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DEVELOPMENT OF PROTOCOL FOR TRANSGENERATIONAL STRESS IN WISTAR RATS

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ABSTRACT

Parental stress increases diseases in their children. In order to determine how offspring of stressed and non-stressed parents respond to chronic stress at different stages of life, a rat model was devised.130 healthy Wistar albino rats, 11 weeks of age were selected. Behavioural testing was done on all and stressed rats were removed. Four rats were dissected for histopathology and 10 rats were sacrificed for baseline corticosterone and other hormones. The rats were divided into two groups of parent generation. One was case parents (n=70) and the other control (n=40). Parent cases were exposed to chronic unpredictable stress for three weeks. Behavioural tests were carried out to assess induction of stress in cases parents. Blood and histopathology samples were taken from 10 case parents. Rest of them were allowed to breed. Control parents were also mated at the same time. Offspring of both the groups were exposed to chronic stress, some in early life, some in late life, and some in both early and late life (all stressors for three weeks). Behavioural tests, blood for biomarkers and histology specimens were obtained after exposure to chronic stressors from all the offspring groups. Corticosterone was high in all the control offspring group compared to case offspring. Case offspring showed greater locomotion, rearing and central entries compared to control showing anxiolytic behaviour. Offspring of stressed parents were more resistant/resilient to the effects of stress as compared to the offspring of control parents.

Keywords: Early life stress, late life stress, restraint stress, chronic stress, cortisol, behavioural tests.

1. Introduction

"Stress" is a threat to homeostasis (Pearlin *et al.*, 1981). An organism's response to it is called a "stress response"(Kemeny, 2003). It is an adaptive process but severe, extended stress response may result in disease conditions e.g., anxiety, depression, cardiovascular issues, depression, diabetes, autoimmune diseases, etc.,(Asalgoo *et al.*, 2015), (Chu *et al.*, 2021), (Mariotti, 2015). Stress response is mediated by stimulation of sympathetic adrenal medullary axis, hypothalamic-pituitary-adrenal (HPA) axis leading to activation of hormone systems (Mifsud and Reul, 2018). Chronic stress raises baseline cortisol levels. cortisol levels take longer to return to pre-stress levels (Juster, McEwen and Lupien, 2010). Exposure to a stress hormone for a long period of time during intrauterine life or in early infancy may lead to upregulation of genes involved in the hypothalamic-pituitary adrenal (HPA) response to stress and a down regulation of genes that exert a dampening effect on these pathways (Ladd *et al.*, 2004). Modification to gene expression such as methylation of DNA and modifications of histones was observed in offspring as a result of exposure to stress in prenatal period (Champagne, 2010).

As a result, antenatal stress may cause behavioural changes in offspring for which duration and timing of antenatal stress is important. (Moisiadis and Matthews, 2014)(Glover, 2015). Higher cortisol levels were reported in infants (Davis *et al.*, 2011) and children five years of age whose mothers had chronic psychosocial stress in first two trimesters of pregnancy (Gutteling, de Weerth and Buitelaar, 2005). Similarly, social defeat stress in rats in second half of gestation caused an elevated corticosterone in stressful situation in male offspring along with depression and anxiety (Brunton, 2013). In another study, exposure to odour of predator in second half of gestation resulted in an increase in anxiety in adult offspring in both genders(St-Cyr *et al.*, 2017). Getting exposed to restraint stress antenatally caused anxiety in adult mice (Gur *et al.*, 2017).

Paternal stress encountered early in life or during adulthood resulted in altered reactivity to stress in offspring (Chan, Nugent and Bale, 2018). Offspring of mice exposed to early life chronic stress showed altered behaviour in male offspring and reduction in sperm count. Similarly, decreased sperm count was reported in human males with unfavourable childhood (Dickson *et al.*, 2018).

Literature shows that various stressful situations for parents can affect their offspring as well. To evaluate the transgenerational (from parents to offspring generation) effect of stress in humans require many years, cannot be in a controlled environment and exorbitant. For these reasons rats were used as they get mature and develop reproductive capability much earlier than humans, can be evaluated in a controlled environment and cost-effective. Specific methodology was designed to carry out this transgenerational research.

2. Methodology

2.1 Experimental Design for stress protocol

A total of 130 adult healthy Wistar rats weighing, 280-300 g; age 11 weeks, male to female ratio 1:1, were raised at the Peshawar Medical College Animal House. The rats were housed in cages with 5 rats per cage at 25±2˚C temperature in a humidity of 40-60% under a 12-hour light/dark cycle. There was free availability of food (standard laboratory diet and water was ensured (Radahmadi *et al.*, 2017). Ethical guidelines for animal care, UK were followed. Ethical approval was taken by the Ethical Committee of Peshawar Medical College: IRB approval number: Prime/IRB/2019-167 and Khyber Medical University No. Dir/KMU-EB/HM/000713.

Preliminary behavioural tests were carried out on all rats (figure 1) to remove any stressed rats. Ten rats showed stress so they were removed. Those exhibiting normal behaviour (i.e., no anxiety) were selected (n=120) (figure 1). For baseline values, ten rats were sacrificed for blood collection(Beeton, Garcia and Chandy, 2007)(Supplementary A) after giving anaesthesia(Risling, Caulkett and Florence, 2012) (Supplementary B). Blood samples were stored for corticosterone, adiponectin, leptin and other blood hormones analysis. Tissues (subcutaneous, perinephric fat, liver, gonads)

were collected for histology from 4 male and 4 female rats for analysis. P1 (the first parent generation) was divided into case parents; P1A (exposed to chronic stress), 70 in number and control parents; P1B (unexposed to chronic stress), 40 in number (figure 1).

Seventy rats from case parents' group were exposed to chronic stress at 11 weeks of age for three weeks i.e., from 11 to 14 weeks of age (figure 1 and 2). The chronic stressors applied were: (one stressor per day for a short time) alteration of day-night cycle, cold water immersion stress, followed by restraint stress on the third day to avoid adaptation (Grissom and Bhatnagar, 2009),(Campos *et al.*, 2013). Details of stressors are explained in the article, "Developing Chronic Unpredictable/Alternating Stress Model In Wistar Albino Rats" by Khattak et al, (sent for publication).

After applying stressors, open field test for behavioural testing was performed on P1A, the case parents' group in order to ensure the induction of stress. It was carried out by the same person at 9 a.m., the following day after the exposure to stressors ended. Rats that did not exhibit stress were removed. Sixty rats revealed anxiety and stress (figure 1). After confirmation of anxiety and stress, 10 rats were anaesthetised for approximately one minute (Supplementary B)(Risling, Caulkett and Florence, 2012). Blood was collected for blood biomarkers (Supplementary A)(Beeton, Garcia and Chandy, 2007), the rats were immediately euthanized by exsanguination (Beeton, Garcia and Chandy, 2007). Blood samples were cold centrifuged (- 4◦C) at 4,000 revolutions per minute for 20 minutes to separate sera. The supernatant was transferred to Eppendorf tubes and preserved at −80◦C until taken out for running ELISA kits for assaying various hormones in sera. Histopathology specimens were collected from four rats and kept in 10% formalin.

Fifty rats remaining in case parents' group and forty in control parents' group were allowed to mate. Parents of cases and controls were kept in respective cages, housing 6 rats per cage, 3 male and 3 female rats, under standard conditions of water, food and temperature. There were approximately 19 pregnancies out of 25 couples in the cases parents while in the control parents, there were 18 pregnancies out of 20 couples (figure 1). Same stress protocol was followed for the offspring of both groups. After birth, litter was kept with the mother for four weeks as they attain sexual maturity at the age of 6 weeks (Blunn, 1939). A litter had 8-10 rats at an average that were divided into two groups of approximately four each. After 4 weeks the pups were separated from the mother and the male pups were kept separate from the female pups (both cases and control rat's offspring). These pups formed the first filial (F1) generation. Offspring of parents exposed to stress were labelled as case offspring (F1A) and those of parents unexposed to stress were named as control offspring (F1B) (figure 1 and 2). Case and control offspring were housed in separate cages with male offspring separate from the female ones. 4 to 5 rats were kept in a single cage to prevent overcrowding. The cages were labelled with their group name and their birth date written on the cage.

They were assigned randomly to one of the six groups (separate six groups each for cases and control offspring). Offspring of cases were assigned to 6 different groups (F1A, F1A1, F1A2, F1A3, control of F1A1 and control of F1A2) and similarly, offspring of control parents were selected to fill F1B, F1B1, F1B2 and F1B3, control of F1B1 and control of F1B2) groups. Number of rats in each group was 14. For blood specimen's 10 rats were required, 12 for behavioural tests and 4 male and 4 female for histology. 2 were added to adjust for attrition. Behavioural tests were done, blood and histology specimens were collected for each of cases and control offspring groups.

