsihotika u mozgu pacova (Parikh i sar., 2003), ni i humanim eritrocitima (Miljević i sar., 2013).

Izolacija je smanjila aktivnost GPx u citosolu hipokampusa iako nije uticala na nivo ovog enzima. Smanjena aktivnost GPx je verovatno posledica posttranslacione modifikacije ili nedostupnosti kosupstrata. Imajući u vidu da NO može inaktivirati GPx (Asahi i sar., 1995; Miyamoto i sar., 2003), smanjena aktivnost ovog enzima se može objasniti trostrukim povećanjem nivoa NO koje je ranije uočeno u hipokampusu izolovanih pacova (Zlatković i sar., 2014b). Smanjena GPx aktivnost, uprkos nepromenjenom nivou iRNK i proteinskog produkta, pokazana je ranije u hipokampusu pacova u modelu ponovljenog stresa fizičkog sputavanja (Popović, 2016). Smanjenje aktivnosti ovog antioksidativnog enzima povećava podložnost ćelija hipokampusa oksidativnom stresu s obzirom da kompromituje njihovu odbranu od peroksida. To potvrđuju detektovana oksidativna oštećenja hipokamusa izolovanih pacova (Zlatković i sar., 2014b).

Aktivnost GPx je ostala snižena i u hipokampusu izolovanih pacova tretiranih kako fluoksetinom, tako i klozapinom. Osim toga, oba leka su doprinela kompromitovanju GSH-zavisnog sistema, smanjujući nivo i aktivnost GLR u

hipokampusu izolovanih pacova. Interesantno je to što su oba leka smanjila aktivnost, ali ne i nivo enzima GPx u hipokampusu nestresiranih životinja. GSH je kosupstrat kako za GPx, tako i za GST (Dringen, 2000), enzim odgovoran za metabolizam ksenobiotika, u koje se ubrajaju i lekovi (Sastre, J., Pallardo, F.V., Viña, 2005). Tako smanjena GPx aktivnost u hipokampusu nestresiranih pacova tretiranih fluoksetinom ili klozapinom može biti posledica smanjene dostupnosti GSH usled njegove povećane potrošnje od strane GST. U prilog ovoj pretpostavci govori podatak da hronični tretman psihoaktivnim lekovima povećava GST aktivnost u ćelijama mozga (Bakare i sar., 2009; Da Silva i sar., 2015).

Hronična izolacija nije aktivirala NF-κB u hipokampusu pacova, niti je dovela do promena u nivou COX-2, što je očekivano jer je ovaj enzim regulisan pomenutim jedarnim faktorom (Kaltschmidt i sar., 2002). Sa druge strane, izolacija je dovela do značajnog povećanja nivoa TNF-α. Ovaj citokin je veoma važan sa aspekta izučavanja patogeneze psihijatrijskih oboljenja generalno, a konkretno i depresije (Berthold-Losleben i Himmerich, 2008). TNF-α utiče na serotoninsku signalizaciju tako što aktivira serotoninski transporter i na taj način pozitivno utiče na preuzimanje serotonina u izolovanim sinaptozomima miša (Zhu i sar., 2006). Pored toga, pokazano je da ovaj citokin stimuliše enzim indolamin 2,3-dioksigenazu, što rezultuje smanjenom količinom triptofana, prekursora za sintezu serotonina (Wichers i Maes, 2002). Povrh svega navedenog, pokazano je da TNF-α antagonisti ublažavaju simptome depresije i smanjuju kognitivne deficite kako na eksperimentalnim modelima, tako i u kliničkim studijama (Bortolato i sar., 2015). Tako je pokazano da infliksimab, TNF-α inhibitor, smanjuje anhedoniju i ponašanje nalik očajanju kod pacova u modelu hroničnog nepredvidivog blagog stresa (Sahin i sar., 2015). Pored toga, infliksimab je ublažio simptome depresije kod pacijenata rezistentnih na antidepresivnu terapiju (Felger i Lotrich, 2013).

Zanimljivo je to što povećanje nivoa TNF-α nije posledica aktivacije NF-κB uprkos tome što je ovaj citokin pod njegovom regulacijom, a ujedno je i njegov aktivator (Mercurio i sar., 1997; Berti i sar., 2002). Međutim, pored NF-κB, ekspresiju TNF-α na nivou transkripcije reguliše mreža transkripcionih faktora, koregulatora i modifikatora hromatina (Moelants i sar., 2013). Bitno je pomenuti da je u prethodnoj studiji naše laboratorije pokazano da je inducibilna forma proteina toplotnog šoka 70,

iHSP70 (engl. *inducible heat shock protein 70*), koja inhibira proinflamatorni faktor NF-κB (Heck i sar., 2011; Kim i sar., 2012) pojačano eksprimirana u hipokampusu, u uslovima hronične izolacije (Zlatković i sar., 2014a). To makar delimično može objasniti odsustvo aktivacije NF-κB uprkos povećanom nivou TNF-α. Takođe je zanimljivo to što promene u TNF-α nisu praćene promenama u IL-1β nivou. Naime, nakon 21 dana izolacije detektovan je nepromenjen nivo IL-1β u hipokampusu, a skorašnja studija je pokazala odsustvo promene u nivou ovog citokina i nakon 6 nedelja izolacije (Perić i sar., 2017).

Tretman fluoksetinom, odnosno klozapinom tokom izolacije, u dozama koje odgovaraju donjim granicama terapijskih doza kod pacijenata, sprečili su porast nivoa TNF-α u hipokampusu izolovanih pacova. Ovaj rezultat je u saglasnosti sa rezultatima *in vitro* studija koji su pokazali negativan efekat fluoksetina na nivo TNF-α (Baumeister i sar., 2015), kao i *in vivo* studija koji su demonstrirali antiinflamatorni efekat ovog antidepresiva (Liu i sar., 2015; Perić i sar., 2017). Što se tiče literaturnih podataka o klozapinu, Hu i saradnici (2012) su pokazali da on smanjuje aktivaciju mikroglije koju izaziva LPS u primarnoj neuron-glijskoj kulturi ćelija, kao i u ćelijskoj liniji mikroglije pacova. Takođe je pokazano da ovaj antipsihotik smanjuje produkciju TNF-α u aktiviranim mikroglijskim ćelijama. Rezultati ove disertacije koji pokazuju da tretmani fluoksetinom, odnosno klozapinom, sprečavaju povećanje produkcije TNF-α u hipokampusu indukovano izolacijom, zajedno sa promenama u ponašanju izolovanih pacova, ukazuju na značajnu ulogu ovog citokina u patogenezi depresivnih i anksioznih simptoma. S obzirom da povećana ekspresija TNF-α nije posredovana NF-κB, jasno je da protektivni efekti koje su ispoljili fluoksetin i klozapin nisu posredovani ovim jedarnim faktorom. Antiinflamatorni efekat fluoksetina ostvaren inhibicijom NF-κB puta u *in vitro* uslovima pokazan je u studiji na primarnoj kulturi glijskih ćelija pacova aktiviranih pomoću LPS (Obuchowicz i sar., 2014). Međutim, u ovoj doktorskoj disertaciji je po prvi put pokazan *in vivo* antiinflamatorni efekat fluoksetina koji nije posredovan sa NF-κB. Dalja istraživanja su potrebna za dobijanje jasnije slike o molekularnim mehanizmima koji se nalaze u osnovi ovog protektivnog efekta, a njeno sagledavanje bi moglo doprineti, pre svega razumevanju patogeneze, a zatim i uspešnijem tretmanu depresije.

5.2.2 Efekti fluoksetina i klozapina na GSH-zavisni sistem i parametre inflamacije u prečeonoj zoni kore cerebruma

Rezultati istraživanja su pokazali da hronična izolacija kompromituje GSH-zavisni antioksidativni sistem i izaziva proinflamatorni odgovor i u prečeonoj zoni kore cerebruma adultnih mužjaka pacova Wistar soja. Apliciranje fluoksetina, ili klozapina, tokom izolacije imalo je protektivan efekat, pri čemu je fluoksetin bio delotvorniji. Naime, oba leka su pokazala zaštitni efekat u okviru parametara inflamacije ali samo fluoksetin je održao nivo GSH u prečeonoj zoni kore cerebruma na nivou zabeleženom kod nestresiranih kontrola i time ispoljio antioksidativno svojstvo.

U prečeonoj zoni kore cerebruma, izolacija je dovela do značajnog smanjenja nivoa GSH, glavnog "hvatača" (engl. *scavenger*) slobodnih radikala u mozgu. Smanjen nivo ovog tripeptida uočen je u mozgu laboratorijskih životinja izlaganih različitim tipovima hroničnog stresa (Hong i sar., 2014; Samarghandian i sar., 2016), kao i u prečeonoj zoni kore cerebruma psihijatrijskih pacijenata (Gawryluk i sar., 2011). To ukazuje na važnost ispitivanja osetljivosti GSH-zavisnog sistema u uslovima stresa u moždanim regionima, naročito onim koji su posebno osetljivi na stres. Smanjen nivo GSH je verovatno direktna posledica povećane aktivnosti GPx enzima, koja je uočena kod izolovanih pacova. Prethodna studija na ovom modelu je pokazala smanjenu antioksidativnu odbranu posredovanu SOD, kao i povećanu aktivnost neuronske i inducibilne NO sintaze (nNOS i iNOS) u prečeonoj zoni kore cerebruma (Zlatković i Filipović, 2013). Ove promene za posledicu imaju povećanje koncentracije $O_2^{\bullet -}$ i NO što favorizuje produkciju veoma toksičnog peroksinitrita. Takođe je u ovom regionu mozga pokazana i povećana peroksidacija lipida kod pacova u uslovima hronične izolacije (Zlatković i sar., 2014b). S obzirom da GPx vrši detoksifikaciju peroksinitrita (Arteel i sar., 2000) i lipidnih peroksida, povećana ekspresija i aktivnost ovog enzima je očekivana. Sa druge strane nivo proteina i aktivnost GLR nisu značajno promenjeni usled izolacije, što zajedno sa povećanom GPx aktivnošću, za posledicu ima smanjenje GSH nivoa. Naime, povećana oksidacija GSH nije praćena povećanom redukcijom GS-SG, tako da se rezerve redukovanog glutationa smanjuju. To slabi antioksidativni kapacitet i povećava osetljivost i podložnost prečeone zone kore cerebruma oksidativnom stresu.

Tretman fluoksetinom, ali ne i klozapinom, održao je GSH na nivou zabeleženom u kontrolnim životinjama u prečeonoj zoni kore cerebruma izolovanih pacova, ali nije uticao na nivo i aktivnost GPx enzima, kao i GLR kod ovih životinja. To sugeriše na mogućnost da fluoksetin utiče na *de novo* sintezu GSH. U prilog ovoj pretpostavci govori podatak da u kori cerebruma miša fluoksetin povećava nivo glutamat-cistein ligaze (Mendez-David i sar., 2015), enzima koji katalizuje prvi korak u biosintezi GSH (Sastre i sar. 2005). Sa druge strane, tretman klozapinom nije ispoljio protektivno svojstvo u uslovima hronične izolacije, čak je smanjio nivo GSH kod nestresiranih pacova. U prilog razumevanju ovog rezultata govori podatak da klozapin može biti oksidovan do nitrenijum jona koji, u procesu detoksifikacije, reaguju sa sulfhidrilnom grupom GSH (Yang i sar., 2011).

Pored toga što je kompromitovala antioksidativnu odbranu, 21-dnevna izolacija je pokrenula proinflamatornu signalizaciju u prečeonoj zoni kore cerebruma pacova. Povećani nivo medijatora inflamacije u moždanim regionima koji regulišu raspoloženje zabeleženi su u različitim psihijatrijskim stanjima, u brojnim prekliničkim i kliničkim studijama (Kiecolt-Glaser i sar., 2015; Réus i sar., 2015). Izvor inflamacije kod depresivnih pacijenata kod kojih ne postoji infektivni, autoimunski ili inflamatorni proces, dugo je bio upitan. Veliki doprinos u tom smislu je donelo saznanje da psihosocijalni stres može da aktivira inflamatorni odgovor kako na periferiji, tako i u mozgu (Miller i sar., 2009). Da aktivacija NF-κB može biti posledica neuroendokrinog odgovora na psihosocijalni stres pokazano je povećanim vezivanjem NF-κB za DNK u mononuklearnim ćelijama krvi zdravih ljudi koji su bili izloženi stresu usled javnog nastupa (Bierhaus i sar., 2003). Pored toga, poznato je da kateholamini povećavaju ekspresiju citokina na periferiji i u mozgu pacova (Johnson i sar., 2005). Zanimljivo je da je povišen nivo noradrenalina i adrenalina u plazmi pacova uočen tek nakon 12 nedelja izolacije (Gavrilovic i sar., 2010), ali ne nakon 3 nedelje (Gavrilovic i Dronjak, 2005). Takođe, iako je poznato da je KORT snažan antiinflamatorni hormon, pokazano je da u kontekstu hroničnog stresa ili depresije imunski sistem može postati rezistentan na glukokortikoide. Tako je povišena ekspresija gena koji sadrže sekvencu za vezivanje NF-κB, a smanjena ekspresija onih koji sadrže sekvencu za vezivanje GR uočena kod individua izloženih stresnim situacijama, pri čemu su koncentracije KORT u pljuvački bile iste kao kod kontrolnih individua (Miller i sar., 2008).

Rezultati studije pokazuju povećanu translokaciju NF-κB-p65 subjedinice iz citoplazme u jedro u ćelijama prečeone zone kore cerebruma pacova, u uslovima hronične izolacije. S obzirom da je NF-κB transkripcioni faktor osetljiv na promene redoks statusa, njegova aktivacija može biti posledica oksidativnog i nitrozativnog stresa čije je prisustvo potvrđeno u prethodnim studijama u ovom moždanom regionu, na ovom modelu (Zlatković i Filipović, 2013; Zlatković i sar., 2014b). Akivaciji ovog faktora može doprineti i disregulacija HHA osovine i smanjena translokacija GR iz citoplazme u jedro koja je takođe ranije opisana u prečeonoj zoni izolovanih pacova (Dronjak i sar., 2004; Filipović i sar., 2005). Poznato je da glukokortikoidi inhibiraju NF-κB tako što indukuju ekspresiju IκB, inhibitorne subjedinice koja zadržava NF-κB u citoplazmi (Auphan i sar., 1995). U uslovima narušene translokacije GR u jedro, smanjena produkcija IκB može uzrokovati povećanu NF-κB aktivnost. Pored aktivacije NF-κB, u prečeonoj zoni kore cerebruma izolovanih pacova uočen je povećan nivo COX-2, IL-1β i TNF-α u citosolu. COX-2 katalizuje prevođenje arahidonske kiseline u prostaglandine i na taj način promoviše inflamaciju. Aktivnost ovog enzima je ne samo proinflamatorna, već i prooksidativna zato što dovodi do oslobađanja $O_2^{\bullet -}$ kao sporednog prozvoda (Morgan i Liu, 2011). To povećava potrošnju GSH i samim tim doprinosi smanjenju nivoa ovog tripeptida kod izolovanih pacova o čemu je diskutovano ranije u tekstu. Povećan nivo COX-2, kao i povećani nivo NF-κB-p50 i p65 subjedinica u jedru, nađeni su *postmortem* u čeonoj zoni kore cerebruma pacijenata sa bipolarnim poremećajem (Rao i sar., 2010). Značaj COX-2 enzima u patologiji depresije istaknut je ranije i u studijama na životinjskim modelima. Nedavno je pokazano da hronični nepredvidivi blagi stres koji smanjuje zainteresovanost za zaslađeni rastvor ujedno povećava nivo iRNK, proteinskog produkta, kao i aktivnost COX-2 enzima i koncentraciju prostaglandina E_2 u prečeonoj zoni kore cerebruma (Yao i sar., 2015). Takođe, na modelu hroničnog nepredvidivog stresa pokazan je antidepresivni efekat selektivnog COX-2 inhibitora celekoksiba (Guo i sar., 2009). Inhibicija COX-2 pokazala se delotvornom i kod pacijenata obolelih od MDD, kod kojih je ublažila depresivne simptome (Köhler i sar., 2014). Citokini IL-1β i TNF-α su regulisani od strane NF-κB, a ujedno i aktiviraju ovaj jedarni faktor (Mercurio i sar., 1997; Berti i sar., 2002). Oni imaju sposobnost da stimulišu sopstvenu sintezu i unakrsnu sintezu, kao i sintezu drugih citokina ili nekih drugih medijatora inflamacije

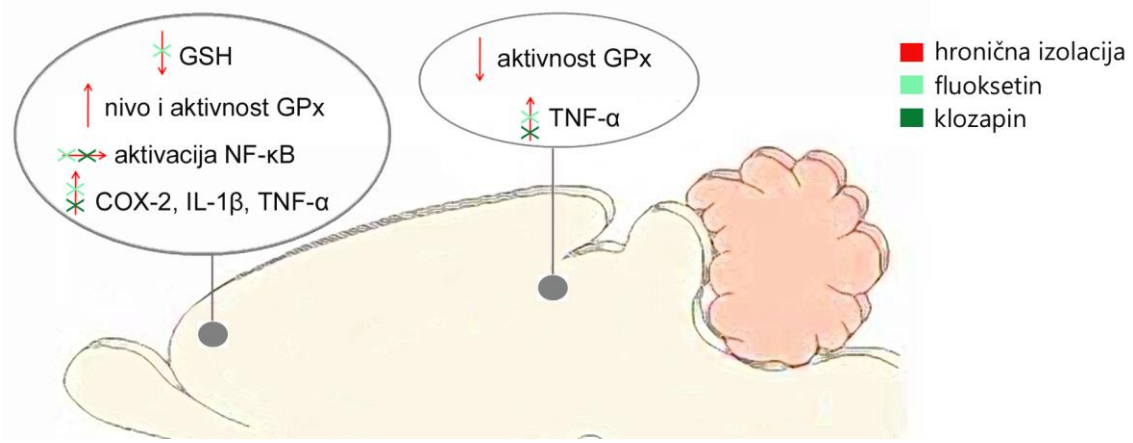
poput iNOS i COX-2, i to upravo posredstvom NF-κB (Vitkovic i sar., 2000). U skladu sa tim, prethodna studija naše laboratorije je pokazala da izolacija izaziva nitrozativni stres u prečeonoj zoni kore cerebruma tako što povećava ekspresiju iNOS enzima (Zlatković i Filipović, 2013). Nedavno je pokazano da 4 nedelje izolacije odmah po završetku perioda sisanja dovode do povećanja nivoa proinflamatornih citokina IL-1β, IL-6, TNF-α i INF-γ u plazmi pacova (Ko i Liu, 2015). Takođe, povećani nivo IL-1β i TNF-α u čeonoj zoni kore cerebruma detektovan je kod pacova koji ispoljavaju anhedoniju nakon 7 nedelja nepredvidivog blagog stresa (Liu i sar., 2014). To je u saglasnosti sa brojnim drugim studijama koje se tiču periferne i centralne sekrecije proinflamatornih citokina u modelima depresije (Kubera i sar., 1998; Leonard i Song, 2002; García-Bueno i sar., 2005; Grippo i sar., 2005).

Tretman fluoksetinom je onemogućio aktivaciju NF-κB indukovanu izolacijom, i održao nivo COX-2, IL-1β i TNF-α u prečeonoj zoni kore cerebruma izolovanih pacova na nivou izmerenom kod nestresiranih kontrola i time ispoljio antiinflamatorno dejstvo. Nedavna studija na mikroglijskim ćelijama pacova je pokazala da fluoksetin smanjuje translokaciju NF-κB-p65 subjedinice u jedro, kao i koncentraciju IL-1β i TNF-α u ćelijama tretiranim sa LPS (Obuchowicz i sar., 2014). Van den Berga i saradnici (2014) sumirali su literaturne podatke koji ističu antiinflamatorni efekat fluoksetina, međutim mehanizmi koji se nalaze u osnovi tog efekta nisu do danas utvrđeni. Jedan od potencijalnih mehanizama, koji ističu i pomenuti autori, uključuje supresiju NF-κB. Rezultati ove doktorske teze govore u prilog tome. Imajući u vidu da GR svoj antiinflamatorni efekat ispoljava delom kroz antagonizam sa proinflamatornim transkripcionim faktorima kao što je NF-κB (Rao i sar., 2011), opravdano je pretpostaviti da fluoksetin sprečava zadržavanje GR u citoplazmi prouzrokovano izolacijom i na taj način omogućava transrepresiju NF-κB. Ovu pretpostavku podržava i podatak da fluoksetin pozitivno utiče na signalnu transdukciju posredovanu glukokortikoidima (Herr i sar., 2003).

Klozapin je, takođe, ispoljio antiinflamatorno dejstvo u prečeonoj zoni kore cerebruma pacova u uslovima hronične izolacije. Ranije je pokazano da klozapin smanjuje oštećenja koja LPS izaziva u primarnoj kulturi neurona i glijskih ćelija kore cerebruma (Hu i sar., 2012). Tom prilikom je demonstrirano da je neuroprotektivno dejstvo klozapina povezano sa njegovim antiinflamatornim efektom, ostvarenim putem

inhibiranja produkcije ROS od strane NOX enzima mikroglijskih ćelija. Zanimljivo, pokazano je da izolacija povećava ekspresiju NOX-2 u prečeonoj zoni kore cerebruma i da apocinin, inhibitor NOX sprečava promene u ponašanju koje su karakteristične za izolovane pacove (Schiavone i sar., 2012). Imajući to u vidu, može se pretpostaviti da je protektivan efekat klozapina koji je uočen u ovoj studiji posredovan inhibicijom NOX-2, ali provera te pretpostavke zahteva dalja istraživanja.

Opisani rezultati daju uvid u molekularne promene koje se podudaraju sa promenama ponašanja pacova u uslovima hronične izolacije. Oni ukazuju na veću osetljivost prečeone zone kore cerebruma na primenjeni psihosocijalni stresor, u odnosu na hipokampus (Slika 41). Takođe, produbljuju fundamentalna znanja o efektima fluoksetina i klozapina u ovim moždanim strukturama koje su veoma značajne sa aspekta psihijatrijskih oboljenja. Iako su oba leka bila efikasna u suzbijanju promena ponašanja kod izolovanih pacova, fluoksetin se pokazao kao protektivniji na molekularnom nivou.



Slika 41. ***Molekularne promene u prečeonoj zoni kore cerebruma (levo) i hipokampusu (desno) izolovanih pacova i protektivni efekti fluoksetina i klozapina***

5.3 EFEKAT FLUOKSETINA I KLOZAPINA NA BROJ PV+ ĆELIJA

Imajući u vidu sve prihvaćenije mišljenje da disregulacija GABA sistema ima važnu ulogu u psihijatrijskim poremećajima, jedan od ciljeva studije bio je da se ispitaju uticaj hronične izolacije i potencijalni protektivni efekti fluoksetina i klozapina, na broj PV+ GABA interneurona. Analiziran je broj PV+ ćelija u podregionima medijalne prečeone zone kore cerebruma, moždanog regiona koji se smatra ključnim za razvoj i progresiju simptoma depresije (Riga i sar., 2014).

5.3.1 Efekat izolacije na broj PV+ ćelija u medijalnoj prečeonoj zoni kore cerebruma

Smanjenje broja PV+ ćelija detektovano je kod hronično izolovanih pacova u svim ispitivanim podregionima medijalne prečeone zone kore. Najveće smanjenje zabeleženo je u IL podregionu u kom je broj PV+ ćelija manji za 33% kod izolovanih u odnosu na nestresirane pacove. IL podregion kod glodara odgovara Brodmanovom polju 25 (engl. *Brodmann Area 25, BA25*) humanog mozga i projektuje se na brojne moždane regione uključene u visceralnu i neuroendokrinu kontrolu odgovora na stres, poput centralnog bademastog jedra (Mcklveen i sar., 2015). Povećanje metaboličke aktivnosti u BA25 polju uočeno je kod pacijenata obolelih od depresije, kao i smanjenje aktivnosti tog polja nakon tretmana različitim antidepresivima (Hamani i sar., 2011). Kod depresivnih pacijenata koji ne odgovaraju na antidepresivnu terapiju, dubinska stimulacija polja BA25 normalizovala je metaboličku aktivnost u ovom regionu, što je rezultiralo antidepresivnim efektom (Mayberg i sar., 2005). Mehanizmi delovanja dubinske stimulacije mozga nisu u potpunosti poznati ali se smatra da aktivacija inhibitornih GABA interneurona u BA25 posreduje u tom procesu (Mayberg i sar., 2005). Studija na pacovima je pokazala da farmakološka inaktivacija IL podregiona skraćuje periode plutanja u testu prinudnog plivanja, odnosno ima antidepresivni efekat (Slattery i sar., 2011). Uzimajući u obzir navedeno ovaj podregion medijalne prečeone zone je veoma značajan sa aspekta patofiziologije depresije kod ljudi i stanja nalik depresiji kod pacova. U saglasnosti sa tim podacima su i rezultati ovog istraživanja koji su pokazali da je upravo IL podregion najosetljiviji na hroničnu izolaciju, u smislu

promene broja PV+ ćelija. Može se pretpostaviti da je smanjen broj funkcionalnih PV+ inhibitornih interneurona, makar u izvesnoj meri, odgovoran za hiperaktivnost ovog podregiona koja je ranije uočena u depresiji.

Smanjen broj PV+ ćelija detektovan je i u PrL podregionu medijalne prečeone zone kore cerebruma izolovanih pacova. PrL se projektuje na strukture uključene u kontrolu emocija i kognitivnih funkcija poput dorzalnog striatuma i bazolateralnog bademastog jedra. Projektuje se i na *nukleus akumbens*, moždanu strukturu koja ima važnu ulogu u regulaciji hedonističkog ponašanja (Peciña i sar., 2006), kao i u patofiziologiji depresije (Mcklveen i sar., 2015). Ovaj podregion medijalne prečeone kore takođe učestvuje u regulaciji anksioznosti. Pokazano je da privremeno inaktiviranje ovog podregiona pomoću kobalta intenzivira istraživanje otvorenih ručica od strane pacova u testu izdignutog plus lavirinta, što je pokazatelj smanjene anksioznosti (Stern i sar., 2010). Smanjen broj inhibitornih PV+ neurona u ovom podregionu kod izolovanih pacova koincidira sa ponašanjem nalik anksioznom, koje je uočeno u testu zakopavanja klikera. Može se pretpostaviti da povećana aktivnost ovog podregiona, kao posledica smanjenja inhibicije posredovane PV+ interneuronima, doprinosi anksioznom ponašanju izolovanih pacova. U prilog ovoj pretpostavci govori studija koja je pokazala da farmakoaktivacija PrL podregiona stimuliše anksiozno ponašanje u testu otvorenog polja kod miševa (Saitoh i sar., 2014).

Studije koje ispituju uticaj hroničnog stresa na ponašanje životinja nalik depresivnom, kao i studije koje se bave ispitivanjem patofiziologije psihijatrijskih oboljenja uopšte, su pre svega fokusirane upravo na IL i PrL, dok je potencijalni značaj Cg1 i DP podregiona nedovoljno istražen. Rezultati ove studije pokazuju da su i ova dva podregiona osetljiva na psihosocijalni stres, jer je i u njima hronična izolacija dovela do smanjenja broja PV+ ćelija. Zanimljiv je podatak da 15 nedelja izolacije odmah po završetku perioda sisanja ne dovodi do značajnih promena u broju PV+ interneurona, ukupnom broju neurona, glijskih ćelija, niti u zapremini medijalne prečeone zone kore cerebruma (Kaalund i sar., 2013). Ove nedoslednosti ukazuju da su dužina trajanja izolacije i starosna dob pacova u izolaciji značajne determinante efekata ovog stresa.

