JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY 2015, 66, 4, 529-537
www.jpp.krakow.pl

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MOLECULAR BACKGROUND AND PHYSIOLOGICAL CONSEQUENCES OF ALTERED PERIPHERAL SEROTONIN HOMEOSTASIS IN ADULT RATS PERINATALLY TREATED WITH TRANYLCYPROMINE

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Serotonin (5-hydroxytryptamine, 5-HT) is a biologically active molecule present in mammals in the brain and peripheral tissues where it exerts many physiological functions. Developmental exposure to 5-HT-enhancing agents has been reported to induce long-lasting changes in the brain, but the long-term effects of perinatal 5-HT enhancement on 5-HT balance and function in the peripheral compartment have not been explored. Perinatal treatment of rats with monoamine oxidase (MAO) inhibitor tranylcypromine (TCP), leads to persistent imbalance in central (increased 5-HT degradation and decreased 5-HT concentrations in the brain) and peripheral (increased platelet and decreased plasma 5-HT concentrations) 5-HT homeostasis. In this study, we explored the molecular background of peripheral 5-HT imbalance, and its possible consequences on bone remodeling and hematopoiesis. Jejunum, liver and blood samples were collected from TCP- and saline-treated rats on post-natal day 70. Relative mRNA levels for tryptophan hydroxylase 1 (TPH1) and MAO A were analyzed using quantitative RT-PCR, femoral trabecular bone parameters were measured using micro-computed tomography, while peripheral blood cell number was determined by cell counter. TCP-treated rats displayed significant decrease in expression of *Tph1*, and significant increase in percentage of bone volume, trabecular number, connectivity density, and leukocyte number. In addition, significant negative correlation was observed between relative concentrations of TPH1 mRNA and trabecular bone parameters. Our results: a) show that perinatal exposure to tranylcypromine leads to long-lasting compensatory decrease in *Tph1* expression in the peripheral compartment, accompanied with alterations in bone remodeling and hematopoiesis, b) suggest that peripheral and central 5HT compartment use different strategies to compensate for 5-HT imbalances of the same cause, and c) indicate dominant role of peripheral over central 5-HT in the regulation of bone maintenance, as well as possible negative *in vivo* influence of peripheral 5-HT on leukocyte development and/or sustainment.

Key words: *serotonin, tryptophan hydroxylase, bone remodeling, hematopoiesis, monoamine oxidase, platelets*

INTRODUCTION

Serotonin (5-HT) is present in the mammalian organism in two so called compartments - central, which refers to the central nervous system, and peripheral, which refers to somatic tissues. Since the two compartments are separated by the blood-brain-barrier, 5-HT synthesis, degradation and action are independently controlled in each of them by 5-HT-regulating proteins. 5-HT synthesis starts with the conversion of L-tryptophan into 5-hydroxytryptophan (5-HTP) by the rate-limiting enzyme tryptophan hydroxylase (TPH), followed by reduction into 5-HT by aromatic L-amino acid decarboxylase. Two TPH isoforms have been identified: TPH2 in the neuronal tissues and TPH1 in the somatic tissues and pineal gland (1). 5-HT is degraded into 5-hydroxy-3-indoleacetic acid through the oxidative deamination catalyzed by the enzyme monoamine oxidase (MAO), preferentially by its MAO A isoform (2).

In the central 5-HT compartment, 5-HT synthesizing neurons are clustered in discrete regions of the brain stem, projecting their axons into various cortical and subcortical regions of the brain. In the developing brain, 5-HT acts as a neurodevelopmental signal regulating outgrowth of its own neurons and maturation of target regions (3), while later it modulates brain function and plasticity by acting as a neurotransmitter at the serotonergic synapse (4).

Midbrain 5-HT is also considered to be involved in the regulation of mammalian biological rhythms, as activation of 5-HT receptors was shown to modulate the activity of single neurons isolated from the intergeniculate leaflet of the thalamus, a structurally and functionally important component of the rodent biological clock (5).

In the peripheral compartment, most 5-HT is synthesized in the enterochromaffin cells of the intestinal mucosa, stored in secretory granules through the active transport by the monoamine vesicular transporter, and released into portal circulation *via* 5-HT receptor mediated exocytosis (6). Most of the released 5-HT is actively accumulated in platelets through the action of the membrane-bound serotonin transporter (5-

HTT), and is used to promote platelet aggregation during the blood clotting process. The remaining, free plasma 5-HT can exert hormonal actions by activating 5-HT receptors (HTR) located on various peripheral tissues. Circulating 5-HT is catabolized primarily in the endothelial cells of lungs and liver. Peripheral 5-HT is mostly known as a mediator of cardiovascular and gastro-intestinal functions (7). However, the recent discovery of local 5-HT synthesis in several organs (e.g. mammary gland, pancreas, and bone) points to an additional paracrine/autocrine function of this monoamine (8).

There has been growing evidence for the role of 5-HT in the regulation of bone remodeling, although the relationship between the actions of the central, peripheral and locally-synthesized 5-HT is far from clear (9, 10). Inverse relationship between serum 5-HT concentrations and bone mineral density and structure in women (11), and negative effect of selective serotonin reuptake inhibitors (SSRI) on bone mineral density (12-14) were observed. Yadav *et al.* (15) proposed that free plasma 5-HT levels, originating from enterochromaffin cells, negatively control osteoblast proliferation and bone formation by acting through the HTR_{1B}-CREB signaling pathway. This theory was supported by observations of a high bone mass phenotype in *Tph1* and *Htr1b* knock-out mice. On the other hand, Chabbi-Achengli *et al.* (16) provided evidence that osteoclast precursors express TPH1 and synthesize serotonin which promotes osteoclastogenesis. They showed markedly decreased bone resorption in *Tph1* knock-out mice, as well as decreased osteoclast differentiation in their spleen and bone-marrow cell cultures, that could be rescued through the addition of serotonin. Findings of a decrease in bone formation parameters and an increase in bone resorption parameters in *Tph2*-defficient mice added to the complexity of the picture by introducing a role of the central 5-HT in bone accrual (17).

