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Perinatal Exposure of Rats to 5-Hydroxytryptophan Affects Midbrain Serotonin Homeostasis

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Abstract

Background: A considerable number of depressed women continue to use 5-hydroxytryptamine (5-HT) targeted antidepressants throughout pregnancy. The immediate 5-HT precursor, 5-hydroxytryptophan (5-HTP), is being increasingly offered as a natural and safer alternative to antidepressant medication. However, consequences of developmental exposure to increased 5-HTP concentrations on brain development and behaviour have not been studied in animal models or humans. During the perinatal period, 5-HT acts as a modulator of neural development. Growing evidence suggests the role of 5-HT, originating from the dorsal raphe nuclei (DRN), in the formation of the barrel field in the rodent somatosensory cortex. Topographically organized barrels, contained within the posteromedial barrel subfield (PMBS), represent the major facial whiskers, which rodents use to explore their environment.

Methods and Findings: We examined consequences of perinatal treatment of Wistar rats with 25 mg/kg 5-HTP, from gestational day 13 until postnatal day (PND) 21, on brain development. Compared to controls, 5-HTP treated rats displayed decreased birth-weight and postnatal weight-gain. ELISA revealed increased serum but not cortical 5-HT concentrations at the end of treatment. Nissl staining of tangentially oriented serial sections across the dorsolateral telencephalic wall displayed unaffected cytoarchitecture of the PMBS, but significantly smaller barrel size on PND70, possibly leading to previously observed impairments in whisker-mediated perception. 5-HT immunostaining of the DRN region revealed significantly lower signal intensity in 5-HT positive cells, pointing to possible compensatory reduction of 5-HT content in DRN.

Conclusions: Our results suggest a need for examination of the potential neurological/behavioural effects in children prenatally exposed to 5-HTP.

Keywords: Barrel field; Brain development; Pregnancy; 5-HT enhancer

Abbreviations

5-HT: 5-hydroxytryptamine, serotonin; 5-HTP: 5-Hydroxytryptophan; AADC: Aromatic Amino Acid Decarboxylase; BF: Barrel Field; CNS: Central Nervous System; CO: Cytochrome Oxidase; DA: Dopamine; DAB: 3,3': Diaminobenzidine; DRN: Dorsal Raphe Nuclei; GD: Gestational Day; M: Mean; MAO: Monoamine Oxidase; NA: Noradrenaline; PFA: Paraformaldehyde; PMBS: Posteromedial Barrel Subfield; PND: Postnatal Day; SEM: Standard Errors of Mean; SSRI: Selective Serotonin Reuptake Inhibitors; TCA: Thalamocortical Axons; Trp: L-tryptophan; VMAT: 2-Vesicular Monoamine Transporter 2.

Introduction

Serotonin (5-hydroxytryptamine, 5-HT) is a multifunctional signalling molecule involved in the regulation of cardiovascular and gastrointestinal functions, haemostasis, circadian rhythms, cognition, mood, and social behaviour. During brain development, 5-HT modulates processes such as neurogenesis, apoptosis, axon branching and dendritogenesis [1]. In both, rodents and humans the main source of 5-HT in the first half of pregnancy is the placenta, and this function is later assumed by the developing serotonergic neurons of the raphe nuclei [2]. Genetic or environmental disruption in 5-HT homeostasis at any stage of development may lead to subtle structural and functional changes in brain circuitry, which can result in an increased vulnerability to behavioural disorders [3].

Drugs targeting the 5-HT system are widely used in treatments of affective and anxiety disorders, and a considerable number of depressed women continue to use antidepressants throughout pregnancy [4]. Selective serotonin reuptake inhibitors (SSRI) are the most commonly used 5-HT

enhancers during pregnancy [5], and the consequences of perinatal exposure to SSRI have been thoroughly studied in rodents and humans (as reviewed in [6]). For several decades, the immediate 5-HT precursor 5-hydroxytryptophan (5-HTP) is being widely offered as a natural alternative to SSRI. 5-HTP is claimed to be effective not only in treating depressive symptoms but also in alleviating various difficulties such as binge-eating, headache or insomnia [7]. However, whereas its therapeutic efficiency has been questioned, the safety of its use has hardly been studied in human population or in animal models [8-10].

