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RESEARCH ARTICLE

Effect of Prenatal Stress and Serotonin Depletion on Postnatal Serotonin Metabolism in Wistar Rats

SAMPATH MADHYASTHA, PRABHU LATHA V, NAYAK SOUBHAGYA R, PAI MANGALA, RAI RAJALAKSHMI, PRASHANTHI MADHYASTHA

For author affiliations, see end of text.

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ABSTRACT

Prenatal stress in rats results in structural, physiological and behavioral alterations that persist in adulthood. Serotonin (5-HT) is an important neurotransmitter known to be involved in these prenatal stress-induced behavioral alterations. The aim of the study was to investigate the effects of interrupted synthesis of 5-HT and immobilization stress during different gestational period on brain serotonergic system of male and female neonatal (postnatal day 15) and adult rats (60 days old). Pregnant rats were subjected to restraint stress three times daily for 45 min during day 3-14 (G 3-14) or day 14-21 (G 14-21) of pregnancy. Another group of pregnant rats were injected with the inhibitor of 5-HT synthesis, parachlorophenylalanine (p-CPA, 400 mg/kg/2ml, single dose, ip) on day 9 or 17 of pregnancy. Following sacrifice, tissue concentrations of 5-HT and its metabolite 5-hydroxy indole acetic acid (5-HIAA) were analyzed in whole brains of neonatal pups and in brainstem, hypothalamus, hippocampus, and frontal cortex of adults. Stress during G 14-21 days showed a significant reduction of 5-HT and 5-HIAA levels in early neonatal development but not later during adulthood. Decreases in whole brain concentrations of 5-HT and 5-HIAA were observed in p-CPA 9 and G 14-21 neonatal pups. The concentration of 5-HT was decreased in frontal cortex and hypothalamus of adult rats receiving p-CPA. Prenatal stress affects tissue concentrations of 5-HT and 5-HIAA in neonatal pups and adults and it is possible that such changes may underlie the reported behavioral deficits in offspring of stressed female rats. These data also provide evidence that the critical period for prenatal stress-induced changes in brain 5-HT neurons were between days 14-21 (during final trimester of pregnancy).

Keywords: Prenatal stress, Prenatal 5-HT depletion, Serotonin, 5-hydroxy indole acetic acid

Prenatal or intrauterine development plays critical role in normal physical, mental and behavioral development of an individual. A vast number of factors such as genetic makeup [1-2], epigenetic factors like nutrition [3], environmental toxins [4-8] and stressful conditions [9-17] play a major role on normal growth in the intrauterine development. In most of such instances, affect of these insults will be carried to perinatal and young age and even to whole lifespan of individual [8,18]. Though any system of body may be the target of flawed development, the involvement of nervous system is of concern. Stressful experiences in humans result in a spectrum of long-term changes in behavioral, autonomic and normal responsivity [19].

A substantial body of evidence indicates that prenatal stress adversely affects human development, increasing susceptibility to diseases later in life as well as altering behavioral and cognitive development. Prenatal stress is also known to increase anxiety, behavioral and cognitive functions, in postnatal life [20-24]. Prenatal stress in rats results in structural, physiological and behavioral alterations that persist into adulthood. In males, prenatal stress alters analgesic sensitivity, taste preference and sexually dimorphic behaviors like sex behavior and partner preference [25-26]. Many reports signify that prenatal stress exhibit increased anxiety, emotional responses to the open field and a decreased tendency to explore [27-29].

There are many reports suggesting the possible mechanisms like altered hypothalamo-pituitary-adrenal axis, neurotransmitters, decreased brain cell proliferations and brain corticosterone level during prenatal development. A substantial body of evidences indicates that prenatal stress-induced behavioral alteration involves brain monoamines specially norepinephrine, dopamine and 5-HT. Serotonin is involved in neuronal

proliferation, migration and differentiation during early development [30]. The role of 5-HT is less studied when compared to dopamine and norepinephrine.

