Utjecaj perinatalne primjene prekursora sinteze ili inhibitora razgradnje serotonina na serotoninsku homeostazu u odraslih štakora

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THE EFFECTS OF PERINATAL TREATMENT WITH SEROTONIN SYNTHESIS PRECURSOR OR SEROTONIN DEGRADATION INHIBITOR ON THE SEROTONIN HOMEOSTASIS IN ADULT RATS

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UTJECAJ PERINATALNE PRIMJENE PREKURSORA SINTEZE ILI INHIBITORA RAZGRADNJE SEROTONINA NA SEROTONINSKU HOMEOSTAZU U ODRASLIH ŠTAKORA

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SAŽETAK

Serotonin (5HT) regulira razvoj vlastitih neurona i ciljnih tkiva tijekom neurogeneze pa odstupanje od optimalnih koncentracija 5HT tijekom razvoja mozga može dovesti do strukturnih i funkcionalnih anomalija. U ovom smo istraživanju kroničnom perinatalnom primjenom neposrednog prekursora sinteze 5HT, 5-hidroksitriptofana (5HTP, 25mg/kg), ili inhibitora razgradnje 5HT, tranilcipromina (TCP, 2mg/kg) povisili razinu 5HT u štakora s ciljem istraživanja posljedica po homeostazu 5HT u odraslom mozgu. Tijekom primjene 5HTP je značajno povisio razinu 5HT samo u perifernom odjeljku (hiperserotoninemija), a TCP i u perifernom i u središnjem odjeljku. Obje primjene uzrokovale su (dugo)trajne posljedice po funkciju središnjeg 5HT-odjeljka, koje su se očitovale kroz promjene u razini i metabolizmu serotonina, ekspresiji 5HT-regulirajućih gena te ponašanja vezanog uz serotonin. Činjenica da su razvojni poremećaji u metabolizmu serotonina (dugo)trajno narušili funkciju središnjeg 5HT-odjeljka upućuje na gene koji reguliraju metabolizam 5HT kao na moguće kandidate u istraživanjima poremećaja ponašanja vezanima uz 5HT sustav. Posljedice dviju primjena bile su analogne, ali su odražavale efekt doze: središnja homeostaza 5HT bila je diskretno narušena u životinja s razvojnim promjenama samo u perifernoj 5HT-homeostazi (5HTP), a opsežno u životinja s razvojnim promjenama i u središnjoj i u perifernoj 5HT-homeostazi (TCP). To upućuje na zaključak da je za ozbiljno narušavanje funkcije 5HT, kakvo je opaženo u psihijatrijskim bolestima, nužna razvojna neuravnoteženost u središnjem 5HT-odjeljku i govori u prilog teoriji o hiperserotoninemiji kao o biljegu, a ne uzročniku promjena u središnjoj homeostazi serotonina.

(92 stranica, 5 slika, 2 tablice, 278 literaturnih navoda, jezik izvornika: hrvatski)

Ključne riječi: serotonin, 5-hidroksitriptofan, tranilcipromin, hiperserotoninemija, perinatalna primjena

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Doctoral thesis

THE EFFECTS OF PERINATAL TREATMENT WITH SEROTONIN SYNTHESIS PRECURSOR OR SEROTONIN DEGRADATION INHIBITOR ON THE SEROTONIN HOMEOSTASIS IN ADULT RATS

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ABSTRACT

Serotonin (5HT) regulates the development of its own neurons and target tissues during neurogenesis, therefore deviations from optimal 5HT concentrations during brain development can lead to structural and functional anomalies. In this thesis, through the chronic perinatal treatment with the immediate serotonin synthesis precursor, 5-hydroxytryptophan (5HTP, 25 mg/kg), or with an inhibitor of 5HT degradation, tranylcypromine (TCP, 2 mg/kg), we have increased 5HT concentrations in rats with the aim of investigating the effects on adult brain 5HT homeostasis. During the treatment 5HTP significantly increased 5HT levels in the peripheral compartment (hyperserotonemia), and TCP in the peripheral and central compartment. Both treatments had long-lasting/permanent consequences on the function of the central 5HT compartment, evident through changes in 5HT concentration and metabolism, expression of 5HT-regulating genes and serotonin related behavior. The fact that developmental disturbances in 5HT metabolism induced permanent disturbances in function of central 5HT compartment points to the genes that regulate 5HT metabolism as potential candidates for research in 5HT-related behavioral disorders. The effects of both treatments where analogous, showing a dose-effect: central 5HT homeostasis was mildly altered in animals with developmental disturbances only in peripheral 5HT homeostasis (5HTP), and drastically altered in animals with developmental disturbances in both central and peripheral 5HT homeostasis (TCP). These findings suggest that severe alterations in 5HT function observed in psychiatric diseases may be caused by developmental disbalance in the central 5HT compartment and speak in favor of the theory of hyperserotonemia as a marker, not a cause, of disturbances in central serotonin homeostasis.

(92 pages, 5 figures, 2 tables, 278 references, original in: Croatian)

Key words: serotonin, 5-hydroxytryptophan, tranylcypromine, hyperserotonemia, perinatal treatment

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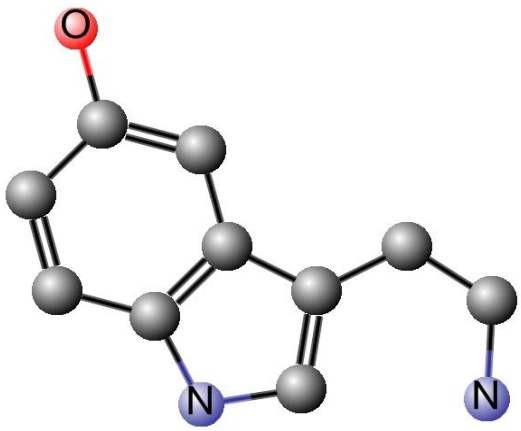
SADRŽAJ

1. UVOD

1.1. Serotonin

Serotonin (5-hidroksitriptamin, 5HT) je biološki aktivan monoamin koji stanice sintetiziraju iz esencijalne aminokiseline triptofana. Nalazi se u skupini biogenih amina kojoj također pripadaju histamin, koji se sintetizira iz histidina te katekolaminski neurotransmiteri adrenalin, noradrenalin i dopamin, koji se sintetiziraju iz tirozina. Serotonin je indolamin (slika 1.) s filogenijski očuvanom ulogom u neurotransmisiji (Turlejski, 1996.), a poznat je kao neurotransmiter koji sudjeluje u modulaciji aktivnosti neurona te u širokom rasponu neurofizioloških procesa. Međutim, većina serotonina se nalazi izvan središnjeg živčanog sustava (SŽS) te je ekspresija 15 poznatih serotoninskih receptora uočena i na periferiji i u mozgu (Berger i sur., 2009.). 5HT je široko je rasprostranjen u životinjskom i biljnom svijetu (Azmitia, 1999.), a nalazi se i u većini jednostaničnih organizama (Azmitia, 2007.). Sudjeluje u regulaciji raznih fizioloških procesa kao što su raspoloženje, motoričke i kognitivne funkcije te cirkadijani i neuroendokrini ritmovi poput regulacije spavanja, hranjenja i spolne aktivnosti (Di Pino i sur., 2004.; Lesch i Moessner, 1998.). Zbog svoje široke distribucije u središnjem živčanom sustavu i utjecaja na veliki broj fizioloških procesa, brojni poremećaji vežu se uz promijenjenu serotoninsku transmisiju (Sodhi i Sanders-Bush, 2004.).



IUPAC ID	3-(2-aminoetil)-1H-indol-5-ol
Molekularna formula	C10H12N20
Molekularna masa	176,22 g/mol

Slika 1. 3D struktura i značajke serotonina

(MarvinSketch, JChem for Excel (ChemAxon, 2013.))

1.1.1. Povijest

Prve naznake da se u krvi nalazi tvar koja uzrokuje vazokonstrikciju pojavile su se sredinom 19. stoljeća (Green, 2006.). Još 1918. godine, Janeway i suradnici pretpostavili su da se ta tvar nalazi u trombocitima (Janeway i sur., 1918.), iako je njihova teorija potvrđena tek 1954. godine (Humphrey i Jaques, 1954.).

Kasnih 40-ih godina, Maurice Rapport, Arda Green i Irving Page identificirali su *serotonin*, molekulu izoliranu iz seruma koja utječe na tonus krvnih žila (Rapport i sur., 1948). Nakon što je utvrđena kemijska struktura serotonina, ustanovljeno je da se radi o 5-hidroksitriptaminu (Rapport, 1949.).

U neovisnim istraživanjima, Vialli i Erspamer (1937.) prvi put su izolirali i opisali amin koji uzrokuje kontrakciju mišića te ga nazvali *enteramin*. Erspamera je zanimao učinak raznih amina prisutnih u koži i crijevu različitih vrsta životinja na kontrakciju glatkih mišića (Whitaker-Azmitia, 1999.). Naročito ga je zanimala tvar koja se nalazila u enterokromafinim stanicama crijeva, a čiji je acetonski izolat uzrokovao kontrakciju glatkih mišića, posebice maternice štakora. Više se godina pisalo o *enteraminu*, dok se nije uspostavilo da se također radi o 5-hidroksitriptaminu (Erspamer i Asero, 1952.).

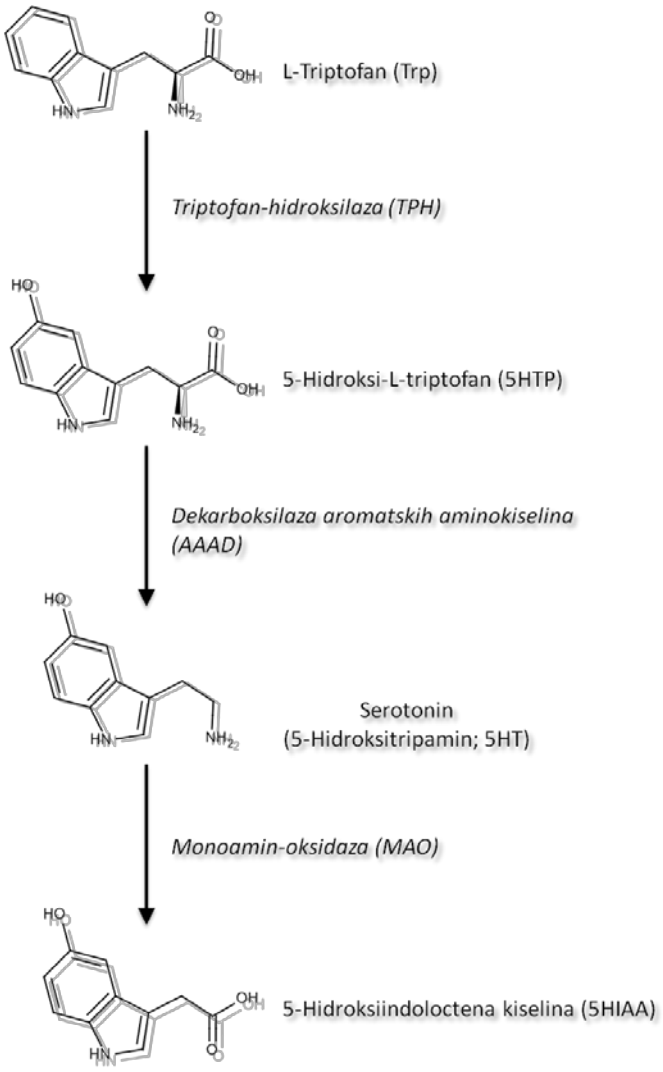
Iako je već 1951. godine 5-hidroksitriptamin bio komercijalno dostupan, još se nije znala njegova točna uloga. Veliki korak naprijed je bilo njegovo otkriće u mozgu. Godine 1953. Twarog i Page objavili su da se serotonin nalazi u mozgu sisavaca i tako postavili osnovu za uvrštenje serotonina među neurotransmitere, u doba kada je teorija neurotransmitera još bila kontroverzna. Amin i suradnici su gotovo istovremeno potvrdili postojanje 5HT u mozgu psa, te odredili njegovu koncentraciju u različitim dijelovima mozga (Amin i sur., 1954.).

Dilworth Wayne Woolley je svojim radom učvrstio teoriju o ulozi serotonina kao važnog neurotransmitera. Woolley je razvijao teoriju o odnosu između pojedinih supstanci i specifičnih bolesti u ljudi. U svojim istraživanjima je pokazao da LSD može poništiti učinke serotonina, te predložio da bi se korištenjem LSD-a u istraživanjima mogla otkriti uloga serotonina. Zatim je predložio da bi serotonin mogao igrati ulogu u razvoju mozga, jer je sličan biljnom hormonu rasta, auksinu (Woolley i Shaw, 1954.). Najzad, 1963. godine Woolley je publicirao ključnu knjigu, *The Biochemical Bases of Psychoses or the Serotonin Hypothesis about Mental Illness*, u kojoj sažima svoju ranije objavljenu teoriju o ulozi serotonina u duševnim bolestima (Woolley, 1962.).

Tako se u manje od 25 godina, 5-hidroksitriptamin, od nepoznatog amina u crijevima različitih životinja, pretvorio u sveprisutni neurotransmiter ključan u razvoju mentalnih bolesti u ljudi (Whitaker-Azmitia, 1999.). Daljnji razvoj istraživanja samo je produbio i detaljnije razjasnio njegovu funkciju.

1.1.2. Metabolizam serotonina

Koncentracija serotonina u pojedinom tkivu ovisi o omjeru sinteze i razgradnje.



Slika 2. Metabolizam 5-hidroksitriptamina (JChem for Excel (ChemAxon, 2013.))

U sisavaca razlikujemo dva serotoninska odjeljka: središnji, koji podrazumijeva središnji živčani sustav, te periferni koji se odnosi na ostala tkiva (v. poglavlje 1.2). Odjeljci

su odvojeni krvno-moždanom barijerom koja je nepropusna za serotonin (Diksic i Young, 2001.). Metabolizam serotonina je u oba odjeljka isti (slika 2., tablica 1.): 5HT se sintetizira iz aminokiseline L-triptofan (Trp) djelovanjem enzima triptofan-hidroksilaze (TPH) i dekarboksilaze aromatskih aminokiselina (AAAD, engl. *aromatic L-amino acid decarboxylase*), a razgrađuje se u 5-hidroksiindoloctenu kiselinu (5-HIAA, engl. *5-hydroxy-3-indoleacetic acid*) oksidativnom deaminacijom koju katalizira enzim monoamin-oksidaza (MAO).

1.1.2.1. Sinteza serotonina

5-hidroksitriptamin se sintetizira iz esencijalne aminokiseline L-triptofana. Sisavci nemaju sposobnost sinteze triptofana, te ga mogu isključivo dobiti prehranom (Cansev i Wurtman, 2007.). Prehrambeni proizvodi bogati triptofanom su zob, suho voće, mlijeko, tunjevina, sir, kruh, piletina, puretina, koštunjavo voće, čokolada, banane, jaje, itd. Većina apsorbiranog Trp se koristi u sintezi proteina, te je često i njezin ograničavajući čimbenik jer predstavlja najmanje zastupljenu aminokiselinu (Sainio i sur., 1996.). Osim u sintezi proteina, Trp je bitan u sintezi serotonina i kinurenina (Wurtman i sur., 1980.). U perifernom odjeljku, serotonin se sintetizira u enterokromafinim stanicama gastrointestinalne sluznice, a u središnjem odjeljku u serotonergičnim neuronima. Iako se pretpostavlja da samo 3% Trp unesenog prehranom sudjeluje u sintezi 5HT, sinteza serotonina je jedan od najvažnijih anaboličkih sintetskih puteva vezanih za triptofan (Richard i sur., 2009.). Više od 95% metabolizma Trp otpada na enzimsku konverziju Trp u kinurenin pomoću triptofan-pirolaze (Tyce, 1990.). Pošto 5HT ne prelazi krvno-moždanu barijeru, sinteza serotonina u SŽS ovisi o dostupnosti triptofana. Lijevi (L) izomer triptofana je jedini koji se koristi u sintezi proteina i jedini koji može prelaziti krvno-moždanu barijeru. Za apsorpciju Trp je odgovoran prijenosnik velikih neutralnih aminokiselina (LAT, engl. *large neutral amino acid transporter*) (Boado i sur., 1999.). Udio Trp koji se unosi u središnji odjeljak ovisi ne samo o dostupnosti Trp u cirkulaciji nego i o koncentraciji ostalih aminokiselina (fenilalanin, tirozin, leucin, izoleucin, histidin, metionin, treonin i valin) koje koriste LAT, a s kojima se Trp natječe za ulazak u SŽS (Fernstrom i Wurtman, 1972.).

U oba odjeljka, prvi korak u sintezi serotonina, a ujedno i ograničavajući čimbenik sinteze, je hidroksilacija triptofana u 5-hidroksitriptofan (5-HTP) pomoću enzima triptofan-hidroksilaze (TPH) (Lovenberg i sur., 1967.; Udenfriend i sur., 1956.). TPH je monooksigenaza koja koristi kisik (O_2) i tetrahidrobiopterin (BH_4) kao kosupstrate te željezo (Fe^{+2}) kao kofaktor u stvaranju neposrednog prekursora serotonina, 5-hidroksi-L-triptofana

(5-HTP) i vode, hidroksilacijom triptofana na 5' mjestu (Cansev i Wurtman, 2007.; Sato i sur., 1967.). Enzim TPH je osjetljiv na oksidaciju; izloženost enzima oksidativnim uvjetima uništava njegovu katalitičku funkciju što rezultira značajnim promjenama u razinama 5HT kao i u serotonergičnoj neurotransmisiji (Hufton i sur., 1995.; Kuhn i sur., 1979., 1980.; Rahman i Thomas, 2009.; Thomas i sur., 2007.). Dušikov monoksid (NO), a vjerojatno i drugi reaktivni kisikovi spojevi, inaktiviraju TPH (Kuhn i Arthur, 1996., 1997.). Stoga je TPH dobar biomarker za promjene u serotonergičnom sustavu uslijed oksidativnog stresa (Rahman i sur., 2011.). TPH postoji kod kralježnjaka u dvije molekularne izoforme, TPH1 i TPH2 (Darmon i sur., 1988.; Walther i Bader, 2003.). Izoforma TPH1, prvotno okarakterizirana u epifizi zeca, regulira sintezu 5HT na periferiji (u timusu, slezeni i crijevu) i u epifizi u kojoj se sintetizirani 5HT metabolizira u melatonin (Grenett i sur., 1987.; Walther i sur., 2003.). *Tph2* se eksprimira u serotonergičkim neuronima, gdje ima važnu ulogu u regulaciji središnje serotoninske homeostaze (Alenina i sur., 2009.; Walther i sur., 2003.). Gen za TPH1 se nalazi na 11. kromosomu čovjeka te na 1. kromosomu štakora, dok se gen za TPH2 nalazi na 12. kromosomu čovjeka, odnosno na 7. kromosomu štakora. Istraživanja su pokazala različitu regulaciju ekspresije ove dvije izoforme TPH na transkripcijskoj i translacijskoj razini (Sakowski i sur., 2006.). Poznata je inhibicija TPH uslijed stresa, rezistencije na inzulin, manjka piridoksina (vitamin B6), nedostatka magnezija, te visoke razine triptofana (Birdsall, 1998.).

5HTP je prisutan u stanici u malim količinama (Long i sur., 1982.), jer se brzo pretvara u 5HT (Boadle-Biber, 1993.). Njegov poluživot je relativno kratak (4,3 ± 2,8 h) (Westenberg i sur., 1982.), a vrijeme postizanja maksimalne koncentracije je 1-2h (Magnussen i Van Woert, 1982.). Također se dobro apsorbira u krvotok, djelomično zbog toga što prisutnost drugih aminokiselina ne ometa njegovu apsorpciju, te zato što se ne koristi u sintezi proteina (Magnussen i Nielsen-Kudsk, 1980.). 5HTP prolazi krvno-moždanu barijeru bez posebne molekule prijenosnika (Udenfriend i sur., 1957.), iako se može koristiti i LAT-om. S obzirom na to, razina 5HT u mozgu ovisi o razinama Trp i 5HTP u SŽS (Birdsall, 1998.). Ovdje 5HTP prvenstveno povisuje razinu serotonina, ali i razinu melatonina, dopamina, noradrenalina i β-endorfina (den Boer i Westenberg, 1990.; Guilleminault i sur., 1973.; van Praag i Lemus, 1986.). 5HTP, izoliran iz sjemenki afričke biljke *Griffonia simplicifolia*, komercijalno je dostupan za primjenu u medicini (Birdsall, 1998.).

Drugi korak u sintezi serotonina kataliziran je, manje specifičnim, široko rasprostranjenim enzimom ovisnom o piridoksinu, dekarboksilazom aromatskih aminokiselina (AAAD, engl. *aromatic L-amino acid decarboxylase*). AAAD

dekarboksilacijom prevodi 5HTP u 5HT, te sudjeluje u dekarboksilaciji mnogih drugih kemijskih spojeva. Gen za AAAD se u čovjeka nalazi na 7. kromosomu, a u štakora na 14. kromosomu.

U ranim farmakološkim studijama dokazano je da se tijekom sinteze serotonina hidroksilacija i dekarboksilacija događaju neposredno jedna iza druge, odnosno gotovo istovremeno, u prisutnosti triptofana (Clark i sur., 1954.). Smatra se da je TPH, a ne AAAD, enzim koji ograničava brzinu reakcije sinteze serotonina i to zbog sljedećih razloga. Prvo, iako su oba enzima nužna za sintezu 5HT, aktivnost AAAD uvelike premašuje aktivnost triptofan-hidroksilaze (Moore i sur., 1985.). Drugo, TPH ima slab afinitet za druge aminokiseline (Noguchi i sur., 1973.), dok AAAD ima visoki afinitet za mnoge L-amino kiseline. Treće, visokospecifična aktivnost TPH je u kontrastu s nespecifičnom aktivnošću AAAD. Četvrto, dok se AAAD nalazi u većini tkiva (Clark i sur., 1954.; Tyce, 1990.), raspodjela TPH je ograničena isključivo na tkiva koje sadrže 5HT (Champier i sur., 1997.; Noguchi i sur., 1973.; Tyce, 1990.). Najzad, teško je smanjiti razine serotonina inhibicijom AAAD (Mohammad-Zadeh i sur., 2008.).

1.1.2.2. Pohrana i otpuštanje serotonina

Novosintetizirani serotonin se aktivno pohranjuje u vezikule pomoću vezikularnog monoaminskog prijenosnika (VMAT, engl. *vesicle monoamine transporter*), koji je također odgovoran za pohranu dopamina, noradrenalina i histamina. Gen za VMAT se nalazi u čovjeka na kromosomu 10, a u štakora na kromosomu 1. Zanimljivo je da se pri zasićenju TPH triptofanom poveća i razgradnja serotonina, jer je količina sintetiziranog serotonina toliko velika da ne uspijeva sav ući u vezikule i zaštititi se od oksidativne razgradnje, te se tada povećava i količina metabolita, 5HIAA (Cansev i Wurtman, 2007.).

U oba odjeljka, otpuštanje serotonina vrši se egzocitozom ovisnom o Ca^{2+} i regulirano je aktivacijom receptora. U neuronima su to presinaptički receptori iz obitelji $5HT_1$ (tzv. autoreceptori). U enterokromafinim stanicama su do sada identificirane dvije klase receptora koje moduliraju otpuštanje 5HT: stimulacijski $5HT_3$ receptori, te inhibicijski $5HT_4$ receptori (Racke i sur., 1995.), a također se sugerira i uloga $5HT_{1A}$ te $5HT_2$ receptora (Schwörer i Ramadori, 1998.).

1.1.2.3. Ponovni unos serotonina

Za dobro funkcioniranje sinapse, neophodno je da neurotransmiter ima ograničeno vremensko djelovanje unutar sinaptičke pukotine. Prekid djelovanja 5HT i povrat u

presinaptički (serotonergični) neuron se odvija aktivnim transportom preko serotoninskog prijenosnika (5HTt, engl. *5HT transporter*) kako bi se reciklirao (tj. ponovno spremio u vezikule) ili razgradio u 5HIAA (Holz i Fisher, 2011.; Kolb i Whishaw, 2011.; Melikian, 2004.). Serotonin se na isti način unosi u trombocite, kao i u sve druge stanice koje pohranjuju ili razgrađuju serotonin. 5HTt identificiran je u SŽS, gastrointestinalnom traktu, plućima i krvožilnom sustavu, te u trombocitima. Pripada velikoj obitelji prijenosnika biogenih amina ili aminokiselina, ovisnih o natriju s 12 transmembranskih regija (Ni i Watts, 2006.; Sneddon, 1973.; Torres i sur., 2003.). Gen za 5HTt nalazi se na 17. kromosomu u čovjeka, odnosno na 10. kromosomu u štakora. Neuronski 5HTt posreduje ponovni unos 5HT u presinaptički neuron, regulirajući tako jačinu i trajanje djelovanja 5HT u sinapsi. Broj prijenosnika u pojedinom neuronu nije stalan, mijenja se ovisno o aktivnosti sinapse, što pridonosi plastičnosti neurona (Melikian, 2004.). 5HTt koji se nalazi na trombocitnoj membrani unosi u trombocit 5HT otpušten iz enterokromafinih stanica u krvnu plazmu. U trombocitima se 5HT pohranjuje u tzv. guste granule pomoću VMAT. Dakle, serotoninski prijenosnik igra važnu ulogu u održavanju središnje serotoninske homeostaze i jedini je izvor 5HT u trombocitima.

1.1.2.4. Razgradnja serotonina

Serotonin se razgradi u 5-hidroksiindoloctenu kiselinu (5HIAA) oksidativnom deaminacijom (esencijalni korak u katabolizmu aminskih neurotransmitera), te se prvenstveno izlučuje urinom (McIsaac i Page, 1958.). Ovu reakciju katalizira enzim monoamin-oksidaza (MAO) koji može biti zastupljen u dvije varijante: MAO A i MAO B. Radi se o flavoproteinima koji se nalaze na vanjskoj membrani mitohondrija neurona i perifernih stanica (Shih i sur., 1999.). MAO katalizira oksidativnu deaminaciju raznih amina u mozgu i perifernim tkivima proizvodnjom vodikovog peroksida (H_2O_2) (Shih i sur., 1999.; Thorpe i sur., 1987.). U fiziološkim uvjetima, ovi izoenzimi imaju različite afinitete za supstrate. MAO A primarno oksidira monoaminergične neurotransmitere serotonin i noradrenalin (Sandler i sur., 1981.), MAO B prvenstveno oksidira feniletilamin (Shih i sur., 1999.), dok su dopamin i tiramin supstrati oba izoenzima (Billett, 2004.). Međutim, obje izoforme MAO mogu razgraditi sve supstrate, te jedna izoforma može preuzeti funkciju druge izoforme kada je ona kompromitirana, npr. u slučaju farmakološke inhibicije (Billett, 2004.). MAO prevodi 5HT u 5-hidroksiindolacetaldehid, koji se zatim oksidira u 5HIAA pomoću aldehid-dehidrogenaze (Hensler, 2011.). Nakon egzogene primjene serotonina, većina se 5HIAA izlučuje unutar 24h, što ukazuje na brzi metabolizam serotonina (Mohammad-Zadeh i sur., 2008.). U SŽS su

prisutna oba izoenzima, dok je na periferiji MAO A zastupljeniji u jetri i gastrointestinalnom traktu, a MAO B u trombocitima (Sandler i sur., 1981.; Shih i sur., 1999.; Tyce, 1990.). Geni za obje izoforme MAO se i u čovjeka i u štakora nalaze na kromosomu X. *Ex vivo* mjerenje aktivnosti enzima MAO moguće je samo za izoformu B koja se nalazi u lako dostupnim trombocitima. Međutim, neposredno mjerenje metabolizma serotonina provodi se mjerenjem koncentracije 5HIAA - u perifernom odjeljku u urinu, a u središnjem odjeljku u cerebrospinalnoj tekućini (CSF, engl. *cerebro-spinal fluid*) (Sarrias i sur., 1990.). Drugi manje zastupljeni metabolički putevi serotonina su glukuronidacija i sulfatacija, koje se odvijaju u jetri, plućima, bubrezima i mozgu (Tyce, 1990.). Prvenstveno u epifizi, ali i u mrežnici**,** crijevima i štitnjači, serotonin se prevodi u melatonin acetilacijom i metilacijom. Manji dio serotonina reducira se u 5-hidroksitriptofol djelovanjem aldehid-reduktaze, te u 5-hidroksiindol-tiazoladin-karboksilnu kiselinu (Bortolato i sur., 2010.).

Tablica 1. Elementi serotoninskog sustava

Ime	**Odjeljak**	Kratica	Smještaj	Funkcija	Gen (kromosom čovjeka)
Triptofan-hidroksilaza	**SREDIŠNJI**	TPH 2	serotonergični neuroni	glavni enzim u sintezi 5HT	*TPH1* (11)
	PERIFERNI	TPH 1	stanice crijeva i epifize		*TPH2* (12)
Monoamin-oksidaza A	**SREDIŠNJI**	MAO A	ne-serotonergični neuroni	glavni enzim u razgradnji 5HT	*MAOA* (X)
	PERIFERNI		stanice jetre i pluća		
Monoamin-oksidaza B	**SREDIŠNJI**	MAO B	serotonergični neuroni	razgradnja monoamina	*MAOB* (X)
	PERIFERNI		trombociti	nije poznata	
Serotoninski prijenosnik	**SREDIŠNJI**	5HTt	serotonergični neuroni	prekid sinaptičkog djelovanja 5HT	*SLC6A4* (17)
	PERIFERNI		trombociti	nakupljanje 5HT	
Receptor 5HT1a	**SREDIŠNJI**	5HT1Ar	presinaptički 5HT neuroni	posreduje otpuštanje 5HT	*HTR1A* (5)
	PERIFERNI		stanice crijeva		
Receptor 5HT2a	**SREDIŠNJI**	5HT2Ar	postsinaptički neuroni	ekscitacijski receptor	*HTR2A* (13)
	PERIFERNI		stanice crijeva; trombociti	otpuštanje 5HT; trombocitna agregacija	

1.1.3. Serotoninski receptori

Zajedno sa svojim receptorima, serotonin je jedan od najstarijih molekularnih sustava koji su preuzeli funkciju međustanične komunikacije, i prisutan je od trenutka nastanka evolucijski najjednostavnijih živčanih sustava (Bockaert i sur., 2010.). Gaddum i Picarelli (1957.) su prvi objavili postojanje više podvrsta serotoninskih receptora, koje su nazvali 5-HT-M i 5-HT-D, prema antagonistima koji su na njih djelovali, morfinu i dibenzilinu. Peroutka i Snyder (1980.) su ih ponovno klasificirali prema studijama vezivanja radio-liganda na homogenatima živčanog tkiva. Razvitkom molekularnih metoda, te kloniranjem receptora, omogućeno je jasnije identificiranje svih 7 obitelji serotoninskih receptora koji se označavaju brojevima od 5HT1 do 5HT7 (Bockaert i sur., 2010.).

Do današnjeg dana, identificirano je 15 različitih serotoninskih receptora u čovjeka (tablica 2.). Gotovo svi receptori 5HT su metabotropni receptori povezani s G-proteinima (GPCR, engl. *G-protein-coupled receptors*). To su integralni membranski proteini sa sedam hidrofobnih transmembranskih domena, tri unutarstanične i tri izvanstanične petlje, s amino-krajem orijentiranim prema izvanstaničnom prostoru, dok je karboksilni kraj orijentiran prema citoplazmi (Hoyer i sur., 2002.). Razlikuje se samo obitelj $5HT_3$, kojoj pripadaju ionotropni receptori, tj. ionski kanali. Velika raznolikost serotoninskih receptora omogućuje da 5HT utječe na razvoj stanica na razne načine, od brze neurotransmisije otvaranjem receptorskih kanali, do kratko i dugotrajne neuromodulacije preko kaskada unutarstaničnih reakcija (Gaspar i sur., 2003.). K tome, sve je više dokaza da raznolikosti doprinosi i veliki broj različito izrezanih varijanti (engl. *splice variants*) s različitim modulatornim aktivnostima (Hannon i Hoyer, 2008.).