Rats actively explore the environment by sweeping their facial whiskers back and forth to generate tactile sensory information on the texture, shape and location of the encountered objects [11]. The major facial whiskers are represented in the posteromedial barrel subfield (PMBS)-region of the somatosensory cortex containing distinguishable, topographically organized structures (barrels) consisting of clustered presynaptic thalamocortical axons (TCA) surrounded by postsynaptic layer IV neurons. The cytoarchitecture of the barrel field, i.e. TCA clustering, layer IV neuron aggregation into barrel walls and orientation of their dendrites towards barrel hollows is regulated by a combination of internal genetic programs and communication with TCA [12,13]. Growing evidence suggests the role of 5-HT signalling in the modulation of both, the prenatal navigation of TCA through the forebrain and the postnatal barrel pattern formation in layer IV of the somatosensory cortex [14].

We previously studied the effects of chronic perinatal exposure of rats to 5-HTP on 5-HT-homeostasis and behaviour, and found that it induces hyperserotonemia in pups [15] and significantly increases exploratory behaviour in adult rats [16]. With these results in mind, we now explored possible consequences of perinatal exposure to 5-HTP on brain development. We compared cytoarchitecture and size of barrels in the posteromedial barrel subfield of the somatosensory cortex, as well as size, number and signal intensity of the 5-HT positive cells in the dorsal raphe nuclei between rats prenatally treated with 5-HTP and control rats treated with saline.

Materials and Methods

Animal housing and breeding

Six 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 removed, and the females randomly assigned to one of the treatment groups (3 with 5-HTP and 3 with saline).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 (Mucedola) and tap water. Two days before parturition, females were separated and remained singly housed. On postnatal day (PND) 21, some pups were sacrificed while other pups were weaned and left to reach adulthood in a 2-4 animals-per-cage setting.

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 minimize the number of animals used, as well as to reduce animal suffering.

Pharmacological treatment

Animals were treated with 25 mg/kg of 5-hydroxytryptophan (5-HTP, Sigma-Aldrich, St. Louis, MO, USA), or saline, by daily subcutaneous injections in the nape, from GD 12 until delivery to the dams, and from PND 1 until PND 21 directly to the pups. This way of administration was used to avoid the risk of damaging foetuses during the prenatal treatment, and to reduce discomfort in pups. 5-HTP was dissolved in acidified saline, neutralized with NaOH and warmed to body temperature prior to injection. Pups were weighed every day before injections. Solutions were delivered in volumes of 3.3 mL per kg of body mass, by a 50 µL glass syringe (Hamilton) with disposable 30G needles (BD, Drogheda, Ireland), until pups reached 15 g, and in volumes of 5 mL per kg of body mass, by disposable 0.5 mL plastic syringes with 30G needles (BD Micro-Fine Plus), until the end of treatment. The control group was treated with saline in the same manner. All injections were performed between 10 and 11 AM.

Tissue sampling

Eight pups (4 males, 4 females) from the control and ten pups (5 males, 5 females) from the experimental group were sacrificed on PND 21. Four hours after the last injection, a pup was separated from the litter, carried in a small cage to the laboratory and put into an exicator containing a cotton pad soaked with isoflurane (Abbott). Anesthesia was maintained by applying a small beaker with a cotton swap soaked in isofluorane to the snout. About 1.5 mL of blood was withdrawn from the vena cava of each pup into an anticoagulant-free syringe and immediately transferred into a microtube. The animal was then decapitated and the brain quickly removed from the scull. Brains from three males and three females from each experimental group were used for monoamine concentration measurement. The cortex encompassing all cortical areas anterior to Bregma was peeled off. Samples were weighted and frozen at -80°C for later analysis. The remaining brains (two of control and four of experimental group), which were used for the barrel field analysis, were fixed by immersion in 4% paraformaldehyde (PFA) for one week and then transferred to 30% sucrose (both in 0.1M PBS, pH7).

The remaining six animals (3 males and 3 females) from each experimental group were weaned and let to reach young adulthood. At PND 70, anesthetized animals were decapitated and the brains were stored (as described above) for barrel field analysis and 5-HT immuno-staining.