Prenatal stress by subcutaneous saline injection throughout pregnancy has decreased 5-HT level of hypothalamus on day 16 but re-elevated on day 60. Interestingly, the level of 5-HIAA did not altered on both day 16 and 60, but it decreased on day 9 and increased on day 23. In the same experiment, frontal cortex 5-HT and 5-HIAA levels were increased on day 16 but not in adulthood [31]. The same author in another experiment claimed an increase in 5-HT and 5-HIAA levels of fetal cerebral cortex of prenatally-stressed rats. This increase was observed up to postnatal day 10 [32]. Prenatal stress resulted in an increased level of 5-HT and decreased level of 5-HIAA in frontal cortex of female offspring but not in male. In the same experiment, the level of amygdala 5-HIAA was higher in female prenatally-stressed rats [17]. A recent experiment by Van den Hove et al [33] using immunocytochemical analysis showed a decrease in 5-HT_1 receptors in ventral hippocampus of four week-old male rats but not 5-HT_{2a} receptor. Prenatal restraint stress during gestational day 18-22 did not alter 5-HT, 5-HIAA, norepinephrine and dopamine levels in hypothalamus of rats tested at postnatal day 75. But dopamine, 5-HT and 5-HIAA levels increased significantly in striatum [34]. Lauder et al [35] have reported that 5-HT axons appear to have a close relationship with some proliferating cells during the late gestation and extending into the postnatal period and they suggest that 5-HT neurons may influence the development of less differentiated cells they contact.

From these findings it is unclear; weather brain serotonergic system plays a crucial role either in early development or during late gestation period. The results reported conflicts in postnatal 5-HT and 5-HIAA levels in different part of the brain. The method and gestational days used for prenatal stress also varies in these experiments. Hence, this study was planned to see the effect of different gestational period stress on the level of tissue concentration of 5-HT and 5-HIAA in different areas of the adult rat brain and whole brain of neonatal rats.

Studies comparing the effects of prenatal stress and prenatal 5-HT depletion on brain 5-HT metabolism have interesting findings. Though, there are indications like chronic restraint stress in mother's leading to conditions similar to 5-HT depletion, but its effects are manifested in different directions. Prenatal stress increased pain sensitivity while prenatal 5-HT depletion decreased pain sensitivity in rat offspring at the age of 90 days [36]. A recent study in the mice indicated that maternal p-CPA treatment on gestational day 9 has a significant influence on development of neurons and astoglial cells of neocortex [37-38]. There is a need to evaluate the brain 5-HT metabolism under prenatal stress condition as well as prenatal 5-HT depletion. It is important to emphasize that reported studies are limited to the analysis of effects of prenatal 5-HT depletion on morphological and behavioral changes in the brain, there is little known about 5-HT turnover in different parts of the brain.

METHODS

Animals

Inbred albino rats of Wistar strain of either sex of 105-120 days old, weighing 220-250 g were selected. The rats were maintained in 12-hour light and dark cycle in temperature- and humidity-controlled environment. The animals were fed with standard food pellet and water *ad libitum.* Breeding and maintenance of the animals were done as per the guidelines of Government of India for use of Laboratory animals (Government of India notifies the rules for breeding and conducting animal experiments, proposed in the gazette of India Dec 15, 1998: which was reproduced in Indian Journal of Pharmacology, 1999; 31:92-95).

Mating of rats and animal groups

Female rats (n=40) were divided into five groups. Rats from each group were allowed to mate with one fertile sexually-active male for 4 hours per day (separate male rats for each group). At the end of 4 hours, females were separated and vaginal smears were taken to detect the presence of sperm for the confirmation of pregnancy and the rats were designated as day 0 of pregnancy. The pregnant rats were housed individually in separate cages and were randomly allocated into 5 groups of eight each (n=8).

One group of female rats were stressed from gestational day 3-14 (G 3-14) and another group stressed between day 14-21 (G 14-21)

Stressing procedure

The pregnant rats were stressed (restraint stress) using a wire mesh restrainer [39] for three times daily for 45 min. The wire mesh restrainer will have a wooden base and stainless steel wire mesh restrainer hinged to the base. A pad lock and latch will help to secure the rat in the restrainer. The restrainer with dimensions 11cm (L) × 6cm (B) × 6cm (H) was used for G 3-14 groups. Restrainer of 11cm (L) × 8cm (B) × 8cm (H) was used for G 14-21 group. This type of restrainer will only restrict the animal movement without any pain, discomfort or suffocation.