Six groups of case offspring included

F1A: 5 weeks of age unexposed to chronic stress and sacrificed at 5 weeks of age.

F1A1: early life stress (ELS) was given for 3 weeks that started at 5 weeks of age till 8 weeks of age and were sacrificed at 8 weeks.

F1A2: exposed to chronic stress both early life stress (ELS) and late life stress (LLS). ELS extended from 5 to 8 weeks of age and then LLS given from 11 to 14 weeks of age. These were sacrificed at 14 weeks of age

F1A3: rats of 11 weeks of age, exposed to chronic stressors,(LLS) for 3 weeks that extended from 11 weeks to 14 weeks of age, and sacrificed at 14 weeks,

Control of F1A1: not exposed to stress and sacrificed at 8 weeks of age
Control for F1A2: not exposed to stress and sacrificed at 14 weeks of age.
Control offspring had also six groups (F1B, F1B1, F1B2 and F1B3, control of F1B1 and control of F1B2) having same number of experimental rats, and ages, exposure to stress at the same time and same procedure was followed for every counterpart. (figure 2 and legend)
This research work is a part of my PhD thesis titled, "Hormonal and metabolic alterations in response to stress in a chronic stress rat model and their offspring."

2.2 Defining timings of chronic stress

2.2.1 Early life stress (ELS) for three weeks

As the offspring reached the age of 5 weeks, they were given early life stress for three weeks i.e., from 5^{th} to 8^{th} week of age.

2.2.2 Late life stress (LLS)

At the age of 11 weeks, they were given late life stress for three weeks i.e., from 11 to 14 weeks.

2.2.3 Both early and late life stress (both ELS and LLS)

For both early and late life stress, offspring were exposed to early life stress from 5^{th} to 8^{th} week of age, kept normally for three weeks and then stressors were applied from 11^{th} to 14^{th} week of age

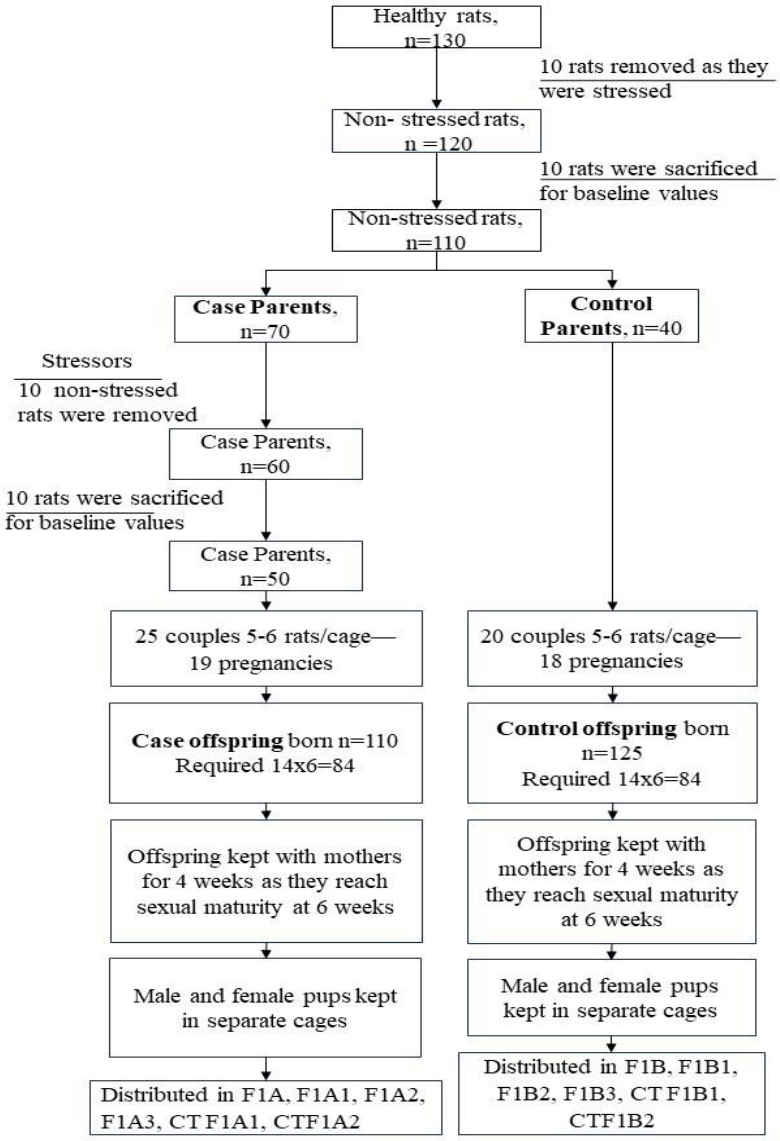

Figure 1: Methodology

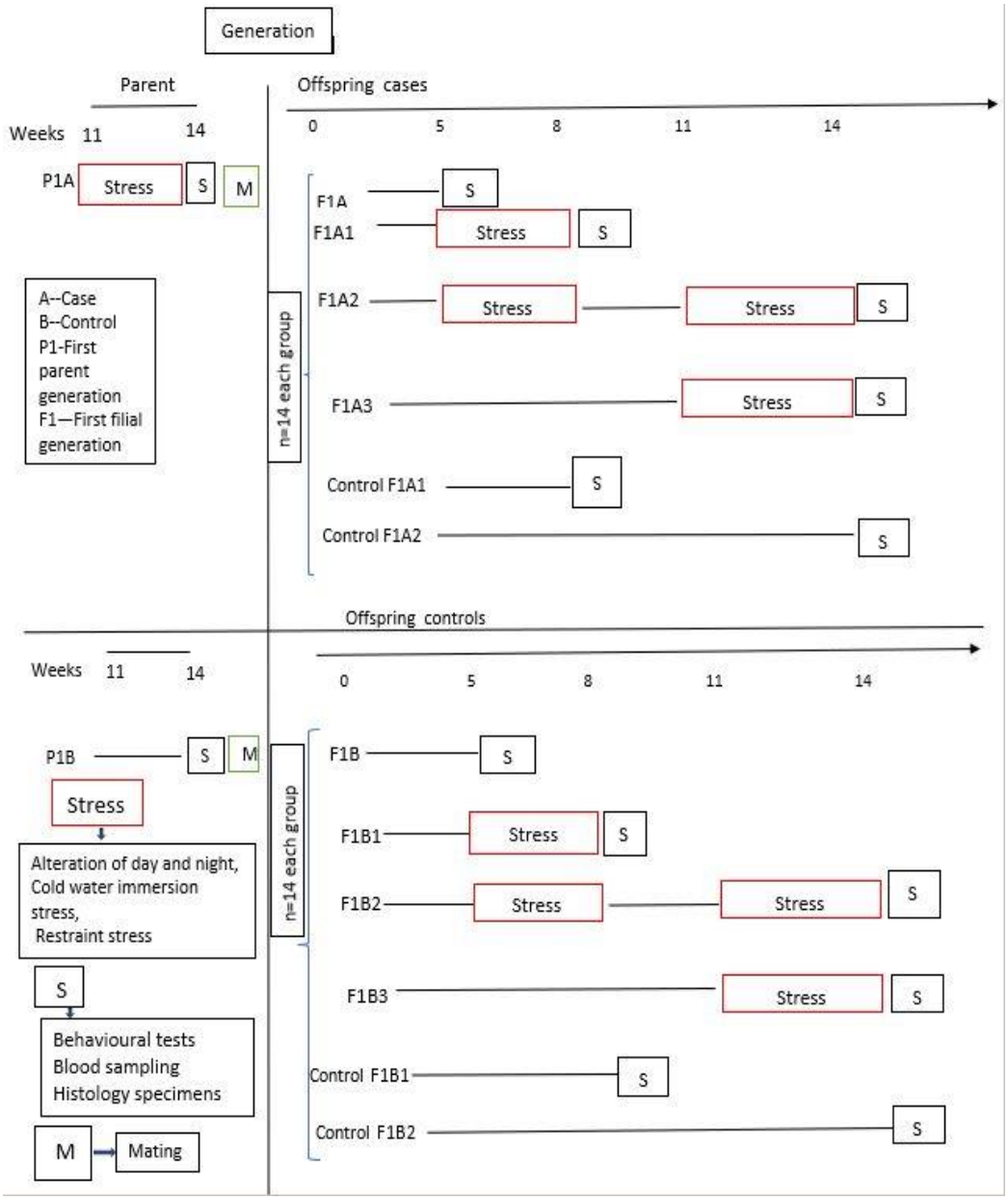

Figure 2: Methodology

Groups of experimental Wistar rats
Healthy rats, n=130
Sample size n=300
P1A,Case parents, n=70, age=11 weeks, exposed to chronic stress for 3 weeks
F1A Off-spring of case parents, n=14, age=5 weeks (Preliminary tests done without being exposed to chronic stress)
F1A1, Off-spring of case parents, n=14, age=8 weeks (after early life stress). Stressors started at 5 weeks till 8th week of age and sacrificed at 8 weeks
F1A2, Off-spring of case parents, n=14, age=14 weeks (after early and late life stress). Stressors applied at 5 weeks of age for three weeks and then again at 11 weeks for three weeks and sacrificed at 14 weeks
F1A3, Off-spring of case parents, n=14, age= 14 weeks (after late life stress). Stressors given at 11 weeks to 14 weeks of age and sacrificed at 14 weeks
Control for F1A1, n=14, age=8 weeks (unexposed to stress), sacrificed at 8 weeks
Control for F1A2, n=14, age=14 weeks (unexposed to stress), sacrificed at 14 weeks
P1B, Control parents, n=40, age=11 weeks, unexposed to chronic stress