Hematopoiesis is a complex yet unraveled process influenced by the microenvironment of hematopoietic stem and progenitor cells (18, 19), as well as by the nervous system, involving mostly the sympathetic innervation of the bone marrow parenchyma (20, 21). Observations of simultaneous decrease in hematopoiesis and trabecular bone structure in aplastic anemia and primary osteoporosis in the human population (22) were explained by animal model studies which located hematopoietic stem cells within a trabecular region of the bone, where osteoblasts seem to play a critical role in their sustainment (23, 24). Indirectly, by affecting osteoblast function, or directly, by acting through HTRs expressed on hematopoietic cells (25), serotonin might represent one of the regulators of hematopoiesis. Effects of 5HT on the promotion of development of $CD34^+$ cells into multilineage committed progenitors *in vitro* (26), as well as stimulation of megakaryocytopoiesis and erythropoiesis (27-29) have been reported, while its effect on lymphocyte proliferation seems to be inhibitory (30, 31).

In order to study the consequences of perinatal exposure to increased 5-HT levels on central 5-HT homeostasis, we have previously treated developing Wistar rats with the immediate 5-HT precursor 5-HTP, which significantly raised peripheral but not central 5-HT concentrations, or the MAO inhibitor tranylcypromine (TCP), which induced significant 5-HT elevations in both compartments (32). We have shown that the exposure of the developing brain to increased 5-HT concentrations induced long-lasting alterations in central 5-HT homeostasis, as reflected in decreased 5-HT concentrations (32), accompanied with anxiolytic behavior (33), probably caused by increased MAO expression/activity (34). All of the mentioned 5-HT-related changes were much more pronounced in TCP-treated than in 5-HTP-treated animals. In the peripheral compartment, 5-HT homeostasis in 5-HTP-treated rats was re-established after a wash-out period, while TCP-treated rats remained hyperserotonemic at adult age (32).

In the present study, we explored the molecular background and physiological consequences of altered peripheral 5-HT homeostasis in TCP-treated animals. Relative mRNA levels for genes regulating 5-HT metabolism were analyzed using quantitative (q) RT-PCR. Femoral trabecular bone parameters were measured using micro-computed tomography (micro CT) to determine the effects on bone parameters. Finally, peripheral blood cell number was used to indicate possible effects on hematopoiesis.

MATERIALS AND METHODS

Breeding and housing of animals

Nulliparous Wistar females supplied 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" or "TCP" group. Two days before parturition, females were separated and remained singly housed until weaning of the pups at postnatal day (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.

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 and 37/2013). All efforts were made to reduce the number of animals used and to minimize animal suffering.

Pharmacological treatments

Pharmacological treatment is thoroughly described elsewhere (35). In short, the experimental group of pups was treated with 2 mg/kg of the monoamine oxidase inhibitor tranylcypromine (TCP, Sigma-Aldrich, St. Louis, MO, USA) by subcutaneous injections in the nape, of pregnant females from GD12 until parturition, and of the pups from PND1 until PND21. 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.

Collection of tissue samples

A total of 13 TCP-treated and 11 saline-treated animals were sacrificed for tissue sample collection on PND 70 ± 1. However, not all of the samples were used for all measurements. From TCP-treated animals 11 samples (5 females, 6 males) were taken for the measurement of 5-HT concentration, 13 samples (6 males, 7 females) for relative gene expression, and 8 samples (5 females, 3 males) for trabecular bone parameters. From saline-treated animals 10 samples (5 males, 5 females) were used for the measurement of 5-HT concentration, 11 samples (6 males, 5 females) for relative gene expression, and 8 samples (4 males, 4 females) for trabecular bone parameters. Additional blood samples for blood cell counting were taken from male animals only (6 saline-treated and 6 TCP-treated). Since some samples were lost during processing, the final number of samples on which the statistical analyses were performed is given in *Table 1*.

Five ml of blood was withdrawn from the vena cava of an anesthetized animal into syringes preloaded with 1 mL of 3.13% trisodium citrate anticoagulant for the measurement of platelet 5-HT concentrations and intended measurement of platelet 5-HT transporter mRNA expression (unfortunately, quantity of isolated RNA was not sufficient for further processing). Additionally, 1.5 mL of blood was collected from males for hematological analysis. After decapitation, approximately 85 mg of liver and 65 mg of jejunum samples were immediately cut with a scalpel, washed in cold saline, placed in microtubes and frozen in liquid nitrogen for mRNA expression analysis. Complete femoral bone including knee and adjacent muscles were cleaned of surplus fat tissue and skin and fixed in 10% paraformaldehyde for a week. The samples were then transferred to 70% alcohol until analysis.

