ISSN: 0976-3031

Available Online at http://www.recentscientific.com

CODEN: IJRSFP (USA)

International Journal of Recent Scientific Research
Vol. 8, Issue, 5, pp. 17153-17158, May, 2017

International Journal of Recent Scientific Research

DOI: 10.24327/IJRSR

Research Article

EFFECT OF CHRONIC PRENATAL RESTRAINT STRESS ON HIPPOCAMPAL NEURONAL CELL DENSITY IN MALE AND FEMALE WISTAR RATS AT WEANING

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DOI: http://dx.doi.org/10.24327/ijrsr.2017.0805.0294

ARTICLE INFO

Article History:

Received 17th February, 2017
Received in revised form 12th March, 2017
Accepted 04th April, 2017
Published online 28th May, 2017

Key Words:

Prenatal stress, restraint stress, hippocampus, cresyl- violet staining

ABSTRACT

Background: Maternal psychosocial stress during fetal brain development results in increased risk for impaired behavioral and emotional development and affective disorders in childre. Controversies exist in literature regarding sexual dimorphism in the effects of prenatal stress on behavioral development. This study was designed with the view to examine the gender- specific effects of chronic maternal stress on hippocampal neuronal cell density in male and female wistar rats at weaning.

Methods: Pregnant wistar rats were subjected to restraint stress from embryonic day 11 till delivery. Male and female rat pups were sacrificed. Brains from different groups were processed for cresyl violet staining on postnatal 22nd day.

Result: It was seen that prenatal stress caused significant reduction in the numerical cell density in CA1, CA3, dentate gyrus and dentate hilar regions of hippocampus in stressed males when compared to the control and did not show any effect on stressed females.

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.

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INTRODUCTION

Intrauterine development plays critical role in normal physical, mental and behavioral development of an individual. The prenatal environment is known to influence the development of the nervous, endocrine, and immune systems, with long-lasting effects on offspring postpartum (Nyirenda and Seckl, 1998). Maternal nutrition (Firth *et al*, 2008), exposure to environmental toxicants (Gilbert *et al* and Wormser *et al*, 2005) and stressful disturbances (Igosheva *et al* and Bowman *et al*, 2004) of the pregnant female are among the many variables that can affect *in utero* conditions and impair the maturational trajectory of the fetus. All sorts of early environmental influences can leave indelible 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 (Roman and Nylander 2005, Coe *et al*, 2003).

Deleterious life events during pregnancy induce neurobiological and behavioral defects in offspring, some of them involving the hippocampal formation (Vall'ee *et al*, 1999), a highly plastic brain region. Prenatal stress evokes a cascade of neurohumoral events which triggers HPA axis hyperactivity in response to stress throughout life. Gestational stress is reported to increase the anxiety like behavior in elevated plus maze or in open field (Kohman, *et al* 2008) and decrease the spatial learning and memory in T-maze (Son, *et al* 2006), diminution of time spent in target quadrant in the water maze, spontaneous alternation test in Y-maze (Darnaudéry 2006) and passive avoidance learning (Wu, *et al* 2007). Thus there are many instances in which neural function and cognition are either facilitated by prenatal stress (Yang, *et al* 2006) or even not affected (Schwalbe, *et al* 2010). Hence there is a paucity in prenatal stress and cognitive (sense of right and wrong) behavioral literature and the mechanisms underlying these lasting developmental and behavioral

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teratology. Therefore, this study was designed to investigate the effect of prenatal stress on hippocampal neuronal cell density in various regions of hippocampus and also to look into gender-specific effects if any.

MATERIALS AND METHODS

Experimental animals and housing conditions

Male and female rats of Wistar strain were used in the study. Animals were bred in Central Animal Reseach 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.

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 had 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 cresyl violet staining on 22nd postnatal day and the rest were utilized for behavioral studies.

Timed pregnancy in rats

All female rats were subjected to vaginal smear test (Lesage, *et al* 2004), in order to get the rats of known gestational age. 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 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.