Interesantno, pokazano je da je ekscitatorna sinaptička transmisija na PV+ interneurone u medijalnoj prečeonoj zoni kore cerebruma smanjena kod miševa koji ispoljavaju bespomoćnost (Perova i sar., 2015). U istoj studiji je pokazano da selektivna supresija aktivnosti PV+ interneurona promoviše bespomoćno stanje koje nalikuje humanoj depresiji. Skupa, ova zapažanja ukazuju da inaktivacija PV+ neurona u medijalnoj prečeonoj zoni kore promoviše razvoj ponašanja koje nalikuje depresivnom, a njihova aktivacija otpornost miševa na promene ponašanja izazvane stresom.

Jedan od mogućih uzroka smanjenja broja PV+ ćelija koje je uočeno u svim podregionima medijalne prečeone zone kore cerebruma izolovanih pacova jeste redoks disregulacija. Pokazano je da deficit GSH negativno utiče na morfološki i funkcionalni integritet PV+ interneurona, u hipokampusu (Steullet i sar., 2010). Smanjen nivo GSH koji je detektovan u prečeonoj zoni kore cerebruma izolovanih pacova svakako povećava podložnost ovog moždanog regiona oksidativnom stresu. PV+ interneuroni su naročito osetljivi na redoks disregulaciju zbog svoje brzookidajuće prirode koja zahteva veliku metaboličku aktivnost, što podrazumeva povećanu produkciju ATP ali i, posledično, ROS u mitohondrijama (Gulyás i sar., 2006). U uslovima intenzivne produkcije ROS i nedostatka GSH dolazi do smanjenja PV-, ali ne i kalbidin- niti kalretinin-imunoreaktivnosti u anteriornoj cingulatnoj kori (Cabungcal i sar., 2006), što ukazuje da su PV+ interneuroni osetljiviji na oksidativni stres od drugih GABA interneurona.

Smanjen broj PV+ interneurona može dovesti do poremećaja inhibicije kore, desinhronizovane aktivnosti, kao i prekomerne ekscitacije njihovih ciljnih neurona. Poremećaji u inhibiciji kore mogu izazvati glutamatom-posredovanu toksičnost i u samim PV+ neuronima. Ovi neuroni su posebno osetljivi na dezinhibiciju kore i ekscitotoksičnost upravo zbog velike osetljivosti na oksidativni stres koja je diskutovana ranije u tekstu. Njihova aktivacija dezinhibiranim glutamatskim aferentnim vlaknima povećava unutarćelijsku koncentraciju Ca^{2+} što doprinosi daljoj hipofunkciji PV+ neurona i samim tim daljoj kortikalnoj dezinhibiciji.

5.3.2 Podregion-zavisni protektivni efekti fluoksetina i klozapina na broj PV+ ćelija kod izolovanih pacova

Tretman fluoksetinom je održao broj PV+ ćelija na nivou zabeleženom kod kontrolnih pacova u PrL i IL, ali ne u Cg1 i DP podregionima medijalne prečeone zone kore cerebruma u uslovima hronične izolacije pacova. Protektivan efekat ovog antidepresiva na PV+ interneurone pokazan je ranije u hipokampusu (Czeh i sar., 2005; Godavarthi i sar., 2014; Filipović i sar., 2018), dok je u ovoj studiji po prvi put predstavljen njegov podregion-zavisni zaštitni efekat u medijalnoj prečeonoj zoni kore cerebruma. Medijalna prečeona zona kore cerebruma, pre svega cingulatni, prelimbički i infralimbički delovi, bogato je inervisana serotoninskim neuronima iz dorzalnog i medijalnog jedra rafe (Puig i Gulledge, 2011). Snažna inervacija serotoninskim neuronima i veliki broj serotoninskih receptora u ovom moždanom regionu sugerišu da je ovaj neurotransmiter glavni modulator njegove funkcije. Poznato je da 5-HT_{1A} i 5-HT_{2A} serotoninske receptore eksprimiraju i piramidni neuroni i interneuroni prečeone zone kore cerebruma pacova (Santana i sar., 2004). Zanimljivo, pokazano je da fluoksetin povećava ekscitabilnost PV+ brzookidajućih interneurona prečeone zone kore i to na dozno-zavisni način, dok na piramidne neurone ima slab efekat (Zhong i Yan, 2011). To ukazuje da su brzookidajući interneuroni značajna meta ovog leka. Pored toga pokazano je da fluoksetin održava broj PV+ ćelija na kontrolnom nivou u dentatnom girusu hipokampusne kore u uslovima dugotrajnog psihosocijalnog stresa (Czeh i sar., 2005).

Što se tiče efekta klozapina, rezultati su pokazali da ovaj antipsihotik ima protektivan efekat u PrL i IL podregionima, poput fluoksetina. Literaturni podaci o efektima klozapina na PV+ interneurone su malobrojni. Jedna studija je pokazala da tronedeljni tretman klozapinom (25 mg/kg/dan) ne dovodi do promena u PV-imunoreaktivnosti u prečeonoj zoni kore, i hipokampusu nestresiranih pacova (Cahir i sar., 2005), što je u skladu sa rezultatima ove disertacije koji su pokazali da se broj PV+ ćelija u svim ispitivanim podregionima medijalne prečeone zone kore cerebruma nestresiranih pacova tretiranih klozapinom ne razlikuje u odnosu na kontrolne. Inače, pozitivni efekti klozapina i fluoksetina na očuvanje broja PV+ ćelija u PrL, koji je paralelan sa njihovim anksiolitičkim efektima, govori u prilog tome da je ovaj podregion značajan sa aspekta regulacije anksioznosti, o čemu je diskutovano ranije.

Opisani rezultati ukazuju da disregulacija ihnibicije medijalne prečeone zone kore cerebruma može biti jedan od uzroka promena ponašanja pacova u uslovima hronične izolacije. Protektivno delovanje antidepresiva fluoksetina i antipsihotika klozapina na PV+ interneurone u PrL i IL ukazuje da je GABA signalizacija u ovim podregionima medijalne prečeone zone kore veoma značajna za promene ponašanja izazvane hroničnom izolacijom.

5.4 EFEKAT FLUOKSETINA I KLOZAPINA NA JETRU NESTRESIRANIH I IZOLOVANIH PACOVA

Jetra je organ u kome se u najvećoj meri odvija metabolizam, detoksifikacija i bioaktivacija lekova. Stoga je jedan od ciljeva disertacije bio da se ispitaju efekti hroničnog apliciranja fluoksetina, odnosno klozapina na jetru pacova u uslovima hronične izolacije.

Rezultati istraživanja su pokazali da i fluoksetin i klozapin izazivaju oksidativni stres i dovode do oštećenja proteina i lipida u ćelijama jetre nestresiranih i izolovanih pacova, sudeći po povišenim nivoima karbonilnih grupa proteina i MDA. Oksidacija aminokiselinskih ostataka, pre svega lizina, arginina i prolina dovodi do formiranja reaktivnih aldehida i ketona, odnosno karbonilnih derivata proteina. Karbonilne grupe su glavni produkt oksidacije posredovane ROS, pa one predstavljaju opšti i najčešće korišćen indikator oksidativnih oštećenja proteina (Stadtman i Levine, 2000). Oksidacija aminokiselinskih ostataka dovodi do malih strukturnih modifikacija proteina koje mogu u velikoj meri uticati na njihovu funkciju u ćeliji. ROS mogu reagovati i sa polinezasićenim masnim kiselinama i na taj način inicirati peroksidaciju lipida u ćelijama i formiranje aldehidnih sporednih proizvoda poput MDA i 4-hidroksi-2-nonenal (HNE) (Esterbauer i sar., 1991). ROS su, zbog velike reaktivnosti, kratkoživeći, te imaju veoma ograničen radijus difuzije. Međutim, MDA i HNE imaju duži poluživot i mogu difundovati dalje od mesta nastanka i na taj način pogoršati efekte oksidativnog stresa (Rolo i sar., 2012). Oštećenja proteina i lipida uočena u jetri pacova tretiranih fluoksetinom ili klozapinom mogu biti posledica povećane produkcije $O_2^{\bullet -}$ usled aktivnosti CYP enzima odgovornih za metabolizam ovih lekova. CYP enzimi koriste molekulski kiseonik za kombinovanu oksidaciju target ksenobiotika (u ovom slučaju

leka) i NADPH, pa predstavljaju značajne potencijalne izvore ROS (Bondy i Naderi, 1994). Pored toga, značajan izvor ROS u jetri su mitohondrije. Na mitohondrijama izolovanim iz jetre je pokazano da fluoksetin i norfluoksetin remete proces oksidativne fosforilacije (Souza i sar., 1994) što može prouzrokovati povećanu proizvodnju prooksidanata. Zatim, Kupferove ćelije, aktivirane u odgovoru na hepatocelularna oštećenja inicirana metabolizmom leka, mogu doprineti oštećenjima povećanom produkcijom ROS i RNS molekula, kao i inflamatornih citokina i hemokina (Barnes i sar., 2013). Inače, u uslovima oksidativnog stresa može doći i do peroksidacije lipida, koji se nalaze u membranama organela i ćelija, što za posledicu može imati njihovu povećanu permeabilnost i narušavanje integriteta ovih struktura (Wong-Ekkabut i sar., 2007; Yajima i sar., 2009). Zanimljivo, povećani nivo karbonilnih grupa i MDA uočen je i u jetri izolovanih pacova koji su primali fiziološki rastvor što znači da sama izolacija uzrokuje oksidativna oštećenja proteina i lipida u jetri. Ovi rezultati su u saglasnosti sa studijom koja je pokazala da ponovljeno izlaganje fizičkom sputavanju i prinudnom plivanju smanjuje aktivnost antioksidativnih enzima i povećava koncentraciju MDA u jetri pacova (Devaki i sar., 2013).

Deficit GSH, uočen u jetri svih eksperimentalnih grupa sa izuzetkom nestresiranih životinja tretiranih fluoksetinom, glavni je indikator prooksidativnog stanja i često je spregnut sa oštećenjima jetre (Han i sar., 2006). Generalno, smanjene zalihe redukovanog oblika glutationa mogu biti posledica njegove povećanje potrošnje usled (a) hvatanja slobodnih radikala, (b) održavanja -SH grupa proteina u redukovanom stanju, (c) njegovog korišćenja kao ko-faktora GST enzima u procesu detoksifikacije ksenobiotika, (d) njegove oksidacije u procesima detoksifikacije H_2O_2 ili lipidnih peroksida koji su posredovani GPx enzimom (Gupta i sar., 2005). Đorđević i saradnici (2010) su pokazali da 21-dnevna izolacija adultnih mužjaka Wistar pacova uzrokuje smanjenje nivoa i aktivnosti GLR enzima u jetri. Na taj način izolacija narušava obnavljanje pula redukovanog GSH, što objašnjava niže vrednosti GSH u sve tri grupe izolovanih pacova. Kompromitovanje antioksidativnog sistema u vidu smanjenja nivoa GSH i aktivnosti GLR enzima, kao i povećanje nivoa MDA u jetri pacova, zapaženo je i u modelu ponovljenog fizičkog sputavanja (Zafir i Banu, 2007). U jetri nestresiranih pacova, samo je klozapin redukovao zalihe GSH. Vrlo je verovatno da je uzrok tome povećana potrošnja GSH na detoksifikaciju metabolita ovog leka.

Konjugacija reaktivnih metabolita lekova i GSH je značajan detoksifikacioni mehanizam koji se može odvijati spontano ili posredstvom GST enzima. U odsustvu GST, identifikovana su tri GSH konjugata sa nitrenijum jonom poreklom od klozapina, a prisustvo GST enzima intenzivira proces konjugacije (Dragovic i sar., 2010). Dakle, GST ima značajnu ulogu u inaktivaciji reaktivnih metabolita klozapina, te je značajno povećanje aktivnosti ovog enzima u jetri obe grupe životinja tretiranih ovim lekom očekivano. Porast aktivnosti GST zabeležen je u jetri svih eksperimentalnih grupa, sa izuzetkom izolovanih pacova tretiranih fluoksetinom. Ovaj rezultat je neočekivan s obzirom da i izolacija i fluoksetin pojedinačno dovode do značajnog povećanja aktivnosti GST. Međutim, kod izolovanih pacova tretiranih fluoksetinom izmerene su najniže vrednosti nivoa GSH, pa je možda njegova nedostupnost, kao ko-faktora GST enzima, odgovorna za odsustvo porasta aktivnosti ovog enzima. Inače, povećana aktivnost GST enzima detektovana je u plazmi pacova koji su mesec dana tretirani ovim antidepresivom u dozi od 24 mg/kg/dan (Inkielewicz-Stêpniak, 2011). Povećana aktivnost GST u jetri izolovanih pacova tretiranih fiziološkim rastvorom može biti posledica angažovanja ovog enzima u detoksifikaciji produkata lipidne peroksidacije koja je kod ovih životinja intenzivirana sudeći po povećanom nivou MDA. Naime, poznato je da GST izoenzimi, pored toga što detoksifikuju elektrofilne ksenobiotike, takođe imaju peroksidaznu aktivnost (Yang i sar., 2001).

U sklopu ispitivanja antioksidativnog kapaciteta u jetri, praćen je i nivo CuZnSOD enzima. Značajno povećanje nivoa ovog antioksidativnog enzima, u odnosu na kontrolnu grupu, primećeno je samo kod izolovanih pacova tretiranih fiziološkim rastvorom. Međutim, prethodni rezultati naše laboratorije su pokazali da izolacija ne dovodi do promene u aktivnosti CuZnSOD enzima u citosolu jetre (Zlatković i Filipović, 2011). Bazalna aktivnost, uprkos povećanom nivou CuZnSOD može biti posledica posttranslacione modifikacije koja utiče na aktivnost enzima, nagomilavanja produkta reakcije koju katalizuje (H_2O_2), ili drugih ROS molekula, ili pak potrošnje aktivnog enzima zbog produženog oksidativnog stresa (Pajovic i sar., 2006).

Oštećenju jetre, pored oksidativnog, može doprineti i nitrozativni stres. Povećan nivo NO metabolita koji sugeriše povećanu produkciju NO, detektovan je kod izolovanih pacova koji su primali fiziološki rastvor i kod obe grupe pacova koje su tretirane klozapinom (nestresirani i izolovani). NO u jetri sintetišu endotelna NO sintaza

(eNOS) Kupferovih ćelija, kao i inducibilna forma (iNOS) hepatocita, Kupferovih i stelatnih ćelija pod dejstvom odgovarajućeg stimulusa (Muriel, 2000). Signalni molekuli, NO i $O_2^{\bullet-}$, koje stvaraju aktivirane Kupferove ćelije mogu aktivirati transkripcioni faktor NF-κB, što promoviše dalje oslobađanje ovih reaktivnih vrsta. Aktivacija ovog proinflamatornog transkripcionog faktora, i njegova translokacija u jedro je upravo i uočena kod izolovanih pacova tretiranih fiziološkim rastvorom ili klozapinom, kod kojih je detektovan povećan nivo NO. S obzirom da NF-κB reguliše ekspresiju iNOS, njegova aktivacija favorizuje dalju povećanu produkciju NO. Uz to, kod ovih životinja je uočena povećana ekspresija COX-2 enzima koji katalizuje sintezu prostaglandina, potentnih proinflamatornih medijatora. Međutim, kod nestresiranih životinja tretiranih klozapinom nije došlo do aktivacije NF-κB i posledične povećane ekspresije COX-2 upkos povećanoj produkciji NO u jetri. Zanimljivo, fluoksetin je sprečio kako povećanje nivoa NO, tako i aktivaciju NF-κB i pojačanu ekspresiju COX-2 u jetri izolovanih pacova. Antiinflamatorno svojstvo fluoksetina uočeno je ranije u *in vitro* sistemima. Pokazano je da inhibira degradaciju IκB, fosforilaciju i translokaciju NF-κB-p65 u jedro i smanjuje ekspresiju TNF-α i iNOS, kao i produkciju NO u mikroglijskim ćelijama aktiviranim pomoću LPS (Liu i sar., 2011). Takođe, imunosupresivan efekat preko inhibicije NF-κB demonstriran je u ćelijskoj kulturi mišjih peritonealniih makrofaga (Ghosh i sar., 2015). Inače, aktivacija NF-κB praćena povećanom ekspresijom proinflamatornih medijatora smatra se kritičnim korakom u inicijaciji oštećenja u jetri. Na modelu alkoholne bolesti jetre je pokazano da ove molekularne promene prethode pojavi histopatoloških promena u jetri (Jokelainen i sar., 2001). U skladu sa tim, analiza histoloških karakteristika jetre je pokazala da su najizraženije promene u strukturi tkiva uočene upravo kod izolovanih pacova koji su tretirani klozapinom.

Histopatološka analiza je pokazala da sama izolacija ne izaziva velike promene na nivou strukture tkiva jetre, jer je spektar histoloških karakteristika uočenih kod izolovanih pacova sličan onom koji je viđen kod nestresiranih životinja. Razlika je u tome što su neke promene, poput inflamatornih infiltrata u portnim trijadama i makrovezikularnih masnih promena, naglašenije kod izolovanih pacova. U jetri nestresiranih životinja koje su tretirane fluoksetinom ili klozapinom takođe su uočeni inflamatorni infiltrati u portnim trijadama koji su sačinjeni od limfocita, makrofaga i,

retko, od neutrofila. Veliki broj različitih tipova ćelija urođenog imuniteta kao što su Kupferove ćelije, NK i NKT ćelije, su rezidentne ćelije jetre, međutim drugi leukociti poput neutrofila i monocita, mogu biti regrutovani tokom inflamacije. Poznato je da je jetra mesto u kom, u okviru završne faze perifernog imunskog odgovora, dolazi do apoptoze limfocita, što je bitno sa aspekta održavanja homeostaze imunskog sistema i sprečavanja autoimunosti (Ikeda i Yoshikawa, 2003). Međutim, infiltriranje aktivnih B i T limfocita u jetri može biti u vezi sa histopatološkim procesima. Oštećenja jetre izazvana lekovima (DILI) su često povezana sa infiltratima limfocita, a od prirode i obima inflamacije zavisi progresija i ozbiljnost oštećenja tkiva. Neutrofili, čija je pojava u infiltratima nestresiranih životinja tretiranih fluoksetinom ili klozapinom zabeležena, mada retko, često su uočljivi tokom ranog odgovora na oštećenje tkiva (Jaeschke i Tadashi, 2006). Ove ćelije mogu imati značajnu ulogu u hepatotoksičnom efektu leka jer predstavljaju značajan izvor ROS, pre svega $O_2^{\cdot -}$ koji nastaje aktivnošću NOX-2 enzima (Adams i sar., 2010). H_2O_2 koji nastaje dismutacijom $O_2^{\cdot -}$ može da difunduje direktno u hepatocite gde izaziva unutarćelijski oksidativni stres (Jaeschke i sar., 1999), ili se posredstvom mijeloperoksidaze prevodi u hipohlornu kiselinu koja je takođe snažan oksidant koji može difundovati u ćelije i dovesti do oksidativnih oštećenja lipida, proteina i nukleinskih kiselina (Gujral i sar., 2004; Malle i sar., 2006). Povećane količine ROS, zajedno sa lizozomalnim enzimima koje oslobađaju neutrofili, mogu dovesti do oštećenja tkiva jetre.

Hronične terapije fluoksetinom i klozapinom uzrokovale su fokalnu nekrozu hepatocita kako nestresiranih, tako i izolovanih pacova. Zanimljivo je da su kod jedne nestresirane životinje tretirane klozapinom uočene spojna i konfluentna nekroza zajedno sa endotelitisom centralne venule, što ukazuje na idiosinkratsku reakciju te jedinke na primljeni lek. Idiosinkratska reakcija podrazumeva neželjenu reakciju preosetljivosti na lek koja se javlja kod malog broja pacijenata i nije u očiglednoj relaciji sa dozom ili trajanjem terapije. Spojna nekroza je zonalna nekroza koja se prostire na više zona jednog lobulusa i zahvata i susedni, dok konfluentna nekroza obuhvata veći broj lobulusa (Krishna, 2017). Idiosinkratsku reakciju na lek najčešće izazivaju reaktivni metaboliti leka. Klozapin je lek koji se gotovo u potpunosti metaboliše pre ekstrakcije. Glavni metaboliti su derivati N-demetilacije, N-oksidacije i hidroksilacije aromatičnog prstena (Walgren i sar., 2005). Kako je pomenuto ranije u diskusiji, klozapin podleže

bioaktivaciji u toksičan, reaktivan nitrenijum jon posredstvom CYP enzima. Reaktivni metabiliti lekova se mogu kovalentno vezivati za proteine formirajući lek-protein komplekse koji mogu uzrokovati toksičnost direktno, ili posredstvom imunskog sistema (Zhou i sar., 2005).

Dakle, bez obzira da li su u pitanju nestresirane ili izolovane životinje, značajnije histopatološke promene jetre su uočene kod pacova tretiranih klozapinom, nego kod onih tretiranih fluoksetinom. To se odnosi i na masne promene koje su posledica abnormalnog zadržavanja lipida u ćeliji, koje su takođe izraženije kod pacova koji su primali klozapin. Antipsihotici utiču na ekspresiju brojnih gena povezanih sa biosintezom lipida i masnih kiselina (Foley i Mackinnon, 2014). Lipogena svojstva klozapina pokazana su u primarnoj kulturi hepatocita pacova (Lauressergues i sar., 2010), kao i u kulturi imortalizovanih humanih hepatocita (Lauressergues i sar., 2012). Ferno i saradnici su otkrili da samo jedna i.p. doza klozapina (50 mg/kg) može poremetiti homeostazu lipida u jetri (Fernø i sar., 2009). Masne promene u hepatocitima mogu biti makrovezikularne i mikrovezikularne prirode. U slučaju makrovezikularne steatoze jedna velika masna vakuola ispunjava hepatocit i dislocira jedro na periferiju ćelije. Ukoliko ne postoje druge promene, makrovezikularna steatoza ima dobru dugoročnu prognozu. Ove masne promene su uočene nakon primene oba leka. Sa druge strane, difuzna, mikrovezikularna steatoza koju karakterišu uvećani hepatociti sa malim lipidnim vezikulama (manje od 1 μm u prečniku) je često asocirana sa insuficijencijom jetre i encefalopatijom (Tira i sar., 2011). Mikrovezikularna steatoza je u vezi sa ozbiljnim narušavanjem procesa β-oksidacije masnih kiselina u mitohondrijama (Fromenty i sar., 1997). U takvim uslovima masne kiseline većinom bivaju esterifikovane u trigliceride koji se akumuliraju u vidu malih vezikula (Fromenty i Pessayre, 1997). Poznato je da lekovi mogu narušiti oksidaciju masnih kiselina i na taj način indukovati mikrovezikularnu steatozu (Begriche i sar., 2011). U ovoj studiji, mikrovezikularne promene su uočene u hepatocitima nestresiranih pacova tretiranih klozapinom. To ne iznenađuje s obzirom da je ranije pokazano da jedna doza klozapina dovodi do smanjene ekspresije gena čiji su produkti uključeni u procese β-oksidacije masnih kiselina i lipolize (Fernø i sar., 2009).

Histopatološke promene u jetri nestresiranih pacova tretiranih fluoksetinom ili klozapinom, kod kojih nije detektovana aktivacija NF-κB i pojačana ekspresija COX-2,

ukazuju da proinflamatorni odgovor nije nužan za oštećenja tkiva jetre uzrokovano lekovima. U prilog tome govori i to što je idiosinkratsku reakciju na lek razvila životinja tretirana klozapinom koja nije podvrgavana izolaciji. Postoje indicije da inflamacija u jetri povećava hepatoksičnost nekog agensa. Tako je pokazano da neki ksenobiotici ispoljavaju veću hepatotoksičnost kada se primenjuju paralelno sa LPS koji podstiče skromnu inflamaciju u vidu ekspresije citokina i COX-2 (Roth i sar., 2003). Može se reći da su histopatološke promene kod životinja tretiranih klozapinom izraženije kod izolovanih pacova u odnosu na nestresirane, ukoliko se zanemari jedinka sa idiosinkratskom preosetljivošću na lek. U slučaju fluoksetina, neke promene poput fokalne nekroze, apoptotičnih hepatocita i makrovezikularnih masnih promena izraženije su kod nestresiranih nego kod izolovanih pacova. Međutim, kod pacova tretiranih ovim antidepresivom, parametri inflamacije nisu povećani kako u jetri izolovanih, tako i nestresiranih pacova.

Dakle, uprkos tome što izaziva oksidativna oštećenja u jetri, izolacija ne dovodi do narušavanja strukture tkiva ovog organa. Hronična primena fluoksetina u dozi od 15 mg/kg/dan, odnosno koncentraciji ~250 ng/mL seruma, uzrokuje oksidativna oštećenja i blage histopatološke promene, ali ne narušava ozbiljno strukturu jetre kod nestresiranih i izolovanih pacova. Hroničan tretman klozapinom u dozi od 20 mg/kg/dan, odnosno koncentraciji ~100 ng/mL seruma, koja odgovara donjoj granici terapijskih doza kod pacijenata, dovodi do upadljivih histopatoloških promena jetre obe grupe životinja. Ovi rezultati ukazuju da se hepatotoksični efekti ne smeju zanemariti prilikom određivanja optimalnih doza fluoksetina, a naročito klozapina.

6 ZAKLJUČCI

Hronična izolacija odraslih Wistar pacova, u trajanju od 21 dana, izaziva promene u ponašanju koje nalikuju depresiji. Ponašanja izolovanih pacova koja odgovaraju očaju, anhedoniji i anksioznosti, koincidirala su sa smanjenjem efikasnosti GSH-zavisnog antioksidativnog sistema i povećanjem nivoa proinflamatornih medijatora u hipokampusu i prečeonoj zoni kore cerebruma. Dobijeni rezultati ukazuju da je prečeona zona osetljivija na psihosocijalni stresor izolacije od hipokampusa. Kod izolovanih pacova uočen je i smanjen broj PV+ ćelija u medijalnoj prečeonoj zoni kore cerebruma.

Oba leka, aplicirana tokom stresa u dozama koje su bliske donjim granicama terapijskih doza kod pacijenata, sprečavaju razvoj promena u ponašanju koje izaziva izolacija, ali različito utiču na neke od parametara praćene u ovoj studiji.