Tablica 2. Serotoninski receptori

Obitelj	Receptor	Vrsta
5HT1	$5\text{-}HT_{1A}$ $5\text{-}HT_{1B}$ $5\text{-}HT_{1D}$ $5\text{-}HT_{1E}$ $5\text{-}HT_{1F}$	**Receptori povezani s G-proteinima**
5HT2	$5\text{-}HT_{2A}$ $5\text{-}HT_{2B}$ $5\text{-}HT_{2C}$	
5HT3	$5\text{-}HT_{3A}$ $5\text{-}HT_{3B}$ $5\text{-}HT_{3C}$	**Ionski kanali**
5HT4	$5\text{-}HT_{4}$	**Receptori povezani s G-proteinima**
5HT5	$5\text{-}HT_{5A}$ $5\text{-}HT_{5B}$	
5HT6	$5\text{-}HT_{6}$	
5HT7	$5\text{-}HT_{7}$	

1.2. Serotoninski odjeljci u organizmu

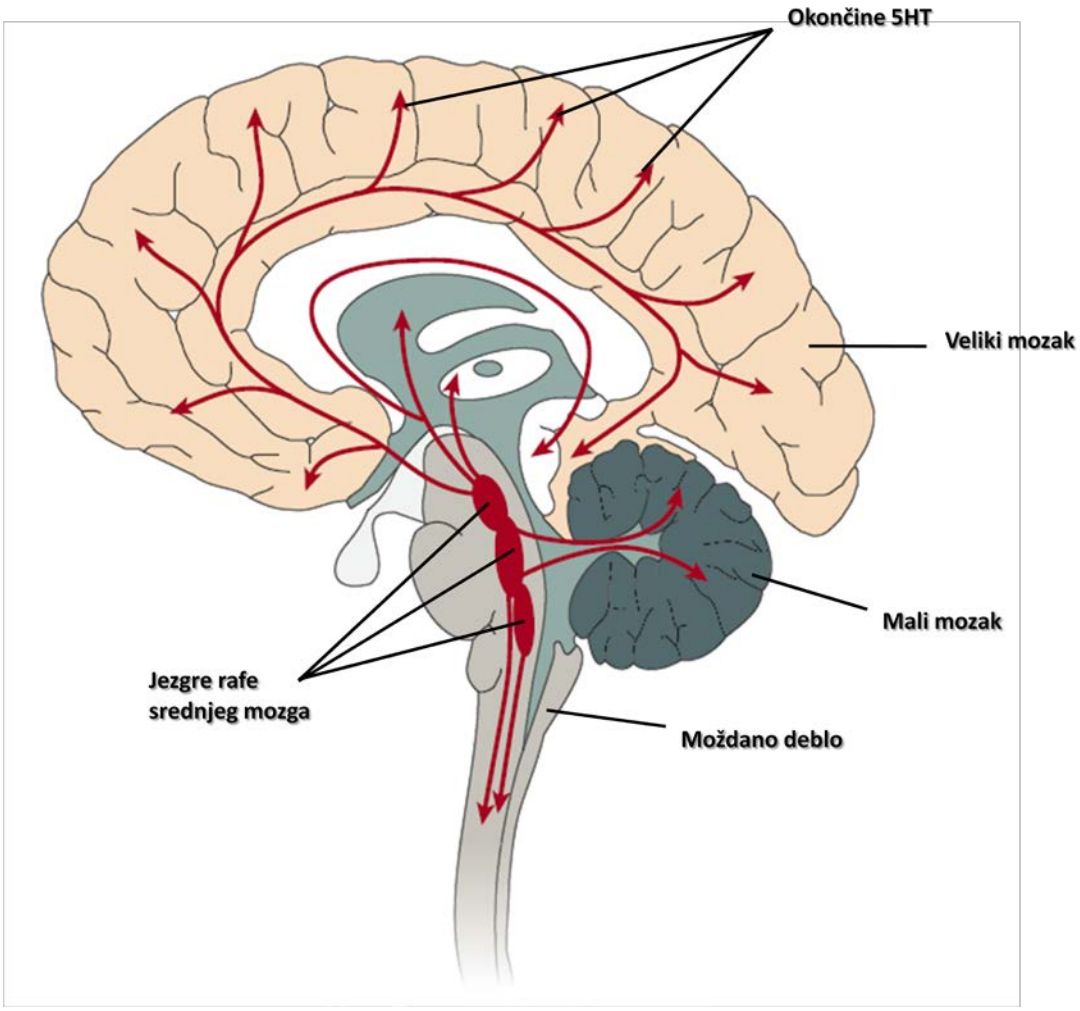
1.2.1. Serotoninski sustav u mozgu

U sisavaca razlikujemo dva serotoninska odjeljka: središnji, koji se odnosi na središnji živčani sustav te periferni, koji čine ostali organski sustavi. Ova dva odjeljka su međusobno odvojena krvno-moždanom barijerom (BBB, engl. *blood-brain barrier*), a većina (90%) 5HT nalazi se u perifernom odjeljku (Nakatani i sur., 2008.). Metabolizam serotonina u oba odjeljka je isti, ali enzimi koji sudjeluju u njegovom metabolizmu, kao i funkcije koje 5HT ima u svakom odjeljku se razlikuju.

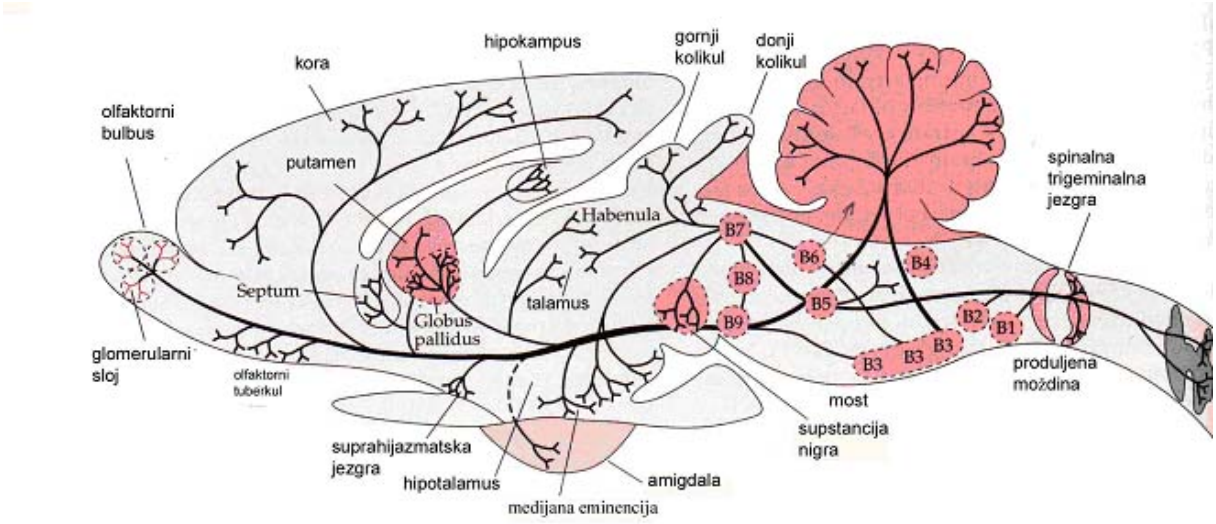
U središnjem živčanom sustavu, serotonin djeluje kao neurotransmiter posredujući mnoge fiziološke procese, uključujući cirkadijane ritmove, unos hrane i spolno ponašanje (Lucki, 1998.). Zbog mnogih funkcija se serotoninu pripisuje ključna uloga u različitim psihijatrijskim poremećajima kao što su anksioznost, depresija, ovisnosti i autizam (Brawman-Mintzer i Yonkers, 2004.; Johnson, 2004.; Joiner i sur., 2005.).

U središnjem odjeljku novosintetizirani serotonin pohranjuje se u vezikulama serotonergičnih neurona. U čovjeka se serotonergični neuroni nalaze u devet izoliranih skupina načinjenih od tijela neurona, tzv. jezgara rafe, smještenih u ponsu i srednjem mozgu (Dahlström i Fuxe, 1964.). Jezgre rafe označene su s B1-B9, a iz njih se odašilju projekcije u skoro sve dijelove mozga. Kaudalni sustav serotonergičnih neurona čine jezgre B1-B5, iz kojih izlaze eferentna vlakna koja se proširuju u moždano deblo i leđnu moždinu. Iz grupe B6-B9 jezgara rafe izlaze aferentna rostralna serotonergična vlakna koja odašilju svoje projekcije u prednji mozak (Dahlström i Fuxe, 1964.). Najveće su dorzalne (skupina B6 i B7) i medijane (skupina B8) jezgre rafe (Feldman i sur., 1997.; Gaspar i sur., 2003.; Jacobs i Azmitia, 1992.). Unutar moždanog debla nalazi se manji broj serotonergičnih jezgara prisutnih u mrežastoj tvorevini (Dahlström i Fuxe, 1964.). Organizacija serotonergičnih neurona je slična i u štakora. U usporedbi s ukupnim brojem neurona u SŽS štakora (10^{10} neurona), broj serotonergičnih neurona je relativno mali (oko 20 tisuća). Međutim, ovi neuroni pružaju gustu mrežu aksona koja inervira gotovo sva područja mozga kao i leđnu moždinu (Gaspar i sur., 2003.) (slika 3).

a)



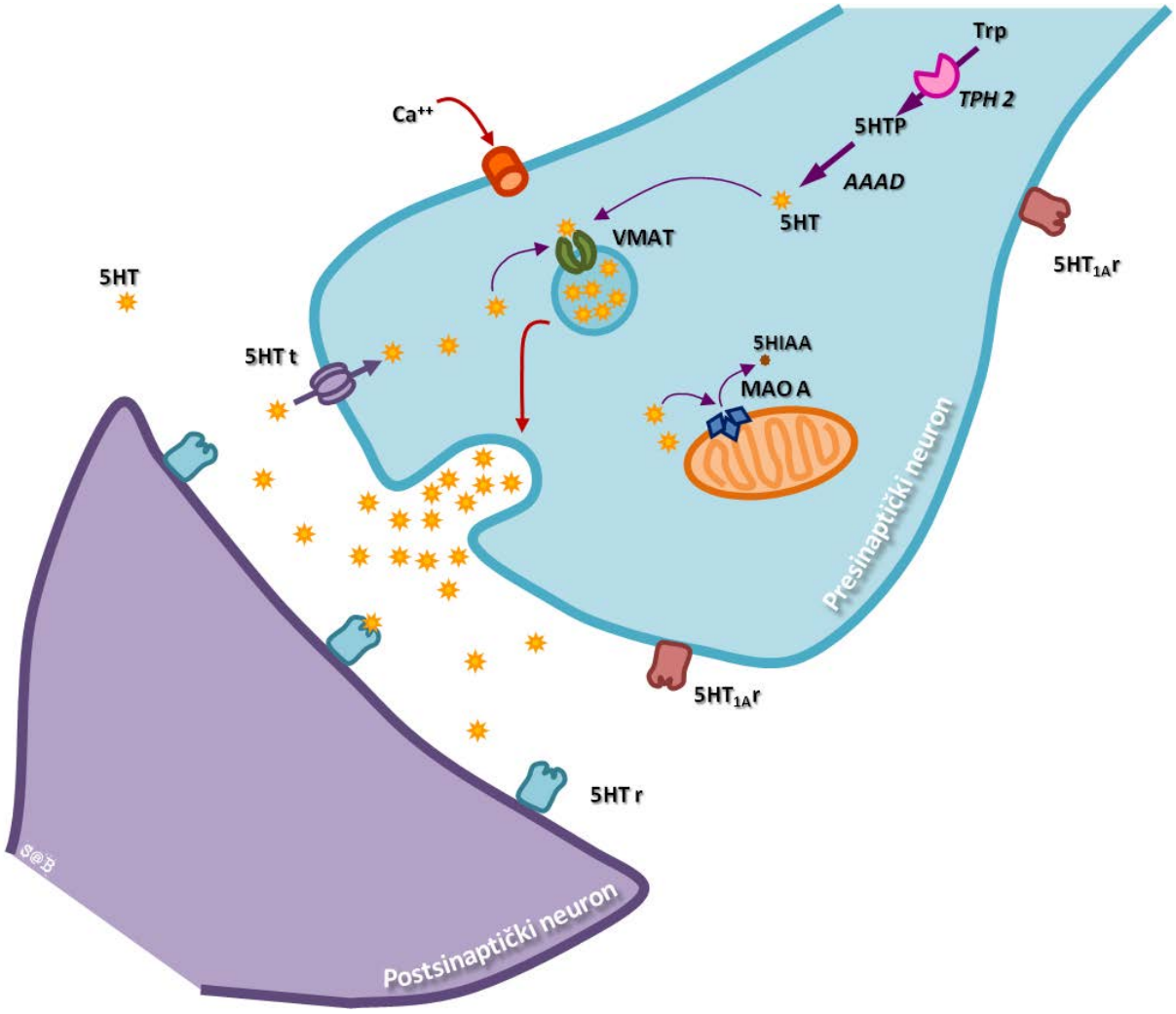
b)



Slika 3. Serotonergične jezgre i njihova projekcijska područja u mozgu (a) čovjeka (prema Berger i sur., 2009.) i (b) štakora (prema Bordukalo Nikšić, 2008.).

Tijekom razvoja mozga serotonin djeluje kao neurorazvojni signal regulirajući rast vlastitih neurona kao i sazrijevanje ciljnih regija (Whitaker-Azmitia, 2001., 2005.). U mozgu sisavaca serotoninski sustav je jedan od neurotransmiterskih sustava koji se najranije razvijaju (Aitken i Törk, 1988.). Serotoninska se vlakna pojavljuju među prvima te od samog početka formiranja mozga inerviraju korteks (Azmitia, 1999.). U čovjeka, serotonergični neuroni su vidljivi krajem petog gestacijskog tjedna, a do desetog tjedna ubrzano raste njihov broj. Već je tijekom 15. tjedna vidljiva njihova tipična organizacija unutar jezgara rafe (Whitaker-Azmitia, 2005.). Aksoni serotonergičnih neurona, iako formirani prije rođenja, tek će tijekom prvih godina života doseći svoj funkcionalni maksimum. Od 2. do 5. godine razina 5HT spušta se na razinu karakterističnu za odraslu dob i iznosi samo 50% od najveće koncentracije u ranom djetinjstvu (Chugani i sur., 1999.). U štakora, prvi serotonergični neuroni pojavljuju se već 12. gestacijskog dana, kada započinje razvoj jezgara rafe (Gaspar i sur., 2003.; Krinke, 2000.), projekcija ovih neurona u druga područja mozga (Wallace i Lauder, 1983.), kao i ekspresija specifičnih receptora. U glodavaca, neuroni jezgara rafe počinju otpuštati 5HT samo dan nakon što se razviju, dok potpuno sazrijevanje serotoninskih aksonskih završetaka zahtijeva više vremena i završava u postnatalnom periodu (Lidov i Molliver, 1982.). Serotonergični receptori, koji se pojavljuju u raznim razvojnim stadijima, moduliraju različite razvojne procese kao što su neurogeneza, apoptoza, nastanak i grananje aksona i dendrita (Azmitia, 2001.). Prvi se pojavljuju $5HT_{1A}$, $5HT_{1B}$ i $5HT_{2B}$ receptori, a tek se kasnije eksprimiraju $5HT_{2A}$ i $5HT_{2C}$ receptora (Gaspar i sur., 2003.). Serotonin stimulira rast živčanih vlakana tako što, između ostalog, potiče lučenje čimbenika rasta S-100β koji također sudjeluje u održavanju citoskeleta neurona (Azmitia, 2007.; Whitaker-Azmitia, 2001.).

Serotonin u razvijenom mozgu djeluje kao klasični monoaminski neurotransmiter (slika 4.). To su tvari koje se skladište u presinaptičkim vezikulama, oslobađaju nakon indukcije akcijskim potencijalom ulaskom kalcijevih iona u presinaptičke završetke, vežu na pre- i postsinaptičke receptore i odgovorne su za izravnu (preko ionskih kanala) ili neizravnu (preko sustava drugih glasnika) depolarizaciju ili hiperpolarizaciju postsinaptičkog neurona (Di Pino i sur., 2004.). Receptori $5HT_{1A}$ važni su za održavanje homeostaze sustava 5HT, te se nalaze pre- i post-sinaptički, kao autoreceptori u neuronskim tijelima jezgara rafe, odnosno kao heteroreceptori u ciljnim tkivima koje inerviraju serotoninski neuroni. Kad se poveća razina serotonina, 5HT se veže za autoreceptor $5HT_{1A}$ inhibirajući daljnju transmisiju, te se veže i za postsinaptičke receptore $5HT_{1A}$ aktivirajući postsinaptički neuron (Artigas, 2013.).



Slika 4. Serotonergična sinapsa

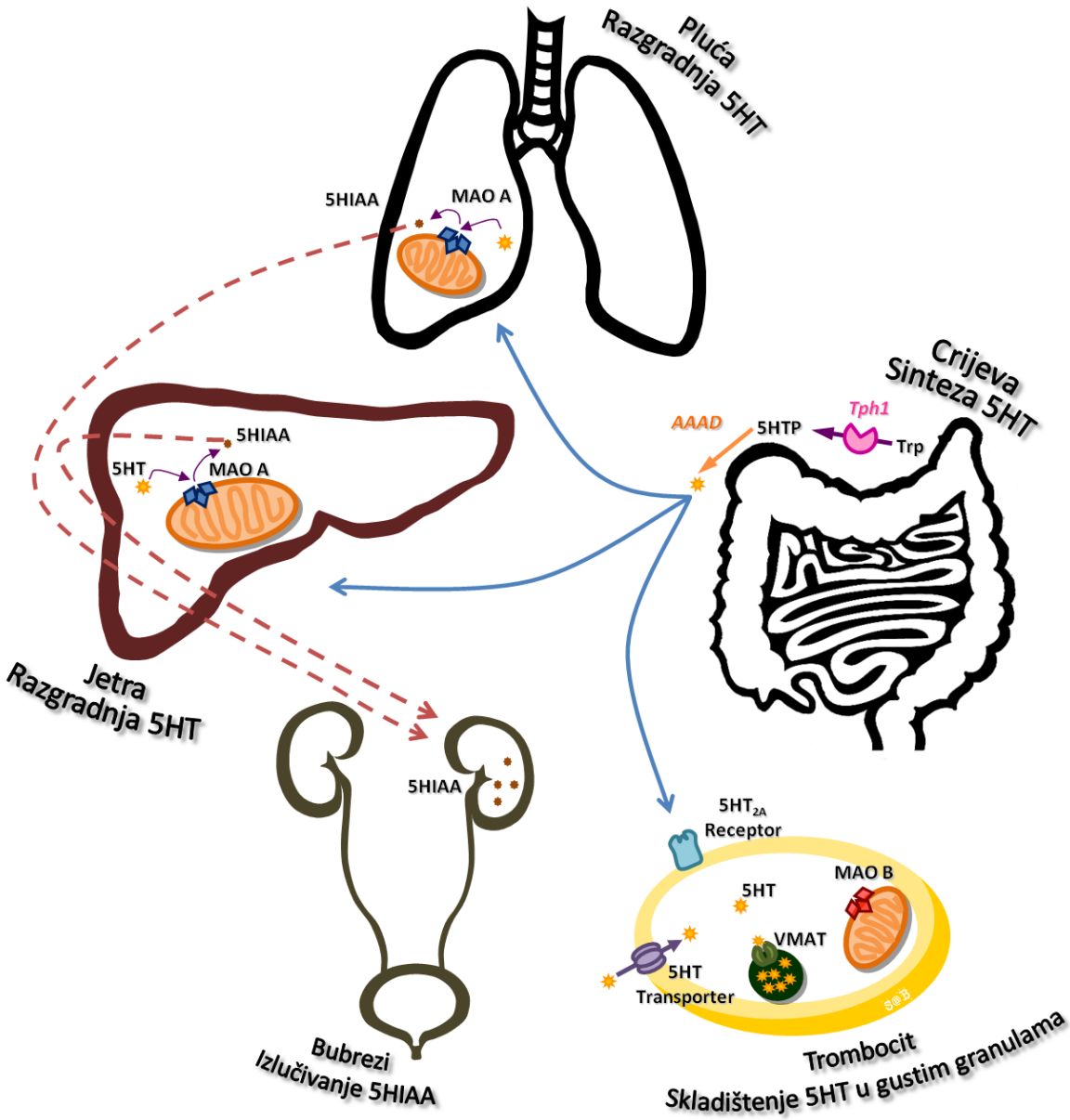
Iako serotonin djeluje u sinapsama, nedavno je otkrivena šira parakrina transmisija 5HT u nekoliko regija SŽS (Bunin i Wightman, 1999.; Ridet i Privat, 2000.; Zoli i sur., 1999.). Naime, mnogi presinaptički završetci koji sadrže 5HT se ne nalaze u neposrednoj blizini postsinaptičkih elemenata (Ridet i Privat, 2000.). Također, 5HT prijenosnik se često nalazi uzduž aksona, izvan sinapse (Tao-Cheng i Zhou, 1999.), a također i u astrocitima (Dave i Kimelberg, 1994.). U prilog izvansinaptičkoj funkciji serotonina govori i raspodjela 5HT receptora (Zoli i sur., 1999.). Također, i tipična dugačka i bogato razgranata morfologija serotonergičnih aksona koji spajaju pojedine 5HT-neurone s mnogim ciljnim stanicama (neuroni, glija, endotelne i ependimske) omogućuje 5HT da postigne globalne efekte (Grimaldi i Fillion, 2000.; Verge i Calas, 2000.). Ova difuzija neurotransmitera na udaljenost od nekoliko μm od mjesta otpuštanja modulira električne, metaboličke i neuroplastične

odgovore okolnih stanica, naročito tijekom razvoja (Di Pino i sur., 2004.). Čini se da bi ovakvo parakrino djelovanje serotonina moglo biti češće od klasične neurotransmisije (Ridet i Privat, 2000.; Zoli i sur., 1999.), što objašnjava ulogu serotonina u finoj regulaciji fizioloških funkcija, osobito ponašanja.

1.2.2. Periferni serotoninski odjeljak

Na periferiji, 5HT primarno posreduje kardiovaskularne i gastrointestinalne funkcije te aktivaciju trombocita (Berger i sur., 2009.).

Periferni 5HT se sintetizira u enterokromafinim stanicama crijeva (slika 5.). Djeluje lokalno kao neurotransmiter i hormon u tankom crijevu (Gershon, 1999.), gdje posreduje reflekse peristaltike i sekrecije (Gershon, 2003., 2004.). 5HT se iz tankog crijeva otpušta u krvotok prema jetri (Racke i sur., 1995.). Većina serotonina koji uđe u krvotok (čak 99%) se aktivno unosi u trombocite pomoću 5HTt smještenog na trombocitnoj membrani. Unutar samih trombocita, 5HT se nalazi u tzv. gustim granulama, u koje se pohranjuje pomoću VMAT, na sličan način kao i u središnjem odjeljku (Launay i sur., 1994.). Pohranjeni 5HT igra važnu ulogu pri aktivaciji trombocita u procesu zgrušavanja krvi (Hilton i Cumings, 1971.; Mitchell i Sharp, 1964.; O'Brien, 1964.), u čemu sudjeluje $5HT_{2A}$ receptor ($5HT_{2A}$r) smješten na membrani trombocita. $5HT_{2A}$r je GPCR koji djeluje preko metaboličkog puta fosfatidilinozitola, povisujući razinu iona Ca^{++} u citosolu. Aktivacija trombocita se odvija u dva koraka, a potiče je adenozin-5'-difosfat (ADP), adrenalin, trombin i kolagen. Nakon aktivacije, trombociti mijenjaju oblik i započinju s agregacijom. U drugom koraku slijedi otpuštanje sadržaja gustih granula, uključujući i 5HT koji se zatim veže za $5HT_{2A}$r, potičući daljnju agregaciju (Puri i Colman, 1997.). Ulogu serotonina u amplifikaciji agregacije trombocita preko $5HT_{2A}$r potkrepljuje činjenica da dodavanje antagonista $5HT_{2A}$r skoro potpuno zaustavlja agregaciju (Cerrito i Raiteri, 1979.; De Clerck, 1990.; Noble i Drake-Holland, 1990.). Serotonin se uklanja iz plazme u endotelnim stanicama pluća i jetre, gdje se djelovanjem enzima MAO razgrađuje u 5HIAA (Stolz, 1985.).



Slika 5. Periferni serotoninski odjeljak.

Druga lokacija produkcije serotonina u perifernom odjeljku je epifiza. Ovdje se serotonin prevodi u melatonin (*N*-acetil-5-metoksitriptamin) koji igra bitnu ulogu u regulaciji cirkadijanih ritmova (Feldman i sur., 1997.). Melatonin se otpušta u krvotok, a odstranjuje se u jetri gdje se metabolizira u 6-sulfatoksi-melatonin (6-SM).

5HIAA, 6-SM i male količine slobodnog 5HT se na kraju izlučuju urinom (Brzezinski, 1997.).

1.2.3. Povezanost središnjeg i perifernog serotoninskog sustava

Središnji i periferni serotoninski odjeljci su dvije fiziološki odvojene, ali funkcijonalno povezane cjeline. S jedne strane, 5HT se neovisno sintetizira i regulira u svakom odjeljku. Enterokromafine stanice i neuroni koriste različite enzime (TPH1 i TPH2) za sintezu serotonina (Walther i Bader, 2003.; Walther i sur., 2003.). Također, krvno-moždana barijera odvaja krvotok od izvanstanične tekućine središnjeg živčanog sustava, što sprječava prelazak 5HT iz jednog odjeljka u drugi. S druge strane, proteini koji reguliraju djelovanje serotonina u oba odjeljka kodirani su istim genima, imaju identične primarne strukture i funkcioniraju na isti način (Chen i sur., 1993.; Cook i sur., 1994.; Lesch i sur., 1993.). Uz to, u vrijeme perinatalnog razvoja mozga, krvno-moždana barijera još nije razvijena pa 5HT slobodno može prelaziti iz jednog odjeljka u drugi.

Pojam krvno-moždane barijere je uveo njemački liječnik Lewandowsky početkom 20. stoljeća**.** Primarna funkcija BBB je sprječavanje ulaska mnogih toksičnih tvari i neurotransmitera koji cirkuliraju krvotokom (npr. noradrenalin, glutamat, serotonin, i sl.) u mozak te omogućavanje ulaska esencijalnih hranjivih tvari, radi održavanja homeostaze koja je neophodna za rad neurona. BBB se sastoji od endotelnih stanica moždanih kapilara međusobno spojenih mnogim čvrstim spojevima (engl. *tight junctions*) i nastavcima („nožicama“) astrocita koji predstavljaju mjesto izmjene tvari između krvi i izvanstanične tekućine mozga (Laterra i sur., 1999.). Najčešći mehanizmi izmjene tvari između krvi i cerebrospinalne tekućine preko BBB su jednostavna difuzija malih nenabijenih molekula topivljih u mastima (npr. kisik, ugljikov dioksid i lipofilni hormoni) i olakšana difuzija sa specifičnim prijenosnicima ili kanalima, kojom prolaze tvari potrebne za normalno funkcioniranje živčanog sustava poput glukoze, ketonskih tijela, L-aminokiselina, vitamina, itd. (Laterra i sur., 1999.). BBB sprječava paracelularni transport molekula i mikroorganizama.

Serotonin je pri fiziološkom pH hidrofilna molekula jer zbog hidroksilne grupe i dušika djeluje kao akceptor protona, i kao takva ne može samostalno proći kroz BBB. Posljedica toga je već spomenuta funkcionalna odijeljenost središnjeg i perifernog serotoninskog sustava (Laterra i sur., 1999.; Whitaker-Azmitia, 2001., 2005.). U odraslih jedinki razina 5HT u krvi nije nužno odraz razine 5HT u mozgu, kako zbog krvno-moždane barijere, tako i zbog različitih enzima TPH koji sudjeluju u sintezi 5HT u svakom od odjeljaka (Veenstra-Vanderweele i Cook, 2003.). Međutim, tijekom fetalnog i ranog postnatalnog razvoja krvno-moždana barijera nije potpuno razvijena, što omogućuje da serotoninski odjeljci

„komuniciraju“ i utječu jedan na drugoga. U čovjeka to razdoblje traje do 2. godine života, a u štakora do 20. postnatalnog dana (PND) (Whitaker-Azmitia i Azmitia, 1986.; Whitaker-Azmitia, 2001., 2005.; Whitaker-Azmitia i sur., 1994.).

1.3. Fiziološka uloga serotonina

Veći udio serotonina, čak 90%, se nalazi u perifernom odjeljku organizma gdje sudjeluje u mnogim fiziološkim procesima. 5HT pohranjen u trombocitima igra važnu ulogu u procesu zgrušavanja krvi. Razvoj srca, kao i kompleksna regulacija srčanog ritma, je također pod kontrolom serotonina, koji sudjeluje i u kontroli krvnog tlaka te regulira konstrikciju i dilataciju krvnih žila. Serotonin utječe na dišni sustav izravno, preko centara za regulaciju disanja u moždanom deblu, i neizravno, preko konstrikcije i dilatacije krvnih žila. U crijevima, 5HT regulira gastrointestinalnu peristaltiku i pražnjenje želuca, sekreciju enzima gušterače i osjećaj mučnine. Serotonin također regulira genitourinarne funkcije kao što su sekrecija testosterona, inhibicija ejakulacije, kontrakcija epididimisa i inhibicija mokrenja kod muškaraca te razvoj mliječnih žlijezda, vazokonstrikciju i kontrakciju glatkih mišića maternice, sazrijevanje jajnih stanica i sekreciju progesterona kod žena. Najzad, serotonin je također uključen u nocicepciju, tj. mogućnost percepcije boli te igra ulogu u ukupnoj brzini metabolizma i kontroli tjelesne temperature (Berger i sur., 2009.).

Iz gore navedenog je vidljivo da je 5HT važan za mnoge procese u tijelu. Međutim, u ovom radu ćemo se osvrnuti na funkciju 5HT u središnjem odjeljku i njegovom utjecaju na razvoj živčanog sustava i regulaciju ponašanja.

1.3.1. Uloga serotonina u razvoju mozga

Prije nego što poprimi funkciju neurotransmitera u zrelom mozgu, serotonin je uključen u regulaciji razvoja živčanog sustava (Sodhi i Sanders-Bush, 2004.). Sve je više dokaza za to da je serotoninska homeostaza kritična za stvaranje, diferencijaciju i sazrijevanje živčanih stanica i njihovih mreža u regijama mozga koje kontroliraju primanje osjetilnih signala, procesiranje podražaja i motoriku (Lesch, 2001.). 5HT je mitogeni i morfogeni čimbenik, kao i signal u diferencijaciji korteksa.

U prenatalnom mozgu štakora 5HT-receptori su eksprimirani u serotonergičnim neuronima kao i u glija-stanicama uzduž rastućih serotonergičnih puteva, pri čemu svaki podtip receptora pokazuje različiti profil ekspresije tijekom razvoja ovisno o razvojnom stadiju i regionalnoj raspodjeli (Borella i sur., 1997.; Hellendall i sur., 1992.; Lauder, 1990.; Lauder i sur., 2000.; Morilak i Ciaranello, 1993.; Rho i Storey, 2001.; Roth i sur., 1991.; Ruiz i sur., 1999.; Talley i Bayliss, 2000.; Talley i sur., 1997.; Verge i Calas, 2000.; Zec i sur., 1996.). U glodavaca, serotonergični rastući čunjići eksprimiraju proteine koje vežu serotonin, 5HTt i VMAT-2, 20. gestacijskog dana (Ivgy-May i sur., 1994.). Ekspresija 5HTt i VMAT-2 jako varira tijekom razvoja mozga, a u nekim se razdobljima drastično razlikuje od njihove ekspresije u odraslim jedinkama (Hansson i sur., 1999.; Mansour-Robaey i sur., 1998.; Verney i sur., 2002.). Iako još nije utvrđeno koji neuroni privremeno izražavaju 5HTt, čini se da bi serotonin mogao ostvarivati svoju razvojnu ulogu upravo preko 5HTt, naročito tijekom oblikovanja živčanih veza, kao što je već dokazano u glodavaca (Verney i sur., 2002.). Mnoga istraživanja ukazuju na ulogu serotonina kao integralne signalne molekule tijekom razvoja živčanog sustava, gdje serotonin djeluje kao koordinator razvoja mozga, modulirajući živčanu aktivnost (Di Pino i sur., 2004.).

Tijekom embrionalnog i ranog postnatalnog razvoja, krvno-moždana barijera nije potpuno razvijena, te je propusna za serotonin koji može slobodno prelaziti iz perifernog odjeljka u mozak i tako utjecati na razvoj živčanog serotoninskog sustava (Whitaker-Azmitia, 2001.). Za pravilnu funkciju serotonina tijekom razvoja potrebno je da ovaj neuromodulator bude prisutan u optimalnim koncentracijama u različitim regijama mozga, a ta se regulacija postiže strogom kontrolom sinteze i razgradnje. Stoga, promijenjena serotoninska homeostaza u bilo kojem od odjeljaka može dovesti do odstupanja od optimalne koncentracije 5HT koje bi zatim moglo utjecati na razvoj živčanog serotoninskog sustava i tako uzrokovati promjene u ponašanju.