F1B Off-spring of control parents, n=14, age=5 weeks, preliminary tests done, unexposed to early life stress
F1B1, Off-spring of control parents, n=14, age=8 weeks (after early life stress). Exposed to stressors from 5^{th} till 8^{th} week of age, sacrificed at 8^{th} week.
F1B2, Off-spring of control parents, n=14, age=14 weeks (after early and late life stress). Exposed to stress first from 5^{th} to 8^{th} week and then from 11^{th} to 14^{th} week of age and sacrificed at 14^{th} week.
F1B3, Off-spring of control parents, n=14, age=14 weeks (after late life stress). Exposed to stress from 11^{th} till 14^{th} week and sacrificed at 14^{th} week.
Control of F1B1 n=14, age=8 weeks (unexposed to stress), sacrificed at 8 weeks.
Control of F1B2 n=14, age=14 weeks (unexposed to stress), sacrificed at 14 weeks.

2.7 Behavioral Tests

2.7.1 Open field test (OFT)

(Patki *et al.*, 2015)(Makori Arika *et al.*, 2019)
The rat was put in the centre in the open field. Testing session lasted for 5 minutes and was done by direct observation recorded by a digital camera, Sony super steady shot D5C-H50(Schmatz *et al.*, 2009). Experimenter was distant and didn't move once the test session started.
Observations included total locomotion in cm, number of rearing responses, time spent in rearing in seconds, number of entries into centre, time spent in centre in seconds, percentage of time spent in centre and periphery respectively. A 5 minute test session was recorded (Bailey and Crawley, 2009). Total distance (cm) travelled in all the quadrants was the measure of locomotor activity (Bailey and Crawley, 2009). Avoiding the centre or staying close to the walls revealed anxiety (Kallai *et al.*, 2007),(Ohl, 2003). Floor was swept with 30% ethanol and dried with a dry paper towel to clean the area for the next rat (Ramos *et al.*, 2008).

2.7.2 Parameters of behavioural tests

Parameters to be measured included locomotion (cm), number and duration of rearing (seconds), time spent in centre in seconds, percentage of time spent in centre and periphery respectively.

2.8 Statistical analysis

Data was analysed through SPSS Version 25. Normality of the data was checked using tests of normality, Kolmogorov-Smirnov and Shapiro-Wilk test. Non normal data was transformed accordingly.
Normal data was presented as mean ± SD, whereas non-normal data were presented as geometric means and confidence interval. Comparison between different groups was done through ANOVA and $p \leq 0.05$ was considered statistically significant.
Among variables of open field, locomotion showed normal distribution. Rest of the markers, number and duration of rearing, time spent in centre and periphery in seconds, percentage of time spent in centre and periphery respectively. Kruskal Wallis test (for non-normal data) was applied for comparison among the groups.
For individual comparison between different groups, MANN-Whitney U test was used. Graphs were made through graph pad prism version 9.1.0 for all the blood markers and variables of behavioural tests.

3. RESULTS

In order to check if our protocol for induction of stress was successful or not, we compared case parents' (P1A) group to control parent's group (P1B). It showed that P1A was stressed compared to P1B. Reduced locomotion and rearing in P1A depict that P1A had increased anxiety compared to P1B. Rearing time in seconds, number of central entries and percentage of time in centre were all significantly higher in PIB than P1A. Percentage of time in periphery was lower in P1B compared to P1A (figure 3),(Supplementary C).
Control offspring showed higher corticosterone compared to the case offspring. Controls of offspring of control parents had decreased corticosterone level compared to controls of offspring of

case parents. Lesser locomotion was observed in control offspring. Highest locomotion was observed in offspring of case parents exposed to both early and late life stress, F1A2. The control offspring groups had more or less comparable locomotion. Rearing and rearing time was increased in F1A2. It was higher in F1A2 (27.83±16.87) than F1B2(13.00±9.77). Furthermore, it was more in F1A2 than F1A1. It was more in F1A compared to F1A1. Rearing was more in F1A3 than F1B3 significant at a lower level. The offspring of case parents had greater central entries than their counter parts. F1A3 had most central entries among case offspring. F1A2 had greater number of central entries than F1A. Among offspring of cases and controls, highest time spent in centre was observed in offspring of case parents before exposure to stress. F1A had highest percentage of time in centre (Supplementary D).

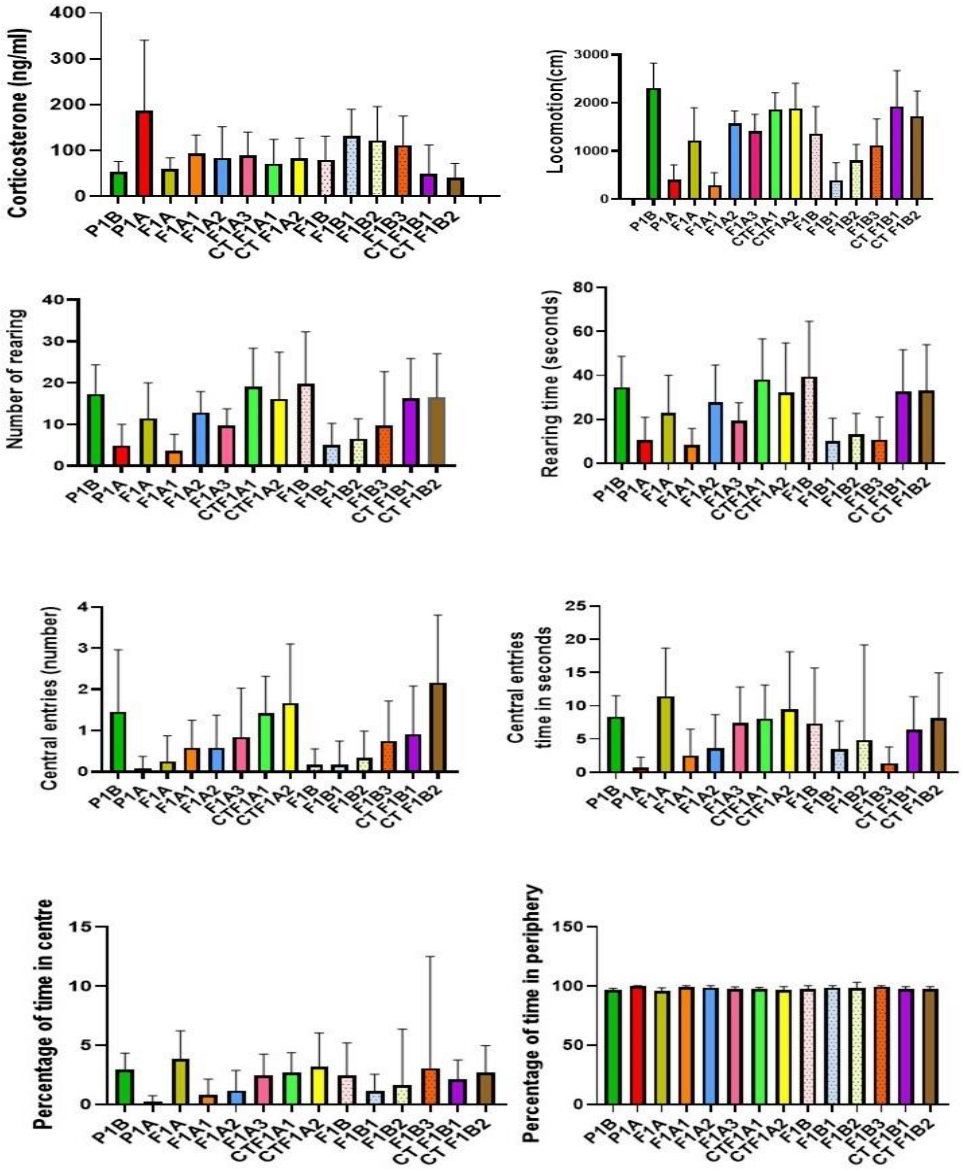

Figure 3: Corticosterone and open field markers, locomotion, number of rearing, rearing time in seconds, number of central entries, central entries in seconds, percentage of time in centre and periphery in all the offspring experimental groups

