Physiological consequences of perinatal treatment of rats with 5-hydroxytryptophan

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, which 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 oxidase 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 gravity 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«, 13/2006).

**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 3 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).

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 treated rats needed less time to return to their dam after separation, a significant difference was observed only in the 5HTP treated group (p < 0.05).

**TABLE 1**

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

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Saline treated group</th>
<th>5HTP treated group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of pups born</td>
<td>19</td>
<td>24</td>
</tr>
<tr>
<td>Offspring per dam</td>
<td>9.5</td>
<td>8</td>
</tr>
<tr>
<td>Number of died pups</td>
<td>1</td>
<td>14</td>
</tr>
<tr>
<td>Litter size per dam</td>
<td>9.33 ± 0.58</td>
<td>42 ***</td>
</tr>
<tr>
<td>Survival rate (%)</td>
<td>95</td>
<td>42 ***</td>
</tr>
<tr>
<td>Birth weight (g)</td>
<td>7.3 ± 0.8</td>
<td>6.0 ± 0.8 ***</td>
</tr>
<tr>
<td>Adult weight at PND 44 (g)</td>
<td>162.0 ± 17.5</td>
<td>140.6 ± 22.4 **</td>
</tr>
</tbody>
</table>

**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 (square). 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.

**Figure 2.** Return to dam test. Bars show time needed for saline (N=18) and 5HTP (N=10) treated pups to return to their dams after separation. Values are expressed as M ± SD; * p<0.05, Mann Whitney test.
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 consequently, 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 pre-treated rats (48). Regarding the second, increased anxiety-like behavior was observed in a rat sublime with high platelet 5HT level in comparison to the sublime 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.

REFERENCES
15. HUETHER G, THOMKE F, ADLER L 1992 Administration of tryptophan-enriched diets to pregnant rats retards the development of the serotonergic system in their offspring. Dev Brain Res 68: 175–181
18. HERNANDEZ-RODRIGUEZ J, CHAGOYA G 1986 Brain serotonin synthesis and Na+,K+-ATPase activity are increased postnatally after prenatal administration of L-tryptophan. Brain Res 350: 221–226
extracellular levels of 5-HT and 5-HIAA in the hippocampus of the rat using microdialysis. Eur J Pharm Sci 4: 247–256
35. JOYCE D, HURWITZ H M B 1964 Aspiration behaviour in the rat after 5-hydroxytryptophan (5-HTP) administration. Psychopharmacology 5: 424–430