Numerical cell density and morphological study of brain at weaning period (On 22nd postnatal day)

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.

Tissue processing for paraffin sectioning

Dehydration

Brain part with hippocampus cut and dehydrated in ascending grades of alcohol: 50% alcohol-24 hours, 70% alcohol-24 hours, 90% alcohol-12 hours, absolute alcohol 12 hours.

Clearing

Tissue was cleared in xylene for 1-2 hours

Embedding

Tissue was infiltrated with paraffin wax (4 changes of 1 hour each) and embedded in fresh paraffin wax.

Sectioning

Five micron thick paraffin section was cut in coronal plane in a rotary microtome.

Sections were cut from the entire hippocampus using a rotary microtome (Leica RM2245, Leica Microsystems, Germany). Every 20th section was selected for staining and mounted serialy on gelatinized, air dried slides (About 25-30 sections from each brain). Sections were stained with cresyl violet stain (0.01%) as detailed below.

Preparation of cresyl violet stain (0.1%)

100mg of cresyl violet (Sigma chemicals, USA) is dissolved in 100 ml distilled water. To this 0.5 ml of 10% acetic acid is added to give a Ph of 3.5 to 3.8. The stain was filtered before use.

Staining protocol

Sections were deparafinized in xylene (1 minute), hydrated in descending grades of alcohol (100%, 90% 70%and 50% for 2 min each) Distilled water(5 min). Sections were stained in prewarmed 0.1% cresyl violet stain(30 min at 60ºC).Sections were cooled to room temperature,and placed in distilled water(5min) and dehydrated in ascending grades of alcohol (70%, 80%, 90% and 100% for 1-2 min each). Clearing was done with xylene (1-2 min).Mounted with DPX.

Observations

In each section cornu ammonis subregions (CA1, CA3 and dentate hilus) and dentate gyrus areas of hippocampus were

observed for any morphological changes under a light microscope (Magnus, Olympus (India) Pvt. Ltd. New Delhi).

Neuronal quantification

In each section number of neurons in cornu ammonis subregions (CA1, CA3 and dentate hilus) and dentate gyrus areas of hippocampus (Figure R1) were quantified using sterioinvestigator principle. In each section number of neurons in in about 4000 - 6000 square micron area CA1, CA3, dentate hilus and dentate gyrus were quantified under 40X magnification in a light microscope (Magnus, Olympus (India) Pvt. Ltd. New Delhi). Slides from different groups of animals were coded to avoid the experimenter bias in counting the cells. Total area of each sub region in each section was measured. Total number of neurons in the entire given sub region was calculated 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.

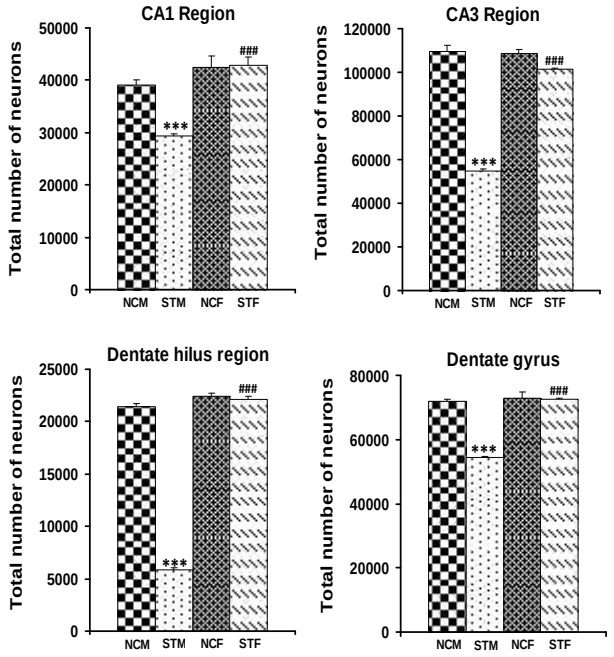

Figure R1 Numerical cell density in different sub regions of hippocampus at weaning (on 22nd postnatal day)**.** NCM-normal control male (n=6), NCF-normal control female(n=6), STM -stressed male(n=6), STF-stressed female(n=6). Note (i) stressed males had significantly less number of neurons in all the sub regions of the hippocampus compared to control males, and stressed females did not differ from control females,(ii) Stressed females had significantly more number of neurons in all regions 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).