Measurement of monoamine concentrations

Blood samples were allowed to clot and serum was separated by centrifugation 10 min at 500 × g. The frozen cortical samples were thawed and homogenized in 4 volumes (w/v) of a solution of 0.01 N hydrochloric acid containing 1 mM EDTA and 4 mM $Na_2S_2O_5$. Tissue homogenates were then centrifuged at 24,000 × g for 20 min at 4°C, and aliquots of the clear supernatant were used for the measurements.

5-HT concentration in cortex and serum was determined using the Serotonin Research ELISA kit, and DA and NA concentrations using the 2-CAT Research ELISA kit (Demeditec Diagnostics GmbH, Germany), according to the kit instructions. A calibration curve was drawn based on the absorbance measured at 450 nm on the microplate reader (Bio Rad 550, Germany) and known concentrations of standard solutions. Concentration values of samples were obtained by interpolating them to the calibration curve, using 4-parameters non-linear regression curve fitting using GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). Results were expressed in ng 5-HT per mL of serum, in pg of DA/NA per mL of serum, and in pg of 5-HT/DA/NA per mg of wet cortical tissue.

Barrel field analysis

Dorsolateral cerebral cortices were peeled off with a scalpel (pup cortices were additionally flattened between two glass slides, leaving 1.2 mm space between, to get as much flattened cortical barrel fields on a single plane as possible), and placed in 4% PFA solution for 48h. Tissue was then embedded in 3% agarose and cut tangentially to the pial surface in serial 60 µm thin slices with a vibratome (Leica). Nissl staining was used to reveal overall cytoarchitectonic structure of the barrel cortex and boundaries of individual barrels. Cytochrome oxidase (CO) staining was used to measure the size of the barrels in the PMBS. The tangential sections were collected in 0.1 M phosphate buffer saline (pH 7.4, PBS) and treated with cytochrome C and 3,3'-diaminobenzidine (DAB). Sections were incubated for 2.5 hours at 37°C in 7.5% sucrose in 0.1 M PBS containing 5 mg DAB (D0426 Sigma), 5 mg cytochrome C (C2506 Sigma) and 20 µg catalase per 10 mL PBS, rinsed in three changes of PBS, mounted on subbed slides, dehydrated in histoclear and coverslipped.

Qualitative analysis of stained subsequent adjacent histological sections was performed using an upright microscope Olympus Provis AX70. Quantitative analysis was performed on pictures captured at 2x magnification using a Zeiss AxioCam mounted on a Nikon Eclipse E600 microscope. The external contour of each barrel was outlined and areas measured with the NIH ImageJ software. Treatment group affiliation was not known to the researchers performing qualitative or quantitative analysis.

5-HT immuno-staining

5-HT immunohistochemical staining was performed on 40 µm transversal slices containing dorsal raphe nuclei. Briefly, sections were incubated 1h at room temperature (RT) in pretreatment solution (15 mL MetOH, 5 mL dH20, 15 µL H2O2) to quench endogenous peroxidase. Sections were incubated overnight in primary anti 5-HT antibody diluted 1:15000 (5-HT, Serotonin, Rabbit Antibody, Immunostar, 20080) at 4°C. Subsequently, after 10' rinsing in PBS (3 times), sections were incubated 1h at RT in secondary anti-rabbit (diluted 1:200), and after rinsing (3 × 10' in PBS) incubated 1h in ABC complex (diluted 1:200) all from Vectastain ABC Kit Peroxidase, IgG, (Vector PK-4001). The reaction was visualized by using DAB (3,3'-diaminobenzidine, D0426 Sigma) as the chromogen. Images of slices were captured at 2x magnification using a Zeiss AxioCam, were processed with Zeiss Zen 2 software, and plate levels were defined according to the rat brain atlas of Paxinos and Watson [17]. The number and size of 5-HT neurons, as well as mean signal intensity per cell (corresponding to 5-HT content) were analyzed in slices corresponding to plates 95, 96 and 97 [17] of each animal, using NIH ImageJ software. 5-HT positive cells were determined with ImageJ "Analize Particle" function, limiting particle size to 70-320 µm, circularity to 0.40-1.0, and adjusting all pictures to same threshold.

