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Original Research Article

Sexually Dimorphic Effects of Chronic Prenatal Restraint Stress on Hippocampal Neurogenesis Using Doublecortin Immunostaining Technique

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ABSTRACT

Introduction: Maternal stress can have a profound effect on physiology; behavior and cognitive function of the offspring, but the mechanism affect brain function of offspring when they are adult remains primarily unknown. Doublecortin (DCX), a microtubule associated phosphoprotein is used as a marker of newly born neurons in the dentate gyrus (DG). This study was designed with the view to examine the gender- specific effects of prenatal stress on neurogenesis in the dentate gyrus of hippocampus in male and female wistar rats at weaning.
Materials and Methods: Pregnant rats of known gestational age were subjected to restraint stress from 11th gestational day till delivery. Rat pups belonging to different groups were sacrificed on 22nd postnatal day and brains were processed for Doublecortin Immunostaining and quantification.
Result: It was observed that prenatal stress caused significantly less number of DCX positive neurons in stressed males but not in stressed females. There was no difference in neuronal density between normal male and female rats.
Conclusion: These data reinforce the view that prenatal stress affects cognitive development in a sex-specific manner and the diminished effect seen in females could be due to the oestrogen- mediated neuroprotection on hippocampal function.

Key Words: Doublecortin Immunostaining, prenatal stress, hippocampus

INTRODUCTION

The prenatal environment is known to influence the development of the nervous, endocrine, and immune systems, with long-lasting effects on offspring postpartum. [1] Early environmental influences can leave ineradicable imprints and influence the development of an offspring. In most of the cases, affects of such insults will be carried to the young age or even to the whole life span of the individual. [2,3] Invariably, Nervous system becomes the main target of this faulty development.

Stress is the physical and psychological reactions of an individual's body to a specific situation that evokes feelings of anger, anxiety or stress. Experiments with rats, mice and monkeys have revealed that voluntary nervous system, autonomic nervous system and neuroendocrine systems gets activated during a stressful situation. The main components of the stress response system are the hypothalamus-pituitary-adrenal (HPA) neuroendocrine axis and locus coeruleus/ norepinephrine autonomic

system. HPA axis is sensitive to fluctuations in circulating corticosteroids. [4,5]

Neurogenesis (birth of neurons) is the process by which neurons are generated. Most active during pre-natal development, neurogenesis is responsible for populating the growing brain. New neurons are continually born throughout adulthood in subventricular zone lining the lateral ventricles and subgranular zone of dentate gyrus of hippocampus. Doublecortin (DCX) is a microtubule-associated phosphoprotein and has been utilized as a marker of newly born neurons in the adult dentate gyrus.

The functional relevance of adult neurogenesis is uncertain [6] but there is some evidence that hippocampal adult neurogenesis is important for learning and memory. [7] It has also been hypothesized that hippocampal-mediated learning [8] may be related to the generation of new neurons in the adult dentate gyrus. [9] Glucocorticoid levels regulate de novo cell proliferation in the dentate gyrus. Hence an attempt has been made here to find out the gender – specific effects of chronic prenatal stress paradigm on the neurogenesis and the mechanisms underlying these effects in both male and female rat pups.

MATERIALS AND METHODS

1.1 Experimental animals and housing conditions

In-house bred male and female Wistar strains of rats were used in the study. Animals were bred in Central Animal Research Facility of Manipal University, Manipal. Adult rats (3 months old) were housed in air conditioned animal rooms with constant light-dark cycle (12:12 h), controlled temperature (22±3ºC) and humidity (50±5%). Polypropylene cage with paddy husk as bedding materials was used for housing the rats. The animals had free access to food (Gold Mohur; Lipton India Ltd.) and water *ad libitum*.

Breeding and maintenance of animals were done according to the guidelines of Committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEA). Institutional Animal Ethical Committee (I.A.E.C) approval was obtained before the conduct of the study (IAEC/KMC/06/2005-2006) and care was taken to handle the rats in humane manner.