Blood sample analyses

After thorough mixing, samples were transferred from syringes into microtubes. 5-HT concentrations in platelet-rich plasma (PRP) of adult rats were determined using a commercial enzyme immuno-assay kit (Serotonin EIA kit, Demeditec Diagnostics GmbH, Germany), according to the kit instructions. A calibration curve was drawn based on the absorbance measured at 450 nm on a microplate reader (Microplate reader 550, Bio-Rad) and known concentrations of the standard solutions. Concentration values of samples were obtained by interpolating them to the calibration curve, using 4-parameters non-linear regression curve fitting.

For hematological analysis, samples were transferred from syringes into microtubes. The number of erythrocytes, platelets and leukocytes was determined in an automatic cell counter (Cell-Dyn® 3200, Abbott, USA).

qRT-PCR

Samples were disrupted and homogenized with an ultrasonic homogenizer (Bandelin electronic, Mecklenburg-Vorpommern, Germany) in 500 µL of guanidinium thiocyanate solution and frozen at –80°C until RNA isolation.

Total RNA was isolated using the phenol-free RNAqueous-4PCR kit (Ambion, Inc., Austin, TX, USA), and genomic DNA removed, according to manufacturer's instructions. RNA concentration and quality was measured in a spectrophotometer (Biochrome), and assessed through agarose gel electrophoresis. From 1 µg of total RNA, 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 20 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 *Tph1* (Rn01476869_m1, Applied Biosystems) and *MaoA* (Rn01430961_m1, Applied Biosystems) was assessed through qRT-PCR using the TaqMan gene expression master mix (Applied Biosystems, Foster City, CA, USA) according to manufacturer's instructions. Serial dilutions were used to determine starting cDNA concentrations, to test whether amplification efficiencies in duplex and simplex reactions were the same, and to confirm that the efficiencies of target and reference amplifications were approximately equal. All reactions were performed in a duplex setup with primer limited rat β-actin (ACTB, VIC labelled, 4352340E, Applied Biosystems) or glyceraldehyde 3-phosphate dehydrogenase (GAPDH, VIC labelled, 4352338E, Applied Biosystems) as an endogenous control reference. 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, MGB, FAM labelled, Applied Biosystems), 6 µL of nuclease free H_2O and 2 µL of cDNA in the range of 50 to 60 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, 10 minutes at 95°C, followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. The amplification results were analyzed with the 7300 System SDS v1.4. software (Applied Biosystems, Foster City, CA, USA). The amounts of TPH1 and MAO A mRNA were normalized to endogenous references and expressed as $2^{-\Delta Ct}$, ΔCt being a difference between a reference gene and target gene threshold cycle.

Bone structure analysis

CT analyses were performed using SkyScan 1076 (SkyScan, Belgium) micro CT scanner. Bone samples were scanned at 50kV/200 µA corresponding to a 18 µm spatial resolution throughout 198° with a 0.6° rotation step. To reduce beam hardening a 0.5 mm aluminum filter was employed, while noise reduction was accomplished by setting the frame averaging to a value of two. Collected datasets were reconstructed using NRecon software (SkyScan, Belgium) while data analysis was carried out using CTAn software (SkyScan, Belgium). Trabecular bone was analyzed at the distal femur site in 200 slices with an offset of 100 slices to growth plate to avoid primary spongiosa where it was manually delineated from the cortical bone.

Statistical analysis

Data were processed with GraphPad Instat 3.01 (GraphPad Software, Inc., La Jolla, CA, USA) and JMP 11.2 (SAS Institute Inc., Cary, NC, USA) Software. Due to small sample sizes after stratification for treatment and gender, calculations were performed using parametric tests (two-way analysis of variance or Student's t-test) on logarithmically (ln) transformed data. Values of the integral sample were correlated using Spearman correlation. Values in the text were presented as means (M) ± standard deviations (S.D.). The level of significance was set to 0.05 (two-tail P value).

RESULTS

The influence of treatment and gender on platelet 5-HT concentration, *Tph1* and *MaoA* gene expression and trabecular bone parameters, as well as their possible interaction, was examined by two-way analysis of variance. Mean values of the mentioned parameters for each sub-group are given in *Table 1*.

Platelet serotonin (5-HT) concentration

As expected, the effect of treatment on platelet 5-HT concentration was significant ($F(1,16) = 6.46$, $P < 0.02$), with TCP-treated animals (755 ± 292 ng/mL) displaying higher mean value than the saline-treated (423 ± 290 ng/mL) rats. The effects of gender ($F(1,16) = 0.997$, $P = 0.20$) and of treatment × gender interaction ($F(1,16) = 0.348$, $P = 0.40$) were not significant.

Expression of genes for tryptophan hydroxylase 1 and monoamine oxidase A

Expression of genes encoding TPH1 and MAO A was calculated in relation to the two reference genes, *Gapdh* and *Actb*.

Significant effect of treatment on relative amounts of TPH1 mRNA in jejunum was observed, using either *Gapdh* ($F(1,14) =$

Table 1. Values (M ± S.D.) of measured parameters in the investigated sample of rats stratified by treatment and gender.

		saline		tranilcypromine	
		female	male	female	male
5-HT conc. (ng/mL)	N	5	5	5	5
		304 ± 247	518 ± 312	725 ± 372	780 ± 243
gene expression (AU)	N	4	4	5	5
Tph1/Gapdh		4.49 ± 1.71	6.62 ± 0.69	3.35 ± 1.23	4.32 ± 1.08
Tph1/Actb		1.32 ± 0.38	1.59 ± 1.1	0.67 ± 0.11	0.82 ± 0.32
MaoA/Gapdh		3.23 ± 4.44	0.80 ± 0.99	1.23 ± 1.17	0.37 ± 0.05
MaoA/Actb		2.04 ± 0.94	0.80 ± 0.19	1.49 ± 0.34	0.65 ± 0.05
trabecular parameters	N	4	4	5	3
connectivity density (mm^{-3})		67.4 ± 18.7	47.7 ± 1.8	98.8 ± 6.9	62.6 ± 10.9
trabecular number (mm^{-1})		2.29 ± 0.50	1.80 ± 0.02	3.04 ± 0.23	2.11 ± 0.23
bone volume (%)		20.1 ± 4.8	15.2 ± 0.4	26.9 ± 2.6	18.1 ± 2.3