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. When the number of samples per subgroup was five or less, log-transformed values were used in the analyses. Mean difference in pup body mass at birth was compared by Student t test. Independent two-way ANOVA was employed to check for influences of treatment and gender on body mass at PND 21 and PND 70, and on monoamine concentrations. Univariate Split-Plot approach of repeated measures ANOVA was used to analyze barrel size, as well as 5-HT neuronal cell number, size and signal intensity after 5-HT immuno-staining. Kenward-Roger first order approximation was used to calculate degrees of freedom of the denominator, due to missing values. Tukey's honest significance test was used for post-hoc analyses. Values were presented as means (M) ± standard errors of mean (SEM). The level of significance was set to 0.05 (two-tail P value).

Results

Physiological parameters

Litter-related parameters are shown in **Table 1**. 5-HTP-treated pups had significantly lower body mass at birth ($t=6.50$, 39 d.f., $p<0.0001$). Although the average litter sizes were comparable between 5-HTP-treated and saline-treated groups, the effect of treatment on body mass was still significant at PND 21 ($F(1,26)=17.41$, $p=0.003$). Males were somewhat heavier than females (41.95 ± 1.92 g vs. 38.52 ± 1.48 g), but there was no significant effect of gender ($F(1,26)=2.64$, $p=0.117$), as well as of treatment x gender interaction ($F(1,26)=0.310$, $p=0.583$).

Significant effects of treatment ($F(1,8)=176$, $p<0.0001$), gender ($F(1,8)=1441$, $p<0.0001$), and their interaction ($F(1,8)=5.78$, $p=0.043$) was observed at PND 70, with significant differences among all four subgroups: control females (218 ± 1.86 g), control males (332 ± 2.40 g), 5-HTP-treated females (182

± 2.40 g) and 5-HTP-treated males (292 ± 4.58 g), revealed by the post-hoc analysis.

In order to check the efficacy of 5-HTP treatment in inducing hyperserotonemia, 5-HT concentrations were measured in sera of the saline-treated and 5-HTP-treated pups, at the end of the treatment period (PND21). As can be seen in **Figure 1** and **Table 2**, the effect of treatment on serum 5-HT concentration was significant ($F(1,14)=114.6$, $p<0.0001$), while there was no significant effect of gender ($F(1,14)=0.661$, $p=0.430$) or treatment x gender interaction ($F(1,14)=2.85$, $p=0.113$). On the contrary, 5-HTP treatment did not have significant influence on cortical 5-HT concentration ($F(1,8)=0.103$, $p=0.756$), as well as catecholamine ($F(1,8)=0.030$, $p=0.868$ for DA, and $F(1,8)=0.013$, $p=0.911$ for NA) levels (**Table 2**). Also, no significant influence of gender or gender x treatment interaction was observed for 5-HT ($F(1,8)=0.388$ $p=0.551$ and $F(1,8)=0.005$, $p=0.944$, respectively), DA ($F(1,8)=0.614$, $p=0.456$ and $F(1,8)=0.852$, $p=0.383$, respectively), or NA ($F(1,8)=0.345$, $p=0.573$ and $F(1,8)=0.310$, $p=0.593$, respectively).

Table 1 Litter-related physiological parameters of saline treated and 5-hydroxytriptophan (5-HTP) treated rats.

	Treatment group	
	saline	**5-HTP**
Number of dams	3	3
Pre-pregnancy body mass (g)	233 ± 9.24	238 ± 7.81
Pregnancy weight gain (g)	84.3 ± 5.86	58.5 ± 7.60
Litter size per dam	4.7 ± 0.94	5.3 ± 2.36
Gender ratio (females / males)	“7	“8
Body mass (g) at PND 0	6.81 ± 0.08	5.24 ± 0.22***
Body mass (g) at PND 21	44.6 ± 0.61	36.2 ± 1.87**

***p<0.001; Student t-test; ** p<0.01, significant effect of treatment as revealed by two-way ANOVA

Table 2 Serum concentrations (ng/mL) of serotonin (5-HT) in saline-treated (N=4 males, 4 females) and 5-hydroxytryptophan-treated (5-HTP, N=5 males, 5 females) rats, and cortical concentrations (pg/mg) of serotonin (5-HT), dopamine (DA) and noradrenaline (NA) in saline-treated (N=3 males, 3 females) and 5-hydroxytriptophan (5-HTP, N=3 males, 3 females) treated rats on PND 21.

