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Cerebellar level of neurotransmitters in rats exposed to paracetamol during development

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ABSTRACT

Background: The present study was designed to clarify the effect of prenatal and postnatal paracetamol administration on the neurotransmitter level and balance of amino acids in the cerebellum.
Methods: Biochemical analysis to determine the concentration of neurotransmitters in this brain structure was performed on two-month-old Wistar male rats previously exposed to paracetamol in doses of 5 (P5, $n = 10$) or 15 mg/kg (P15, $n = 10$) throughout the entire prenatal period, lactation and until the completion of the second month of life, when the experiment was terminated. Control animals were given tapped water (Con, $n = 10$). The cerebellar concentration of monoamines, their metabolites and amino acids were assayed using High Performance Liquid Chromatography (HPLC).
Results: The present experiment demonstrates that prenatal and postnatal paracetamol exposure results in modulation of cerebellar neurotransmission with changes concerning mainly 5-HIAA and MHPG levels.
Conclusion: The effect of paracetamol on monoaminergic neurotransmission in the cerebellum is reflected by changes in the level of catabolic end-products of serotonin (5-HIAA) and noradrenaline (MHPG) degradation. Further work is required to define the mechanism of action and impact of prenatal and postnatal exposure to paracetamol in the cerebellum and other structures of the central nervous system (CNS).

Introduction

Paracetamol (acetaminophen, *N*-acetyl-*p*-aminophenol) is at the top of the list of over-the-counter medications taken prenatally. The drug passes freely through the placenta and can affect fetal development, but most clinical trials have not confirmed the association between exposure to paracetamol in the first trimester of pregnancy and an increased risk of congenital abnormalities [1–4].

Although the drug has been used for many years as a first-choice analgesic and antipyretic during pregnancy and in infancy, its effect on the early neural development of the central nervous system (CNS) has not been sufficiently explored. In particular, the impact of prenatal and early postnatal exposure on cerebellar development and function is little known. In the course of ontogenesis, cerebellar structures develop relatively early and from the beginning operate in close connection with the motor centers in the CNS. The cerebellar neurons arise in a sequential order: deep nuclei neurons in charge of output are generated first, interneurons are produced later [5,6]. Meanwhile, the cerebellum is responsible for maintaining body balance, control of eye movements and participation in the planning, initiating and proper coordination of movements. This part of the brain allows to adjust incorrect movements, as well as learn and memorize the new motor skills and their automation.

Paracetamol has a multidirectional effect on the CNS and displays a complex mechanism of action, which definitely makes it hard to speculate on possible long-term consequences of the drug on neural development and cognition. Its analgesic and antipyretic properties are probably associated with inhibition of the centrally situated isoform of the cyclooxygenase enzyme COX-3 and suppression of elementary prostaglandin synthesis. Other

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aspects of central actions are also identified, e.g. stimulation of transient receptor potential cation channel A1 (TRPA1) protein by the active metabolites of paracetamol, interaction with opioid receptors and with serotonergic, noradrenergic and cholinergic systems [7–10].

Even though human studies indicate paracetamol innocuousness, further neurotoxicological and behavioral studies are needed to demonstrate the harmlessness of paracetamol administration in the prenatal and early postnatal periods. In general, in infants the metabolic clearance and elimination capacity of paracetamol is below the expected values and for full safety use of the drug should be limited to 48–72 h [8–14].

An alarming cohort clinical study concerning the effects of early exposure to paracetamol on motor skills and the risks of adverse psychomotor and behavioral outcomes confirm a possible connection between prenatal drug exposure and neurodevelopmental disorders [15]. At the same time, it has been shown that early postnatal exposure to COX inhibitors alters cerebellar Purkinje cell development [16]. In rats paracetamol affects dendritic length and lead to sex-dependent changes in the cerebellum, e.g. a decrease of dendritic spines, cell atrophy and loss of cerebellar volume in males. This indicates subtle sex-based differences in cerebellar responses. The cerebellum integrates sensory information and cognitive tasks such as working memory [17]. In the present study, we have decided to see how paracetamol administered prenatally and in the early ontogenesis affects the cerebellar neurotransmission.