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

Numerical cell density in hippocampus at weaning (On22nd postnatal day)

Cresyl violet staining of serial sections of hippocampus and quantification of neurons in CA1, CA3, dentate hilus and dentate gyrus regions of the hippocampus revealed significant difference in the number of cells in stressed males and female. Figure R1 is low magnification photograph of dorsal hippocampus, from different group indicating the area shown in subsequent figures.

Hippocampus (22nd postnatal day)

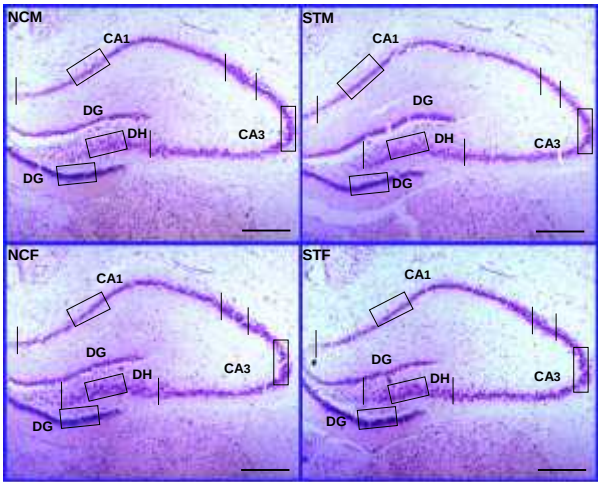

Figure R2 Low magnification (4x) photomicrographs of dorsal hippocampal region in different groups on 22nd postnatal day (Cresyl violet stain). NCM - normal control male,NCF- normal control female, STM-stressed male, STF-stressed female. CA1,CA3-cornu ammonis subregions, DH-dentate hilus, DG- dentate gyrus. Limits of different subregions are indicated by vertical lines. Rectangular boxes indicate the region shown in Fig12, 13, 14 and 15. Scale bar=200µm.

Numerical cell density in the CA1 region

Stressed male rats (STM) showed significantly less (P<0.001) number of neurons in the CA1 region of the hippocampus, but not the female (STF) rats. Stressed females had significantly more (P<0.001) number of neurons in the CA1 region compared to stressed males. However normal males and females are not significantly different from each other (Fig. R3).

CA1 Region (22nd postnatal day)

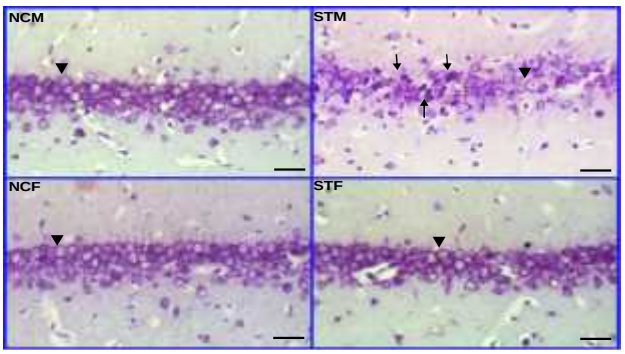

Figure R3 Photomicrographs of CA1 region of the hippocampus in different groups on 22nd postnatal day (Cresyl violet stain). NCM - normal control male, NCF- normal control female, STM-stressed male, STF-stressed female. Note less compactly arranged (dispersed cell layer), and darkly stained degenerating cells (arrows) among the healthy neurons(arrow heads) in stressed males but not in stressed females. There was no difference in cell density between normal male and normal female rats. Scale bar=20µm.

