EFFECTS OF CHRONIC PRENATAL RESTRAINT STRESS ON ANXIETY IN POST WEANED MALE AND FEMALE WISTAR RATS

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Summary

Stress in adulthood can have a profound effect on physiology and behavior, but the extent to which prolonged maternal stress affect brain function of offspring when they are adult remains primarily unknown. Controversies exist in literature regarding sexual dimorphism in the effects of prenatal stress on the postnatal cognitive behavioral development. To investigate the effect of prenatal stress on locomotor, exploratory and emotional development, pregnant rats of Wistar strain were subjected to restraint stress from E11 till delivery. Male and female pups born to these stressed rats were subjected to open field test on 21st day of postnatal life. Results were compared with rats of the same age and sex born to control mothers, which were not stressed. The results showed that prenatal maternal restraint stress affected both male and female offsprings during young age. These results suggests that prolonged maternal stress leads to long lasting malfunction of the hippocampus, which extends to and is manifested in adulthood. Prenatally stressed males exhibited higher anxiety levels when compared to the stressed females suggesting that prenatal stress effects are gender- specific.

Key words: Prenatal stress, open field test, hippocampus

Introduction

Development is shaped by a highly complex process involving the interplay of complex biological and environmental factors. Prenatal or intrauterine development plays critical role in normal physical, mental and behavioral development of an individual. Maternal nutrition (**1**), exposure to environmental toxicants (**2,3)**, and stressful disturbances(**4,5**) 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 **(6).** Though any system of the body is the target of flawed development, nervous system becomes the main target of faulty development.

A substantial body of evidence indicates that prenatal stress is known to increase anxiety, behavioral and cognitive functions, in postnatal life **(7).** Gestational stress is reported to increase the anxiety like behavior in elevated plus maze or in open field (**8**) and decrease the spatial learning and memory in T-maze (**9),** diminution of time spent in target quadrant in the water maze, spontaneous alternation test in Y-maze **(10)** and passive avoidance learning (**11).**Thus there are many instances in which neural function and cognition are either facilitated by prenatal stress (**12**) or even not affected (**13**). 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 teratology.

In male rats, prenatal stress is reported to decrease the learning ability in water maze (**14)** and increase the tight rope test score (**15),** increase the emotionality in open field (**Abe H et al 2007)**,and depression like behavior in forced swim test (**16)**. In female rats, prenatal stress results in increased learning in water maze (**14)**, decreased learning ability and memory (**17)** and elevated anxiety like behavior (**18)**. Both male and female prenatally stressed rats showed decreased performance in spontaneous alternation and delayed alternation in Y-maze **(19)**, delayed memory deficit, spatial and non spatial memory and short and long term memories. Thus controversies exist in the literature regarding differences in behavior according to the gender of offspring and mechanisms underlying these cognitive behavioral teratology. Hence an attempt was made here to find out the effect of chronic prenatal stress paradigm on anxiety and the mechanisms underlying these effects in both male and female rat pups.

Materials and Methods

In-house bred male and female rats of Wistar strain were used in the study. Animals were bred in Central Animal House of Kasturba Medical College, Manipal. 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 (IAEC/KMC/06/2005-2006) was obtained before the conduct of the study and care was taken to handle the rats in humane manner.

Adult female rats (3 months old) were housed in presence of a male rat in polypropylene cage using paddy husk bedding. The animals had free access to food and water under a

constant light-dark cycle (12:12 h) with controlled temperature (22±3ºC) and approximately 50±5% humidity in an air conditioned animal house. Animals were fed on laboratory feed (Gold Mohur; Lipton India Ltd.) and water *ad libitum*.

Timed pregnancy in rats

To get the pregnant rats of known gestational day, all female rats were subjected to vaginal smear test (**20).** The rats in the estrus cycle were placed with adult males overnight for breeding. A vaginal smear was examined on the next day of breeding. The presence of sperms in the smear confirmed the mating and that day is taken as day zero of pregnancy for further counting the days. Each pregnant female was separated and kept in an individual cage and fed with standard feed. Pregnant females were assigned randomly into control and stressed mothers groups.

Prenatal stress protocol

Pregnant rats in the stressed group were stressed daily from embryonic day E11 till delivery. They were exposed to a regimen of restraint stress by placing in a wire mesh restrainer for 6 hours per day (**21).**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 restrainer with dimensions11cm×6cm (B) ×6cm (H) (**modified from 22)** was used for rats with gestation E11 to E17. Restrainer of 11cm (L) x 8cm (B) x 8cm (H) was used to stress the pregnant rats from E18 till delivery. This type of restrainer restricts the animal's movement only without any pain discomfort or

suffocation. Control mothers were left undisturbed in the home cage for the duration of their pregnancies. All dams delivered at term (21-22 days of gestation). The offspring of both groups were raised by their biological mothers until weaning (21 days after birth).