Neurotransmitter	**Gender**	**Treatment**		**Gender mean**
		Saline	5-HTP	
Serum 5-HT	male	519 ± 17.5	684 ± 8.59	614 ± 29.4
	female	558 ± 10.3	673 ± 10.2	622 ± 21.3
	Treatment mean	542 ± 12.4	678 ± 6.55***	
Cortical 5-HT	male	177 ± 8.20	199 ± 46.0	188 ± 21.5
	female	179 ± 85.6	172 ± 40.1	176 ± 42.3
	Treatment mean	178 ± 38.5	186 ± 28.0	
Cortical DA	male	5.06 ± 1.79	5.97 ± 1.58	5.52 ± 1.10
	female	7.18 ± 0.05	5.68 ± 1.49	6.43 ± 0.75
	Treatment mean	6.12 ± 0.93	5.80 ± 0.97	
Cortical NA	male	28.0 ± 2.31	31.8 ± 4.25	29.9 ± 2.33
	female	41.8 ± 11.8	36.0 ± 12.8	38.9 ± 7.87
	Treatment mean	34.9 ± 6.18	33.9 ± 6.10	

***p<0.001; Student t-test; two-way ANOVA

Barrel cortex

In order to examine the possible effect of perinatal 5-HTP treatment on the development of the barrel fields, the formation of barrel pattern in layer IV was analysed in PND21 pups after Nissl staining (**Figure 2**). Examination of successive tangential slices did not reveal obvious differences in barrel organization and delineation in the barrel fields of 5-HTP-treated pups (**Figure 2B**) although individual barrels appeared to be smaller in comparison to those of the saline-treated pups (**Figure 2A**).

In adult rats, we concentrated on the PMBS representing the major facial whiskers. Again, barrels of 5-HTP-treated rats appeared to be organized and well defined but smaller. We attempted to quantify the observed difference by measuring average size of the individual barrels in rows A-E after CO staining (**Figure 3A**).

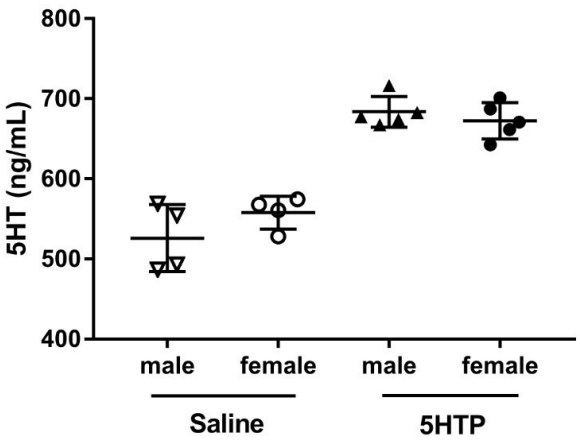

Figure 1 Concentration of serotonin (in ng/mL) in serum of saline-treated (N=4 males, 4 females) and 5-hydroxytryptophan (5-HTP, N=5 males, 5 females) treated rats on PND 21. Two-way ANOVA revealed significant effect of treatment.

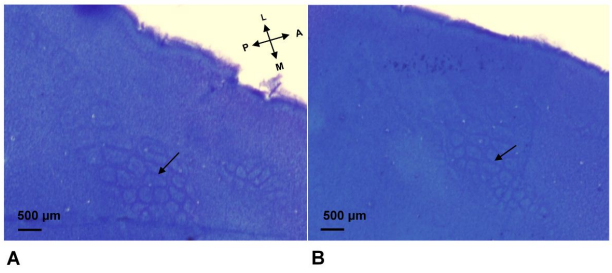

Figure 2 Representative tangential sections of somatosensory cortex, stained by Nissl, showing barrel fields of saline-treated (A) and 5-hydroxytryptophan (5-HTP)-treated (B) rats at PND 21. In 5-HTP-treated pups, individual barrels (marked with an arrow) appear delineated and well-defined, but somewhat smaller in comparison to the control pups.

We were able to reconstruct the entire PMBS from 2-3 consecutive slices in 6 animals from each experimental group, and average barrel size per column was entered as the data for repeated measure two-way ANOVA, with treatment representing between-subject variable, and barrel rows representing within-subject variable. There was significant effect of treatment ($F(1,10)=10.71$, $p=0.0084$) on barrel size, with barrels of 5-HTP treated rats being smaller (about 20% in rows A-C and about 25% in rows D-E) than those of the control rats (**Figure 3B**). As expected, the size of the barrel also significantly depended on its location ($F(4,40)=49.15$, $p<0.0001$), with average barrel sizes in rows A and B being significantly smaller than the average barrel sizes in rows C-E. The effect of treatment x row interaction on barrel size was not significant ($F(4,40)=1.49$, $p=0.224$).