U skladu sa ciljevima postavljenim u ovoj doktorskoj disertaciji ustanovljeno je da:

- Hronična izolacija pacova u trajanju od 21 dana dovodi do:

    1. Razvoja ponašanja nalik depresivnom;

    2. Kompromitovanja GSH-zavisnog sistema u hipokampusu i prečeonoj zoni kore, porasta nivoa TNF-α u hipokampusu, kao i nivoa COX-2, IL-1β i TNF-α u prečeonoj zoni kore cerebruma;

    3. Smanjenja broja PV+ ćelija u svim ispitivanim podregionima medijalne prečeone zone kore cerebruma;

    4. Oksidativnog stresa, oštećenja proteina i lipida jetre ali ne i histoloških promena.

- Hronični tretman fluoksetinom ili klozapinom u trajanju od 21 dana:

    1. Sprečava razvoj ponašanja sličnih depresiji u uslovima hronične izolacije;

    2. Ostvaruje protektivno dejstvo kada je reč o proinflamatornim promenama indukovanim izolacijom u oba ispitivana moždana regiona, a u slučaju fluoksetina i u okviru GSH-zavisnog sistema u prečeonoj zoni kore cerebruma;

    3. Održava broj PV+ ćelija u prelimbičkom i infralimbičkom podregionu medijalne prečeone zone kore cerebruma u uslovima hronične izolacije na nivou zabeleženom kod kontrolnih pacova;

    4. Izaziva oksidativna oštećenja u jetri, s tim da klozapin indukuje upadljivije histopatološke promene što znači da je hepatotoksičan.

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Ova studija potvrđuje validnost hronične izolacije adultnih pacova kao modela za ispitivanje patofiziologije depresije. Dobijeni rezultati pokazuju ulogu proinflamatornih promena u hipokampusu i prečeonoj zoni kore cerebruma, kao i disregulacije GABA signalizacije u medijalnoj prečeonoj zoni kore mozga pacova u etiopatogenezi simptoma koji nalikuju depresivnim. Fluoksetin i klozapin inhibiraju proinflamatorne promene u hipokampusu i prečeonoj zoni kore cerebruma, i sprečavaju narušavanje inhibicije u prelimbičkom i infralimbičkom podregionu medijalne prečeone zone kore. Osnovano je pretpostaviti da pomenuti protektivni efekti lekova uočeni na molekularnom nivou doprinose njihovim antidepresivnim i anksiolitičkim dejstvima.

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БИОГРАФИЈА АУТОРА

Невена Р. Тодоровић је рођена 20. августа 1986. године у Горњем Милановцу, где је завршила основну школу и гимназију општег смера. Биолошки факултет Универзитета у Београду, смер Молекуларна биологија и физиологија, уписала је 2005 године. Дипломирала је 2012. године, са просечном оценом 9,39, одбранивши дипломски рад под називом „Анализа аутоантитела након хиперимунизације тетанус токсоидом код два соја лабораторијских мишева" са оценом 10. Докторске студије је уписала 2012. године на Биолошком факултету Универзитета у Београду, студијски програм Биологија, модул Анимална и хумана физиологија.

Невена Тодоровић је од 1.9.2012. године запослена у Институту за нуклеарне науке „Винча" у Лабораторији за молекуларну биологију и ендокринологију, као истраживач приправник. У звање истраживача сарадника први пут је изабрана 17.7.2014., а реизабрана 30.5.2017. године одлукама Научног већа Института за нуклеарне науке „Винча", Универзитета у Београду.

Научно-истраживачки рад Невене Тодоровић реализован је кроз пројекте: „Молекуларни механизми патофизиолошких промена у ћелијама централног нервног система и периферног ткива код сисара" (173044) и „Ћелијске и молекулске основе малигних и кардиоваскуларних обољења" (III41027), финансиране од стране Министарства просвете, науке и технолошког развоја Републике Србије. До сада је објавила четири научна рада из категорије М21, три рада из категорије М22, један рад категорије М23, и имала тринаест саопштења на међународним и домаћим научним скуповима.

Прилог 1.

Изјава о ауторству

Потписани-а Невена Тодоровић

број индекса Б3008/2012

Изјављујем

да је докторска дисертација под насловом

Ефекти флуоксетина и клозапина на антиоксидативни систем и параметре инфламације у мозгу и јетри пацова у условима хроничне изолације

- резултат сопственог истраживачког рада,
- да предложена дисертација у целини ни у деловима није била предложена за добијање било које дипломе према студијским програмима других високошколских установа,
- да су резултати коректно наведени и
- да нисам кршио/ла ауторска права и користио интелектуалну својину других лица.

Потпис докторанда

У Београду, 24.9.2018.

Невена Тодоровић

Прилог 2.

Изјава о истоветности штампане и електронске верзије докторског рада

Име и презиме аутора Невена Тодоровић

Број индекса Б3008/2012

Студијски програм Биологија

Наслов рада Ефекти флуоксетина и клозапина на антиоксидативни систем и параметре инфламације у мозгу и јетри пацова у условима хроничне изолације

Ментор др Драгана Филиповић и проф. др Јелена Ђорђевић

Потписани/а Невена Тодоровић

Изјављујем да је штампана верзија мог докторског рада истоветна електронској верзији коју сам предао/ла за објављивање на порталу **Дигиталног репозиторијума Универзитета у Београду.**

Дозвољавам да се објаве моји лични подаци везани за добијање академског звања доктора наука, као што су име и презиме, година и место рођења и датум одбране рада.

Ови лични подаци могу се објавити на мрежним страницама дигиталне библиотеке, у електронском каталогу и у публикацијама Универзитета у Београду.

Потпис докторанда

У Београду, 24.9.2018.

Невена Тодоровић

Прилог 3.

Изјава о коришћењу

Овлашћујем Универзитетску библиотеку „Светозар Марковић“ да у Дигитални репозиторијум Универзитета у Београду унесе моју докторску дисертацију под насловом:

Ефекти флуоксетина и клозапина на антиоксидативни систем и параметре инфламације у мозгу и јетри пацова у условима хроничне изолације

која је моје ауторско дело.

Дисертацију са свим прилозима предао/ла сам у електронском формату погодном за трајно архивирање.

Моју докторску дисертацију похрањену у Дигитални репозиторијум Универзитета у Београду могу да користе сви који поштују одредбе садржане у одабраном типу лиценце Креативне заједнице (Creative Commons) за коју сам се одлучио/ла.

1. Ауторство

2. Ауторство - некомерцијално

3. Ауторство – некомерцијално – без прераде

4. Ауторство – некомерцијално – делити под истим условима

5. Ауторство – без прераде

6. Ауторство – делити под истим условима

(Молимо да заокружите само једну од шест понуђених лиценци, кратак опис лиценци дат је на полеђини листа).

Потпис докторанда

У Београду, 24. 9. 2018.

Невена Ајдачић

1. Ауторство - Дозвољавате умножавање, дистрибуцију и јавно саопштавање дела, и прераде, ако се наведе име аутора на начин одређен од стране аутора или даваоца лиценце, чак и у комерцијалне сврхе. Ово је најслободнија од свих лиценци.

2. Ауторство – некомерцијално. Дозвољавате умножавање, дистрибуцију и јавно саопштавање дела, и прераде, ако се наведе име аутора на начин одређен од стране аутора или даваоца лиценце. Ова лиценца не дозвољава комерцијалну употребу дела.

3. Ауторство - некомерцијално – без прераде. Дозвољавате умножавање, дистрибуцију и јавно саопштавање дела, без промена, преобликовања или употребе дела у свом делу, ако се наведе име аутора на начин одређен од стране аутора или даваоца лиценце. Ова лиценца не дозвољава комерцијалну употребу дела. У односу на све остале лиценце, овом лиценцом се ограничава највећи обим права коришћења дела.

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5. Ауторство – без прераде. Дозвољавате умножавање, дистрибуцију и јавно саопштавање дела, без промена, преобликовања или употребе дела у свом делу, ако се наведе име аутора на начин одређен од стране аутора или даваоца лиценце. Ова лиценца дозвољава комерцијалну употребу дела.

6. Ауторство - делити под истим условима. Дозвољавате умножавање, дистрибуцију и јавно саопштавање дела, и прераде, ако се наведе име аутора на начин одређен од стране аутора или даваоца лиценце и ако се прерада дистрибуира под истом или сличном лиценцом. Ова лиценца дозвољава комерцијалну употребу дела и прерада. Слична је софтверским лиценцама, односно лиценцама отвореног кода.

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Chronic administration of fluoxetine or clozapine induces oxidative stress in rat liver: A histopathological study

Jelena Zlatković [a,1], Nevena Todorović [a,1], Nada Tomanović [b], Maja Bošković [a], Snežana Djordjević [c], Tamara Lazarević-Pašti [d], Rick E. Bernardi [e], Aleksandra Djurdjević [f], Dragana Filipović [a,*]

[a] Laboratory of Molecular Biology and Endocrinology, Institute of Nuclear Sciences "Vinča", University of Belgrade, Serbia
[b] Institute of Pathology, School of Medicine, University of Belgrade, Serbia
[c] Poisoning Control Centre, Military Medical Academy, Belgrade, Serbia
[d] Department of Physical Chemistry, Institute of Nuclear Sciences "Vinča", University of Belgrade, Serbia
[e] Central Institute of Mental Health, Institute of Psychopharmacology, Mannheim, Germany
[f] Medicines and Medical Devices Agency of Serbia, Belgrade, Serbia

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ABSTRACT

Chronic exposure to stress contributes to the etiology of mood disorders, and the liver as a target organ of antidepressant and antipsychotic drug metabolism is vulnerable to drug-induced toxicity. We investigated the effects of chronic administration of fluoxetine (15 mg/kg/day) or clozapine (20 mg/kg/day) on liver injury via the measurement of liver enzymes, oxidative stress and histopathology in rats exposed to chronic social isolation (21 days), an animal model of depression, and controls. The activity of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST), the liver content of carbonyl groups, malonyldialdehyde (MDA), reduced glutathione (GSH), cytosolic glutathione S-transferase (GST) and nitric oxide (NO) metabolites were determined. We also characterized nuclear factor-κB (NF-κB), cyclooxygenase-2 (COX-2) and CuZn-superoxide dismutase (CuZnSOD) protein expression as well as histopathological changes. Increased serum ALT activity in chronically-isolated and control animals treated with both drugs was found while increased AST activity was observed only in fluoxetine-treated rats (chronically-isolated and controls). Increased carbonyl content, MDA, GST activity and decreased GSH levels in drug-treated controls/chronically-isolated animals suggest a link between drugs and hepatic oxidative stress. Increased NO levels associated with NF-κB activation and the concomitant increased COX-2 expression together with compromised CuZnSOD expression in clozapine-treated chronically-isolated rats likely reinforce oxidative stress, observed by increased lipid peroxidation and GSH depletion. In contrast, fluoxetine reduced NO levels in chronically-isolated rats. Isolation induced oxidative stress but histological changes were similar to those observed in vehicle-treated controls. Chronic administration of fluoxetine in both chronically-isolated and control animals resulted in more or less normal hepatic architecture, while clozapine in both groups resulted in liver injury. These data suggest that clozapine appears to have a higher potential to induce liver toxicity than fluoxetine.

1. Introduction

Chronic psychosocial stress contributes to the pathogenesis of mood and anxiety disorders (Strekalova et al., 2005), which may be caused by serotonin, norepinephrine or dopamine deficiency in the brain (Feighner, 1999; Trujillo, 1996). Fluoxetine, an antidepressive drug, inhibits the reuptake of serotonin by the serotonin reuptake transporter, thus enhancing and prolonging serotonin signaling, while the atypical antipsychotic clozapine (Meltzer, 1995) has affinity for both serotonergic and dopaminergic receptors (Bymaster et al., 1996). As the liver is the primary site of drug metabolism, the effect of drugs on the integrity of the liver is important. Fluoxetine and clozapine are extensively metabolized in the liver by the isoenzymes of the mixed-function oxidase cytohrome P450 system, primarily CYP1A2 and CYP2D6 isozymes,

* Corresponding author. Address: Laboratory of Molecular Biology and Endocrinology, Institute of Nuclear Sciences "Vinča", University of Belgrade, P.O. Box 522-090, 11001 Belgrade, Serbia. Tel./fax: +381 (11) 6455 561.
E-mail address: dragana@vinca.rs (D. Filipović).
URL: http://www.vinca.rs (D. Filipović).
[1] These authors contributed equally to this work.

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respectively (Dumortier et al., 2002; Inkielewicz-Stepniak, 2011). Fluoxetine undergoes extensive biotransformation to the active metabolite norfluoxetine (Hiemke and Hartter, 2000), which is pharmacologically comparable to fluoxetine (Wong et al., 1995), while clozapine is metabolized to its active metabolite, norclozapine (desmethylclozapine) (Prior et al., 1999). Intraperitoneally-administered drugs, together with their metabolites, rapidly reach peak levels in the liver (Ferno et al., 2009; Parli and Hicks, 1974). And although these treatments generate acceptable outcomes in mood and anxiety disorders, they may result in hepatic toxicity and undesirable side effects.

It has been shown that drugs may cause liver injury via oxidative/nitrosative stress (Bautista and Spitzer, 1990). Souza et al. (1994) reported that fluoxetine (as well as norfluoxetine) effects energy metabolism in rat liver mitochondria and is potentially toxic in high doses. Its administration in mice has been reported to cause changes such as steatosis (fatty change) and hepatocyte enlargement (Bendele et al., 1992). Studies with clozapine have demonstrated oxidative stress and oxidative cell injury via increased levels of membrane lipid peroxidation and total protein oxidation in the brain and other organs (Barakauskas et al., 2010; Polydoro et al., 2004). Furthermore, hepatotoxicity may be mediated by toxic intermediates of drug metabolism (Castiella and Arenas, 1994).

Biochemical changes in the tissue can be determined by biomarkers of oxidative stress. Thus, lipid peroxidation indicates oxidative damage via an increase in malonyldialdehyde (MDA) that contributes to significant toxicity (Devbhuti et al., 2009), while carbonyl group content is an indicator of protein oxidation (Dalle-Donne et al., 2003; Halim et al., 2004). Alterations in reduced glutathione (GSH) and glutathione S-transferase (GST), which participate in the conjugation of toxic electrophiles with GSH, indicate deleterious oxidative changes (Stadtman, 1992). In addition, oxidative stress may affect protein expression and the activity of antioxidant enzymes such as CuZn-superoxide dismutase (CuZnSOD) (Zlatković and Filipović, 2011). One factor that may link oxidative stress and liver injury is the transcription factor nuclear factor-κB (NF-κB). NF-κB can stimulate the expression of a variety of genes, such as cyclooxygenase-2 (COX-2), detected in Kupffer cells that are believed to be important factor in liver injury (Nieto et al., 2000).

However, the roles of fluoxetine and clozapine on liver injury via liver enzymes, oxidative stress and histopathology of rat liver cells have yet to be determined. In the present study, we investigated the effects of chronic (21 days) administration of these drugs on serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities, liver content of carbonyl group, MDA, GSH, cytosolic GST activity and NO metabolites, as well as cytosolic NF-κB, COX-2 and CuZnSOD protein expression, in addition to histological analysis, following chronic (21 days) isolation (IS), an animal model of depression, and controls. We observed significantly increased oxidative stress in the liver of chronically-isolated rats as compared to vehicle-treated controls. Interestingly, the administration of clozapine and fluoxetine drugs in both chronically-isolated and control animals resulted in a potentially harmful increase in markers of oxidative stress and a compromised antioxidative system. Chronic IS had no significant effect on hepatic histopathological changes, and only the chronic administration of clozapine induced liver injury.

2. Materials and methods

2.1. Drugs

Flunisan tablets (containing 20 mg of fluoxetine-hydrochloride) and Leponex tablets (containing 25 mg of clozapine) were purchased from Hemofarm "Zorka Pharma" Šabac, Serbia and Novartis Pharmaceuticals UK, respectively. Fluoxetine-hydrochloride and clozapine reference standards were obtained from the same company from which the tablets were purchased. To prepare the fluoxetine solution for treatment, the Flunisan tablets were crushed and the content was dissolved in distilled, sterile water with the aid of ultrasound, and filtered through Whatman No. 42 filter paper. Clozapine solution was prepared daily by dissolving 140 mg of Leponex tablets in 0.6 ml of 1 N HCl with gentle heating, then diluting the solution with distilled water to 20 mg/ml. Solutions were neutralized with 1 N NaOH to a pH of 5.1 (Halim et al., 2004). Concentrations of fluoxetin-hydrochloride and clozapine solutions were determined using Ultra Performance Liquid Chromatography (UPLC) analysis (Kovacevic et al., 2006).

2.2. Animals and drug treatments

Adult male Wistar rats, 2.5 months old, weighting 300–350 g at the onset of the experiment served as subjects. Rats were maintained under standard conditions in a temperature-controlled environment (21–23 °C) on a 12 h/12 h light/dark cycle, with food (commercial rat pellets) and water available *ad libitum*. All experimental procedures were carried out in accordance with the Ethical Committee for the Use of Laboratory Animals of the Institute of Nuclear Sciences, "Vinča," which follows the guidelines of the registered "Serbian Society for the Use of Animals in Research and Education." Prior to stress exposure, the animals were housed in groups of four per cage and randomly divided into six groups. Control groups consisted of four animals per cage, while rats underwent chronic social IS stress were housed individually for 21 days, during which animals had normal auditory and olfactory experiences, but were deprived of any visual or tactile contacts with other animals. Fluoxetine-hydrochloride and clozapine were administered daily by intraperitoneal (i.p.) injections of 15 mg/kg and 20 mg/kg, respectively, during the 21 days in both control (Control + Fluox and Control + Cloz groups) and chronically-isolated (IS + Fluox and IS + Cloz groups) rats. The doses of fluoxetine (15 mg/kg/day) and closapine (20 mg/kg/day) were selected on the basis of the previous study in our laboratory, in which both drugs prevented chronic stress-induced depressive- and anxiety-like behaviors in rats (manuscript in preparation). Moreover, used dose of fluoxetine, in the literature data, has shown ability to achieve therapeutic plasma levels within the dose range for the treatment of depression (Czeh et al., 2005), while used dose of clozapine was in accordance with its receptor occupancy (Halim et al., 2004). The serum concentrations of fluoxetine or clozapine were determined by a Liquid Chromatography–Mass Spectrometry (LC–MS) (Djordjevic et al., 2005) and Liquid Chromatography–Tandem Mass Spectrometry (LC–MS–MS) method (Song et al., 2009; Waters, 2008), respectively. For the 15 mg/kg/day of fluoxetine in fluoxetine-treated controls, serum concentrations were 280 ± 50 ng/ml, while in chronically-isolated animals they were in the range of 203 ± 28 ng/ml, similar to those reported in human patients treated with therapeutically effective doses of 20–80 mg/day Prozac (100–700 ng/ml) (Dulawa et al., 2004). There was no significant difference between the Control + Fluox and IS + Fluox groups, that is in agreement with the findings of Czéh et al. (2007). Serum levels for the 20 mg/kg/day of clozapine, was in the range of 103 ± 18 ng/ml in clozapine-treated controls and 123 ± 18 ng/ml in chronically-isolated animals, that were comparable to therapeutic levels (100–700 ng/ml) (Sadock and Sadock, 2008). Vehicle-treated (Control and IS) groups received daily i.p. injections of normal saline (0.9% NaCl).

2.3. Serum hepatospecific markers

Blood samples were collected directly from the heart by cardiac puncture. Serum was obtained by centrifugation at 1500*g* for 10 min at 4 °C. Serum ALT and AST activity were measured by

the method Reitman-Frankel (Crowley, 1967) in all experimental groups. Enzyme activity is expressed in International Units per liter (IU/L).

2.4. Preparation of cytosolic and nuclear extracts from liver tissue

Animals were anesthetized with ketamine/xylazine (100/5 mg/kg i.p.) and the livers of animals from each group were perfused *in situ*, excised and kept frozen (−70 °C) until further analyses. For each subject, on the day of analysis, liver tissue was thawed, weighed and homogenized at 4 °C using 20 strokes of a Potter–Elvehjem glass homogenizer with a Teflon pestle to a ratio 1:4 (w/v) tissue in 50 mM Tris–HCl pH 7.4 buffer (containing 100 mM NaCl, 5 mM $MgCl_2$, 250 mM sucrose, 1 mM Na_2EDTA, 1 mM EGTA, 1 mM DTT, 0.15 mM spermidine, 0.1 mM PMSF). The pellets obtained after centrifugation of the homogenate (10 min, 2000*g*, 4 °C, SS-34 Sorvall centrifuge) was used to prepare the nuclear fraction (Czeh et al., 2005). The obtained supernatant was further centrifuged at 100,000*g* for 60 min at 4 °C in a Beckman L8-M Ultracentrifuge Ti50, to obtain the cytosolic fraction. The protein concentration was measured by the method of Lowry et al. (1951) using purified bovine serum albumin as a standard.

2.5. Determination of protein oxidation

The protein oxidation level was monitored by determination of the carbonyl content via the method of Levine using 2,4-dinitrophenylhydrazine (DNPH) (Levine et al., 1994). Spectrophotometric measurement of reactive carbonyl derivatives values was performed and calculated using the extinction coefficient of DNPH-reactive carbonyl derivatives at 380 nm = 22 mM^{-1} cm^{-1} and expressed as nmol/mg protein.

2.6. Measurement of lipid peroxidation

Lipid peroxidation was estimated by measuring MDA with its reaction with thiobarbituric acid at 535 nm (Albro et al., 1986) using a standard curve prepared with MDA (Sigma Aldrich; lot 10838-3). The results are expressed as nmol/mg protein.

2.7. Determination of reduced glutathione

GSH was determined in the liver homogenate according to Ellman's method (1959) and modified by Hissin and Hilf (1976). The procedure is based on the reduction of Ellman's reagent by –SH groups of GSH to form 2-nitro-s mercaptobenzoic acid. The yellow color developed was read immediately at 405 nm. A calibration curve was performed with standard GSH (0.001–0.1 mM), and GSH concentrations were calculated as nmol/mg protein.

2.8. Determination of cytosolic glutathione-S-transferase activity

GST activity was assessed using the method of Habig et al. (1974). The enzyme was assayed by its ability to conjugate GSH and 1-chloro-2,4-dinitrobenzene (CDNB), the extent of conjugation causing a proportionate change in the absorbance at 340 nm. The specific activity of GST is expressed as μmol of GSH – CDNB conjugate formed per minute per milligram of protein, using an extinction coefficient of 9.6 mM^{-1} cm^{-1}.

2.9. NO metabolites (NO_x^-): nitrite–nitrate (NO_2^- and NO_3^-) levels in the rat liver

NO is a highly reactive molecule that is rapidly converted to the more stable nitrates/nitrites (NO_x^-), which are good markers for NO activity levels. Therefore, for NO assay estimation, NO_x^- levels were measured in the cytosol of liver, where NO_3^- was previously transformed into NO_2^- in the presence of Cd (Cortas and Wakid, 1990). NO_2^- was determined by colorimetric assay using Griess reagent [1% sulfanilamide, 2.5% H_3PO_4, 0.1% N-(1-naphthyl) etylenediamine dihydrochloride] (Navarro-Gonzálvez et al., 1998). The optical density at 550 nm was measured using an ELISA microplate reader. The standard was prepared with several concentrations of $NaNO_2$ (ranging from 0.5 to 10 μM) and the final NO level was expressed as nmol/mg protein. The measurement of NO_x^- levels has been found to be a reliable technique for determination of the synthesizing capacity of NOS in the brain (Salter, 1996).

2.10. Western blot of NF-κB, COX-2 and CuZnSOD

Thirty micrograms of cytosolic proteins per lane were fractionated by reducing 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electroblotted to a polyvinylidene difluoride membrane. Immunodetection was performed with specific primary antibodies against NF-κB (SC-372, Santa Cruz, CA, USA), COX-2 (SC-1747, Santa Cruz, CA, USA) and CuZnSOD (SOD-100, Stressgene Biotechnologies, Victoria, BC, Canada), followed by 2 h with anti-rabbit HRP-conjugated secondary antibody (Santa Cruz Biotechnology). Antigen–antibody complexes were visualized by chemiluminescence using Immobilion western chemilum HRP substrate. To confirm a consistent protein loading for each lane, the membranes were stained for β-actin (SC-1616-R, Santa Cruz Biotechnology). The signals were electronically digitized by scanning and the image was processed for quantification using Image software. Protein molecular mass standards (Pierce Prestained protein Molecular Weight Marker, Thermo Scientific, USA) were used for calibration. Results are expressed as NF-κB, COX-2 and CuZnSOD/β-actin ratio and levels are expressed as % change in relation to those in controls animals taken as 100%.

2.11. Histopathological investigation

Rats were sacrificed by decapitation 24 h after the last fluoxetine and clozapine administration. Fragments of the liver were fixed for 48 h in buffered 10% formalin solution and then embedded in paraffin and sectioned at 5 μm; the sections were stained with hematoxylin and eosin (H&E), coded and evaluated for any histopathological changes using an Olympus BX50F4 microscope and microphotographs were taken using an Olympus DP70 digital camera by a pathologist (TN).

2.12. Statistical analysis

Results were analyzed using a two-way analysis of variance (ANOVA) [the factors were drug treatment (levels: vehicle, fluoxetine and clozapine) and stress (levels: (IS) stress or control]. Duncan's post hoc test was used to evaluate differences between groups. Statistical significance was set at $p < 0.05$. The data are expressed as mean ± standard error of the mean (S.E.M.) of 5–6 animals per group.

3. Results

3.1. Drugs-induced liver transaminases leaking to serum in chronically-isolated rats and controls

The changes in serum AST and ALT activities are shown in Table 1. A two-way ANOVA showed a significant effect of drug treatment on serum ALT ($F_{2,24} = 15.306$, $p < 0.001$) and AST ($F_{2,30} = 9.130$, $p < 0.001$) activity. Post hoc test revealed a statistically significant increases in ALT activity in drug-treated IS and

Table 1
Effect of chronic administration of fluoxetine or clozapine on serum alanine transaminase (ALT) and aspartate transaminase (AST) activity.

	Treatments					
	Control			Isolation		
	Vehicle	Fluox	Cloz	Vehicle	Fluox	Cloz
ALT IU/L	30.17 ± 2.00	43.51 ± 2.05***	36.85 ± 2.34*	32.91 ± 1.80	43.31 ± 1.48***	32.27 ± 2.77*
AST IU/L	62.24 ± 1.01	70.22 ± 2.39*	66.14 ± 2.07	61.96 ± 1.19	69.72 ± 2.10*	63.76 ± 1.42

Results are expressed as mean ± S.E.M. of 5–6 animals per each group. Significant differences between groups obtained from two-way ANOVA analysis followed by Duncan post hoc test are indicated as follows: $^{*}p < 0.05$, $^{***}p < 0.001$ between vehicle-treated rats and drug-treated IS animals or drug-treated controls.

drug-treated control groups ($^{*}p < 0.05$; $^{***}p < 0.001$), as compared to vehicle-treated control animals. With regard to serum AST activity, a significant increase was observed in animals treated with fluoxetine (both chronically-isolated and control animals) ($^{*}p < 0.05$), as compared to vehicle-treated control rats.

3.2. Protein carbonyl content

The protein carbonyl content is presented in Fig. 1. A two-way ANOVA revealed significant main effects of stress ($F_{1.26} = 6.09$, $p < 0.05$) and drug treatment ($F_{2.26} = 6.06$, $p < 0.01$). Significant increase was observed following IS as compared to vehicle-treated controls ($^{*}p < 0.05$). Chronic administration of fluoxetine or clozapine in IS and control groups induced a significant increase in protein carbonyl content as compared to vehicle-treated control rats ($^{**}p < 0.01$, $^{*}p < 0.05$). Post hoc test did not show any significant differences between vehicle- or drug-treated chronically-isolated animals ($p > 0.05$).

