Housing under the pyramid reduces susceptibility of hippocampal CA3 pyramidal neurons to prenatal stress in the developing rat offspring

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Mother-offspring interaction begins before birth. The foetus is particularly vulnerable to environmental insults and stress. The body responds by releasing excess of the stress hormone cortisol, which acts on glucocorticoid receptors. Hippocampus in the brain is rich in glucocorticoid receptors and therefore susceptible to stress. The stress effects are reduced when the animals are placed under a model wooden pyramid. The present study was to first explore the effects of prenatal restraint-stress on the plasma corticosterone levels and the dendritic arborisation of CA3 pyramidal neurons in the hippocampus of the offspring. Further, to test whether the pyramid environment would alter these effects, as housing under a pyramid is known to reduce the stress effects, pregnant Sprague Dawley rats were restrained for 9 h per day from gestation day 7 until parturition in a wire-mesh restrainer. Plasma corticosterone levels were found to be significantly increased. In addition, there was a significant reduction in the apical and the basal total dendritic branching points and intersections of the CA3 hippocampal pyramidal neurons. The results thus suggest that, housing in the pyramid dramatically reduces prenatal stress effects in rats.

Keywords: CA3 pyramidal neurons, Chronic restraint-stress, Corticosterone, Gestation, Hippocampus, Offspring, Prenatal stress, Pyramid

Stress is part and parcel of everyday living. The body responds to stress with a physiological fight-or-flight reaction. Stress hormones surge throughout the body to help cope with the stress situation by increasing the heart rate and metabolism to meet the extra energy needs.

Stress activates the hypothalamic–pituitary–adrenal (HPA) axis that leads to elevation in the levels of corticotrophin-releasing hormone (CRH), in response to input from extra-hypothalamic sources. This in turn, causes the secretion of more adrenocorticotropic hormone (ACTH) from the anterior pituitary, which subsequently leads to the release of cortisol in humans (corticosterone in rodents) from the adrenal cortex. The hippocampus is rich in glucocorticoid receptors, which make it most vulnerable to long-term stress compared to most other areas of the brain. Stress related steroids affect the hippocampus in different ways such as: reducing the excitability of some hippocampal neurons, inhibiting the genesis of new neurons in the dentate gyrus and by causing atrophy or shrinkage of dendrites in the CA3 pyramidal region, all of which could affect memory, learning and mood.

Stress can be of short-term or acute which is the most common form of stress. Acute-stress is reaction to an immediate threat commonly known as the fight-or-flight response. Once the treat has passed away the responses are inactivated and the stress hormones return to their normal levels. It is in small doses and does not have enough time to cause extensive damage. Long-term or chronic stress on the other hand persists for long durations in which the individual cannot see a way out of the stressful situation. The fight-or-flight response needs to be suppressed such as seen in highly demanding pressured work situations, persistent abuse etc. These can be traumatic and have deleterious effects on the brain and behaviour of the individual.

Chronic stress leads to a number of neural changes which could precipitate the onset of psychiatric disorders. Considerable amount of research both human epidemiological and experimental have shown maternal stress during gestation is associated with juvenile and adult offspring and abnormal behaviour, mental and cognitive disorders.

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Parent-infant or mother-offspring relationship begins even before birth, continues through gestation and then develops and matures during the postnatal period. Interaction between genes and environment determines the functional development of an organism. The foetus because of its rapid growth is particularly vulnerable to insults and hormones.

Glucocorticoid levels are used as measures of the level of stress the mothers’ experience. In humans, stress can be of different kinds, such as lack of social support, child abuse, anxiety, fear, and environmental stressful conditions. Pyramids influence the energy fields generated within them and placing within the pyramid enclosure (Pyramid of Giza or its small-scale replicas) result in bettering effects on these objects such as longer preservation of fruits and vegetables, faster growth of plants, greater relaxation and the electromagnetic frequencies are converted by the direct influence of the pyramid shape causing them to harmonize the energy. This energy cannot be recorded with the available instruments despite all the efforts to build antennas or receivers.