1.3.2. Uloga serotonina u regulacji ponašanja

Usprkos tome što se većina tjelesnog serotonina nalazi izvan SŽS i što manje nego jedan od milijun neurona SŽS proizvodi 5HT, serotonin modulira skoro sve bihevioralne procese (Gershon i Tack, 2007.). Serotoninski receptori se eksprimiraju u svim regijama mozga, a u svakoj regiji na specifičan način. Pojedini neuron može eksprimirati više podtipova receptora, koji mogu imati čak i suprotne efekte na neurotransmisiju (Araneda i Andrade, 1991.).

Općenito govoreći, serotonin inhibira ili smanjuje odgovor na okolišne podražaje, dok njegove smanjene koncentracije dovode do pojačanja odgovora (Lucki, 1998.). Serotonin ima ulogu u cirkadijanom ritmu, što podrazumijeva kontrolu ciklusa spavanja i tjelesne temperature (Lucki, 1998.). Drugačiji obrasci spavanja uočeni su nakon promjene u koncentraciji 5HT ili promjene u aktivnosti $5HT_1$, $5HT_{2C}$ ili $5HT_7$ receptora (Frank i sur. 2002, Morrow i sur. 2008, Thomas i sur. 2003).

Serotonin utječe na osjećaj sitosti, kratkoročno ga povećavajući uslijed distenzije želuca i prisutnosti nutrijenata u crijevima, te modulirajući odgovore izazvane uvjetovanim poticajima koji vode do započinjanja hranjenja (Lee i Clifton, 2010.). Osjećaj sitosti i prekid hranjenja slijedi nakon povećanja koncentracije 5HT u medijalnim jezgrama hipotalamusa, dok smanjenje koncentracije dovodi do pretjeranog unosa hrane (Lucki, 1998.). Promijenjena serotoninska transmisija u hipotalamusu je prisutna u osoba koje boluju od anoreksije i bulimije (Jimerson i sur., 1990.).

Uloga 5HT u spolnom ponašanju je kompleksna, ovisno o spolu, 5HT receptoru uključenom u proces te bihevioralnom parametru koji se ispituje, no generalno ima inhibitornu ulogu. Povećana razina 5HT inhibira spolno ponašanje u oba spola, najčešće posredovanjem $5HT_{1A}$ receptora, dok smanjena razina 5HT potiče spolno ponašanje (Uphouse i Guptarak, 2010.).

Serotoninu se pripisuje regulacija anksioznosti, agresije i impulzivnog ponašanja. Otpuštanje 5HT u amigdali, hipokampusu i prednjem korteksu posreduje odgovor na stresne situacije i aktivira obrambene mehanizme (Viana i sur., 2008.). Smanjene razine serotonina uzrokuju smanjenu pažnju i prepoznavanje pozitivnog emotivnog poticaja te veće usmjeravanje pažnje prema negativnim podražajima u zdravih ljudi. Povećanje razine 5HT rezultira pojačanom pažnjom i prepoznavanjem pozitivnog emotivnog poticaja (Hensler, 2010.). Serotonergični putevi aktivirani su anksiogenim podražajima koji uključuju psihosocijalni stres, uvjetovani strah i konfliktne situacije (Millan, 2003.). Vezano za impulzivnost i kompulzivnost, smanjena razina serotonina potiče odgovor na podražaj u situacijama u kojima je odgoda reakcije poželjna te uzrokuje povećani averzivni odgovor na emotivne podražaje (Robbins i Crockett, 2010.). Manjak serotonina povećava agresivno ponašanje te smanjuje anksioznost (Mosienko i sur., 2012.). Serotonin također igra ulogu i u socijalnom ponašanju gdje je manjak serotonina povezan sa sniženom bihevioralnom inhibicijom (McNamara i sur., 2008.). Također, smanjena razina serotonina tijekom razvoja uzrokuje poremećaje u učenju u odraslih jedinki (Mazer i sur., 1997.).

1.4. Poremećaji serotoninske homeostaze

Zbog uključenosti serotonina u mnoge fiziološke procese, možemo pretpostaviti da promjene u serotoninskoj homeostazi uzrokuju značajne poremećaje u mozgu i na periferiji, koji se mogu odraziti i na ponašanje u odrasloj dobi, uključujući i razvoj poremećaja poput anksioznosti, depresije, ovisnosti, itd. Stoga je precizna regulacija serotoninske homeostaze, koja uključuje sintezu, razgradnju, unos, pohranu i otpuštanje serotonina, ključna za pravilan razvoj i funkciju živčanog sustava.

1.4.1. Farmakološki utjecaj na serotoninsku homeostazu

Postoje različiti načini na koji se farmakološki može poremetiti serotoninska homeostaza u organizmu. Jedan od njih je triptofanska deplecija koja se sastoji u prehrani bez triptofana. Ovim se putem potiče iskorištenje Trp prisutnog u organizmu za sintezu proteina, a smanjuje dostupnost Trp za sintezu 5HT u mozgu. Akutne promjene u dostupnosti triptofana korištene su u svrhu proučavanja raznih vrsta temeljnih psiholoških, bihevioralnih i fizičkih procesa kao i mnogih psihijatrijskih poremećaja u kojima serotonin igra važnu ulogu: depresije, opsesivno-kompulzivnog poremećaja, bulimije nervoze, bipolarnog poremećaja, sezonskog afektivnog poremećaja, shizofrenije, itd. (Richard i sur., 2009.).

S druge strane, povišenje koncentracije 5HT moguće je postići primjenom prekursora sinteze, Trp ili 5HTP. Da bi povećali razinu 5HT pomoću Trp, razine ostalih neutralnih aminokiselina trebaju biti niže jer je ulazak Trp u SŽS kompetitivan, te ne ovisi toliko o dostupnosti veće količine Trp koliko o razini ostalih aminokiselina koje koriste isti prijenosnik (Fernstrom i Wurtman, 1972.). Primjenom 5HTP efikasnije se povisuju razine 5HT jer je on neposredni prekursor serotonina i postoji isključivo u njegovom sintetskom putu. 5HTP se prodaje kao dodatak prehrani u svrhu poboljšanja raspoloženja, suzbijanja apetita ili pomoći pri spavanju (Birdsall, 1998.).

Osim dodavanjem prekursora, razina serotonina može se povisiti inhibicijom njegove razgradnje ili inhibicijom njegovog ponovnog unosa u presinaptički neuron. Inhibitori monoamin-oksidaze (MAOi, engl. *monoamin oxidase inhibitors*) djeluju unutar stanica vezujući se za enzim monoamin-oksidazu koja se nalazi na vanjskoj membrani mitohondrija. Mogu biti selektivni ili neselektivni, odnosno djelovati samo na jednu od izoformi ili na obje, te mogu biti reverzibilni ili ireverzibilni, ovisno o tome vežu li se privremeno ili ostaju vezani

do razgradnje enzima (Youdim i sur., 2006.). Nakon inhibicije MAO serotonin se ne razgrađuje, te se povisuje njegova razina, a time i njegovo djelovanje u sinapsi. Također se povisuju razine katekolamina koji isto tako trebaju MAO za svoju razgradnju. Iako su MAOi prvi razvijeni antidepresivi, danas se koriste uglavnom u liječenju depresije rezistentne na SSRI (Nolen i sur., 1985.; Thase i sur., 1995.), zbog većeg broja nuspojava. I dalje se učestalo koriste u liječenju poremećaja anksioznosti, ADHD, Parkinsonove i Alzheimerove bolesti (Bortolato i sur., 2008.).

Selektivni inhibitori ponovnog unosa serotonina (engl. *selective serotonin reuptake inhibitor*, SSRI) djeluju na serotoninski prijenosnik (5HTt) i onemogućuju njegovu funkciju. Inhibicijom ponovnog unosa serotonina u stanicu, 5HT ostaje u sinaptičkoj pukotini gdje nastavlja aktivirati svoje receptore pojačavajući serotoninsku neurotransmisiju (Grzeskowiak i sur., 2012.). Osim za ublažavanje simptoma depresije, djeluju također i pri liječenju shizofrenije i opsesivno-kompulzivnog i drugih poremećaja (Dutton i Barnes, 2008.).

Razvijeni su razni životinjski modeli za proučavanje ovih farmakološki aktivnih supstanci koji su omogućili detaljno upoznavanje njihovog djelovanja, kako na odrasli organizam, tako i na organizam u razvoju.

1.4.2. Utjecaj promijenjene serotoninske homeostaze na razvoj mozga

Tijekom prenatalnog i ranog postnatalnog razdoblja, 5HT modulira proliferaciju, migraciju, i programiranu smrt stanica te utječe na njihov oblik i stvaranje veza s drugim stanicama. Dobri modeli za proučavanje utjecaja promijenjene razine serotonina na razvoj mozga su farmakološki i *knock-out* (KO) modeli. Antagonist receptora $5HT_2$, ritanserin, uzrokuje kraniofacijalne i srčane mane u kultiviranim mišjim embrijima (Colas i sur., 1997.; Lauder i sur., 1994.; Nebigil i Maroteaux, 2001.; Nebigil i sur., 2000a., 2000b.). Sličan efekt ima i selektivni inhibitor ponovnog unosa serotonina (SSRI, engl. *selective serotonin reuptake inhibitor*), fluoksetin, čiji se teratogeni učinci pripisuju prekomjernoj stimulaciji receptora 5HT uslijed visokih razina izvanstaničnog 5HT (Di Pino i sur., 2004.). Visoke razine 5HT tijekom razvoja nastaju također i u slučaju inaktivacije gena za MAOA i 5HTt, što uzrokuje prekomjernu stimulaciju receptora $5HT_{1B}$ i niz drugih molekularnih promjena. U navedenim modelima, povišena razina 5HT uzrokuje drastične promjene u citoarhitekturi regija somatosenzoričke moždane kore (tzv. bačvasta polja, engl. *barrel fields*), te abnormalni razvoj projekcija koje iz talamusa dolaze u korteks. Opažene su također i promjene u ponašanju u smislu povećane agresivnosti životinja (Cases i sur., 1995.). Inhibitorna uloga

serotonina u razvoju vlastitih neurona dokazana je na životinjskim modelima s perinatalno povišenom koncentracijom serotonina izazvanom agonistom 5HT-receptora, 5-metoksitriptaminom (McNamara i sur., 2008.; Shemer i sur., 1991.), prekursorom serotonina, triptofanom (Thomke i sur., 1992.), kombinacijom selektivnih inhibitora MAOA i MAOB (Whitaker-Azmitia i sur., 1994.) i inhibitorima ponovnog unosa serotonina (Cabrera-Vera i sur., 1997.).

S druge strane, inaktivacijom gena za VMAT, koji pohranjuje sintetizirani 5HT u vezikule, izazvan je nedostatak serotonina tijekom razvoja mozga. U ovih životinja dolazi do poremećene neurotransmisije, prvenstveno zbog nedostatka aktivacije 5HT receptora te one ne prežive dugo nakon okota (najduže do 7 PND). Uočen je i kasniji razvoj somatosenzoričke moždane kore dok je razvoj talamičkog korteksa normalan (Di Pino i sur., 2004.). Transkripcijski faktor s domenom ETS, Pet1 [*pheochromocytoma 12 ETS* (E26 *transformation-specific*)] sudjeluje u kontroli embrionalne diferencijacije neurona 5HT. Pet1-KO miševi također imaju manju koncentraciju 5HT tijekom razvoja mozga. Kod njih makroskopska struktura SŽS izgleda normalno, ali pokazuju značajno smanjenu ekspresiju serotoninskih elemenata i promjene u ponašanju u smislu povećane anksioznosti i agresivnog ponašanja (Hendricks i sur., 2003.). Smanjena razina serotonina tijekom razvoja, postignuta farmakološkim tretmanima majki tijekom gestacije (p-klorofenilalanin (Lauder i Krebs, 1978.)) ili mladunaca na dan okota (5,7-dihidroksitriptamin (Durig i Hornung, 2000.)), uzrokuje značajne promjene u neurogenezi, migraciji stanica i sazrijevanju dendrita (Gaspar i sur., 2003.).

Navedeni podaci ukazuju na to da nagla odstupanja od normalnih razina serotonina tijekom razvoja mozga, bilo to povećanje ili smanjenje iste, uzrokuju temeljite promjene u strukturi SŽS i u ponašanju (Di Pino i sur., 2004.; Gaspar i sur., 2003.).

1.4.3. Hiperserotoninemija

Pojam hiperserotoninemija označava veću koncentraciju serotonina u krvi, a može nastati uslijed poremećenog metabolizma perifernog serotonina (povećana sinteza i/ili smanjena razgradnja) ili poremećene fiziologije trombocita (povećani unos i/ili smanjeno otpuštanje). Ova je pojava uočena u slučaju karcinoidnog tumora tankog crijeva uzrokovanog prekomjernim rastom i dijeljenjem enterokromafinih stanica koje zatim sintetiziraju više serotonina. Taj višak serotonina cirkulira krvlju i unosi se u trombocite. Osim u karcinoidnim tumorima, u kojima je uzrok hiperserotoninemije jasan, ovaj fenomen je uočen i u osoba

oboljelih od autizma, u kojih mehanizam nastanka hiperserotoninemije još uvijek nije razjašnjen.

1.4.3.1. Hiperserotoninemija i autizam

Promjene u serotonergičnoj neurotransmisiji smatraju se biološkim temeljem niza neuropsihijatrijskih poremećaja uključujući i autizam – neurorazvojni poremećaj odlikovan trima glavnim značajkama: a) poremećenom socijalnom interakcijom, b) poremećenom komunikacijom i c) repetitivnim stereotipnim ponašanjem (Owley i sur., 2006.). Drugi simptomi su: nemogućnost prepoznavanja osjećaja, manjak mimike, smanjeno snalaženje u društvenim situacijama, smanjeno socijalno vezivanje, pretjerana osjetljivost na dodirne i zvučne podražaje, kao i stereotipni pokreti (Crawley, 2004.; Moy i sur., 2009.). Prevalencija autizma u općoj populaciji procjenjuje se na 2 oboljela na 1000 osoba (Brugha i sur., 2011.; Owley i sur., 2006.), pri čemu obolijeva četiri puta više muškaraca negoli žena (Eisenberg, 1956.; Kanner, 1943.).

Iako uzroci autizma još nisu razjašnjeni, obiteljske analize i studije blizanaca pokazale su postojanje snažne genetičke podloge ovog poremećaja (Belzung i sur., 2005.; Crawley, 2004.). Koincidencija obolijevanja od autizma u braće i sestara iznosi 1-8%, u dvojajčanih blizanaca 10-20%, dok u jednojajčanih blizanaca ona raste na čak 60-90% (Bailey i sur., 1995.; Hallmayer i sur., 2002.; Le Couteur i sur., 1996.; Rosenberg i sur., 2009.). Smatra se da do pojave autizma dolazi ukoliko postoji genska predispozicija, te okolišni čimbenik koji djeluje kao okidač simptoma bolesti. Pretpostavlja se da je više od 200 gena uključeno u razvoj ove bolesti, dok se kao okolišni čimbenici spominju talidomid, valproična kiselina, kokain, alkohol kao i izloženost nekim virusnim infekcijama (DiCicco-Bloom i sur., 2006.).

Postoje razni dokazi koji upućuju na ulogu serotonina u nastanku poremećaja autističnog spektra. Pokazalo se da primjena lijekova koji utječu na neurotransmisiju 5HT utječe na simptome autizma. Ukoliko se smanji razina 5HT deplecijom triptofana, dolazi do pogoršanja simptoma u oboljelih od autizma (McDougle i sur., 1996.). Suprotno tome, primjenom selektivnih inhibitora povratnog unosa serotonina dolazi do smanjenja ponavljajućih i stereotipnih obrazaca ponašanja (Hollander i sur., 2005.; McDougle i Naylor, 1996.). PET (engl. *positron emission tomography*) studije, u kojima se koristi radioaktivno obilježen prekursor serotonina, pokazale su smanjenu sintezu 5HT u kori mozga i talamusu oboljelih od autizma (Chugani i sur., 1997., 1999.). Također, povišene razine serotonina u krvi konzistentno se nalaze kod trećine oboljelih bez obzira na etničku pripadnost (Cook i Leventhal, 1996.; Owley i sur., 2006.; Schain i Freedman, 1961.).

U istraživanju složenih bihevioralnih poremećaja, kao što je autizam, kod kojih nije poznat jasan genski ili neurobiološki uzrok, korisna je podjela bolesti prema specifičnim simptomima koji su dio kliničke slike i imaju u osnovi biološki mehanizam. Takve simptome nazivamo endofenotipovima. Oni predstavljaju nasljedna svojstva koja visoko koreliraju s bolešću koju istražujemo, a regulirana su manjim brojem gena (Gottesman i Gould, 2003.). Tako se hiperserotoninemija, koja jasno odražava promjene u regulaciji serotoninske homeostaze, može smatrati jednim od endofenotipova autizma (Hranilovic i Blazevic, 2012.). Hiperserotoninemija je najkonzistentniji nalaz u autizmu povezan za serotoninom i podrazumijeva povećanje razine 5HT u krvi od prosječno 50% (Anderson i sur., 1990.). Kao moguće fiziološke promjene koje bi mogle uzrokovati hiperserotoninemiju u autizmu spominju se povećana sinteza 5HT u enterokromafinim stanicama crijeva (Croonenberghs i sur., 2005.) ili njegovo pojačano otpuštanje u portalni krvotok (Janusonis, 2005.), zatim povećani unos 5HT u trombocite (Marazziti i sur., 2000.) ili njegovo smanjeno otpuštanje iz trombocita (Cook i Leventhal, 1996.) i najzad, smanjena periferna razgradnja 5HT (Anderson, 1987.). Genska komponenta hiperserotoninemije istraživana je studijama vezanosti (engl. *association studies*) uz gene koji reguliraju perifernu serotoninsku homeostazu, prvenstveno serotoninskog prijenosnika. Rezultati su prilično nekonzistentni i pokazuju kako povezanost s genom za 5HTt – samim (Coutinho i sur., 2004.; Weiss i sur., 2005.) ili u kombinaciji s genom koji kodira podjedinicu integrina beta 3 (proteina koji sudjeluje u trombocitnoj agregaciji) (Coutinho i sur., 2007.), tako i nepostojanje izravnog utjecaja varijanti ovog gena na hiperserotoninemiju (Anderson i sur., 2002.; Betancur i sur., 2002.; Hranilovic i sur., 2008.; Persico i sur., 2002.). Geni za enzime koji sudjeluju u regulaciji metabolizma serotonina, TPH1 i MAO A, također su pokazani kao mogući regulatori razina serotonina u krvi (Hranilovic i sur., 2008.).

Bez obzira na mnoga istraživanja, mehanizam nastanka hiperserotoninemije kao i njegova povezanost s disfunkcijom serotonina u središnjem odjeljku su i dalje nejasni. Trenutačno postoje dvije glavne teorije o povezanosti hiperserotoninemije i autizma. Prva teorija predlaže da najprije dolazi do promjene u perifernoj serotoninskoj homeostazi koja uzrokuje hiperserotoninemiju. Tijekom fetalnog i ranog postnatalnog razdoblja, dok još nije potpuno razvijena krvno-moždana barijera, ove visoke razine 5HT u krvi mogu prelaziti u SŽS i negativnom povratnom spregom inhibirati razvoj 5HT neurona te uzrokovati anatomske i funkcionalne promjene u mozgu karakteristične za autizam (Whitaker-Azmitia, 2005.). Druga teorija pretpostavlja da istovremeno, u mozgu i na periferiji, dolazi do promjene u ekspresiji jednog ili više gena koji reguliraju serotoninsku homeostazu (npr. receptore ili

enzime). Promjene u proteinima koje reguliraju funkciju 5HT narušile bi pravilnu serotoninsku neurotransmisiju utječući tako na rani razvoj mozga i rezultirajući bihevioralnim simptomima svojstvenima za autizam, dok bi se te promjene na periferiji očitovale kao hiperserotoninemija (Janusonis, 2005.). Prema prvoj teoriji, hiperserotoninemija bi bila uzrok promjenama u mozgu, dok bi prema drugoj teoriji, hiperserotoninemija bila tek periferni biljeg za promjene koje se događaju u središnjem serotoninskom odjeljku.

1.4.3.2. Modeli hiperserotoninemije

S ciljem razumijevanja načina nastanka hiperserotoninemije i njezine uloge u autizmu, razvijeno je nekoliko životinjskih modela.

Prva dva modela dobivena su mutacijom/inaktivacijom gena i podupiru teoriju da promjene u autizmu ovisne o serotoninu mogu biti uzrokovane simultanim promjenama u serotonergičnim elementima u središnjem i perifernom odjeljku, od kojih bi hiperserotoninemija bila samo biljeg. Jedna grupa znanstvenika nedavno je razvila model koji eksprimira najčešću mutaciju u genu 5HTt (Glatt i sur., 2001.; Sutcliffe i sur., 2005.), u kojem je aminokiselina glicin (Gly) zamijenjena alaninom (Ala) na položaju 56. U ovim životinjama 5HTt pokazuje veću aktivnost, veći udio fosforiliranog oblika, i povećan unos 5HT. U mozgu dolazi do promijenjene aktivacije serotonergičnih neurona jezgara rafe kao i pre-osjetljivosti receptora $5HT_{1A}$ i $5HT_{2A}$, dok se na periferiji javlja hiperserotoninemija. Autori su uočili da su ove fiziološke promjene popraćene promjenama u socijalnom ponašanju, komunikaciji, i repetitivnim ponašanjima, što čini ovaj model dobrim za izučavanje povezanosti hiperserotoninemije i autizma (Veenstra-Vanderweele i sur., 2012.). Modelu još nedostaju analize metabolizma i koncentracije serotonina u središnjem živčanom sustavu, moguće anatomske i druge promjene. Drugi model je miš s inaktiviranim genom za receptor $5HT_{1A}$, koji igra važnu ulogu u razvoju mozga. U mozgu odrasle jedinke djeluje kao autoreceptor koji regulira otpuštanje 5HT u sinaptičkoj pukotini, a eksprimira se i u crijevima koja su glavni izvor perifernog 5HT. $5HT_{1A}$r KO miševi su hiperserotoninemični te pokazuju povećanu anksioznost (Janusonis i sur., 2006.).

Sljedeći model je farmakološki i naziva se modelom razvojne hiperserotoninemije (DHS, engl. *developmental hyperserotonemia*). Dobiven je tretiranjem Sprague-Dawley štakora neselektivnim agonistom 5HT receptora, 5-metoksitriptaminom (5-MT; 1mg/kg), u razdoblju razvoja serotonergičnih neurona od gestacijskog dana (GD) 12 do PND 20 (Shemer

i sur., 1991.). U skladu s prvom teorijom, smatra se da tijekom rane faze razvoja, visoke razine serotonina iz krvi ulaze u mozak fetusa, negativnom povratnom spregom uzrokuju gubitak serotoninskih neuronskih završetaka i tako ometaju serotonergičnu funkciju u zrelom mozgu. Tretirane životinje su pokazale metaboličke promjene u mozgu istraživane oslikavanjem mozga *in vivo*. U postmortalnim analizama tkiva DHS životinja uočen je gubitak stanica koje sadržavaju oksitocin u paraventrikularnoj jezgri hipotalamusa, i povišenu razinu peptida povezanog s genom za kalcitonin u amigdali. DHS-mladunci proveli su manje vrijeme s majkom tijekom aktivne faze između 15. i 17. PND i pokazali su manju privrženost majci 17. PND u testu povratak majci, kao i smanjenu vokalizaciju uslijed odvajanja od majke. Tretirane životinje također su pokazale prekomjernu reakciju na zvučni ili dodirni senzorički podražaj, kao i dezinhibiciju ponašanja, bez promjene u anksioznom ponašanju. Promjene u socijalnom i drugim ponašanjima konzistentne su s onima uočenima u osoba oboljelih od autizma, te ukazuju da bi ovaj model mogao biti dobar za proučavanje autizma (Kahne i sur., 2002.; McNamara i sur., 2008.). Međutim, treba spomenuti da ovaj model ima dva ozbiljna nedostatka. Prvo, primjenjuje se 5-MT, koji neselektivno aktivira sve 5HT receptore, pa je teško odrediti mehanizam nastanka hiperserotoninemije. Drugo, iako se, deklarativno, u ovom modelu izaziva hiperserotoninemija, koja zatim negativno djeluje na središnji odjeljak 5HT, zapravo već sama primjena utječe i na 5HT receptore u samom mozgu pa je nemoguće razlučiti neizravni utjecaj (putem hiperserotoninemije) od izravnog utjecaja (aktivacijom pre- i post-sinaptičkih receptora) tretmana na razvoj i funkciju serotoninskog sustava u mozgu.

1.5. Modeli farmakoloških tretmana koji utječu na metabolizam serotonina

S obzirom da su istraživanja hiperserotoninemije u autističnih osoba, provedena u okviru našeg projekta, ukazala na promjene u metabolizmu serotonina (tj. sintezi i/ili razgradnji) kao na mogući uzrok povišenih koncentracija serotonina u krvi (Hranilovic i sur., 2008., 2009.), odlučili smo načiniti dva razvojna modela hiperserotoninemije: jedan s povećanom sintezom serotonina, a drugi sa smanjenom razgradnjom serotonina.

Prva skupina dobila bi prekursor sinteze 5-hidroksitriptofan - produkt djelovanja TPH i neposredni prekursor serotonina. Korištenje 5HTP ima nekoliko prednosti nad Trp. Prvo,

5HTP se isključivo nalazi u sintetskom putu serotonina te se kvantitativno prevodi u 5HT (Birdsall, 1998.; Magnussen i Nielsen-Kudsk, 1980.; Udenfriend i sur., 1957.). Drugo, dodavanjem 5HTP preskače se djelovanje TPH, koje predstavlja ograničavajući čimbenik u reakciji sinteze serotonina. Kliničke studije pokazale su da je 5HTP efikasan u povećanju razine serotonina u krvi (Thorre i sur., 1996.; Turner i sur., 2006.; van Praag, 1982.; van Praag i sur., 1972.; Zmilacher i sur., 1988.), te da prelazi krvno-moždanu i placentarnu barijeru bez posebnog prijenosnika (Birdsall, 1998.). Hirai i Nakajima su pokazali da je 20 mg/kg 5HTP minimalna doza koja uzrokuje mjerljivi porast koncentracije serotonina u krvi u Wistar štakora, zadržavajući pri tom fiziološki odnos između 5HT i njegovog metabolita 5HIAA (Hirai i Nakajima, 1979.). S druge strane, visoke doze 5HTP (100 mg/kg i veće), iako učinkovite u povišenju razine 5HT u krvi, pokazale su se neurotoksičnima u glodavaca, uzrokujući serotoninski sindrom (Casal i sur., 2000.; Mokler i sur., 1992.; Nisijima i sur., 2000., 2001.). Za naša smo istraživanja odabrali dozu od 25 mg/kg 5HTP koja nije toksična, a koja se pokazala vrlo efikasnom u povišenju koncentracije serotonina u krvi odraslih štakora (Engleman i sur., 1995.; Joyce i Hurwitz, 1964.; Park i sur., 1991.; Penn i sur., 1977.). Kako dodavanje supstrata za sintezu 5HT povećava njegovu razinu bez izravnog utjecaja na proteine koji reguliraju funkciju serotonina, smatrali smo da bi ovaj model mogao ispitati teoriju hiperserotoninemije kao uzroka promjena serotoninske homeostaze u mozgu (Whitaker-Azmitia, 2005.).

U drugoj eksperimentalnoj skupini htjeli smo ispitati teoriju hiperserotoninemije kao perifernog biljega promjene serotoninske homeostaze u mozgu, uzrokovane alteracijom jednog ili više serotoninskih elemenata (Janusonis, 2008.) Odlučili smo inhibirati razgradnju serotonina djelovanjem na obje izoforme enzima monoamin-oksidaze. Iako MAO A ima veći afinitet za serotonin nego MAO B (Izumi i sur., 2007.), oba enzima su jednako zastupljena u mozgu štakora (Lang i sur., 2004.). Kad bismo inhibirali samo jedan izoenzim, drugi bi djelovao kompenzatorno i tako uklanjao višak serotonina. Iz tog razloga, izabrali smo ireverzibilni neselektivni MAOi tranilcipromin (TCP) koji efikasno inhibira MAO A i MAO B u oba odjeljka (Weinstock i sur., 2003.). U odraslih štakora su izmjerene značajne promjene u serotoninskom metabolizmu uzrokovane primjenom TCP: smanjena aktivnost MAO A (Celada i Artigas, 1993.), povišene koncentracije 5HT (Ferrer i Artigas, 1994.; Green i Youdim, 1975.; Malyszko i sur., 1993.; McKim i sur., 1983.), i snižene razine 5-hidroksiindoloctene kiseline (Celada i Artigas, 1993.; Malyszko i sur., 1993.). Ovi učinci postignuti su nakon akutne ili kronične intraperitonealne primjene TCP u dozama od 0,5 do 15 mg/kg. Hiperaktivnost je uočena pri dozi od 20 mg/kg i većima (Foldes i Costa, 1975.;

Murphy i Kalin, 1980.), dok je serotoninski sindrom uočen isključivo nakon dodatka TCP u kombinaciji s prekursorom 5HT (Shioda i sur., 2004.; Sleight i sur., 1988.). Maki i suradnici (2000.) su akutnom dozom od 3 mg/kg TCP efikasno utjecali na ponašanje štakora, dok je njegova kronična primjena u dozama od 0,5 mg/kg i 3 mg/kg bila učinkovita u povišenju razine serotonina za više od 200% (Ferrer i Artigas, 1994.; Greenshaw i sur., 1989.). U ovom istraživanju, izabrali smo dozu od 2 mg/kg za koju smo očekivali da će u velikoj mjeri, ali ne i potpuno, blokirati razgradnju serotonina.

Cilj ove disertacije bio je istražiti utjecaj povišene koncentracije serotonina tijekom perinatalnog razvoja na homeostazu serotonina u odraslih štakora. Farmakološka primjena 5HTP i TCP izvršena je u razdoblju intenzivnog razvoja serotonergičnih neurona u štakora: od 13. prenatalnog do 21. postnatalnog dana. Treća grupa štakora tretirana je u istom razdoblju fiziološkom otopinom te je poslužila kao kontrolna skupina. Po okotu, uslijedila je detaljna analiza svakog modela. Najprije su ustanovljene neposredne fiziološke posljedice farmakološke primjene mjerenjem veličine legala, porođajne mase, stope preživljenja, prirasta mase, te koncentracije 5HT u krvi i mozgu mladih štakora. Ovi parametri ukazuju na neposredni učinak povišenih razina serotonina za vrijeme trajanja tretmana. Zatim je izvršeno ispitivanje dugoročnih/trajnih posljedica perinatalne izloženosti povišenim koncentracijama serotonina na homeostazu 5HT u organizmu odraslih štakora, mjerenjem koncentracije 5HT u perifernom i središnjem odjeljku, testiranjem emocionalnog, socijalnog i kognitivnog ponašanja, kao i određivanjem razine ekspresije gena za proteine koji reguliraju funkciju 5HT. Rezultati provedenog istraživanja trebali bi pomoći u razjašnjavanju odnosa između poremećene homeostaze serotonina u perifernom i središnjem odjeljku, te doprinijeti razumijevanju uloge hiperserotoninemije u razvoju autizma kao i fiziološke uloge serotonina u razvoju mozga i ponašanja.

2. ZNANSTVENI RADOVI

2.1. Physiological consequences of perinatal treatment of rats with 5-hydroxytryptophan (rad 1)

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Physiological consequences of perinatal treatment of rats with 5-hydroxytryptophan

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Key words: Serotonin (5-hydroxytryptamine, 5HT), 5-hydroxytryptophan (5HTP), Perinatal Rats, Physiology

Abbreviations:

5HT	5-hydroxytryptamine (Serotonin)
5HTP	5-hydroxytryptophan
Trp	Tryptophan
G	Gestation day
PND	Postnatal day

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Abstract

Background and Purpose: *Serotonin (5-hydroxytryptamine, 5HT) is present in brain and peripheral tissues and mediates various physiological functions. It also regulates perinatal development of serotonergic neurons and target tissues. It is assumed that dysregulation of the peripheral 5HT--homeostasis, which causes elevated blood 5HT concentrations, could inhibit development of serotonergic neurons and lead to anatomical/functional alterations of the brain. In this study we have investigated the physiological consequences of perinatal treatment with the immediate 5HT precursor, 5-hydroxytryptophan (5HTP) in young rats.*

Materials and Methods: *Rats were treated with 25 mg/kg 5HTP from gestational day 13 until postnatal day 21. The number of born and survived pups in each litter, body mass increase over time, level of anxiety produced by separation of pups from their mother, and blood 5HT concentrations were determined in the experimental group of rats and compared with values obtained in the saline-treated control group.*

Results: *Although a similar number of pups were born to each litter in both groups, 5HTP-treated pups, in comparison with saline-treated pups, had significantly lower body mass at PND1, significantly lower survival rate, significantly higher blood 5HT concentrations, and returned to their dam significantly faster in the separation anxiety test. They gained weight at slower rate than the control rats and maintained significantly lower body mass.*

Conclusion: *Temporary increase in peripheral 5HT concentrations during the critical phase of brain development has caused physiological disturbances in pups. Possible permanent changes in the central 5HT compartment are also indicated and will be explored in further studies.*

INTRODUCTION

Serotonin (5-hydroxytryptamine, 5HT) is a biologically active amine present both in the brain and the peripheral tissues where it mediates various physiological functions *(1)*. Before it assumes the function of a neurotransmitter in the mature brain, it regulates the perinatal development of serotonergic neurons and target tissues *(2)*. Alterations in the system that regulates 5HT metabolism and function might therefore represent a biological basis of several behavioral disorders *(3)*.

