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EFFECT OF RESTRAINT STRESS DURING PREWEANING PERIOD ON THE DEVELOPMENT OF NEURONS OF SUBSTANTIA NIGRA IN ALBINO MICE

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ABSTRACT

Brain is highly dynamic organ and plastic structure. It can be affected by stress, toxins and malnutrition. This study was conducted to see the effect of restraint stress during preweaning period on the development of neurons of substantia nigra.In the present experiment on new born pups of albino mice of BALB/C strain were restrained for 6 hours daily from birth till postnatal day 21 (21 days stress) and from postnatal day 16 to 21 day (5 day stress) in a restraining device, which consists of a wooden platform to which a wire-mesh was attached. At the postnatal day 22 (P22) these pups were sacrificed. Substantia nigra was dissected out and processed for Golgi staining.Well stained nigral neurons were quantified by concentric circle method, data was analyzed by unpaired 't' test.both in control and experimental groups. In mice subjected to 21 days restraint stress throughout preweaning age resulted in significant decrease in dendritic intersections in all the concentric circles, dendritic branching points at 1^{st} (0-20µ), 2^{nd} (20-40µ), 3^{rd} (40-60µ) and 4^{th} (60-80µ) concentric zones. It also induced gastric ulcers and there was increase in wet weight of suprarenal gland. However, 5 days of stress during postnatal day 16^{th} to 21^{st} (P_{16}-P_{21}) did not affect any of the parameters, except increase in suprarenal weight.Stress from day of birth has more effect on the development of nigral neurons than during later part. Altered dendritic arborization may affect the afferent projections on these neurons and subsequently on the normal functioning of the neurons.

Keywords

Restraint Stress, Neurodegeneration, Substantia nigra, Albino Mice, Dendritic morphology.

1. INTRODUCTION

Stress is a highly individualized response of an organism to external or internal challenges which individual cannot control or can control with difficulty. Any stimulus that displaces the state of normal physiological function can cause "stress". Brief early life experiences, pre or postnatal can cause significant changes in the stress response system and emotionality that persist into adulthood (e.g. susceptibility to disease, Barker, 1996; depression, Phillips, 2002; anxiety-type behavior, Heim and Nemeroff, 2001).The current interest in early life experience stems from the observation that specific early events can apparently programme the "set-point'' of the hypothalamic–pituitary–adrenal (HPA) axis by

GLOBAL JOURNAL OF ADVANCED RESEARCH
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altering the effective feedback.Ganong (1995) defines " stress" as those stimuli which increases ACTH is an intricate chain which begins with the inputs at the higher centre, that triggers the hypothalamus. In response to stressfull stimuli hypothalamus releases corticotropin releasing hormone (CRH) to stimulate the pituitary, which inturn releases ACTH. Thus, adrenal cortex is stimulated for release of cortisol and androgen precursors. ACTH and cortisol are secreted in episodic manner in response to stress.

Observations at birth and thereafter, day by day progress in growth of body, head and tail in the postnatal life of man and animals are amazing experience. Enormous studies on birth and growth phenomenon in the postnatal life of animals have been compiled over the years (Bruno et al 1990). However, most of the investigators followed the traditional cross sectional method of study in which groups of animals generally mice or rats were sacrificed periodically (Tanner, 1955). Different strains of laboratory rats have been used for various types of investigations (Asling and Frank, 1963; Harel, 1995).

Over the decades, many longitudinal studies were reported, e.g. Saxton and Silberg (1947) and Williams et al (1974). Any such studies were related to development and growth (Morgan and Naismith, 1982).Specifically related with postnatal development and growth, some researchers have shown proclivity to study certain skeletal elements (Groot, 1963; Harkness and Trotter, 1980). Growth and development manifest by increment in size of the different parts of the body like head length, body length and tail length (Hughes, 1970). In the present study two parameters were considered for investigation, (i) growth of normal albino mice, (ii) growth of albino mice reared under stress. As far as the stress is concerned restraint stress was inflicted.