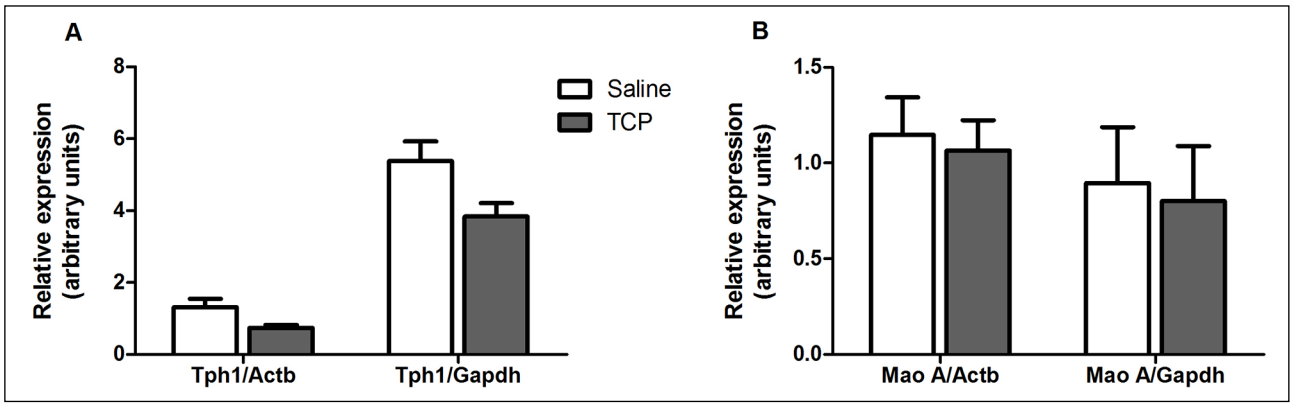

Fig. 1. Relative expression of tryptophan hydroxylase 1 (Tph1) gene in jejunum (A) and monoamine oxydase A (Mao A) gene in liver (B) of rats perinatally treated with saline (N = 8) and tranylcypromine (TCP, N = 10), in reference to beta actin (Actb) and glyceraldehyde 3-phosphate dehydrogenase (Gapdh). Results are shown as Mean ± S.E.M. Two-way ANOVA on logarithmically transformed values revealed significant effect of treatment on *Tph1* expression (P < 0.01 for Tph1/Actb, P < 0.03 for *Tph1/Gapdh*), but not on *MaoA* expression.

6.13, P < 0.03) or *Actb* (F1,14) = 10.58, P < 0.01) as a reference gene. Mean expression of *Tph1* was significantly lower in jejuna of TCP-treated rats than in control rats (*Fig. 1A*). There was also a significant effect of gender on *Tph1* expression when using *Gapdh* as a reference (F(1,14) = 6.02, P < 0.03) with females showing lower level of expression than males. However, this significance was lost when using *Actb* as a reference (F(1,14) = 0.282, P = 0.60), probably due to a high dispersion of values in the male control sample. There was no significant effect of treatment × gender interaction with either reference gene (F(1,14) = 0.333, P = 0.57 for *Gapdh*, and F(1,14) = 0.021, P = 0.89 for *Actb*).

On the other hand, there was no significant effect of treatment on relative amounts of *Mao A* in liver, using either *Gapdh* (F(1,14) = 1.24, P = 0.28) or *Actb* (F(1,14) = 3.56, P = 0.08) as a reference, although mean values of TCP-treated animals were slightly lower than the values in control animals (*Fig. 1B*). The effect of gender on relative expression of the *Mao A* gene was significant in both cases (F(1,14) = 5.31, P < 0.04 using *Gapdh*, and F(1,14) = 47.4, P < 0.01 using *Actb*), with higher mean expression in females than in males. The effect of treatment × gender interaction was not significant with either of reference genes (F(1,14) = 0.173, P = 0.68 for *Gapdh*, and F(1,14) = 0.065, P = 0.80 for *Actb*).

Trabecular bone parameters

Femoral trabecular bone was analyzed in order to check for possible influences of decreased *Tph1* expression on bone remodeling. TCP-treated rats displayed an increase in trabecular structure (*Fig. 2A*) which was reflected in several trabecular bone parameters (*Fig. 2B-2D*). There were significant effects of both, treatment (F(1,12) = 9.37, P < 0.01) and gender (F(1,12) = 18.45, P < 0.01) on percentage of bone volume. In comparison to the saline-treated rats, TCP-treated rats displayed significant increase in percentage of bone volume (17.6 ± 4.1% vs. 23.6 ± 5.1%; *Fig. 2B*), as did females in comparison to males (23.9 ± 4.96% vs. 16.4 ± 2.07%). Trabecular bone number was also significantly affected by treatment (F(1,12) = 11.28, P < 0.01) and gender (F(1,12) = 18.45, P < 0.01), with TCP-treated rats displaying increased mean value over saline-treated rats (2.0 ± 0.42 mm^{-1} and 2.7 ± 0.53 mm^{-1}, respectively; *Fig. 2C*), and females displaying increased values over males (2.7 ± 0.53 mm^{-1} and 1.9 ± 0.21 mm^{-1}, respectively). Both parameters significantly affected connectivity density as well ((F(1,12) = 14.23, P < 0.01 for treatment, and (F(1,12) = 18.73, P < 0.01 for gender). Connectivity density was increased in TCP-treated rats in comparison to the controls (57.6 ± 16.2 mm^{-3} vs. 85.3 ± 20.3 mm^{-3}; *Fig. 2D*), as well as in females in comparison to males