Materials and methods

Animals and paracetamol treatment

Virgin, three-month old Wistar (Albino Glaxo) rat females were mated and the day when the vaginal plug was detected was considered the first gestation day. Administration of paracetamol began with the first day of pregnancy. Pregnant females ($n = 15$) were randomly assigned to paracetamol treated groups or control group and placed individually in plastic breeding cages in a room with controlled temperature (24–26 °C) and 12 h dark-light cycle. During pregnancy and lactation mothers were given free access to standard food (Labofeed, Kcynia, Poland) and tap water or solution of paracetamol. Drugs solutions were administered orally in drinking water, and concentrations were adjusted to an average daily intake of 5 (group P5) and 15 mg/kg (group P15) per rat. In order to minimize dosing error, we have controlled the quantity of a drinking drug solution each day to properly adjust the medication dose.

The dams were checked daily in the morning for pups. After birth, male offspring was left with dams until 28 days of age. After weaning five animals per cage were placed and oral administration of paracetamol at doses of 5 (P5, $n = 10$) and 15 mg/kg (P15, $n = 10$) was continued for the second month of life. Control pups received water (Con, $n = 10$). The effect of maternal and early life exposure to low doses of paracetamol was assessed in two-month old male rats. All procedures were carried out in compliance with the ethical standards of the Directive 2010/63EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes.

Biochemical assessment

The cerebellar concentrations of monoamines, their metabolites and amino acids were estimated in 30 rats. Control and treated rats were sacrificed at two months of age. Rats offspring were decapitated and samples for evaluation of cerebellum neurotransmitters by high-performance liquid chromatography (HPLC) were immediately sectioned on ice. Cerebellar tissue was rapidly weighed, flash frozen and stored in a deep freezer (−80 °C) for future biochemical analysis. To precipitate proteins, tissues were homogenised in ice-cold 0.1 N perchloric acid and centrifuged at 13,000 × g for 15 min at 4 °C. The supernatant was collected, filtered (0.2 μm pore size filter; Whatman) and a 20 μL aliquot was injected onto the HPLC.

The concentration of cerebellar dopamine (DA) and corresponding metabolites: 3,4-dihydroxyphenyl acetic acid (DOPAC), homovanillic (HVA), 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA), noradrenalin (NA) and 3-methoxy-4-hydroxyphenylglycol (MHPG) was determinated using a HPLC procedure, as previously described by our group [18]. In summary, L-3500 A detector (Merck) with electrochemical detection and glassy carbon electrode was employed, the electrochemical potential was set at 0.8 V, the mobile phase comprised 32 mM sodium phosphate, 34 mM citric acid, 1 mM octane sulfonic acid and 54 μM ethylenediaminetetraacetic acid in deionised water containing 12% methanol. Monoamines were separated on a C-18 column (Nucleosil, Macherey-Nagel, Germany) and the mobile phase flow rate was maintained at 0.8 ml/min. Data were collected and analysed by Eurochrom 2000 for Windows (Knauer, Germany). Contents of neurotransmitters and metabolites were expressed as ng/g of tissue.

The protocol for assessment of concentrations of amino acids neurotransmitters; glutamic acid (GLU), aspartate (ASP), alanine (ALA), histidine (HIS), γ-aminobutyric acid (GABA) and taurine (TAU), followed the previously described procedure [18]. The final amount of amino acids in the tissue sample were expressed as ng/mg brain tissue. All standards were purchased from Sigma-Aldrich (St. Louis, USA).

Statistical analysis

Comparison of monoamines, metabolites and amino acids concentration between-groups was performed by using one-way analysis of variance ANOVA. Significant effects were examined subsequently by Newman-Keuls test (NK) to determine specific differences. All data are presented as mean ± SE. The accepted level of significance for all tests was $p < 0.05$. All results without statistical significance with p above this value were marked as not significant (N.S.).

Results

Cerebellar monoamines concentration

The level of monoamines and their metabolites in the cerebellum are summarised in Table 1.

NA and metabolites

ANOVA did not demonstrate any statistically significant difference between the content of NA in the cerebellum between all experimental groups ($F_{(2,27)} = 1.61$, N.S.). Statistically significant differences between groups in the cerebellar MHPG concentration were observed ($F_{(2,27)} = 3.49$, $p < 0.05$). The P15 group had significantly higher level of MHPG compared with that in the control group ($p < 0.05$, NK) (Fig. 1). The ANOVA demonstrated statistically significant differences between groups in the MHPG/NA ratio in the cerebellum ($F_{(2,27)} = 4.14$, $p < 0.05$). Analysis of differences between groups showed that cerebellar MHPG/NA ratio was significantly higher in the P15 group compared to the P5 group ($p < 0.05$, NK). There were no significant differences between the control and any of the paracetamol treated groups in the MHPG/NA ratio.