Restraint stress has been proposed as an animal model of depression and anorexia nervosa, as many investigators have shown that stress suppresses food intake and body weight gain in rats (Ganong WF 1995). The stress-induced reduction in food intake has been demonstrated both as maintained decrease in 24-hr food intake during and after repeated daily restraint stress, (Ganong WF 1995) and as an acute response in stress has ended, restraint rats fail to return to the body weight of control animals (Ganong WF 1995 and Kratin DD et al 1990). In the rat, repeated separation from the mother for 3 hrs. has been shown to have potentially deleterious effects like reduced hippocampal glucocorticoid receptors (GR), elevated basal plasma glucocorticoids (Plotsky and Meaney, 1993) and became hyper responsive to stressors during behavioral development (Ladd et al 2000). There are reports that maternal separation of 3 hrs. can lead to adult rats having reduced cognitive performance (Laddet al 2000). The mechanism underlying this hyper-responsiveness appears to be a reduction in the negative feedback to the HPA axis provided by the hippocampal brain region. Separated rat pups have a reduced density of glucocorticoid receptors in the hippocampus and consequently reduced capacity to inhibit the responsiveness of the HPA axis. In contrast, rat pups exposed to a positive post-natal experience (i.e. born to mothers that show higher levels of maternal grooming) show positive effects of this experience. Pups born to more maternal mothers have increased hippocampal GR expression and reduced hypothalamic corticotropin-releasing hormone messenger ribo-nucleic acid (CRH mRNA) expression, suggesting that these pups had a less responsive HPA axis (Liu et al 1997). These pups also showed changes in the neural circuitry controlling fear behavior (Caldji et al 1998), suggesting that they should be less behaviorally and physiologically responsive to fear and stress-inducing stimuli. Other work where pups were removed from the mother for a short period (15 min) and then returned (a procedure now known to cause an increase in maternal grooming (Macri et al., 2003)), resulted in pups which were more easily handled, and showed reduced ACTH and glucocorticoid responses to an open-field test (Nunez et al 1996),the parameters are precise, but extensive, literature analyzed were substantial (Amarillo et al 1985a and b; Alario et al 1987; Hennessy et al 1989; Avishai-Ephner et al 1995). Thus the present work consists of neuronal degeneration in stressed and normal albino mice observed from birth to 3 weeks postnatal age (21 days stress) and from postnatal day 16 to postnatal day 21 (5 days stress). The study was conducted in normal health of growing albino rats and stress like restraint stress. To the best of our knowledge and endeavor we could not come across any single study which describes the effect of chronic restraint stress on albino rats in during preweaning age.

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2. MATERIALS AND METHODS

A Animals

In the present study albino mice of the the BALB/C strain of both sexes were used. Control and experimental of both sexes consisting of twelve albino mice in each group were formed randomly.The mice were maintained in the institutional animal house at Manipal University.

B Environment

The mice were maintained in well-ventilated room. Temperature ranged between and 27± 3^0C.Doors and windows were closed during morning, evening and night hour to prevent them from colds. They were kept in natural source of light which was12:12 hour L: D cycle. Size of the cages for housing the mice were 40 X 25 X 16cm. Cages contained paddy husk (Sterilized), which were changed on every 2nd day.

C Diet Regimen

The animals were fed with synthetic food pellets and tap water adlibitum. The food pellets contained mainly wheat and 22.05 crude proteins, 3.99% crude oil, 2.62% crude fiber, 1.34% sand silica and 7.81% ash. Drinking water was acidified with hydrochloric acid to give a pH of 2.0 -2.5 this was achieved by adding 2 ml of hydrochloric acid to 3 liters of tap water. The purpose of adding acid to water is to prevent massive bacterial proliferation in the water bottle.

D Stress Regimen

The new born pups (P_0) were divided into two subgroups (a) Control (C) (b) Restraint stress (RS)

Control group:

New born pups in this group remained undisturbed with their mothers till the postnatal day 21.