Studies in rats have shown that stress shortly after birth can affect hippocampal function in ways that persist throughout life. The effects of restraint-stress in rodents on the adrenal glands showed hypertrophy of the adrenals while the CA3 pyramidal neurons of the hippocampus showed a decrease (atrophy) in the dendritic arborisations. There is paucity of available data concerning the effect of prenatal stress (intrauterine-environment) on the offspring. In the present research the aim is to first explore the effects of prenatal restraint-stress on the plasma corticosterone, and the stress-induced structural remodelling of dendritic arborisation of the CA3 pyramidal neurons of the offspring at postnatal day (PND) 10, 21, 40 and 60. Also it is proposed to confirm whether housing inside a wooden pyramid during gestation would counter the stress-induced effects in the offspring as evidenced earlier in adult rodents that showed housing in the pyramid counteracts the neuroendocrine and oxidative stress caused by chronic restraint stress. Earlier studies also showed that it is the geometric shape of the pyramid, and not the mere enclosure that was responsible for the beneficial effects to counter the stress as the effects were not seen when the animals were restrained in a square box with similar dimensions.

Materials and Methods

Animals—Sexually mature 3-month-old female Sprague Dawley rats weighing 180-250 g used in the study were housed individually in polypropylene cages (25 × 47.5 × 20 cm). The animals were housed in a controlled environment at 23±2 °C with 50±5% RH on a 12:12 h light/dark cycle. Food and water were provided ad libitum. All procedures were performed in accordance with the Guidelines of National Institute of Health Guide for the Care and Use of Laboratory Animals and with the approval of the Animal Experiments Ethics Committee of the Institution. All efforts were made to minimize the number of animals used and their suffering.

Prenatal stress—The pregnant dams of the restrained control (RC) and restrained under the pyramid (RP) groups were exposed to restraint stress outside and under the pyramid respectively from gestation day 7 (GD7) until day of parturition while the control (NC) group of dams were left undisturbed in their home-cage. The experimental group of dams were restrained in the wire-mesh restrainer (15 cm length, 7 cm wide, and 7 cm height) for 9h/day. After delivery, the pups were culled to maintain 7 pups per respective dam until day of weaning or day of killing for experiment. The pups (n=8-10/group) of each group were later killed on PND 10, 21, 40 and 60 by cervical dislocation.

Pyramid model design—A wooden pyramid model was locally fabricated with a height of 30", base 45" and 41.5" sides. Holes were drilled for ventilation and a small glass window was provided for observation. The four triangular sides of the pyramid angled upwards at 51° to the base and met at the apex of the pyramid.

Housing in the pyramid—The pyramid was aligned such that the four sides faced the four cardinal north, south, east and west directions. It was placed to face in the magnetic north-south axis as the beneficial effects are dependent on the alignment of the pyramid. Pregnant dams were restrained and placed under the pyramid in the north-south axis on a platform at one-third the height (10") from the base as the maximum effect is believed to be exerted at this height.
Corticosterone measurement—Trunk blood was collected in EDTA tubes after decapitation for estimation of the plasma corticosterone using Abnova-Corticosterone ELISA kit (Cat #KA0468 V.02) according to the manufacturer’s instructions. The sensitivity of the assay with the limit of detection was 0.3 ng/mL. The corticosterone ELISA kit employs competitive enzyme immunoassay technique that measures corticosterone. A polyclonal antibody specific for corticosterone is precoated onto a 96-well micro plate with removable strips. Corticosterone in standards and samples is competed by a biotinylated corticosterone sandwiched by the immobilized antibody and streptavidin-peroxidase conjugate. All unbound material is then washed away and a peroxidase enzyme substrate is added. The colour development is stopped and then the intensity of the colour is measured at 450 nm wavelength.