A disorder in which 5HT homeostasis is disturbed both centrally and peripherally is autism, a neurodevelopmental syndrome with onset in early childhood, characterized by impairment in social interaction and communication, and by the presence of restricted and repetitive behaviors and interests *(4)*. Elevated blood 5HT levels (hyperserotone-

mia) have been consistently found in about one third of autistic patients *(5),* while at the same time brain 5HT activity was found to be decreased *(6)*.

Although 5HT is synthesized in the central and peripheral compartments via different tryptophan hydroxylase enzymes *(7)* and its two pools are separated by the blood-brain barrier, proteins that control 5HT function in both compartments are encoded by the same genes, have identical primary structures and follow the same kinetics *(8–10)*. It is therefore possible that alterations in the expression of one or more of the serotonergic elements could lead to the dysregulation of 5HT transmission in the brain, affecting so its early development and resulting in autistic behavioral symptoms, while it is at the same time reflected in the periphery as hyperserotonemia *(11)*.

Alternatively, dysregulation of the peripheral 5HT--homeostasis could lead to high concentrations of 5HT in blood. During fetal development, before the formation of the blood-brain barrier, these high 5HT levels could inhibit development of serotonergic neurons and lead to the anatomical and functional alterations of the brain, characteristic for autism *(12)*. Inhibitory function of 5HT on development of serotonergic neurons has so far been investigated on animal models using pharmacological treatment with 5HT receptor agonist 5-methoxytryptamine *(13, 14),* 5HT precursor tryptophan *(15),* monoamine oxydase inhibitors *(16),* and 5HT uptake inhibitors *(17)*.

We have recently started studies of the effects of hyperserotonemia on the developing rat brain by administering immediate 5HT precursor, 5-hydroxytryptophan (5HTP) perinatally from gestational day 13 until postnatal day 21, the period of most intensive development of serotonergic neurons. With the hypothesis that the mentioned treatment will cause hyperserotonemia and lead to measurable physiological consequences in young rats, we have determined the number of born and survived pups in each litter, body mass increase over time, anxiety-like behavior of pups and blood 5HT concentrations in rats treated with 25 mg/kg 5HTP and compared the measured parameters with those of the saline treated control rats.

MATERIALS AND METHODS

Housing and breeding of animals

Out of five nulliparous Wistar females from the animal facility of the Croatian Institute for Brain Research (University of Zagreb, Zagreb, Croatia), weighing 260–291 g, two were assigned to a »saline group«, and three to a »5HTP group«. Females were mated with males of the same strain and age in 2:1 and 3:1 ratio, respectively. Vaginal smears were taken daily at 10 a.m. to check for the presence of sperm. Weight was monitored daily and progressive increase during the following week was considered as a confirmation of pregnancy. The day sperm was found in the smear was considered as day 0 of gestation (G0). After gravidity was confirmed in all females, the male was removed from the cage. Females remained together until two days before parturition when they were separated and remained singly housed until weaning of the pups (at postnatal day 21, PND 21). After weaning, animals were kept 3 per cage. Females were closely observed during parturition to determine the number of pups born to each litter. Pups were weight daily during treatment and three times weekly after treatment. Animals were housed in polycarbonate cages under 12-h light:12-h dark conditions at a temperature of 22 ± 2 °C, with free access to rat chow and tap water. The study was approved by the Ethic committee of the Faculty of Science, University of Zagreb, and was conducted in accordance with the Croatian Animal Protection Law (»Narodne novine«, 135/2006).

Pharmacological treatment

The experimental group of pups was treated with 5HTP (Sigma-Aldrich), from GD 13 until birth by injecting 25 mg/kg of 5HTP subcutaneously to pregnant females, and from PND1 until PND 21 by receiving subcutaneous injections in the nape at a dose 25 mg/kg. 5HTP was dissolved in acidified saline. Before treatment, the solution was neutralized with NaOH and warmed to the body temperature. The control group was treated with saline in the same manner. All injections were performed at 2 pm. A 50 μL syringe (Hamilton) and disposable 30G needles (BD, Drogheda, Ireland) were used to treat the pups.

Behavioral test – Return to dam

The return to dam test was adapted from McNamara *et al. (14)*. The test was performed on PND 17 in a cage with a dark non translucent wall inserted in the middle. The wall contained a 2.5 × 2.5 cm opening at the bottom with a tunnel-like extension on the mother's side, so the pups could pass but she could not reach out for them. A maximum of 5 pups per litter were placed on one side of the wall and the dam on the other. The pups were allowed ten minutes to return to their dam and the time when their hind legs crossed through the opening in the wall onto the mother's side was scored.

Blood 5HT concentration

Blood 5HT concentrations were measured at the end of the treatment (PND 22) in five randomly chosen pups from each treatment group. Under light ether narcosis, 800 μL of blood was withdrawn from the jugular vein into syringes preloaded with 200 μL of 3.13% trisodium citrate anticoagulant. Animals were sacrificed after blood sampling. Blood samples were transferred to microtubes after a thorough mixing and centrifuged at 200 × g for 10 min to generate platelet rich plasma. 5HT concentration in both, platelets and platelet-free plasma was determined using a commercial enzyme immunoassay kit (Serotonin ELISA kit, DRG Instruments GmbH, Germany), according to the kit instructions. A calibration curve was drawn based on the absorbances measured at 450 nm on the microplate reader (Bio Rad 550, Germany) and known concentrations of the standard solutions. Concentration values of samples were obtained by

interpolating them to the calibration curve, using the 4-parameters non-linear regression curve fitting. Results were counted as a sum of concentration in platelets and concentration in platelet-free plasma, and were expressed in ng 5HT per mL of blood.

Statistical analysis

Data was processed using GraphPad InStat 3.01 software. Normality of distributions of the measured parameters was tested by Kolmogorov/Smirnov method, while the equality of SDs was tested by Bartlett's test. Mean values of normally distributed parameters were compared using unpaired t-test, and of those that were not normally distributed using non-parametric Mann-Whitney test. Statistical significance of difference in survival rate was compared using two-sided Fisher's exact test. The level of significance was set to 0.05. Values were expressed as means ± standard deviations (M ± SD).

RESULTS

Several physiological parameters were determined in rats perinatally treated with the serotonin precursor 5HTP and compared to those of the saline treated rats (Table 1).

Two dams from the control group gave birth to 10 and 9 pups, respectively, one of which died during the first 24 hrs. Three dams from the 5HTP treated group gave birth to a total of 24 pups (8 per dam) out of which only ten survived after the first 24 hrs. The difference in survival rate was very significant (p = 0.0003), with a relative risk of dying for the 5HTP treated pups being 2.6 (95% CI 1.6–4.4).

Although the number of pups born per dam (9.5 in saline treated and 8 in 5HTP treated group), as well as the maternal weight gain during pregnancy (121,9% in the saline treated and 109,7% in the 5HTP treated group), was very similar between the groups, body mass of the surviving pups on PND 1 was significantly lower in the 5HTP treated (6.0 ± 0.7 g) than in the saline treated (7.3 ± 0.8 g) group (t = 4.222, df = 22, p = 0.0004) (Figure 1).

TABLE 1

Physiological parameters determined in saline- and 5HTP-treated rats.

	Saline treated group	5HTP treated group
Number of pups born	19	24
Offspring per dam	9.5	8
Number of died pups	1	14
Litter size per dam	9	3.33 ± 0.58
Survival rate (%)	95	42 ###
Birth weight (g)	7.3 ± 0.8	6.0 ± 0.8 ***
Adult weight at PND 44 (g)	162.0 ± 17.5	140.6 ± 22.4 **

** p<0.01, *** p<0.001, unpaired t-test; ### p<0.001, Fisher's exact test.

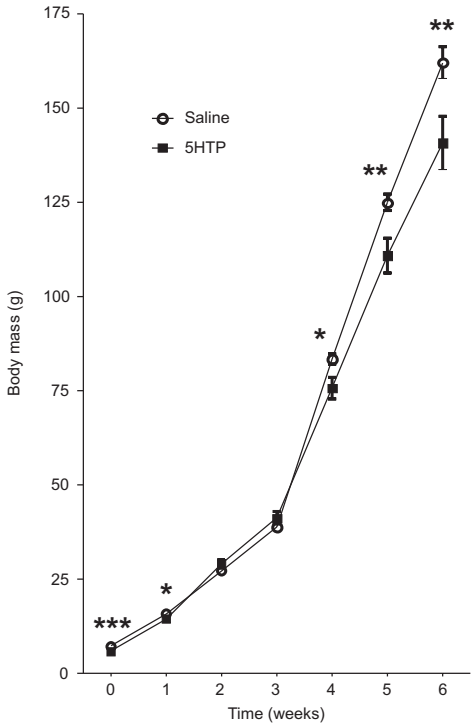

Figure 1. *Increase in body mass in rats perinatally treated with saline N=17 (circles), or 5HTP N= 10 (squares). Values are expressed as M ± SD. Differences in mean values of the body mass between the groups were compared at different time points using unpaired t-test; **p<0.01, *** p<0.001, p<0.05.*

Weight gain during breast-feeding period was constant in both groups and the average body mass of 5HTP treated pups reached that of saline treated pups after the second week of age (PND 15, 29.09 ± 1.080 g and 27.34 ± 0.3086 g, respectively), probably due to much smaller litter sizes. However, after weaning, the body mass of the 5HTP treated rats increased at a slower rate than the body mass of the saline treated rats, resulting in a significantly lower weight at adult age (PND 44, 140.6 ± 22.4 g and 162.0 ± 17.5 g, respectively; U = 32; p = 0.0067).

On PND 17, possible differences in behavior between the saline and 5HTP treated pups were determined by the return-to-dam test (Figure 2). While the saline trea-

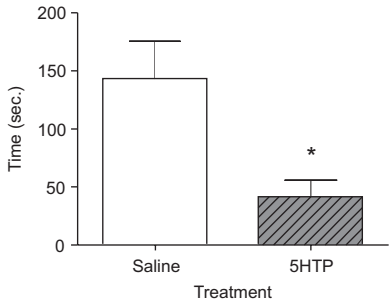

Figure 2. *Return to dam test. Bars show time needed for saline (N=18) and 5HTP (N=10) treated pups to return to their dam after separation. Values are expressed as M ± SD; * p<0.05, Mann Whitney test.*

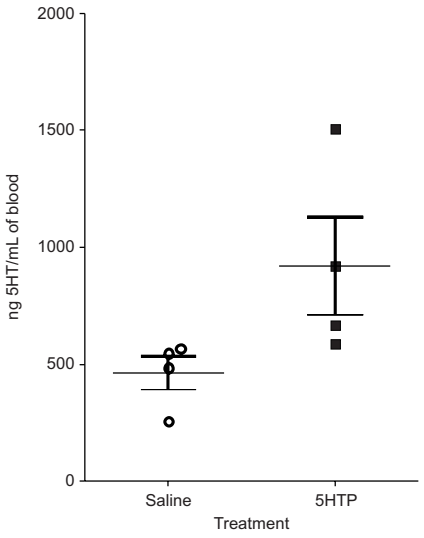

Figure 3. *Blood 5HT concentrations in rats perinatlly treated with saline or 5HTP at the end of treatment (PND 22). N=4 per group. Values are expressed as M ± SD; * p<0.05, Mann Whitney test.*

ted pups needed 143 ± 32 s on average to return to their mothers, the 5HTP treated pups performed the given task significantly faster needing on average only 41 ± 14 s (U = 37; p = 0.0118).

Finally, in order to determine whether the perinatal 5HTP treatment did indeed cause hyperserotonemia in the experimental group, blood 5HT concentrations were measured in five randomly chosen pups from both groups at the end of treatment. One sample from each group was lost during processing, leaving us with four samples per group (Figure 3). Although the number of samples was low, it was evident that pups from the 5HTP treated group have elevated blood 5HT concentrations in comparison to those of the saline treated group, mean values being 463 ± 142 ng/mL and 920 ± 416 ng/mL, respectively (U=16, p=0.0286).

DISCUSSION

By the use of the described method, we have caused hyperserotonemia in the experimental group of rats, with the mean blood 5HT concentration amounting to about 200% of the mean 5HT concentration in the saline treated group. In the body, 5HTP is the intermediate in the synthesis of 5HT from its precursor tryptophan (Trp) through a rate-limiting step mediated by the enzyme tryptophan hydroxylase. 5HTP is then reduced by aromatic amino acid decarboxylase to serotonin. Although Trp has been used by some groups to increase 5HT levels perinatally *(18–20),* we consider the use of 5HTP to have several advantages. First, Trp is an essential amino acid with many functions in the body (mainly a precursor in protein synthesis) while only 1–2% is consumed in the synthesis of 5HT *(21)*; on the other hand, 5HTP is only found in the 5HT synthesis pathway and is quantitatively converted to 5HT *(21–23)*. Secondly, administration of 5HTP allowed us to elude the rate-limiting step in the synthesis of 5HT and to mimic the effect of increased 5HT synthesis through a chosen 5HTP dose. Indeed, clinical studies have shown that 5HTP is effective in increasing blood 5HT levels *(24–27)* and is more effective than Trp in increasing brain 5HT levels *(28)*. The advantage of using 5HTP over 5HT itself is that it readily crosses the placental barrier *(21),* which is crucial for the prenatal part of the treatment. Hirai and Nakajima *(29)* have shown that a dose of 20 mg/kg 5HTP is minimal to cause a measurable increase in blood 5HT levels in Wistar rats, while maintaining a physiological proportion between 5HT and 5HIAA content. On the other hand, higher doses of 5HTP (100 mg/kg and above), although effective in rising 5HT blood levels, proved to be neurotoxic for rodents, causing the 5HT syndrome *(30–33)*. We have chosen a dose of 25 mg/kg 5HTP which is reported to be quite effective in raising blood 5HT concentrations in adult rats *(34–37)*. The injections were given subcutaneously in the nape both to gravid dams and pups. This way of drug administration, used to avoid the risk of damaging the fetuses during the prenatal treatment, and to reduce discomfort in the pups, enabled 100% of pup survival during treatment.

The physiological consequences of the perinatal treatment with 5HTP in young rats were evident. Although a similar number of pups were born to each litter in both, 5HTP treated and saline treated dams, pups from the 5HTP treated group had significantly lower body mass at PND1 and significantly lower survival rate. Pups which did not survive were either still born or died within 24 hours after birth. Research on the influence of 5HTP in pregnant rats, showed that an acute dose of 100 mg/kg of 5HTP caused reduced fetal weight and increased fetal reabsorption *(38),* presumably caused by the vasoconstricting effect of serotonin, especially on the umbilical and chorionic arteries. The negative effect of the reduced uteroplacental blood flow on fetal growth has been demonstrated in several studies *(39–41)*. The chronic treatment with 5HTP used in this experiment, although at a much lower dose, could have reduced placental blood flow and induced slower fetal growth and, consequently, lower survival rate. Another possible explanation might be that the increased 5HT concentrations caused by 5HTP treatment have impaired development of serotonergic brain regions, which was reflected in death of some pups and lower birth weight of others.

The influence of the 5HTP treatment on body mass was obvious after weaning. Free-feeding rats from the experimental group gained weight at slower rate than the control rats and retained significantly lower body mass after the wash-out period. This indicates that 5HTP treatment has induced changes in the central serotonergic compartment resulting in reduced food intake or increased metabolic rate. A number of studies reported the influence of centrally and peripherally increased 5HT concentrations on decreased food ingestion, and consequentially, lower body mass in both, rats and humans *(42–47)*.

The return to dam test was conducted in order to determine the level of anxiety produced by the separation of the pups from their mother. Although all pups from both groups returned to their dam within the experimental time, 5HTP treated pups did it significantly faster than the control pups. This might be the result of increased locomotor activity, increased anxiety, or the combination of both. Regarding the first, increased frequencies in basic locomotor patterns have been observed after the acute administration of 55 mg/kg of 5HTP in carbidopa pretreated rats *(48)*. Regarding the second, increased anxiety-like behavior was observed in a rat subline with high platelet 5HT level in comparison to the subline with low platelet 5HT level *(46)*. Also, the reduction in brain 5HT levels induced through tryptophan depletion has been reported to cause panic attacks and anxiety in patients with panic syndrome as well as in healthy subjects *(49, 50)*. Hence, it is possible that the results of our behavioral test represent the consequence of reduced activity and/or number of serotonergic neurons caused by peripheral 5HT increase after 5HTP treatment.

At this point it is hard to distinguish between the indirect 5HTP effects, acting through hyperserotonemia, and direct 5HTP effects acting through the increased 5HT synthesis in the brain, which represents the main limitation of the study. In any case, the brain was exposed to elevated 5HT levels during the development of serotonergic neurons.

In conclusion, we have shown that the perinatal treatment of rats with 25 mg/kg of the serotonin precursor 5HTP has caused physiological disturbances in pups. Differences in body mass between 5HTP and saline treated animals, which remained significant after the wash-out period, indicate that the changes in the central 5HT compartment might have been permanent. Whether the temporary increase in peripheral 5HT concentrations during the critical phase of brain development has left permanent changes in the central serotonergic compartment, and to what extent, will be explored in further studies.

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2.2. Perinatal treatment of rats with MAO inhibitor tranylcypromine (rad 2)

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PERINATAL TREATMENT
of rats with MAO inhibitor tranylcypromine

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Abstract

Serotonin (5-hydroxytryptamine, 5HT) regulates the development of 5HT neurons and target tissues during neurogenesis, while later it assumes the function of a neurotransmitter. Alterations in serotonin neurotransmission are indicated as biological substrates in several neuropsychiatric disorders, including autism. The most consistent 5HT-related finding in autistic disorder is hyperserotonemia, but the mechanism of its development and its relation to central 5HT dysfunction are still unclear. In an attempt to pharmacologically induce hyperserotonemia during the period of most intensive development of 5HT neurons, and to later investigate its effects on central 5HT functions, we have treated rats from gestational day 13 until postnatal day 21 with 2 mg/kg of the non-selective irreversible MAO inhibitor tranylcypromine (TCP). The control group received saline in the same manner. TCP treated rats displayed a long-lasting significant increase in platelet 5HT concentrations compared to the control rats. The TCP treated group had smaller litters, significantly lower pup survival rate, and slower weight gain during the post-weaning free-feeding period than the control group. Pups from the TCP group returned to their dams significantly slower than the control pups suggesting lower separation anxiety. Our results indicate that the perinatal treatment of rats with tranylcypromine has induced both, disregulation of the peripheral 5HT homeostasis and disturbances in central 5HT physiology in pups and young rats. The extent of the changes in the central serotonergic compartment in adult rats will be explored in our further studies.

Keywords

TCP • monoamine oxidase • serotonin • development • platelets • survival • feeding • weight • anxiety

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1. Introduction

Serotonin (5-hydroxytryptamine, 5HT) is present in the mammalian organism both, in the brain and the peripheral tissues. In the brain, it regulates the development of serotonergic neurons and target tissues during neurogenesis [1], while later it assumes the function of a neurotransmitter that controls a broad range of physiological systems [2]. In the periphery, 5HT mediates gastro-intestinal functions and platelet activation [3]. Peripheral 5HT is synthesized in enterochromaffin cells of the intestinal mucosa and released into portal circulation [4]. More than 99% of whole blood serotonin is contained in platelets [5]. Serotonin concentration in platelets is regulated by several elements that control either the 5HT level in plasma through the rate of 5HT synthesis (tryptophan hydroxylase, TPH) and metabolism (monoamine oxidase, MAO) or the rate of its accumulation into (5HT transporter) and release from (5HT2A receptor) the platelets. 5HT pools in the central and the peripheral compartments are separated by the blood-brain barrier and, in each of them, 5HT is synthesized by the action of a different TPH enzyme [6]. On the other hand, proteins that control 5HT function in both compartments are encoded by the same genes, have identical primary structures and follow the same kinetics [7-9].

Alterations in serotonin neurotransmission have been indicated as biological substrates in several neuropsychiatric disorders including autism – a neurodevelopmental syndrome characterized by disturbances in social interactions, language and communication, and by the presence of stereotyped behaviors and interests [10]. The most consistent 5HT-related finding in autistic disorder is hyperserotonemia. For several decades, elevated blood 5HT levels have been found in about one third of autistic patients [5,10], but the mechanism of the observed phenomenon and its relation to central 5HT dysfunction have remained unclear. One explanation lies in possible alterations in the expression of one or more of the 5HT elements that could lead to the dysregulation of 5HT transmission in the brain (affecting so its early development and resulting in autistic behavioral symptoms), while it is at the same time reflected in the periphery as hyperserotonemia [11]. Alternatively, dysregulation of the peripheral 5HT-homeostasis could happen first, leading to the high concentrations of serotonin in blood. During fetal and early post-natal development, before the formation of the blood-brain barrier, these high 5HT levels could inhibit development of 5HT neurons and lead to the anatomical and functional alterations of the brain, characteristic for autism [12]. The inhibitory function of 5HT on the development of serotonergic neurons has been demonstrated on animal models using pharmacological treatment with the 5HT receptor agonist 5-methoxytryptamine [13,14], 5HT precursor tryptophan [15], combination of selective MAOA and MAOB inhibitors [16], and 5HT reuptake inhibitors [17].

In an attempt to pharmacologically induce hyperserotonemia during the period of the most intensive development of 5HT neurons, and to later investigate its effects on the central 5HT functions, we have treated rats with the non-selective irreversible MAO inhibitor tranylcypromine (TCP) from gestational day 13 until postnatal day 21. In this paper we describe the perinatal treatment with TCP that led to

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the elevated platelet 5HT concentrations. We also report the physiological consequences of TCP treatment in young rats, reflected in the number of born and survived pups in each litter, body mass increase over time, and anxiety-like behavior of pups.

2. Experimental Procedures

2.1 Housing and breeding of animals

Five nulliparous Wistar females from the animal facility of the Croatian Institute for Brain Research (University of Zagreb, Zagreb, Croatia), weighing 260-290 g, were used in the experiment. Two were randomly assigned to a "saline group", and three to a "TCP group". Females were mated with males of the same strain and age in 2:1 and 3:1 ratio, respectively. Vaginal smears were taken daily at 10 a.m. to check for the presence of sperm. The day sperm was found in the smear was considered as day 0 of gestation (G0). Body mass was monitored daily and progressive increase during the following week was considered as a confirmation of pregnancy. After gravidity was confirmed in all females, the male was removed from the cage. Females remained together until two days before parturition when they were separated and remained singly housed until weaning of the pups (at postnatal day 21, PND 21). After weaning, animals were kept 3-4 per cage. Females were closely observed during parturition to determine the number of pups born to each litter. Pups were weighted daily during treatment and three times weekly after treatment. Animals were housed in polycarbonate cages under 12-h light:12-h dark conditions at a temperature of 22 ± 2 °C, with free access to rat chow and tap water. The study was approved by the Ethic committee of the Faculty of Science, University of Zagreb, and was conducted in accordance with the Croatian Animal Protection Law ("Narodne novine", 135/2006).

2.2 Pharmacological treatment

The experimental group of pups was treated with TCP (Sigma-Aldrich), from GD 13 until birth by injecting 2 mg/kg of TCP subcutaneously to pregnant females, and from PND1 until PND 21 by receiving subcutaneous injections in the nape at a dose 2 mg/kg. TCP was dissolved in ethanol and saline. Before treatment, the solution was neutralized with HCl and warmed to body temperature. Solutions were delivered in volumes of 1.51 mL per kg of body mass to dams, in volumes of 3.3 mL per kg of body mass to pups until they reached 15g, and in volumes of 5 mL per kg of body mass until the end of treatment on PND21. The control group was treated with saline in the same manner. All injections were performed at 2 pm. A 50 µL glass syringe (Hamilton) with disposable 30G needles (BD, Drogheda, Ireland) were used to treat the pups until they reached a body mass of 15 g, while disposable 0,5 mL plastic syringes with 30G needles (BD Micro-Fine Plus) were used to treat pregnant females and older pups.

2.3 Platelet 5HT concentration

Platelet 5HT concentrations were measured in seven TCP-treated and nine saline-treated rats at the age of 10 weeks. Under light ether narcosis, 1.5 mL of blood was withdrawn from the jugular vein into syringes preloaded with 500 µL of 3.13% trisodium citrate anticoagulant. Animals were sacrificed after blood sampling for the purpose of tissue collection. After thorough mixing, blood samples were transferred to microtubes and centrifuged at 200 x g for 10 min to generate platelet rich plasma. 5HT concentration in platelets was determined using a commercial enzyme immunoassay kit (Serotonin ELISA kit, DRG Instruments GmbH, Germany), according to the kit instructions. A calibration curve was drawn based on the absorbances measured at 450 nm on the microplate reader (P-Lab IASON, Austria) and known concentrations of the standard solutions. Concentration values of samples were obtained by interpolating them to the calibration curve, using the 4-parameters non-linear regression curve fitting. Results were expressed in ng 5HT per mL of platelet rich plasma.

2.4 Behavioral test – Return to dam

The return to dam test was adapted from McNamara et al. [14]. The test was performed on PND 17 in a cage with a dark non translucent wall inserted in the middle. The wall contained a 2.5 x 2.5 cm opening at the bottom with a tunnel-like extension on the mother's side, so the pups could pass but the dam could not reach out for them. A maximum of 5 pups per litter were placed on one side of the wall and the dam on the other. The pups were allowed ten minutes to return to their dam and the time when their hind legs crossed through the opening in the wall onto the mother's side was scored. If a pup did not finish a task within a 10 minute period, a time of 600 seconds was scored.

2.5 Statistical analysis

Data was processed using GraphPad InStat 3.01 software. Normality of distributions of the measured parameters was tested by Kolmogorov/Smirnov method, while the equality of SDs was tested by Bartlett's test. Mean values of normally distributed parameters were compared using unpaired t-test, and of those that were not normally distributed using non-parametric Mann-Whitney test. Statistical significance of difference in survival rate was compared using two-sided Fisher's exact test. The level of significance was set to 0.05. Values in the text were expressed as means ± standard deviations (M ± SD).

3. Results

Several physiological parameters were determined in rats perinatally treated with the MAO inhibitor TCP and compared to those of the saline treated rats (Table 1).

Two dams from the control group gave birth to 10 and 9 pups, respectively, one of which died during the first 24 hrs. Three dams from the TCP treated group gave birth to a total of 18 pups (6 per dam) out of which only 9 survived during the first 24 hrs. The difference between the survival rates of pups from the control and TCP treated groups are shown in Fig 1. The difference was quite significant ($p = 0.003$), with a relative risk of dying for the TCP treated pups being 1.9 (95% CI 1.2-3.0).

Although the mean body mass of the survived pups on PND1 did not significantly differ between the TCP treated (7.9 ± 0.8 g) and the saline treated (7.3 ± 0.6 g) groups ($t = 2.004$, $df = 21$, $p = 0.0581$), weight gain during the

Table 1. Physiological parameters determined in saline- and TCP-treated rats. M – males, F – females; * p<0.05, Mann Whitney test; ** p<0.01, Unpaired t-test; ## p<0.01, Fisher's exact test.

	Saline treated group	TCP treated group
Number of pups born	19	18
Offspring per dam	9.5	6
Number of survived pups	18 (8 M, 10F)	9 (5 M, 4 F)
Litter size per dam	9	4.5 ± 0.5
Survival rate (%)	95	50 ##
Birth weight (g)	7.3 ± 0.8	7.9 ± 0.6
Adult weight (g)	162.0 ± 17.5	150.6 ± 17.4 *
Return to dam time (s)	143 ± 32	452 ± 82 *
Platelet 5HT level (ng/mL)	381 ± 74	612 ± 153 **

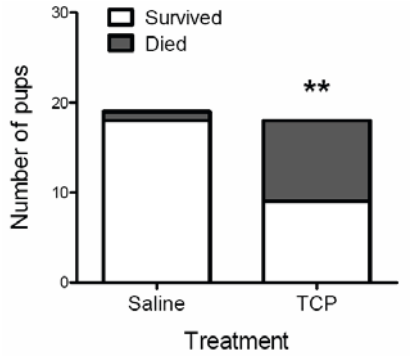

Fig 1. Survival rate of saline- and TCP-treated rats. ** p<0.01, Fisher's exact test.

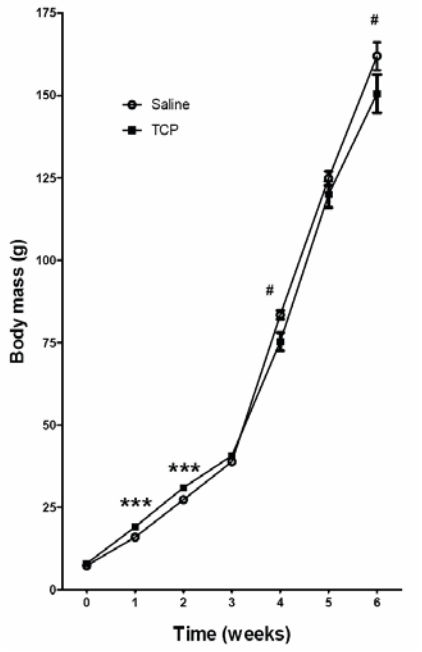

Fig 2. Increase in body mass over time in rats perinatally treated with saline (N=18, circles) or TCP (N=9, squares). Values are expressed as M ± SEM; *** p<0.001, Unpaired t-test; # p<0.05, Mann Whitney test.

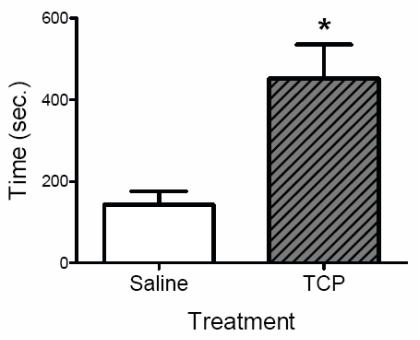

Fig 3. Return to dam test. Bars show time needed for saline (N=18) and TCP (N=9) treated pups to return to their dam after separation. Values are expressed as M ± SEM; * p<0.05, Mann Whitney test.

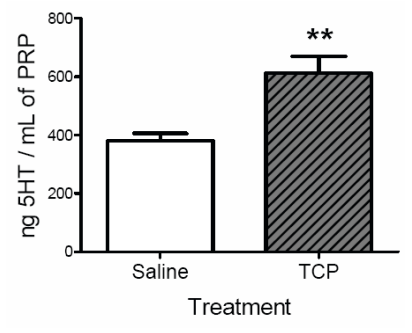

Fig 4. Platelet 5HT concentrations in adult rats perinatally treated with saline (N=9) or TCP (N=7). Values are expressed as M ± SEM; ** p<0.01, Unpaired t-test.

breast-feeding period was more pronounced in the TCP treated group, probably due to much smaller litter sizes (4.5 vs. 9 pups per dam) (Fig 2). However, with the onset of free-feeding, during the third week of age, the average body mass of the saline treated pups reached that of the TCP treated pups (38.87 ± 0.7 g and 40.64 ± 0.9 g on PND21, respectively). After weaning, the body mass of the TCP treated rats increased at a slower rate than the body mass of the saline treated rats, resulting in a significantly lower weight at the age of six weeks (150.6 ± 17.4 g and 162.0 ± 17.5 g at PND 44, respectively; U = 39, p = 0.0461).

On PND 17, possible differences in anxiety-like behavior between the saline and TCP treated pups were determined by the return-to-dam test (Fig 3). While the saline treated pups needed 143 ± 32 s on average to return to their mothers, the TCP treated pups performed the given task significantly slower needing on average 452 ± 82 s (U = 34, p = 0.0168). Five out of nine TCP treated pups did not return to their dam in the given time period.

Finally, in order to determine whether the perinatal TCP treatment caused long-lasting/permanent hyperserotonemia in the experimental group, platelet 5HT concentrations were measured in rats from both groups at the age of 10 weeks (Fig 4). Rats from the TCP treated group had significantly elevated platelet 5HT concentrations in comparison to those of the saline treated group, mean values being 612 ± 153 ng/mL and 381 ± 74 ng/mL, respectively (t=4.003, df=14, p=0.0013).