Legend:
P1A, Case parents, age=11 weeks,
F1A Off-spring of case parents, age=5 weeks (unexposed to chronic stress), sacrificed at 5 weeks

F1A1, Off-spring of case parents, age=8 weeks (after early life stress). Stressors started at 5 weeks till 8th week of age and sacrificed at 8 weeks
F1A2, Off-spring of case parents, age=14 weeks (after early and late life stress). Stressors applied at 5 weeks of age for three weeks and then again at 11 weeks for three weeks and sacrificed at 14 weeks
F1A3, Off-spring of case parents, age= 14 weeks (after late life stress). Stressors given at 11 weeks to 14 weeks of age and sacrificed at 14 weeks
Control for F1A1, age=8 weeks (unexposed to stress), sacrificed at 8 weeks
Control for F1A2, age=14 weeks (unexposed to stress), sacrificed at 14 weeks
P1B, Control parents, age=11 weeks
F1B Off-spring of control parents, age=5 weeks, unexposed to stress, sacrificed at 5 weeks
F1B1, Off-spring of control parents, n=14, age=8 weeks (after early life stress). Exposed to stressors from 5th till 8th week of age, sacrificed at 8th week.
F1B2, Off-spring of control parents, age=14 weeks (after early and late life stress). Exposed to stress first from 5th to 8th week and then from 11th to 14th week of age and sacrificed at 14th week.
F1B3, Off-spring of control parents, age=14 weeks (after late life stress). Exposed to stress from 11th till 14th week and sacrificed at 14th week.
Control of F1B1, age=8 weeks (unexposed to stress), sacrificed at 8 weeks.
Control of F1B2, age=14 weeks (unexposed to stress), sacrificed at 14 weeks.

4. DISCUSSION

Modern way of life entails encountering everyday stressful circumstances, and for some individuals, the stress becomes persistent and hence chronic. Parental stress may result in the development of both metabolic and psychological problems in the next generation. We planned this study to evaluate the transgenerational (from parents to offspring generation) effect of stress. We used rats to evaluate this phenomenon in a controlled environment. We found lower corticosterone levels in offspring of stressed parents. Moreover, the behavioural tests of stressed parent's offspring showed less anxiety and more resilience when exposed to prolonged stress. In addition, the offspring's which were given repeated stress became more resilient.

For this study we prepared a stress protocol of three weeks to which the parent generation was subjected. The effectiveness of stress was checked by evaluating corticosterone which was raised in stressed parents. Moreover, the behavioural tests also showed anxiety and stressed behaviours. Our results of open field behavioural test were similar to another study which showed a reduction of locomotion in the open field test (OFT) after exposure to chronic unpredictable stress (CUS) (Sequeira-Cordero *et al.* 2019). The stressed rats showed decreased rearing which was also observed by another research (Seibenhener and Wooten, 2015). Similar chronic stress protocols have been used by others and have also reported an increase in corticosterone and behavioural modifications (Chen *et al.*, 2021),(Dal-Zotto, Martí and Armario, 2000), (Vagnerová *et al.*, 2023). The same stress model that we used in parents was also used in offspring generation and showed the development of stress by alteration in behavioural tests and rise in corticosterone levels. To limit the effect of environmental confounding factors both the parents and offspring were given same housing environment, food and water.

Our main objective was to check if the chronic stress given to parents have psychological effects in offspring and if the parent generation stress alters response to stress in their offspring. To explore this, we made six groups each in the offspring of case and control parents. We used early life, late life, both early and late life stress to explore this. Moreover, there were controls for all the time points of sacrifice in both cases and control offspring groups which were not given stress. This model covered all the possible alterations in responses which could be due to chance.

Stress causes activation of HPA axis by activating neurons present in paraventricular nucleus (PVN) of the hypothalamus resulting in corticotropin releasing factor (CRF) and arginine vasopressin (AVP) secretion into the portal circulation via the median eminence. Thus stimulation of anterior pituitary gland releases adrenocorticotropic hormone (ACTH) which acts on adrenal cortex and

glucocorticoids get released from the adrenal cortex resulting in mobilization of energy stores. (Pfau and Russo, 2015). Glucocorticoid receptors are present in several areas of the brain and in various organs and tissues. In chronic stress, the negative feedback depending on glucocorticoids controlling stress response is compromised. Furthermore, there is resistance to glucocorticoid receptor leading to long term multiple organ damage (MN, 2012). The reason of chronically high cortisol/corticosterone in chronic stress is due to a continuous stress-induced activation of the HPA axis resulting in altered negative feedback and inappropriate regulation of feedforward signalling. This leads to inefficient dynamic regulation of the stress response (Herman, 2013)(Mariotti, 2015). In our study the effect of chronic stress given to parents was limited to parent generation and harmful effects were not transmitted to the next generation. This could be due to the fact that sufficient time was given to rats (around four to six weeks) for mating and pregnancy. This four to six weeks' time may have attenuated the stress effect but kept the beneficial effect which was seen in the offspring generation.

In offspring generation, an interesting finding in our study was a little raised corticosterone in controls of cases (not given stress), as compared to control offspring which were not given stress (non-significant difference). Similarly, case offspring not stressed also had similar corticosterone. On exposure to stressful situation, there is an increase in corticosterone to make the body cope up with stress. In relation to our data, when considering offspring generation, there was an increase in corticosterone in both case and control offspring after exposure to stress. However, there was lesser increase in corticosterone in case offspring. This shows that previous generation stress may induce resilience in next generation (Supplementary D, figure D.1). The individual perception of a certain stressor to be able to predict it in order to control it, plays an important role in the stress response to that stressor i.e., in promoting adaptation (Koolhass, Bartolomucci and Buwalda, 2011).This explains that response to stress depends on anticipation of that stressor by an individual and its efficiency in causing adaptation. Moreover, on integrating both corticosterone and behavioural test parameters, we saw a pattern that in cases offspring corticosterone was raised but lesser than control offspring who were also given stress. Similarly, behavioural tests show more adaptation with lesser increase in corticosterone in cases offspring as compared to control offspring's which showed higher corticosterone and less adaptation or more influence of stress on behavioural tests. When we integrated corticosterone values with behavioural tests, we found that control offspring had lower locomotion, rearing, central entries as compared to cases offspring when stressed (Supplementary D, figure D.2,D.3,D.5). A similar study also showed that case offspring exposed to early and late life stress (ELS+LLS) observed a consistent increase in locomotion in rodents exposed to chronic unpredictable stress (CUS) (Watt *et al.*, 2009). However, in our study control offspring (not stressed) had similar locomotion as cases offspring (not stressed) (figure 3). When considering baseline corticosterone, control parents and offspring of controls not given stress gave us the base line corticosterone level (figure 3),(Supplementary D, figure D.2). Among all the case and control offspring, the most stressed group was control offspring (8 weeks of age having a raised corticosterone compared to other offspring (Supplementary D, figure D.1). Contrary to our finding, chronic mild stress (CMS) did not alter corticosterone level in different age groups of experimental rats, rather resilience was observed in younger age (Toth *et al.*, 2008). Moreover, a blunted cortisol response was observed in chronically stressed populations (MacDonald and Wetherell, 2019).

Modulation of the central and peripheral systems concerned with stress pathways and negative feedback systems have been observed in cases of human resilience. Resilience or susceptibility to stress is influenced by environmental and genetic factors (Feder, Nestler and Charney, 2009). Adaptation occurs at many levels of organisation facilitated by developmental plasticity to environmental responsive and epigenetic memory (Coffman, 2020). Association of neural circuits, signalling and genetics have been associated with resilience (Cathomas *et al.*, 2019),(Osório *et al.*, 2017). Corticotropin-releasing hormone and brain-derived neurotropic factor are examples of factors associated with resilience (Osório *et al.*, 2017),(Swaminathan *et al.*, 2023).