Table 1
Effect of the prenatal and postnatal paracetamol administration on the level of monoamines and their metabolites (mean ± SE) in the cerebellum of rat pups.

Monoamine and metabolite levels in the cerebellum ng/g wet tissue (mean ± SE)				
	Con	P5	P15	F value
NA	400.47 ± 39.50	463.69 ± 19.50	451.48 ± 12.57	$F_{(2,27)} = 1.61$
MHPG	**48.47 ± 6.37**	46.47 ± 2.91	**62.54±4.11***	$F_{(2,27)} = 3.49$
MHPG/NA	0.12 ± 0.01	**0.10±0.01**	**0.14±0.01**●	$F_{(2,27)} = 4.14$
DA	26.42 ± 2.84	27.30 ± 1.18	30.89 ± 1.67	$F_{(2,27)} = 1.38$
DOPAC	6.06 ± 0.81	8.15 ± 1.29	9.86 ± 1.13	$F_{(2,27)} = 3.02$
DOPAC/DA	0.35 ± 0.15	0.30 ± 0.04	0.33 ± 0.04	$F_{(2,27)} = 0.10$
HVA	11.87 ± 2.36	16.32 ± 1.77	17.18 ± 2.21	$F_{(2,27)} = 1.80$
HVA/DA	0.51 ± 0.10	0.60 ± 0.06	0.56 ± 0.07	$F_{(2,27)} = 0.32$
5-HT	30.03 ± 5.40	47.90 ± 6.05	34.61 ± 4.18	$F_{(2,27)} = 3.11$
5-HIAA	**29.09±3.84**	**49.68±7.28***	**28.37±4.40**●	$F_{(2,27)} = 5.05$
5-HIAA/5-HT	1.20 ± 0.19	1.02 ± 0.08	0.83 ± 0.10	$F_{(2,27)} = 1.89$

* P5, P15 *vs.* Con, $p < 0.05$ (Newman-Keuls).
● P5 *vs.* P15, $p < 0.05$ (Newman-Keuls).

DA and metabolites

The ANOVA did not reveal statistically significant differences between groups in DA concentration ($F_{(2,27)} = 1.38$, N.S.), DOPAC content ($F_{(2,27)} = 3.02$, N.S.) and DOPAC/DA ratio ($F_{(2,27)} = 0.10$, N.S.) in the cerebellum. Overall ANOVA did not demonstrate significant differences between groups in HVA content ($F_{(2,27)} = 1.80$, N.S.) and HVA/DA ratio in this structure ($F_{(2,27)} = 0.32$, N.S.).

5. -HT and metabolites

There was no statistically significant differences between groups in the 5-HT content ($F_{(2,27)} = 3.11$, N.S.) and in the cerebellar 5-HIAA/5-HT ratio ($F_{(2,27)} = 1.89$, N.S.). ANOVA demonstrated significant difference between tested groups in the content of 5-HIAA in the cerebellum ($F_{(2,27)} = 5.05$, $p < 0.05$). Cerebellar level of 5-HIAA was higher in P5 group compared to the P15 group and control rats ($p < 0.05$, NK) (Fig. 1).

Cerebellar amino acids concentration

Effects of the prenatal and early life paracetamol administration on the amino acids in the cerebellum are given in Table 2. In the cerebellum the significant increase of alanine concentration was observed in the group treated with paracetamol at dose 15 mg/kg body weight (b.w.) (P15: 344.29 ± 7.82 ng/mg tissue) compared to the group received paracetamol at dose 5 mg/kg b.w (P5: 299.65 ± 11.86 ng/mg tissue) ($F_{(2,27)} = 3.99$, $p < 0.05$). There was no significant differences between the control and any of the paracetamol treated groups in the alanine level. The level of glutamic acid ($F_{(2,27)} = 2.26$, N.S.) and aspartic acid ($F_{(2,27)} = 3.02$, N.S.) did not change significantly after paracetamol administration. The level of GABA ($F_{(2,27)} = 1.60$, N.S.), histidine ($F_{(2,27)} = 1.07$, N.S.) and taurine ($F_{(2,27)} = 1.83$, N.S.) did not change significantly in paracetamol treated groups.