Experimental design

After weaning, two male pups, and two female pups were selected from each of the control mother and designated as normal control (NC, n=12) group. Similarly, two male pups and two female pups were selected from each of the stressed mother and designated as stressed (ST, n=12) group. Rats in both NC, and ST group were subjected to open field test at 21st postnatal day as described below:

Open field test

To assess the locomotor,exploratory activities and emotional reactivity, neonatal rats at 21 days were subjected to open field test as described by **(23)**.The apparatus consists of a rectangular box(100×100×40 cms),the floor area marked into 25 squares out of which 9 are central and 16 are peripheral. In this novel environment, the fraction of total exploratory time spent in peripheral area (close to the wall) and in the central area was measured for individual rats. The critical measure was the time the animal spent exploring the inner area of the novel arena. The open field was cleaned between each subject to prevent olfactory cues from affecting the behavior of subsequently tested rats. Total time for exploration in each session was 5 minutes and each rat had 3 sessions with 30 minutes interval.

In addition in this novel open field, Rearing (elevated hind limb & pelvis with elevation of fore limb) and grooming (use of head, tongue and fore limb for the process of cleaning various part of the body) behaviour and fecal pellets was also quantified .

Statistical analysis:

Data were presented as Mean ± SE. Results obtained from the present study were correlated and analyzed by one way Analysis of Variance (ANOVA) followed by Bonferroni's post hoc test. Values of $P<0.05$ were considered statistically significant.

Results

Number of peripheral squares entered during 5 minutes

Stressed rats [both male (STM) and female (STF)] entered significantly less number of peripheral squares compared to respective control rats [control male (NCM), and control female (NCF)] in all trial days. Stressed females entered significantly higher number of peripheral squares compared to stressed male rats in all trials (Table R-1 and Fig. R- 1). NCM vs STM: ***P<0.001**; NCF vs STF: **$ P<0.05, $$ P<0.01, $$$ P<0.001;** STM vs STF: **# P<0.05, ## P <0.01, ### P <0.001**

Number of central squares entered during 5 minutes

Stressed rats [both male (STM) and female (STF)] entered significantly less number of central squares compared to respective control rats [control male(NCM),and control female(NCF)] in all trial days. Stressed females entered significantly higher number of central squares compared to stressed male rats in all trials (Table R-2 and Fig. R- 2). NCM vs STM: *****P<0.001**; NCF vs STF: **$ P<0.05, $$ P<0.01**; STM vs STF**:## P <0.01,** NCM vs NCF **:@@@ P< 0.001**

Number of grooming events during 5 minutes

Stressed rats [both male (STM) and female (STF)] showed less number of grooming compared to respective control rats [control male(NCM),and control female(NCF)] in all trial days. Stressed females showed significantly higher number of grooming events compared to stressed male rats in all trials (Table R-3 and Fig. R- 3).NCM vs STM:, ****P<0.01***P<0.001;** NCF vs STF**: $ P<0.05, $$ P<0.01;** STM vs STF**: # P<0.05,## P <0.01**

Number of rearing events during 5 minutes

Stressed male rats (STM) showed significantly less number of rearing compared to respective control rats,control male(NCM) in all trial days. Stressed females showed significantly more number of rearing compared to stressed male rats (Table R-4 and Fig. R- 4). NCM vs STM**: *P<0.05;**P<0.01, ***P<0.001** ; STM vs STF: **# P<0.05, P<0.05, ### P <0.001**

Defecation scores during 5 minutes

Stressed rats [both male (STM) and female (STF)] showed significantly more number of fecal pellets compared to respective control rats[control male(NCM),and control female(NCF)] in all trial days. Stressed females showed significantly lower number of fecal pellets compared to stressed male rats (Table R-5 and Fig. R- 5). NCM vs STM**: ***P<0.001**, ***P<0.05** ; NCF vs STF: **$ P<0.05,$$$ P<0.001**; STM vs STF: **# P<0.05, ### P <0.001**