Numerical cell density in the CA3 region

Quantitative data on number of cells in CA3 region is similar to CA1 region. Stressed male rats (STM) showed significantly less (P<0.001) number of neurons in the CA3 region of the hippocampus, but not the female (STF) rats. Stressed females had significantly more (P<0.001) number of neurons in the CA3 region compared to stressed males. However normal males and females are not significantly different from each other (Fig. R4).

CA3 Region (22nd postnatal day)

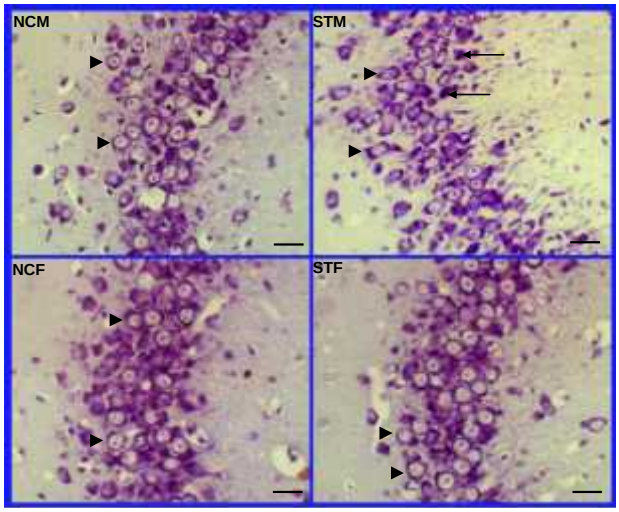

Figure R4 Photomicrographs of CA3 region of the hippocampus in different groups on 22nd postnatal day (Cresyl violet stain). NCM - normal control male, NCF- normal control female, STM- stressed male, STF- stressed female. Note less compactly arranged (dispersed cell layer), smaller neurons and darkly stained degenerating cells (arrows) among the healthy neurons (arrow heads) in stressed males but not in stressed females. There was no difference in cell density between normal male and normal female rats. Scale bar=20µm.

Dentate hilus (22nd postnatal day)

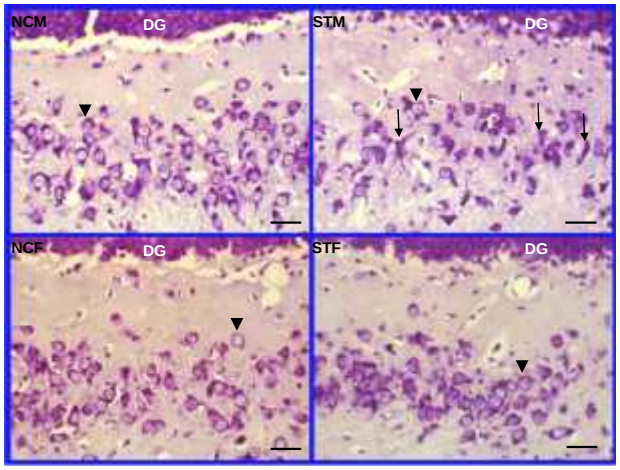

Figure R5 Photomicrographs of dentate hilus region of the hippocampus in different groups on 22nd postnatal day (Cresyl violet stain). NCM - normal control male,NCF- normal control female, STM- stressed male, STF- stressed female, DG-dentate gyrus. Note less compactly arranged (dispersed cell layer), smaller neurons and darkly stained degenerating cells (arrows) among the healthy neurons (arrow heads) in stressed males but not in stressed females. There was no difference in cell density between normal male and normal female rats. Scale bar=20µm.

Numerical cell density in the dentate hilus

Quantitative data on number of cells in dentate hilus region is similar to CA1, and CA3 region. Stressed male rats (STM) showed significantly less (P<0.001) number of neurons in the dentate hilus region of the hippocampus, but not the female (STF) rats. Stressed females had significantly more (P<0.001) number of neurons in the dentate hilus region compared to stressed males. However normal males and females are not significantly different from each other (Fig. R5)

Numerical cell density in the dentate gyrus

Stressed male rats (STM) showed significantly less (P<0.001) number of neurons in the dentate gyrus region of the hippocampus, but not the female (STF) rats. Stressed females had significantly more (P<0.001) number of neurons in the dentate gyrus region compared to stressed males. However normal males and females are not significantly different from each other (Fig. R6).