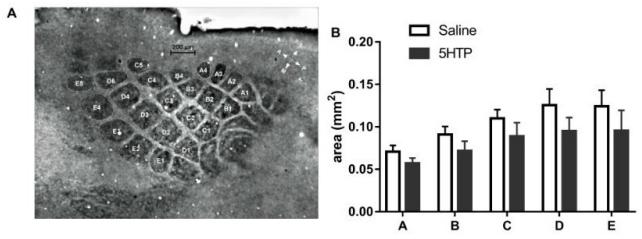

Figure 3 Tangential section through posteromedial portion of the somatosensory cortex stained for cytochrome oxidase, showing barrels organized into rows A-E (A). Comparison of the mean barrel size in each row between saline-treated rats (N=6) and 5-hydroxytriptophan-treated (5-HTP) rats (N=6) (B). Significant effects of both, treatment and row, were revealed by 2-way ANOVA.

5-HT immuno-staining

Finally, we performed 5-HT immuno-staining of the raphe nuclei area to check for potential alterations in the serotonergic cell bodies, induced by perinatal 5-HTP treatment (**Figure 4A**). Samples of four control and five 5-HTP-treated animals were of sufficient quality to allow us comparison of number and size of 5-HT-positive cells, as well as signal intensity per cell (corresponding to the 5-HT content). Comparisons were made in three consecutive plates (95, 96 and 97 [17]) using repeated measures two-way ANOVA, with treatment representing between-subject variable, and plate representing within-subject variable.

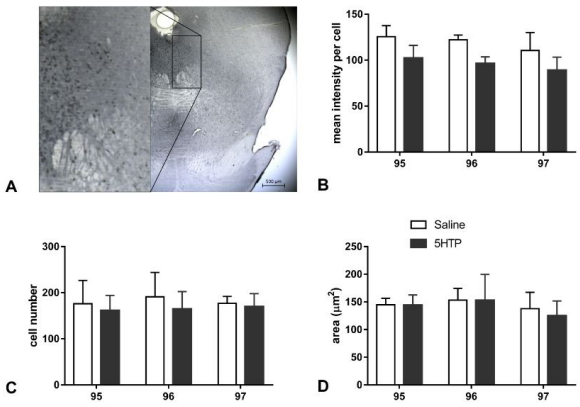

Figure 4 5-HT immuno-staining of the raphe nuclei area. Number, size and signal intensity of 5-HT-positive cells in each plate were examined in a rectangular area of fixed position and size (A). Comparison of signal intensity per cell (B), cell number (C), and cell size (D) in the three consecutive plates of the dorsal raphe nuclei of saline-treated (N=4) and 5-hydroxytryptophan (5-HTP)-treated (N=5) rats. Repeated measures two-way ANOVA revealed significant effect of 5-HTP treatment on the mean intensity of 5-HT immunoreactivity.

While treatment had no significant effect on the number ($F(1,7)=0.944$, $p=0.366$; **Figure 4C**) and size ($F(1,7)=0.132$, $p=0.727$; **Figure 4D**) of the 5-HT-containing cells in the examined area, it did affect signal intensity ($F(1,7)=27.9$, $p=0.001$; **Figure 4B**), pointing to a lower 5-HT content (about 20% in each plate) in adult animals perinatally treated with 5-HTP. Plate, as well as treatment x plate interaction did not have significant effect on either cell number ($F(2,14)=0.164$, $p=0.850$ and $F(2,14)=0.123$, $p=0.887$, respectively), cell size ($F(2,14)=1,326$, $p=0.298$ and $F(2,14)=0.137$, $p=0.873$, respectively), or signal intensity per cell ($F(2,14)=2.38$, $p=0.129$ and $F(2,14)=0.059$, $p=0.943$, respectively).