3.3. Lipid peroxidation

Lipid peroxidation is indicated by the presence of MDA (Fig. 2). A two-way ANOVA revealed significant main effects of stress ($F_{1.30} = 77.41$, $p < 0.001$) and drug treatment ($F_{2.30} = 33.87$, $p < 0.001$) and a significant drug treatment × stress interaction ($F_{2.30} = 4.33$, $p < 0.05$) on MDA levels. MDA content was increased in drug-treated controls ($^{*}p < 0.05$, $^{***}p < 0.001$) and chronically-isolated animals (vehicle- or drug-treated) ($^{***}p < 0.001$), as compared to vehicle-treated controls. Significant differences in MDA between drug-treated chronically-isolated animals and drug-treated controls ($^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$) were revealed. Post hoc test also showed significant changes in MDA between vehicle- and clozapine-treated IS groups ($^{\wedge\wedge\wedge}p < 0.001$).

3.4. Reduced glutathione (GSH)

Levels of GSH are presented in Fig. 3. A two-way ANOVA revealed significant main effects of stress ($F_{1.30} = 29.93$, $p < 0.001$) and drug treatment ($F_{2.30} = 23.66$, $p < 0.001$), and a significant drug treatment × stress interaction ($F_{2.30} = 10.22$, $p < 0.001$). Post hoc test revealed a significant decrease in GSH levels following both vehicle and drug treatment in chronically-isolated animals, as compared to vehicle-treated controls ($^{**}p < 0.01$, $^{***}p < 0.001$). GSH levels were significantly decreased in chronically-isolated animals treated with clozapine or fluoxetine compared to vehicle-treated IS group ($^{\wedge\wedge}p < 0.01$, $^{\wedge\wedge\wedge}p < 0.001$). Moreover, a significant decrease in GSH levels in fluoxetine-treated IS group relative to fluoxetine-treated controls was revealed ($^{\#\#\#}p < 0.001$) (i.e., IS + Fluox vs. Control + Fluox). Post hoc test did not show any significant changes in GSH levels between clozapine-treated IS group and clozapine-treated controls ($p > 0.05$) (i.e., IS + Cloz vs. Control + Cloz). In comparison to vehicle-treated control animals, fluoxetine-treated controls did not show any significant changes in reduced GSH level.

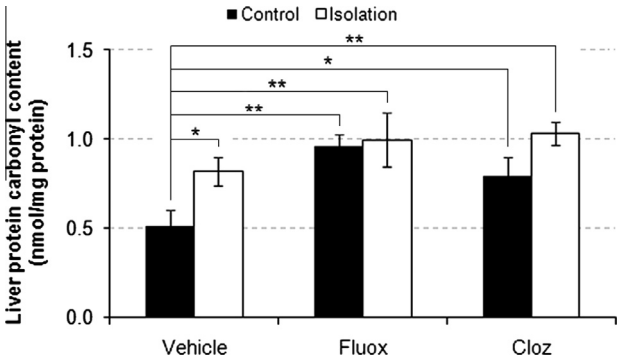

Fig. 1. Protein carbonyl contents (nmol/mg protein) in liver of control and chronically-isolated male rats treated either with vehicle (0.9% NaCl), 15 or 20 mg/kg/day doses of fluoxetine or clozapine, respectively. Results are expressed as mean ± S.E.M. Significant differences between groups obtained from two-way ANOVA analysis followed by post hoc Duncan test are: $^{**}p < 0.01$ and $^{*}p < 0.05$ treated experimental groups vs. vehicle-treated control group.

3.5. Glutathione S-transferase

A two-way ANOVA revealed a significant main effects of drug treatment ($F_{2.30} = 9.50$, $p < 0.001$) and a significant drug treatment × stress interaction ($F_{2.30} = 7.79$, $p < 0.001$) on cytosolic GST activity. Duncan's post hoc test revealed a significant increase in GST activity in vehicle- and clozapine-treated chronically-isolated animals as compared to vehicle-treated controls ($^{*}p < 0.05$, $^{***}p < 0.001$) (Fig. 4), as well as in clozapine-treated IS group as compared to IS alone ($^{\wedge}p < 0.05$). Also, a significant increase in cytosolic GST activity in drug-treated control animals as compared to vehicle-treated control animals was found ($^{**}p < 0.01$). Furthermore,

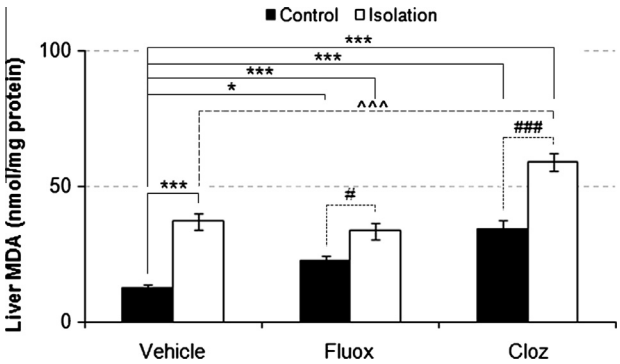

Fig. 2. MDA content (nmol/mg protein) in liver of control and chronically-isolated male rats treated either with vehicle (0.9% NaCl), 15 or 20 mg/kg/day doses of fluoxetine or clozapine, respectively. Results are expressed as mean ± S.E.M. Significant differences between groups obtained from two-way ANOVA analysis followed by post hoc Duncan test are indicated as follows: $^{***}p < 0.001$, $^{*}p < 0.05$ treated experimental groups vs. vehicle-treated control group; $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ between IS + Fluox vs. Control + Fluox, IS + Cloz vs. Control + Cloz respectively; $^{\wedge\wedge\wedge}p < 0.001$ between IS + Cloz and vehicle-treated IS group.

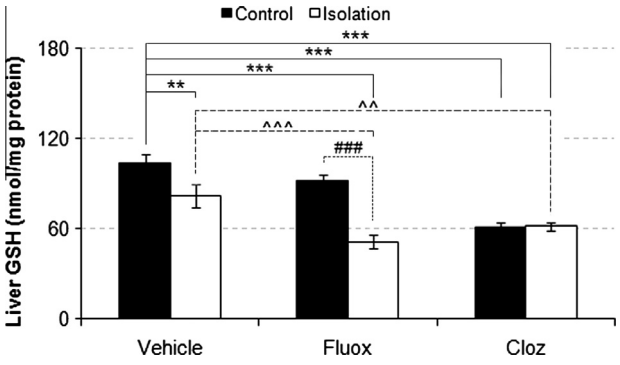

Fig. 3. GSH content (nmol/mg protein) in liver of control and chronically-isolated male rats treated either with vehicle (0.9% NaCl), 15 or 20 mg/kg/day doses of fluoxetine or clozapine respectively. Results are expressed as mean ± S.E.M. Significant differences between groups obtained from two-way ANOVA analysis followed by post hoc Duncan test are indicated as follows: $^{***}p < 0.001$, $^{**}p < 0.01$ treated experimental groups vs. vehicle-treated control group; $^{\#\#\#}p < 0.001$ between IS + Fluox and Control + Fluox group; $^{\wedge\wedge\wedge}p < 0.001$, $^{\wedge\wedge}p < 0.01$ between IS + Fluox or IS + Cloz and vehicle-treated IS group.

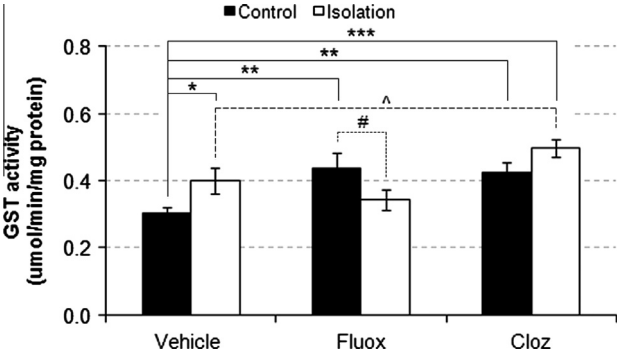

Fig. 4. GST activity (μmol/min/mg protein) in liver of control and chronically-isolated male rats treated either with vehicle (0.9% NaCl), 15 or 20 mg/kg/day doses of fluoxetine or clozapine, respectively. Results are expressed as mean ± S.E.M. Significant differences between groups obtained from two-way ANOVA analysis followed by post hoc Duncan test are indicated as follows: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ treated experimental groups vs. vehicle-treated control group; $^{\#}p < 0.05$ between IS + Fluox vs. Control + Fluox; $^{\wedge}p < 0.05$ between IS + Cloz and vehicle-treated IS group.

post hoc test showed a significant decrease in GST activity in fluoxetine-treated IS group compared to fluoxetine-treated controls ($^{\#}p < 0.05$) (i.e., IS + Fluox vs. Control + Fluox).

3.6. NO metabolites (NO_x^-)

A two-way ANOVA revealed a significant main effect of drug treatment ($F_{1.30} = 6.374$, $p < 0.01$) and a significant stress × drug treatment interaction ($F_{1.30} = 4.216$, $p < 0.05$). Post hoc Duncan's tests showed a significant increase in cytosolic NO_x^- levels following IS stress ($^{*}p < 0.05$). A significant increase in cytosolic NO_x^- levels was seen in both control and chronically-isolated animals treated with clozapine as compared to vehicle-treated controls ($^{**}p < 0.01$, $^{*}p < 0.05$) (Fig. 5).

3.7. Activation of cytosolic NF-κB, COX-2 and CuZnSOD protein expression during chronic IS stress was affected by drugs administration

To examine the NF-κB activation and its nuclear translocation, NF-κB-p65 localization in the cytosolic and nuclear fractions of the liver in all animals was determined (Fig. 6). A two-way ANOVA revealed significant main effects of drug treatment ($F_{2.29} = 4.72$, $p < 0.05$) and stress ($F_{1.29} = 22.96$, $p < 0.001$), as well as a significant drug treatment × stress interaction ($F_{2.29} = 4.68$, $p < 0.05$) on cytosolic NF-κB protein levels. A significant decrease in cytosolic NF-κB protein levels was found in vehicle- and clozapine-treated IS groups ($^{***}p < 0.001$, $^{**}p < 0.01$) as compared to vehicle-treated controls, as well as in clozapine-treated chronically-isolated animals as compared to clozapine-treated controls ($^{\#\#}p < 0.01$) (Fig. 6A). A significant increase in cytosolic NF-κB was seen in fluoxetine-treated IS group as compared to vehicle-treated IS group ($^{\wedge\wedge\wedge}p < 0.001$). In the nuclear fraction, a two-way ANOVA showed a significant main effects of stress ($F_{1.24} = 17.21$, $p < 0.001$) and a significant drug treatment × stress interaction ($F_{2.24} = 4.01$, $p < 0.05$) on NF-κB protein levels. Specifically, a significant increase in NF-κB protein levels was revealed in vehicle- and clozapine-treated chronically-isolated, as compared to vehicle-treated controls ($^{**}p < 0.01$), while a decrease in NF-κB protein levels was found in fluoxetine-treated IS group, as compared to vehicle-treated IS group ($^{\wedge}p < 0.05$) (Fig. 6B).

A two-way ANOVA revealed a significant main effect of stress ($F_{1.26} = 17.965$, $p < 0.001$) on cytosolic COX-2 protein expression. Post hoc Duncan's tests showed an increase in COX-2 protein expression in vehicle- and clozapine-treated IS groups, as compared to vehicle-treated controls ($^{*}p < 0.05$), as well as in drug-treated chronically-isolated animals as compared to drug-treated controls ($^{\#\#}p < 0.01$; $^{\#}p < 0.05$), (i.e., IS + Fluox vs. Control + Fluox; IS + Cloz vs. Control + Cloz) (Fig. 7).

A two-way ANOVA revealed a significant main effect of drug treatment ($F_{2.30} = 12.590$, $p < 0.001$) and a significant drug treatment × stress interaction ($F_{2.30} = 5.174$, $p < 0.05$) on cytosolic CuZnSOD protein levels. A significant increase in CuZnSOD levels was observed in vehicle-treated IS group, as compared to vehicle-treated controls ($^{**}p < 0.01$) (Fig. 8). In contrast, fluoxetine administered to chronically-isolated animals resulted in a decrease in CuZnSOD as compared to vehicle-treated controls ($^{*}p < 0.05$) or the vehicle-treated IS group ($^{\wedge\wedge\wedge}p < 0.001$). Interestingly, there was a decrease in CuZnSOD levels in clozapine-treated IS group relative to vehicle-treated chronically-isolated animals ($^{\wedge\wedge\wedge}p < 0.001$), but these changes did not achieve statistical significance compared to vehicle-treated controls.

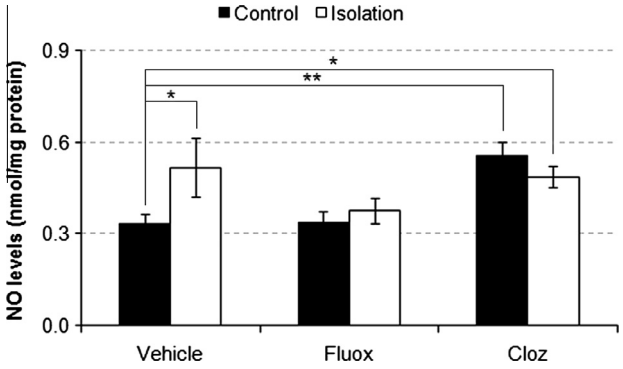

Fig. 5. NO levels in liver of control and chronically-isolated male rats treated either with vehicle (0.9% NaCl), 15 or 20 mg/kg/day doses of fluoxetine or clozapine, respectively. Results are expressed as mean ± S.E.M. Significant differences between groups obtained from two-way ANOVA analysis followed by post hoc Duncan test are indicated as follows: $^{*}p < 0.05$, $^{**}p < 0.01$ treated experimental groups vs. vehicle-treated control group.

3.8. Histopathological changes in rat liver tissue

Histopathological examination showed changes in specific areas of the liver in animals exposed to chronic fluoxetine or clozapine administration. The obtained results are shown in Table 2 and Fig. 9. In vehicle- and drug-treated controls, the presence of minor

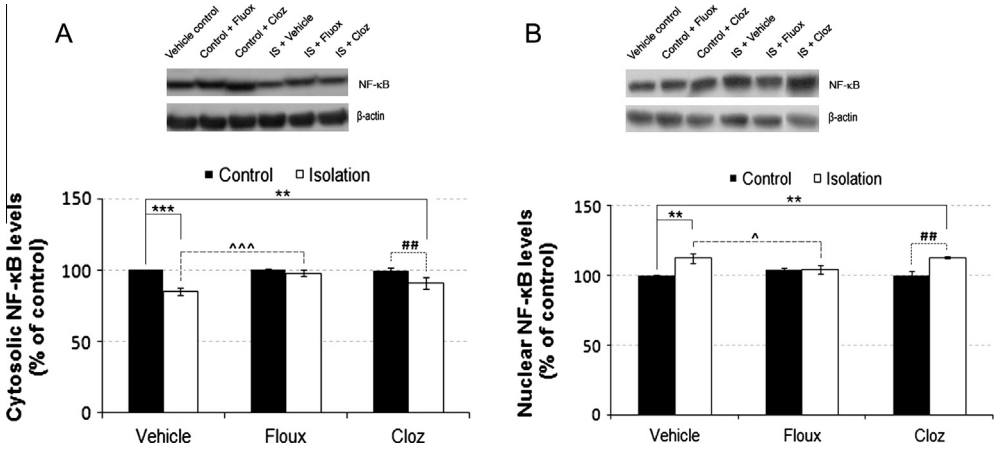

Fig. 6. NF-κB protein levels in liver of control and chronically-isolated male rats treated either with vehicle (0.9% NaCl), 15 or 20 mg/kg/day doses of fluoxetine or clozapine, respectively, in cytosolic (A) and nuclear (B) fraction. Results are expressed as mean ± S.E.M. Significant differences between groups obtained from two-way ANOVA analysis followed by post hoc Duncan test are indicated as follows: $^{***}p < 0.001$, $^{**}p < 0.01$ treated experimental groups vs. vehicle-treated control group; $^{\#\#}p < 0.001$ between IS + Cloz and Control + Cloz; $^{\wedge\wedge\wedge}p < 0.001$ between IS + Fluox and vehicle-treated IS group (A); $^{**}p < 0.01$ treated experimental groups vs. vehicle-treated control group; $^{\#\#}p < 0.001$ between IS + Cloz and Control + Cloz; $^{\wedge}p < 0.001$ between IS + Fluox and vehicle-treated IS group (B).

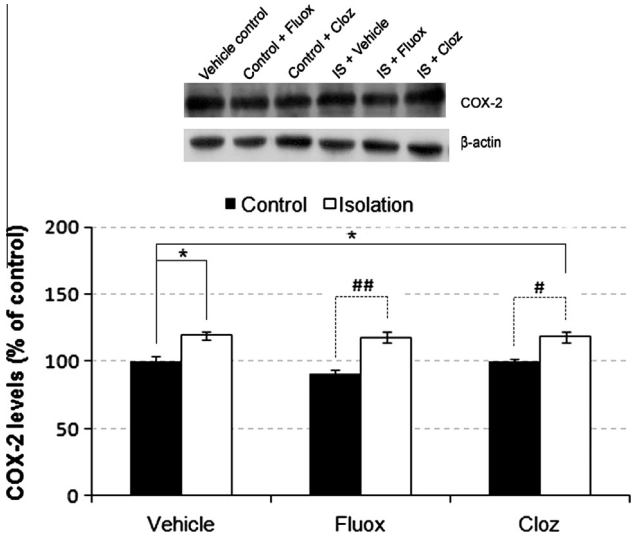

Fig. 7. COX-2 protein levels in liver cytosol of control and chronically-isolated male rats treated either with vehicle (0.9% NaCl), 15 or 20 mg/kg/day doses of fluoxetine or clozapine, respectively. Results are expressed as mean ± S.E.M. Significant differences between groups obtained from two-way ANOVA analysis followed by post hoc Duncan test are indicated as follows: $^{*}p < 0.05$ treated experimental groups vs. vehicle-treated control group; $^{\#\#}p < 0.01$ between IS + Fluox and Control + Fluox; $^{\#}p < 0.05$ between IS + Cloz vs. Control + Cloz group.

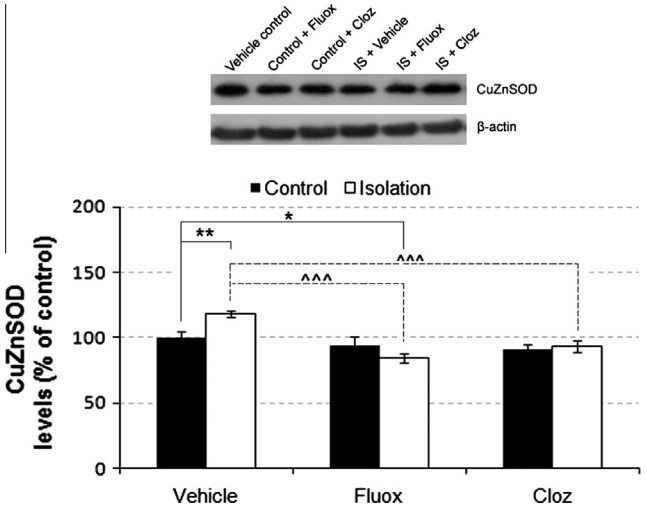

Fig. 8. CuZnSOD protein levels in liver cytosol of control and chronically-isolated male rats treated either with vehicle (0.9% NaCl), 15 or 20 mg/kg/day doses of fluoxetine or clozapine, respectively. Results are expressed as mean ± S.E.M. Significant differences between groups obtained from two-way ANOVA analysis followed by post hoc Duncan test are indicated as follows: $^{**}p < 0.01$, $^{*}p < 0.05$ treated experimental groups vs. vehicle-treated control group; $^{\wedge\wedge\wedge}p < 0.001$ between IS + Fluox and vehicle-treated IS group, IS + Cloz and vehicle-treated IS group, respectively.

portal inflammatory infiltrate that consisted mostly of lymphocytes, macrophages and very rare neutrophils was observed. Infiltrates observed in these animals were very rarely present and inconspicuous in some of the portal tracts (Fig. 9A). The presence of focal necroses (necroses of individual hepatocytes) was noted in all vehicle-treated control animals (Fig. 9B), but were more numerous in control animals treated with clozapine or fluoxetine. Occasional presence of apoptotic hepatocytes was noted in samples treated with fluoxetine, but was usually absent in samples treated with clozapine. In vehicle-treated control livers, there was macrovesicular fatty change that varied in quantity (Fig. 9C), but never encompassed more than 10% of hepatocytes in one sample. Microvesicular fatty change was more often present in control samples treated with clozapine. Very light and inconspicuous micro- and macro-vesicular fatty change was also observed in vehicle-treated control animals, but never encompassed more than 1–3% of hepatocytes. A hepatocyte in mitosis was noted in one vehicle-treated control sample (Fig. 9D). As to lobular changes, there was bridging and confluent necrosis observed in just one animal treated with clozapine (Fig. 9E). A slightly increased number of Kupffer and inflammatory cells was noted in vehicle-treated control samples but was more pronounced in clozapine-treated controls. In one clozapine control (that also had bridging and confluent necrosis), central venule endothelitis was noted (Fig. 9F).

Concerning chronically-isolated animals, histopathological analysis in portal triads indicated the presence of inflammatory infiltrates (lymphocytes, macrophages and rare neutrophils). These changes were morphologically similar in all samples but their quantity was different. In the vehicle-treated IS group, inflammatory infiltrates were only occasionally present (in some portal spaces). The number of Kupffer and inflammatory cells present in liver sinusoids was much more pronounced in clozapine-treated

Table 2
Histopathological changes observed in specific areas of liver tissue.

Histopathological changes	Treatments					
	Control			Isolation		
	Vehicle	Fluox	Cloz	Vehicle	Fluox	Cloz
	Portal triad					
Infiltrate of neutrophils, lymphocytes, macrophages	–	++	++	+/–	++	++
	Sinusoids					
Kupffer and inflammatory cells	+	+	++	+	+	++
Central venules endothelitis	–	–	–*	–	–	–
	Liver lobules					
Bridging/confluent necrosis	–	–	–*	–	–	–
Focal necrosis	+/–	++	++	+/–	+/–	++
Apoptotic hepatocytes	–	+/–	–	–	–	+/–
Microvesicular fatty change	–	–	+/–	–	–	–
Macrovesicular fatty change	–	+	+	+/–	+/–	++
Hepatocytes in mitosis	–	–	–	–	–	–

Results are expressed as mean ± S.E.M. of 6–7 animals per each group; ++ indicates almost always present, + usually present, +/– occasionally present, – usually absent, –* present in one animal.

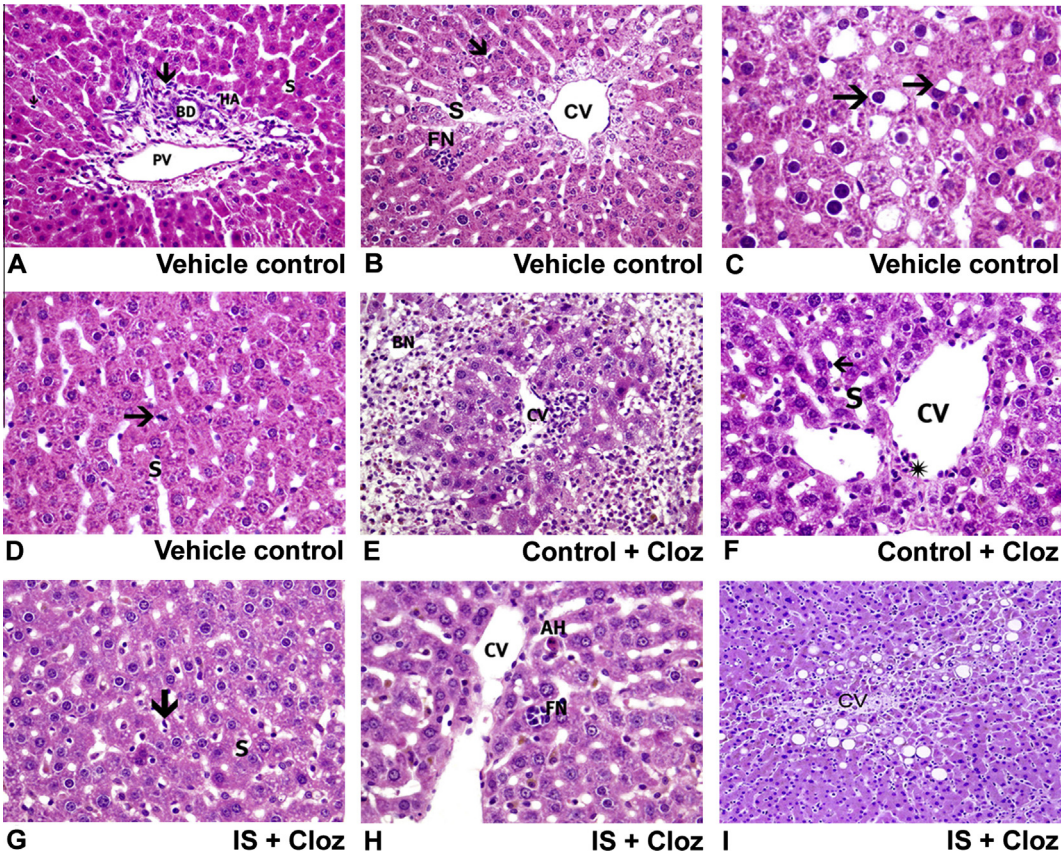

Fig. 9. Histopathological changes in rat liver (H&E, original magnification 400×). Vehicle-treated control liver: (A) Portal triad with branches of hepatic artery (HA), portal vein (PV) and bile duct (BD), with very light, inconspicuous inflammatory infiltrate of lymphocytes and macrophages (large arrow); normal parenchyma architecture and visible Kupffer cells (small arrow) in sinusoids (S); (B) Focal hepatocyte necrosis (FN) in close proximity to central venule (CV). In sinusoids (S), Kupffer cells (arrow) are visible; (C) Focal macrovesicular fatty change in hepatocytes (arrows); (D) Hepatocyte in mitosis (arrow), S-sinusoids. Clozapine-treated control liver: (E) Bridging hepatocyte necrosis (BN) central venule (CV). Microvesicular fatty change in hepatocytes; (F) Central venule endothelitis (asterisk). CV-central venule, S-sinusoids with Kupffer cells (arrow). Chronically-isolated animals treated with clozapine: (G) Increased number of Kupffer cells (arrow) in liver sinusoids (S); (H) Focal hepatocyte necrosis (FN) and apoptotic hepatocyte (AH) in close proximity to central venule (CV); (I) Macrovesicular fatty change in hepatocytes around central venule (CV).

samples, especially near the central venule (Fig. 9G). Focal necroses were present in all samples but were more numerous (up to 3 necroses per one lobule) in samples that were treated with clozapine (Fig. 9H). Rare apoptotic hepatocytes were observed only in samples treated with clozapine (Fig. 9H). Microvesicular fatty change was present in some of the samples but was inconspicuous. Macrovesicular fatty change was also more pronounced in clozapine-treated samples, comprising up to 60% of liver (Fig. 9I). The spectrum of histopathological changes present in vehicle-treated chronically-isolated animals was similar to that in vehicle-treated controls, but was more pronounced, especially regarding macrovesicular fatty change.