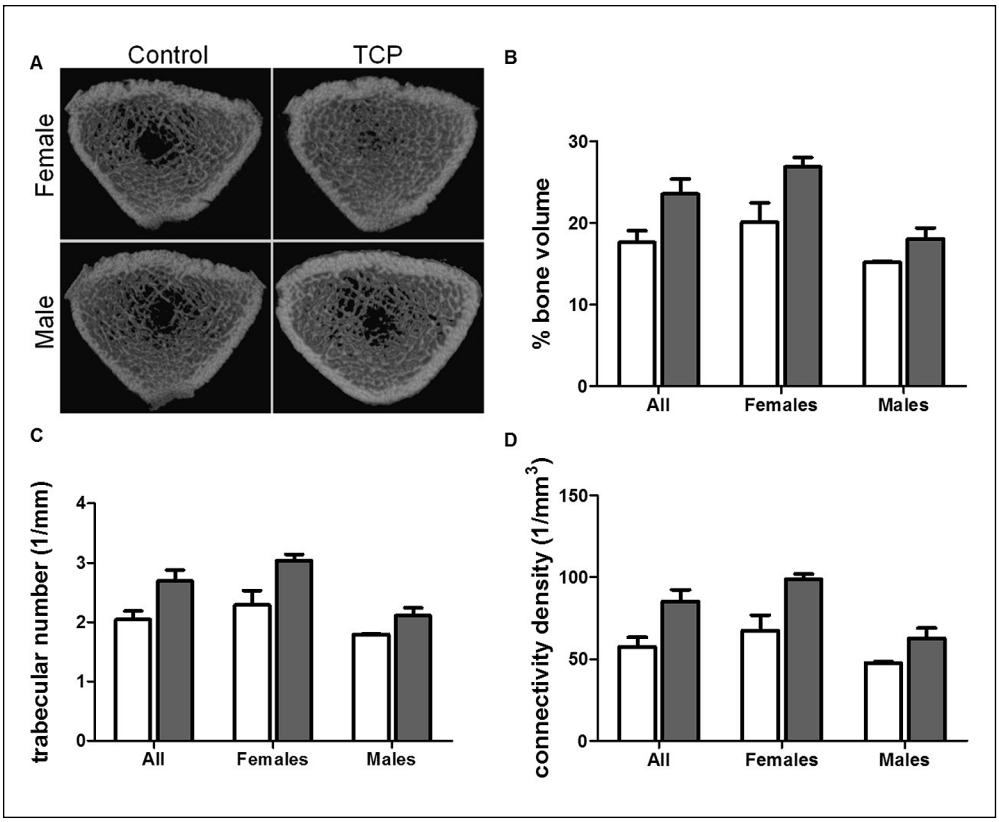

Fig. 2. Trabecular bone section and trabecular bone parameters obtained from 8 saline-treated (4 females, 4 males) and 8 tranylcypromine (TCP)-treated (5 females, 3 males) rats. (A) A typical transaxial section of distal part of femur from a male and a female rat from each experimental group. Increased trabecular bone structure can be observed in TCP-treated rats of both genders. (B) Trabecular bone volume. (C) Trabecular number. (D) Connectivity density. Results are shown as Mean ± S.E.M. Two-way ANOVA on logarithmically transformed values revealed significant effect of treatment ($P < 0.01$) and gender ($P < 0.01$) on all three parameters.