4. Discussion

The aim of the perinatal treatment with TCP was to induce hiperserotonemia through inhibition of 5HT catabolism, during the most intensive phase of development of serotonergic neurons (from gestational day 13 until post natal day 21).

Isoenzymes MAOA and MAOB, located on the outer mitochondrial membranes of neural and peripheral cells, catalyze oxidative deamination - an essential step in the catabolism of exogenous amines and monoamine neurotransmitters, including serotonin [18]. Although the izoenzymes have different substrate affinities under normal physiological

conditions (MAOA preferentially oxidizes serotonin and norepinephrine, MAOB preferentially oxidizes phenylethylamine, while dopamine and tyramine represent substrates for both isoenzymes), both can catabolize the same compounds and are able to take over when the function of the other is compromised through pharmacological inhibition. Accordingly, more pronounced effects on rat 5HT metabolism were observed after the inhibition of both isoforms than after the sole inhibition of MAO A [19-22].

Tranylcypromine is an irreversible MAO A and MAO B inhibitor which inhibits the oxidation of 5HT, norepinephrine and dopamine [23]. In rats, significant effects of TCP on 5HT metabolism in the brain and the periphery was measured as a reduction in MAO A activity [19], an increase in 5HT concentrations [20,24-26], and a decrease in 5-hydroxyindolacetic acid levels [19,25]. The above mentioned effects were obtained in adult rats after acute or chronic administration of TCP intraperitoneally at the doses of 0.5 mg/kg – 15 mg/kg. Hyperactivity was observed after doses of 20mg/kg and above [27,28], while the serotonin syndrome was reported only after combining TCP with other 5HT agonists [22,29].

For the purpose of this study, we chose a dose of 2mg/kg, which was expected to effectively block most of the 5HT degradation. The injections were given subcutaneously in the nape both to gravid dams and pups. This way of drug administration was used to avoid the risk of damaging the fetuses during the prenatal treatment. It also reduced discomfort and damage in the pups and enabled 100% of pup survival during treatment. Adult rats perinatally treated with TCP in the above described manner displayed a significant increase in platelet 5HT concentrations compared to the control group (161% of the control mean value). Although TCP-induced hyperserotonemia was expected in the experimental group during the treatment, significantly elevated 5HT levels observed seven weeks after the cessation of the treatment were somewhat surprising and indicate a long-lasting, if not permanent, 5HT disregulation in the periphery.

A few additional physiological consequences of the TCP treatment were also observed in the experimental group. Since the peripheral administration of TCP was shown to inhibit MAO activity both, in the periphery and in the brain [19], it is hard to distinguish between the direct TCP effects acting through decreased 5HT degradation in the brain, and the indirect TCP effects acting through hyperserotonemia. In any case, the brain of the rats from the experimental group was exposed to elevated 5HT levels during development.

The TCP treated group had smaller litters and significantly lower pup survival rate. Pups which did not survive were either still born or died within 24 hours after birth. In literature, we did not find data on the influence of TCP on pregnancy in either rats or humans. Higher perinatal mortality was reported, however, in mice perinatally treated with MAO A inhibitor clorgyline [30]. The increase in 5HT (and other monoamine) concentrations, caused by TCP treatment, might have impaired brain development, which could have been reflected in the death of some late fetuses and newborn pups. Besides the possible central effects of TCP, the high mortality rate in fetuses and newborns might be due to the vasoconstricting effect of the peripherally increased 5HT levels, especially on the umbilical and chorionic arteries. The negative effect of the reduced uteroplacental blood flow on fetal growth has been demonstrated in several studies [31-33].

The TCP treatment also affected the body mass of the young rats. Although the weight of the TCP treated pre-weanlings was larger than that of the saline-treated pups (probably due to smaller litter sizes in the experimental group), post-weaning free-feeding rats from the experimental group gained weight at slower rate than the control rats and retained significantly lower body mass after a wash-out period and into the adulthood (data not shown). This indicates that TCP treatment might have induced changes in the central serotonergic compartment resulting in reduced food intake and/or increased metabolic rate. A number of studies reported the influence of centrally and peripherally increased 5HT concentrations on decrease in food ingestion, and consequentially, lower body mass in rats and humans [34-38]. Loss of body weight was reported after TCP treatment of rabbits [39].

The return to dam test was conducted in order to determine the level of anxiety produced by the separation of the pups from their mother. There were significant differences in the time it took the pups to return to their dam. Overall, the saline treated pups returned to their dams faster than the TCP treated pups, and most of the TCP treated pups did not return to their dam at all in the allotted time. This might be the result of decreased locomotor activity or decreased separation anxiety. The reported evidence of a significant increase in rat motor activity after single doses of 1 – 15 mg/kg TCP [24,40] makes the first possibility less likely. On the other hand, complete MAO inhibition with tranylcypromine or phenelzine was reported to reduce anxiety after acute treatment in adult rats [41]. TCP is also considered effective in reducing social phobia and anxiety in humans [42,43].

In conclusion, we have shown that the perinatal treatment of rats with 2 mg/kg of the monoamine oxidase inhibitor tranylcypromine has induced both, disregulation of the peripheral 5HT homeostasis reflected in significantly increased blood 5HT levels, and disturbances in the central 5HT physiology reflected in lower perinatal survival rate, lower separation anxiety in pups, and lower body mass increase in young rats after weaning. The extent of the changes in the central serotonergic compartment in adult rats will be explored at the molecular, neurochemical and behavioral levels in our further studies.

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2.3. The effects of the perinatal treatment with 5-hydroxytryptophan or tranylcypromine on the peripheral and central serotonin homeostasis in adult rats

(rad 3)

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The effects of the perinatal treatment with 5-hydroxytryptophan or tranylcypromine on the peripheral and central serotonin homeostasis in adult rats

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ABSTRACT

Serotonin (5HT) is a biologically active amine present in mammals in the brain and the peripheral tissues. Autism is a neurodevelopmental disorder in which 5HT homeostasis is disturbed both centrally and peripherally, but the relationship between the 5HT disturbances in the two compartments is not understood. In an attempt to explore the relationship between the disturbed peripheral 5HT homeostasis and central 5HT functioning, we exposed the developing rat brain to increased 5HT concentrations, by treatment of rats with subcutaneous injections of the immediate 5HT precursor 5-hydroxy-L-tryptophan (5HTP, 25 mg/kg), or the non-selective MAO inhibitor tranylcypromine (TCP, 2 mg/kg), during the period of the most intensive development of 5HT neurons – from gestational day 13 to post-natal day 21. The effects of the mentioned treatments on peripheral and central 5HT levels were then studied in adult rats. Platelet and plasma 5HT concentrations (measured by ELISA), as well as cortical and midbrain 5HT, tryptophan and 5-hydroxyindoleacetic acid levels (measured by HPLC) were determined in twelve 5HTP treated and eight TCP treated rats, and compared with the values measured in 10 control, saline treated rats. Treatment with 5HTP significantly raised peripheral but not central 5HT concentrations. At adult age, peripheral 5HT homeostasis was re-established, while modest decrease in 5HT concentration was observed in frontal cortex, presumably due to hyperserotonemia-induced loss of 5HT terminals during brain development. Treatment with TCP induced significant 5HT elevations in both compartments. At adult age, permanent changes in 5HT homeostasis were observed, both peripherally (as hyperserotonemia) and centrally (as altered 5HT metabolism with decreased 5HT concentrations). Further studies are planned in order to explore the nature of the different disturbances of 5HT homeostasis induced by the two compounds, and their results are expected to shed some light on the role of hyperserotonemia in autism.

1. Introduction

Serotonin (5-hydroxytryptamine, 5HT) is a biologically active amine present in mammals both, in the brain and the peripheral tissues.

In the periphery, 5HT mediates cardiovascular and gastrointestinal functions and platelet activation (Berger et al., 2009). Peripheral 5HT is synthesized from the amino acid tryptophan (Trp) mostly in enterochromaffin cells of the intestinal mucosa, by the action of tryptophan hydroxylase (Tph) and aromatic L-amino acid decarboxylase (Racke et al., 1995). 5HT is released into portal circulation in a receptor-mediated manner and actively accumulated into platelets via the serotonin transporter. The amine is catabolized primarily in lungs and liver by the action of the mitochondrial enzyme monoamine oxidase (MAO) into 5-hydroxyindoleacetic acid (5HIAA) and eliminated through kidneys. More than 99% of whole blood serotonin is contained in platelets in which its concentration is regulated either by the rate of peripheral 5HT synthesis and metabolism (i.e. its concentration in blood plasma), or the rate of its accumulation into- and release from the platelets (Cook and Leventhal, 1996).

In the brain, 5HT synthesizing neurons are located in discrete regions of the brain stem – *raphe nuclei*, but project their axons into various cortical and subcortical regions of the brain (Benes et al.,

Abbreviations: 5HT, 5-hydroxytryptamine; Trp, tryptophan; Tph, tryptophan hydroxylase; MAO, monoamine oxidase; 5HIAA, 5-hydroxyindoleacetic acid; 5HTP, 5-hydroxytryptophan; TCP, tranylcypromine; GD, gestational day; PND, post-natal day; WB, whole blood; PRP, platelet-rich plasma; PFP, platelet-free plasma; RN, raphe nuclei; FC, frontal cortex.

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2000). In the developing brain, 5HT regulates serotonergic outgrowth and maturation of target regions (Whitaker-Azmitia, 2001), while later it assumes the role of a neurotransmitter modulating function and plasticity of the adult brain (Catalano, 2001; Lesch, 2001).

Central and peripheral 5HT compartments are two separated, yet closely related entities. On the one hand, enterochromaffin and neural cells use different Tph enzymes (Tph1 and Tph2, respectively) to synthesize 5HT (Walther et al., 2003), and the blood-brain barrier prevents serotonin entering from one compartment into the other. On the other hand, proteins that control 5HT function in both compartments are encoded by the same genes, have identical primary structures and follow the same kinetics (Chen et al., 1993; Lesch et al., 1993; Cook et al., 1994). Moreover, during the fetal and early postnatal development of the brain, the blood-brain barrier is not formed and the two compartments can freely communicate (Davies et al., 1996).

A disorder in which 5HT homeostasis seems to be disturbed both centrally and peripherally is autism, a neurodevelopmental syndrome with onset in early childhood, characterized by impairment in social interaction and communication, and by the presence of restricted and repetitive behaviors and interests (Owley et al., 2003). Elevated blood 5HT levels (hyperserotonemia) have been found in about one third of autistic patients of different ethnic and age groups (Cook and Leventhal, 1996; Owley et al., 2003; Mulder et al., 2004; Hranilovic et al., 2007). On the other hand, brain serotonin synthesis was found to be decreased in the cortex and thalamus of autistic children (Chugani et al., 1997). Despite decades of research, the mechanism of hyperserotonemia and its relation to central 5HT dysfunction are not fully understood. One explanation lies in possible alterations in the expression of one or more of the 5HT elements that could lead to the dysregulation of 5HT transmission in the brain (affecting so its early development and resulting in autistic behavioral symptoms), while it is at the same time reflected in the periphery as hyperserotonemia (Janusonis, 2005). Alternatively, dysregulation of the peripheral 5HT-homeostasis could happen first, leading to high concentrations of serotonin in blood. During fetal and early post-natal development, before the formation of the blood-brain barrier, these high 5HT levels could inhibit the development of 5HT neurons and lead to the anatomic and functional alterations of the brain, characteristic for autism (Whitaker-Azmitia, 2005).

We have recently started studies on the relationship between the disturbed peripheral 5HT homeostasis and central 5HT functioning on an animal model by pharmacologically inducing hyperserotonemia during the period of most intensive development of the 5HT neurons. For that purpose Wistar rats were treated subcutaneously with either the immediate 5HT precursor 5-hydroxytryptophan (5HTP, 25 mg/kg), or the non-selective MAO inhibitor tranylcypromine (TCP, 2 mg/kg), from gestational day (GD) 13 to post-natal day (PND) 21. In this study we report the effects of the mentioned perinatal treatments on peripheral and central 5HT levels in adult rats. Platelet and plasma 5HT concentrations, as well as cortical and midbrain 5HT, Trp and 5HIAA levels were measured in twelve 5HTP treated and eight TCP treated rats, and compared with the values measured in 10 control rats perinatally treated with saline.

2. Materials and methods

2.1. Breeding and housing of animals

Breeding procedure has been described in detail elsewhere (Blazevic et al., 2010). In short, eight nulliparous Wistar females from the animal facility of the Croatian Institute for Brain Research (University of Zagreb, Zagreb, Croatia), weighing 260–290 g, were randomly assigned to a "saline" (two), "5HTP" (three), or "TCP" (three) group, and mated with males of the same strain and age in 2:1 or 3:1 ratio, respectively. After gravidity was confirmed in all females, the male was removed from the cage. Females remained together until 2 days before parturition when they were separated and remained singly housed until weaning of the pups (at PND 21). After weaning, animals were kept 3–4 per cage. Animals were housed in polycarbonate cages under 12 h light:12 h dark conditions at a temperature of 22 ± 2 °C, with free access to rat chow and tap water.

All efforts were made to reduce the number of animals used and to minimize animal suffering. The study was approved by the Ethics committee of the University of Zagreb, and was conducted in accordance with the European Communities Council Directive (86/609/EEC) and the Croatian Animal Protection Law ("Narodne novine", 135/2006).

2.2. Pharmacological treatments

The experimental groups of pups were treated either with 2 mg/kg tranylcypromine (Sigma–Aldrich), or with 25 mg/kg of 5-hydroxy-L-tryptophan (Sigma–Aldrich), from GD 13 until birth by subcutaneous injections to pregnant females, and from PND1 until PND 21 by receiving subcutaneous injections of the same doses. 5HTP was dissolved in acidified saline. Before treatment, the solution was neutralized with NaOH and warmed to body temperature. TCP was dissolved in ethanol and saline. Before treatment, the solution was neutralized with HCl and warmed to body temperature. Solutions were delivered in volumes of 1.51 mL per kg of body mass to dams, in volumes of 3.3 mL per kg of body mass to pups until they reached 15 g, and in volumes of 5 mL per kg of body mass until the end of treatment. The control group was treated with saline in the same manner. All injections were performed between 2 and 3 pm. A 50 μL glass syringe (Hamilton) with disposable 30G needles (BD, Drogheda, Ireland) were used to treat the pups until they reached a body mass of 15 g, while disposable 0.5 mL plastic syringes with 30G needles (BD Micro-Fine Plus) were used to treat pregnant females and older pups.

2.3. Collection of tissue samples

Blood and brain samples were collected from 5 saline, five 5HTP and 3 TCP treated pups at the end of treatment (on PND 22), and from 10 saline (5 males, 5 females), twelve 5HTP (6 males, 6 females) and 8 TCP (4 males, 4 females) treated adult rats (on PND 70).

Under light ether narcosis, 800 μL of pup blood or 1.5 mL of adult blood was withdrawn from the jugular vein into syringes preloaded with 200 or 500 μL of 3.13% trisodium citrate anticoagulant, respectively. Animals were then decapitated and brains were removed from the sculls and briefly frozen at −20 °C in a freezer. A midbrain region containing serotonergic cell bodies of the dorsal and median raphe nuclei was obtained by a 3 mm thick coronal brain slice (plates 43 and 55 in the rat brain atlas, Paxinos and Watson, 2007), followed by a 3 mm diameter punch into the midbrain area. A 4 mm coronal cut was then made at the frontal lobes (plate 11) and cortex (all cortical areas anterior to bregma + 1.7 mm) was peeled off. Samples were weighted and frozen at −80 °C for later analysis.

2.4. Measurement of the peripheral 5HT concentrations

After thorough mixing, samples were transferred from syringes into microtubes. 5HT concentrations in whole blood (WB) of pups or in platelet-rich plasma (PRP) and platelet-free plasma (PFP) of

adult rats were determined using a commercial enzyme immunoassay kit (Serotonin ELISA kit, DRG Instruments GmbH, Germany), according to the kit instructions. A calibration curve was drawn based on the absorbances measured at 450 nm on the microplate reader (P-Lab IASON, Austria) and known concentrations of the standard solutions. Concentration values of samples were obtained by interpolating them to the calibration curve, using the 4-parameters non-linear regression curve fitting. Results were expressed in ng 5HT per mL of WB, PRP or PFP.

2.5. Measurement of brain Trp, 5HT and 5HIAA concentrations

The frozen samples were thawed and homogenized in 5 vol (w/v) of a solution of 0.1 M perchloric acid containing 0.2 mM EDTA and 0.4 mM $Na_2S_2O_5$. Tissue homogenates were then centrifuged at 14000*g* for 15 min at 0–4 °C, and aliquots of the clear supernatant were used for the high performance liquid chromatography analysis with electrochemical detection (HPLC-ED).

The HPLC system consisted of a delivery pump (Agilent 1100 Series, Agilent Technologies, U.S.A.), a sample injector (Rheodyne 7125, U.S.A.), a C18 reverse phase column (Agilent Technologies Zorbax SB-C8, 75 × 4.6 mm, 5 μm particle size), and a Guard column (4 × 4 mm, Agilent Technologies, U.S.A.). An electrochemical detector (HP-ED 1094A, Hewlett–Packard, U.S.A.) with a glassy carbon electrode was used at −0.55 V versus the reference electrode. All chromatograms were recorded and analyzed using the HPCore ChemStation Software.

Concentrations were determined from peak areas against external standards. The mobile phase contained 0.1 M Na_2HPO_4, 0.05 M citric acid, 5% methanol (v/v), 0.1 mM EDTA, and 1 mM KCl at pH 4.5. The flow rate was maintained at 0.8 mL per min at a pressure of 120 bars. Tissue concentrations of 5HT and 5HIAA were expressed as ng, and that of Trp as μg of substance per g of wet tissue.

2.6. Statistical analyses

Data were processed by the use of GraphPad InStat 3.01 software. Normality of distributions of the measured parameters was tested by Kolmogorov/Smirnov method. Mean values of normally distributed parameters were compared using unpaired *t*-test or one-way analysis of variance (ANOVA) with Tukey's post-test. Mean values of parameters that were not normally distributed were compared using Mann–Whitney test or using non-parametric Kruskal–Wallis method with Dunn's post-test. The level of significance was set to 0.05. The values were expressed as means (M) ± standard error of means (SEM).

3. Results

Direct effects of chronic treatment with 25 mg/kg 5HTP or with 2 mg/kg TCP on blood and brain 5HT levels were measured in pups at the end of treatment (Fig. 1). Both substances efficiently caused hyperserotonemia (KW = 8.65, $p = 0.003$), with 5HT concentrations being 874 ± 168 ng/mL in 5HTP treated and 669 ± 34.5 ng/mL in TCP treated pups, compared to 491 ± 59.5 ng/mL in saline treated pups. The effect of treatment on 5HT levels in frontal cortex was also significant (KW = 6.96, $p = 0.017$), although only TCP (917 ± 32.2 ng/g), and not 5HTP (393 ± 34.2 ng/g) caused considerable raise in 5HT concentrations compared to saline (346 ± 32.4 ng/g).

In order to check whether the perinatal treatment with 5HTP or TCP has permanently affected the peripheral 5HT homeostasis, 5HT concentrations in both PFP and PRP were measured in adult rats. One sample from the TCP treated group and one sample from the saline treated group were lost during processing. 5HT concentrations in PFP did not differ between male and female rats ($t = 0.011$, $p = 0.991$, 26 d.f.), and those in PRP were only indicatively higher in females (Welch corrected $t = 1.995$, $p = 0.061$, 19 d.f.). This allowed the results from males and females to be analyzed jointly (Fig. 2).

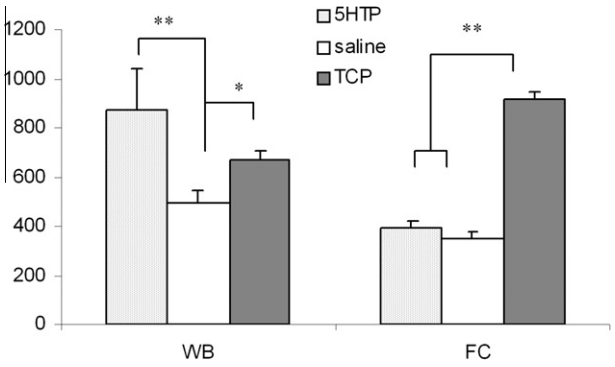

Fig. 1. The direct effects of chronic treatment with 25 mg/kg 5-hydroxytryptophan (5HTP) or 2 mg/kg tranylcypromine (TCP) on 5HT concentrations in whole blood expressed as ng 5HT per mL of blood (WB), and in frontal cortex expressed as ng 5HT per g of wet tissue (FC). $N = 5$ in saline and 5HTP treated, and $N = 3$ in TCP treated group. Values are expressed as M ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$; Dunn's multiple comparison after Kruskal–Wallis test.

The effect of treatment on 5HT concentrations was significant in both PFP ($F_{(2,27)} = 5.99$, $p = 0.008$) and PRP ($F_{(2,27)} = 6.89$, $p = 0.004$). Post-hoc analysis revealed significantly higher mean 5HT concentration in PRP of TCP treated rats (612 ± 57.8 ng/mL) than in PRP of saline treated (381 ± 24.6 ng/mL) or 5HTP treated (432 ± 42.6) animals, indicating the persistence of hyperserotonemia at adult age (Fig. 2B). On the other hand, mean 5HT concentrations in PFP of animals from 5HTP treated (5.08 ± 0.83 ng/mL) and TCP treated (3.92 ± 0.14 ng/mL) rats did not significantly differ from that of the control animals (4.67 ± 0.24 ng/mL), although they differed from each other (Fig. 2A).

We further searched for possible effects of the perinatal treatment with 5HTP or TCP on 5HT metabolism in the brain of adult rats by measuring 5HT, Trp and 5HIAA concentrations and their ratios in midbrain raphe region and frontal cortex. There were no gender influence on any of the measured parameters in either raphe region ($t = 0.186$, $p = 0.854$ for 5HT, $t = 0.252$, $p = 0.803$ for Trp, and $t = 0.641$, $p = 0.527$ for HIAA) or frontal cortex ($t = 0.260$, $p = 0.797$ for 5HT, $t = 0.554$, $p = 0.584$ for Trp, $t = 0.025$, $p = 0.980$ for 5HIAA) in an integral sample of ten 5HTP treated, 10 saline treated and 8 TCP treated adult rats (26 d.f.). Therefore, results obtained in males and females were pooled together for the analyses (Fig. 3).

There was a strong influence of treatment on 5HT concentration (Fig 3A) in both, the raphe region ($F_{(2,27)} = 73.69$, $p < 0.0001$) and frontal cortex ($F_{(2,27)} = 90.34$, $p < 0.0001$). While the mean 5HT levels of the TCP treated animals were markedly decreased compared to the controls in both regions (74.4 ± 6.11 ng/g vs. 306 ± 17.3 ng/g in RN, and 48.1 ± 1.20 ng/g vs. 185 ± 7.11 in FC), 5HTP treatment seemed to significantly lower only 5HT levels in the frontal cortex (155 ± 9.23 ng/g), but not in the raphe region (309 ± 16.2 ng/g).

The mean concentration of the 5HT precursor tryptophan (Fig 3B) was markedly increased in the raphe region of the TCP treated animals (18.9 ± 2.76 μg/g) in comparison to the saline treated (4.98 ± 0.70 μg/g) and 5HTP treated (4.39 ± 0.69 μg/g) rats (KW = 16.74, $p = 0.0002$). No significant differences among the mean values of Trp concentrations of 5HTP treated (4.29 ± 0.50 μg/g), saline treated (5.32 ± 0.63 μg/g) and TCP treated (6.32 ± 0.60 μg/g) rats were found in frontal cortices (KW = 5.70, $p = 0.058$).

The mean concentrations of the main 5HT metabolite 5HIAA (Fig 3C) were similar in raphe regions (663 ± 45.6 ng/g for 5HTP

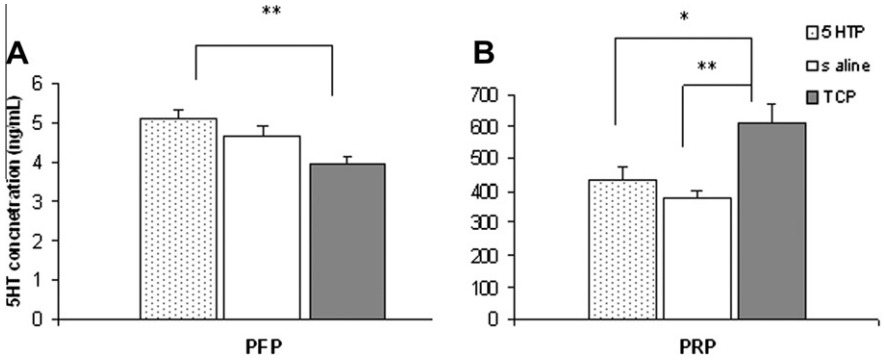

Fig. 2. Permanent effects of perinatal treatment with 5-hydroxytryptophan (5HTP) or tranylcypromine (TCP) on 5HT concentrations in (A) platelet-free plasma (PFP) and (B) platelet-rich plasma (PRP). $N = 12$ in 5HTP treated, $N = 9$ in saline treated, and $N = 7$ in TCP treated group. Values are expressed as M ± SEM. $^{**}p < 0.01$, $^{*}p < 0.05$, Tukey–Kramer multiple comparisons after one-way ANOVA.

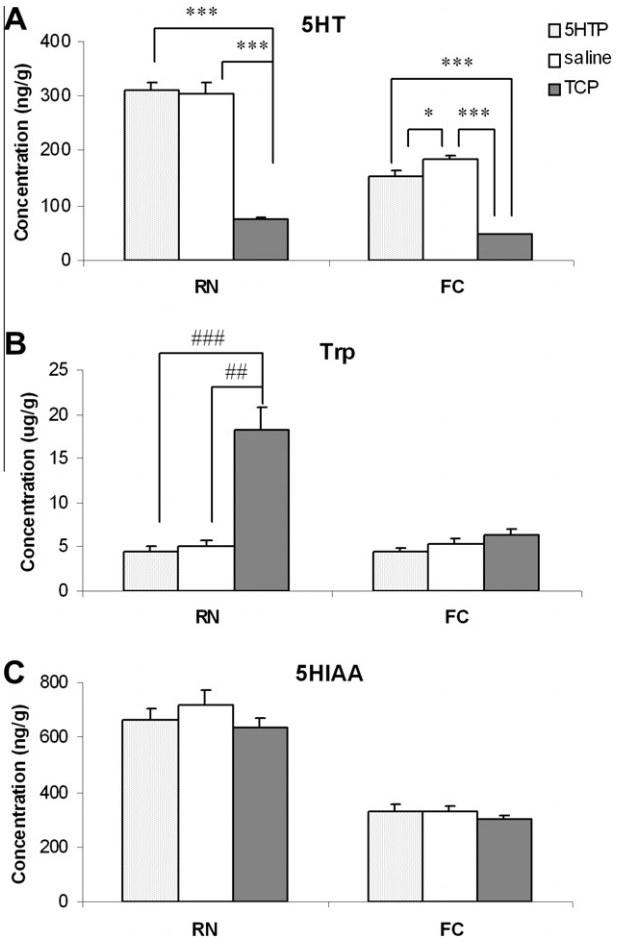

Fig. 3. Concentrations of (A) serotonin (5HT), (B) tryptophan (Trp), and (C) 5-hydroxyindol acetic acid (5HIAA) in midbrain raphe nuclei (RN) and frontal cortices (FC) of 5-hydroxytryptophan (5HTP) treated ($N = 10$), saline treated ($N = 10$) and tranylcypromine (TCP) treated ($N = 8$) rats at PND 70. Values are expressed as M ± SEM. $^{*}p < 0.05$, $^{***}p < 0.001$, Tukey–Kramer multiple comparison after one-way ANOVA. $\#\#p < 0.01$, $\#\#\#p < 0.001$, Dunn's multiple comparison after Kruskal–Wallis test.

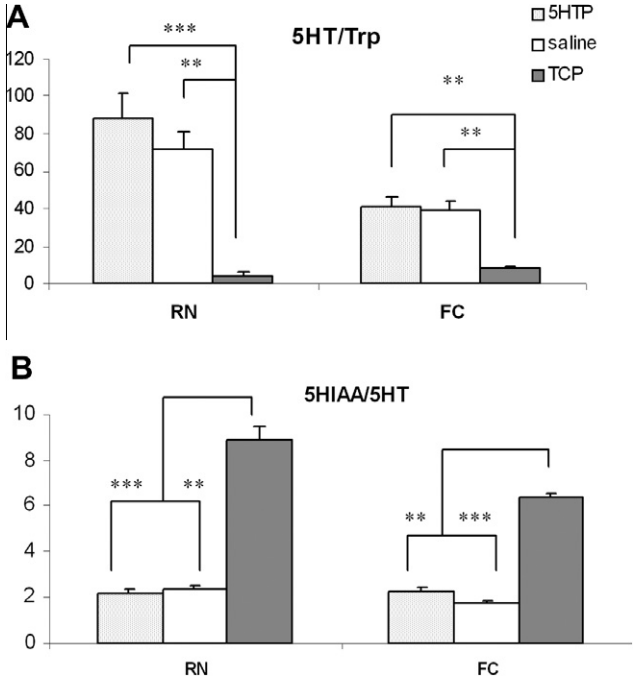

Fig. 4. (A) 5HT/Trp ratio ($\times 10^{-3}$), and (B) 5HIAA/5HT ratio in midbrain raphe nuclei (RN) and frontal cortices (FC) of 5-hydroxytryptophan (5HTP) treated ($N = 10$), saline treated ($N = 10$) and tranylcypromine (TCP) treated ($N = 8$) rats at PND 70. Values are expressed as M ± SEM. $^{**}p < 0.01$, $^{***}p < 0.001$, Dunn's multiple comparison after Kruskal–Wallis test.

treated, 716 ± 60.3 ng/g for saline treated, and 638 ± 36.0 ng/g for TCP treated animals) as well as in frontal cortices (330 ± 21.8 ng/g for 5HTP treated, 328 ± 18.5 ng/g for saline treated, and 303 ± 11.9 ng/g for TCP treated animals) of the investigated groups ($F_{(2,27)} = 0.625$, $p = 0.543$, and $F_{(2,27)} = 0.602$, $p = 0.556$, respectively).

Finally, we determined the 5HT/Trp ratio, which could indicate the activity of Tph, and the 5HIAA/5HT ratio, which indicates the rate of 5HT degradation, in both brain regions (Fig. 4). 5HT/Trp ratio (Fig 4A) was significantly influenced by the treatment in both the raphe region (KW = 16.7, $p = 0.0002$) and the frontal cortex (KW = 16.6, $p = 0.0003$). Post-hoc analysis revealed no significant differences in the 5HT/Trp ratio between 5HTP treated and saline treated animals in either the raphe region ($87.6 \times 10^{-3} \pm 14.3 \times 10^{-3}$ and $71.9 \times 10^{-3} \pm 9.22 \times 10^{-3}$, respectively), or the frontal cortex ($40.9 \times 10^{-3} \pm 5.53 \times 10^{-3}$ and $39.5 \times 10^{-3} \pm 4.77 \times 10^{-3}$, respectively). On the other hand, the 5HT/Trp ratio of TCP treated animals was markedly reduced in both regions ($4.87 \times 10^{-3} \pm 1.10 \times 10^{-3}$ in RN and $8.09 \times 10^{-3} \pm 1.08 \times 10^{-3}$ in FC). The rate of 5HT degradation (Fig 4B) was also significantly influenced by treatment in both brain regions (KW = 16.98, $p = 0.0002$ for RN and KW = 17.86, $p = 0.0001$ for FC). The observed significance was the result of considerable increase in the 5HIAA/5HT ratio of the TCP treated rats in comparison with 5HTP treated

and saline treated rats in both, the raphe region (8.83 ± 0.64 vs. 2.18 ± 0.16 and 2.35 ± 0.16, respectively) and frontal cortex (6.31 ± 0.21 vs. 2.21 ± 0.20 and 1.78 ± 0.08, respectively).

4. Discussion

The aim of our study was to investigate in which way does the perinatal exposure to elevated 5HT levels influence peripheral and central 5HT homeostasis in adult rats. Two different 5HT enhancers were used for this purpose: the immediate 5HT precursor, which affects only the 5HT system and elevates 5HT levels without acting directly on the key regulators of serotonergic transmission (rate-limiting enzymes, transporters, receptors), and a non-selective MAO inhibitor, which inhibits the main enzyme involved in 5HT degradation and also affects other monoaminergic systems.