Golgi staining—On PND 10, 21, 40 and 60 the animals killed by cervical dislocation were decapitated and the hippocampus was carefully dissected out and processed for Golgi staining using silver impregnation technique\textsuperscript{15,17}. Briefly, the tissue pieces were first fixed in a solution of potassium dichromate for two days, later they were exposed to 1% silver nitrate and kept in the dark to avoid oxidation. The hippocampal tissue was rinsed in absolute alcohol, fixed on to a tissue holder, covered with paraffin wax and tissue slices were cut in the coronal plane at 100-120 µm thickness, dehydrated in absolute alcohol, cleared in xylene and then mounted on to clean glass slides, covered with DPX and a coverslip was placed over it. The slides were coded and later examined under bright field microscope under 40 X magnification to view the silver impregnated CA3 hippocampal neurons. The well impregnated neurons were identified and traced with the aid of the drawing tube attachment which were later analysed by placing the camera Lucida drawings on the Sholl’s grid\textsuperscript{18} for quantification of the dendritic branching and the intersections at 20 µm distances from the soma of the neuron. Though the data were collected at 20, 40, 60, 80 and 100 µm from the soma, here only the total number of branching points and the intersections for each group is presented here.

Statistical analysis—Data were analysed for statistics using SPSS version 17 software and the results expressed as mean±SE. The data obtained were analysed using one-way ANOVA test. A $P<0.05$ was considered statistically significant.

Results

Plasma corticosterone levels (Fig. 1)—The plasma corticosterone levels increased with age in all the groups. The plasma corticosterone levels in the offspring of RC group showed a significant ($P < 0.001$) increase when compared to NC group at postnatal days PND 10, 21, 40 and 60 (Fig. 1) reflecting the effect of stress, while there was a significant decrease in the plasma corticosterone levels in the offspring of RP groups when compared to RC group ($P < 0.001$) showing the anti-stress effects of pyramid exposure.

Dendritic intersections and branching points of CA3 hippocampal pyramidal neurons

Apical dendritic arborisation

Total apical dendritic intersections (Fig. 2a)—Analysis of the total apical dendritic intersections by one-way ANOVA showed a significant decrease in the stressed RC group pups compared to the unstressed control NC group pups ($P < 0.001$, $< 0.01$) at PND 10, 21 and 40. At PND 60 also there was a decrease seen but was not statistically significant. In the pyramid stressed RP groups ($P < 0.01$, $< 0.05$) the decrease in the intersections was significantly less compared to the RC group at the respective PND which shows the beneficial effects of the pyramid environment as an anti-stressor.

Total apical dendritic branching points (Fig. 3a) – Analysis of the total apical dendritic branching points by one-way ANOVA showed a significant decrease in the stressed RC group pups compared to the unstressed control NC group pups ($P < 0.001$, $< 0.01$) at PND 10, 21, 40, and 60. In the pyramid stressed RP groups the decrease in the intersections was
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significantly less compared to the RC group ($P < 0.01, < 0.05$) at the respective PNDs except at PND 60 which was less but not significant. This shows the beneficial effects of the pyramid environment as an anti-stressor.

Basal dendritic arborisation

**Total basal dendritic intersections (Fig. 2b)**—Analysis of the total basal dendritic intersections by one-way ANOVA showed a significant decrease in the stressed RC group pups compared to the unstressed control NC group pups ($P < 0.001, < 0.01, < 0.05$) at PND 10, 21, 40 and 60. In the pyramid stressed RP groups the decrease in the intersections was significantly less compared to the RC group ($P < 0.001, < 0.01$) at the PND 10 and 21 but not at PND 40 and 60, showing the beneficial effect of the pyramid environment as an anti-stressor.

**Total basal dendritic branching points (Fig. 3b)**—Analysis of the total basal dendritic branching points by one-way ANOVA showed a significant decrease in the stressed RC group pups compared to the unstressed control NC group pups ($P < 0.001, < 0.01, < 0.05$) at PND 10, 21, 40 and 60. In the pyramid stressed RP groups the decrease in the intersections was significantly less compared to the RC group ($P < 0.05, < 0.01$) at the PND 10 and 21 but not at PND 40 and 60.