Parachlorophenylalanine (p-CPA) administration

The third group of pregnant rats received the inhibitor of the 5-HT synthesis, p-CPA (Sigma, USA) on day 9 (p-CPA-9) and the fourth group on day 17 (p-CPA-17). The dose selected has previously been shown to be effective in reducing rat's midbrain 5-HT concentration by 74% three days after treatment. The day of injection was selected because the maximal effect of the drug (the greatest decrease of 5-HT concentration in the dam and in the embryo) is within 2-3 days after injection at this age [36]. This developmental window is critical for development of serotonergic system, since it is the time when dorsal raphe neurons start to differentiate [36]. The fifth group (control) of pregnant rats received saline vehicle.

After birth, the offspring of all groups were kept in

Figure-1

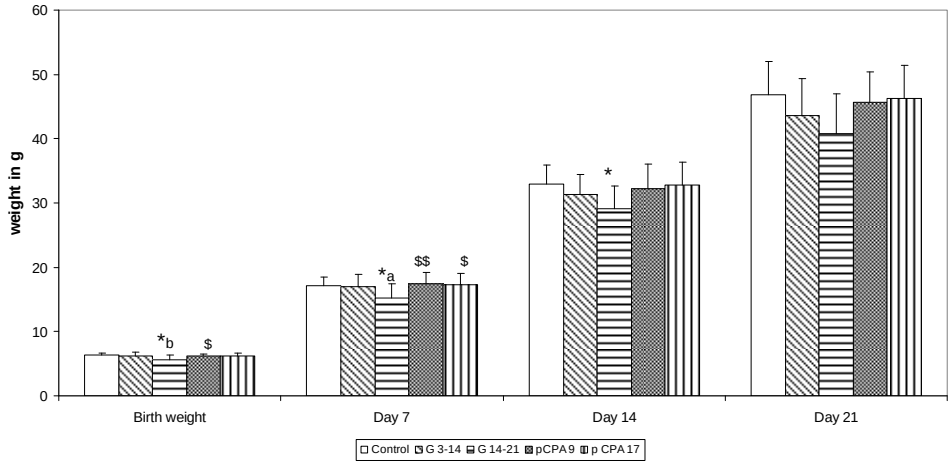

Fig 1. Mean (±SD) of birth weight (in g) and weight gained during different neonatal period following in uteri exposure to stress or 5-HT depletion (n=16). Animal groups: G 3-14: received prenatal stress during day 3 to 14, G 14-21: received stress during day 14 to 21, pCPA-9: received single dose Parachlorophenylalanine (pCPA) treatment (for 5-HT depletion) on day 9 of pregnancy and pCPA-17: received pCPA on day 17 of pregnancy. For comparison with control, * = $p<0.05$, for comparison with G 3-14, a=p<0.05, b=$p<0.01$, for comparison with G 14-21, $=$p<0.05$, $$=$p<0.01$.

same animal room and all pups were kept together with their mothers. The offspring of all groups were weaned 21 days after birth and housed separately based on sex until the completion of the study. Two pups from each litter (n=16) were considered (one male and one female) for 5-HT estimation.

Neonatal study parameters

i) Mortality: Number of still-born pups and total number of pups born to each control or stressed rat was counted at birth to calculate the mortality at birth. During postnatal period (till 21 days), number of death was also counted to get the post-natal mortality rate.

ii) Birth weight and weight gain: Weight of pups was recorded at birth and on day 7, 14 and 21. Comparison at same age between groups is described in the 'Result' section.

Estimation of brain serotonin and 5-hydroxy indole acetic acid

Offspring from all five groups (n=80; males, n=8 per group; females, n=8 per group) were sacrificed on day 15 or 60 by decapitation. In offspring sacrificed on day 15 (males, n=4; females, n=4), the entire brain was processed for estimation of 5-HT and 5-HIAA. In offspring sacrificed on day 60, the brains were rapidly removed and various regions of the brain (frontal cortex, hypothalamus, hippocampus and brainstem) were separated on an ice slab and then transferred to ice-cold saline. The tissues were immediately homogenized in 5 mL 0.4 N ice cold perchloric acid containing 0.1% ethylene diamine tetra acetic acid (EDTA) disodium salt, and 0.05% sodium metabisulphite and internal standard 3,4-dihydroxy benzylamine (DHBA) using a teflon-glass homogenizer (Thomas Scientific, Philadelphia

Fig-2.