5HTP is the intermediate in the synthesis of serotonin from its precursor tryptophan. Unlike Trp which is an essential amino acid with many functions in the body (mainly a precursor in protein synthesis), 5HTP is only found in the serotonin synthesis pathway and is quantitatively converted to 5HT (Udenfriend et al., 1957; Magnussen and Nielsen-Kudsk, 1980; Birdsall, 1998). The advantage of using 5HTP over 5HT itself, is that it readily crosses the placental barrier (Birdsall, 1998), which is crucial for the prenatal part of the treatment. Administration of 5HTP allowed us to elude the rate-limiting step in the synthesis of serotonin and to mimic the effect of increased serotonin synthesis through a chosen 5HTP dose. According to our experience with adult rats, we have chosen a dose of 25 mg/kg 5HTP which was quite effective in raising blood 5HT concentrations, while causing only a slight reduction in body weight and no signs of brain toxicity, during the two weeks of treatment (Jernej and Cicin-Sain, 1986).

TCP is an irreversible MAO A and MAO B inhibitor which inhibits oxidative deamination – an essential step in the catabolism of exogenous amines and monoamine neurotransmitters, including serotonin (Billett, 2004; Frieling and Bleich, 2006). Although the two isoenzymes have different substrate affinities under normal physiologic conditions (MAO A preferentially oxidizes serotonin and norepinephrine, MAO B preferentially oxidizes phenylethylamine, while dopamine and tyramine represent substrates for both isoenzymes), both can catabolize the same compounds and are able to take over when the function of the other is compromised through pharmacological inhibition. Accordingly, more pronounced effects on rat 5HT metabolism were observed after the inhibition of both isoforms than after the sole inhibition of MAO A (Johnston, 1968; Green and Youdim, 1975; Sleight et al., 1988; Celada and Artigas, 1993). Significant effects of TCP were measured in the brain and the periphery of adult rats, after acute or chronic administration at doses of 0.5–15 mg/kg, as a reduction in MAO A activity (Celada and Artigas, 1993), an increase in 5HT concentrations (Green and Youdim, 1975; McKim et al., 1983; Malyszko et al., 1993; Ferrer and Artigas, 1994), or a decrease in 5-hydroxyindolacetic acid levels (Celada and Artigas, 1993; Malyszko et al., 1993). We have chosen a dose of 2 mg/kg, which was expected to effectively block most, but not all of the 5HT degradation.

4.1. The effects of 5HTP

Several pups were sacrificed at the end of treatment to check for the direct effects of 5HTP on blood and brain 5HT concentrations. Although transient increases in 5HTP, 5HT and 5HIAA content were reported in rat serotonergic neurons after a single oral or intraperitoneal dose of 5HTP (Sémont et al., 2000; Lynn-Bullock et al., 2004), it seems that the chronic treatment with 5HTP used in our experiment significantly raised 5HT levels only in blood but not in the frontal cortex. It is possible that, at the administered way and dose, 5HTP was more efficiently converted to 5HT and/or stored in the periphery than in the brain, or that the neuronal compensatory mechanisms were much more efficient in compensating for the excess of newly synthesized 5HT than the peripheral ones. In any case, given the results of its direct effects, we could assume that, under our experimental conditions, the long lasting effects of 5HTP in the adult brain would primarily result from hyperserotonemia during the perinatal period, and to a lesser extent from the increased brain 5HT levels. This condition corresponds to the theory that the excessive 5HT, which causes alterations in brain development, originates from blood.

The effect of 5HTP on blood 5HT levels seemed to be only temporary as the peripheral 5HT homeostasis was established at adult age (presumably after a wash-out period).

Perinatal administration of 5HTP did not seem to affect 5HT levels or metabolism in the serotonergic cell bodies of the adult rats. However, in the region of serotonergic terminals there was modest but significant decrease in 5HT concentration without any changes in 5HT synthesis or degradation rate. This indicates intact expression/activity of the 5HT metabolizing enzymes but suggests possible reduction in number of serotonergic terminals in frontal cortex. This would be in line with the reported inhibitory effects of serotonin on 5HT terminal outgrowth in tissue culture (Whitaker-Azmitia and Azmitia, 1986) as well as on animal models using pharmacological treatment with the 5HT receptor agonist 5-methoxytryptamine (Shemer et al., 1991), 5HT precursor tryptophan (Huether et al., 1992), combination of selective MAOA and MAOB inhibitors (Whitaker-Azmitia et al., 1994), and 5HT reuptake inhibitors (Cabrera-vera et al., 1997).

4.2. The effects of TCP

Measurements of blood and cortical 5HT levels at the end of treatment revealed that TCP, besides inducing hyperserotonemia in the periphery, significantly raised 5HT concentration in the brain, due to efficient inhibition of MAO isoenzymes in both 5HT compartments. This is in line with the findings of Ferrer and Artigas (1994) that chronic treatment with low doses of tranylcypromine increases extracellular 5HT concentration in frontal cortex and dorsal raphe nuclei. Therefore, it would be expected that the developing brain of a TCP treated animal would be exposed to the high levels of 5HT not only from blood but from serotonergic neurons as well. This situation complies more with the theory of simultaneous 5HT dysregulation in the brain and the periphery as a neurobiological basis of autism.

TCP treated animals remained hyperserotonemic at adult age. It is important to note that although a mean 5HT concentration in platelets was increased by about 60%, concentration of 5HT in plasma was indicatively lower than that of the control animals. The lack of parallelism between plasma and platelet 5HT changes point to a long-lasting (or permanent) increase in activity/expression of the 5HT transporter on platelet membranes (Anderson et al., 1987) which probably happened during treatment to compensate for the excess of 5HT left in circulation after the inhibition of the degrading enzyme.

The effect of TCP on brain 5HT levels and metabolism in adult animals was impressive. Compared to the control animals, 5HT concentration and 5HT/Trp ratio in both, raphe nuclei and frontal cortex were significantly decreased while 5HIAA/5HT ratio was significantly increased. In addition, in the raphe region there was an almost fourfold increase in Trp concentrations. We suppose that, in analogy to our results obtained with 5HTP and to the earlier mentioned reports, reduction in number of serotonergic terminals also occurred in brains of TCP treated animals, but the additional consequence of TCP treatment appeared to be permanent changes in 5HT metabolism. We could speculate that, while such low levels of released 5HT in the terminal region have in-

duced increased Trp uptake into the nerve cell bodies, this Trp was either not sufficiently converted to 5HT or the synthesized 5HT was not efficiently protected from MAO activity. A reason for that might lie in the long-lasting/permanent downregulation of tryptophan hydroxylase activity and/or 5HT storage, induced by high 5HT levels during the chronic inhibition of 5HT degradation. Another long-lasting consequence of TCP treatment might be the upregulated degradation of 5HT, since 5HIAA levels reach those of the control animals despite considerably lower 5HT concentrations. Of course, possible effects of increased concentrations of other monoamines during brain development (Andersen, 2003), and of consequential compensatory mechanisms, complicate interpretation of the obtained results and should be kept in mind.

5. Conclusion

Through chronic pharmacological treatments during the perinatal period, we have exposed the developing rat brain to increased 5HT concentrations in two different manners. Simple increase in the availability of the immediate 5HT precursor significantly raised peripheral but not central 5HT levels, leading to decreased 5HT concentration in the cortex of adult animals. On the other hand, inhibition of the 5HT-degrading enzyme simultaneously elevated 5HT levels in the brain and the periphery, and caused permanent changes in 5HT homeostasis of adult animals, both peripherally (hyperserotonemia) and centrally (altered 5HT metabolism with decreased 5HT concentrations). Further anatomic and expressional studies are planned to explore the mechanisms of the different disturbances in 5HT homeostasis induced by the two compounds. We expect that the obtained results will bring some answers to the question whether hyperserotonemia is a cause or a marker of the central 5HT-alterations seen in autism.

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2.4. Anxiety-like behavior and cognitive flexibility in adult rats perinatally exposed to increased serotonin concentrations (rad 4)

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Research report

Anxiety-like behavior and cognitive flexibility in adult rats perinatally exposed to increased serotonin concentrations

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ABSTRACT

Serotonin (5-hydroxytryptamine, 5HT) is a biologically active amine that regulates the development of 5HT neurons and target tissues during neurogenesis, while later it assumes the function of a neurotransmitter. Serotonin mediates many essential behaviors common to all mammals, and is held responsible for anxiety-like behavior and cognitive rigidity. Proper serotonin levels, controlled through 5HT synthesis and metabolism, are crucial for normal brain development. In this study we investigated anxiety-like behavior and cognitive flexibility in adult animals after exposing their developing brains to increased 5HT concentrations. Wistar rats were treated subcutaneously from gestational day 12 to post-natal day 21 with the immediate 5HT precursor 5-hydroxytryptophan (5HTP, 25 mg/kg), a non-selective MAO inhibitor tranylcypromine (TCP, 2 mg/kg), or saline. After reaching adulthood, animals were tested for anxiety-like behavior (exploratory behavior, thigmotactic behavior, social contact, and reaction to stressful stimulus) and cognitive flexibility (ability for reversal learning). Results of the behavioral studies corresponded with our previous neurochemical findings. Treatment with 5HTP, which has induced mild reduction in cortical 5HT concentrations, caused reduction in only one aspect of anxiety-like behavior (increased exploratory activity). Treatment with TCP, which lead to drastic reduction in 5HT concentration/function, resulted in a highly anxiolytic phenotype (reduced thigmotaxis, reaction to stress, and social anxiety) with improved cognitive flexibility. Although further neurochemical, anatomical and gene-expression studies are needed to elucidate the mechanisms underlying the observed behavior, we hope that our results will contribute to the understanding of the role of serotonin in anxiety-like behavior and cognitive rigidity.

1. Introduction

Serotonin (5-hydroxytryptamine, 5HT) is a biologically active amine with diverse roles in the mammalian organism [1]. In the brain, 5HT synthesizing neurons are located in discrete regions of the brain stem – *raphe nuclei*, but project their axons into various cortical and subcortical regions [2]. During ontogenesis, 5HT serves as a developmental signal for both serotonergic neurons and target tissues [3,4]. Later, in the mature brain, 5HT functions as a neurotransmitter modulating neuronal function and plasticity [5,6]. Due to its dual role in brain development and function, serotonin mediates many essential behaviors common to all mammals [7]. It is also held responsible for anxiety-like behavior [8] and cognitive rigidity [9,10] which are often coinciding as symptoms in behavioral disorders such as obsessive-compulsive disorder [11], autism [12], or anorexia nervosa [13].

In order to exert its proper function during development, serotonin must be present in various brain regions in optimal concentrations which are controlled through the levels of 5HT synthesis and metabolism. Serotonin is synthesized from the amino acid tryptophan through the intermediate 5-hydroxytryptophan (5HTP). The rate limiting step in this synthesis pathway is the action of tryptophan hydroxylase (TPH) on tryptophan. The enzyme has two forms, TPH1 and TPH2, one present in the periphery and the other in the brain [14]. In both 5HT compartments, serotonin is metabolized into 5-hydroxyindoleacetic acid starting with oxidative deamination mediated by the enzyme monoamine oxidase (MAO) which also participates in the metabolism of the catecholamines dopamine (DA) and noradrenalin (NA). 5HT cannot cross the blood–brain barrier; hence it is synthesized and regulated independently in each compartment. During perinatal development, however, the blood–brain barrier is not completely formed and serotonin can freely cross from the peripheral compartment into the brain [15]. Peripheral 5HT levels seem to have a role during pregnancy, as maternal [16] and placental [17] serotonin

Abbreviations: MAO, monoamine oxidase; 5HT, 5-hydroxytryptamine, serotonin; DA, dopamine; NA, noradrenalin; TCP, tranylcypromine; 5HTP, 5-hydroxytryptophan; G, gestational day; PND, postnatal day.

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were shown to influence fetal brain development in mice. It could be therefore expected that altered 5HT metabolism in any compartment could lead to deviations from optimal serotonin concentrations affecting the development of the brain serotonergic system and later lead to the related behavioral deficits.

The aim of this study was to investigate anxiety-like behavior and cognitive flexibility in adult animals after exposing their developing brains to increased 5HT concentrations by manipulating the synthesis and degradation of the amine during the period of most intensive development of the 5HT neurons [3,18]. Two alternative approaches were used: (1) treatment with the immediate 5HT precursor which primarily increased 5HT concentrations in the periphery, and (2) treatment with a non-selective MAO inhibitor which altered serotonin metabolism both in the brain and periphery leading to increased 5HT concentrations in both compartments [19]. For that purpose Wistar rats were treated subcutaneously with either 5-hydroxytryptophan (5HTP, 25 mg/kg), or tranylcypromine (TCP, 2 mg/kg), from gestational day (GD) 12 to post-natal day (PND) 21. After reaching adulthood, animals were tested for several aspects of anxiety-like behavior (exploratory behavior, thigmotactic behavior, social contact, and reaction to stressful stimulus) and for cognitive flexibility (measured as ability for reversal learning), and their behavior was compared to that of saline treated control animals.

2. Materials and methods

2.1. Breeding and pharmacological treatment of animals

Wistar rats were bred and raised at the Division of Biology of the Faculty of Science, University of Zagreb. The breeding procedure and pharmacological treatment were described in detail elsewhere [20,21]. In short, nulliparous Wistar females from the animal facility of the Croatian Institute for Brain Research (University of Zagreb, Zagreb, Croatia), weighing 230–275 g, were randomly assigned to a "saline", "5HTP", or "TCP" group, and mated with males of the same strain and age in 2:1 or 3:1 ratio. After gravidity was confirmed in all females, the male was removed from the cage. Females remained together until two days before parturition when they were separated and remained singly housed until weaning of the pups (at PND 22). After weaning, animals were kept 3–4 per cage throughout the experiment. Animals were housed in polycarbonate cages under 12 h light:12 h dark conditions at a temperature of 22 ± 2 °C, with free access to rat chow and tap water.

Animals were perinatally treated with 25 mg/kg of the serotonin precursor 5-hydroxy-L-tryptophan (Sigma–Aldrich), 2 mg/kg of the monoamine oxidase inhibitor tranylcypromine (Sigma–Aldrich) or saline, from GD 12 until birth through subcutaneous injections to pregnant females, and from PND 1 until PND 21 through subcutaneous injections of the same doses.

The study was approved by the Ethics committee of the Faculty of Science, University of Zagreb, and was conducted in accordance with the Croatian Animal Protection Law ("Narodne novine", 135/2006).

2.2. Behavioral testing

2.2.1. Experimental design

All animals were submitted to a battery of behavioral tests in the following order: reversal learning (T-maze), locomotor activity (open field), exploratory and thigmotactic behaviors (hole-board), social contact (social choice apparatus), and reaction to a stressful stimulus (open field), from PND 55 onward. Each animal had 1 day of break between the experiments. The T-maze test was conducted on 10 saline (5 females, 5 males), 7 TCP (4 females, 3 males) and 14 5HTP (7 females, 7 males) treated rats, and all other experiments on 27 saline (14 females, 13 males), 16 TCP (8 females, 8 males), and 24 5HTP (13 females, 11 males) treated rats. Animals were tested in an adjacent isolated room, under illumination of 30 lx, between 13:00 and 18:00 h. Experimenters were blind with respect to the rats' pharmacological treatment.

2.2.2. Apparatuses

Hole-board and open field consisted of a 60 cm × 60 cm × 35 cm enclosure made of clear Plexiglas walls and a removable white Plexiglas floor. The floor of the hole-board contained 16 holes (4 cm in diameter, 4 cm deep), displayed in a 4 × 4 configuration, while the open field floor was divided into 16 squares.

The social choice enclosure was composed of three cages (adapted from Ref. [22]) between which the animal could move freely. The central cage was empty while each of the side cages contained a wire enclosure in which an object or an unfamiliar rat could be placed.

The T-maze consisted of an 85 cm × 7.8 cm × 6 cm central arm and two 37 cm × 7.8 cm × 6 cm side arms. The maze had a wooden bottom and side walls dyed with black color, with the upper side made of a clear Plexiglas.

Testing apparatuses were washed with detergent, rinsed with water, and dried after each animal, or after each trial, in order to remove odor.

2.2.3. Procedures

Horizontal and vertical locomotor activities were tested in the open field as the number of squares entered with the front paws and the number of rearings, respectively, during 10 min.

Exploratory and thigmotactic behaviors were tested in a hole-board. The number of head dips (both eyes in a hole) into four inner and twelve outer holes during 10 min was recorded. Total number of holes visited was used as a measure of exploration, and inner-to-outer hole ratio as a measure of thigmotaxis.

The reaction to a stressful stimulus was tested in the open field apparatus. A dog whistle was used to produce a high pitched sound of a frequency 18–22 kHz, corresponding to rat alarm calls. Animals were placed in the apparatus and were allowed to explore it for 5 min. During the following 5 min, animals were exposed every 30 s to a 3 s-long sound, and a number of freezing behaviors after each whistle was recorded. Total number of freezings was used as a measure of stress-induced anxiety.

In the social choice test, animals were placed in the central cage of the apparatus and allowed to move freely between the compartments during 10 min in order to habituate. Then the animal was simultaneously presented with an object placed in the wired enclosure in one side-cage, and an unfamiliar conspecific placed in the wired enclosure in another side-cage. The wire cage allowed olfactory, visual, auditory, and tactile contact with the conspecific, and only the tested animal could initiate social contact. Time spent exploring the object or the conspecific were measured during a 10 min period.

Reversal learning, tested in a T-maze, consisted of two sets of experiments. In the first set, the animal had to learn which side-arm contains the reward (learning); in the second set the animal had to learn that the reward is now on the opposite side-arm (reversal). Two days prior to testing and throughout the experiments rats were allowed to eat only 1 h a day. On the zero trial, a reward (condensed milk) was placed on both arms of the T-maze, and the arm the animal chose was recorded. During learning trials, the reward was placed in the arm opposite to the one the rat had chosen on the zero trial. The animals were then given 10 trials, each lasting 60 s, per day until they learned where to find the treat. Learning was achieved when the rats scored 8 out of 10 correct choices in one day. During reversal trials, the reward was placed on the opposite arm, and again rats were given 10 trials per day until they learned the new position of the reward. The number of correct choices on consecutive days was used as a measure for both, learning and reversal.

2.3. Statistical analysis

Data were processed by the use of GraphPad Prism 5 Software. The measured parameters were tested for normality of distributions by the method of Kolmogorov and Smirnov. Normally distributed parameters were compared using one-way analysis of variance (ANOVA) with Dunnett's multiple comparison test, which is specific for comparison of more groups against a control group. Parameters that were not normally distributed were compared using the non-parametric Kruskal–Wallis method, with Dunn's multiple comparison post hoc test. Learning ability within each group was analyzed with non-parametric Friedman test with Dunn's multiple comparison post hoc test; while the differences in learning between the groups was compared using one-way analysis of variance (ANOVA), with Dunnett's multiple comparison test. The level of significance was set to 0.05. Values in the text were expressed as means (M) ± standard error of means (S.E.M.).

3. Results

Two experimental groups of animals were tested on five serotonin related paradigms and compared with a control group. First, the interaction between treatment and gender in different behavioral tests was analyzed using two-way ANOVA. Since no interaction was found, groups were analyzed separately for gender and treatment influences in order to allow for more reliable post hoc analysis. Values for most of the examined parameters did not significantly differ between males and females in the integral sample of 67 animals ($p = 0.38$ for total number of holes; $p = 0.35$ for inner-to-outer hole ratio; $p = 0.19$ for number of freezings; $p = 0.35$ for time spent with object; $p = 0.37$, $p = 0.57$, and $p = 0.16$ for days 1, 2 and 3 in learning test; $p = 0.79$, $p = 0.15$, and $p = 0.18$ in days 1, 2, 3 in reversal test), so the treatment groups were analyzed as a whole. Data for the parameters that differed between genders ($p = 0.0068$ and $p = 0.0046$ for horizontal and vertical activity in open field, respectively; $p = 0.0004$ for time spent with conspecific) was

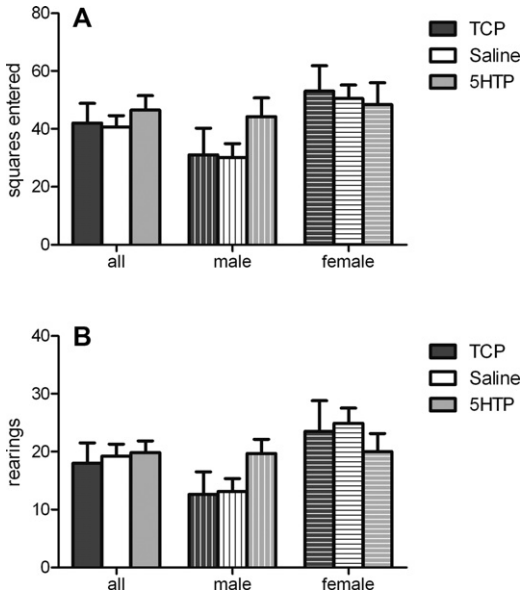

Fig. 1. Horizontal (A) and vertical (B) locomotor activity in the open field, expressed as number of squares entered and number of rearings, respectively, in saline treated ($N = 13$ males, 14 females), tranylcypromine (TCP) treated ($N = 8$ male, 8 females), and 5-hydroxytryptophan (5HTP) treated (11 male and 13 female) rats. Values are expressed as $M \pm$ S.E.M.

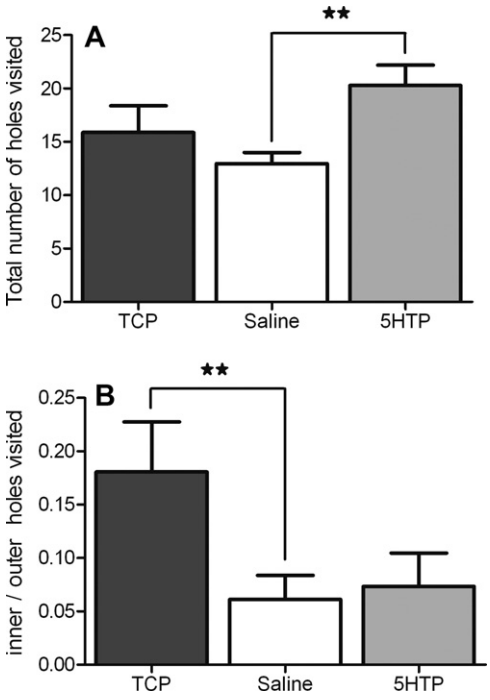

Fig. 2. Exploratory behavior measured as a total number of holes visited (A) and anxiety like behavior measured as the ratio of inner and outer holes visited (B), in the hole board for saline treated ($N = 27$), tranylcypromine (TCP) treated ($N = 16$), and 5-hydroxytryptophan (5HTP) treated ($N = 23$) rats. Values are expressed as $M \pm$ S.E.M. $^{**}p < 0.01$ Dunn's multiple comparison after Kruskal–Wallis test.

also analyzed separately for males and females. Animals differing in values of the tested parameter by more than two standard deviations from the group means were considered as outliers and were eliminated from the analyses.

The open field test was conducted in order to check for the possible treatment-induced differences in locomotor activity (Fig. 1). Females showed higher level of both types of locomotion than the males. However, within each gender, there were no significant influence of treatment on either horizontal activity ($F_{(2,29)} = 1.56$, $p > 0.2$ for males; $F_{(2,32)} = 1.03$, $p > 0.9$ for females) or vertical activity ($F_{(2,29)} = 2.16$, $p > 0.1$ for males; KW = 2.16, $p > 0.3$ for females). Mean values of the whole groups were also very similar (40.7 ± 3.8, 42.1 ± 6.8, and 46.5 ± 5.0 squares entered for saline, TCP, and 5HTP, and 19.3 ± 2.0, 18.1 ± 3.5 and 19.9 ± 2.0 rearings for saline, TCP, and 5HTP).

Exploratory and thigmotactic behaviors were tested on the hole board paradigm (Fig. 2). There was a significant group effect on both, the total number of holes visited (KW = 9.44, $p = 0.0089$), and the ratio of inner and outer holes visited (KW = 9.67, $p = 0.0079$). While the 5HTP treated rats visited significantly higher total number of holes (20.3 ± 1.9) than the saline treated rats (13.0 ± 1.0), the number of holes visited by the TCP treated animals (15.9 ± 2.5 holes) did not differ from that of the control group. On the contrary, TCP treated rats had a significantly higher inner-to-outer visited holes ratio (0.18 ± 0.05) than the saline treated rats (0.06 ± 0.02) which was similar to that of the 5HTP treated animals (0.07 ± 0.03).

The level of anxiety after exposure to a stressful stimulus, measured as freezing behavior, was also significantly influenced by treatment (KW = 8.47, $p = 0.0145$, Fig. 3). When presented with a repeated high pitched sound, rats treated with 5HTP and rats treated with saline freezed to a similar extent (5.3 ± 0.4 and 5.7 ± 0.8, respectively) while TCP treated rats freezed significantly less (3 ± 0.6) than the control animals.

When tested for social contact, rats were given the choice of exploring an object or an age matched unfamiliar conspecific. As expected, rats regardless of treatment spent a considerably larger amount of time exploring an animate than exploring an inanimate object (Fig. 4A). When analyzing each type of object separately, we found significant influence of treatment on both, time spent with inanimate object ($F_{(2,57)} = 6.01$, $p = 0.0043$) and time spent with a conspecific ($F_{(2,60)} = 3.42$, $p = 0.0392$). The TCP treated rats spent an equal amount of time exploring an inanimate object (22.7 ± 2.4 s) as the saline treated rats (22.5 ± 1.3 s), while 5HTP treated rats explored it significantly longer (30.8 ± 2.4 s). On the contrary, rats treated with 5HTP and rats treated with saline spent similar amounts of time investigating a conspecific (93.5 ± 6.0 s and 92.7 ± 7.3 s, respectively) while the TCP treated rats explored it significantly longer (117.6 ± 8.6 s). Since males spent significantly more time in social exploration (139 ± 6.1 s) than females (121 ± 4.9 s), the results were also analyzed separately. Although in separate gender groups the effects of treatment were only indicative in males ($F_{(2,27)} = 2.51$, $p = 0.0997$) and non-significant in females ($F_{(2,30)} = 1.30$, $p = 0.2871$), we could notice the same tendency for higher interest in social contact in TCP treated rats in both, males and females, as in the integral sample (Fig. 4B).

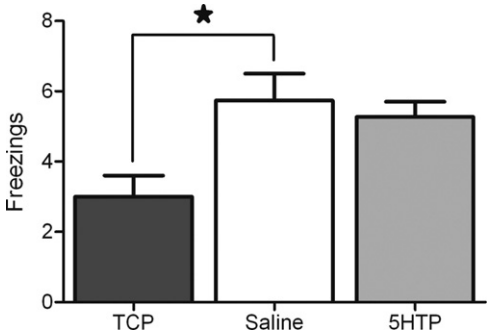

Fig. 3. Stress induced anxiety, measured as number of freezings, in saline treated ($N = 23$), tranylcypromine (TCP) treated ($N = 16$), and 5-hydroxytryptophan (5HTP) treated ($N = 23$) rats. Values are expressed as $M \pm$ S.E.M. $^{*}p < 0.05$ Dunn's multiple comparison after Kruskal–Wallis test.

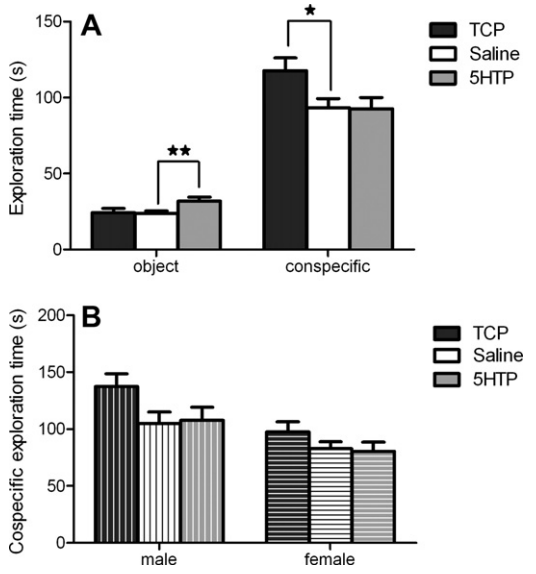

Fig. 4. The social choice test. (A) Time spent with an object and with an unknown conspecific for saline treated ($N = 26$), tranylcypromine (TCP) treated ($N = 15$), and 5-hydroxytryptophan (5HTP) treated ($N = 19$) rats. (B) Time spent exploring the conspecific in males (12 saline, 8 TCP, 9 5HTP) and females (14 saline, 7 TCP, 10 5HTP). Values are expressed as $M \pm$ S.E.M. $^{*}p < 0.05$, $^{**}p < 0.01$, Dunnett's multiple comparison after one-way ANOVA.

Finally, rats were tested for reversal of the learned paradigm in a T-maze. In the learning part of the test, all groups of animals significantly improved the number of correct choices over consecutive days of training (Fr = 28, $p < 0.0001$ for saline, Fr = 18, $p = 0.0004$ for TCP, and Fr = 30, $p < 0.0001$ for 5HTP) (Table 1). Also, all tested animals were able to reverse the learned paradigm over consecutive days of training (Fr = 18.6, $p < 0.0001$ for saline, TCP Fr = 8.7, $p = 0.0084$ for TCP, and 5HTP Fr = 26, $p < 0.0001$ for 5HTP). There were no significant differences among the treatment groups in the number of correct choices on each day of the learning period

Table 1
Number of correct choices over consecutive days of learning and reversal training in saline, tranylcypromine (TCP) and 5-hydroxytryptophan (5HTP) treated rats.

Days	Saline ($N = 10$)	TCP ($N = 7$)	5HTP ($N = 14$)
Learning 1	3.2 ± 0.9	5.0 ± 1.1	4.6 ± 0.8
Learning 2	7.0 ± 0.8	7.3 ± 1.3	7.1 ± 1.0
Learning 3	$9.1 \pm 0.6^{***}$	$9.7 \pm 0.2^{**}$	$8.8 \pm 0.5^{***}$
Learning 4	$9.9 \pm 0.1^{***}$	$10.0 \pm 0^{**}$	$10.0 \pm 0^{***}$
Reversal 1	4.3 ± 0.7	7.1 ± 0.8	4.6 ± 0.3
Reversal 2	$8.8 \pm 0.5^{\#}$	8.6 ± 1.1	$9.1 \pm 0.2^{\#\#}$
Reversal 3	$9.9 \pm 0.1^{\#\#\#}$	$9.4 \pm 0.6^{\#}$	$10.0 \pm 0^{\#\#\#}$

$^{**}p < 0.01$, $^{***}p < 0.001$, against Learning day 1, Dunn's multiple comparison after Friedman test.
$^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, against Reversal day 1, Dunn's multiple comparison after Friedman test.

($F_{(2,28)} = 0.95$, $p > 0.3$ for the first day, KW = 1.1, $p > 0.5$ for the second day, KW = 0.45, $p > 0.7$ for the third day, KW = 2.1, $p > 0.3$ for the fourth day) (Fig. 5A). However, significant influence of treatment on the number of correct choices was observed on the first day of reversal training ($F_{(2,28)} = 6.12$, $p = 0.0062$) due to significantly higher incidence of correct choices in the TCP treated group (Fig. 5B). No differences in learning among the groups were noted on the second or third day of the reversal training (KW = 0.3, $p > 0.8$ and KW = 1.9, $p > 0.3$, respectively).