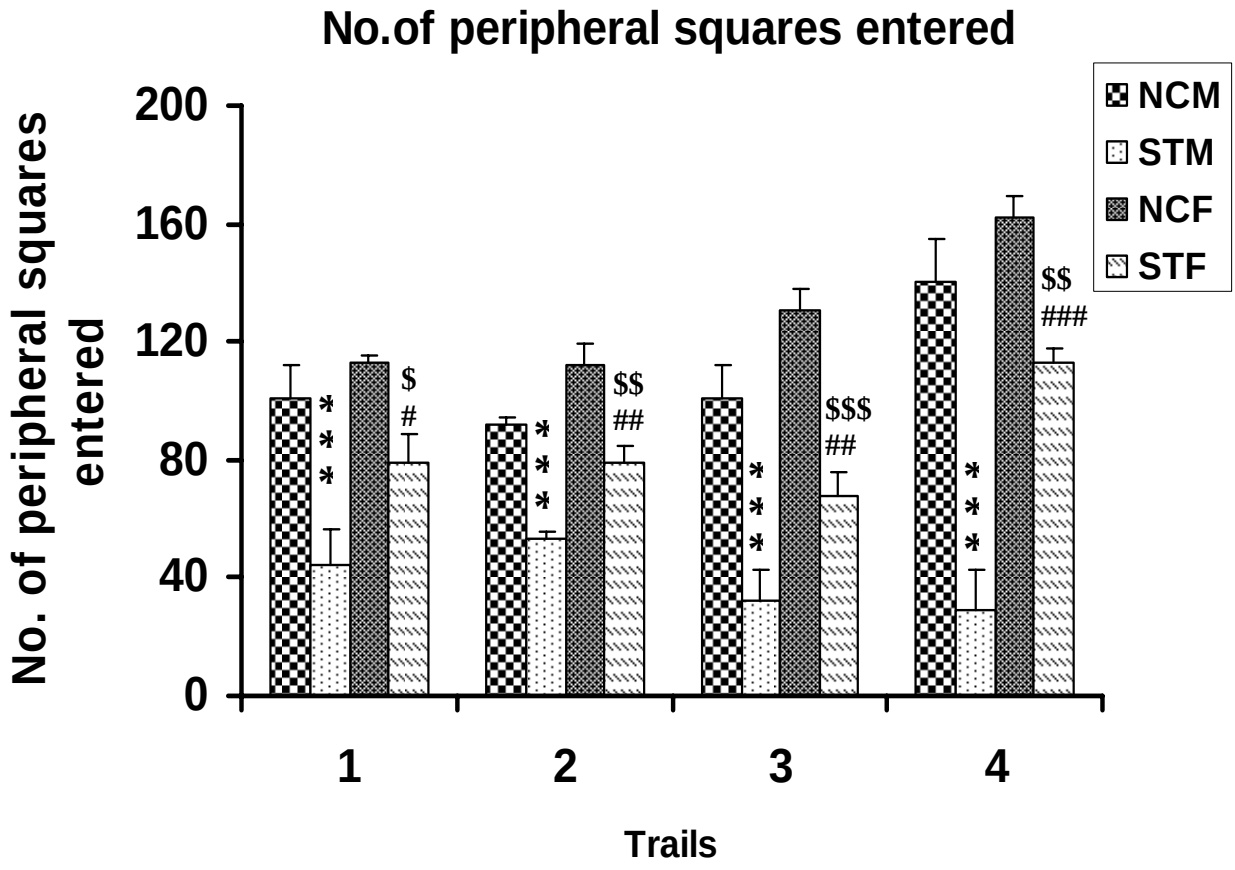

Figure. R- 1 : Number of peripheral squares entered by the control (n=6) and stressed (n=6) rats in the open field test. Note that stressed rats [both male (STM) and female (STF)] entered significantly less number of peripheral squares compared to respective control rats [control male (NCM), and control female (NCF)] in all trial days. Stressed females entered significantly higher number of peripheral squares compared to stressed male rats in all trials, though there was no significant difference between control male and control female. NCM vs STM: ***P<0.001; NCF vs STF: $ P<0.05, $$ P<0.01, $$$ P<0.001; STM vs STF: # P<0.05,## P <0.01, ### P <0.001(One way ANOVA, Bonferroni's test).

Number of peripheral squares entered

Groups	Trial Day			
	Day 1	**Day 2**	**Day 3**	**Day 4**
NCM	100.5±11.90	92.15±2.54	101.0±10.80	140.7±13.83
STM	**44.50 ± 2.46*** **	**53.00±7.39*** **	**32.33±7.79*** **	**29.17±7.54*** **
NCF	112.7±2.20	112.0±3.20	130.3±7.01	161.7±16.00
STF	**79.00±9.49$,#**	**79.33±5.48$$,##**	**68.00±8.06$$$,##**	**113.2±4.39$$,###**
F value	14.72	24.13	24.51	25.85
Anova Significance	P<0.0001	P<0.0001	P<0.0001	P<0.0001

Table R- 1 : Number of peripheral squares entered by the control (n=6) and stressed (n=6) rats in the open field test. Note that stressed rats [both male (STM) and female (STF)] entered significantly less number of peripheral squares compared to respective control rats [control male (NCM),and control female(NCF)] in all trial days. Stressed females entered significantly higher number of peripheral squares compared to stressed male rats in all trials, though there was no significant difference between control male and control female. NCM vs STM: ***P<0.001; NCF vs STF: $ P<0.05, $$ P<0.01,$$$ P<0.001(One way ANOVA, Bonferroni's test, Each data represents mean ± SEM).