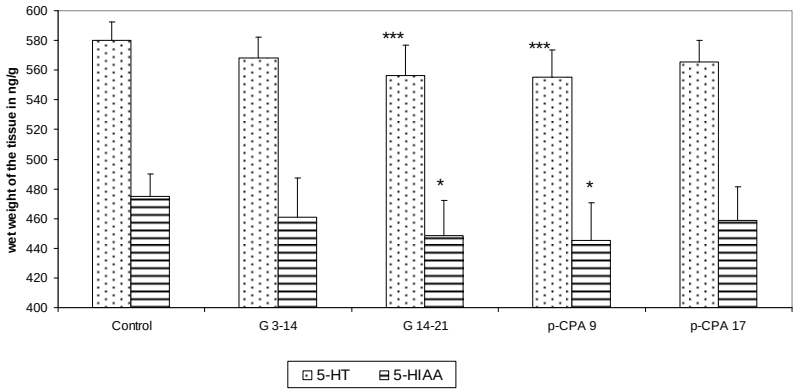

Fig 2. The level of 5-HT and 5-HIAA (ng/g wet weight of the tissue) in the whole brain of the different offspring groups on postnatal day 16. Animal groups: G 3-14: received prenatal stress during day 3 to 14, G 14-21: received stress during day 14 to 21, pCPA-9: received single dose Parachlorophenylalanine (pCPA) treatment (for 5-HT depletion) on day 9 of pregnancy and pCPA-17: received pCPA on day 17 of pregnancy. For comparison with control, * =p<0.05, *** =$p<0.001$.

Table 1. Level of 5-HT and 5-HIAA in different regions of the brain on day 60 (n=16). Values are expressed as mean ± SD

Brain region	Chemical measured	Experimental groups				
		Control	G 3-14[a]	G 14-21[b]	pCPA-9[c]	pCPA-17[d]
Frontal cortex	5-HT	172.2 ± 8.2	170.5 ± 9.5	166.32 ± 9.04	158.75 ± 11.24 **a	168.35 ± 10.65
	5-HIAA	162.34 ± 8.12	160.4 ± 9.2	158.65 ± 9.35	150.84 ± 12.22 *	154.38 ± 9.42
Hippocampus	5-HT	185.34 ± 15.5	188.24 ± 20.3	180.5 ± 18.5	172.62 ± 22.45	174.38 ± 17.5
	5-HIAA	146.45 ± 12.31	154.88 ± 16.22	144.65 ± 16.15	138.86 ± 20.18	144.22 ± 12.11
Hypothalamus	5-HT	510.37 ± 20.55	496.52 ± 23.45	484.50 ± 32.5	478.4 ± 27.35 **	490.42 ± 23.75
	5-HIAA	478.75 ± 18.25	462.38 ± 26.12	460.33 ± 26.11	448.44 ± 28.22 **	464.72 ± 24.36
Brainstem	5-HT	420.68 ± 14.5	426.24 ± 22.8	408.35 ± 24.4	406.71 ± 26.25	415.18 ± 16.3
	5-HIAA	415.75 ± 16.25	418.45 ± 28.15	398.86 ± 22.13	394.96 ± 32.23	406.32 ± 17.08

[a]G 3-14: gestational day 3-14, [b] G 14-21: gestational day 14-21, [c] pCPA-9: pregnant rats received the inhibitor of the 5-HT synthesis, Parachlorophenylalanine (p-CPA) on day 9, [d]pCPA-17: pregnant rats received p-CPA on day 17, *p<0.05, **p<0.01

Penn). The homogenate was centrifuged at 12000 rpm for 10 minutes at 40°C. The supernatant was removed and stored at -20°C until assayed. 5-HT and its metabolite 5-HIAA levels were estimated by HPLC with electrochemical detector (VMD-101a, Yanagimoto manufacturing Co. Ltd. Kyoto Japan).