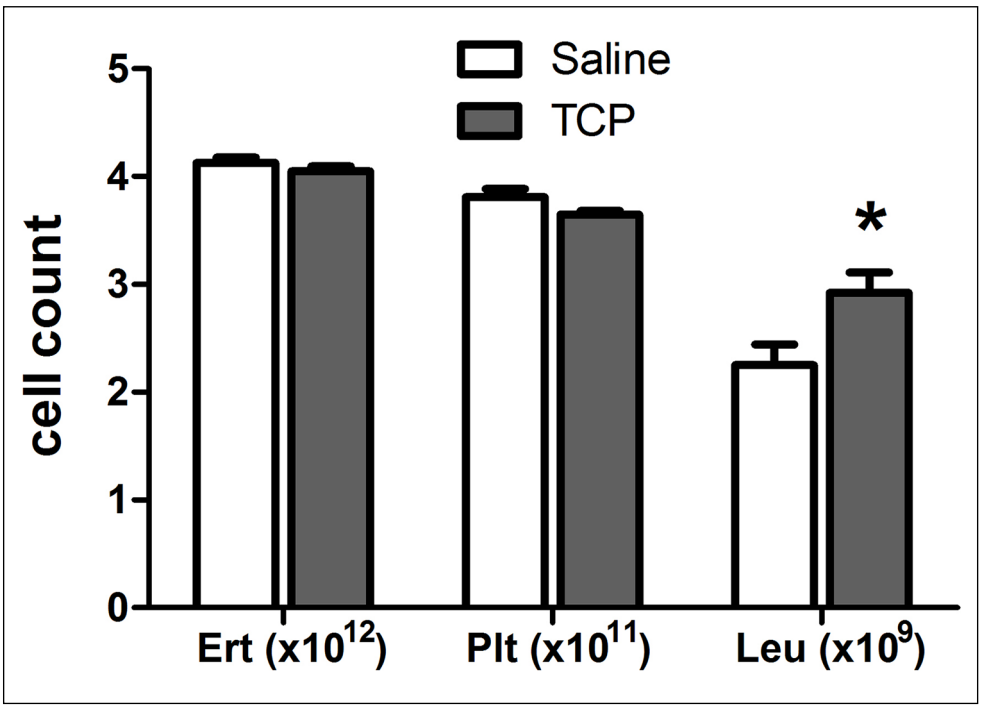

Fig. 3. Number of erythrocytes (Ert), platelets (Plt), and leukocytes (Leu) in peripheral blood obtained from 6 saline-treated and 6 tranylcypromine (TCP)-treated male rats. Results are shown as Mean ± S.E.M. *$P < 0.05$, Student t-test on logarithmically transformed values.

Table 2. Correlation of bone parameters with relative Tph1 gene expression.

parameter	r [1]	P
% bone volume	–0.556	0.04
trabecular number	–0.574	0.03
connectivity density	–0.618	0.02

[1]Spearman correlation; N=14

(84.9 ± 20.7 mm^{-3} vs. 54.1 ± 10.2 mm^{-3}). There was no significant effect of treatment × gender interaction on either trabecular bone volume ($F(1,12) = 0.81$, $P = 0.386$), trabecular bone number ($F(1,12) = 1.12$, $P = 0.312$), or connectivity density ($F(1,12) = 0.71$, $P = 0.416$).

Significant negative correlation was observed between relative concentrations of mRNA for TPH1 in jejunum and all three trabecular bone parameters (*Table 2*).

Table 3. Differential leukocyte count.

Cell Type	Treatment	[1]Cell count × 10^7/L	Statistic parameters[2]
lymphocytes	Saline	181 ± 49.2	t = 1.36, P = 0.21
	TCP	231 ± 49.7	
monocytes	Saline	3.91 ± 2.81	t = 2.71, P < 0.03
	TCP	11.63 ± 7.7	
neutrophils	Saline	27.5 ± 14.1	t = 2.31, P<0.05
	TCP	52.2 ± 18.3	
eosinophils	Saline	1.24 ± 0.47	t = 2.48, P < 0.04
	TCP	2.38 ± 1.03	

[1]Values are expressed as Mean ± S.D.; [2]Student's unpaired t-test on logarithmically transformed values.

Number of peripheral blood cells

Number of erythrocytes, platelets, and leukocytes were analyzed in the peripheral blood of 6 saline treated and 6 TCP-treated male rats (*Fig. 3*). Compared to the control rats, TCP-treated rats displayed similar number of erythrocytes (4.13 ± 0.12 × 10^{12}/L vs. 4.05 ± 0.11 × 10^{12}/L; P = 0.242, t = 1.24, 10 d.f.), and mild, non-significant, reduction in number of platelets (381 ± 18.5 × 10^9/L vs. 365 ± 7.18 × 10^9/L; P = 0.079, t = 1.96, 10 d.f.). On the other hand, increase in total leukocyte number was significant (2.25 ± 0.47 × 10^9/L and 2.93 ± 0.46 × 10^9/L in control and experimental rats, respectively; P < 0.04, t = 2.45, 10 d.f.). Leukocyte differential analysis revealed significant increase in monocyte and granulocyte numbers (*Table 3*).

DISCUSSION

Developmental exposure to 5-HT-enhancing agents has been reported to induce long-lasting cellular and molecular changes in the brain, accompanied by alterations in 5-HT-regulated behavior (36-40). There is, to our knowledge, no data on the possible long-term effects of perinatal 5-HT enhancement on 5-HT balance and function in the peripheral compartment. Perinatal treatment of rats with TCP led to persistent imbalance in both, central 5-HT homeostasis, as reflected by significantly increased 5-HT degradation and significantly decreased 5-HT concentrations in the brain, and peripheral 5-HT homeostasis, as reflected in increased platelet and decreased plasma 5-HT concentrations.

Study limitations

Before we continue with the discussion, we have to mention several drawbacks of our study, which could have affected the reliability and interpretation of our results.

First, we are aware that the inhibition of MAO also affects dopamine and noradrenaline metabolism, and that the degree of this effect could be reliably determined only by measuring their plasma concentrations. However, while 5-HT is catabolized solely through oxidative deamination, catecholamines can be alternatively degraded by catechol-o-methyltransferase present both in the brain and peripheral tissues (41). Since inactivation of both *Mao* genes was reported to have considerably lower effect on catecholamine than on 5-HT levels in the central compartment (42), we did not expect developmental MAO inhibition to significantly alter peripheral catecholamine concentrations. While determination of TCP-effect on catecholamines will be one of the goals in our future studies, in this study we focused only on 5-HT. We will therefore discuss physiological consequences through changes in 5-HT homeostasis, keeping in mind possible influences of other monoamines.