4. Discussion

4.1. The choice of 5HT enhancers

Interference with neurotransmitter homeostasis can be achieved through inhibition/stimulation of receptors, modulation of neurotransmitter reuptake, or interference with neurotransmitter metabolism. Since our earlier findings in autistic subjects indicated that disrupted 5HT homeostasis might be related to alterations in 5HT metabolism [23,24], we decided to use the thirdly mentioned approach in order to study the consequences

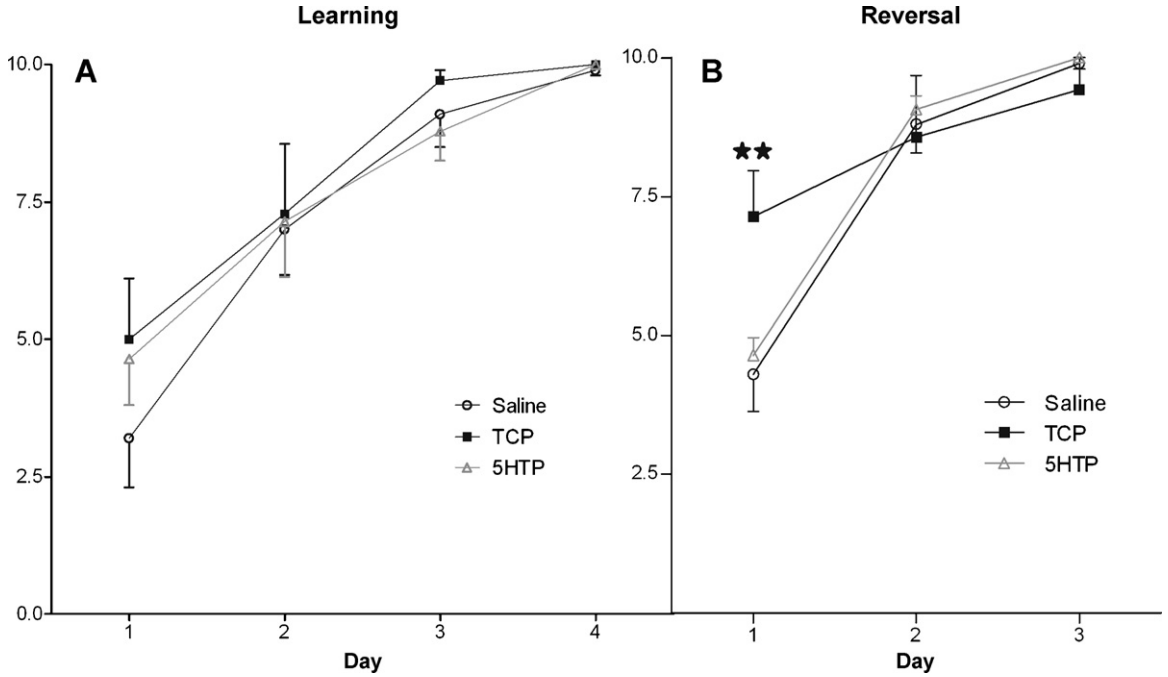

Fig. 5. Comparison of the average number of correct choices on each day of learning (A) and reversal (B) training among saline treated ($N = 10$), tranylcypromine (TCP) treated ($N = 7$), and 5-hydroxytryptophan (5HTP) treated ($N = 14$) rats. Values are expressed as means $M \pm$ S.E.M. $^{**}p < 0.01$ Dunnett's multiple comparison after one-way ANOVA.

of increased 5HT concentrations during brain development in an animal model.

5HTP is the immediate 5HT precursor. It readily crosses the placental barrier, and is only found in the serotonin synthesis pathway [25]. The administration of 5HTP allowed us to elude the rate-limiting step in the synthesis of serotonin and to mimic the effect of increased serotonin synthesis in one group of animals. Treatment with 5HTP significantly raised peripheral but not central 5HT levels. At adult age, a modest decrease in 5HT concentration was observed in the frontal cortex, presumably due to hyperserotonemia-induced loss of 5HT terminals during brain development [19].

TCP is an irreversible MAO A and MAO B inhibitor which inhibits the oxidative deamination of exogenous amines and monoamine neurotransmitters, including serotonin [26]. Although the two isoenzymes have different substrate affinities under normal physiological conditions, both can catabolize the same compounds and are able to take over when the function of the other is compromised through pharmacological inhibition. Since more pronounced effects on rat 5HT metabolism were observed after the inhibition of both isoforms than after the sole inhibition of MAO A [27,28], we decided to use TCP over a specific MAO A inhibitor in a dose which was expected to effectively block most, but not all of the 5HT degradation. Indeed, treatment with TCP caused simultaneous inhibition of MAO enzymes in the brain and the periphery, inducing significant 5HT elevations in both compartments. At adult age, a drastic decrease in 5HT concentrations, as well as altered 5HT metabolism was observed in both, frontal cortex and midbrain raphe region [19]. Although, focused on 5HT research, we did not neurochemically characterize catecholamine homeostases in the treated animals, we could assume that DA and NA levels were also increased during the treatment. This might have caused additional effects in our animals since both catecholamines are considered to have a role in brain development and establishment of essential circuits required for typical adult function [29–31]. On the other hand, while 5HT is catabolized solely through oxidative deamination, cathecolamines can also be degraded by the action of cathecol-o-metil-trasferase which is especially abundant in prefrontal cortex [32]. Therefore increases in DA and NA concentrations were not expected to be as massive as that of 5HT. This view is supported by findings that MAO A/B knock-out mice have about twofold increase in dopamine and noradrenalin levels but more than eightfold increase in 5HT levels in comparison to wild-type animals [33], and that treatment with a combination of MAO A and MAO B inhibitor throughout gestation produced marked changes in the development of 5HT but not of DA and NA terminals in rats [34]. Taken all together, we will discuss behavioral data primarily from the point of view of the well characterized changes in the 5HT homeostasis, while also addressing possible influences of other monoamines.

4.2. Behavioral effects of 5HTP and TCP treatments

Results from our previous studies have shown that treatments with both, 5HTP and TCP have led to a long-lasting/permanent decrease in 5HT concentrations in the serotonergic terminals of the frontal cortex, the one in TCP-treated group being far more pronounced [19]. In this study we explored in which way the decreased 5HT function in frontal cortex has affected anxiety-like behavior and cognitive flexibility in the treated groups of animals. It was hard to predict the outcome of the study. On one hand, one could expect that the non-optimal 5HT concentrations during development would lead to increased anxiety and cognitive rigidity, as reported in the studies with serotonin-deficient rats [9,35,36]. On the other hand, in line with the theory that increased 5HT function is considered to act as inhibitor of behavioral activation, while reduced 5HT function facilitates behavioral response to environmental stimuli [7,37], the opposite effect on the explored features could also be expected.

Results of behavioral studies accorded well with the neurochemical findings – behavioral alterations in the 5HTP-treated group were very mild, while those of the TCP-treated group were prominent. Compared to the saline treated rats, both groups displayed reduced anxiety-like behavior. 5-HTP-treated animals showed significantly higher degree of exploration of both, novel environment (Fig. 2A) and novel object (Fig. 4A), while TCP-treated rats showed significantly reduced degree of thigmotactic (Fig. 2B) and freezing (Fig. 3) behaviors, and spent more time exploring a conspecific (Fig. 4) than did control rats.

Our findings seem to differ from the results obtained after prenatal or neonatal exposure of rodents to 5HT reuptake blockers, which was also shown to alter the long-term chemical profile of the raphe cortical projection system [38]. Groups that investigated anxiety-like behaviors in these animals reported long lasting decrease in exploratory activity [39,40], transient decrease in social exploration [39], and no changes in thigmotactic behavior [40,41]. Although both interference with 5HT metabolism and interference with 5HT uptake increased 5HT availability at the synapse, the observed discrepancies between the anxiolytic profile of our animals and the anxiogenic profile of the animals treated with reuptake blockers might be explained by different compensatory mechanisms induced by interference with presynaptic vs. synaptic events. In any case, the reduced anxiety-like behavior observed in our animals corresponds with the reduced 5HT function found in their cortices, and is in line with the reported findings of reduced anxiety-like behavior after pharmacologically induced [42,43] or lesion-induced [44,45] decrease of 5HT function, as well as with studies on Wistar-Zagreb 5HT rats reporting significantly higher anxiety in the “high 5HT” than in the “low 5HT” subline [46].

Interestingly, different aspects of anxiety-like behavior were affected in the two treated groups, which is probably the result of different effects induced by treatment with 5-HTP or TCP. The observed differences in anxiety-like behavior might be the result of a 5HT-dose effect, in a sense that lower degree of 5HT-reduction might have affected only exploration as a less anxiogenic component, while higher degree of 5HT-reduction would have affected more anxiogenic components such as exposure to open spaces, novel conspecific or stressful stimulus. Alternatively, prominent changes in anxiety-like behavior of TCP-treated rats might be a result of perinatally altered catecholamine homeostasis in addition to the effects of serotonin, as opposed to a solely 5HT-induced effect in 5HTP-treated rats. Dopamine is present in the entire forebrain where it primarily contributes to the regulation of motor and limbic functions, including anxiety-related processes [47]. Indeed, reduced thigmotactic behavior was observed in D3 receptor knockout mice [48], while D2 receptor acting antipsychotics were shown to reduce anxiety [49–51] and increase social investigation [52] in rodents. NA, on the other hand, serves as an essential neurotransmitter to regulate arousal and adapt to environmental and internal stressors, and facilitated function of the NA system has been associated with anxiogenic effects [53]. According to the above mentioned reports, the anxiolytic profile of TCP-treated animals could indicate possible reduction in catecholaminergic function.

Cognitive flexibility is defined as the ability to adjust behavior to changes in the environment or task conditions and is often measured as ability for reversal learning of the acquired task [54]. Animal studies have indicated a major role of 5HT in the modulation of reversal learning [55]. However, reports on the effects of pharmacologically induced decrease of 5HT function on cognitive flexibility are contradictory. Impairments of the reversal learning ability have been reported after 5HT depletion in rats [9,10], while

no effect [56] or even improvement [57] on the reversal learning task have been reported in marmosets after treatment with a 5HT3 antagonist. In our study, all groups of rats displayed similar level of learning of the initial position of the reward, indicating that the treatments with 5HT agonists did not affect general learning ability. However, TCP-treated animals learned the new position significantly faster than the 5HTP-treated and saline-treated rats, suggesting an improvement of cognitive flexibility in a group of rats with drastically reduced cortical 5HT levels. Again, the possibility that alterations in the DA and/or NA systems have contributed to this effect must be taken into consideration. Pharmacological studies using dopamine agonists and antagonists have shown that functional overactivity of the dopamine system impairs reversal learning, both in animal models and in humans, as reviewed in Clark et al. [55]. Also, a β-andrenergic antagonist was shown to improve cognitive flexibility in stressed humans [58], while inhibitors of noradrenalin transporter improved reversal performance in rats and monkeys [59].

5. Conclusion

In conclusion, perinatal treatment of rats with two different 5HT agonists: 5HTP which induced mild reduction in cortical 5HT concentrations, and TCP which lead to drastic reduction in 5HT concentration/function, affected 5HT-related behavior in the two groups of adult animals to different extents. While reduction in only one aspect of anxiety-like behavior (increased exploratory activity) was found in the 5HTP-treated group, TCP-treated rats displayed a profound anxiolytic profile (reduced thigmotaxis, reaction to stress and social anxiety) with improved cognitive flexibility. Further neurochemical, anatomical and gene-expression studies are planned in order to elucidate whether the highly anxiolytic phenotype of TCP-treated vs. 5HTP-treated animals is the consequence of a 5HT-dose effect, or of a reduced cathecolaminergic in addition to serotonergic function. We hope that our results will eventually contribute to the understanding of the role of serotonin and other monoamines in anxiety-like behavior and cognitive rigidity, and to the possibility of treatments of these symptoms in certain behavioral disorders.

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2.5. Effects of perinatal exposure to increased 5HT concentrations on expression of 5HT-regulating genes in adult rat brain (rad 5)

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EXPRESSION OF 5HT-RELATED GENES AFTER PERINATAL TREATMENT WITH 5HT AGONISTS

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Abstract

Serotonin (5HT) is a biologically active amine with diverse roles in the mammalian organism. Developmental alterations in 5HT homeostasis could lead to exposure of the developing brain to non-optimal serotonin concentrations that may result in developmental and behavioral deficits. In order to explore the molecular basis of the effects of developmental disturbances on 5HT metabolism on adult central 5HT homeostasis, observed in our previous studies, we measured changes in gene expression of the neuronal 5HT-regulating proteins in adult animals after perinatal treatment with the immediate 5HT precursor 5-hydroxytryptophan (5HTP, 25 mg/kg), or monoamine oxidase (MAO) inhibitor tranylcypromine (TCP 2 mg/kg), during the period of the most intensive development of 5HT neurons - from gestational day 12 until postnatal day 21. Adult animals were sacrificed and the relative mRNA levels for tryptophan hydroxylase 2, MAO A, MAO B, receptors $5HT_{1A}$ and $5HT_{2A}$, 5HT transporter (5HTT) and vesicular monoamine transporter (VMAT) were determined in the raphe nuclei region and prefrontal cortex using Real-Time Relative qRT-PCR. In comparison to the saline treated animals, treatment with 5HTP caused mild but significant increase in MAO A and MAO B mRNA abundance. TCP-treated animals, besides an increase in mRNA abundance for both MAO genes, displayed significantly increased 5HTT and VMAT2 mRNA levels and significantly decreased $5HT_{1A}$ receptor mRNA levels. Our results suggest that perinatal exposure of rats to 5HTP, and especially TCP, induces long-lasting/permanent changes in the expression of 5HT-regulating genes, that presumably underlie 5HT-related neurochemical and behavioral changes in adult animals.

Keywords

• Serotonin • Tranylcypromine • 5-hydroxytryptophan • mRNA • Rat brain • Perinatal treatment

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1. Introduction

Serotonin (5-hydroxytryptamine, 5HT) is a biologically active amine with diverse roles in the mammalian organism, where it is present both in the brain and peripheral tissues. In the periphery, serotonin serves as a regulator of food intake, gastrointestinal, endocrine and cardiovascular function, and as platelet activator [1]. In the developing brain, serotonin serves as a key regulator of serotonergic outgrowth and maturation of target regions [2]. In the mature brain it acts as a neurotransmitter modulating function and plasticity [3,4]. The blood brain barrier, which is not permeable to 5HT, separates the central and peripheral 5HT-compartments allowing for independent regulation of 5HT homeostases maintained through the action of 5HT-regulating proteins.

Each of the 5HT-regulating proteins in the serotonergic synapse has a homologue in the peripheral compartment. Some are present as isoforms encoded by separate genes. The enzyme tryptophan hydroxylase (TPH) is responsible for the rate-limiting step of serotonin synthesis. TPH1 exists mostly in the enterochromafin cells of the intestinal mucosa [5] and TPH2 in 5HT synthesizing neurons [6]. The vesicular monoamine transporter (VMAT) is responsible for the cellular 5HT accumulation and storage in platelet (VMAT1) or neuronal (VMAT2) vesicles [7]. Other 5HT-elements are encoded by the same genes centrally and peripherally. The serotonin transporter (5HTT) is important for serotonin transport into platelets, and reuptake into the presynaptic neuron [8]. Serotonin receptors 1A ($5HT_{1A}R$) and 2A ($5HT_{2A}R$) act as pre- and post-synaptic regulators of synaptic 5HT action [9], while those present on the cells of the intestinal mucosa may be involved in the regulation of 5HT release from the gut [10]. $5HT_{2A}r$ is also present on the platelet membrane and is involved in the process of aggregation during which 5HT is released from the platelets. In both compartments, 5HT is converted into 5-hydroxyindolacetic acid (5HIAA) through the oxidative deamination catalyzed by the mitochondrial enzyme monoamine oxidase (MAO) [11]. MAO comes in two isoforms: MAO A preferentially oxidizes serotonin and norepinephrine, MAO B phenylethylamine, while dopamine and tyramine represent substrates for both isoenzymes.

During fetal and early postnatal development, the blood brain barrier is not fully formed and serotonin can freely cross from the peripheral compartment into the brain and influence the development of the neuronal 5HT system [12]. In fact, peripheral 5HT levels seem to have a role during pregnancy, as maternal [13] and placental [14] serotonin levels were shown to influence fetal brain development in mice. Therefore, it may be assumed that altered 5HT homeostasis, induced either endogenously (i.e.

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by mutations/polymorphisms of one or more of the 5HT-regulating genes) or exogenously (i.e. by taking 5HT-enhancing agents during pregnancy), could lead to the exposure of the developing brain to non-optimal serotonin concentrations that may result in immediate developmental and later behavioral deficits.

Pharmacological studies on the consequences of perinatal neurotransmitter disbalance can be performed in animal models using three approaches: first, by blocking/stimulating receptors; second, through modulation of the activity of the neurotransmitter's transporter; and third, by interfering with the neurotransmitter's metabolism affecting either its synthesis or degradation. Consequences of the perinatal exposure to excessive serotonin concentrations using the first approach have been thoroughly studied through the developmental hyperserotonemia (DHS) model of autism [15]. The second approach has been widely employed in order to investigate the consequences of perinatal exposure to SSRI [16]. The long-term effects of perinatal alterations in serotonin metabolism have been far less studied. We therefore opted for the third approach using the immediate precursor 5-hydroxytryptophan (5HTP) to facilitate 5HT synthesis, or a MAO inhibitor tranylcypromine (TCP) to impede 5HT degradation, during the period of most intensive development of 5HT neurons [17]. In our previous studies we demonstrated that: a) during treatment, 5HTP significantly elevated 5HT concentrations in the peripheral but not in the central 5HT compartment [18], while its long-term effect (in adult animals) was mild but significant reduction in cortical 5HT levels without any effect on 5HT metabolism [19]; b) during treatment, TCP significantly elevated 5HT levels both, in the brain and the periphery [20], while its long-term effect was a robust decrease in cortical and midbrain 5HT levels accompanied with markedly increased 5HT degradation [19]; and c) animals from both groups displayed reduction in anxiety-like behavior that corresponded with the degree of the reduction in 5HT function [21].

The aim of the present study was to explore molecular changes that underlie the observed neurochemical and behavioral alterations in the treated animals, in order to clarify the effects of developmental disturbances in 5HT metabolism on adult central 5HT homeostasis. For this purpose, Wistar rats were subcutaneously administered either 5HTP (25 mg/kg) or TCP (2 mg/kg), from gestational day (GD) 12 until postnatal day (PND) 21. Adult animals were sacrificed and the relative mRNA levels for TPH2, MAO A, MAO B, $5HT_{1A}R$, $5HT_{2A}R$, 5HTT and VMAT2 were determined in the raphe nuclei region (RNR) - the site of 5HT neuronal cell bodies, and prefrontal cortex (PFC) - the site of 5HT axon terminals, using Real-Time Relative qRT-PCR (RT-qPCR).

2. Experimental Procedures

Wistar rats were bred and raised at the Division of Biology of the Faculty of Science, University of Zagreb, according to the procedure described elsewhere [18,20]. In summary, nulliparous Wistar females from the animal facility of the Croatian Institute for Brain Research (University of Zagreb, Croatia), weighing 230-275 g were mated with males of the same strain and age in a 3:1 ratio. Once gravidity was confirmed, the males were separated, and the females randomly assigned to a "saline", "5HTP", or "TCP" group. Two days before parturition, females were separated and remained singly housed until weaning of the pups (at PND 22). After weaning, animals were kept 3-4 per cage. The animals were housed in polycarbonate cages under 12 h light: 12 h dark conditions at 22 ± 2°C, with free access to rat chow and tap water. All efforts were made to reduce the number of animals used and to minimize animal suffering. The study was approved by the Ethics committee of the University of Zagreb, and was conducted in accordance with the Directive of The European Parliament and of the Council (2010/63/EU) and the Croatian Animal Protection Law ("Narodne Novine", 135/2006).

Pharmacological treatment is thoroughly described elsewhere [18,20]. In short, the experimental groups of pups were treated with either 25 mg/kg of the serotonin precursor 5-hydroxy-L-tryptophan (5HTP, Sigma-Aldrich, St. Louis, MO, USA), 2 mg/kg of the monoamine oxidase inhibitor tranylcypromine (TCP, Sigma-Aldrich, St. Louis, MO, USA) or saline, from GD 12 until birth through subcutaneous injections to pregnant females, and from PND 1 until PND 21 through subcutaneous injections of the same doses.

On PND 70 prefrontal cortex and raphe nuclei region samples were collected from 15 saline (10 males, 5 females), sixteen 5HTP (9 males, 7 females), and 17 TCP (9 males, 8 females) treated rats. After anaesthesia and decapitation, the brains were removed from the skulls and briefly frozen in dry ice. A midbrain region containing serotonergic cell bodies of the dorsal and median raphe nuclei was obtained by a 3 mm thick coronal brain slice (plates 43 and 55 in the rat brain atlas, [22], followed by a 3 mm diameter punch into the mid-brain area. A 4 mm coronal cut was then made at the frontal lobes (plate 11) and cortex (all cortical areas anterior to bregma + 1.7 mm) was peeled off. All samples were placed in microtubes and immediately frozen in liquid nitrogen. Samples were disrupted and homogenized with an ultrasonic homogenizer (Bandelin electronic, Mecklenburg-Vorpommern, Germany) in 500 µL of guanidinium thiocyanate solution. The homogenates were then frozen at -80°C until RNA isolation.

Total RNA was isolated from samples using the phenol-free RNAqueous-4PCR kit (Ambion, Inc., Austin, TX, USA) according to manufactures' instructions. Genomic DNA was removed following the kits DNase treatment. RNA concentration and quality was measured in a spectrophotometer (Biochrome), and assessed through standard agarose gel electrophoresis. From 1.25 µg of total RNA added, mRNA was reversely transcribed using MuLV reverse transcriptase (Applied Biosystems, Foster City, CA, USA) and oligo dT primers (Applied Biosystems, Foster City, CA, USA), following manufacturer's instructions, in a total volume of 25 µL. The performance of the reverse transcription was assessed through PCR with positive intron spanning primers provided in the isolation kit. cDNA was stored at -20°C until further processing.

Relative expression of the genes included in Table 1 was assessed through qRT-PCR using the TaqMan gene expression master mix (Applied Biosystems, Foster City, CA, USA) according to manufacturer's instructions. All reactions were performed in a duplex setup with primer

Table 1. Analyzed genes and their assay ID's.

Gene of interest (GOI)	Predesigned TaqMan gene expression assay ID (FAM):	Probe context Sequence[a]
Tryptophan hydroxylase 2 (TPH2)	Rn00598017_m1	AAACTGGCCACGTGCTATTTCTTCA
Monoamine oxidase A (MAO A)	Rn01430961_m1	TGCCTGCCATCATGGGCTTCATACT
Monoamine oxidase B (MAO B)	Rn00566203_m1	AAGAAGCTCTGCAGCCAGTCCATTA
Serotonin transporter (5HTT)	Rn00564737_m1	GGTGGCCAAAGACGCAGGCCCCAGC
Vesicle monoamine transporter 2 (VMAT2)	Rn00564688_m1	AAGTGGCAGCTGGGCGTTGCTTTCC
5HT1a receptor ($5HT_{1A}R$)	Rn00561409_s1	TAATGGGGCAGTGAGGCAGGGTGAC
5HT2a receptor ($5HT_{2A}R$)	Rn00568473_m1	TCCTGTATGGGTACCGGTGGCCTTT

[a] as reported by vendor

limited rat β-actin (ACTB, VIC labelled, Applied Biosystems) as an endogenous control reference gene. The final volume of 20 µL contained 10 µL of master mix, 1 µL of the primers and probes for the reference gene, 1 µL of the primers and probes for the gene of interest (GOI), 6 µL of nuclease free H_2O and 2 µL of cDNA in the range of 20 to 25 ng per reaction. Each sample was run in duplicate. The qRT-PCR setup in the AB 7300 Real-Time PCR System was two minutes at 50°C and then 10 minutes at 95°C, followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds, according to the manufacturer's instruction manuals. The amplification results were analyzed with the 7300 System SDS v1.4. software (Applied Biosystems, Foster City, CA, USA). The cDNA levels were normalized to the endogenous control and relative differences were calculated according to the relative quantitation method.

Delta Cq data were processed with GraphPad Prism 5 Software (GraphPad Software, Inc., La Jolla, CA, USA). The measured parameters were tested for normality of distributions by the method of Kolmogorov and Smirnov. Normally distributed parameters were compared using one-way analysis of variance (ANOVA) with Dunnett's multiple comparison test, which is specific for the comparison of more groups against a control group. Parameters that were not normally distributed were compared using the non-parametric Kruskal-Wallis method, with Dunn's multiple comparison post hoc test. The level of significance was set to 0.05. Values in the text were expressed as means (M) ± standard error of means (S.E.M.).

3. Results

Relative abundance of mRNAs for serotonin related elements was analyzed in the RNR and PFC of the two experimental groups and compared to a control group. Some samples were lost during processing leaving 36 raphe nuclei region (13 5HTP; 12 TCP; 11 saline) and 40 prefrontal cortex (14 5HTP; 13 TCP; 13 saline) samples for further expression analysis. Reliably measurable levels of mRNAs of all investigated genes were found in both analyzed regions. Using two-way ANOVA, it was established that there is no interaction between gender and treatment. Groups were then analyzed separately for gender and treatment influences allowing for a more reliable *post hoc* analysis. Values of gene expression for the regions and genes of interest did not significantly differ between males and females in the integral sample (data not shown), therefore the groups were analyzed as a whole to assess the influence of the treatment on gene expression.

The effects of treatment on the relative mRNA levels of the analyzed genes in each of the regions are shown in Tables 2 and 3. It should be kept in mind that ΔCq value represents a difference between Cq value of an abundant reference gene and Cq value of a GOI; therefore lower ΔCq values indicate higher gene expression. Regardless of treatment, levels of mRNA for TPH2, 5HTT, VMAT2 and $5HT_{1A}R$ were relatively higher in RNR, mRNA for $5HT_{2A}R$ was more abundant in PFC, while similar concentrations of mRNAs for MAO A and MAO B were found in both regions.

In the raphe nuclei region (Table 2), the treatment had very significant influence on mRNA levels for MAO A (KW = 14.49, p = 0.0007) and MAO B (KW = 13.9, p = 0.001) isoenzymes, due to the significantly increased expression of both genes in the groups treated with 5HT enhancers compared to the control group. A similar trend was observed for mRNA levels of TPH2 and VMAT2, although in this case the influence of treatment was only indicative (KW = 5.27, p = 0.0718 for TPH2, and KW = 5.94; p = 0.0513 for VMAT2). The rest of the genes analyzed ($5HT_{1A}R$, $5HT_{2A}R$ and 5HTT) showed no significant changes in relative mRNA abundance (KW = 1.85, p = 0.40; KW = 1.67, p = 0.43; and KW = 1.8, p = 0.41 respectively).

In the frontal cortex (Table 3), the mRNA abundance was significantly influenced by treatment for 5HTT (KW = 9.31, p = 0.0095) and $5HT_{1A}R$ (KW = 7.24, p = 0.027). *Post hoc* analysis revealed that only TCP treated rats had significantly increased 5HTT expression and significantly decreased $5HT_{1A}R$ expression in comparison with the control group of rats. The influence of treatment on TPH2 mRNA levels was only indicative ($F_{(2,35)}$ = 0.139, p = 0.0737), with a tendency for a higher expression in both experimental groups. The abundance of other mRNAs analyzed (for MAOA, MAOB, VMAT2, and $5HT_{2A}R$) was not influenced by treatment with 5HT enhancers ($F_{(2,37)}$ = 0.1136, p = 0.89; KW = 0.77, p = 0.68; $F_{(2,37)}$ = 0.109, p = 0.119; and KW = 1.73, p = 0.42 respectively).

Table 2. ΔCq values# of genes analyzed in raphe nuclei regions of treated animals.

Treatment	N	TPH2	MAOA	MAOB	5HTT	VMAT2	5HT1aR	5HT2aR
5HTP	13	1.06±0.40	4.68±0.20**	4.51±0.18*	5.85±0.52	4.38±0.36	6.01±0.43	9.77±0.23
saline	11	1.77±0.31	5.88±0.21	5.12±0.13	5.35±0.22	5.20±0.43	5.60±0.22	10.1±0.24
TCP	12	0.81±0.48	4.34±0.26***	4.08±0.22***	5.26±0.47	2.86±0.98*	5.30±0.25	9.89±0.34

#Values are expressed as M ± S.E.M. Lower ΔCq numbers indicate higher expression of the genes.
* p < 0.05; ** p < 0.01; *** p < 0.001; experimental vs. control; Dunns *post hoc* analysis after Kruskal-Wallis test

Table 3. ΔCq values# of genes analyzed in prefrontal cortices of treated animals.

Treatment	N	TPH2	MAOA	MAOB	5HTT	VMAT2	5HT1aR	5HT2aR
5HTP	14	7.04±0.30	4.10±0.18	3.49±0.14	15.0±0.23	12.0±0.26	7.60±0.16	7.26±0.19
saline	13	7.94±0.22	4.07±0.18	3.42±0.18	15.4±0.24	12.6±0.32	7.21±0.16	6.96±0.47
TCP	13	7.29±0.30	4.19±0.21	3.36±0.16	14.0±0.47**	11.7±0.33	7.74±0.15**	7.47±0.35

Values are expressed as M ± S.E.M. Lower ΔCq numbers indicate higher expression of the genes.
** p < 0.01; experimental vs. control; Dunns *post hoc* analysis after Kruskal-Wallis test

4. Discussion

4.1 Choice of treatments

We considered 5HTP to be the most suitable choice for the facilitation of 5HT synthesis for the following reasons. First, it readily crosses the placental barrier [23], which is crucial for the prenatal part of the treatment. Second, unlike tryptophan (Trp) which is involved in other metabolic processes, 5HTP is quantitatively converted to serotonin. Third, it allowed us to bypass the rate-limiting step in the synthesis of serotonin (i.e. the action of TPH).

The choice of the MAO inhibitor was somewhat harder. Although MAO A and MAO B under normal physiological conditions have different substrate affinities, both can catabolize the same compounds and are able to take over when the function of the other is compromised through pharmacological inhibition. Accordingly, more pronounced effects on rat 5HT metabolism were observed after the inhibition of both isoforms than after the sole inhibition of MAO A [24–27]. We therefore opted for tranylcypromine, a non-selective irreversible MAO inhibitor.

In addition, both compounds are widely used in the human population: TCP as an antidepressant for treatment of depression resistant to SSRI, and 5HTP as an over-the-counter dietary supplement acting as a mood enhancer, appetite suppressant or sleep aid. However, long-lasting consequences of perinatal exposure to tranylcypromine have hardly been explored, and even less is known about the long-term effects of 5HTP.

Although it would have been more informative to explore dose dependencies of the induced effects, both substances were administered only in single chosen concentrations due to long duration of treatment, low number of pups in experimental groups, and complexity of experimental design. As described earlier [18,20], doses were determined according to literature data and to the results of our experiments on adult animals. 5HTP was administered in a dose sufficient to raise 5HT concentrations without causing the serotonin syndrome, while the given dose of TCP was expected to effectively block most, but not all of the 5HT degradation.

4.2 Sites of expression of 5HT-regulating genes

There is little data available on the levels of expression of genes coding for the 5HT-regulating elements in various brain regions. We therefore first compared relative mRNA abundance for these genes between the site of 5HT neuronal somas and the site of 5HT neuronal endings in the control group of rats. All of the investigated genes were expressed both in the RNR and PFC. Our findings are in line with the reported detection of mRNA in both regions for 5HTT [28], TPH2 [29], $5HT_{1A}R$ [30], $5HT_{2A}R$ [31], and MAO A [32], suggesting that 5HT-regulating proteins are synthesized both, presynaptically in the neuronal somas, and at the target projection sites after axonal transport of mRNA. The presence of mRNA for MAO B [32] and VMAT2 [33] was previously reported only in the RNR of adult animals, but a reason for these discrepancies might lie in different methodologies used to detect mRNA (*in situ* hybridization vs. qRT-PCR) between the mentioned studies and ours. Differences in regional mRNA abundance for each 5HT-regulating gene might reflect different regulation mechanisms of protein synthesis (local vs. central), possibly related to the physiological role of the 5HT-regulating element.

4.3 The effects of pharmacological treatments on the expression of 5HT-regulating genes

Two different 5HT enhancers interfering with 5HT metabolism were used in this study. The first one was the immediate 5HT precursor, which elevated 5HT levels without acting

directly on the 5HT-regulating proteins, and significantly disturbed 5HT-homeostasis only in the peripheral compartment. Still, this transient hyperserotonemia was sufficient to induce mild but significant increases in MAO A and MAO B mRNA abundance in the RNR of adult animals. Upregulation in MAO gene expression was presumably a reaction to the chronic increase in 5HT concentrations during treatment, and was apparently sufficient to prevent a robust increase in brain 5HT concentrations during development. However, gene expression remained upregulated after the wash-out period, and likely represents a cause of a mild reduction in 5HT levels in the cortices of these animals observed in our previous study [19].

The second 5HT-enhancer inhibited the main enzyme involved in the degradation of 5HT and other monoamines, and significantly disturbed 5HT homeostasis both peripherally and centrally. As expected, treatment with TCP induced more robust changes in the brains of adult animals: besides an increase in mRNA abundance for both MAO genes, they displayed significantly increased levels of 5HTT and VMAT2 mRNA and significantly decreased level of $5HT_{1A}R$ mRNA. While we found no literature data on the influence of chronic treatment with MAO inhibitors on the expression of 5HT-regulating elements in the rat brain, dysregulation in both presynaptic and postsynaptic serotonergic mechanisms was found in mice with inactivated MAO A gene [34,35]. The postsynaptic effects, including downregulation of $5HT_{1A}$ receptors, were comparable to ours, while the presynaptic effects, involving downregulation of VMAT2 and 5HTT, were the opposite. This discrepancy is not unexpected considering differences between permanent MAO inactivation at the gene level (knock-out model) and temporary MAO inactivation at the protein level (our model).

During treatment, TCP presumably induced extensive upregulation of MAO gene expression. Since every new dose of TCP also inhibited newly synthesized MAO proteins, this mechanism was not sufficient to maintain 5HT homeostasis during brain development. The attenuation of the excessive 5HT concentrations was probably attempted through the upregulation of 5HTT and VMAT2 expression, in order to increase removal from the synapse and storage in vesicles, and through the downregulation of post-synaptic $5HT_{1A}$ receptors. Regulation of all of the mentioned genes remained altered at adult age. This seems to underlie the drastically reduced 5HT levels with markedly increased 5HT degradation in the brains of these animals observed in our previous study [19].