The brain amines were isolated on a C18 reverse phase column of particle size 5 micron meter and 25 cm length using the isocratic elution method. The mobile phase was composed of 70 mM sodium acetate buffer (pH 4.5), containing 1 ml of 10% EDTA and 0.05 M hexane sulfonic acid in one litre of buffer. The buffer was filtered through a 0.45 micron millipore membrane filter and then mixed with methanol in 87:13 (v/v) ratios before use. The mobile phase was degassed using an online degasser (ERC-3310, Tokyo-Japan) before passing through the column. The flow rate was kept at 1.5 ml/min. The voltametric detector with a glassy carbon electrode was used for electrochemical detection of the amines. The detector potential was set at 0.8 V versus an Ag/AgCl reference electrode with a sensitivity of 1nA. A volume of 20 microL of the filtered homogenate was injected without prior processing. Peak recording and quantitation was done using the chromatocorder 11 (Alphatech Corporation Limited, Tokyo, Japan). The actual concentration of 5-HT and its metabolite was calculated by comparing the recovery of each standard amine from the crude homogenate with that of the internal standard. The amounts of 5-HT and 5-HIAA were expressed in nanograms per gram wet weight of the tissue [40-41].

Statistical analysis

The data were expressed as mean ± SD. Effects of prenatal environment (control, G 3-14, G 14-21, p-CPA-9, or p-CPA-17) on 5-HT and 5-HIAA concentrations were analyzed independently for each brain region or whole brain using one-way analysis of variance (ANOVA) test. Significant effects of prenatal environment were further analyzed with multiple pair-wise comparisons using Bonferroni's correction for multiple comparisons. The p values < 0.05 were considered significant. Comparison of data between male and female group was assessed by Mann-Whitney's unpaired "t" test.

Table 2. Comparison of levels of significance in different regions of the brain among groups of control, G 3-14, G 14-21, pCPA-9 and pCPA-17

	Frontal cortex		Hippocampus		Hypothalamus		Brainstem	
Values	P	F	P	F	P	F	P	F
5-HT	0.0023	4.568	0.0999	F=2.022	0.0097	3.602	0.0599	F=2.37
	Control Vs pCPA-9 **p<0.01, G 3-14 Vs pCPA-9 p<0.05		No significant difference among the groups		Control Vs pCPA-9 **p<0.01		No significant difference among the groups	
5-HIAA	0.0152	3.299	0.0801	2.174	0.0220	3.048	0.0268	2.915
	Control Vs pCPA-9 *p<0.05		No significance difference among the groups		Control Vs pCPA-9 **p<0.01		No significance difference among the groups	

G 3-14: gestational day 3-14, G 14-21: gestational day 14-21, pCPA-9: pregnant rats received the inhibitor of the 5-HT synthesis, Parachlorophenylalanine (p-CPA) on day 9, pCPA-17: pregnant rats received p-CPA on day 17.

RESULTS

No gender differences were observed in any measures and therefore, data from males and females were collapsed into one group. Prenatal environment (control, G 3-14, G14-21, p-CPA-9, p-CPA-17) had no effect on gestational length {F=0.6557; p=0.62} or litter size {F=1.946; *p*=0.124]. There was one still born female offspring in stress group (G 14-21). A male offspring died on postnatal day 1 in the late stress group.

Birth weight and weight gain

There was a main effect of prenatal environment (control, G 3-14, G14-21, p-CPA-9 or p-CPA-17) on birth weight {F=4.689; *p*=0.0020}, and on weight recorded on day 7 {F=4.255; *p*=0.0037}, day 14 {F=3.492; *p*=0.0114}, but not on day 21{F=2.33; *p*=0.0628}. Post hoc pair wise comparisons revealed that birth weight and weight on day 7 was lower in G 14-21 pups as compared to control ($p<0.05$), G 3-14 ($p<0.01$ for birth weight; $p<0.05$ for day 7) and p-CPA (9 or 17) ($p<0.05$). G 14-21 rats at day 14 were significantly lighter than controls ($p<0.05$) but not any of the other groups. There was no significant difference ($p=0.0628$, F=2.33) in the body weight when calculated at day 21 (Fig. 1).