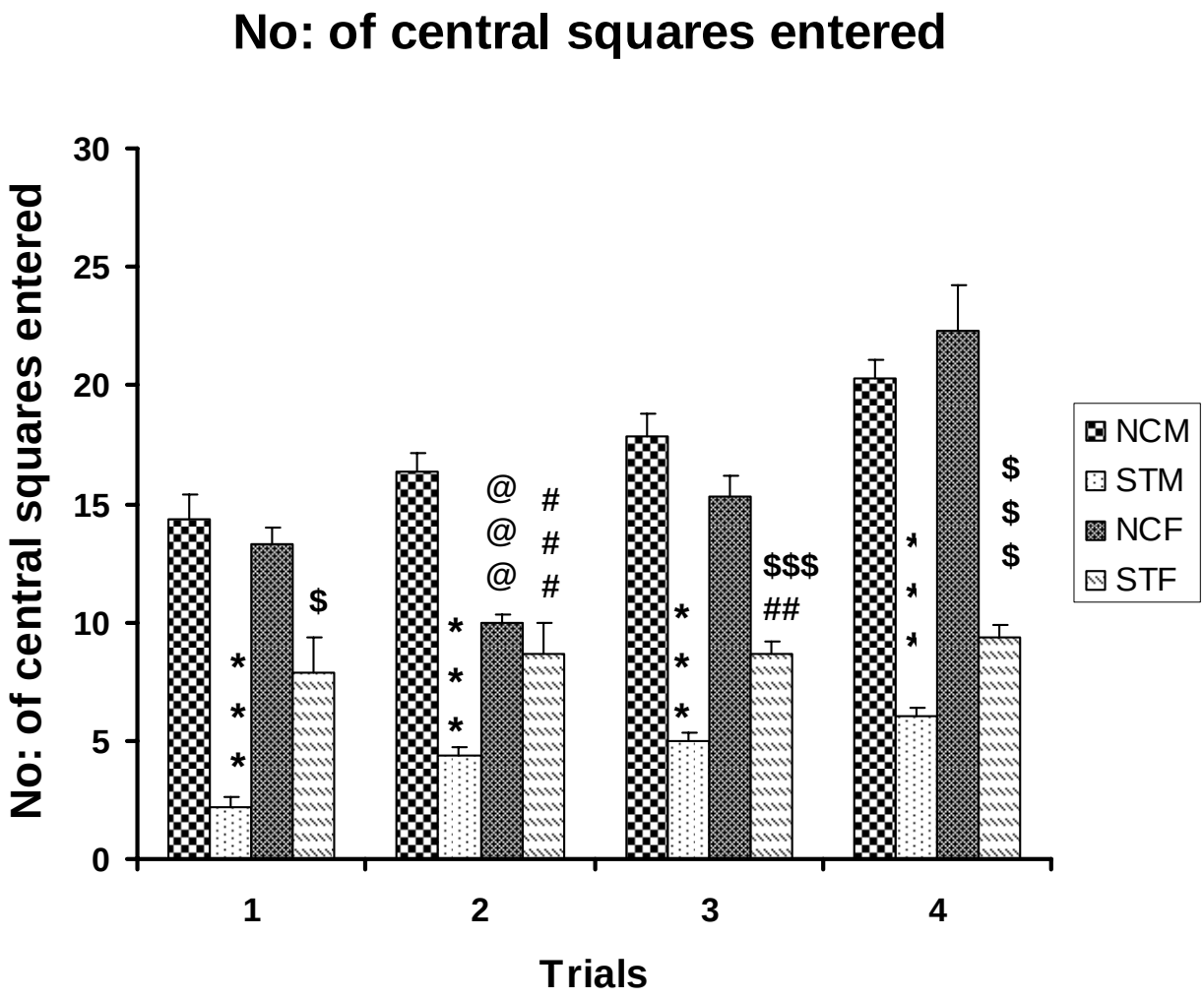

Figure R- 2: Number of central squares entered by the control (n=6) and stressed (n=6) rats in the open field test. Note that stressed rats [both male (STM) and female (STF)] entered significantly less number of central squares compared to respective control rats [control male(NCM),and control female(NCF)] in all trial days. Stressed females entered significantly higher number of central squares compared to stressed male rats in all trials. NCM vs STM: ***P<0.001; NCF vs STF: $ P<0.05, $$ P<0.01; STM vs STF:## P <0.01, NCM vs NCF :@@@ P< 0.001 (One way ANOVA, Bonferroni's test).

P

Number of central squares entered

Groups	Trial Day			
	Day 1	Day 2	Day 3	Day 4
NCM	14.33±1.03	16.33±0.84	17.83±0.94	20.33±0.71
STM	**2.16 ± 0.47*** **	**4.33±0.42*** **	**5±0.36*** **	**6±0.36*** **
NCF	13.33±0.66	**10±0.36@@@**	15.33±0.88	22.33±1.9
STF	**7.83±1.53$**	**8.66±1.33###**	**8.66±0.55$$$,###**	**9.33±0.55###**
F value	15.88	35.19	66.08	56.33
Anova Significance	P<0.0001	P<0.0001	P<0.0001	P<0.0001

Table R- 2: Number of central squares entered by the control (n=6) and stressed (n=6) rats in the open field test. Note that stressed rats [both male (STM) and female (STF)] entered significantly less number of peripheral squares compared to respective control rats [control male(NCM),and control female(NCF)] in all trial days. Stressed females entered significantly higher number of central squares compared to stressed male rats in all trials, though there was no significant difference between control male and control female. NCM vs STM: ***P<0.001; NCF vs STF: $ P<0.05, $$ P<0.01,$$$ P<0.001(One way ANOVA, Bonferroni's test, Each data represents mean ± SEM).