1.2 Experimental design

Gestation day 11 pregnant rats were divided into two groups: i) No stress group (n=20) and ii) Stress group (n=20). Pregnant rats in the "No stress" group remained without any procedure till delivery. Two Male and two female pups born to these rats were randomly selected and grouped as normal control-male (NCM, n=40) and normal control-female (NCF,n=40). Pregnant rats in the stress group were restraint stressed in a wire mesh restrainer 6hrs/ day till they deliver the pups. Two male and two female pups born to the stressed mothers were selected randomly and were grouped as stressed-male (STM,n=30) and stressed- female (STF,n=30). Thus we have four experimental groups-(i) Normal control-Male(NCM), (ii) Normal control-female(NCF), (iii) Stressed male(STM), and (iv) stressed female(STF) (n=30 in each group. Six pups in each group were used for studying neurogenesis in dentate gyrus of hippocampus by doublecortin immunostaining technique

1.3 Timed pregnancy in rats

To get the pregnant rats of known gestational days, all female rats were subjected to vaginal smear test. [10] The rats in the estrus cycle were mated with adult male rats overnight. Vaginal smear was examined within 12 hours after mating. The presence of sperms in the smear confirms the mating (Figure1), and that day was taken as day zero of pregnancy for further counting the days. Pregnant female rat was separated from other rats and housed individually with proper label indicating the day of conception. Pregnant females were assigned randomly into 'No stress' and 'stress groups' (n=20 in each group). The rats in 'No stress group' remained without any further procedures and allowed to

deliver the pups. The rats in the 'Stress group' were subjected to restraint stress.

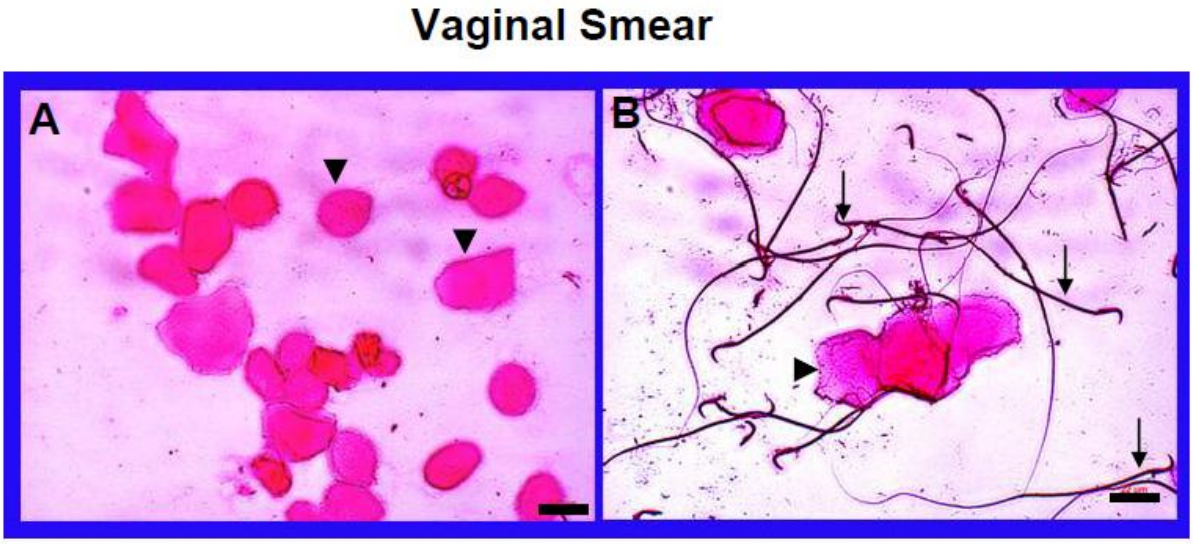

Figure 1: A-Vaginal smear of rat in estrous cycle. B-Vaginal smear of a rat, 8 hours after mating. Note the presence of sperms in B (arrows). Arrow heads indicate the vaginal squamous cells. Eosin stain. Scale bar =20µm