4. Discussion

As the liver is a primary organ for drug activation and detoxification, we investigated the effects of chronic administration of fluoxetine or clozapine on liver injury via liver enzymes, oxidative stress and histological analysis following chronic IS stress, an animal model of depression (Fuchs and Flugge, 2006; Hall, 1998; Scaccianoce et al., 2006; Serra et al., 2007; Spasojević et al., 2007). Hepatic dysfunction was monitored by serum ALT and AST activity, markers of drug-associated hepatotoxicity (Kuester et al., 2002). The increased activity of ALT, an enzyme localized in the cytoplasm, in drug-treated animals (both chronically-isolated and controls) indicated hepatic dysfunction probably due to drug-induced damage in the cell membrane. Human studies have shown that approximately 40% of subjects receiving clozapine have ALT levels twice the upper limit of normal (Gaertner et al., 2001). As several drug failures have been associated with mitochondrial dysfunction (Chan et al., 2005), the observed increase in the activity of AST, an enzyme localized in mitochondria, in fluoxetine-treated rats (both chronically-isolated and controls) may have resulted from a fluoxetine-induced loss of functional integrity of mitochondrial liver cell membranes (Feldman et al., 2006; Sastry, 1985). Our results are in agreement with those of Souza et al. (1994), who found that fluoxetine alters the energy metabolism of liver mitochondria and causes potentially toxic effects when administered in high doses.

The significant increase in hepatic protein carbonyl content and MDA levels in both drug-treated controls and chronically-isolated (vehicle- or drug-treated) rats suggests increased oxidative stress caused by clozapine and fluoxetine or chronic IS stress alone. Moreover, lipid peroxidation may be coupled to deleterious effects to the cell membrane, causing increased permeability (Yajima et al., 2009), observed by ALT and AST leakage into the serum. It has been reported that psychological stress enhances lipid peroxidation (Hibbeln and Salem, 1995), as clinical studies have demonstrated elevated MDA levels in patients with affective disorders (Ozcan et al., 2004). These results suggest that oxidants produced by clozapine or fluoxetine, and/or chronic IS, attack both lipids and proteins. Our data are in agreement with those of Inkielewicz-Stepniak (2011), who found that chronic high doses of fluoxetine increased the levels of thiobarbituric acid reactive substances, carbonyl groups in the liver, and transaminase activity in the serum. In addition, increased MDA in clozapine-treated IS group compared to vehicle-treated IS group or clozapine-treated controls suggests a synergistic action of clozapine and IS-induced lipid peroxidation.

One of the main intracellular mechanisms of drug-induced liver injury is the depletion of GSH, which was detected in all treated groups except controls treated with fluoxetine. This decrease in GSH levels may be due to several possible mechanisms, such as its oxidation during the detoxification of hydrogen peroxide and/or lipid peroxides (Gupta et al., 2005), its participation in the maintenance of non-GSH sulfhydryl proteins in a reduced state, or its increased consumption via GST (Bymaster et al., 1996; Lang et al., 2008; Lesurtel et al., 2008; Tew and Ronai, 1999). Interestingly, fluoxetine administration depleted GSH levels only in chronically-isolated animals. We have previously reported compromised mitochondrial function in the liver of chronically-isolated rats, as indicated by the decreased protein expression of mitochondrial MnSOD (Filipović et al., 2010) and its activity (Zlatković and Filipović, 2011). As the effects of fluoxetine on rat liver is, in part, a consequence of drug and/or metabolite solubilization in the inner membrane of the mitochondria (Souza et al., 1994), we hypothesize that fluoxetine interferes with signaling during IS stress and contributes to more pronounced changes in GSH content in chronically-isolated animals than in controls. As conjugation of reactive drug metabolites to GSH is an important detoxification mechanism mediated by GST (Strange et al., 2000), our observed increase in cytosolic GST activity in drug-treated controls and clozapine-treated chronically-isolated rats is likely a defensive response to drug-induced oxidative stress and toxic drug metabolites. Furthermore, increased GST activity in IS group suggests an ability of this enzyme to detoxify lipid peroxidation products accumulated during chronic IS stress (Meltzer, 1995; Trujillo, 1996). Interestingly, decreased GST activity in fluoxetine-treated chronically-isolated animals compared to fluoxetine-treated controls was found. As GST requires GSH as co-substrate, whereby cellular GSH in a reduced state is controlled, in part, by glutathione reductase (GLR) (Nagaoka et al., 2004), it is tempting to speculate that the lack of GST response to fluoxetine treatment in chronically-isolated rats may be a consequence of the depletion of GSH content and the disturbance of its regeneration by GLR. Previous data have also shown that rats exposed to the chronic IS procedure used here (21 days) showed a decrease in GLR protein expression and its activity (Djordjevic et al., 2010), during which the lack of GLR may further deteriorate oxidative damage, compromising GSH restoration and adversely affecting drug detoxification via hepatic GST activity. Future experiments targeting the hepatic activity of GSH-related defense enzyme systems following the chronic administration of drugs may reveal the precise functional role of GST enzymatic activity.

Previous studies have shown that chronic stress causes a significant decrease in SOD in rodents. In contrast, antidepressant treatment has been found to prevent psychological stress-induced oxidative damage and restore the levels of SOD (Zafir and Banu, 2007). In our study, increased CuZnSOD protein level in chronically-isolated animals is not consistent with our previously published observations of its activity (Zlatković and Filipović, 2011), suggesting that the liver is susceptible to chronic IS stress. A lack of consistency between protein levels of CuZnSOD may suggest posttranslational protein modifications affecting its activity (Hopper et al., 2006), or partially non-coupled activity of another hydrogen-peroxide removing enzyme such as catalase (Pajović et al., 2006), which may lead to the accumulation of hydrogen peroxide, resulting in the inhibition of CuZnSOD. Furthermore, increased oxidative stress markers that are not accompanied by increased CuZnSOD protein expression in drug-treated chronically-isolated animals may reinforce the oxidative stress. Moreover, NO may contribute to drug-induced oxidative stress and liver injury, given that some antidepressants are able to induce NO synthesis (Dhir and Kulkarni, 2007; Ha et al., 2006). In the present study, chronic IS and the chronic administration of clozapine (both chronically-isolated and controls) caused an increase in NO levels. NO is synthesized by inducible or endothelial nitric oxide synthase found in hepatocytes, Kupffer cells, and endothelial cells. Drug-activated Kupffer cells that produce signaling molecules such as NO and superoxide anion activate NF-κB, resulting in an increased synthesis of ROS and NO. Formation of peroxynitrite by the reaction of superoxide anions and NO may cause cellular toxicity, observed by increased lipid peroxidation and GSH depletion (Squadrito and Pryor, 1995). The main source of superoxide anion is the cytochrome P-450, enzyme system involved in the metabolism of clozapine and fluoxetine (Dumortier et al., 2002; Inkielewicz-Stepniak, 2011), as well as NADPH oxidase, an enzyme located in Kupffer cells and neutrophils (Hinson et al., 2004). Furthermore, increased generation of NO activates COX-2 expression, which is regulated by transcriptional factor NF-κB (Na et al., 2006). In our study, activated NF-κB and the concomitant increase in COX-2 following chronic IS may be due to the accumulation of peroxide, causing perpetual NF-κB activity (Kobayashi et al., 2008).

Moreover, the activation of NF-κB and COX-2 are considered critical steps in the initiation of liver injury (Jokelainen et al., 2001; Nanji et al., 1997), as indicated by increased serum ALT activity. Interestingly, fluoxetine in chronically-isolated rats reduced NO levels and suppressed the NF-κB activation which was reflected in unchanged COX-2 levels.

To examine whether the alterations of serum transaminases, together with oxidative stress biomarkers, were accompanied by comparable changes in the tissue, the liver was subjected to histological analysis. The liver histopathological picture of chronically-isolated and control animals that received either clozapine or fluoxetine showed the appearance of neutrophil infiltration, which occurs as an early response to tissue damage or cellular stress (Jaeschke and Tadashi, 2006). Neutrophils may play an important role in drug toxicity, because they represent a powerful source of ROS (Adams et al., 2010; Jaeschke et al., 1993). Neutrophils release lysosomal enzymes and generate superoxide anion through the enzyme NADPH oxidase. Hydrogen peroxide, which is influenced by the neutrophil-derived enzyme myeloperoxidase, generates hypochlorus acid, a potent oxidant, that causes oxidation of lipids and increases MDA (Malle et al., 2006). With regard to necrosis, fluoxetine and clozapine treatments did not cause loss of masses of cells (no confluent necrosis), but only necrosis of individual hepatocytes (focal necrosis) (Özden et al., 2005). Interestingly, one clozapine-treated control animal showed bridging/confluent necrotic damage in conjunction with central vein endothelitis, a failure obviously caused by an excessive reaction to clozapine. This pattern of necrosis, which may lead to hepatitis in humans, could be, in part, caused by drug bioactivation in the liver (Dumortier et al., 2002; Inkielewicz-Stepniak, 2011). A clozapine reaction with hypochlorous acid generated by neutrophils forms its reactive metabolite (Kalgutkar et al., 2005), where its covalent binding to cellular macromolecules are involved in the occurrence of idiosyncratic drug toxicity (Walgren et al., 2005; Zhou et al., 2005; Uetrecht, 2007). Moreover, GSH depletion may also be associated with clozapine-induced idiosyncratic hepatotoxicity (Hadi et al., 2013). The clozapine-induced histopathological changes in our study have been reported in human case studies during clozapine therapy (Dorta et al., 1989; Schmidt et al., 1987). Thus, it is in limited use due to its adverse effects in liver, including agranulocytosis and hepatotoxicity (Lu et al., 2008) resulting from the accumulation of reactive metabolites of clozapine (Maggs et al., 1995). Moreover, more pronounced histopathological changes in the liver of clozapine-treated rats has been found than those treated with fluoxetine (Castiella and Arenas, 1994).

Fatty change is the process of abnormal retention of lipids within a cell, described as macrovesicular or microvesicular steatosis. A single i.p. dose of clozapine has been shown to cause perturbation of lipid homeostasis in liver (Ferno et al., 2009); hence, steatosis observed in our clozapine-treated animals is not surprising. Medications are one of the causes of these changes in steatosis, but this macrovesicular change usually has no effect on the function of the hepatocyte. More serious, toxic, microvesicular steatosis, demonstrated only in the clozapine-treated control group, is likely associated with hepatotoxicity produced by administered drugs (Berson et al., 1998). A severe drug-induced impairment of mitochondrial fatty acid oxidation can induce accumulation of free fatty acids and triglycerides that could play a major role in the pathophysiology of microvesicular steatosis (Begriche et al., 2011). Here, focal necrosis and macrovesicular fatty changes were less pronounced in fluoxetine-treated animals exposed to chronic IS compared to fluoxetine-treated controls, which is consistent with previous reports (Özden et al., 2005). Evidenced mitosis in the hepatocytes of one control rat may be caused by hormones, polypeptides, and metabolites, as potential regulators of liver cell proliferation (Bucher and Malt, 1971).

In conclusion, the present study has shown that the chronic administration of fluoxetine or clozapine have the ability to cause hepatotoxicity, as indicated by serum transaminase activity. Increased MDA, protein carbonyl contents, GST activity and decreased GSH levels in drug-treated controls/chronically-isolated animals suggest a link between drugs and hepatic oxidative stress. Chronic administration of clozapine in chronically-isolated rats likely reinforce hepatic oxidative stress, judged by increased NO levels, *NF-κB activation*, increased COX-2 and compromised CuZn-SOD protein expression, which was observed by increased lipid peroxidation and GSH depletion. In contrast, rise in NO levels due to chronic IS was markedly reduced by fluoxetine treatment. Moreover, clozapine appears to have a higher potential to induce liver toxicity than fluoxetine (Lucena et al., 2003). Furthermore, this is the first *in vivo* study revealing evidence of histopathological changes caused by fluoxetine or clozapine at the light microscopic level. Although chronic IS caused oxidative stress, no significant effects on hepatic histopathological changes were found. Chronic administration of fluoxetine in both chronically-isolated and control animals resulted in more or less normal hepatic architecture, while clozapine in both groups resulted in liver injury. Histopathological changes identified in our study may help in determining the appropriate dose of clozapine to reduce its hepatotoxic effects.

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PREFRONTAL CORTICAL GLUTATHIONE-DEPENDENT DEFENSE AND PROINFLAMMATORY MEDIATORS IN CHRONICALLY ISOLATED RATS: MODULATION BY FLUOXETINE OR CLOZAPINE

NEVENA TODOROVIĆ AND DRAGANA FILIPOVIĆ *

Laboratory of Molecular Biology and Endocrinology, Institute of Nuclear Sciences "Vinča", University of Belgrade, 11001, Serbia

Abstract—Chronic psychosocial stress modulates brain antioxidant systems and causes neuroinflammation that plays a role in the pathophysiology of depression. Although the antidepressant fluoxetine (FLX) represents the first-line treatment for depression and the atypical antipsychotic clozapine (CLZ) is considered as a second-line treatment for psychotic disorders, the downstream mechanisms of action of these treatments, beyond serotonergic or dopaminergic signaling, remain elusive. We examined behavioral changes, glutathione (GSH)-dependent defense and levels of proinflammatory mediators in the prefrontal cortex (PFC) of adult male Wistar rats exposed to 21 days of chronic social isolation (CSIS). We also tested the ability of FLX (15 mg/kg/day) or CLZ (20 mg/kg/day), applied during CSIS, to prevent stress-induced changes. CSIS caused depressive- and anxiety-like behaviors, compromised GSH-dependent defense, and induced nuclear factor-kappa B (NF-κB) activation with a concomitant increase in cytosolic levels of proinflammatory mediators cyclooxigenase-2, interleukin-1beta and tumor necrosis factor-alpha in the PFC. NF-κB activation and proinflammatory response in the PFC were not found in CSIS rats treated with FLX or CLZ. In contrast, only FLX preserved GSH content in CSIS rats. CLZ not only failed to protect against CSIS-induced GSH depletion, but it diminished its levels when applied to non-stressed rats. In conclusion, prefrontal cortical GSH depletion and the proinflammatory response underlying depressive- and anxiety-like states induced by CSIS were prevented by FLX. The protective effect of CLZ, which was equally effective as FLX on the behavioral level, was limited to proinflammatory components. Hence, different mechanisms underlie the protective effects of these two drugs in CSIS rats.

Key words: chronic social isolation, fluoxetine, clozapine, prefrontal cortex, glutathione, proinflammatory mediators.

INTRODUCTION

An increasing body of evidence suggests that oxidative stress in the brain caused by chronic psychosocial stress contributes to the development of psychiatric disorders, including depression (Van Winkel et al., 2008; Maes et al., 2011; Schiavone et al., 2012). The brain is highly susceptible to oxidative stress due to high oxygen consumption and a lipid-rich environment; thus, maintaining a balanced redox status is crucial for proper brain functioning (Noseworthy and Bray, 1998). In addition, neuroinflammation has been proposed to mediate the association of psychosocial stressors and psychiatric disorders (Maes, 2008; Réus et al., 2015; Calcia et al., 2016). Stress-related activation of inflammatory mediators, as well as modifications of oxidative/nitrosative pathways in the brain, have been implicated in the pathophysiology of psychiatric diseases and may also represent pharmacological targets for their treatment (Munhoz et al., 2008). Chronic social isolation stress (CSIS) is an animal model that has been shown to be reliable for studying the pathophysiology of depression, as it has good face, construct, and predictive validity (Abelaira et al., 2013). It is also widely used as a neurodevelopmental animal model of schizophrenia that produces long-lasting behavioral alterations, such as deficient sensorimotor gating and working memory, locomotor hyperactivity, increased anxiety, and aggression (Fone and Porkess, 2008; Möller et al., 2013). CSIS also produces a variety of neurochemical changes consistent with schizophrenia, including lower frontal cortical dopamine turnover (Heidbreder et al., 2000), altered frontal cortical dopamine D1 and glutamate N-methyl-D-aspartate receptor binding (Toua et al., 2010), and increased striatal dopamine D2 receptor density (King et al., 2009). Together, these behavioral and neurochemical alterations resemble and correspond to features of the human disorder.

The prefrontal cortex (PFC) is one of the most stress-sensitive brain regions (Arnsten, 2009; Sandi, 2013), and

*Corresponding author. Address: Laboratory of Molecular Biology and Endocrinology, Institute of Nuclear Sciences "Vinča", University of Belgrade, P.O. Box 522-090, 11001 Belgrade, Serbia. Fax: +381-(11)-6455-561.

E-mail address: dragana@vinca.rs (D. Filipović).

URL: http://www.vinca.rs (D. Filipović).

Abbreviations: ANOVA, analysis of variance; CLZ, clozapine; COX-2, cyclooxygenase-2; CSIS, chronic social isolation; FLX, fluoxetine; GLR, glutathione reductase; GPx, glutathione peroxidase; GSH, glutathione; IL-1β, interleukin-1 beta; iNOS, inducible nitric oxide synthase; IκB, inhibitor kappa B; MB, marble burying; NADPH, nicotinamide adenine dinucleotide phosphate; NF-κB, nuclear factor-kappa B; NOX, nicotinamide adenine dinucleotide phosphate oxidase; PFC, prefrontal cortex; SP, sucrose preference; TNF-α, tumor necrosis factor-alpha; Veh, vehicle.

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a rapidly growing body of evidence implicates this brain structure in the pathophysiology of depression in both clinical and animal models (Ménard et al., 2016; Negrón-Oyarzo et al., 2016). The PFC participates in cognitive, socio-emotional and executive functions and modulates autonomic and neuroendocrine responses to stress (Lucassen et al., 2014). A high density of glucocorticoid receptors (GR), as well as engagement in hypothalamic–pituitary–adrenal (HPA) axis regulation (Smith and Vale, 2006), indicate an important role of the PFC in the response to stress.

Depression is a complex, multifactorial disorder that likely results from the interplay of multiple genetic and environmental factors. Changes in the synthesis, metabolism, reuptake or receptors of neurotransmitters, primarily serotonin, are the focus of a majority of studies concerning the neurobiology of depression (Aan het Rot et al., 2009; Albert et al., 2014). Selective serotonin reuptake inhibitors, which prolong serotonin signaling, are the most widely used treatments for depression, and fluoxetine (FLX) was the first available on the market and is the most commonly used drug of this class (Brambilla et al., 2005; Wagner, 2015). However, 30–40% of patients suffering from major depressive disorder never achieve symptom resolution via standard antidepressant therapy. In some of these cases, atypical antipsychotics used as monotherapy or adjunctively with antidepressants may be effective (Rogóż, 2013; Wang and Si, 2013). Clozapine (CLZ), an atypical antipsychotic with high affinity for serotonin, dopamine, muscarinic, adrenergic and other biogenic amine receptors (Roth et al., 2004), is mainly used in the treatment of psychotic disorders, including schizophrenia, but has been shown to improve symptoms in treatment-resistant bipolar disorder (Li et al., 2015). With regard to FLX, growing evidence demonstrates antioxidative (Behr et al., 2012) as well as immunomodulatory (Baumeister et al., 2015) effects, while CLZ has been reported to induce both pro- and anti-inflammatory activities (Baumeister et al., 2015).

Glutathione (GSH), a non-enzymatic component of GSH-dependent antioxidative defense, plays a central role in maintaining physiological redox status in the brain. It is a substrate for glutathione peroxidase (GPx), which catalyzes the reduction of hydrogen peroxide and a wide variety of organic peroxides to water and the corresponding stable alcohols. Simultaneously, GSH is oxidized to glutathione disulfide, which is reduced back to GSH by glutathione reductase (GLR) (Sastre et al., 2005). Numerous studies have revealed compromised antioxidant defense in the brain of chronically stressed rats (Eren et al., 2007; Ahmad et al., 2010; Che et al., 2015). The results from a proteomic analysis of the PFC revealed that GSH metabolism is among the most altered biological pathways resulting from chronic unpredictable mild stress, a rat model of depression (Yang et al., 2013). Furthermore, Gawryluk et al. (2011) reported reduced GSH levels in post-mortem PFC from patients with bipolar disorder, major depressive disorder and schizophrenia.

In addition to oxidative stress, neuroinflammation plays an important role in the pathophysiology of psychiatric disorders (Smith, 1991; Maes, 2008; Calcia et al., 2016). Patients with depressive disorder exhibit increased expression of proinflammatory cytokines and their receptors in peripheral blood and cerebrospinal fluid (Miller et al., 2009). Moreover, increased expression of interleukin-1 beta (IL-1β) and tumor necrosis factor (TNF) in post-mortem brain samples from depressed patients has been found (Miller and Raison, 2016). IL-1β and TNF-α, major proinflammatory cytokines, are constitutively expressed in healthy, adult brain by neurons and glial cells and act as neuromodulators mediating normal neuronal functions, such as sleep regulation (Vitkovic et al., 2000). Upregulation of these cytokines in the brain is associated with numerous diseases, including mood disorders (McNamara and Lotrich, 2012). A recent study demonstrated that social isolation rearing for 4 weeks elevated rat plasma levels of IL-1β, IL-6 and TNF-α (Ko and Liu, 2015). More importantly, the expression of IL-1β and TNF-α were increased in the frontal cortex of rats that demonstrated anhedonia after 7 weeks of unpredictable mild stress (Liu et al., 2014). These findings are consistent with others regarding the peripheral and central secretion of proinflammatory cytokines in rodent models of depression (Kubera et al., 1998; Leonard and Song, 2002; García-Bueno et al., 2005; Grippo et al., 2005).

The interrelationship between oxidative stress and inflammation in the brain may be mediated by nuclear factor-kappa B (NF-κB), a redox sensitive transcriptional factor that may be activated by both oxidative stress and proinflammatory mediators (Van den Berg et al., 2001; Fischer and Maier, 2015). The activity of NF-κB is under the control of inhibitor kappa B (IκB), which prevents its translocation into the nucleus and subsequent activation of NF-κB target genes (Auphan et al., 1995; Baldwin, 1996; Hayden and Ghosh, 2008). NF-κB is an important positive regulator of inflammatory responses, as it enhances the transcription of proinflammatory cytokines, including TNF-α and IL-1β (Grilli and Memo, 1999; Jin et al., 2008), as well as enzyme cyclooxygenase 2 (COX-2) (Inoue and Tanabe, 1998), which catalyzes the production of prostaglandins, important mediators of inflammation (Marnett et al., 1999; Morgan and Liu, 2011). Interestingly, hydrogen peroxide induces the expression of COX-2, which contributes to further production of reactive oxygen species (Hsieh and Yang, 2013).

Our previous study showed that 21 days of social isolation in adult, male Wistar rats produced features that resemble a depressive-like state, such as anhedonia, anxiety and despair (Zlatković et al., 2014a). Social isolation also compromised antioxidative defense by decreasing the activity of cytosolic copper-zinc superoxide dismutase and mitochondrial manganese superoxide dismutase (Zlatković and Filipović, 2013), and caused oxidative damage of lipids in the PFC (Zlatković et al., 2014a). Here we investigated whether CSIS negatively affects GSH-dependent antioxidative defense by monitoring GSH levels, as well as protein levels and activities of GPx and GLR enzymes. Keeping in mind the close link between oxidative stress and inflammation, we also investigated NF-κB activation and the protein levels of proinflammatory mediators (COX-2, TNF-α and IL-1β) in

the PFC of CSIS rats. We found that depressive- and anxiety-like behaviors of CSIS rats coincided with compromised GSH-dependent defense and proinflammatory signaling in the PFC. Furthermore, we demonstrated that either FLX or CLZ applied simultaneously with CSIS prevented behavioral changes observed in CSIS rats and showed neuroprotective effects in the PFC, although FLX was shown to be more beneficial.

EXPERIMENTAL PROCEDURES

Animals

Adult male Wistar rats, 2.5 months old, weighting 300 to 350 g were used as subjects. Rats were maintained under standard conditions (temperature 21–23 °C, 12-h/12-h light/dark cycle), with food (commercial rat pellets) and water available *ad libitum.* All experimental procedures were conducted in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and approved by the Ethics Committee for the Use of Laboratory Animals of the Institute of Nuclear Sciences "Vinča", Belgrade, Serbia (Application No. 02/11). All efforts were made to minimize animal suffering and to reduce the number of animals used.

Preparation of drug solutions

Flunisan (containing 20 mg of fluoxetine-hydrochloride) and Leponex tablets (containing 25 mg of CLZ) were purchased from Hemofarm, Serbia and Novartis Pharmaceuticals UK, respectively. Reference standards were obtained from the same companies. Flunisan tablets were crushed and dissolved in distilled, sterile water with the aid of ultrasound. CLZ solution was prepared daily by dissolving Leponex tablets in 1 N HCl with gentle heating. The solution was then diluted with distilled water and neutralized with 1 N NaOH to a pH of 5.1 (Halim et al., 2004). Since CLZ is insoluble at neutral pH, it requires a low pH to be solubilized. Abrams et al. (2008) revealed that CLZ solution (1 mg/ml) precipitated between pH 6 and 7. The pH values of CLZ solutions administered intraperitoneally are in the range of 4–6 (Halim et al., 2004; Goudie et al., 2007; Fernø et al., 2009; Jassim et al., 2012; Brown et al., 2014). In order to minimize the tissue damage, the site of i.p. injection was varied between right and left side of the rat during the period of treatment. Both drug solutions were filtered through Whatman No. 42 filter paper and concentrations of the respective drugs were determined using Ultra Performance Liquid Chromatography analysis (Kovacevic et al., 2006). These concentrations, together with the weight of each rat, determined once a week, were used to calculate the volume of drug solution administered to each animal in order to achieve the target doses.

Study design

Animals were divided into non-stressed (No stress) rats, housed in groups of four animals per cage, and rats that underwent chronic social isolation (CSIS) stress for 21 days. CSIS rats, housed one animal per cage, were deprived of any visual or tactile contacts with other animals, but had normal auditory and olfactory experiences. Both groups were subdivided into groups treated with FLX (15 mg/kg/day), CLZ (20 mg/kg/day) or physiological saline (0.9% NaCl) (Vehicle-Veh). Drugs were administered daily by intraperitoneal (i.p.) injection during the 21 days in non-stressed (No stress + FLX and No stress + CLZ) and chronically isolated (CSIS + FLX and CSIS + CLZ) rats. CSIS rats received drug treatment simultaneously with stress exposure. The dose of FLX used in this study was previously shown to result in a range of plasma drug concentrations similar to that reported for patients under FLX treatment (Czeh et al., 2006). The dose of CLZ was also chosen to emulate the therapeutic dose given to patients (Halim et al., 2004; Larsson et al., 2015). To confirm the validity of these doses, serum concentrations of FLX and CLZ were determined by liquid chromatography-mass spectrometry (Djordjevic et al., 2005) and liquid chromatography-tandem mass spectrometry method (Song et al., 2009), respectively, in No stress and CSIS groups, 24 h after the last drug i.p. injection. The obtained serum drug concentrations were found to be comparable to therapeutic levels in human patients (Table 1) (Zlatković et al., 2014b).

Behavioral testing

All animals were separated into individual cages during behavioral testing. Tests were performed one day prior to the first day of experimental protocol described in the Study design (baseline) and on the 21st day of the treatment (21d) (before last vehicle/drug administration to avoid the effect of injection-related stress on behavior). Behavioral tests were performed on the same day, with the sucrose preference test preceding the marble burying test.