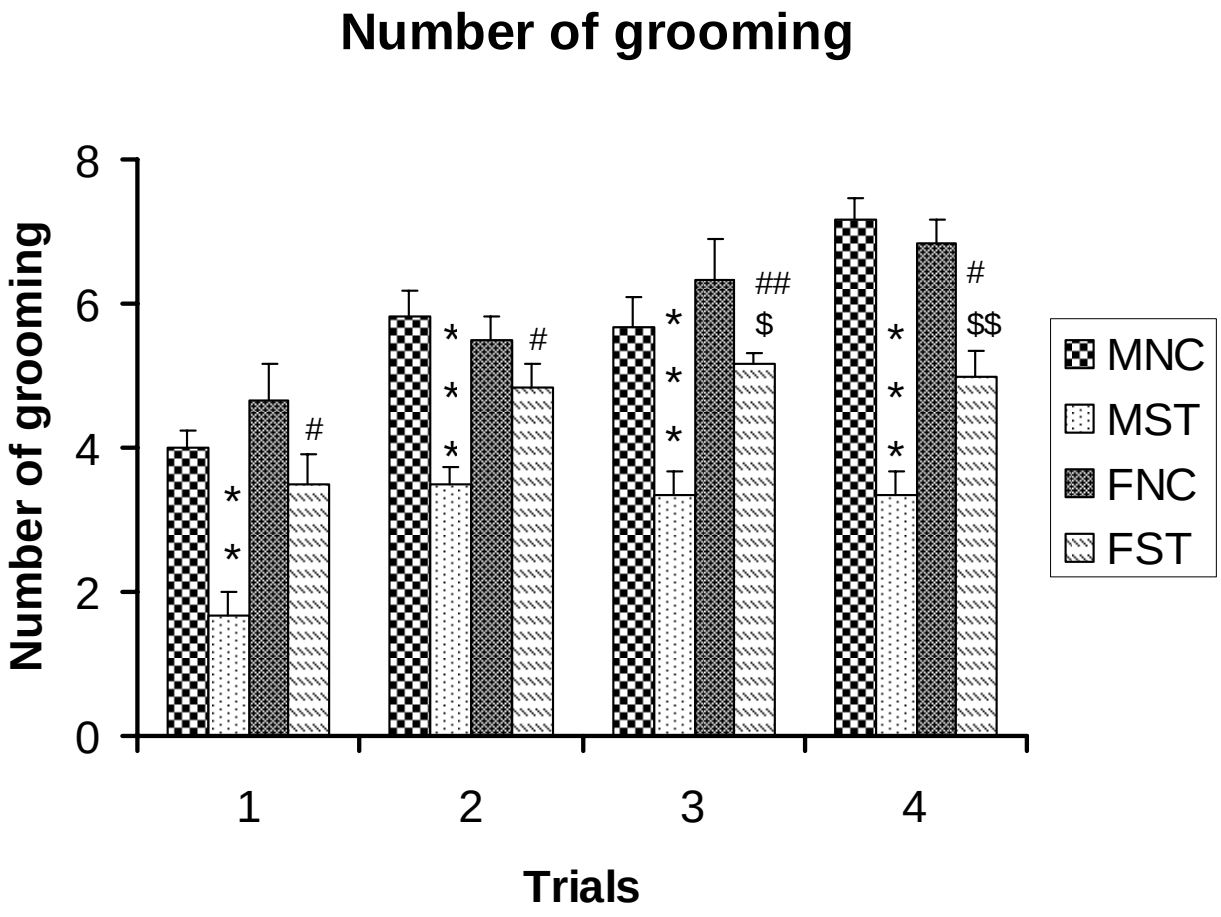

Figure R- 3: Number of grooming events by the control (n=6) and stressed (n=6) rats in the open field test. Note that stressed rats [both male (STM) and female (STF)] showed less number of grooming compared to respective control rats[control male(NCM),and control female(NCF)] in all trial days. Stressed females showed significantly higher number of grooming events compared to stressed male rats in all trials, though there was no significant difference between control male and control female. NCM vs STM:, **P<0.01*P<0.001; NCF vs STF: $ P<0.05, $$ P<0.01; STM vs STF: # P<0.05,## P <0.01 (One way ANOVA, Bonferroni's test).**

Number of grooming events

Groups	Trial Day			
	Day 1	**Day 2**	**Day 3**	**Day 4**
NCM	4±0.25	5.83±0.3	5.66±0.42	7.16±0.30
STM	**1.66± 0.33****	**3.5±0.22*****	**3.33±0.33*****	**3.33±0.33****
NCF	4.66±0.49	5.5±0.22	6.33±0.33	6.83±0.4
STF	**3.5±0.42**#	**4.83±0.3** #	**5.16±0.3**##	**5±0.36**$,##
F value	10.93	14.74	13.39	25.29
Anova Significance	P<0.001	P<0.001	P<0.001	P<0.001