Additionally, in humans, dehydroepiandrosterone (DHEA) has emerged to be a biomarker having a role in resilience. DHEA is released from the adrenal cortex with cortisol in response to stress and

can counter the effects of glucocorticoids (Yehuda *et al.*, 2006)Cortisol on binding to glucocorticoid receptors inhibits HPA axis by negative feedback. This action of cortisol is suppressed by DHEA (Wu *et al.*, 2013) An elevated DHEA: cortisol ratio has been shown in a study carried on military men, to be associated with lesser stress induced symptoms. Furthermore, DHEA has anxiolytic and antidepressant properties (Averill *et al.*, 2018). Certain epigenetics changes as methylation of DNA reduces HPA activation resulting in altered resilience to stress (Bick *et al.*, 2012). We did not check DHEA or DNA methylation in our study and may be a good area to further explore resilience mechanism in this model.

Mesocorticolimbic reward circuitry may be involved in promoting resilience to chronic stress. (Christoffel, Golden and Russo, 2011). Chronic unpredictable stress causes inhibition of dopamine release (Baik, 2020). However, a different observation after exposure to chronic stress for three to seven weeks was an increase in dopamine level in nucleus accumbens (Stamford *et al.*, 1991). Among all the offspring groups, number of rearing, rearing time, central entries were more in case offspring exposed to both early and late life stress and late life stress compared to control offspring revealing lesser anxiety in cases offspring.(figure 3), (Supplementary D, figure D.3, D.4, D.5).This may be due to resilience produced in the case offspring group. Similar to our study, human studies have observed resilience in response to exposure to chronic stress in early life (Espejo *et al.*, 2007)(Luthar, 2015)(Wyman *et al.*, 1991). Some children may be less susceptible to ELS than other children observed in the differential reactivity model(Wachs, 1992). Children showing resilience after stress exposure, demonstrated a relatively positive adaptation in spite of challenges (Luthar, 2015).

Exposure to stressors throughout early life predicted higher resilience in later life. This may seem to be different from the well-established relationships between early-life trauma and later behavioural and emotional problems, and psychiatric disorders (Espejo *et al.*, 2007). However, stress does not lead to such outcomes in all cases (Wyman *et al.*, 1991). The same view was supported by another study that not even most of the cases exposed to stress develop psychiatric problems (Bonanno, 2004). Furthermore, exposure to stress is required to learn to adapt to stress effectively (Martin and Martin, 2002). Even a severe and prolonged stressor as childhood physical abuse can lead to higher resilience (Cicchetti and Rogosch, 2007). A study differentiated between different stressors such as 'toxic stress' in childhood for e.g., a long-term neglect or abuse; 'tolerable stress', as the death of a dear one and 'positive stress', e.g., minor stressors on daily bases and suggested that positive stress facilitates in developing coping mechanisms to deal with stress that one can encounter later in life. It was concluded that in the presence of support, tolerable stress can act as positive stress too. Although some of the early-life stressors counted in this study seem severe, none would be categorized by Middlebrooks and Audage as toxic, so their model suggests all could have benefited resilience (Audage and Middlebrooks, 2008).

Conclusion:

Offspring of stressed parents were more resilient to early and late life stress as compared to control (non-stressed) parents. On exposure to stress corticosterone was less increased and behavioural tests were less altered in stressed parent's offspring. Offspring given both early and late life stress were more resilient as compared to offspring given only early or only late life stress.

Future Recommendation: Finding the mechanism of adaptation in transgenerational model is recommended for further research.

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Manuscript write up, Idea of study, lab work, statistics, critical reading

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Idea of study, critical reading

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Critical reading, lab work, data collection.

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Annexure (s):

Supplementary "A"

Blood sampling through cardiac puncture (Beeton et al., 2007)

A syringe of 5 ml having 23 Gauge needle was used. The rat was placed on its back with its ventral side facing us. Position of the heart was located by index finger of left hand, kept at the lowest ribs' level, without applying pressure. Heart lies slightly to right and a cm above. Syringe was held at an angle of 45 degrees. Needle was inserted till a drop of blood appeared in the syringe. Then, the plunger of the syringe was pulled to fill the syringe which was disconnected carefully. Blood was emptied into a tube. If more blood was required, the syringe was again connected to the needle. Approximately, 5-10 ml of blood was obtained from a rat weighing 120-180 g.

Supplementary "B"

Open drop method (Risling et al., 2012)

1. After putting on the gloves, gauze was soaked with approximately less than 1cc of 30% v/v isoflurane in propylene glycol mixture per 500cc volume of anesthesia container.
2. A wire mesh was placed at the base of the glass container and the gauze was placed under it in order to prevent direct contact with isoflurane as irritation and reabsorption through the skin can occur.
3. Rat was placed in the container and covered with the lid tightly. Rat took approximately 2 minutes to get anesthetized that was ensured by absent righting reflex.
4. For 10 seconds, it was allowed to remain in deep anesthesia and then taken out from the container which was covered with the lid immediately.
5. Procedure was started if toe pinch was absent otherwise it was returned to the container.
6. By open drop method, rats were anesthetized for a minute during which blood was collected through cardiac puncture and rat was euthanized by exsanguination.

Supplementary "C"

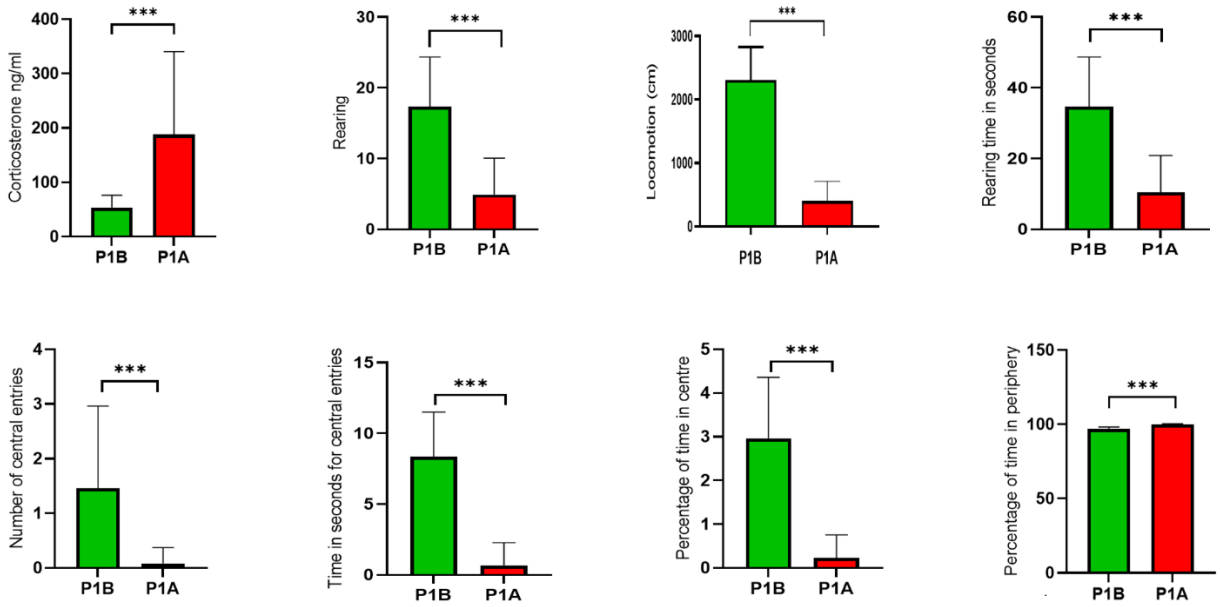

Figure C.1 Comparison of corticosterone and open field parameters between case and control parents

Key:
p* ≤ 0.05
p**≤ 0.01
p***≤0.001
P1A, Case parents, age=11 weeks
P1B, Control parents, age=11 weeks

Corticosterone level was raised in P1A(155.09 (101.15-237.79) ng/ml compared to P1B 49.22 (35.88- 67.54) ng/ml. It was statistically significant with p value<0.001 Locomotion was 2307.45 ± 518.00 cm in P1B compared to 400.50±310.45cm in P1A. P value<0.001.Rearing in P1B was 17.36 ± 6.99 times compared to 4.92 ± 5.14 times with p-value<0. 001.Rearing time in seconds was 34.73 ±13.97 compared to 10.42 ± 10.47 (p<0.001)in P1B compared to P1A .Number of central entries was 1.91 ± 0.83 compared to 0.17±0.38 in P1B and P1A respectively, statistically significant(p<0.001).Time in seconds for central entries was 8.36 ± 3.13 seconds in P1B compared to 0.66 ± 1.61 seconds in P1A (p<0.001). Percentage of time in centre was 2.96 ±1.39 % in P1B compared to 0.22 ±0.53% in P1A (p<0.001). Percentage of time in periphery was 96.84 ±1.33 in P1B compared to 99.77 ± 0.53 in P1A (p<0.001) (Figure C.1).