Discussion

5-HTP is the intermediate product in the synthesis of serotonin from its precursor L-tryptophan (Trp). It represents the result of the first, rate-limiting step catalyzed by the enzyme tryptophan hydroxylase and is sequentially reduced by the aromatic amino acid decarboxylase (AADC), a less specific enzyme involved in the synthesis of monoamines, catecholamines and trace amines. 5-HTP is quantitatively transferred into 5-HT and was shown to cross the placental and the blood-brain barrier [18,19].

In our study 5-HTP was administered to rats perinatally, during the period of development of serotonergic neurons, in a dose comparable to therapeutic doses in human population [7], which was proven to effectively raise blood 5-HT concentrations in adult rats without inducing the serotonin syndrome [20].

Effects on physiological parameters

As expected from our previous study [15], 5-HTP treatment significantly raised 5-HT concentration in blood. It did not, however, significantly affect cortical 5-HT concentrations, indicating that it might have been completely (or mostly) consumed in the periphery. Since AADC acts both in the periphery and in the CNS, the amount of 5-HTP reaching the central nervous system is affected by the extent to which 5-HTP is converted to serotonin in the periphery, and this site of 5-HTP conversion seems to be dominant. Indeed, the vast majority of successful clinical trials involving 5-HTP actually used the co-administration with the peripheral AADC inhibitor in order to achieve significant central effects [10]. Alternatively, 5-HTP reaching the central compartment might have induced effective compensatory mechanisms in serotonergic neurons of the midbrain that resulted in unchanged cortical levels of serotonin. 5-HTP treatment did not seem to have a significant effect on the cortical catecholamine levels. Although somewhat higher concentrations of DA (15%) and NA (25%) were observed in females in comparison to males, the influence of gender on catecholamine levels was also not significant presumably due to the small number of samples per subgroup and relatively high deviations in female NA levels. Indeed, brain catecholamine levels in rats were reported to be positively regulated by ovarian hormones, and to vary in females during the estrus cycle [21,22].

First observable effects of the treatment were indicatively lower weight-gain in dams and significantly lower birth weight of the newborn pups in the 5-HTP treatment group (**Table 1**). Similar findings were reported after the application of an acute dose of 100 mg/kg of 5-HTP to pregnant rats [19], and might be explained by the combination of: 1) the reduction of utero-placental blood flow probably induced by increased placental 5-HT, and 2) anorectic effects of increased peripheral 5-HT concentrations on both, dams and pups during the respective prenatal and postnatal part of the treatment. Regarding the former, 5-HT was shown to have a marked vasoconstrictor effect on the capacitance vessels of the human placenta, suggesting a role in the mediation of fetal circulation [23]. Regarding the latter, experimental peripheral administration of 5-HT was shown to decrease food consumption in adult rats [24], or to enhance lipid metabolism in mice [25], pointing at a role of 5-HT in energy balance. Body mass of the 5-HTP treated rats remained significantly lower not only at the end of treatment, but also in adulthood, long after a wash-out period suggesting permanent metabolic changes.

Effects on brain development

The expression of the 5-HT transporter, vesicular monoamine transporter 2 (VMAT 2) and 5-HT1B receptor in rodent TCA terminals during the first three postnatal weeks [26,27], and co-localization of 5-HT axons and TCA patterns in the early postnatal somatosensory cortex [28] suggest that 5-HT may play an important role in the modulation of BF formation. 5-HT originating from the raphe nuclei axons is presumably taken up into TCA terminals and used to regulate thalamocortical fiber segregation, as well as glutamatergic neurotransmission between thalamic axons and layer IV neurons, through the activation of 5-HT1B auto-receptors [13].

The conditions of an increased extracellular 5-HT concentration such as those existing in the 5-HT transporter or MAO A knock-outs [29,30], as well as the selective activation of 5-HT1B receptors without raising 5-HT concentrations [31], seem to considerably disrupt BF formation by altering the segregation of thalamocortical fiber patches through overactivation of 5-HT1B receptors. On the other hand, pharmacological 5-HT depletion [27,32], or inactivation of VMAT 2 gene [29], leading to a decrease in the extracellular 5-HT concentrations, do not appear to have an adverse impact on the BF structure but rather lead to a decrease in barrel size. Combination of modestly altered barrel organization and reduced barrel size presumably due to the reduced number of branch tips on the thalamocortical afferents, was observed in rat pups after early postnatal SSRI treatment [33,34].