Table R- 3 . Number of grooming events by the control (n=6) and stressed (n=6) rats in the open field test. Note that stressed rats [both male (STM) and female (STF)] showed less number of grooming compared to respective control rats[control male(NCM),and control female(NCF)] in all trial days. Stressed females showed significantly higher number of grooming events compared to stressed male rats in all trials, though there was no significant difference between control male and control female. NCM vs STM:, **P<0.01*P<0.001; NCF vs STF: $ P<0.05, $$ P<0.01; STM vs STF: # P<0.05,## P <0.01 (One way ANOVA, Bonferroni's test, Each data represents mean ± SEM).**

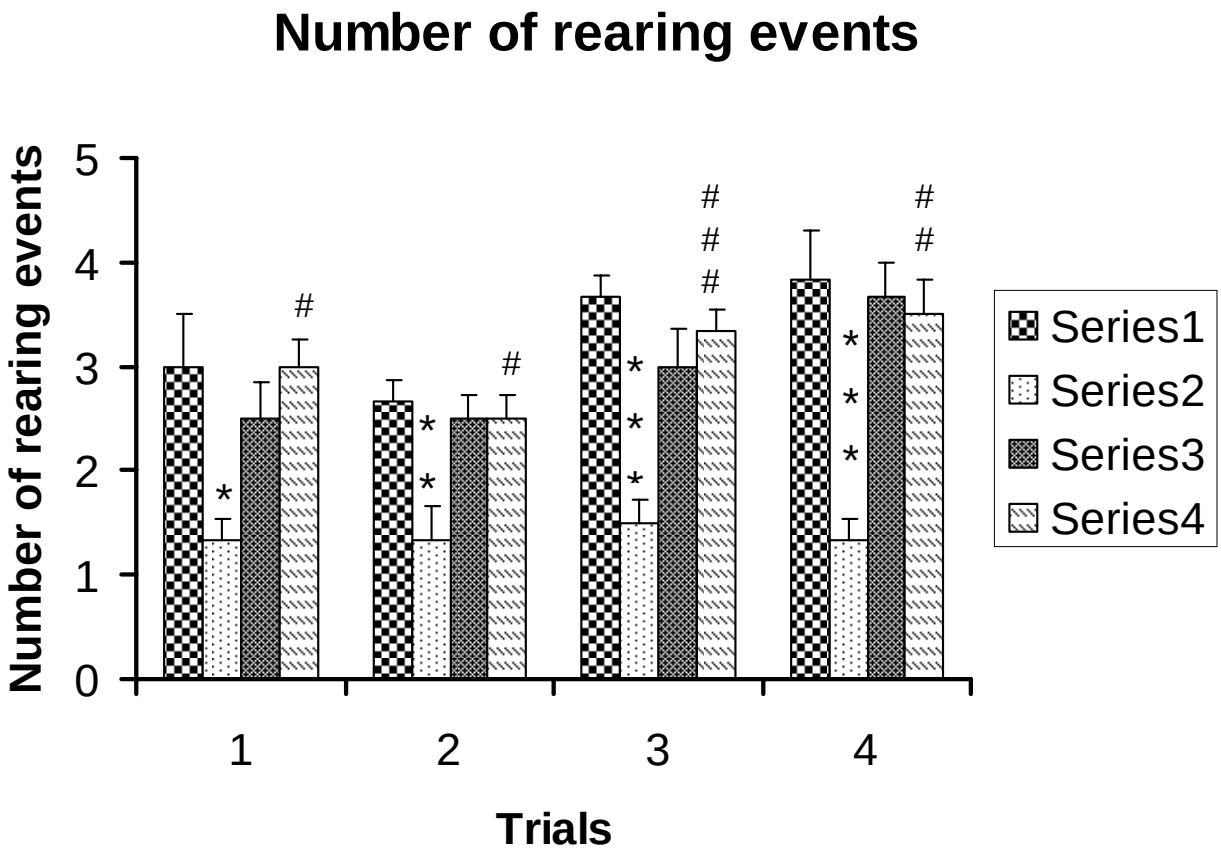

Figure R-4: Number of rearing events by the control (n=6) and stressed (n=6) rats in the open field test. Note that stressed male rats (STM) showed significantly less number of rearing compared to respective control rats[control male(NCM) in all trial days. Stressed females showed significantly more number of rearing compared to stressed male rats. NCM vs STM: *P<0.05;P<0.01, ***P<0.001 ; STM vs STF: # P<0.05, P<0.05, ### P <0.001 (One way ANOVA, Bonferroni's test).**

Number of rearing events

Groups	Trial Day			
	Day 1	**Day 2**	**Day 3**	**Day 4**
NCM	3.0±0.51	4.33±0.66	2.16±0.40	2.33±0.55
STM	1.33± 0.21	**8.16±0.30*** **	**4.0±0.36***	**6.66±0.33*** **
NCF	2.5±0.34	2.83±0.60	**4.83±0.47@@@**	1.66±0.47
STF	3.0±0.25	**5.5±0.67$,#**	**2.16±0.16$$$,#**	**3.16±0.40###**
F value	5.00	15.05	13.11	38.73
Anova Significance	P<0.01	P<0.0001	P<0.0001	P<0.0001