Supplementary "D"

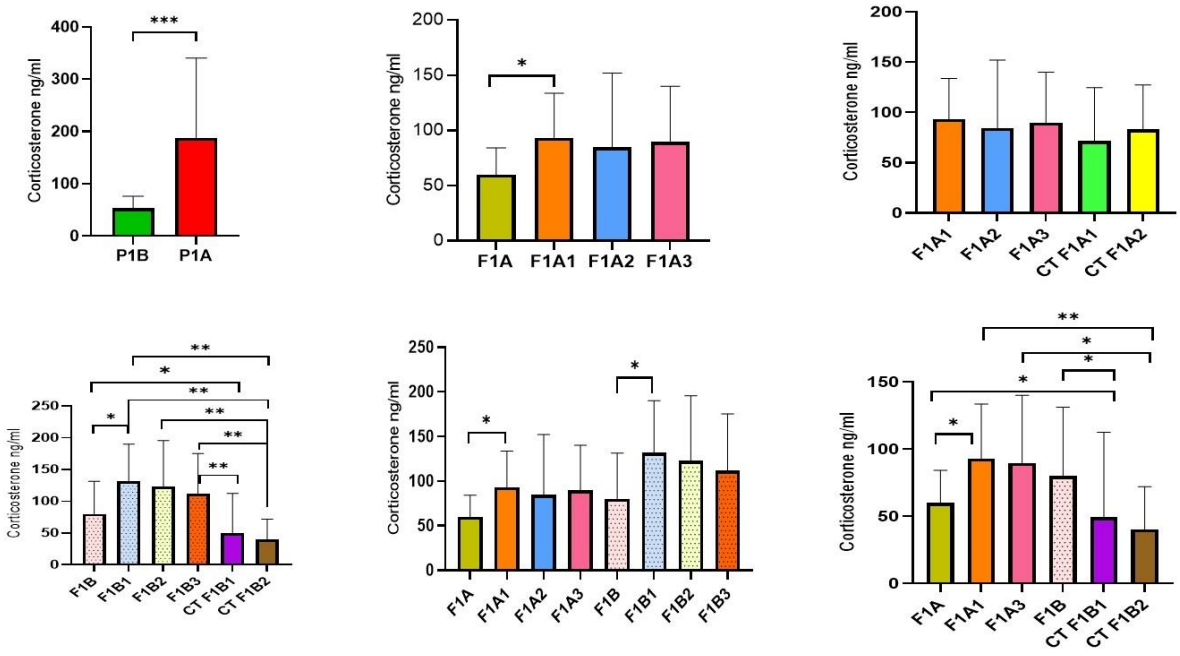

Figure D.1: Comparison of corticosterone in offspring of case parents and offspring of control parents

Key:
p* ≤ 0.05
p**≤ 0.01
p***≤0.001

ELS – Early life stress
LLS – Late life stress

F1A, Off-spring of cases, age= 5 weeks (unexposed to chronic stress)
F1A1, Off-spring of cases, age=8 weeks (after ELS)
F1A2, Off-spring of cases, age=14 weeks (after both ELS, LLS)
F1A3, Off-spring of cases, age= 14 weeks (after LLS)
Control for F1A1, n=14, age=8 weeks (No stress)
Control for F1A2, n=14, age=14 weeks (No stress)

F1B Off-spring of controls, age=5 weeks, (No stress)
F1B1, Off-spring of controls, age=8 weeks (after ELS)
F1B2, Off-spring of controls, age=14 weeks (after both ELS, LLS)
F1B3, Off-spring of controls, age=14 weeks (after LLS)
Control of F1B1, age=8 weeks (No stress)
Control of F1B2, age=14 weeks (No stress)

The most stressed group was F1B1(5 weeks age, offspring of non-stressed parents) among the offspring of case and control parents having the highest level of corticosterone (ng/ml) 115.42(74.94-177.76) compared to the level of corticosterone in F1B i.e., group of offspring 5 weeks of age, unexposed to stress 67.90(44.55-103.48) with statistical significance of $p<0.05$. Off-spring of control parents after early, late and both early and late life stress had high corticosterone level compared to their controls that was statistically significant (figure D.1).

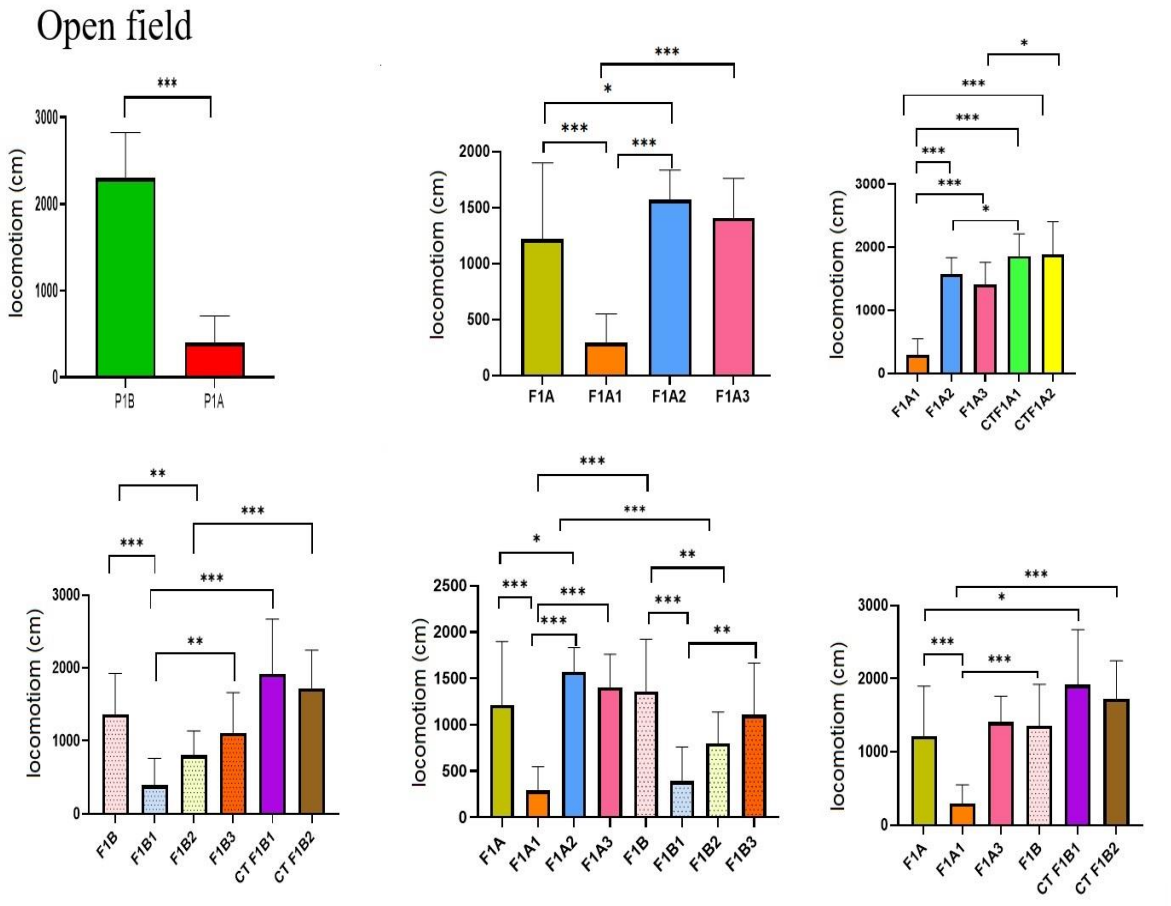

Figure D.2: Comparison of locomotion in offspring of case parents and offspring of control parents in open field test

Key:
p* ≤ 0.05
p**≤ 0.01
p***≤0.001

Among F1A, F1A1, F1A2, F1A3, F1B, F1B1, F1B2, F1B3 group, F1A2(1569.00±265.74) had higher locomotion compared to F1B2 801.00±335.65 with a p<0.001, F1A3(1405.50 ±355.92) revealed greater locomotion than F1A1(291.00 ±258.55 cm) p< 0.001, F1A (1215±683.37cm) showed greater locomotion than F1A1(291.00 ±258.55 cm) (p<0.001), F1A2 showed greater locomotion than F1A1, p<0.001, F1A2 (1569.00±265.74) also showed greater locomotion than F1A(1215±683.37cm) p<0.05 F1B (1359.00±565.08 cm) had greater locomotion compared to F1B1391.50±367.46, F1B (1359.00±565.08 cm)also had greater locomotion than F1B2 (801.00±335.65 cm) p<0.01. F1B3 (1109.83±555.14 cm) had more locomotion than F1B1(391.50±367.46 cm) p<0.01 (figure D.2). We deduce that the most locomotion was observed in F1A2, after getting exposed to both early and late life stress.