Interestingly, a common effect of both treatments seems to be the upregulation in expression of both MAO genes. This finding suggests that: 1) both isoforms might have a role in 5HT degradation - although under physiological conditions 5HT is not a preferential substrate for MAO B, it might become a substrate for this enzyme under conditions of high 5HT concentrations; and 2) upregulation of MAO expression might represent the main or the "first-line" mechanism to fight exposure to chronic excessive 5HT concentrations, regardless of the way of interference with 5HT metabolism. Also, both experimental groups displayed indicative increase in TPH2 gene expression, which could represent an attempt to counterbalance the increase in 5HT degradation in the brains of the treated animals which remained after the wash-out period.

4.4 Possible implications

Disbalance in monoaminergic concentrations during brain development appears to have a significant impact on functions of the mature brain [36]. On one hand, disturbances in 5HT transmission have been suggested as an underlying cause of several behavioral disorders, including autism, alcohol dependence and suicidal behavior [37]. On the other hand, if taken during pregnancy, 5HT-enhancing agents such as antidepressants or drugs of abuse could later on lead to behavioral abnormalities in the offspring. Long-lasting behavioral, cellular and molecular changes have been reported in animal models and children perinatally exposed to selective serotonin reuptake inhibitors, cocaine, amphetamine, MDMA, p-chlorophenylalanine and 5-methoxytryptamine [38–44]. Our study is the first to report significant long-lasting/permanent changes in the expression of 5HT-regulating genes induced by chronic perinatal treatment with 5HTP or TCP, that presumably underlie the respective mild or robust neurochemical and behavioral changes observed in our previous studies [19,21]. The obtained results might have the following implications. First, the fact that developmental disturbances in 5HT synthesis or degradation induced permanent alterations in the central 5HT homeostasis, suggests that genes which regulate 5HT metabolism should be considered as potential candidates in 5HT-related behavioral disorders. Indeed, our findings in autistic subjects indicated that disrupted 5HT homeostasis is related to alterations in 5HT metabolism rather than 5HT uptake [45,46]. Second, the developmental disbalance induced in both 5HT-compartments (i.e. by the TCP treatment) leads to more robust permanent alterations of 5HT homeostasis than the developmental disbalance induced only in the peripheral 5HT-compartment (i.e. by the 5HTP treatment). Still, this transient fetal/neonatal (probably along with maternal) hyperserotonemia was sufficient to induce measurable molecular and behavioral 5HT-related changes, indicating the need for further experiments on animal models, as well as prospective studies in humans, which would thoroughly explore the safety of the use of these two 5HT enhancers by pregnant and lactating women.

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Conflict of interest

The authors declare that they have no conflict of interest.

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3. RASPRAVA

3.1. Prednosti i nedostaci eksperimentalnog pristupa

Smatramo da eksperimentalni pristup koji smo primijenili u ovoj disertaciji ima nekoliko prednosti:

1) Posljedice perinatalne izloženosti prekomjernim koncentracijama serotonina nakon izlaganja tvarima koje djeluju putem 5HT receptora temeljito su proučene u razvojnom modelu hiperserotoninemije (Hadjikhani, 2010.). Posljedice perinatalne izloženosti eksperimentalnih životinja i ljudi SSRI-ima, koji djeluju putem transportera 5HT, također su temeljito istražene (Nijenhuis i sur., 2012.). Međutim, vrlo se malo zna o dugoročnim posljedicama perinatalnih promjena u serotoninskom metabolizmu. Stoga smo u svojim istraživanjima farmakološki promijenili metabolizam serotonina u vrijeme perinatalnog razvoja mozga primjenom 5HTP koji potiče sintezu serotonina ili TCP koji onemogućuje razgradnju serotonina.

2) U literaturi ne postoje podaci o dugo(trajnom) učinku povišenih koncentracija serotonina tijekom razvoja mozga na ekspresiju gena koji reguliraju funkciju 5HT i to je po prvi puta istraženo u ovoj disertaciji.

3) Tretman je izvršen tijekom prenatalnog (od 13. gestacijskog dana do okota) i ranog postnatalnog (od okota do 21. postnatalnog dana) razvoja mozga, čime je posve obuhvaćeno razdoblje intenzivnog razvoja serotonergičnih neurona u štakora (Lauder, 1990.).

4) Tvari su injicirane subkutano, u nabor kože zatiljka, čime se mogućnost oštećenja fetusa za vrijeme prenatalne primjene izbjegla, a mogućnost ozljede mladunaca svela na najmanju moguću mjeru.

5) Spoznaja da 5HTP uzrokuje značajne promjene homeostaze 5HT samo u perifernom, a TCP u oba odjeljka, omogućila nam je usporedbu dugotrajnih promjena u homeostazi serotonina nakon razvojne neuravnoteženosti inducirane u perifernom odjeljku 5HT naspram one inducirane u središnjem odjeljku 5HT i testiranje hipoteza o hiperserotoninemiji kao uzroku ili kao biljegu promjena 5HT u mozgu.

6) Činjenica da se obje tvari koriste za povišenje razine serotonina u ljudi, uključujući i trudnice, doprinosi relevantnosti dobivenih rezultata za humanu populaciju.

Ovdje moramo spomenuti i glavni nedostatak istraživanja, a to je činjenica da TCP inhibicijom obje izoforme enzima MAO povisuje i razinu katekolamina, što može utjecati na jasnoću interpretacije dobivenih rezultata. Međutim, s obzirom da se 5HT razgrađuje isključivo oksidativnom deaminacijom, a da se katekolamini mogu razgraditi i djelovanjem

katekol-O-metil-transferaze (koja dominira nad MAO u prefrontalnom korteksu), pretpostavljamo da su promjene u razini dopamina i noradrenalina bile znatno manje nego promjene u razini serotonina. Tome u prilog govore i podaci iz literature o drastičnim promjenama u razvoju serotonergičnih, ali ne i katekolaminergičnih živčanih završetaka nakon primjene inhibitora MAO A i MAO B tijekom gestacije (Whitaker-Azmitia i sur., 1994.), te o dvostrukom porastu razine katekolamina u odnosu na osmerostruki porast razine serotonina u miševa s inaktiviranim genima za MAO A/B (Chen i sur., 2004.).

3.2. Posljedice perinatalne izloženosti povećanoj sintezi serotonina

3.2.1. Neposredni učinak 5HTP

Opisana primjena 5HTP je bila vrlo učinkovita u izazivanju hiperserotoninemije, pri čemu su koncentracije 5HT u krvi eksperimentalnih životinja bile u prosjeku 200% veće nego u krvi kontrolnih životinja tretiranih fiziološkom otopinom (v. radove 1 i 3). Međutim, primjena nije izazvala značajni porast razine 5HT u mozgu, mjereno u prefrontalnoj kori (v. rad 3). Ova periferna, no ne i središnja značajna promjena razine serotonina mogla je biti uzrokovana odabranom dozom i načinom primjene 5HTP, koji su efikasnije potaknuli konverziju u 5HT i njegovo skladištenje na periferiji nego u mozgu. Druga mogućnost je da su kompenzatorni mehanizmi u mozgu bili mnogo efikasniji u otklanjanju prekomjernog novosintetiziranog serotonina nego što je to bilo na periferiji, na što ukazuju podaci iz literature o promjeni koncentracije serotonina unutar prvih sati nakon primjene 5HTP, a koja se 24h nakon injekcije vraća na bazalne razine (Lynn-Bullock i sur., 2004.; Sémont i sur., 2000.). Razvojna hiperserotoninemija bila je privremena pa se u odrasloj dobi srednja vrijednost razine 5HT u krvi eksperimentalnih životinja nije razlikovala od srednje vrijednosti u krvi kontrolnih životinja (v. rad 3).

Tijekom primjene 5HTP došlo je i do drugih fizioloških posljedica. Tako su porođajna masa i stopa preživljenja bile niže u eksperimentalnih nego u kontrolnih životinja, iako je broj mladunaca po okotu bio jednak. Moguće je da su ove promjene posljedica indirektnog povišenja razine 5HT u mozgu mladunaca, izazvanog hiperserotoninemijom, koje je moglo negativno utjecati na razvoj fetalnog mozga. Značajne razlike u tjelesnoj masi koje su se održavale tijekom rasta sve do odrasle dobi, govore u prilog promjenama u središnjoj serotonergičnoj transmisiji tretiranih životinja, koje su se mogle očitovati smanjenim unosom hrane i/ili pojačanim metabolizmom. Korelacija između razine serotonina i tjelesne mase

dokumentirana je u ljudi i životinja (Broqua i sur., 1992.; Curzon, 1990.; Fletcher, 1988.; Halford i sur., 2005.; Hranilovic i sur., 2005.; Pollock i Rowland, 1981.). Druga je mogućnost da je zbog kroničnog tretmana majki došlo do smanjenog protoka krvi u maternici i posteljici s obzirom da serotonin regulira tonus krvnih žila. Smanjena opskrba krvlju mogla se negativno odraziti na fetalni razvoj, kao što je to pokazano u ljudi (Duvekot i sur., 1995.; Salas i sur., 2006., 2007.), te uzrokovati povećanu smrtnost, odnosno nižu tjelesnu masu u tek okoćenih mladunaca.

3.2.2. Dugoročne posljedice perinatalne primjene 5HTP

S obzirom na uočene direktne posljedice primjene 5HTP, a to je izražena hiperserotoninemija bez značajnih promjena u središnjem odjeljku 5HT, smatrali smo da je ovaj model prikladan za ispitivanje teorije o hiperserotoninemiji kao uzroku razvojnih poremećaja u središnjem serotoninskom odjeljku (Whitaker-Azmitia, 2005.). Stoga smo u odraslih životinja istražili razinu i metabolizam serotonina te ekspresiju 5HT-regulatornih gena u središnjem odjeljku, kao i ponašanje regulirano serotoninom.

S neurokemijskog stanovišta, u prefrontalnoj kori je došlo do blagog, ali značajnog smanjenja razine serotonina, bez promjena u njegovom metabolizmu. Pretpostavljamo da je u prefrontalnoj kori došlo do gubitka serotonergičnih završetaka uslijed perinatalne hiperserotoninemije. Naime, inhibitorna uloga serotonina na razvoj vlastitih neurona pokazana je u kulturi stanica (Whitaker-Azmitia i Azmitia, 1986.) kao i u životinjskim modelima nakon farmakološke primjene agonista receptora 5HT, 5-metoksitriptamina (Shemer i sur., 1991.); prekursora 5HT, triptofana (Thomke i sur., 1992.); kombinacija selektivnih inhibitora MAO A i MAO B (Whitaker-Azmitia i sur., 1994.) te inhibitora ponovnog unosa 5HT (Cabrera-Vera i sur., 1997.).

Određivanje ekspresije gena koji reguliraju funkciju 5HT (v. rad 5) sugerira da je kronično povišenje razine serotonina tijekom tretmana prvenstveno uzrokovalo povećanu ekspresiju gena za MAO A i MAO B u jezgrama rafe te da je navedena promjena u ekspresiji bila dovoljna da se izbjegnu značajna povećanja razine 5HT u mozgu tijekom razvoja. Ekspresija ovih gena ostala je, međutim, trajno povećana što vjerojatno doprinosi opaženoj smanjenoj razini serotonina u prefrontalnoj kori odraslih štakora. U ovih je životinja uočena i indikativno povećana razina ekspresije gena za enzim TPH2, kojom se vjerojatno pokušava kompenzirati povećana razgradnja 5HT u tijelima neurona i/ili smanjena koncentracija serotonina u aksonskim završetcima.

Bihevioralne studije pokazale su da je perinatalna primjena 5HTP rezultirala povećanom eksploratornom aktivnosti (istraživanje nove okoline i novih predmeta) u odrasloj dobi (v. rad 4). Stupanj eksploratorne aktivnosti smatra se jednim od aspekata anksioznog ponašanja pa možemo zaključiti da je primjena 5HTP djelovala blago anksiolitički. S obzirom da, u pravilu, smanjene koncentracije serotonina dovode do pojačanog odgovora na podražaj iz okoline (Lucki, 1998.), opažena promjena u ponašanju u skladu je sa sniženom koncentracijom serotonina izmjerenom u našim neurokemijskim istraživanjima. Smanjeni stupanj anksioznog ponašanja također je uočen pri oslabljenoj serotonergičnoj funkciji izazvanoj farmakološkim tretmanom (Artaiz i sur., 1998.; Borsini i sur., 2002.; Kshama i sur., 1990.), ozljedom (Ciobica i sur., 2010.; Darlington i sur., 2009.), inaktivacijom gena (Mosienko i sur., 2012.) ili usmjerenom selekcijom (Hranilovic i sur., 2005.).

Zaključujemo da je razvojna hiperserotoninemija uzrokovana primjenom 5HTP zaista dovela do (dugo)trajnih posljedica po razvoj i funkciju središnjeg serotoninskog odjeljka, premda su one vrlo blage i diskretne.

3.3. Posljedice perinatalne izloženosti smanjenoj razgradnji serotonina

3.3.1. Neposredni učinak TCP

Primjena TCP izazvala je značajni porast koncentracije serotonina, ne samo u perifernom odjeljku (krvi), nego i u središnjem odjeljku (prefrontalna kora) (v. rad 3). Ovaj rezultat ukazuje na učinkovitu inhibiciju enzima MAO u oba odjeljka i u skladu je s literaturnim podacima o povišenju razine serotonina u prefrontalnoj kori i u jezgrama rafe nakon kronične primjene TCP u odraslih životinja (Ferrer i Artigas, 1994.).

Prenatalni dio tretmana uzrokovao je smanjeni broj okoćenih mladunaca po majci kao i nisku stopu preživljenja s relativnim rizikom ugibanja od čak 1,9 (v. rad 2). Dosta mladunaca je okoćeno mrtvo ili je uginulo unutar 24h nakon okota. Zanimljivo, prosječna tjelesna masa preživjelih mladunaca tretiranih TCP-om nije se razlikovala od one u kontrolnih mladunaca. U literaturi nema podataka o utjecaju TCP na tijek trudnoće u ljudi ili štakora, iako je veći perinatalni mortalitet uočen u miševa perinatalno tretiranih klorgilinom, inhibitorom MAO A (Vitalis i sur., 1998.). Povišene razine 5HT, kao i drugih monoamina, uzrokovane primjenom TCP, mogle su utjecati na razvoj mozga što se zatim moglo odraziti na ugibanje u kasnoj fetalnoj i ranoj neonatalnoj dobi. Međutim, kao i pri primjeni 5HTP, i

ovdje je, zbog visokih perifernih razina 5HT, moglo doći do vazokonstrikcije žila maternice i posteljice te negativnog utjecaja na razvoj fetusa (Duvekot i sur., 1995.; Salas i Rosso, 1998.; Salas i sur., 2006.).

Iako je anoretički efekt 5HT uočen u odraslih štakora nakon primjene različitih agonista 5HT (Curzon, 1990.; Fletcher, 1988.; Halford i sur., 2005.; Hranilovic i sur., 2005.; Pollock i Rowland, 1981.) mladunci su u doba primjene TCP imali značajno veću masu nego kontrolni štakori, vjerojatno zbog značajno manjeg broja mladunaca po leglu. Međutim, nakon odvajanja od majki, mladunci iz eksperimentalne skupine sporije su dobivali na tjelesnoj masi (v. rad 2) što ukazuje na dugoročne posljedice primjene TCP u središnjem odjeljku serotonina.

3.3.2. Dugoročne posljedice perinatalne primjene TCP

Kako je primjena TCP uzrokovala značajno povišenje koncentracije serotonina i u krvi i u mozgu, ovaj smo model smatrali prikladnim za ispitivanje teorije o istovremenoj disregulaciji serotoninskih elemenata u središnjem i perifernom odjeljku, koja hiperserotoninemiju smatra biljegom, a ne uzrokom središnje promjene homeostaze 5HT (Janusonis, 2005.).

(Dugo)trajne posljedice primjene TCP uočene su u oba odjeljka 5HT. Životinje su bile hiperserotoninemične i sedam tjedana nakon završetka tretmana (v. radove 2 i 3), što ukazuje na trajnu promjenu homeostaze u perifernom serotoninskom odjeljku. S obzirom da je značajno povišena razina serotonina uočena u trombocitima (161% srednje vrijednosti kontrolne skupine) dok je u plazmi ona bila indikativno niža, pretpostavljamo da je u odraslih životinja hiperserotoninemija rezultat povećanog unosa 5HT u trombocite (Anderson i sur., 1987.), a ne promjena u perifernom metabolizmu serotonina.

Još značajniji učinci primjene TCP uočeni su u mozgu eksperimentalnih životinja i u jezgrama rafe, u kojima su smještena tijela neurona i u prefrontalnoj kori, u kojoj se nalaze aksonski završetci. U obje promatrane regije razina serotonina bila je trajno smanjena, a njegov metabolizam značajno promijenjen. Nizak omjer 5HT/Trp ukazao je na smanjenu sintezu i/ili pohranu novosintetiziranog serotonina, a visok omjer 5HIAA/5HT na njegovu povećanu razgradnju (v. rad 3). Budući da se TCP ireverzibilno veže za obje izoforme enzima MAO, sustav je morao sintetizirati nove enzime kako bi razgradio neurotransmitere. Ekspresijske studije ukazuju na to da se nedostatak razgradnog enzima pokušao kompenzirati povećanom ekspresijom gena za enzime MAO u regiji jezgara rafe, dok se višak serotonina u

sinapsi pokušao kompenzirati povećanom ekspresijom gena za 5HTt (uklanjanje iz sinapse) i VMAT (transport u vezikule) i smanjenom ekspresijom gena za postsinaptičke receptore $5HT_{1A}$ (slabija aktivacija post-sinaptičkog neurona) u prefrontalnoj kori (v. rad 5). Pošto su životinje svaki dan dobivale novu dozu TCP, ova prilagodba nije bila dovoljna da se održi homeostaza 5HT tijekom razvoja mozga. Geni za enzime MAO ostali su pojačano eksprimirani dugo nakon što je TCP prestao djelovati (prema Planzu i sur. (1972.), tri tjedna od završetka tretmana) i vjerojatno predstavljaju glavni razlog izrazito niskoj koncentraciji 5HT uz nepromijenjenu koncentraciju 5HIAA u mozgovima eksperimentalnih u odnosu na kontrolne životinje. Uz to, i trajno promijenjena ekspresija gena za 5HTt, VMAT i $5HT_{1A}$r vjerojatno doprinosi narušavanju 5HT-homeostaze u odrasloj dobi, dok je indikativno povećana ekspresija TPH2 nastoji kompenzirati.

Perinatalna primjena TCP imala je snažan anksiolitički učinak na ponašanje u odrasloj dobi u smislu smanjenja tigmotaksije, socijalne anksioznosti i reakcije na akutni stres (v. rad 4). Rezultati su opet u skladu s reduciranom funkcijom 5HT u prefrontalnoj kori, kao što je bio slučaj i kod životinja tretiranih 5HTP-om, samo što je učinak bio znatno jači te je zahvatio druge aspekte anksioznog ponašanja. Jedan uzrok različitom djelovanju 5HTP i TCP na ponašanje može biti efekt doze – značajna redukcija u koncentraciji moždanog serotonina nakon primjene TCP ublažila je jače anksiogene komponente ponašanja poput izlaganja otvorenim prostorima, stresnom podražaju ili drugoj jedinki, dok je blaga redukcija u koncentraciji serotonina u mozgu životinja tretiranih 5HTP-om utjecala na slabije anksiogenu komponentu poput istraživanja okoline ili nepoznatog objekta. Drugi uzrok može biti promjena u razinama dopamina (DA) i noradrenalina (NA), koji reguliraju motoričke i limbičke funkcije, odnosno budnost i reaktivnost na podražaje iz okoline. Ovaj učinak izaziva TCP zbog inhibicije enzima koji sudjeluje u razgradnji svih monoamina, dok 5HTP djeluje samo na serotoninski sustav. Naši rezultati u skladu su s rezultatima istraživanja na ljudskoj populaciji koji pokazuju kako genotip s visokom aktivnošću MAO-A djeluje zaštitno protiv razvijanja antisocijalnog ponašanja (Caspi i sur., 2002.), no razlikuju se od rezultata dobivenih nakon prenatalnog i neonatalnog izlaganja štakora SSRI-ma koji su također promijenili biokemijski profil 5HT u jezgrama rafe (Maciag i sur., 2006.), ali su djelovali ili anksiogeno, u smislu smanjene eksploratorne aktivnosti (Ansorge i sur., 2008.; Rodriguez Echandia i Broitman, 1983.) i socijalnog ponašanja (Rodriguez Echandia i Broitman, 1983.), ili nisu izazvali promjene u tigmotaksiji (Ansorge i sur., 2008.; Popa i sur., 2008.). Razlika između naših i literaturnih podataka se može pripisati različitim načinima izazivanja visoke razine serotonina, tj. sinaptičkom učinku SSRI naspram presinaptčkom učinku 5HTP i TCP.

Životinje tretirane TCP-om također su pokazale povećanu kognitivnu fleksibilnost u vidu značajno bržeg obrata naučenog (engl. *reversal learning*). Iako je poznato da 5HT sudjeluje u ovom procesu (Clark i sur., 2004.), efekt smanjenja 5HT-funkcije na kognitivnu fleksibilnost u provedenim farmakološkim studijama je kontradiktoran: od smanjenja (Clarke i sur., 2004.; Mazer i sur., 1997.), preko nedostatka utjecaja (Arnsten i sur., 1997.) pa do poboljšanja mogućnosti obrata naučenog (Domeney i sur., 1991.). Naravno i u ovaj vid ponašanja je moguća uključenost drugih monoamina čija je razina vjerojatno promijenjena primjenom TCP.

Zaključujemo da je direktno povišenje razine 5HT u mozgu (uz ono indirektno koje je posljedica hiperserotoninemije), uzrokovano primjenom TCP, dovelo do značajnih (dugo)trajnih posljedica po razvoj i funkciju središnjeg serotoninskog odjeljka, u vidu drastičnog smanjenja koncentracije serotonina u mozgu, pojačane ekspresije i funkcije metaboličkog enzima te snažnog anksiolitičnog fenotipa.

3.4. Mogući značaj dobivenih rezultata za humanu populaciju

Poznato je da odstupanje od optimalnih koncentracija monoamina tijekom razvoja mozga može imati utjecaj na funkcije odraslog mozga (Thompson i Stanwood, 2009.). S jedne strane, pretpostavlja se da bi promjene u serotonergičnoj transmisiji mogle biti podloga raznih poremećaja ponašanja, kao što su autizam, ovisnost o alkoholu ili suicidalno ponašanje (Lesch i Moessner, 1998.). S druge strane, uzimanje pripravaka kao što su antidepresivi ili droge koje povisuju razine 5HT tijekom trudnoće, moglo bi kasnije uzrokovati poremećaje u ponašanju potomstva. Dugoročne, bihevioralne, stanične i molekularne promjene uočene su u životinjskim modelima, kao i kod djece, perinatalno izloženima selektivnim inhibitorima ponovnog unosa 5HT, kokainu, amfetaminu, MDMA, p-klorofenilalaninu i 5-metoksitriptaminu (Baum i Misri, 1996.; Borue i sur., 2007.; Frederick i Stanwood, 2009.; Henderson i McMillen, 2005.; Kelly i sur., 2002.; Lauder i sur., 2000.; Pawluski, 2012.). U sklopu ove doktorske disertacije, po prvi put je pokazano postojanje značajnih promjena u fiziologiji, biokemiji, ekspresiji gena i ponašanju uslijed kroničnih perinatalnih primjena 5HTP ili TCP. Dobiveni rezultati mogli bi biti značajni za humanu populaciju iz sljedećih razloga.

Prvo, činjenica da su promjene u sintezi ili razgradnji 5HT tijekom razvoja inducirale trajne promjene u središnjoj homeostazi serotonina, sugerira moguću ulogu gena koji

reguliraju metabolizam serotonina u poremećajima ponašanja povezanim s 5HT. Doista, naša istraživanja u osoba oboljelih od autizma pokazala su da je poremećena homeostaza 5HT vezana uz promjene metabolizma, a ne ponovnog unosa 5HT (Hranilovic i sur., 2008., 2009.).

Drugo, razvojna neuravnoteženost 5HT inducirana u središnjem odjeljku (primjena TCP) vodi ka znatno jačim trajnim promjenama homeostaze 5HT u odnosu na razvojnu neuravnoteženost 5HT inducirana samo na periferiji (primjena 5HTP). Stoga se čini da sama hiperserotoninemija nije dovoljna za induciranje ozbiljnih poremećaja serotonergične funkcije kakvi su opaženi u određenim psihijatrijskim bolestima, što govori u prilog teoriji o hiperserotoninemiji kao o biljegu, a ne uzročniku promjena u središnjoj homeostazi serotonina (Janusonis, 2005.).

Također, važno je naglasiti da je, fetalna/neonatalna, a vjerojatno i majčinska hiperserotoninemija uzrokovana primjenom 5HTP, ipak bila dovoljna da izazove mjerljive promjene u središnjem odjeljku 5HT na molekularnoj i bihevioralnoj razini. Kako su dugoročne posljedice perinatalne izloženosti 5HTP-u (Mokler i sur., 1992.; Salas i sur., 2007.) slabo istražene, a još manje se zna o posljedicama primjene TCP, smatramo da je nužno provesti daljnja istraživanja njihovog utjecaja kako na životinjskim modelima, tako i u humanoj populaciji, kako bi se preispitala sigurnost primjene ovih spojeva u trudnica i dojilja.

4. ZAKLJUČCI

1) **Perinatalna primjena** neposrednog **prekursora sinteze serotonina** (25mg/kg 5HTP), ili **inhibitora razgradnje serotonina** (2mg/kg TCP), uzrokovala je **značajni poremećaj homeostaze serotonina**:

- 5HTP je izazvao hiperserotoninemiju bez značajnih promjena razine 5HT u mozgu
- TCP je, uz hiperserotoninemiju, izazvao i značajni porast razine 5HT u mozgu

To nam je omogućilo **usporedbu dugotrajnih promjena u 5HT-homeostaz**i **nakon razvojne neuravnoteženosti** inducirane u **perifernom** odjeljku 5HT **naspram** one inducirane u **središnjem 5HT-odjeljku**.

2) U mozgu koji se razvijao u uvjetima povišene koncentracije serotonina došlo je do **(dugo)trajnih posljedica na funkciju središnjeg serotoninskog odjeljka** koje su se očitovale **u**:

a) **Razini i metabolizmu serotonina**

- Primjena **5HTP** je uzrokovala **blago**, ali značajno **smanjenje razine serotonina** samo **u prefrontalnoj kori**, bez promjena u njegovom metabolizmu, do koje je vjerojatno došlo zbog gubitka serotonergičnih završetaka uslijed perinatalne hiperserotoninemije.
- Primjena **TCP** je uzrokovala **drastično smanjenje razine serotonina** i **promjenu njegovog metabolizma** u regiji **jezgra rafe** i **prefrontalnoj kori**. Nizak omjer 5HT/Trp ukazuje na smanjenu sintezu i/ili pohranu novosintetiziranog serotonina, a visok omjer 5HIAA/5HT na njegovu povećanu razgradnju.

b) **Ekspresiji 5HT-regulirajućih gena**

- Životinje tretirane **5HTP**-om imaju **pojačanu ekspresiju gena za MAO A i MAO B** u regiji jezgara rafe
- Životinje tretirane **TCP**-om imaju **pojačanu ekspresiju gena za MAO A i MAO B** u regiji jezgara rafe te **pojačanu ekspresiju gena za 5HTt i VMAT** i **smanjenu ekspresiju gena za $5HT1_a$r** u prefrontalnoj kori.
- Činjenica da je zajednički učinak oba tretmana značajno povećana ekspresija gena za MAO A i MAO B upućuje na zaključak da **promjene u ekspresiji gena za razgradni enzim predstavljaju „prvu liniju obrane" od kronično visokih razina 5HT**, bez obzira jesu li inducirane povećanom sintezom ili smanjenom razgradnjom, te da **pri značajno povišenim koncentracijama serotonina obje izoforme igraju ulogu u njegovoj razgradnji.**

c) **Ponašanju reguliranom serotoninom**

- Životinje tretirane **5HTP**-om pokazale su **blago anksiolitički profil**, koji se očitovao u značajno povećanom eksploratornom ponašanju.
- Životinje tretirane **TCP**-om pokazale su **snažan anksiolotički profil** u smislu smanjenja tigmotaksije, socijalne anksioznosti i reakcije na akutni stres te **povećanu kognitivnu fleksibilnost**.

3) **Dobiveni rezultati upućuju** na sljedeće zaključke:

- S obzirom da razvojni poremećaj u metabolizmu serotonina (dugo)trajno narušava funkciju središnjeg serotoninskog odjeljka, **gene koji reguliraju metabolizam 5HT** trebalo bi uzeti u obzir kao **moguće kandidate** u istraživanjima **poremećaja ponašanja vezanima uz sustav 5HT.**

- Perinatalne primjene **5HTP** i **TCP** inducirale su **analogne (dugo)trajne promjene u mozgu** tretiranih životinja na biokemijskoj (snižena razina 5HT), ekspresijskoj (pojačana ekspresija gena za MAO) i bihevioralnoj (anksiolitički učinak) razini, koje **odražavaju efekt doze**: središnja homeostaza 5HT je diskretno narušena u životinja s razvojnim promjenama samo u perifernoj homeostazi 5HT (5HTP), a opsežno u životinja s razvojnim promjenama i u središnjoj i u perifernoj homeostazi 5HT (TCP).

- Opaženi efekt doze upućuje na zaključak da je **za ozbiljno narušavanje serotonergične funkcije**, kakvo je opaženo u određenim psihijatrijskim bolestima, **nužna razvojna neuravnoteženost u središnjem odjeljku 5HT** i govori u prilog teoriji o **hiperserotoninemiji** kao **biljegu**, a ne uzročniku **promjena u središnjoj homeostazi serotonina**.

- Ipak, fetalna/neonatalna (i majčinska) **hiperserotoninemija** kojoj je tijekom razvoja bio izložen mozak životinja tretiranih 5HTP-om, bila je **dovoljna** da u odrasloj dobi uzrokuje **mjerljive promjene u središnjem odjeljku 5HT**, što ukazuje na potrebu za ispitivanjem mogućih **posljedica uporabe 5HTP i TCP** u žena **tijekom trudnoće i dojenja**.

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6. ŽIVOTOPIS AUTORA

Sofia Ana Blažević, rođena 30.05.1983. u Buenos Airesu, treće je od petero djece Josipa Mariana Blaževića i Vere Katarine Crnko. U Buenos Airesu je pohađala osnovnu školu do 10. godine života kad se cijela obitelj preselila u Puerto Rico gdje je završila osnovnu i srednju školu u Baldwin School of Puerto Rico. Studij Biologije je započela 2001. na Sveučilištu u Navarri, Pamplona, Španjolska. U Hrvatsku se preselila 2003. te na Sveučilištu u Zagrebu završava studij molekularne biologije. Diplomski rad pod naslovom „*Blokiranje signalnog puta Hh-Gli u primarnim kulturama dermoida ovarija*" izradila je pod vodstvom dr. sc. Sonje Levanat u Laboratoriju za nasljedni rak, Zavoda za molekularnu medicinu Instituta Ruđer Bošković. Od siječnja 2008. radi kao znanstvena novakinja-asistent na projektu prof. dr. sc. Dubravke Hranilović u Zavodu za animalnu fiziologiju Biološkog odsjeka, Prirodoslovno-matematičkog fakulteta Sveučilišta u Zagrebu. Te iste godine upisuje poslijediplomski doktorski studij Biologije pri Sveučilištu u Zagrebu. Sudjeluje u izvedbi nastave predmeta: „Neurofiziologija", „Neurofiziologija i endokrinologija", „Metode u imunologiji" i „Laboratorijske životinje u biološkim istraživanjima". Godine 2012., magistrirala je Bioetiku na Sveučilištu u Navarri u Pamploni, Španjolska s radom pod naslovom „*Epidemiological or social factors that contribute to the increase in autism incidence: epigenetics of the brain"*. Pohađala je tečajeve *Master course Advanced qPCR Techniques for Publication Success: Following MIQE Recommendation* (EMBL, Heidelberg); *Course in Laboratory Animal Science* (FELASA-C; Sveučilište u Uppsali, Švedska); te tečaj Bioinformatike (Sveučilište u Zagrebu). Koautorica je 7 znanstvenih radova, jednog poglavlja u knjizi i 13 sažetaka u zbornicima skupova te bila neposredni voditelj 5 diplomskih radova.

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