Table R-4 . Number of rearing events by the control (n=6) and stressed (n=6) rats in the open field test. Note that stressed rats [both male (STM) and female (STF)] showed significantly less number of rearing events compared to respective control rats[control male(NCM),and control female(NCF)] in all trial days. Stressed females showed significantly more number of fecal pellets compared to stressed male rats . NCM vs STM: ***P<0.001, *P<0.05 ; NCF vs STF: $ P<0.05, $$$ P<0.001; STM vs STF: # P<0.05, P<0.05, ### P <0.001(One way ANOVA, Bonferroni's test, Each data represents mean ± SEM).

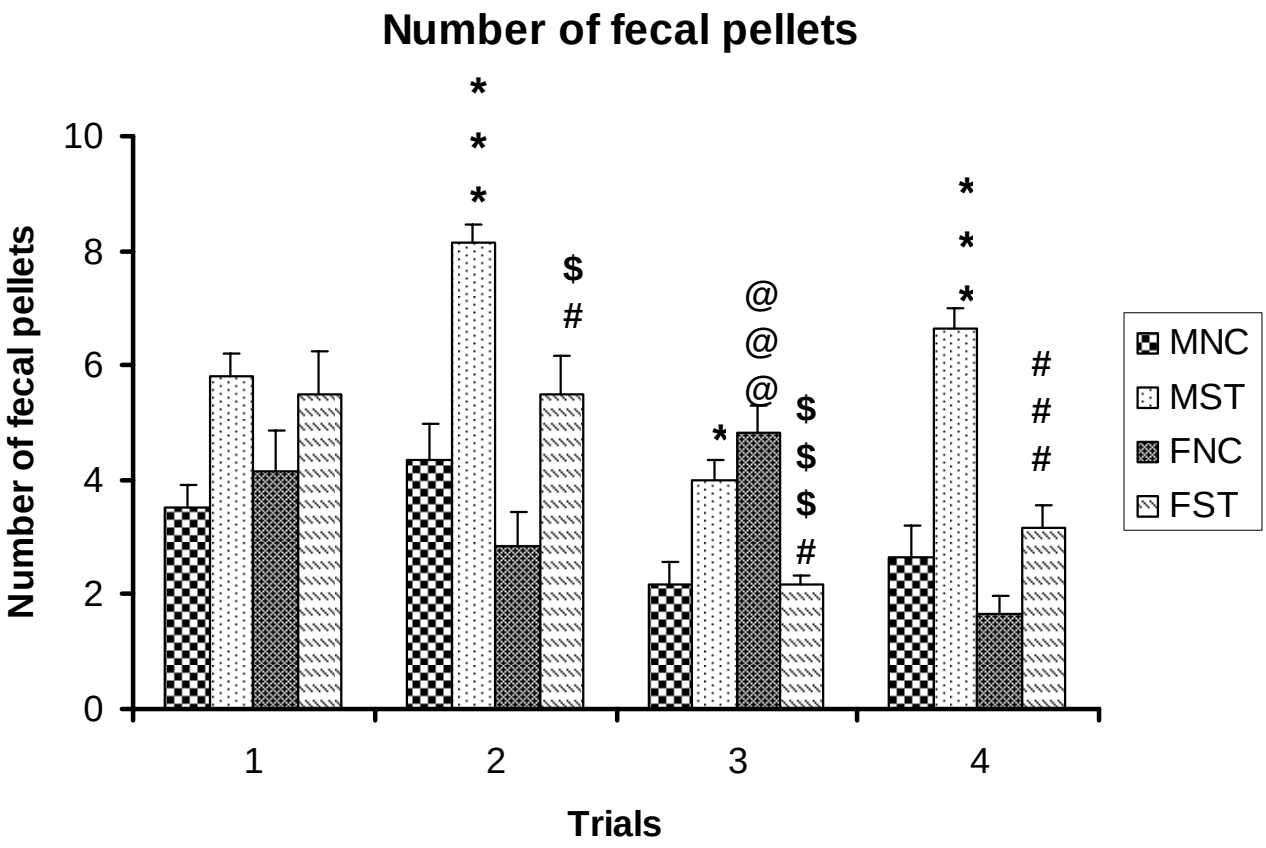

Figure R-5: Defecation scores by the control (n=6) and stressed (n=6) rats in the open field test. Note that stressed rats [both male (STM) and female (STF)] showed significantly more number of fecal pellets compared to respective control rats[control male(NCM),and control female(NCF)] in all trial days. Stressed females showed significantly lower number of fecal pellets compared to stressed male rats . NCM vs STM: ***P<0.001, *P<0.05 ; NCF vs STF: $ P<0.05,$$$ P<0.001; STM vs STF: # P<0.05, ### P <0.001(One way ANOVA, Bonferroni's test).