Under our experimental conditions, 5-HTP treatment did not change the organization of the barrel field or the cytoarchitecture of individual barrels, but it did reduce the size of the barrels in the PMBS of the treated pups – a change that remained through adulthood. There are two possible explanations for this observation.

Since our findings resembled those of 5-HT depletion studies, one possible explanation is induction of compensatory mechanisms in the midbrain 5-HT neurons that would

counterbalance high blood-borne concentrations of 5-HT and/or its precursor, and eventually lead to smaller amounts of 5-HT available to TCA. Results from 5-HT immunostaining of the raphe nuclei region allow for this possibility. While there were no alterations in the number and size of neuronal cell bodies, 5-HT signal intensities were modestly but significantly decreased, pointing at lower 5-HT content in the somas of serotonergic neurons. This result is in line with our previous finding of significantly increased levels of mRNA for MAO genes in the midbrain raphe region after the perinatal 5-HTP treatment, suggesting an increased degradation of the internalized/synthesized 5-HT as a compensatory mechanism to maintain central 5-HT homeostasis [35]. Persistence of increased MAO mRNA levels and decreased 5-HT concentrations in adulthood point to a permanent alteration in the central 5-HT homeostasis as a result of perinatal exposure to 5-HTP.

Another possible explanation would be in line with the argument of Persico et al. [29] that the reduced barrel size is a consequence of growth-impairment induced by pharmacological or genetic manipulation rather than of altered central 5-HT concentrations. Indeed, 5-HTP treatment in our study significantly reduced body mass of the pups, as did the earlier mentioned 5-HT depletion manipulations and SSSRI treatment, and might have indirectly, rather than directly, affected the size of the barrels.

Regardless of the cause, smaller barrel size may explain increased time spent in object exploration (hole-board and social choice tests) without alterations in other aspects of anxiety-related behaviour, previously observed in the 5-HTP treated animals [16], as possibly blunted tactile response would require prolonged time of exposure to the explored objects. Altered somatosensory response was also observed in neonatally SSRI-treated rat pups as decreased performance [34] and in 5-HTT knock-out mice as increased performance [36] in the whisker-dependent gap test, as well as in infants prenatally exposed to SSRI as reduced response to pain [37].

Study limitation

Number of females entering the experiment was chosen according to the Three R principle. It was determined from our previous experience and considered sufficient to provide us with 6-10 pups per treatment group for each analysis (i.e. biochemical, histological and immunohistochemical). However, rather small litter sizes, as well as loss of several samples, resulted in sub-optimal sample number, especially in immunohistochemical analysis, making some of our findings only indicative and requiring further confirmation.

Conclusion

Due to an increase of public interest in natural therapies, 5-HTP regained its popularity as a natural alternative to antidepressant drugs. The versatility of potential therapeutic effects, easy availability over-the-counter or through the internet and the convenience of unsupervised use, have increased probability of prenatal exposure to this 5-HT precursor.

We have demonstrated that perinatal treatment of rats with 5-HTP induced hyperserotonemia, a decrease in birth-weight and postnatal weight-gain, and a reduction in size of the PMBS barrels (possibly leading to impairments in whisker-mediated perception), and indicated a possibility of a long-lasting compensatory reduction of 5-HT content in the cell bodies of serotonergic neurons in the midbrain. Our results suggest a need for further, more thorough, examination of the perinatal 5-HTP effects in animal models.

Taking into account that the period of treatment used in our study corresponds to the second and third trimester of human pregnancy [38], analogous effects on the exposed human fetus cannot be ruled out. The possibility of impeded growth, impairments in sensory processing, and other potential behavioural alterations caused by subtle structural changes but visible only after exposure to environmental triggers, should also be systematically examined in human population before further widening of 5-HTP use.

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Competing Interests

The authors declare no conflict of interest.

Author Contributions

DH, SAB, and NJM designed the study; SAB performed pharmacological treatment; SAB and DH collected samples and performed ELISA; SAB, DS and BN performed Nissl staining and 5-HT immunostaining; NJM performed qualitative and DS, BN and KI quantitative analyses of the brain slices. DH performed statistical analyses of data and wrote first draft of the manuscript. DS, BN and KI prepared figures. All authors participated in writing of the manuscript and in its critical revision.

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