Serotonin and 5-Hydroxy indole acetic acid levels on day 15

There was a main effect of prenatal environment (Control, G3-14, G14-21, p-CPA-9, or p-CPA-17) on tissue concentration of 5-HT (F=6.143) and 5-HIAA (F=3.559). Post hoc pair wise comparisons revealed that tissue concentration of 5-HT was lower in G 14-21 pups as well as p-CPA-9 pups as compared to control pups ($p<0.001$). The concentration of 5-HIAA was also lower in G 14-21 pups as well as p-CPA-9 pups as compared to control pups ($p<0.05$) (Fig. 2).

Serotonin and 5-Hydroxy indole acetic acid levels on day 60

There was a main effect of prenatal environment (Control, G3-14, G14-21, p-CPA-9, or p-CPA-17) on tissue concentration of 5-HT of frontal cortex (F=4.568; $p=0.0023$) and on hypothalamus (F=3.602; $p=0.0097$), but not on hippocampus (F=2.022) and brainstem (F=2.37). Post hoc pair wise comparison revealed that tissue concentration of 5-HT of frontal cortex in p-CPA-9 group was lower as compared to control ($p<0.01$) and G 3-14 group ($p<0.05$). The hypothalamic 5-HT concentration of p-CPA-9 was lower as compared to control ($p<0.01$) group. There was also a main effect of prenatal environment (Control, G3-14, G14-21, p-CPA-9, or p-CPA-17) on tissue concentration of 5-HIAA of frontal cortex (F=3.299, $p=0.0152$) and of hypothalamus (F=3.048, $p=0.0220$). Post hoc pair wise comparison revealed that tissue concentration of 5-HIAA of frontal cortex of p-CPA-9 group was lower as compared to control ($p<0.05$) groups. The hypothalamic concentration of 5-HIAA was also lower as compared to control ($p<0.01$) group (Tables 1&2).

DISCUSSION

The major finding of the present study is that prenatal stress during last embryonic week and prenatal 5-HT depletion on day 9 of gestation had similar effects (reduced brain 5-HT levels) on neonatal rat brain. Reduced 5-HT levels were not documented during adulthood in (early or late gestation period stress) parentally stressed rats. But the prenatal 5-HT depletion on day 9 had long term effect, since this effect (reduced 5-HT level) was observed in hypothalamus and frontal cortex even during adulthood. So from the present study it is clear that prenatal 5-HT depletion on day 9 involve mainly tissue concentration of 5-HT of hypothalamus and frontal cortex of the adult rats. The early prenatal stress and prenatal 5-HT depletion on day 17 did not have significant effect on postnatal 5-HT metabolism.

The statistically significant decrease in the birth weight (Figure-1) of the offspring subjected to late gestational stress and prenatal 5-HT depletion was consistent with previous studies in which both stress and p-CPA treatment (on day 9 of gestation) led to decrease in birth weight [31& 42] but inconsistent with Gerardin et al [34]. The offspring of late gestational period stress showed a delay in the weight gain up to second week of development but not during later period. p-CPA 9 did not have a delayed weight gain on day 7, instead gained weight slightly higher than control (Fig.1). The reduced birth weight and followed by delayed weight gain in prenatal stressed offspring could be due to involvement of many physiological system of the developing offspring. This could also be due to the involvement of 5-HT depletion, which is involved in gluco-regulation in the hypothalamus because the CNS is said to control blood glucose in highly complex process involving several brain areas [43].

Both late gestational stress and 5-HT depletion on day 9 of gestation resulted in a very significant reduction of whole brain 5-HT level in neonatal rats (Figure-2). Similar results were obtained by Plaut et al [44] in which the whole brain 5-HT level was decreased in 21 old day offspring after prenatal stress. In the present study the reduced level of 5-HT did not differ between late gestational period stress as well as prenatal 5-HT depletion on day 9. These results prove that the effects are similar between late prenatal stress and prenatal 5-HT depletion. There are reports which claim that the effects on behavior after prenatal stress and prenatal 5-HT depletion [45] were similar. There is a report claiming the effect of p-CPA treatment on 5-HT metabolism was maximum on gestational day 9 with reduction of whole brain 5-HT content on day 12 of prenatal development [46]. A recent study in mice indicated that maternal p-CPA treatment on gestational day 9 has a significant influence on development of neurons and neuroglial cells of neocortex [38-39]. Since the effect of late gestational stress had a similar effect to p-CPA treatment on day 9, it is possible to influence the development of neurons and neroglial cells.