Number of fecal pellets

	Trial Day			
Groups	**Day 1**	**Day 2**	**Day 3**	**Day 4**
NCM	3.5±0.42	4.33±0.66	2.16±0.40	2.33±0.55
STM	5.8± 0.4	**8.16±0.30*** **	**4.0±0.36***	**6.66±0.33*** **
NCF	4.16±0.70	2.83±0.60	**4.83±0.47@@@**	1.66±0.47
STF	5.5±0.76	**5.5±0.67$,#**	**2.16±0.16$$$,#**	**3.16±0.40###**
F value	3.41	15.05	13.11	38.73
Anova Significance	P<0.05	P<0.0001	P<0.0001	P<0.0001

Table R- 5. Defecation scores by the control (n=6) and stressed (n=6) rats in the open field test. Note that stressed rats [both male (STM) and female (STF)] showed significantly more number of fecal pellets compared to respective control rats[control male(NCM),and control female(NCF)] in all trial days. Stressed females showed significantly lower number of fecal pellets compared to stressed male rats . NCM vs STM: ***P<0.001, *P<0.05 ; NCF vs STF: $ P<0.05, $$$ P<0.001; STM vs STF: # P<0.05, P<0.05, ### P <0.001(One way ANOVA, Bonferroni's test, Each data represents mean ± SEM).

Discussion

The results of the present study revealed that prenatal stress affected the locomotor, exploratory and emotional reactivity in both male and female young adult rats. There is significantly decreased exploration in the peripheral and central squares by the stressed male and female with reduced grooming and rearing effects and more number of fecal pellets .But when the stressed males were compared with the stressed females, it was observed that the male rats exhibited higher anxiety levels with less exploration and more number of fecal pellets when compared to the stressed females.

The method of stress used in the present study is one of the well known methods of stress(**19,22**). Different methods of stress procedures have been used such as forced immersion in cold water(**24**), social stress by exposing the rats to cat (**25,26**), electric foot shock(**27,12**). Stress by restrainer method used in the present study is convenient and animals will not suffocate, but at the same time stress them. Stress by this method is known to increase the adrenal gland weight and glucocorticoid hormone level(**19,22**).

Open field activity is one of the behavioral assays for emotionality. Emotional states are also accompanied by various vegetative phenomena (acceleration of heart rate, dilatation of pupils, etc.) An autonomic function, which can be conveniently evaluated together with the activity measurement, is defecation **(28**). Those animals which ambulate less and defecate more in the open field situation are considered more emotional than animals with high ambulation and low defecation scores (**23**). The results of our study showed that stressed rats [both male (STM) and female (STF)] were more emotional as they exhibited less exploration and more fecal pellets when compared to the normal rats.

Gestational stress increases circulating maternal hormones that produces changes in behavior **(29**). Adult rats exposed to excess endogenous glucocorticoids *in utero* display decreased grooming and rearing in an open field and increased immobility in a forced swim test (**30**). It has been shown that HPA axis of prenatally stressed rats is dysregulated which is manifested by increase plasmacorticosterone in response to stress (**31**). Dysregulation of the HPA axis and abnormal behaviour in response to a stressful situation is a prominent feature of patients with mental illness. Chronic elevation of plasmacorticosterone can impair the negative feedback regulation of the HPA axis by reducing the number of mineralocorticoid (MR) and glucocorticoid (GR) receptors in the hippocampus and other brain regions (**32**). High levels of corticosterone also facilitate conditioned fear- induced freezing behavior by increasing corticotropin –releasing hormone (CRH) gene expression in the central nucleus of amygdala and in the bed nucleus of stria terminalis (**33)**. These brain regions were known to be involved in the generation of fear – related behavior (**34**) It is therefore possible that exposure of the developing fetal brain to higher than normal concentrations of glucocorticoid at a critical period during development can sensitise it to fear- inducing stimuli at adulthood which would be the possible mechanism underlying our findings.

Increased anxiety-related behaviour following prenatal stress has been reported in rats and primates(**30,6**). Anxiety, shows sex-dependent changes following chronic prenatal stress--stress is anxiolytic in males and anxiogenic in females. **(35**). In our study, females were less anxious than males as indicated by more exploration, less number of fecal pellets and more of rearing and grooming behaviors. There is a consistent pattern of

masculinized behavior of the STF (stressed female) rats when compared to their NCF (normal control female counterparts). Prenatal stress influenced this measure of anxiety in a sex- specific manner. This PS influence on anxiety is similar to previous reports(**5**). PS appears to have masculinized the female performance and this finding would be consistent with others who have observed PS- induced masculinization of the female offspring (**36,37**). Changing levels of estradiol in the sexes over the lifespan appear to contribute to the differences in response to stress **(35**). It is also becoming increasingly clear that maternal stress during pregnancy can influence childhood behaviour (**36**).

Conclusion

Our results reinforce the hypothesis that many psychopathological affections have 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 anxiety disorder and depression in children and that these effects are gender- specific, 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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