**Discussion**

The present study was designed to study the effect of chronic restraint-stress on the offspring of pregnant dams subjected to the chronic-stress during gestation. It was also aimed to evaluate the anti-stress effects of the pyramid on these effects. The results demonstrated that, housing under the pyramid during the prenatal stress significantly reduced the stress effects on the plasma corticosterone levels as well as the total dendritic arborisation of the hippocampal CA3 pyramidal neurons in the offspring at all the postnatal days studied compared to prenatal restraint stress outside.

**Plasma corticosterone levels**—Cortisol (corticosterone in rodents) is the major stress hormone mediating the effects of prenatal stress. In rats, chronic stress during gestation increases the levels of maternal and foetal plasma corticosterone, thereby leading to a reduction in glucocorticoid receptors (GR) and mineralocorticoid receptors (MR) in the foetal hippocampus and limbic system$^{19}$. Increased activity of corticosterone and
CRH in the developing brain could also alter the synaptic development and neurotransmitter activity which results in altered behaviour in adulthood. Li et al.\textsuperscript{20} found prenatal stress greatly reduced the expression of GRs in the CA1 field and whole hippocampus. Prenatal stress is one of the critical factors that affect the development of the brain in the offspring\textsuperscript{21}, while environmental enrichment during gestation improves behaviour consequences and synaptic plasticity in hippocampus of prenatal stressed rat’s offspring\textsuperscript{20}. The results showed a significant increase in the plasma corticosterone levels of the experimental RC group compared to the control NC, while the experimental pyramid exposed RP group showed significant decrease as compared to the RC group. Plasma corticostrone levels of the respective mothers (unpublished data) also showed the same trend. Thus, the enriched pyramid environment supresses the stress affects and lowers the levels of the stress hormones, not only in the mothers but also its transfer across the placenta into the foetus.

**Dendritic arborisation**—Neurons receive and integrate information from the environment through the dendrites. The dendrites form synapses with the somas, axons or dendrites of other cells and express their function. Amount of information that a neuron can receive from the environment depends on the number of branching points of the dendrites and the size of the dendritic field. There is evidence to show change in the dendritic morphology with age and the phase of an individual especially very early in life\textsuperscript{22}. Chronic stress is found to produce consistent and reversible changes in the dendritic arborisation of CA3 hippocampal neurons, characterized by a decrease in the dendritic length as well as reduction in the number of branches as shown by Rao et al.\textsuperscript{17}. They found complete reversal of dendritic atrophy in CA3 neurons of hippocampus following rehabilitation in restraint stress in rats. Hossein-Sharifabad\textsuperscript{23} reported retraction in apical dendrites of CA3 and granule cells whereas morphology of apical dendrites of CA1 neurons remained unaffected in two-month old offspring.

Prenatal stress serves or provides as an important and useful model to study the influence of early exposure to stress on the physical development,
brain development, cognitive skills as well as any alterations in behaviour of the offspring later in life. There is evidence to show prenatal stress induces hippocampal-dependent learning and memory loss. The results showed a significant decrease in the dendritic arborisation (decrease in the dendritic branching points and intersections) in the hippocampus of the offspring to the chronic restraint-stress induced during gestation (Figs 4-7). This shows that the stress hormone crosses the placental barrier to produce these deleterious effects. These effects were significantly reduced in the pyramid-exposed groups. Mychasiuk et al. using stereological techniques in combination with Golgi-Cox methods studied the effect of prenatal stress on the dendritic morphology and the synaptic connectivity in the prefrontal cortex and hippocampus in the developing offspring. Their results also suggest alteration in both regions in development, similar to what we observed.