Dentate gyrus (22nd postnatal day)

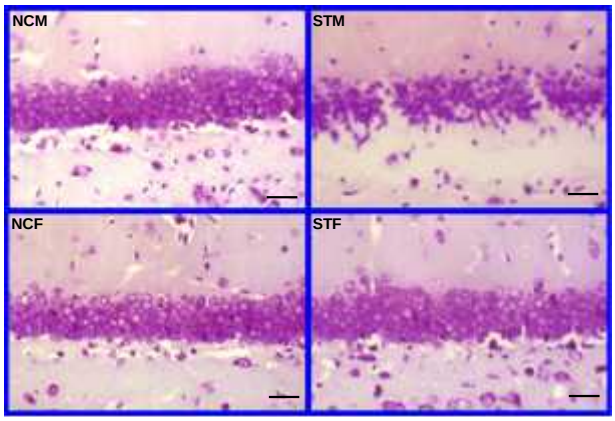

Figure R6 Photomicrographs of dentate gyrus of the hippocampus in different groups on 22nd postnatal day (Cresyl violet stain). NCM - normal control male, NCF- normal control female, STM- stressed male, STF- stressed female. Note less compactly arranged (dispersed cell layer), in stressed males but not in stressed females. There was no difference in cell density between normal male and normal female rats. Scale bar=20µm.

DISCUSSION

Morphological effect of prenatal stress on hippocampus

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 (Takahashi 1998). Previous studies have shown that prolonged stressful periods can result in cell death (Tsankova *et al*, 2007). 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. The decreased number of cells seen in other regions of hippocampus could be the consequence of this alteration in cell proliferation. Collectively there is clear

evidence that chronic stress can significantly alter the hippocampal structure. The decreased number of cells seen in other regions of hippocampus could be the consequence of this alteration in cell proliferation. Collectively there is clear evidence that chronic stress can significantly alter the hippocampal structure.

Stress during pregnancy sensitizes hypothalamo- pituitary-adrenal (HPA) axis, increasing stress induced corticosterone secretion in preweaning rats (Uno, *et al* 1989) and prolonged stress induced corticosterone secretion in the adult (Takahashi, *et al* 1998). Prenatal stress also decreases the number of hippocampal corticosteroid receptors (Takahashi, *et al* 1998), 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) (Maccari *et al* 1995 and McEwen *et al*, 1986). Elevated levels of corticosteroid hormones on MRs and GRs assume opposite roles in regulation of synaptic plasticity after acute exposure to stressors (De Kloet, *et al* 1987). Glucocorticoids (GCs) are secreted by the adrenal cortex and mediate adaptation to acute stress **(**Avi Avital *et al*, 2006). 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 (Avi Avital *et al*, 2006 and Munck *et al*, 1984). Glucocorticoids are very liposoluble and easily cross placental and blood-brain barrier (Krieger 1982). Glucocorticoids appear capable of damaging or destroying hippocampal neurons (Zarrow *et al*, 1970). A hallmark of GC action is its inhibition of glucose uptake by peripheral target tissues (Munck 1971) which is particularly seen in hippocampus. 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 (Siesjo 1978). Thus glucocorticoids through their catabolic effects on neuronal energy metabolism exacerbate the state of energy depletion in hippocampal neurons and thus increase their toxicity. These could be the various possible mechanisms that can cause hippocampal damage by gestational stress leading to behavioral changes manifesting into adulthood.

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How to cite this article:

Cherian SB, Bairy KL and Rao MS.2017, Effect of Chronic Prenatal Restraint Stress on Hippocampal Neuronal Cell Density In Male and Female Wistar Rats At Weaning. *Int J Recent Sci Res.* 8(5), pp. 17153-17158.
DOI: http://dx.doi.org/10.24327/ijrsr.2017.0805.0294

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