Sucrose preference (SP) test. The SP test measures a rat's appetite for a highly palatable, rewarding substance, and it has been extensively used to measure

Table 1. Concentrations of FLX and CLZ measured in the serum of treated rats (Zlatković et al., 2014b)

Drug	Used dose (mg/kg/day)	Measured concentrations in rat serum (ng/ml)	Therapeutic levels in patients (ng/ml) (related ref)
Fluoxetine	15	No stress + FLX 280 ± 50 CSIS + FLX 203 ± 28	100–700 (Dulawa et al., 2004)
Clozapine	20	No stress + CLZ 103 ± 18 CSIS + CLZ 123 ± 18	100–700 (Sadock and Sadock, 2008)

FLX = fluoxetine; CLZ = clozapine; CSIS = chronic social isolation.

anhedonia (decreased ability to experience pleasure) in chronic mild stress models of depression (Willner et al., 1987). Two pre-weighed bottles, containing 0% (tap water) or 1% sucrose solution, were placed in each cage. After 1 h, bottles were removed, re-weighed and SP was calculated using the formula SP = [consumed sucrose solution/total liquid consumed (sucrose solution + water)] 100.

Marble burying (MB) test. Object burying is a rodent-specific defense reaction to aversive stimulation which is suppressed by anxiolytic drugs, and is therefore considered an indicator of anxiety (Ho et al., 2002; Farley et al., 2010). Six glass marbles (2.5 cm in diameter) were placed evenly across the surface of the sawdust bedding in each cage. After 30 min, the number of buried marbles (with at least two-thirds of the surface covered with bedding) was assessed blindly to group conditions. The results are shown as a mean number of buried marbles.

Preparation of cytosolic and nuclear extracts from the PFC

Rats were sacrificed 24 h after the last FLX or CLZ administration. Animals were anesthetized with ketamine/xylazine (100/5 mg/kg i.p.), *in situ* perfused with physiological saline and sacrificed by guillotine decapitation. The whole brain was immediately removed and the PFC was dissected on ice. To obtain cytosolic fractions, PFC was homogenized in 2 vol. (w/v) of cold homogenization buffer by 40 strokes in the Potter–Elvehjem Teflon-glass homogenizer. Cytosolic and nuclear fractions were obtained by differential centrifugation, as previously described (Zlatković et al., 2014a). The protein concentration was measured according to the modified Lowry method (Markwell et al., 1978), using purified bovine serum albumin as a standard.

GSH content and GPx and GLR activity in the cytosol of PFC

GSH content in cytosol was determined according to Ellman's method (1959) and modified by Hissin and Hilf (1976), based on the reduction of Ellman's reagent by –SH groups of GSH, formation of 2-nitro-s mercaptobenzoic acid and production of intense yellow color measured at 405 nm. A calibration curve was performed with standard GSH (0.25–2 mM), and GSH concentrations in the samples were expressed as nmol/mg protein. Cytosolic GPx activity was assayed using a Ransel kit (Randox Laboratories, Crumlin, UK) according to manufacturer's protocol, while GLR activity was determined according the method of Halliwell and Foyer (1978). The molar extinction coefficient of nicotinamide adenine dinucleotide phosphate (NADPH) (6.22 mM ^{1} cm ^{1}) was used for calculations of GPx and GLR activities, expressed as mU per mg protein.

Western blot analysis of GPx, GLR, NF-κB p65, COX-2, IL-1β and TNF-α

Cytosolic and nuclear protein fractions of the PFC were prepared with denaturing buffer according to Laemmli (1970), separated on a SDS–polyacrylamide gel (Mini-Protean II Electrophoresis Cell, Bio-Rad, Hercules, CA, USA), and electroblotted to a polyvinylidene difluoride membrane using Mini Trans-blot apparatus (Bio-Rad). After 1-h blocking in a TBS-T buffer pH 7.6 containing 5% BSA, membranes were incubated overnight (4 °C) with rabbit antibody raised against GPx (sc-30147, Santa Cruz Biotechnology), GLR (sc-32886, Santa Cruz Biotechnology), NF-κB p65 (sc-372, Santa Cruz Biotechnology), COX-2 (sc-1747, Santa Cruz Biotechnology), IL-1β (AB1832P, Millipore) or TNF-α (AB1837P, Millipore), which was followed by 2-h incubation with anti-rabbit secondary antibody (sc-2004, Santa Cruz Biotechnology). The membranes were also stained for β-actin (sc-1616-R, Santa Cruz Biotechnology) to confirm a consistent protein loading. The blots were developed by enhanced chemiluminescence (Immobilon™ Western, Millipore Corporation, Billerica, USA), evaluated using a Chemidoc-MP System (Bio-Rad) and analyzed with Image Lab 5.0 software (BioRad). Protein molecular mass standards (Pierce Prestained protein Molecular Weight Marker, Thermo Scientific, USA) were used for calibration. All results were normalized against β-actin and expressed as the percent change relative to Veh-treated non-stressed rats (100%).

Statistical analysis

The results of behavior tests were analyzed using two-way repeated measures analysis of variance (ANOVA) (STATISTICA Release 7) [the factors were drug treatment (levels: Veh, FLX and CLZ), stress (levels: No stress and CSIS) and within-subject factor time (levels: baseline and 21d). Biochemical data were analyzed using a two-way ANOVA [the factors were drug treatment (levels: Veh, FLX and CLZ) and stress (levels: No stress and CSIS)]. Duncan's post hoc test was used to evaluate differences between groups and $p < 0.05$ was considered statistically significant. The data are expressed as mean ± S.E.M. of 5–6 animals per group.

RESULTS

FLX and CLZ prevented CSIS-induced changes in hedonic- and anxiety-like behaviors

A two-way repeated measures ANOVA was used to analyze the results of SP test and post hoc test analysis demonstrated that only Veh-treated CSIS group showed significantly decreased SP after day 21 compared to appropriate baseline value ($^{*}p < 0.05$) (Fig. 1A). Also, FLX- and CLZ-treated CSIS rats showed significantly higher SP values compared to Veh-treated CSIS rats at the end of experiment (21d) ($^{\wedge\wedge}p = 0.01$, $^{\wedge}p < 0.05$, respectively). With regard to marble burying, a two-way repeated measures ANOVA revealed a significant main effect of CSIS ($F_{1.30} = 5.05$, $p < 0.05$) and drug ($F_{2.30} = 5.49$, $p < 0.01$), as well as a significant main effect of time ($F_{1.30} = 19.56$, $p < 0.001$) and

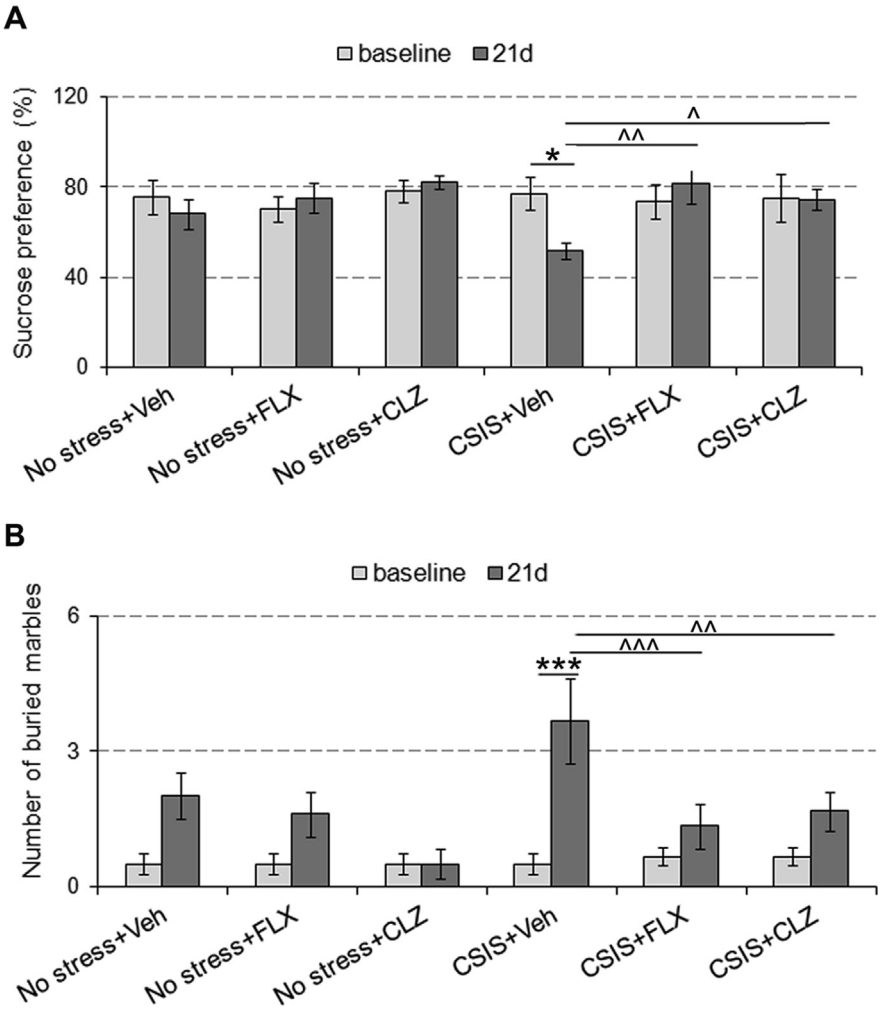

Fig. 1. Sucrose preference (A) and number of buried marbles (B) in No stress and CSIS rats treated with vehicle (0.9% NaCl), fluoxetine (15 mg/kg/day) or clozapine (20 mg/kg/day) at baseline and after 21d of stress exposure or/and drug treatment. CSIS = chronic social isolation; Veh = vehicle; FLX = fluoxetine; CLZ = clozapine. $^{*}p < 0.05$, $^{***}p < 0.001$ values measured at the end of the experiments (21d) vs appropriate baseline values for CSIS + Veh;, $^{\wedge\wedge}p = 0.01$, $^{\wedge\wedge\wedge}p < 0.001$ CSIS + FLX (21d) vs CSIS + Veh (21d); $^{\wedge}p < 0.05$, $^{\wedge\wedge}p < 0.01$ CSIS + CLZ (21d) vs CSIS + Veh (21d).

time drug interaction ($F_{2.30} = 3.97$, $p < 0.05$). Comparing day 21 to the appropriate baseline values, post hoc test analyses revealed an increased number of buried marbles in the Veh-treated CSIS group ($^{***}p < 0.001$) (Fig. 1B). In other experimental groups, there was no statistical significance between these two time points. A significant decrease in buried marbles was found in FLX- and CLZ-treated CSIS rats compared to Veh-treated CSIS on day 21 ($^{\wedge\wedge\wedge}p < 0.001$, $^{\wedge\wedge}p < 0.01$, respectively).

GSH content and GPx and GLR activity

A two-way ANOVA revealed a significant main effect of drug on GSH levels ($F_{2.24} = 6.90$, $p < 0.01$) (Table 2). Post-hoc tests showed a significant decrease in GSH levels in the Veh-treated CSIS group compared to the Veh-treated non-stressed rats ($^{*}p < 0.05$). Also, significantly lower GSH levels were found in both CLZ-treated rats (No stress and CSIS) compared to Veh-treated non-stressed rats ($^{**}p < 0.01$). With regard to GPx activity, a two-way ANOVA demonstrated significant main effect of CSIS ($F_{1.27} = 8.63$, $p < 0.01$) and drug ($F_{2.27} = 3.64$, $p < 0.05$). Post-hoc tests showed significant increases in GPx activity in Veh-treated CSIS ($^{*}p < 0.05$), CLZ-treated rats (No stress and CSIS) and FLX-treated CSIS rats ($^{**}p < 0.01$) compared to Veh-treated non-stressed rats. Also, a significant difference was demonstrated between the FLX-treated CSIS and FLX-treated non-stressed groups ($^{\#}p < 0.05$). In the case of GLR activity, a two-way ANOVA did not show any significant differences.

Results from Western blot analysis

GPx and GLR protein levels. A two-way ANOVA revealed a significant main effect of drug treatment ($F_{2.25} = 4.78$, $p < 0.05$), and post hoc testing showed a significant increase in GPx protein levels in Veh- and FLX-treated CSIS ($^{*}p < 0.05$) groups, as well in CLZ-treated animals (No stress and CSIS) ($^{**}p$ 0.01) compared to Veh-treated non-stressed animals (Fig. 2A). In contrast, no significant change was seen in the protein levels of GLR (Fig. 2B).

NF-jB activation. Cytosolic and nuclear protein levels of NF-κB p65 were measured to test NF-κB activation and nuclear translocation. A two-way ANOVA demonstrated a significant CSIS drug interaction on cytosolic NF-κB levels ($F_{2.29} = 4.69$, $p < 0.05$), and post hoc tests showed a significant decrease in Veh-treated CSIS rats compared to Veh-treated non-stressed animals ($^{**}p = 0.01$) (Fig. 3A). Also, it was shown that treatment with both FLX and CLZ significantly increased cytosolic NF-κB levels in CSIS rats ($^{\wedge}p < 0.05$). In the case of nuclear NF-κB levels, a two-way ANOVA demonstrated significant main effect of CSIS ($F_{1.26} = 4.71$, $p < 0.05$) and drug ($F_{2.26} = 3.84$, $p < 0.05$). Post-hoc analysis showed a significant increase in NF-κB protein levels in the nuclear fraction of Veh-treated CSIS rats compared to Veh-treated non-stressed rats ($^{**}p = 0.01$) (Fig. 3B). In addition, a significant decrease in FLX- and CLZ-treated CSIS compared to Veh-treated CSIS was demonstrated ($^{\wedge\wedge}p = 0.01$).

Table 2. Effect of CSIS or/and chronic administration of FLX (15 mg/kg/day) or CLZ (20 mg/kg/day) on GSH content, GPx and GLR activity in the cytosol of rat prefrontal cortex

Groups	GSH levels (nmol/mg protein)	GPx activity (mU/mg protein)	GLR activity (mU/mg protein)
No stress + Veh	70.9 ± 4.0	87.0 ± 1.1	24.7 ± 0.4
No stress + FLX	62.5 ± 4.2	92.1 ± 1.8	25.1 ± 0.9
No stress + CLZ	54.9 ± 2.9**	104.6 ± 5.0**	24.2 ± 1.3
CSIS + Veh	60.2 ± 4.1*	100.9 ± 5.1*	24.6 ± 1.1
CSIS + FLX	65.9 ± 1.8	106.2 ± 3.4**,#	24.6 ± 0.8
CSIS + CLZ	54.0 ± 0.7**	103.1 ± 3.8**	22.6 ± 1.6

Veh = vehicle; FLX = fluoxetine; CLZ = clozapine; CSIS = chronic social isolation; GSH = glutathione; GPx = glutathione peroxidase; GLR = glutathione reductase.
* p 0.05.
** $p < 0.01$ treated experimental groups vs No stress + Veh.
# $p < 0.05$ CSIS + FLX vs No stress + FLX.

Protein levels of proinflammatory mediators. A two-way ANOVA revealed a significant CSIS drug interaction ($F_{2.25} = 4.63$, $p < 0.05$) on cytosolic COX-2 protein levels. Post-hoc tests showed a significant increase in cytosolic COX-2 in Veh-treated CSIS compared to Veh-treated non-stressed animals ($^{*}p < 0.05$) (Fig. 4). Also, post hoc analyses demonstrated a significant decrease in FLX- and CLZ-treated CSIS ($^{\wedge}p < 0.05$, $^{\wedge\wedge}p < 0.01$, respectively) as compared to Veh-treated CSIS animals. With regard to IL-1β, a two-way ANOVA revealed a significant main effect of drug ($F_{2.30} = 3.52$, $p < 0.05$). Post-hoc tests showed a significant increase in IL-1β protein levels in Veh-treated CSIS compared to Veh-treated non-stressed rats ($^{*}p < 0.05$) (Fig. 5A), as well as a significant decrease in FLX- and CLZ-treated CSIS as compared to Veh-treated CSIS rats ($^{\wedge\wedge}p < 0.01$, $^{\wedge}p < 0.05$, respectively). A two-way ANOVA revealed a significant main effect of drug on TNF-α protein levels ($F_{2.30} = 4.70$, $p < 0.05$), as well as a significant CSIS drug interaction ($F_{2.30} = 7.80$, $p < 0.01$). Post-hoc tests showed significantly increased levels of this cytokine in Veh-treated CSIS compared to Veh-treated non-stressed animals ($^{**}p < 0.01$) (Fig. 5B). In FLX- and CLZ-treated CSIS rats, a significant decrease was seen in comparison to Veh-treated CSIS animals ($^{\wedge\wedge\wedge}p < 0.001$, $^{\wedge\wedge}p < 0.01$, respectively). FLX and CLZ-treated non-stressed rats did not show a significant difference in the levels of proinflammatory mediators compared to Veh-treated non-stressed animals.

DISCUSSION

In the present study, we investigated GSH-dependent defense and proinflammatory mediators in the PFC of adult male rats exposed to CSIS with concomitant FLX or CLZ treatment, in order to examine stress-induced prefrontal cortical changes associated with depressive- and anxiety-like behaviors and the potential neuroprotective effects of these drugs.

CSIS induced depressive-like behavior in rats, as demonstrated by decreased sucrose preference, indicative of anhedonic-like behavior and impaired sensitivity to reward, as well as anxiety-like behavior assessed by increased burying behavior. Despair behavior, as indicated by increased immobility and less time swimming and climbing in the forced swim test, was previously detected in this model (Zlatković et al., 2014a). Anxiety-like behavior in chronically isolated adult male rats was also demonstrated in the elevated plus

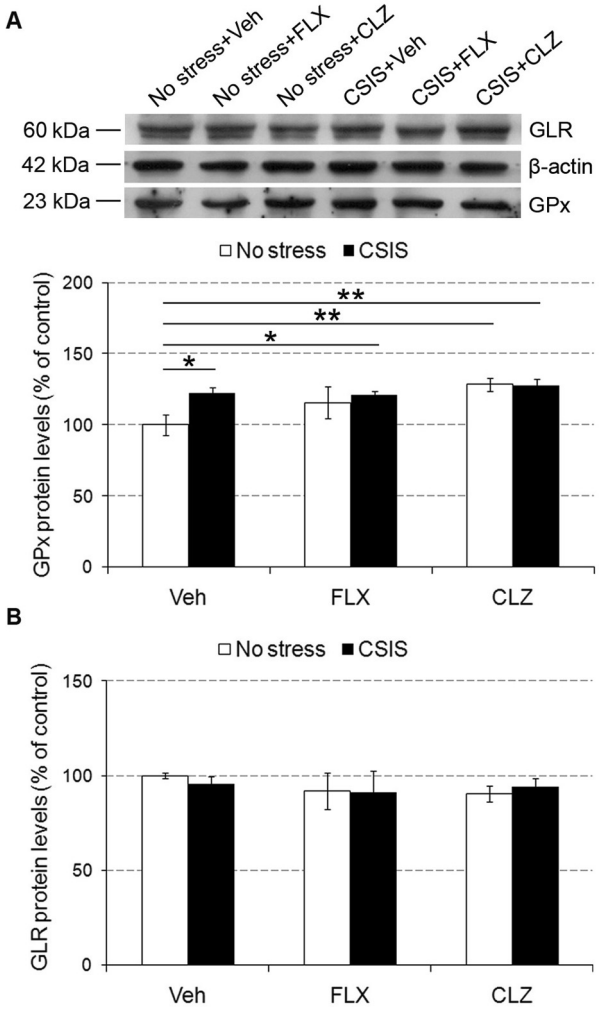

Fig. 2. Cytosolic GPx (A) and GLR (B) protein levels in the prefrontal cortex of No stress and CSIS rats treated with vehicle (0.9% NaCl), fluoxetine (15 mg/kg/day) or clozapine (20 mg/kg/day). CSIS = chronic social isolation; Veh = vehicle; FLX = fluoxetine; CLZ = clozapine, GPx = glutathione peroxidase; GLR = glutathione reductase. Asterisks indicate statistically significant difference between treated groups and vehicle-treated non-stressed rats $^{*}p < 0.05$; $^{**}p$ 0.01.

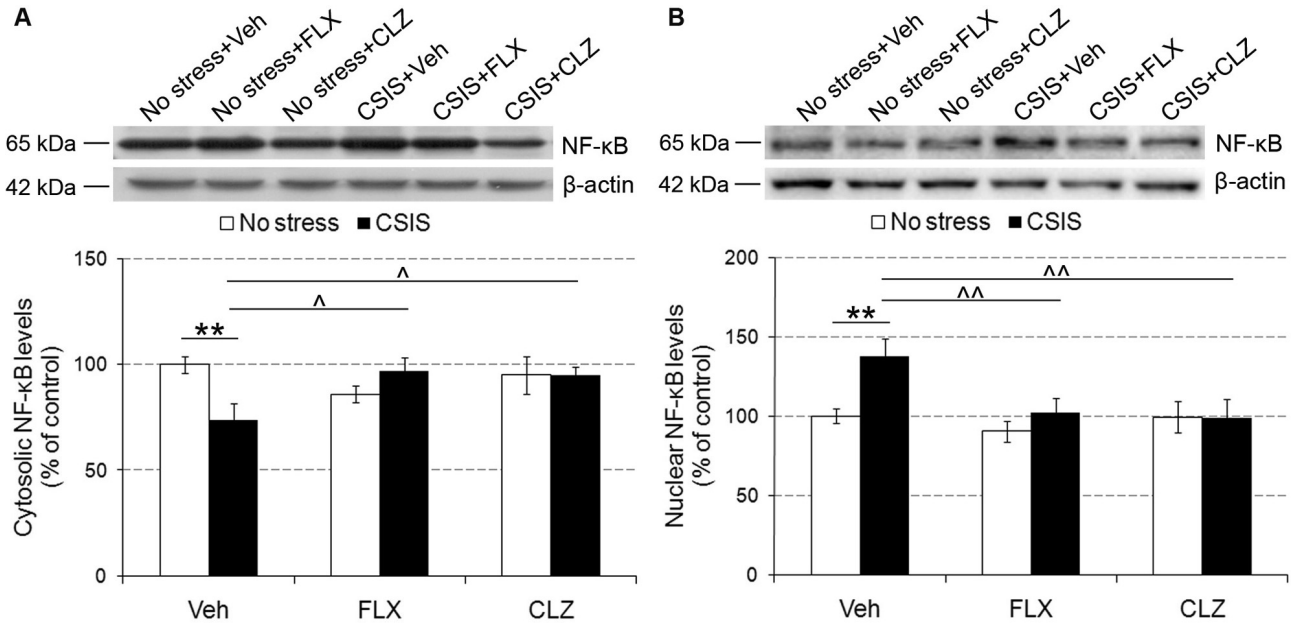

Fig. 3. Cytosolic (A) and nuclear (B) NF-κB p65 protein levels in the prefrontal cortex of No stress and CSIS rats treated with vehicle (0.9% NaCl), fluoxetine (15 mg/kg/day) or clozapine (20 mg/kg/day). CSIS = chronic social isolation; Veh = vehicle; FLX = fluoxetine; CLZ = clozapine; NF-κB = nuclear factor-kappa B. $^{**}p$ 0.01 CSIS + Veh vs No stress + Veh; $^{\wedge}p < 0.05$, $^{\wedge\wedge}p < 0.01$, CSIS + FLX or CSIS + CLZ vs CSIS + Veh.

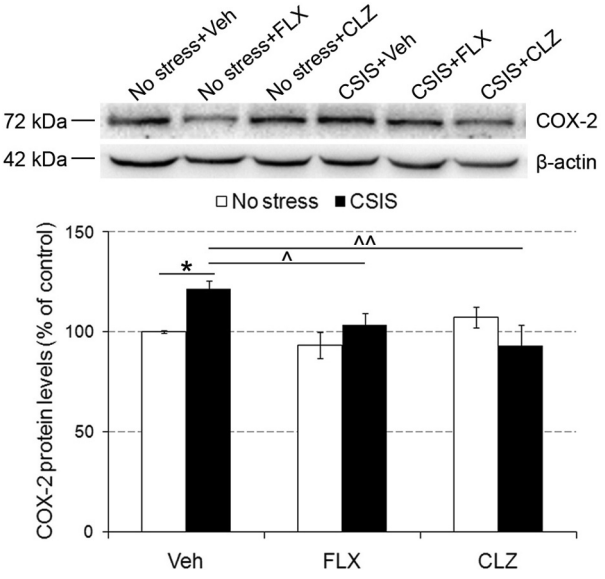

Fig. 4. Cytosolic COX-2 protein levels in the prefrontal cortex of No stress and CSIS rats treated with vehicle (0.9% NaCl), fluoxetine (15 mg/kg/day) or clozapine (20 mg/kg/day). CSIS = chronic social isolation; Veh = vehicle; FLX = fluoxetine; CLZ = clozapine; COX-2 = cyclooxygenase 2. $^{*}p < 0.05$ CSIS + Veh vs No stress + Veh; $^{\wedge}p < 0.05$, $^{\wedge\wedge}p < 0.01$ CSIS + FLX and CSIS + CLZ vs CSIS + Veh, respectively.

maze (Djordjevic et al., 2015), elevated platform test (Spasojevic et al., 2007) and light–dark box test (Carrier and Kabbaj, 2012). Treatment with FLX (15 mg/kg/day) or CLZ (20 mg/kg/day) applied during CSIS prevented changes in hedonic- and anxiety-like behaviors observed in chronically isolated rats. Previous studies have shown that FLX treatment significantly increased sucrose preference in rats exposed to various chronic stress paradigms (Rong et al., 2010; Yang et al., 2014; Chen et al., 2015). Vardigan et al. (2010) demonstrated that CLZ reversed deficits associated with sucrose preference in one model of schizophrenia. With regard to the anxiolytic properties, both FLX and CLZ were previously shown to reduce the number of buried marbles in mice (Bruins Slot et al., 2008).