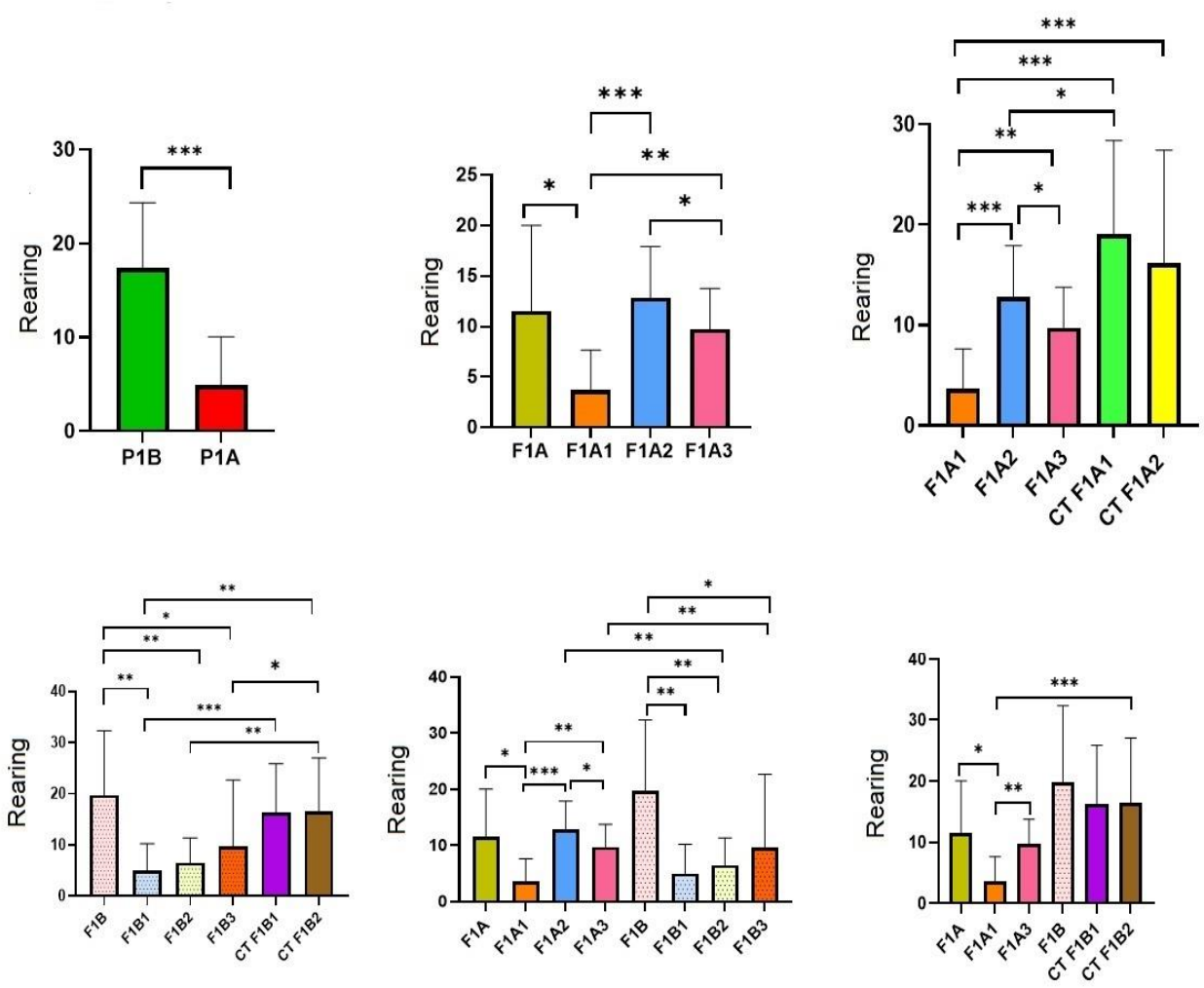

Figure D.3: Comparison of rearing in OFT in offspring of case parents and offspring of control parents in open field test

Key:
p* ≤ 0.05
p**≤ 0.01
p***≤0.001

In F1A, F1A1, F1A2, F1A3, Group F1A1(3.67 ±3.98) had least number of rearing and the difference was highly significant, p<0.001 compared to F1A2 (12.83±5.09) and F1A3 (9.75 ±4.02) (i.e., exposure to chronic early life stress had decreased rearing the most. (Figure 5) In F1B, F1B1, F1B2, F1B3 number of rearing was 19.75±12.61, 5.00±5.25, 6.50±4.89 and 9.67±13.04 respectively. In F1A, F1A1, F1A2, F1A3, F1B, F1B1, F1B2, F1B3 group, number of rearing was more in F1A2 than F1B2 p<0.01 They were observed more in F1A3 than F1B3, p<0.01. Most stressed group was F1A1(least rearing) and F1A2 was the most resilient. figure D.3)

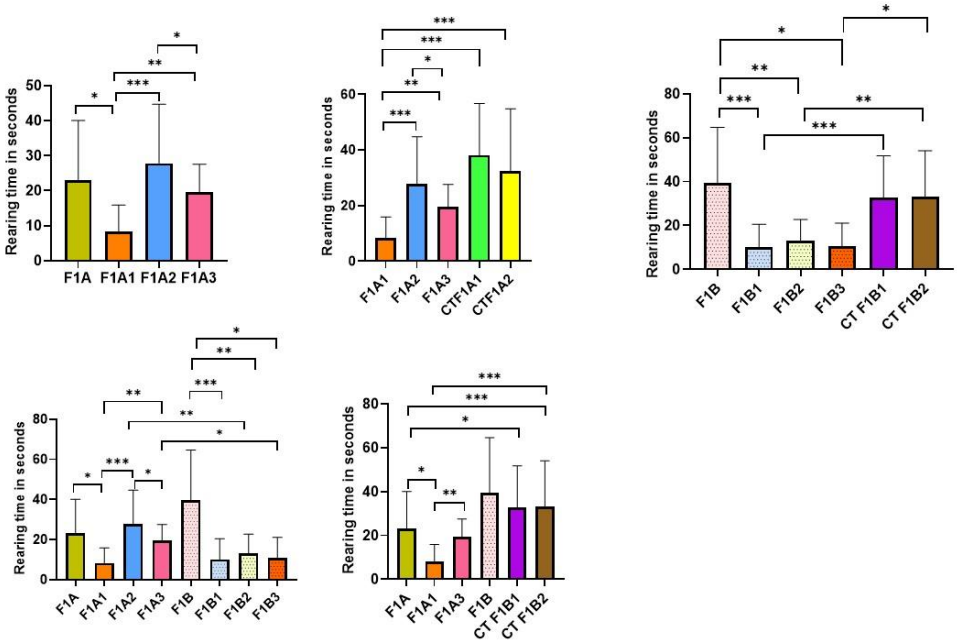

Figure D.4: Comparison of rearing time in seconds in offspring of case parents and offspring of control parents in open field test

Key:
p* ≤ 0.05
p**≤ 0.01
p***≤0.001

Rearing time in seconds was least in F1A1(8.25±7.64) group compared to F1A2(27.83±16.87) (p<0.001). Significant difference, p<0.01 existed between F1A1 (8.25±7.64) and F1A3(19.50±8.05) i.e., the group exposed to late life stress. In F1A, F1A1, F1A2, F1A3, F1B, F1B1, F1B2, F1B3 group, rearing time was more in F1A3 than F1B3 significant at a lower-level p<0.05. Higher in F1A2 (27.83±16.87) than F1B2(13.00±9.77) p<0.01. It was more in F1A2 than F1A1 (p<0.001). It was more in F1A compared to F1A1, p<0.05 (figure D.4).