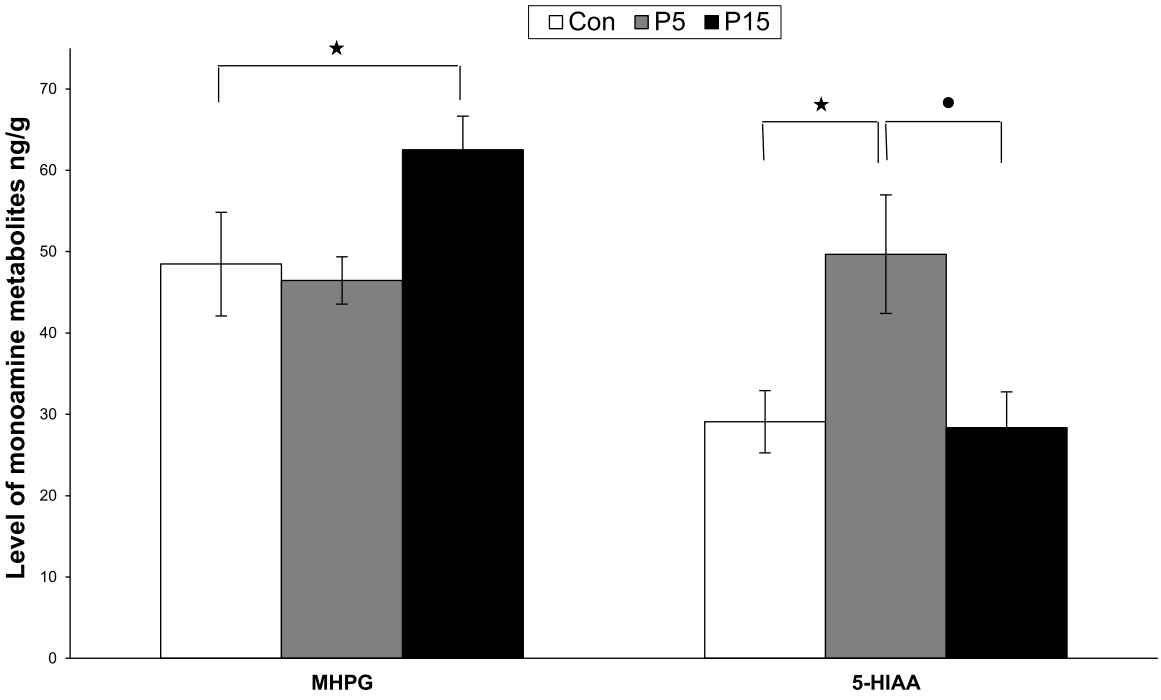

Fig. 1. Changes in the cerebellar MHPG and 5-HIAA concentration (mean ± SE) in Control group and rats exposed to paracetamol during fetal development and in postnatal period. *P5, P15 *vs.* Control, $p < 0.05$ (Newman-Keuls); •P5 *vs.* P15, $p < 0.05$ (Newman-Keuls).

Table 2
Effect of the prenatal and postnatal paracetamol administration on the level of amino acids (mean ± SE) in cerebellum of the rat pups.

Amino acid levels in the cerebellum ng/mg wet tissue (mean ± SE)				
	Con	P5	P15	F value
Glutamic acid	1728.10 ± 80.28	1773.71 ± 37.72	1891.43 ± 39.62	$F_{(2,27)} = 2.26$
Taurine	765.94 ± 25.60	762.04 ± 12.85	804.93 ± 10.94	$F_{(2,27)} = 1.83$
Alanine	315.55 ± 13.55	**299.65 ± 11.86**	**344.29 ± 7.82**•	$F_{(2,27)} = 3.99$
γ-Aminobutyric acid	302.95 ± 14.50	322.55 ± 8.12	330.10 ± 9.68	$F_{(2,27)} = 1.60$
Aspartic acid	278.61 ± 18.76	294.50 ± 10.23	326.10 ± 11.20	$F_{(2,27)} = 3.02$
Histidine	10.89 ± 0.81	12.11 ± 0.31	11.58 ± 0.54	$F_{(2,27)} = 1.07$

• P5 *vs.* P15, $p < 0.05$ (Newman-Keuls).

Discussion

The present study demonstrates that fetal and early exposure to low doses of paracetamol in rats is changing cerebellar neurotransmission in small extent. The potential risk of deleterious effects on central nervous system function due to prolonged prenatal and early postnatal paracetamol treatment is still unknown. The drug is widely used throughout the human population because there is a widespread belief that it is safer than the other over-the-counter analgesics.