Because GSH is the major scavenger of free radicals in the brain, its depletion after various chronic psychological stress exposures (Hong et al., 2014; Samarghandian et al., 2016), as well as in post-mortem PFC from patients with psychiatric disorders (Gawryluk et al., 2011), indicate the importance of investigation of the vulnerability of GSH-dependent defense in stress conditions. Here, we found decreased cytosolic GSH levels in the PFC of CSIS rats, likely a consequence of the increased expression and activity of GPx, also found in these animals. Our previous studies revealed increased risk for peroxynitrite production (Zlatković and Filipović, 2013), as well as elevated lipid peroxidation in the PFC of CSIS rats (Zlatković et al., 2014a). Since GPx reduces peroxynitrite to nitrite using GSH (Arteel Gavin and Briviba Karlis, 2000), and also detoxifies lipid peroxides, increased expression and activity of this protective enzyme were expected. Surprisingly, protein levels and activity of GLR were unchanged, which compromised compensation of GSH consumed by GPx. This resulted in overall depleted GSH levels. A CSIS-induced decrease in GSH levels was prevented only by FLX treatment. Interestingly, FLX treatment did not prevent a CSIS-induced increase in the level and activity of GPx and did not affect GLR levels, which suggests that basal levels of GSH found in these animals was probably maintained by its *de novo* synthesis. The positive influence of chronic

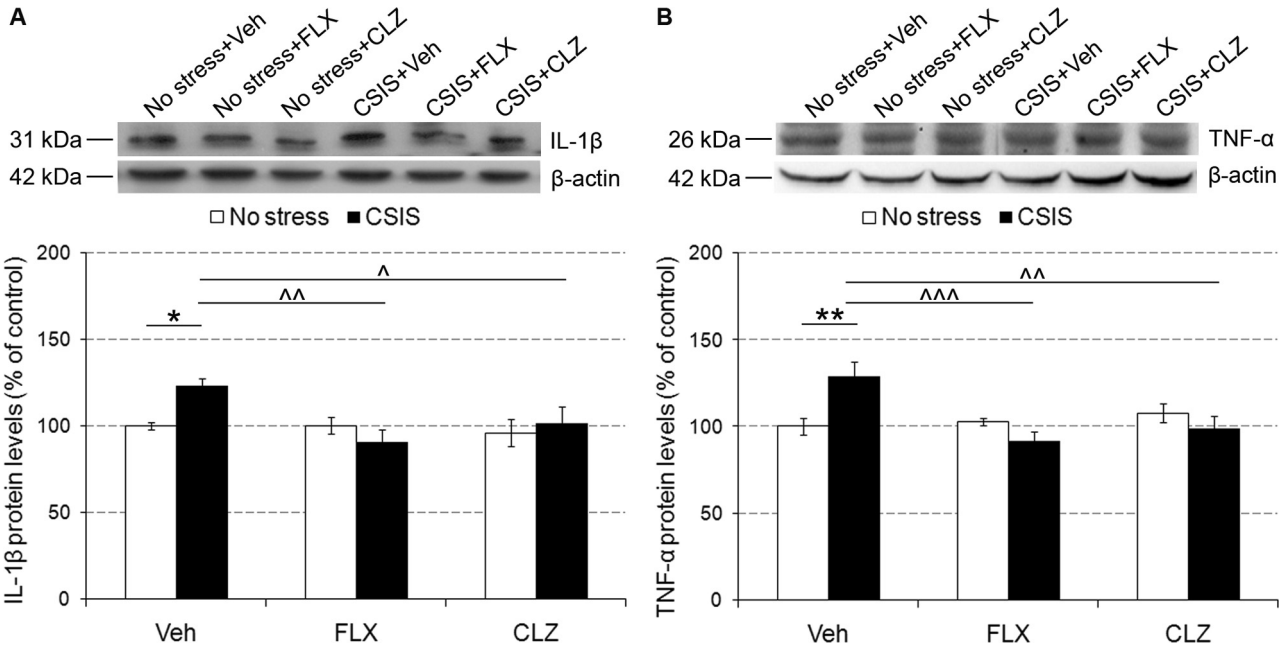

Fig. 5. IL-1β (A) and TNF-α (B) protein levels in cytosol of the prefrontal cortex of No stress and CSIS rats treated with vehicle (0.9% NaCl), fluoxetine (15 mg/kg/day) or clozapine (20 mg/kg/day).CSIS = chronic social isolation; Veh = vehicle; FLX = fluoxetine; CLZ = clozapine; IL-1β = interleukin-1beta; TNF-α = tumor necrosis factor-alpha. $^{*}p < 0.05$, $^{**}p < 0.01$ CSIS + Veh vs No stress + Veh; $^{\wedge}p < 0.05$, $^{\wedge\wedge}p < 0.01$, $^{\wedge\wedge\wedge}p < 0.001$ CSIS + FLX or CSIS + CLZ vs CSIS + Veh.

FLX treatment on cortical levels of glutamate-cysteine ligase (Mendez-David et al., 2015), the enzyme which catalyzes the first step and is rate-limiting for GSH biosynthesis (Sastre et al., 2005), support this conclusion. In contrast, CLZ not only failed to prevent a CSIS-induced GSH decrease, but caused its decline in non-stressed animals. Previous data regarding the effect of CLZ on antioxidative systems in the brain are inconsistent, but its negative impact on GSH levels was reported previously (Ebuehi and Odiba, 2013). The fact that CLZ can be oxidized to the reactive and toxic nitrenium ion, which is further detoxified by GSH, may partly explain its decrease in CLZ-treated non-stressed rats (Yang et al., 2011).

Elevated inflammatory signaling in mood-relevant brain regions, including the PFC, has been reported by preclinical and clinical studies of psychiatric conditions (Kiecolt-Glaser et al., 2015; Réus et al., 2015). In the present study, increased levels of NF-κB in the nuclear fraction, accompanied by increases in cytosolic protein levels of COX-2, IL-1β and TNF-α in the PFC of CSIS rats, were probably induced by oxidative and nitrosative stress previously reported in this animal model (Zlatković and Filipović, 2013; Zlatković et al., 2014a). In addition, dysregulated HPA axis activity and incomplete nuclear translocation of cytosolic GR found in CSIS rats (Dronjak et al., 2004; Filipović et al., 2005) may also contribute to NF-κB activation, as glucocorticoids induce the transcription of IκB, which keeps NF-κB in an inactive state in the cytoplasm (Miller et al., 2008). IL-1β and TNF-α induce cytokine cascades via self- and cross-stimulation, as well as the stimulation of other cytokines, and also induce the activity of nitric oxide synthase (NOS) and COX-2 via NF-κB (Vitkovic et al., 2000). This is consistent with our previous results which revealed CSIS-induced nitrosative stress in the PFC mediated by increased inducible NOS expression (Zlatković and Filipović, 2013). COX-2-derived prostaglandins promote inflammation, in which superoxide, generated as a side product of prostaglandin production (Morgan and Liu, 2011), contributes to oxidative stress and GSH depletion. Rao et al. (2010) found significantly increased protein levels of COX-2 and nuclear NF-κB p50 and p65 in post-mortem frontal cortex from bipolar disorder patients, while the blockade of cytokines or COX-2, an inflammatory signaling pathway component, has been shown to reduce depressive symptoms in patients with major depressive disorder (Köhler et al., 2014). A significant role for COX-2 in stress-induced depressive-like behavior in rats has been demonstrated in previous studies (Guo et al., 2009; Yao et al., 2015), as has the antidepressant effect of selective COX-2 inhibition (Müller, 2013).

FLX treatment prevented NF-κB activation and concomitant COX-2 upregulation and elevation of IL-1β and TNF-α in the PFC of CSIS rats. A recent study on microglial cells in rat glial cultures showed that FLX diminished lipopolysaccharide-induced nuclear translocation of the NF-κB p65 subunit and an increase in IL-1β and TNF-α concentrations (Obuchowicz et al., 2014). Van den Berg et al. (2014) summarized the current knowledge of the anti-inflammatory effect of FLX on activated microglia in the brain; however, the underlying mechanisms of these effects remain unknown. The suppression of NF-κB was stated as one of the possible mechanisms, which is in accordance with our data. Considering antagonism between GR and NF-κB (Rao et al., 2011), it is tempting to speculate that FLX prevents CSIS-induced GR retention in the cytoplasm and enables

transrepression of NF-κB. This is supported by the previous findings of Herr and coworkers (2003), who showed that FLX positively affects the efficiency of corticosteroid signal transduction.

CLZ treatment also prevented the CSIS-induced proinflammatory response in the rat PFC, as demonstrated by the absence of NF-κB nuclear translocation and by the basal cytosolic levels of COX-2, IL-1β and TNF-α. Hu et al. (2012) showed that CLZ attenuated lipopolysaccharide-induced neuronal damage in cortical cultures in a microglia-dependent manner. They revealed that CLZ-mediated neuroprotection was associated with its anti-inflammatory effect, primarily through the inhibition of microglial NADPH oxidase (NOX). As Schiavone et al. (2012) previously showed that CSIS elevated the NOX2 complex in the PFC and that apocynin/NOX inhibitor prevented behavioral changes associated with CSIS, we speculate that the protective effect of CLZ observed in this study was NOX2 mediated, but this requires further research.

CONCLUSION

Our data reveal that CSIS induced depressive- and anxiety-like behaviors, compromised GSH-dependent defense and triggered proinflammatory responses in the PFC of adult male Wistar rats. Treatment with FLX or CLZ, applied simultaneously with CSIS, showed protective effects against these observed stress-induced detrimental changes, though FLX was shown to be more beneficial for the PFC in CSIS than CLZ. In contrast to CLZ, which diminished GSH levels even in non-stressed rats, FLX offered protection from CSIS-induced GSH depletion. However, both FLX and CLZ prevented the increase in protein levels of proinflammatory mediators in the PFC, as well as behavioral changes, observed in CSIS rats. These results indicate the role of proinflammatory components in the pathophysiology of depressive- and anxiety-like states and the significance of anti-inflammatory properties of effective treatments. These findings also highlight the need for further experiments to achieve a full understanding of differences in the molecular mechanisms of the actions of FLX and CLZ, which could lead to personalized and more efficient use in patient treatment.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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The antidepressant- and anxiolytic-like effects of fluoxetine and clozapine in chronically isolated rats involve inhibition of hippocampal TNF-α

Nevena Todorović, Dragana Filipović*

Laboratory of Molecular Biology and Endocrinology, Institute of Nuclear Sciences "Vinča", University of Belgrade, 11001, Serbia

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ABSTRACT

Brain oxidative stress and neuroinflammation are implicated in psychiatric disorders. Thus, it is important to investigate the effects of individual psychotropic agents on antioxidative defense and proinflammatory mediators in brain regions associated with these disorders. Psychosocial stress is recognized as a threat to mental health, and the hippocampus is a primary target of stress-related damage. Chronic social isolation (CSIS) is a mild psychosocial stress used to model the pathophysiology of depression. We examined the antioxidative and anti-inflammatory potential of the antidepressant fluoxetine (FLX) and atypical antipsychotic clozapine (CLZ) in the hippocampus in the CSIS model of depression. We measured the effects of FLX and CLZ on depressive- and anxiety-like behaviors in non-stressed rats and rats exposed to 21d of CSIS. We further evaluated the content of reduced glutathione (GSH), the protein expression and activity of the GSH-related enzymes, the subcellular localization of nuclear factor-kappa B (NF-κB) and protein levels of proinflammatory mediators cyclooxygenase-2 (COX-2), interleukin-1beta (IL-1β), and tumor necrosis factor-alpha (TNF-α) in these groups of rats. CSIS resulted in an increase in depressive- and anxiety-like behaviors that corresponded with compromised glutathione peroxidase (GPx)-mediated antioxidative defense and increased TNF-α, but not with changes in NF-κB, IL-1β and COX-2 levels. FLX and CLZ, applied during CSIS, prevented the behavioral changes associated with CSIS, and inhibited the increase in TNF-α, but did not affect GPx-mediated antioxidative defense. Furthermore, both drugs decreased hippocampal GPx activity when applied to non-stressed rats. These results emphasize the significance of hippocampal TNF-α-mediated proinflammmatory signaling in the pathophysiology of depressive symptoms and the importance of the anti-inflammatory action of both FLX and CLZ in the prevention of these symptoms.

1. Introduction

Depression is a multifactorial disorder with a complex neurobiological basis that includes changes in the synthesis, metabolism, and reuptake of neurotransmitters and receptor expression, primarily serotonin (Aan het Rot et al., 2009; Albert et al., 2014). Treating depression remains challenging, as despite a large number of available medications, only 60–70% of patients with depression respond to antidepressant therapy (Al-Harbi, 2012). Fluoxetine (FLX), one of the most commonly used antidepressants, belongs to the group of selective serotonin reuptake inhibitors that prolong serotonin signaling (Di Rosso et al., 2015; Wagner, 2015). Clozapine (CLZ) is an atypical antipsychotic with high affinity for serotonin, dopamine, muscarinic, adrenergic and other biogenic amine receptors (Roth et al., 2004) mainly used in the treatment of psychotic disorders like schizophrenia, but also associated with the improvement of symptoms of depression in treatment-resistant bipolar disorder (Li et al., 2015). The atypical antipsychotics, including CLZ, enhance the antidepressant-like activity of antidepressants, with serotonin 5-HT1A, 5-HT2A and adrenergic α2 receptors likely mediating their action (Rogóż, 2013). Despite extensive research, the downstream molecular mechanisms of FLX and CLZ action beyond serotonergic and dopaminergic signaling are not fully understood. Their antioxidative and immunomodulatory potential are of particular interest, as a growing body of evidence suggests that oxidative stress and inflammation play important roles in stress-induced neuropsychiatric disorders, including depression (Bakunina et al., 2015;

Abbreviations: ANOVA, analysis of variance; CLZ, clozapine; COX-2, cyclooxygenase-2; CSIS, chronic social isolation; EDTA, ethylenediaminetetraacetic acid; FLX, fluoxetine; GLR, glutathione reductase; GPx, glutathione peroxidase; GR, glucocorticoid receptor; GSH, glutathione; GSSG, glutathione disulfide; GST, glutathione-S-transferase; IL-1β, interleukin-1 beta; NADPH, nicotinamide adenine dinucleotide phosphate; NF-κB, nuclear factor-kappa B; NO, nitric oxide; TBP, TATA binding protein; TNF-α, tumor necrosis factor-alpha; Veh, vehicle

* Corresponding author at: Laboratory of Molecular Biology and Endocrinology, Institute of Nuclear Sciences "Vinča", University of Belgrade, P.O. Box 522-090, 11001 Belgrade, Serbia.
E-mail address: dragana@vinca.rs (D. Filipović).
URL: http://www.vinca.rs (D. Filipović).

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Kim et al., 2016; Maes et al., 2009; Munhoz et al., 2008). The information regarding this issue is limited by inconsistent findings between *in vitro* and scarce *in vivo* experimental studies (Baumeister et al., 2015; Caiaffo et al., 2016; Sadowska-Bartosz et al., 2016). New findings, especially from *in vivo* studies, may be beneficial for future treatment strategies.

The hippocampus is a brain structure particularly sensitive to stress stimuli and strongly associated with psychiatric disorders (McEwen, 1999; Sala et al., 2004). Chronic social isolation (CSIS) is a mild psychosocial stress demonstrated to be a reliable and useful model of depressive disorders (Abelaira et al., 2013) and schizophrenia (Möller et al., 2013). It causes depressive- and anxiety-like behaviors in adult, male Wistar rats in as few as 21 days (Zlatković et al., 2014b), and the social nature of this stress makes it more naturalistic than those that include physical stress (Heinrichs and Koob, 2006).

The glutathione (GSH)-dependent defense system acts as a first line of defense against oxidative stress and includes GSH, glutathione peroxidase (GPx) and glutathione reductase (GLR). GPx catalyzes the reduction of hydrogen peroxide, lipid peroxides and peroxynitrite, using GSH as a substrate. The resulting oxidized glutathione (GSSG) is reduced back to GSH by nicotinamide adenine dinucleotide phosphate (NADPH) in a reaction catalyzed by GLR (Sastre et al., 2005). A postmortem study of bipolar disorder patients revealed a significant downregulation of some antioxidative enzymes, including GPx, in the hippocampus (Benes et al., 2006), and increased markers of oxidative stress have repeatedly been reported in the blood of depressed patients (Lopresti et al., 2014). In addition, elevated levels of proinflammatory cytokines, primarily interleukin-1beta (IL-1β) and tumor necrosis factor-alpha (TNF-α), were reported in the cerebrospinal fluid and blood of patients suffering from depression (Raison et al., 2006). In animals, administration of these cytokines may cause depressive- and anxiety-like behaviors (Bluthé et al., 2000; Dantzer et al., 2008). Furthermore, the inhibition of proinflammatory mediators and reduction of oxidative stress in the brain were shown to attenuate behavioral abnormalities in a lipopolysaccharide (LPS)-induced animal model of depression (Gawali et al., 2016; Huang et al., 2016). Pro- and antioxidative, as well as inflammatory events, are regulated and coordinated by several transcriptional factors, including nuclear factor-kappa B (NF-κB) (Bakunina et al., 2015). NF-κB induces the expression of various genes, including those that mediate the expression of cyclooxygenase-2 (COX-2), the enzyme that catalyzes the conversion of arachidonic acid to prostaglandin H_2 (Morgan and Liu, 2011), as well as proinflammatory cytokines (Bakunina et al., 2015; Kaltschmidt et al., 2002) and GPx (Morgan and Liu, 2011).

Recently, we demonstrated that 21 days of CSIS caused depressive- and anxiety-like behaviors, a compromised hypothalamic–pituitary–adrenal axis, and increased hippocampal levels of nitric oxide (NO) and protein oxidation (Zlatković et al., 2014b). In addition, compromised GSH-dependent defense and proinflammatory signaling were found in the prefrontal cortex in this model (Todorović and Filipović, 2017). In the current study, we examined whether the antidepressant FLX and atypical antipsychotic CLZ demonstrate antioxidative and anti-inflammatory effects in the hippocampus in the CSIS model. We monitored hippocampal GSH-dependent antioxidative defense, subcellular localization of transcriptional factor NF-κB, and protein levels of proinflammatory mediator COX-2 and cytokines IL-1β and TNF-α.

2. Materials and methods

2.1. Animals

Adult male Wistar rats (2.5 months old, 300–350 g body weight) were housed under standard conditions in a temperature-controlled environment (21–23 °C) with a 12 h/12 h light/dark cycle, with food (commercial rat pellets) and water available *ad libitum*. All experimental procedures were conducted in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and approved by the Ethical Committee for the Use of Laboratory Animals of the Institute of Nuclear Sciences "Vinča", Belgrade, Serbia (Application No. 02/11).

2.2. Preparation of drugs solutions

Flunisan (20 mg of fluoxetine-hydrochloride per tablet) and Leponex tablets (25 mg of CLZ per tablet), as well as corresponding reference standards, were purchased from Hemofarm, Serbia and Novartis Pharmaceuticals UK, respectively. Flunisan tablets were dissolved in distilled, sterile water with the aid of ultrasound. Leponex tablets were dissolved daily in 1 N HCl with gentle heating and the solution obtained was diluted with distilled water and neutralized with 1 N NaOH to a pH of 5.1 (Halim et al., 2004). The drug solutions obtained were filtered through Whatman No. 42 filter paper. The drug concentrations in the prepared solutions were determined using Ultra Performance Liquid Chromatography analysis (Kovacevic et al., 2006). The concentrations obtained were used to determine the volume of drug solution administered to each animal in order to achieve doses of 15 mg/kg/day FLX or 20 mg/kg/day CLZ, based on rat weight, measured once per week. These doses were chosen to emulate the therapeutic doses given to patients (Czeh et al., 2006; Halim et al., 2004; Larsson et al., 2015). The validity of doses was confirmed by measuring serum concentrations of FLX or CLZ 24 h after the last administration using liquid chromatography-mass spectrometry (Djordjevic et al., 2005) and the liquid chromatography-tandem mass spectrometry method (Song et al., 2009), respectively, in No stress and CSIS groups. The measured serum drug levels (100–300 ng/ml) corresponded to those reported in human patients treated with therapeutically effective doses (100–700 ng/ml) (Zlatković et al., 2014c).

2.3. Study design

Prior to stress exposure, animals were housed in groups of four per cage and randomly divided into 6 groups (Table 1). Non-stressed rats (No stress) continued to be housed in groups of four animals per cage, and CSIS rats were housed individually for 21 days with normal auditory and olfactory experiences but without visual or tactile contact with each other. Non-stressed and CSIS rats were intraperitoneally (i.p.) treated either with vehicle (Veh, 0.9% NaCl), FLX (15 mg/kg/day) or CLZ (20 mg/kg/day) for 21 days.

2.4. Behavioral assessments

Behavioral tests were conducted one day prior to the first day of the experimental protocol (baseline) and on the 21st day of treatment (21 d). All animals were separated into individual cages during testing.

Table 1
Experimental groups and respective treatments.

Group	Stress	Drug
No stress + Veh	No stress	Vehicle (0.9% NaCl)
No stress + FLX	No stress	Fluoxetine-hydrochloride (15 mg/kg/day)
No stress + CLZ	No stress	Clozapine (20 mg/kg/day)
CSIS + Veh	21 d social isolation	Vehicle (0.9% NaCl)
CSIS + FLX	21 d social isolation	Fluoxetine-hydrochloride (15 mg/kg/day)
CSIS + CLZ	21 d social isolation	Clozapine (20 mg/kg/day)

Veh = vehicle; FLX = fluoxetine; CLZ = clozapine; CSIS = chronic social isolation.

2.4.1. Sucrose preference test

The sucrose preference test measures anhedonia, described as a decreased ability to experience pleasure (Willner et al., 1987). Two pre-weighed bottles, one containing 0% (tap water) and the other containing 1% sucrose solution, were placed in each cage. After 1 h, bottles were removed and re-weighed and liquid consumption was calculated as the difference in the weight (g) of each bottle before and after the test. Sucrose preference was calculated using the formula Sucrose preference = [consumed sucrose solution/total liquid consumed (sucrose solution + water)] × 100.

2.4.2. Marble burying test

This test is related to object burying, which in rodents represents a specific defense reaction to aversive stimuli. Although Thomas et al. (2009) argued that marble burying behavior is a reflection of repetitive and perseverative digging behavior more than novelty-induced anxiety, previous data strongly suggest that anxiolytic drugs suppress this behavior, and therefore this test is indicative of anxiety (Farley et al., 2010; Ho et al., 2002). Six glass marbles (2.5 cm in diameter) were placed evenly across the sawdust bedding. After 30 min, the animals were removed from the cages and the number of buried marbles (with at least two-thirds of the surface covered with bedding) was assessed, with the experimenter blind to the group conditions. The results are shown as the mean number of buried marbles.

2.5. Preparation of hippocampal cytosolic and nuclear fractions

Rats were sacrificed 24 h after the last FLX or CLZ administration. Animals were deeply anesthetized with ketamine/xylazine (100/5 mg/kg i.p.), transcardially perfused with physiological saline and then sacrificed by guillotine decapitation. Whole brains were immediately removed and hippocampi were dissected on an ice-cold plate. Cytosolic and nuclear hippocampal fractions were obtained by differential centrifugation, as previously described (Zlatković et al., 2014b). Protein concentrations were measured by a modified Lowry method (Markwell et al., 1978) using purified bovine serum albumin as a standard. The purity of the subcellular fractions was confirmed by Western blot analysis (Fig. 1). Cytosolic and nuclear probes (20 μg of proteins per lane) were loaded onto a SDS-polyacrylamide gel. The membrane blot was incubated with primary antibodies raised against α-tubulin (sc-8035, Santa Cruz Biotechnology), and TATA binding protein (TBP, ab51841, Abcam), used as cytosolic and nuclear markers, respectively, and β-actin (sc-47778, Santa Cruz Biotechnology) as loading control. Protein bands for specifically sublocalized proteins were detected in corresponding subcellular fraction, confirming the relative purity of these preparations. β-actin, although more abundant in cytosol, was also detected in nuclear fraction, as expected (Visa and Percipalle, 2010).

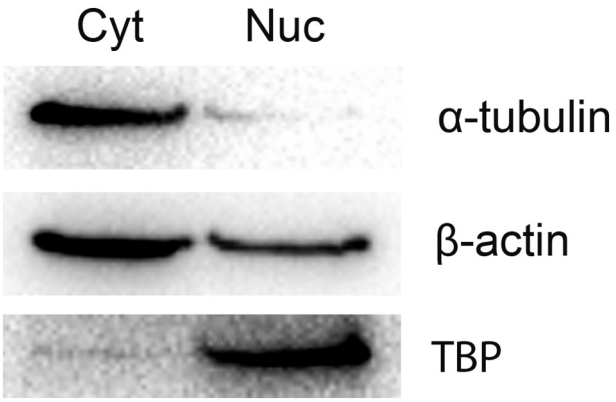

Fig. 1. The purity of the cytosolic (Cyt) and nuclear (Nuc) protein fraction of the hippocampus. TBP = TATA binding protein

2.6. Spectrophotometric analyses of components of GSH-dependent defense

2.6.1. GSH levels

Cytosolic GSH content was determined spectrophotometrically according to Ellman's method (1959) and modified by Hissin and Hilf (1976). The assay is based on the Ellman's reagent reduction by –SH groups of GSH and 2-nitro-s-mercaptobenzoic acid formation. This resulted in the production of a yellow-colored mixture with absorbance measured at 405 nm. Standard GSH (0.25–2 mM) was used for the calibration curve. GSH concentrations in the samples are expressed as nmol/mg protein.

2.6.2. GPx and GLR activity

Cytosolic GPx activity was measured using a Ransel kit (Randox Laboratories, Crumlin, UK) according to the manufacturer's protocol. GLR activity was determined according to the method of Halliwell and Foyer (1978). GPx and GLR activities were calculated using the molar extinction coefficient of NADPH (6.22 mM^{-1} cm^{-1}) and are expressed as mU/mg protein.

2.7. Western blot analyses of GPx, GLR, NF-κB p65, COX-2, IL-1β and TNF-α

Cytosolic and nuclear fractions of the hippocampus were prepared with denaturing buffer according to Laemmli (1970) and separated on a SDS-polyacrylamide gel using a Mini-Protean II Electrophoresis Cell (Bio-Rad, Hercules, CA, USA). The separated proteins were electroblotted to a polyvinylidene difluoride membrane using Mini Trans-blot apparatus (Bio-Rad). Membranes were blocked in a TBS-T buffer pH 7.6 containing 5% BSA for 1 h and incubated overnight (4 °C) with rabbit antibody raised against GPx (sc-30147, Santa Cruz Biotechnology), GLR (sc-32886, Santa Cruz Biotechnology), NF-κB p65 (sc-372, Santa Cruz Biotechnology), COX-2 (sc-1747, Santa Cruz Biotechnology), IL-1b (AB1832P, Millipore) or TNF-α (AB1837P, Millipore). Afterwards, membranes were washed (TBS-T buffer pH 7.6) and incubated with appropriate secondary antibody (sc-2004, Santa Cruz Biotechnology) for 2 h. After washing steps, the blots were developed by enhanced chemiluminescence (Immobilon™ Western, Millipore Corporation, Billerica, USA), evaluated using a Chemidoc-MP System (Bio-Rad) and analyzed with Image Lab 5.0 software (BioRad). Protein molecular mass standard (Pierce Prestained protein Molecular Weight Marker, Thermo Scientific, USA) was used for calibration. All results were normalized against β-actin (sc-1616-R, Santa Cruz Biotechnology) and are expressed as the percent change relative to Veh-treated non-stressed rats (100%).

2.8. Statistical analysis

The results of behavioral tests were analyzed using two-way repeated measures analyses of variance (ANOVA) (STATISTICA Release 7) [the factors were drug treatment (levels: Veh, FLX and CLZ), stress (levels: No stress and CSIS) and within-subject factor time (levels: baseline and 21d). The results obtained from spectrophotometric and western blot analyses were analyzed using two-way ANOVA [the factors were drug treatment (levels: Veh, FLX and CLZ) and stress (levels: No stress and CSIS stress)]. Differences between groups were evaluated using Duncan's post-hoc test, with statistical significance set at $p < 0.05$. The data are expressed as mean ± S.E.M. of 5–6 animals per group.

3. Results

3.1. The effects of CSIS and/or FLX or CLZ treatment on anhedonic- and anxiety-like behaviors

Duncan's post-hoc tests revealed a significantly decreased sucrose preference in the Veh-treated CSIS group after 21 d compared to the baseline value ($^{*}p < 0.05$) (Fig. 2A).