1.4 Prenatal stress protocol

Pregnant rats in the 'stressed group' were subjected to daily restraint stress from 11thgestational day, till they deliver the pups. The pregnant rats were restraint stressed by placing them individually in a wire mesh restrainer, 6 hours per day. [11] This type of restrain is known to induce stress in rats as indicated by increased serum cortisol level and adrenal gland weight in them. The wire mesh restrainer has a wooden base and stainless steel wire mesh restrainer hinged to the base. A padlock and latch will help to secure the rat in the restrainer. The restrainers of two different dimensions were used. The restrainer with 11cm (Length) × 6cm (Breadth) × 6cm (Height) dimensions for restraining the pregnant rats from E11-E17, and restrainer with 11cm (Length) x 8cm (Breadth) x 8cm (Height) dimensions was used to stress the pregnant rats from E18 till delivery. [11] This type of restrainer claimed to restricts the animal's movement without any pain, discomfort or suffocation.

1.5 Experimental animal groups

The pups born to 'No stress' and 'Stress' group of pregnant rats remained with their respective mothers until 21 days after birth. Number of pups for each dam was culled to six on 8th post delivery day by removing the extra pups if any in both groups of dams. The male and female pups from 'No stress' dams were designated as normal control-male (NCM) and Normal control-female (NCF) groups. The male and female pups from 'Stressed' dams were designated as normal Stressed-male (STM) and Stressed-female (STF) groups.

1.6 Brain fixation

Brains from different groups of animals were fixed by transcardial perfusion with 4% paraformaldehyde and processed for histological studies on the postnatal 22nd day. The rats were deeply anesthetized with ether and were placed on its back, and its rib cage was opened by cuts to the left and right of the sternum to expose the heart. A cannula, fastened to the rubber tube, is then inserted into the left ventricle and perfused with 100ml of saline. This is followed by perfusion with 250 ml of 4% cold paraformaldehyde (Prepared in 0.1M phosphate buffer, pH7.2). A successful perfusion can be recognized by the reaction of the perfusion fluid with the proteins of the cells, which causes the muscles to tremble. When the animal becomes stiff, and when about 3 times the animal's weight in perfusion fluid has passed through it, the brain may be removed from the skull. The

brain was postfixed in 4% paraformaldehyde for 48 hours.

1.7 Doublecortin immunostaining and quantification [12]

After transcardial perfusion with 4% paraformaldehyde, brains were removed, postfixed in 4% paraformaldehyde overnight at 4ºC and cryoprotected in 30% sucrose solution in phosphate buffer (PB). Cryostat sections (30µm) thick were cut coronally through the entire hippocampus and collected serially in PB. 10-15 sections from each hippocampus (with intersection interval of 45µm) was processed for doublecortin (DCX) immunostaining using a polyclonal antibody to DCX. Sections were treated with 0.1M PBS containing 20% methanol and 3% hydrogen peroxide, washed in PBS, blocked in 3% Normal horse serum(NHS) and incubated for 4h at room temperature in the DCX antibody (1:200; Sc-8066, Santa Cruz Biotechnology, Santa Cruz, CA, USA). DCX antibody is an affinity purified goat polyclonal antibody, which was raised against a peptide mapping at the carboxy terminus of human DCX. Following primary antibody treatment, sections were washed in PBS, incubated in the biotinylated horse anti-goat IgG (Vector) for 1 h, washed in PBS and incubated in the ABC reagent (Vector) for 1 h. Following this, the peroxidase reaction was visualized using vector grey (Vector) as the chromogen. Number of DCX positive neurons in the entire dentate gyrus and subgranular zone was quantified using the formula

$$N=1/ssf \,.1/asf \,.\, 1/hsf \,.\, \Sigma\, Q^{-}$$

N- Total number of neurons, ssf - section sampling fraction, asf - area sampling fraction (area sampled/ total area), hsf - height sampling fraction (Section thickness at the time of analysis, Σ Q – Total counts sampled

STATISTICAL ANALYSIS

Data was expressed as mean±SEM. Data were compared with one way ANOVA test using Graph pad in stat software. If the ANOVA test is significant, Bonferroni's multiple comparision tests was applied to determine the significance between the groups.