Our decision to use paracetamol across the whole pregnancy in rats results from the fact that epidemiological data from more than 10,000 pregnant women show that it is the most widely taken drug, with at least 65% of women using it at some point during pregnancy [19]. Overall, the amount of reliable data concerning the safe dosage of paracetamol during pregnancy and early life remains very limited. By selecting the dosage of paracetamol, we have relied on relatively few pharmacokinetic studies in humans. It is assumed that well-tolerated dose in neonates ranges between 10–30 mg/kg b.w. To provide an adequate drug concentration and achieve a desirable analgesic effect in neonates, a loading dose of 20 mg/kg b.w. of paracetamol followed by 10 mg/kg b.w./6h *iv* is required [20,21]. Furthermore, it is reported that prolonged use of paracetamol in pregnancy adversely affects early development, although the sporadic use of the drug for a short period is probably not harmful to the fetus [15]. Breastfed infants receive only a daily maximum of approximately 1–3.6% of the maternal dose [22–24]. The clinical relevance of these observations is immense, although the critical window of exposure is not yet specified [25].

For all of the above-mentioned reasons, in our study the daily dosage was set only slightly below the standard therapeutic level due to a lack of data on drug accumulation in the body of the fetus and to avoid toxicity. Preclinical studies suggest that at the recommended doses paracetamol affects the cognitive processes and behavior of animals by inhibiting the activity of endogenous cyclooxygenase, changing neurotransmission and influencing the endocannabinoid system [26]. *N*-arachidonoylphenolamine (AM404), an active lipid metabolite of paracetamol formed by the enzymatic activity of fatty acid amide hydrolase (FAAH), is a potent activator of cannabinoid receptor type 1 (CB1) [27,28]. It is interesting that cannabinoid binding sites in rodents are concentrated in the basal ganglia and cerebellum – areas of the brain responsible for coordination of movement [29]. Cannabinoid receptors appear early in embryonic development and are expressed presynaptically by motor neurons [30]. In addition, CB1 receptors can modify the neurotransmitter release through the inhibition of adenyl cyclase and protein kinase A [31].

Cerebellar impairment may influence the functioning of the entire nervous system and ultimately affects cognitive abilities and behavior later in life.

Even a single oral administration of a therapeutic dose of paracetamol (100, 200 and 400 mg/kg) in rats causes passage of the drug through the blood-brain barrier and homogeneous distribution in CNS structures [32,33]. Recently published studies indicate that paracetamol influences the development of the neonatal brain and induces long-lasting anomalies in the cognition and locomotor activity of mice [34].

Our study demonstrates that prenatal and early postnatal paracetamol exposure results in significant modulation of cerebellar neurotransmission mainly with respect to the noradrenergic and serotonergic systems. Paracetamol changes the level of 5-HIAA and MHPG – catabolic end products of serotonin and noradrenaline metabolism. We can assume, that alterations in the metabolites concentration observed in 2-month old rats are not related to the impaired neurogenesis. Probably the amendments of the neurotransmitter levels in this brain are related to the influence of paracetamol on the activity of enzymes involved in the degradation of monoamines: aldehyde dehydrogenase, catechol-O-methyltransferase (COMT), monoamine oxidase (MAO) or norepinephrine transporter (NET). The next stage of our research will address this hypothesis.

In vitro investigation demonstrated that paracetamol in concentration up to 1 mM has no effect on the activity of enzymes responsible for catecholamines metabolism [33]. However we believe, that the differences in drug response may result from stimulation of the activity of one or more of these enzymes at lower dose and inhibition of its activity by higher dose of paracetamol.

In vitro studies show that paracetamol inhibits the activity of Na^+K^+ATPase and Mg^{2+}ATPase in the cerebrum and cerebellum of a human fetus from 10 to 32 weeks of gestation. Inhibition of enzyme activity can affect the release and uptake of biogenic amines in the developing CNS, thereby disrupting the maturation of the fetal brain [35]. Simultaneously, we do not exclude that changes in the concentration of 5-HIAA and MHPG can be triggered by indirect action of paracetamol on the level of prostanoids in CNS *via* receptor EP3 located on several monoamine neurons [26].

The cerebellum is the part of the brain responsible for coordination of body movements, muscle balance and tone. The main task of the cerebellum is sensory-motor coordination, which represents the integration of visual, auditory and motor information. Consequently, the cerebellum is also involved in memory processes, learning, attention and detection of sequences of movements [36].