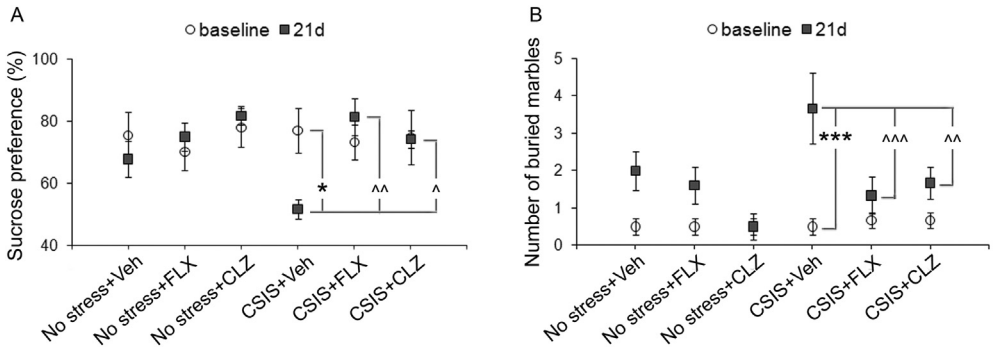

Fig. 2. Sucrose preference (A) and the number of buried marbles (B) (mean ± SEM) in non-stressed and CSIS rats treated with Veh (0.9% NaCl), FLX (15 mg/kg/day) or CLZ (20 mg/kg/day) at the beginning (baseline) and the end (21 d) of the experiment. CSIS = chronic social isolation; Veh = vehicle; FLX = fluoxetine; CLZ = clozapine. Symbols indicate significant difference: $^{*}p < 0.05$, $^{***}p < 0.001$ values measured at the end of the experiments (21 d) *vs* appropriate baseline values for CSIS + Veh; $\tilde{}\tilde{}p = 0.01$, $\hat{}\hat{}p < 0.001$ CSIS + FLX (21 d) *vs* CSIS + Veh (21 d); $\hat{}p < 0.05$, $\tilde{}\tilde{}p < 0.01$ CSIS + CLZ (21 d) *vs* CSIS + Veh (21 d).

No significant differences were observed between these two time points in other experimental groups. FLX- and CLZ-treated CSIS rats had significantly higher sucrose preference than Veh-treated CSIS rats ($\tilde{}\tilde{}p = 0.01$, $\hat{}p < 0.05$, respectively) at the conclusion of the experiment (21 d). With regard to marble burying, a two-way repeated measures ANOVA revealed significant main effects of CSIS ($F_{1.30} = 5.05$, $p < 0.05$), drug ($F_{2.30} = 5.49$, $p < 0.01$), and time ($F_{1.30} = 19.56$, $p < 0.001$), and a significant drug × time interaction ($F_{2.30} = 3.97$, $p < 0.05$). An increased number of buried marbles in Veh-treated CSIS group was revealed after 21d compared to the appropriate baseline ($^{***}p < 0.001$) (Fig. 2B). In other experimental groups, there were no differences between these two time points. A significantly decreased number of buried marbles was found in FLX- and CLZ-treated CSIS rats compared to Veh-treated CSIS at the conclusion of the experiment (21d) ($\hat{}\hat{}\tilde{}p < 0.001$, $\tilde{}p < 0.01$, respectively).

3.2. Hippocampal GSH-dependent defense

3.2.1. GSH content

A two-way ANOVA showed no effect of CSIS or drug on cytosolic GSH levels in rat hippocampus (Fig. 3).

3.2.2. Protein levels and activity of GPx

A two-way ANOVA revealed a significant main effect of drug ($F_{2.29} = 5.66$, $p < 0.01$) on cytosolic GPx levels in rat hippocampus. Post-hoc tests showed a significant decrease in cytosolic protein levels in FLX- and CLZ-treated CSIS rats ($^{*}p < 0.05$, $^{**}p < 0.01$, respectively) compared to Veh-treated non-stressed rats (Fig. 4A). In addition, a significant decrease was seen in CLZ-treated CSIS rats compared to Veh-treated CSIS animals ($\hat{}p < 0.05$). With regard to GPx activity, a two-way ANOVA revealed a significant main effects of CSIS ($F_{1.24} = 13.07$, $p = 0.001$) and drug ($F_{2.24} = 5.72$, $p < 0.01$). Post-hoc tests showed that FLX- and CLZ-treated non-stressed, as well as Veh-, FLX- and CLZ-treated CSIS rats, had significantly lower GPx activities compared to Veh-treated non-stressed rats ($^{*}p < 0.05$, $^{***}p < 0.001$) (Fig. 4B).

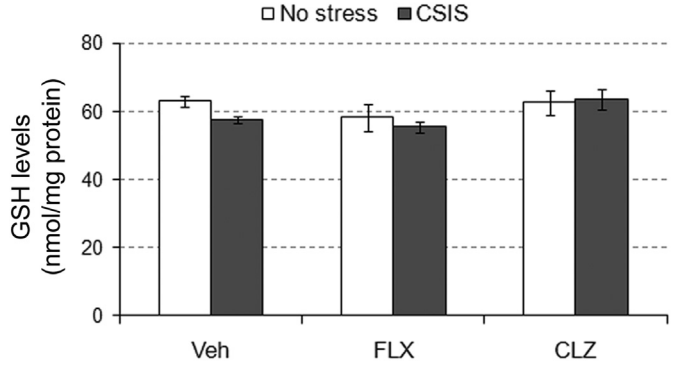

Fig. 3. Hippocampal cytosolic GSH levels in No stress and CSIS rats treated with Veh (0.9% NaCl), FLX (15 mg/kg/day) or CLZ (20 mg/kg/day). CSIS = chronic social isolation; Veh = vehicle; FLX = fluoxetine; CLZ = clozapine; GSH = glutathione.

3.2.3. Protein levels and activity of GLR

A two-way ANOVA revealed a significant main effect of CSIS ($F_{1.30} = 7.40$, $p < 0.05$) on cytosolic GLR levels in rat hippocampus. Post-hoc tests showed significantly decreased GLR levels in FLX- and CLZ-treated CSIS rats ($^{*}p < 0.05$) compared to Veh-treated non-stressed rats (Fig. 5A). In the case of GLR activity, a two-way ANOVA revealed significant main effects of CSIS ($F_{1.24} = 6.93$, $p < 0.05$) and drug ($F_{2.24} = 5.01$, $p < 0.05$). Duncan's post-hoc tests showed significantly lower GLR activity in FLX- and CLZ-treated CSIS rats

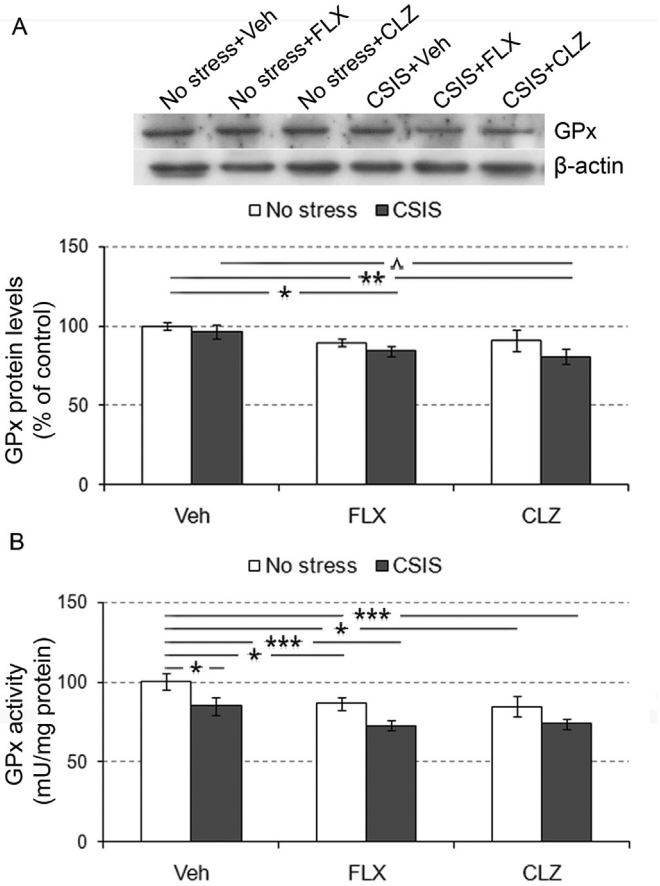

Fig. 4. Protein levels (A) and activity (B) of GPx in the hippocampal cytosol of No stress and CSIS rats treated with Veh (0.9% NaCl), FLX (15 mg/kg/day) or CLZ (20 mg/kg/day). CSIS = chronic social isolation; Veh = vehicle; FLX = fluoxetine; CLZ = clozapine; GPx = glutathione peroxidase. Symbols indicate significant difference: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ from vehicle-treated non-stressed rats; $\hat{}p < 0.05$ from vehicle-treated CSIS.

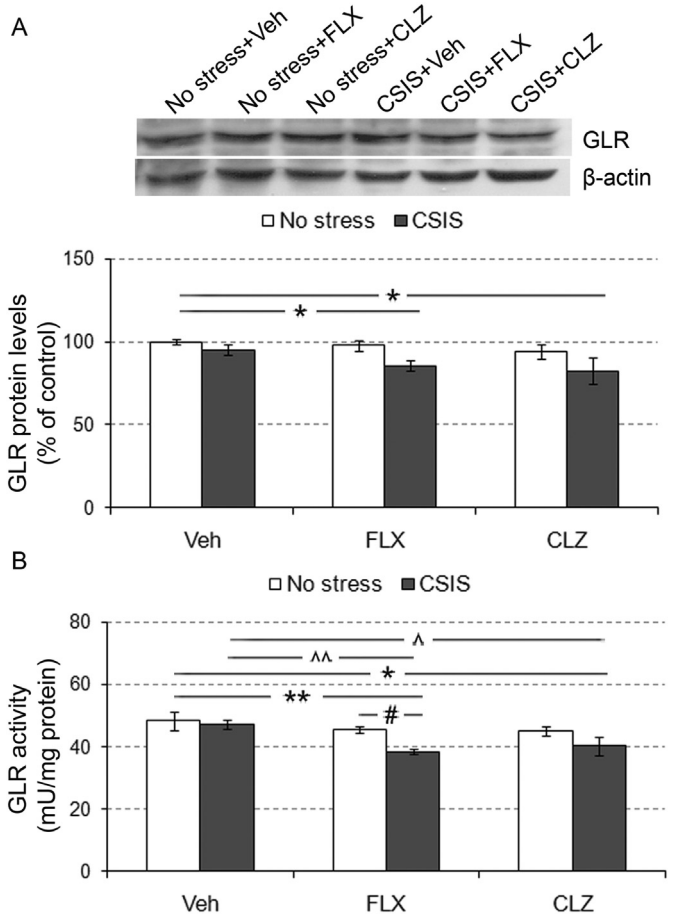

Fig. 5. Protein levels (A) and activity (B) of GLR in the hippocampal cytosol of No stress and CSIS rats treated with Veh (0.9% NaCl), FLX (15 mg/kg/day) or CLZ (20 mg/kg/day). CSIS = chronic social isolation; Veh = vehicle; FLX = fluoxetine; CLZ = clozapine; GLR = glutathione reductase. Symbols indicate significant difference: $^{*}p < 0.05$, $^{**}p < 0.01$ from vehicle-treated non-stressed rats; $\hat{}p < 0.05$, $\tilde{}p < 0.01$ from vehicle-treated CSIS, $^{\#}p < 0.05$ between CSIS + FLX and No stress + FLX.

compared to Veh-treated non-stressed rats ($^{**}p < 0.01$, $^{*}p < 0.05$, respectively) (Fig. 5B). In addition, a significant decrease was seen in FLX- and CLZ-treated CSIS rats compared to Veh-treated CSIS animals ($\tilde{}p < 0.01$, $\hat{}p < 0.05$, respectively), as well as in FLX-treated CSIS rats compared to FLX-treated non-stressed rats ($^{\#}p < 0.05$).

3.3. Cytosolic/nuclear distribution of NF-κB p65 subunit

CSIS did not cause NF-κB activation and its nuclear translocation in rat hippocampus. A two-way ANOVA revealed no significant changes in cytosolic (Fig. 6A) or nuclear protein levels (Fig. 6B) of the NF-κB p65 subunit. Moreover, treatment with FLX or CLZ did not affect NF-κB p65 levels in the cytosol or nucleus in non-stressed or CSIS rats.

3.4. Protein levels of proinflammatory mediators

A two-way ANOVA showed no significant changes in the COX-2 protein levels (Fig. 7).

With regard to IL-1β, a two-way ANOVA revealed a significant main effect of drug ($F_{2.26} = 3.47, p < 0.05$), while post-hoc tests showed no significant differences among experimental groups (Fig. 8A). A significant main effects of drug ($F_{2.24} = 3.71, p < 0.05$) and CSIS × drug interaction ($F_{2.24} = 4.51, p < 0.05$) on TNF-α protein levels were also found. Duncan's post-hoc tests showed significantly increased levels of this cytokine in Veh-treated CSIS rats compared to Veh-treated non-stressed rats ($^{*}p < 0.05$) (Fig. 8B). In addition, a significant decrease was seen in FLX-, as well as in CLZ-treated CSIS rats compared to Veh-treated CSIS rats ($\hat{\hat{}}p = 0.001$, $\hat{}p < 0.05$, respectively).

4. Discussion

Hippocampal sensitivity to various stress paradigms is well-documented. However, the *in vivo* effects of FLX and CLZ on antioxidative defense and proinflammatory mediators in the hippocampus resulting from chronic psychosocial stress remain unclear. In this study, rats that underwent 21 days of CSIS stress displayed depressive- and anxiety-like behaviors, decreased GPx-mediated antioxidative defense, and increased levels of the proinflammatory cytokine TNF-α in the hippocampus. The antidepressive and anxiolytic effects of FLX and CLZ parallelled their inhibitory effect on hippocampal TNF-α-mediated proinflammmatory signaling.

The effects of FLX and CLZ on CSIS were tested behaviorally using sucrose preference and the marble burying test. CSIS resulted in decreased sucrose preference and increased burying behavior, indicative of depressive- and anxiety-like behaviors, respectively. These findings are consistent with our previous results, which also revealed despair behavior following CSIS, as measured with the forced swim test (Zlatković et al., 2014b). It is worth noting that some previous studies have demonstrated that marble burying behavior is not correlated with other measures of anxiety-like traits (Thomas et al., 2009). However, the increased burying behavior reported here has been demonstrated to correspond with less time spent in the light compartment of the light-dark box (Carrier and Kabbaj, 2012), and with a decreased number of arm entries, open arm entries and time spent in the open arms of the elevated plus maze (Djordjevic et al., 2015) following CSIS. Both FLX (15 mg/kg/day) and CLZ (20 mg/kg/day) prevented CSIS-induced depressive- and anxiety-like behaviors. The ability of FLX to increase sucrose preference in rats exposed to various chronic stress paradigms has been shown previously (Chen et al., 2015; Perić et al., 2017; Rong et al., 2010; Yang et al., 2014). In the case of CLZ, there is little data regarding its effects on anhedonia, but it has been demonstrated to reverse deficits in sucrose preference in a model of schizophrenia (Vardigan et al., 2010). The anxiolytic properties of these two drugs have previously been shown using the marble burying test in mice (Bruins Slot et al., 2008), as well as in other behavioral tests (Mead et al., 2008; Zhang et al., 2000). In addition, previous studies have shown that both FLX and CLZ reduces depressive behaviors in the forced swim test, as determined by decreased immobility behavior (Brenes and Fornaguera, 2009; Chatterjee et al., 2012; Ciulla et al., 2007; Weiner et al., 2003).

Hippocampal GSH levels in CSIS rats did not significantly differ from control values, although a trend toward a decrease was observed. In contrast, a significant decrease in GPx activity, despite protein levels being unchanged, was observed in CSIS animals. Considering that NO may inactivate GPx (Asahi et al., 1995; Miyamoto et al., 2003), a threefold increase in NO levels previously reported in the hippocampus of CSIS rats (Zlatković et al., 2014b) may partly explain the decreased GPx activity without a change in protein levels. Decreased GPx activity can promote susceptibility to oxidative stress, as it compromises the capacity of brain cells to protect against peroxide-mediated oxidative damage (Dringen et al., 2005). This is consistent with previously demonstrated oxidative protein damage in the hippocampus of CSIS rats (Zlatković et al., 2014b). FLX and CLZ not only failed to prevent the CSIS-induced decrease in GPx activity, but they further compromised GSH-dependent defense by reducing GLR protein levels and activity in CSIS rats. Despite the antioxidant action of FLX shown previously, there is little information regarding the influence of this drug on antioxidant enzymes (Caiaffo et al., 2016). Previous studies have shown that FLX restores the antioxidant capacity in the brain (Bilici et al., 2001; Novío et al., 2011), while Adzic et al. (2011) showed that FLX negatively affects antioxidant systems in rat erythrocytes by decreasing GLR activity. In the case of CLZ, its direct antioxidant activity may contribute to its

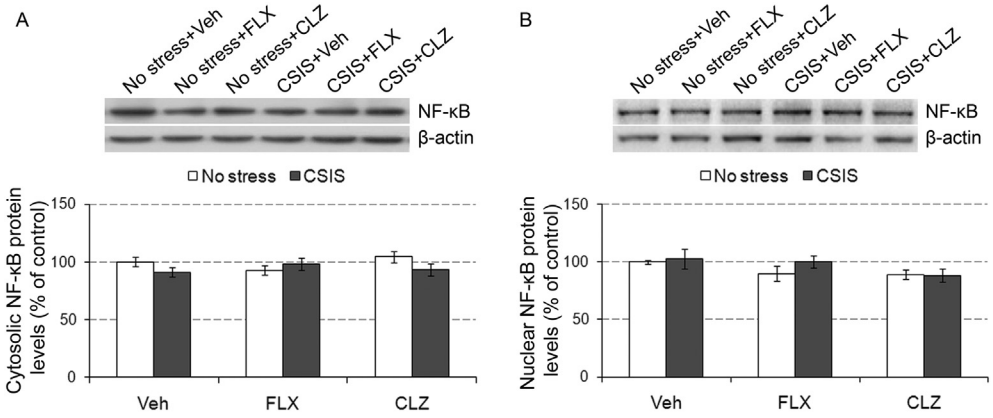

Fig. 6. Protein levels of cytosolic (A) and nuclear (B) NF-κB p65 in the hippocampus of No stress and CSIS rats treated with Veh (0.9% NaCl), FLX (15 mg/kg/day) or CLZ (20 mg/kg/day). CSIS = chronic social isolation; Veh = vehicle; FLX = fluoxetine; CLZ = clozapine; NF-κB = nuclear factor-kappa B.

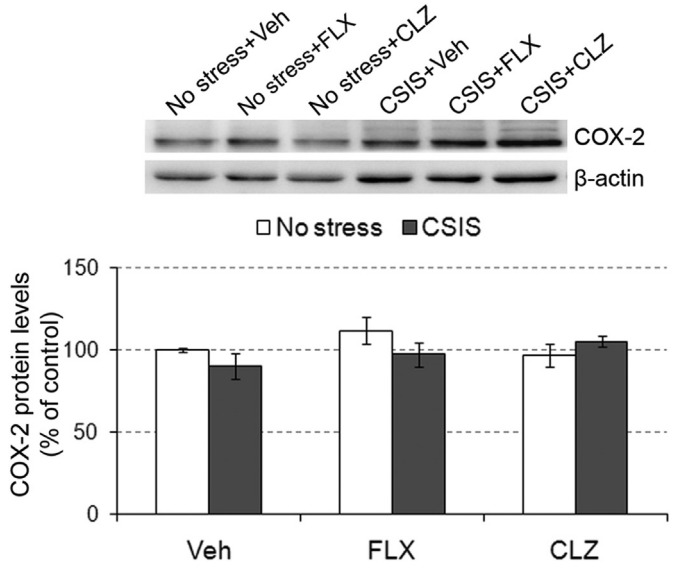

Fig. 7. Protein levels of COX-2 in the hippocampal cytosol of No stress and CSIS rats treated with Veh (0.9% NaCl), FLX (15 mg/kg/day) or CLZ (20 mg/kg/day). CSIS = chronic social isolation; Veh = vehicle; FLX = fluoxetine; CLZ = clozapine; COX-2 = cyclooxygenase 2.

therapeutic action (Sadowska-Bartosz et al., 2016). However, studies of antioxidant enzymes, including GPx and GLR, showed no effect of CLZ in rat brain (Parikh et al., 2003) or human erythrocytes (Miljević et al., 2013). A trend toward a decrease in GPx and GLR protein levels was observed in CSIS rats, as well as in FLX- and CLZ-treated non-stressed rats, but statistical significance was reached only in the combination of stress and drug. This may be partly explained by the incomplete nuclear translocation of glucocorticoid receptors (GR) previously described in the hippocampus of CSIS rats (Filipović et al., 2016), as glucocorticoids induce the expression of GPx (Grier and Halliday, 2004). In addition, pharmacodynamic drug interference with GR-mediated gene transcription that has been shown for some antidepressants, including FLX (Budziszewska et al., 2000), may contribute to the negative effect of the combination of stress and drug observed in the GSH-dependent antioxidative system.

With regard to the proinflammatory response, unchanged protein levels of the NF-κB p65 subunit in cytosolic and nuclear fractions indicate that CSIS failed to activate this transcriptional factor. Thus, a lack of change in COX-2 protein levels in response to CSIS was not surprising, as this enzyme is under NF-κB regulation (Kaltschmidt et al., 2002). However, levels of TNF-α, a proinflammatory cytokine strongly implicated in the pathogenesis of psychiatric disorders (Berthold-Losleben and Himmerich, 2008), was increased in CSIS rats. TNF-α influences serotonergic homeostasis by positively affecting serotonin uptake (Zhu et al., 2006), as well as stimulating the enzyme indoleamine 2,3-dioxygenase, resulting in a depletion of the serotonin precursor tryptophan (Wichers and Maes, 2002). TNF-α and IL-1β are both targets of NF-κB regulation and its activators (Berti et al., 2002; Mercurio et al., 1997). Interestingly, the increase in TNF-α was not NF-κB-mediated, as NF-κB nuclear translocation was not seen. Inducible heat shock protein 70, which inhibits the proinflammatory transcriptional factor NF-κB (Heck et al., 2011; Kim et al., 2012), has been shown to be upregulated in the hippocampus of CSIS rats (Zlatković et al., 2014a), which may, at least in part, explain the absence of NF-κB activation despite increased TNF-α levels. Interestingly, IL-1β levels were not increased after 21 d or even 42 d of CSIS (Perić et al., 2017). In contrast, we recently revealed NF-κB activation and subsequent IL-1β, TNF-α and COX-2 upregulation in the rat prefrontal cortex after 21 d of CSIS (Todorović and Filipović, 2017).

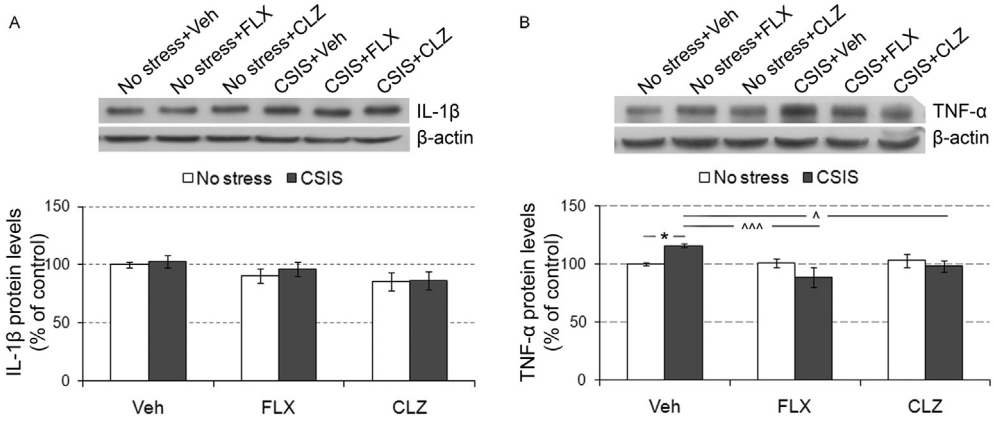

Fig. 8. Protein levels of IL-1β (A) and TNF-α (B) in the hippocampal cytosol of No stress and CSIS rats treated with Veh (0.9% NaCl), FLX (15 mg/kg/day) or CLZ (20 mg/kg/day). CSIS = chronic social isolation; Veh = vehicle; FLX = fluoxetine; CLZ = clozapine; IL-1β = interleukin-1beta; TNF-α = tumor necrosis factor-alpha. Symbols indicate significant difference: $^{*}p < 0.05$ from vehicle-treated non-stressed rats; $\hat{p} < 0.05$, $\hat{\hat{p}} < 0.001$ from vehicle-treated CSIS.

Literature data regarding the immunomodulatory effects of FLX and CLZ are inconsistent, especially when comparing *in vitro* and *in vivo* studies. Inconsistencies appear partly due to different methodological approaches, including the duration of treatments and doses (Baumeister et al., 2015). Nonetheless, the results presented here further the goal of determining whether treatment with FLX or CLZ is effective in preventing or reversing the neurochemical changes in antioxidative defense and proinflammatory signaling induced by CSIS in the hippocampus and other critical brain regions, such as the prefrontal cortex. Although reversing CSIS-induced alterations represents a more translational design, preventing the neurochemical effects of CSIS may provide insight into the mechanisms underlying the pathophysiology of depressive disorder. Our results revealed that 21 days of treatment with FLX or CLZ, when applied at concentrations that produce serum drug levels near the lower end of the therapeutically effective range, prevented an increase in TNF-α in CSIS rats. These findings are consistent with an *in vitro* study investigating the effects of FLX on TNF-α, which reported a trend toward inhibitory effects (Baumeister et al., 2015), as well as *in vivo* studies demonstrating anti-inflammatory effects of FLX (Liu et al., 2015; Perić et al., 2017). The effects of CLZ on TNF-α production have been contradictory with respect to studies conducted on schizophrenic patients (Kluge et al., 2009; Monteleone et al., 1997), although CLZ ameliorated the production of TNF-α in microglia activated by LPS (Hu et al., 2012). Our results suggest that TNF-α plays an important role in the pathogenesis of depressive- and anxiety-like behaviors, as FLX and CLZ attenuated hippocampal TNF-α levels while inhibiting these behaviors. Because the increase in TNF-α was not accompanied by NF-κB activation, the protective effects of FLX and CLZ were not NF-κB-mediated. The anti-inflammatory effect of FLX mediated by the inhibition of LPS-activated NF-kB pathway has been reported previously in primary rat mixed glial cell culture (Obuchowicz et al., 2014). However, here we demonstrated an *in vivo* anti-inflammatory effect of FLX unrelated to NF-κB. One limitation of the present study is the use of a different control solution (0.9% NaCl) in vehicle-treated non-stressed and CSIS rats. We assumed that neither saline nor drug solvents would affect parameters studied in the hippocampus, and this allowed us to minimize the number of animals used in the study by having one set of vehicle-treated controls.

In conclusion, this study demonstrated that depressive- and anxiety-like behaviors in adult male Wistar rats that underwent 21-day CSIS stress coincided with a compromised GPx-mediated antioxidative defense and increased TNF-α protein level in the hippocampus. Thus, CSIS predisposed the hippocampus to oxidative stress and TNF-α-mediated proinflammmatory signaling. FLX and CLZ prevented behavioral changes and the increase in hippocampal TNF-α levels, but not the stress-induced decrease in the efficiency of GSH-dependent defense system. Taken together, the results of this study highlight the importance of TNF-α in the pathophysiology of depressive- and anxiety-like behaviors and the significance of the anti-inflammatory properties of effective psychotropic drugs.

Conflicts of interest

None.

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