RESULTS

Neurogenesis (DCX neuron number) at weaning (On 22nd post natal day)

Quantification of doublecortin positive(DCX) neurons in the immunostained serial sections of dentate gyrus showed a significantly less number of newly generated DCX positive neurons in stressed males($P<0.001$) compared to control males. Stressed females had significantly more ($P<0.001$) number of DCX positive neurons compared to stressed male rats. However, normal males and females are not significantly different from each other (Fig.2&3)

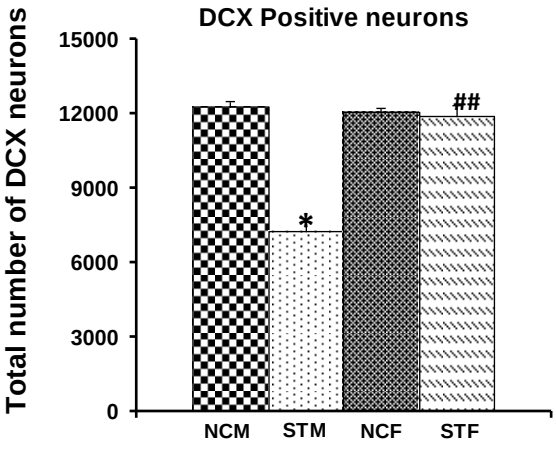

Figure 2: Number of doublecortin (dcx) positive neurons in the dentate gyrus of the hippocampus at weaning (on 22nd postnatal day) in different groups**.** NCM-normal control male(n=6), NCF-normal control female(n=6), STM -stressed male(n=6), STF-stressed female(n=6). Note (i) stressed males had significantly less number of dcx positive neurons in the dentate gyrus of the hippocampus compared to control males, and stressed females did not differ from control females,(ii) Stressed females had significantly more number of dcx positive neurons compared to stressed male rats. NCM vs STM: ***P<0.001; NCF vs STF: not significant; STM vs STF: ### P<0.001, NCM vs NCF: not significant. (One way ANOVA, Bonferroni's test. Each bar represents mean ±SEM).