The metabolite of 5-HT, the 5-HIAA level was also decreased in the whole brain of neonatal rats subjected to late gestational stress and p-CPA treatment on day 9.

These results are inconsistent with results of David Peters [32] in which the level of 5-HIAA was increased on gestational day 21 after prenatal stress and also similar report by Dunn [47].

It was interesting to note that, this effect (5-HT depletion) was observed on postnatal day 60 in frontal cortex and hypothalamus but not in hippocampus and brainstem (Table-1). David Peters [31] demonstrates an increase in the level of 5-HT and 5-HIAA at about 16 days of age and this increase was no longer present at 23rd day of age in cerebral cortex, pons and medulla. In the same experiment, the hypothalamus showed different pattern of monoamine changes. The level of 5-HT and 5-HIAA were reduced on day 9 and 16 and elevated on day 23 or 60 of postnatal life. An increased 5-HT and 5-HIAA levels in corpus striatum on postnatal day 75 of parentally stressed rats was reported by Gerardin et al [34], in which the hypothalamus did not show such increase. In our study, the prenatal stress did not show any change in 5-HT and its metabolite level on postnatal day 60 in stress group animals. The difference in the results could be due to region specific differences in 5-HT development [48], for example the cortex and striatum showed similar pattern of development (decreased turnover), while comparing with hippocampus (increased turnover). In an another study by Sobrain et al [49] claims no change in 5-HT and 5-HIAA levels in the brainstem of offspring of electrically shocked females at 1, 12 and 25 days. In an *in vivo* study by Lauder et al [35], depletion of fetal brain 5-HT by p-CPA was associated with depletion of 5-HT level in hypothalamus. The prenatal 5-HT depletion on day 9 resulted in decreased levels of 5-HIAA in frontal cortex and hypothalamus.

In rats, raphe neurons show evidence of 5-HT synthesis as early as embryonic day 13 and begin to receive synaptic contacts about one week later [50]. Maternal stress during this period may interfere with synapse formation. Serotonergic neurons in the raphe nucleus are among the earliest to be generated in the rat brain at about GD 11 to GD 15 [51]. These cells send axons to the forebrain and may be of importance in the differentiation of neuronal progenitors. Hence prenatal stress or prenatal 5-HT depletion at this critical period can alter the neonatal or adult brain 5-HT system. The effect of reduced brain 5-HT level (after prenatal stress or prenatal 5-HT depletion) in the present study might involve maternal neurotransmitter and endocrine system, which may further affect the fetal brain development. It is also observed that in adult animals, p-CPA treatment produced an elevation in the hormones of hypothalamic-pituitary adrenal axis with increase in plasma corticosterone [52]. Hence maternal hormonal estimation would give more insight into the possible mechanism underlying in it.

From the outcome of the study it is clear that prenatal stress induced neurotoxic effects on postnatal development of CNS, which involves brain 5-HT system especially during early development but not during adulthood. However experimental 5-HT depletion had long term effect on brain 5-HT system especially involving hypothalamus and frontal cortex. The data present further proof that harmful exposure during final trimester of pregnancy may cause severe affects on foetal brain development especially during neonatal development.

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CURRENT AUTHOR ADDRESSES

Sampath Madhyastha, Department of Anatomy, Kasturba Medical College, Mangalore, Karnataka, India. E-mail: madhyast1971@gmail.com (Corresponding author)

Prabhu Latha V, Department of Anatomy, Kasturba Medical College, Mangalore, Karnataka, India.

Nayak Soubhagya R, Department of Anatomy, Kasturba Medical College, Mangalore, Karnataka, India.

Pai Mangala, Department of Anatomy, Kasturba Medical College, Mangalore, Karnataka, India.

Rai Rajalakshmi, Department of Anatomy, Kasturba Medical College, Mangalore, Karnataka, India.

Prashanthi Madhyastha, 2Department of Dental Materials, Manipal College of Dental Sciences, Mangalore.