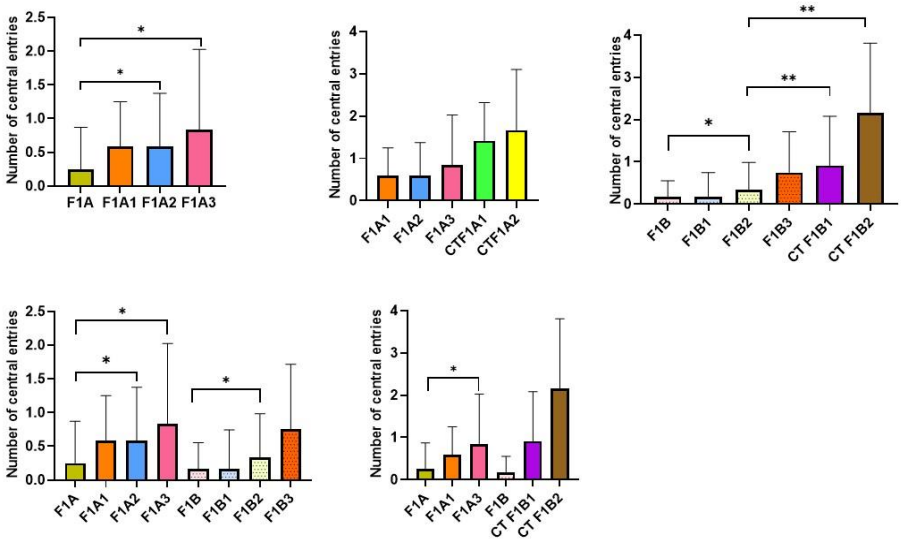

Figure D.5: Comparison of number of central entries in offspring of case parents and offspring of control parents in open field test

Key:
p* ≤ 0.05
p**≤ 0.01
p***≤0.001

In F1A, F1A1, F1A2, F1A3, F1B, F1B1, F1B2, F1B3 group, F1A3 had most central entries that remained statistically significant at a lower level with F1A, p<0.05, F1A2 had greater number of central entries than F1A, p<0.05 (figure D.5).

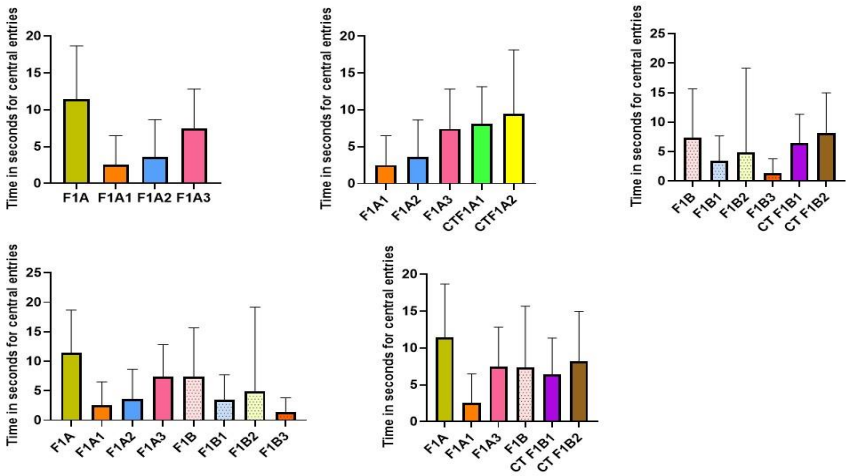

Figure D.6: Comparison of time in seconds for central entries in offspring of case parents and offspring of control parents in open field test

Key:
p* ≤ 0.05
p**≤ 0.01
p***≤0.001

In F1A, F1A1, F1A2, F1A3, F1B, F1B1, F1B2, F1B3 group, time in centre in seconds was highest in F1A but non-significant (Figure D.6)

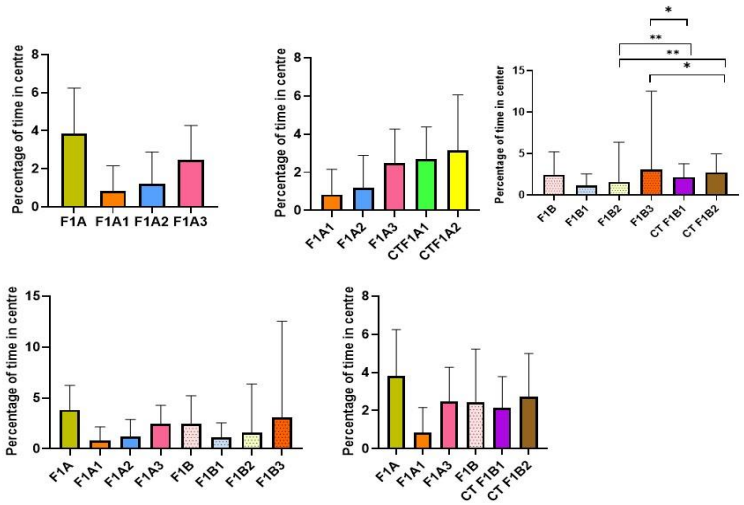

Figure D.7.: Comparison of percentage of time in centre in offspring of case parents and offspring of control parents in open field test

Key:
p* ≤ 0.05
p**≤ 0.01
p***≤0.001

In F1A, F1A1, F1A2, F1A3, highest percentage of time in centre was observed in F1A (3.831 ± 2.408) but no significance with any offspring group of cases. F1A1, F1A2, F1A3 had 0.83 ±1.32, 1.19±1.69, 2.47 ±1.80 percentage of time in centre respectively (figure 9) In F1B, F1B1, F1B2, F1B3, percentage of time in centre was highest in F1B3 but not significant with offspring of controls. The values were 2.44±2.78, 1.13±1.42, 1.61±4.76, 3.08±9.45 in F1B, F1B1, F1B2, F1B3 groups respectively. In F1A, F1A1, F1A2, F1A3, F1B, F1B1, F1B2, F1B3 group, F1A had highest percentage of time in centre but did not reveal any significance. (Figure D.7)

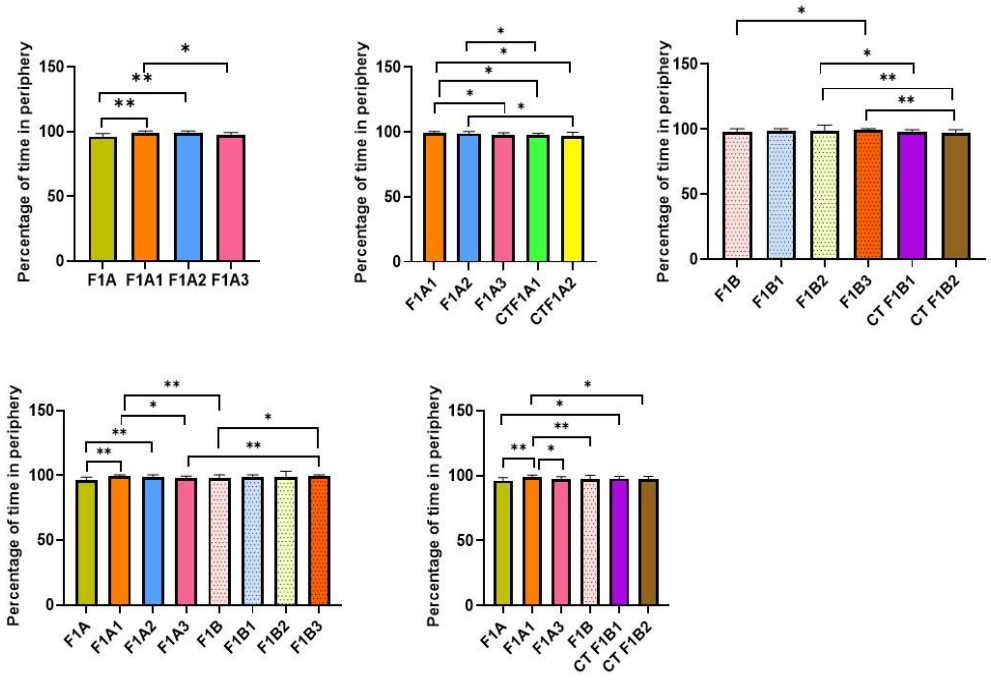

Figure D.8: Comparison of percentage of time in periphery in offspring of case parents and offspring of control parents in open field test

Key:
p* ≤ 0.05
p**≤ 0.01
p***≤0.001

In F1B, F1B1, F1B2, F1B3, percentage of time in periphery for these groups were 97.55±2.78, 98.86±1.42, 98.38±4.77, 99.55±0.82 respectively. F1B3(99.55±0.82) compared to F1A3 (97.52 ±1.80) had a statistical significance with p=0.008. F1B3 was statistically significant with F1B at a low level of significance. It was significant between F1A (96.16 ± 2.41) and F1A1 (99.16 ±1.33), between F1A (96.16 ± 2.41) and F1A2 (98.80 ±1.69) p<0.01 respectively. It was significant between F1A1(99.16 ±1.33), and F1A3(97.52 ±1.80) p<0.05. It showed significance between F1A1 and F1B, p<0.01. Least percentage of time in periphery was spent by F1A and highest percentage of time was spent in periphery by F1A1. Early life stress is worst as maximum time in periphery was spent by F1A1(figure D.8)