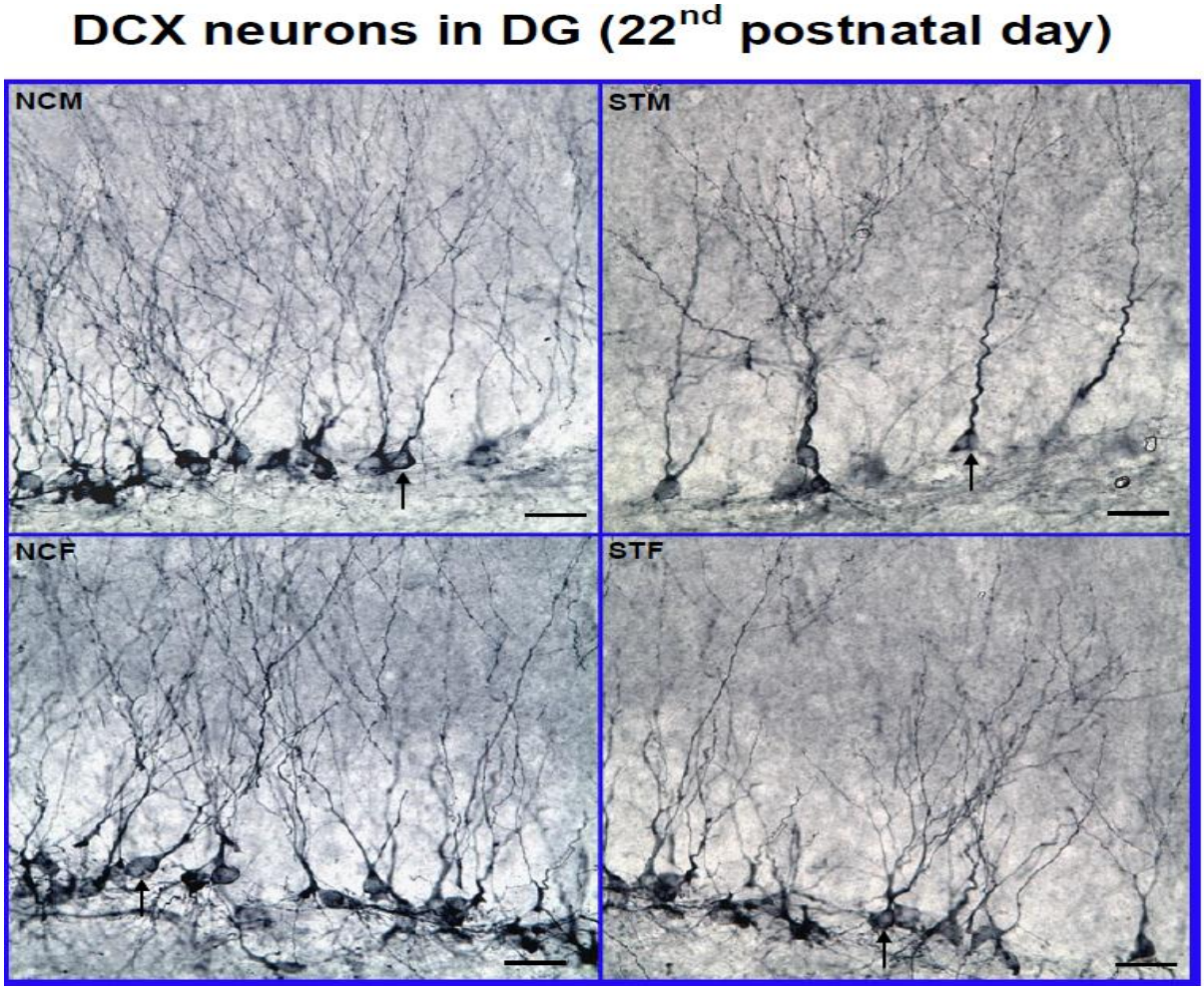

Figure 3: Photomicrographs doublecortin (dcx) positive neurons in the dentate gyrus and subgranular zone in different groups on 22nd postnatal day (arrows, DCX immunostaining and colour developed with vector grey. NCM - normal control male,NCF- normal control female, STM- stressed male, STF-stressed female. Note significantly less number of dcx positive neurons in stressed males but not in stressed females. There was no difference neuronal density between normal male and normal female rats. Scale bar=20µm.

DISCUSSION

Prenatal stress induced structural abnormalities in the hippocampal formation. Our results show that prenatal stress causes a decline in the number of cells in the various regions of hippocampus in the prenatally stressed male offspring. Hippocampal neurons are highly plastic and respond to early environmental challenges with long-lasting changes in the mechanism regulating synaptic plasticity and network organization. [13,14] Previous studies have shown that prolonged stressful periods can result in cell death. [15] Prenatally stressed males exhibited a greater reduction in cell proliferation in the dentate gyrus suggesting that early stressful experience affects cell proliferation in dentate gyrus. The degenerating profiles (pyknotic cells) were characterized by a condensed chromatin and a light or absent cytoplasm. This finding is of particular interest because the dentate gyrus is associated with spatial learning and memory [16,17] which clearly are affected in prenatally stressed male rats in our study and previous reports.