Second, in this study we only determined platelet 5-HT concentration, as an illustrative indicator of the peripheral 5-HT dysregulation while we relied on our previously published findings (31) regarding the 5-HT concentrations in platelet-free plasma (PFP), which might have been too speculative. Still, since the mean platelet 5-HT concentrations in control and experimental animals, as well as the extent of increase in TCP-treated rats, were comparable between the mentioned former experiment and the present study, we hold the assumption that similar analogy between the experiments may also exist with respect to PFP concentrations as plausible.

Finally, the number of animals after stratification for treatment and gender was quite small. While *post-hoc* sample power calculation revealed sufficient power for some analyses (>80% for trabecular bone parameters, *MaoA/Actb* and *Tph1/Gapdh* expression), power for other analyses (*MaoA/Gapdh* and *Tph1/Actb* expression, hematological parameters) was insufficient, potentially causing false negative as well as false positive results. Our results should, therefore, be considered only as preliminary, requiring confirmation on a larger sample of animals.

Molecular background of dysregulation of the peripheral 5-HT homeostasis

Persistence in the dysregulation of peripheral 5-HT homeostasis in early adulthood of TCP-treated animals was demonstrated by an increase of about 80% in 5-HT concentration in PRP. These significantly increased amounts of 5-HT were, however, stored in platelets and were not expected to affect the peripheral functions under study to a notable extent, since the main mediator of 5-HT-induced physiological processes in the bone is free plasma serotonin. In our previous study we found a decrease in free plasma 5-HT of TCP-treated rats (t = 2.54, P = 0.02, TCP-treated vs. saline-treated animals only), which accompanied increase in platelet 5-HT (31). We therefore hypothesized that decreased PFP concentrations in TCP-treated rats may cause alterations in 5-HT-mediated peripheral functions.

This opposite direction of alterations in free plasma 5-HT and platelet 5-HT levels in TCP-treated animals pointed to potential long-lasting changes in the expression of 5-HT-regulating elements (leading to decreased 5-HT synthesis, increased platelet 5-HT accumulation, and/or increased 5-HT degradation), which probably occurred during the treatment to compensate for excess 5-HT left in the circulation after inhibition of the degrading enzyme. Since, unfortunately, the obtained quantity of platelet RNA did not allow us to reliably measure the expression of 5-HTT mRNA, our attempt to reveal the molecular basis of persistent hyperserotonemia in TCP-treated rats was focused on the expression of genes coding for TPH1 in the intestinal cells and MAO A in liver. The expression

of TPH1 mRNA was significantly decreased in the jejuna of TCP-treated rats, while no significant changes were observed in the relative levels of MAO A mRNA. Down-regulation of *Tph1* expression presumably represented compensation for inadequate 5-HT degradation during MAO inhibition, which persisted into young adulthood, long after a wash-out period. On the other hand, lack of compensatory increase in MAO A mRNA levels suggests that accumulation into platelets, rather than up-regulation of *Mao A* expression, was the main way to remove the excess 5-HT from circulation during the treatment. High blood 5-HT concentrations at the end of TCP treatment (32) and immense speed of blood clotting in TCP-treated pups (unpublished observations) speak in favor of such hypothesis. The observed compensatory mechanism is quite different from the one found in the central 5-HT compartment. Our earlier study demonstrated a marked, long-lasting increase in mRNA expression for both MAO isoforms in brains of TCP-treated animals, indicating that up-regulation of *Mao* expression represented the main mechanism to fight exposure of the brain to the chronic excessive 5-HT concentrations (34).

Effects on bone remodeling

The role of peripheral 5-HT in bone remodeling *via* 5-HT receptors expressed on bone cells has been widely accepted but the type of influence (positive vs. negative), the mechanism of its action (osteoblasts vs. osteoclasts) and the origin of its effect (intestinal vs. locally synthetized 5-HT) are still debated. *In vitro* studies revealed both, positive (43-45) and negative (17) effects of 5-HT on osteoblast proliferation, which were attempted to be explained by differential actions of 5-HT at low concentrations - inhibition of osteoblast proliferation through activation of HTR_{1B}, and high concentrations - promotion of osteoblast proliferation through activation of $HTR_{2A/B}$ (46). *In vivo* studies on growing post-pubertal rodents revealed lower trabecular bone volume in rats chronically treated with 5-HT (47), mice chronically treated with an SSRI, and mice with inactivated gene for 5-HT transporter (48), all of which increase the concentration of free circulating 5-HT. On the other hand, inactivation of the *Tph1* gene induced significant increase in trabecular bone volume, trabecular thickness and number, as well as reduced trabecular separation in mice (16), while pharmacological inhibition of TPH1 was shown to prevent bone loss in ovariectomized rodents (49). Our results, showing increased trabecular bone volume in rats with decreased level of free circulating serotonin and decreased expression of *Tph1* gene, are in line with the above mentioned findings and support the negative role of 5-HT in bone remodeling. At this point, we are not able to determine whether this increased bone structure is due to alterations in bone formation, as suggested by Warden *et al.* (48) or to alterations in bone resorption, as suggested by Chabbi-Achengli *et al.* (16), or to distinguish paracrine action of gut-derived serotonin from autocrine action of 5-HT synthetized in osteoclasts. These questions will be addressed in our future studies.