Tellez-Martinez et al. studied the effects of prenatal stress from embryonic day 11 until delivery on locomotor activity and the morphology of hippocampus and the nucleus accumbens neurons in male offspring rats at PND 35 and 65. They found permanent reduction in spine density of CA3 hippocampus at both ages. Jia et al. found prenatal stress decreased the number of branch points of the apical dendrites of pyramidal neurons in the hippocampal CA3 region of offspring rats with shorter apical dendrites suggesting that prenatal stress leads to apical dendritic atrophy of the CA3 pyramidal neurons. This could be caused by corticosterone and its glucocorticoid receptors. It is speculated that the maternal and foetal hypothalamic-pituitary-adrenal (HPA) axes and the placenta are the most likely candidates for the effects of prenatal stress on the offspring.

The mechanism underlying stress-induced CA3 dendritic retraction is that corticosterone levels rather than any other hormones of the HPA axis or enhanced activity of the sympathetic nervous system, mediates CA3 dendritic retraction. Chronic stress interacts
synergistically with a variety of glutaminergic afferents to remodel CA3 dendritic morphology. The CA3 sub region of the hippocampus in the adult rat brain is shown to be extremely vulnerable to the effects of long-term stress, as evidenced by a robust stress-induced decrease in the complexity and retraction of dendrites.

Exposure to stress during critical periods of an organism’s development can result in permanent changes and induced hyper-responsive to aversive stimuli as an adult. Hippocampus is a plastic and vulnerable brain structure that is susceptible to damage during aging and repeated stress. Prenatal restraint-stress regulates the hypothalamic-pituitary-adrenal axis, and the effect is found to be similar to a corticosterone (40 mg/kg) being injected as reported by Afadlal et al. who examined the effect of maternal restraint stress on the level of GAP-43, pGAP43 and synaptophysin in the hippocampus of rat pups. They found significant increase in GAP43 and pGAP43 in the pup’s hippocampus during day 7 and 14, but not at later ages. Since the first two postnatal weeks correlate with the peak (critical) period of synaptogenesis it shows that up-regulation of GAP43 and pGAP43 may alter the axonal growth and the very formation of synapses. On the other hand Zhang et al. observed that the levels of corticosterone were not different in pups at PND10, 20 and 45. The present results on the plasma corticosterone levels in the restrained stress control showed a significant increase at PND 10, 21, 40 and 60 while the effect was significantly less in the PR group at all the time points studied.

The beneficial (anti-stressor) effects of the pyramid environment during gestation could be due to suppression of the hypothalamus leading to decrease in the CRH levels with reduced plasma corticosterone levels thereby preserving the dendritic morphology and function of the hippocampus in the rats.

Fig. 6—Representative photomicrographs of Golgi-stained CA3 pyramidal neurons (upper panel) and Camera Lucida tracings (lower panel) from PND 40 pups. NC = Normal control, RC = Restraint control and RP = Restraint pyramid 40X.
Conclusion

In conclusion, taking into consideration the earlier reports\textsuperscript{10,12,13} on adult rodents, the present results confirm reduction in the effects of prenatal stress on the plasma corticosterone levels as well as on the dendritic field of hippocampus (CA3 neurons) when exposed to the pyramid environment. Thus, the energy field generated within the pyramid when placed in the north-south axis certainly has beneficial effects, serves as an enriched environment and helps ameliorate or reduce the prenatal stress effects probably by suppressing the hypothalamic-pituitary-adrenal (HPA) axis and the transfer of stress hormones across the placenta. The stress effects seem to wear off over time as the pups’ age, seen in the effects with decrease by PND 60, which may be because of the structural adaptation occurring during the development, as they are not under stress after birth. Thus, the pyramid structure could be used as a non-invasive therapy for stress management. Further research is warranted to explore the prenatal-stress effects on three other regions of the brain, namely the dentate gyrus granule cells, prefrontal cortex and the amygdala as they all are connected to the neural circuit of the hippocampus and would impair cognitive aspects of learning and memory. Further studies are also required to elucidate the energy field claimed to be generated under the geometric shape of the pyramid.

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