Stress during pregnancy sensitizes hypothalamo-pituitary-adrenal (HPA) axis, increasing stress induced corticosterone secretion in preweaning rats [18] and prolonged stress induced corticosterone secretion in the adult. [19] Prenatal stress also decreases the number of hippocampal corticosteroid receptors, [20] which are the principal substrate of the negative feedback control of glucocorticoid secretion. Thus, a decrease in these corticosteroid receptors is accompanied by increased glucocorticoid secretion and vice versa. Two different cytosolic receptors contribute to this

control: (1) the type I, or mineralocorticoid receptor (MR); and (2) the type II, or glucocorticoid receptor (GR). [20,21] Elevated levels of **corticosteroid** hormones on MRs and GRs assume opposite roles in regulation of synaptic plasticity after acute exposure to stressors. [22] Glucocorticoids (GCs) are secreted by the adrenal cortex and mediate adaptation to acute stress. [23] Chronic GC exposure as a result of prolonged stress or pathological GC hypersecretion can be profoundly deleterious, due to the catabolic effects of the hormone's actions. [23]

Glucocorticoids are very liposoluble and easily cross placental and blood-brain barriers. [24] Glucocorticoids appear capable of damaging or destroying hippocampal neurons. [25] A hallmark of GC action is its inhibition of glucose uptake by peripheral target tissues [22] which is particularly seen in hippocampus. [26] Furthermore, neurons are markedly dependent on glucose as an energy substrate because of their extremely limited capacity for glycogen storage as well as the limited number of energy sources that can penetrate the blood-brain barrier. [27] Thus glucocorticoids through their catabolic effects on neuronal energy metabolism exacerbate the state of energy depletion in hippocampal neurons and thus increase their toxicity.

Also the atrophy of hippocampal CA3 neurons has been attributed to the increased release of excitatory neurotransmitter, glutamate in the hippocampus. Elevations in the circulating corticosterone levels can increase basal glutamate levels in the hippocampus. [28] Prenatal stress has been shown to increase glutamate release in the hippocampal CA1 region. [29] The major excitatory inputs to the hippocampus originates in the entorhinal area; an ipsilateral (perforant path) pathway activates the granule cells of the dentate gyrus, which in turn innervates the CA3 pyramidal cells [29] and glutamate is the transmitter involved in this pathway. [30] Findings from experiments claimed that lesioning of entorhinal cortex attenuates stress- induced atrophy of hippocampal CA3 neurons. [31] It has been shown that maternal chronic stress applied during gestation can exacerbate excitotoxic neonatal brain damage. [32]

Clinical and experimental evidence suggest that enhanced glutamatergic transmission may lead to the death of the neurons, which have post synaptic glutamatergic receptors. Stress induced secretion of glucocorticoids appear to contribute to this reponse because adrenalectomy markedly attenuates stress-induced elevations in extracellular glutamate levels. [33]

These could be the various possible mechanisms that can cause hippocampal damage by gestational stress leading to behavioral changes manifesting into adulthood.

The results also revealed that prenatal stress did not affect the number of cells in the hippocampal regions of female rats. This could be due to the organizational and activational effects of esterogen. [34] Ovarian steroids are of prime importance in the normal maintenance of brain function. Ovarian cycle regulates cyclic synaptogenesis on excitatory spines in hippocampal CA1 pyramidal neurons in female but not in male rats. [35,36] Synaptogenesis is cyclic, and fluctuations in synapse density occur throughout the estrous cycle of the female rat. [37] Male rats show much less estrogen-induced synapse formation unless they are treated at birth. This enhanced performance seen in stressed female offspring compared to stressed males is due to the estrogen mediated neuroprotection on hippocampal function and thus supports the notion that estrogenic hormones have a wide range protection of hippocampal function. [37]

CONCLUSION

Prenatal stress effects appear to be gender-specific. The various sub regions of stressed male hippocampus also showed inhibition of neurogenesis at weaning which could be due to the elevated levels of corticosterone throughout the life span thus

causing cognitive deficits. Prenatal stress induced low levels of cell death in stressed females which could be due to esterogen - mediated neuro protection on hippocampal function.

Our results reinforce the hypothesis that much psychopathological affection has their origin in early developmental influences. More generally, they show the heuristic value of accurate animal models to better understand the mechanism by which early stress and epigenetic risk factors promote learning disabilities in children thus revealing the decisive importance of nine months of pregnancy for the rest of the child's life and that of the adult it will become.

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