Changes in noradrenergic and serotonergic neurotransmission in the cerebellum can have serious consequences for the proper functioning of the whole nervous system, and can interfere with cooperation between other brain structures. Through the assessment of the variations in the metabolites level in adult rats we can not definitely exclude their impact for the early neurogenesis. The changes established in our experiment, apply to the 2-month-old rats. There is no assurance that these alterations occurred in the earlier stages of development of the cerebellum and whether will last after discontinuation of paracetamol. Dysregulation of serotonergic neurotransmission in the cerebellum may adversely

affect higher cognitive and emotional functions. Furthermore, abnormalities in serotonin homeostasis within the cerebellum may be responsible for some symptoms specific to psychiatric disorders like ADHD or autism [37–39]. Serotonin is a crucial neuromodulator in the early development of the mammalian brain, which is detectable in neurons about 10 days before birth [40,41]. This indeed underlines the key role of serotonin in the physiology of the developing cerebellum. Serotonin affects different development phases of the cerebellum by controlling dendritic growth, as well as the formation and stabilization of synapses. This applies particularly to the first 3 weeks after birth when important stages for cerebellar development take place such as migration, growth and maturation of the neurons [42,43]. Fetal exposure to the drug is controlled via placental drug transporters located in the syncytio-trophoblast and the fetal capillary endothelium. Examples of such transporters are multi-drug resistance proteins (MRPs), which may participate in the removal of acetaminophen sulfate through the placenta [44,45].

These observations are in accordance with the results of our earlier studies that have shown modulation of neurotransmission in the CNS after long-term subcutaneous paracetamol administration in young adult rats. In a similar manner, major changes in the prefrontal cortex, hypothalamus and striatum were related to serotonergic and noradrenergic neurotransmission [46]. Our earlier studies have shown that paracetamol causes a significant reduction of 5-HIAA concentration in the striatum and hypothalamus, as well as hypothalamic 5-HIAA/5-HT turnover. Paracetamol alters the dynamics of the serotonergic system in the CNS in many brain areas e.g. the hippocampus, posterior cortex, striatum and hypothalamus [32]. This variability in the cerebellar neurotransmission of serotonin may be due to changes in the activity of MAO-A or some alterations in 5-HT release/reuptake processes.

Acute intraperitoneal administration of a higher dose of paracetamol (400 mg/kg) also inhibits in the cerebellum and hippocampus creatine kinase activity, which is responsible for the maintenance of brain energy homeostasis [47].

In our study, tested animals received also the drug via their mother's milk. It is proven that recovery of the drug depends on the frequency of milk sampling. Paracetamol administered to breast-feeding women in doses of 1–2 g quickly passes into human milk and reaches an average level of 6.1 ± 1.3 mg/L. In the urine of newborns, glucuronide, sulphate, cysteine and mercapturate conjugates of the drug are detectable and, at the same time, larger proportions of unchanged parent drug and lower proportions of paracetamol sulphate appear as compared to the adult's values. This may indicate a deficiency of sulphate conjugations in newborns and the apparent immaturity of the excretory mechanisms [24].

As a result of our experiments, we have found that paracetamol at a dose of 15 mg/kg produces a significant increase in the cerebellar content of MHPG, a major noradrenaline metabolite. This is particularly relevant since we know that the noradrenergic system is crucial in the regulation of fundamental mammalian brain functions such as attention, memory consolidation and anxiety.

Changes in the level and turnover of cerebellar neurotransmitters may be induced by impaired sulfation due to long-term paracetamol administration during the prenatal and postnatal period [48].

In 2014, we have published data showing that eight weeks of paracetamol treatment (*sc*) affects the balance of amino acids in the striatum, prefrontal cortex and hypothalamus in adult rats [49]. The differences between the current study and research articles published by our team in the years 2013 and 2014 are substantial and include: origin of the animals, age, body weight, time of exposure, the stage of development at which the drug was administered and the route of administration. Therefore it is difficult to compare all these studies and it is hard to develop a coherent hypothesis linking these projects at the moment. In our previous studies the paracetamol in different doses (10 and 50 mg/kg b.w. per day) was administered only postnatally by subcutaneous injections to animals from another breeding pull and according to the different experimental protocol. Impact of 8 weeks long drug treatment on neurotransmitters level in other CNS regions was investigated in aged three-month old male Wistar rats [46,49].

Our study shows that prenatal and early postnatal paracetamol treatment elicits subtle changes in neurotransmission. Significant cerebellar differences in the content of end products of monoamines metabolism: 5-HIAA and MHPG suggests that paracetamol slightly affects neurotransmission and serotonin and noradrenaline catabolism. Further work is required to determine the safety and long-term consequences of paracetamol on fetal and long-term postnatal health. Neurodevelopmental and functional assessment of changes occurring after early paracetamol exposure will be the next stage of our research taken into account.

Conflict of interest

We wish to confirm that there are no known conflicts of interest associated with this publication.

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