We must also discuss the possible contribution of brain-derived serotonin to the regulation of bone structure in our animals. Central regulation of bone remodeling is considered to be mediated mainly by sympathetic activity through the β_2-adrenoreceptors (Adrb2) expressed on osteoblasts, which is in turn influenced by other neuromodulators, such as endocannabinoids and 5-HT (50). Studies on a rat model demonstrated age-related differential roles of cannabinoid receptor type 1 in bone turnover, and suggested therapeutic role of its antagonists in the prevention of corticosteroid-induced osteoporosis at young age (51). 5-HT synthetized in the raphe nuclei presumably acts on ventro-medial hypothalamic neurons through HTR_{2c} to decrease sympathetic activity. A dominant positive role of central 5-HT indirectly acting on both arms of bone remodeling was introduced by Yadav *et al.* (16) after demonstration of severely low bone mass phenotypes in *Tph2*-deficient mice and *Tph1/Tph2* double knock-outs, resulting from decrease in bone formation parameters and increase in bone resorption parameters. On the other hand, the increase in trabecular bone structure of our rat model despite a fourfold reduction in midbrain 5-HT concentrations (32), as well as significant negative correlations between trabecular bone parameters and *Tph1* expression, favors the peripheral over the central 5-HT influence in the regulation of bone accrual. The reason for this discrepancy might lie in the different origin of low midbrain 5-HT concentrations (increased degradation in TCP-treated rats as opposed to decreased synthesis in *Tph2* +/– mice) or in the potential influence of MAO inhibition during the TPC treatment on the adrenergic pathway (as the bone loss in *Tph2* knock-out mice was mediated by significant increase in noradrenalin concentration and was rescued by *Adrb2* inactivation).

Effects on hematopoiesis

Although *in vitro* studies reported positive influence of serotonin on expansion of early progenitor cells and committed colony forming units of the myeloid, erythroid, and megakaryocytic lineage (26), as well as on megakaryocytopoiesis and erythropoiesis (27-29), it is hard to distinguish 5-HT influence from the influence of numerous other regulators of hematopoiesis *in vivo*. While physiological studies correlating 5-HT concentration with the number of blood cells in the peripheral blood or bone marrow are lacking, several studies on animal models may indicate the direction of 5-HT influence on hematopoiesis. Significant decrease in number of erythrocytes, hematocrit and hemoglobin levels (presumably by acting directly on erythroid precursors in the bone marrow), along with increased leukocyte count (due to monocytes and neutrophils) and no difference in platelet count, was reported in *Tph1* –/– mice in comparison to wild-type animals (29, 52). On the other hand, amphetamine derivates, which are potent 5-HT releasers, reduced the number of circulating lymphocytes, while leaving the number of other leukocytes unchanged (31). Similarly to *Tph1* deficient mice, our TCP-treated rats displayed modest but significant increase in leukocyte count, mostly due to the increase in monocyte and granulocyte numbers, and no significant difference in platelet count. As opposed to *Tph1* knock-outs, TCP-treated rats displayed changes neither in erythrocyte number nor in hematocrit and hemoglobin levels (data not shown). Our results suggest that 5-HT might negatively regulate development of granulocyte-macrophage progenitors. They, however, do not exclude possible positive influence of 5-HT on the development of megakaryocyte-erythroid progenitors. It is possible that the number of circulating platelets and erythrocytes in our animals might have been compensated by TCP-induced positive catecholaminergic influence on the development of their precursors in the bone marrow (which is absent in *Tph1*–/– mice due to the sole intervention in the 5-HT system), or by their normal production in spleen (as this organ was suggested to be the main source of circulating platelets and erythrocytes at steady state (53)).

Results of this study show that perinatal exposure to tranylcypromine leads to long-lasting compensatory decrease in *Tph1* gene expression, accompanied with alterations in bone remodeling and hematopoiesis, suggesting a need for thorough exploration of the possible effects of this 5-HT enhancer in pregnant and lactating women.

Significant long-lasting decrease in the intestinal *Tph1* expression, along with accumulation into platelets, seems to be the main mechanism of peripheral compensation for the excess of 5-HT during the TCP treatment, in contrast to the significant

increase in expression of both Mao genes in the brain of TCP-treated animals. Different strategies used in the central and the peripheral 5-HT compartment to compensate for 5-HT imbalances of the same cause suggest consideration of the two compartments as regulatory distinct entities and ask for caution when using results obtained in the peripheral 5-HT compartment to interpret or treat a central 5-HT dysfunction.

Significant increase in trabecular bone structure in rats with decreased *Tph1* expression and decreased concentrations of both, plasma free and midbrain 5-HT support the negative influence of peripheral 5-HT on bone accrual and suggest its dominant role over central 5-HT in the regulation of bone maintenance. Finally, increase in leukocyte number in TCP-treated animals indicates a negative *in vivo* effect of 5-HT in the regulation of leukocyte development and/or sustainment. Further research on bone-marrow-derived cells should provide us with more insight into the mechanisms of the observed 5-HT action.

Abbreviations: 5-HTP, 5-hydroxytryptophan; 5-HTT, serotonin transporter; Adrβ2, β2 adrenergic receptors; ACTB, β-actin; Ct, threshold cycle; CREB, cAMP response element-binding protein; GD, gestational day; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MAO, monoamine oxidase; micro CT, micro-computed tomography; PFP, platelet-free plasma; PRP, platelet-rich plasma; PND, postnatal day; SSRI, selective serotonin reuptake inhibitors; TCP, tranylcypromine; TPH, tryptophan hydroxylase.

Acknowledgements: This study was supported by the grant "Neurobiological basis of autism: the role of serotonin system" (119-1081870-2396) funded by the Ministry of Science Education and Sports of the Republic of Croatia. The authors wish to thank dr.sc. Anica Horvat-Knezevic for assistance in the collection of hematological parameters.

Conflict of interests: None declared.

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Received: February 26, 2015
Accepted: June 4, 2015

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