(b) Restraint stress group (RS): Pups in this group were grouped into two sub groups

(i) 5 days stress group: Pups were stressed in a wire mesh restrainer, for 5 days (6h/day) from P_{16}-P_{21} (postnatal day 16-21) in restrainer no 3 having dimensions-4.8 cm (L) x 2.2cm (B) x 2.4cm (H).

(ii) 21 days stress group: Pups in this subgroup were stressed 6h/day for 21 days in the restrainer No-1 (P_0-P_7) having dimensions (2.5cm (L) x1.4cm (B) X 1.1cm (H), (**Fig.1**) restrainer No-2 (P_8-P_{14}) having dimensions (3.5cm (L) X1.6cm (B) x 1.2cm (H) Fig.1 and restrainer No-3 (P_{15}-P_{21}) having dimensions (4.8 cm (L) x2.2cm (B) x 2.4cm (H). (**Fig.2)**

In both 21 and 5 days restraint stress the mice remained within the restrainer for 6hrs on each day (10.00 A.M-4.00 P.M) The restrainer with mice was kept on a table in a room with sufficient ventilation. After 6hrs of stress, the pups remained with their mother. Control mice pups remained unseparated from the mothers. The food and water were withdrawn from experimental group during stress presentation. After stress presentation mice were returned to their respective cages.

On postnatal day 22, all the pups were anesthetized and substantia nigra obtained from them were processed for Golgi staining

E Data Collection

a. Dendritic morphology-Dendritic intersections and dendritic branching points

b.Wet weight of suprarenal gland

F Data presentation

The data has been tabulated as mean and standard deviation. Unpaired ‘t’ test was used for comparison of data between two groups and a ‘p’ value less than 0.05 was considered as significant.

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3. RESULTS

Results were made on the observations of growths of normal and stressed mices on dendritic morphology, suprarenal weight and presence of gastric ulcers in mucosa

a. Dendritic morphology

i. Dendritic intersections (Fig.3,5)

There was no change in the dendritic intersections at any of the concentric circles in the 5 days restraint stressed mice. In contrast in 21 days restraint stressed mice dendritic intersections are found significantly reduced in all the concentric circles (20µ concentric circle: 4.2 ± 0.75 in control vs 2.67 ± 1.37, in RS P<0.001;40µ concentric circle: 4.1 ± 0.89 in control vs 2.64 ± 0.53, in RS P<0.001;60µ concentric circle: 3.3 ± 0.13 in control vs 2.33 ± 0.66, in RS P<0.001;80µ concentric circle: 2.1 ± 0.74 in control vs 1.14 ± 0.58, in RS P<0.001;100µ concentric circle: 1.2 ± 0.58 in control vs 0.56 ± 0.46, in RS P<0.001.

ii. Dendritic branching points at different concentric zones (Fig3,4)

Like the dendritic intersections no significant change was observed in the dendritic branching points at any concentric zones in 5 days restraint stressed mice group. But in 21 days restraint stressed mice dendritic branching points are significantly decreased in all the concentric zones except the 5th zone (80-100µ). (Concentric zone-1 (0-20µ): 0.90 ± 0.35 in control vs 0.60 ±0.31 in RS, P< 0.01;Zone-2 (20-40µ): 1.5 ± 0.34 in control vs 1.05 ± 0.48 in RS, P< 0.05;Zone-3 (40-60µ): 0.81 ± 0.26 in control vs 0.54 ±0.38 in RS, P< 0.001;Zone-4 (60-80µ): 0.34 ± 0.17 in control vs 0.19 ± 0.12 in RS, P< 0.01.

b. Suprarenal weight: Fig 6

Suprarenal weight was found significantly increased both in 5 days (2.45 ±0.69 mg in control vs 3.57 ± 0.31 in 5 days RS, P<0,001) and in 21 days restraint stressed mice (2.45 ± 0.69 mg in control vs 4.07± 0.51 mg in 21 days RS, P<0.001).

c. Gastric ulcers (Fig.7)

Gastric mucosa of 21 days stressed mice had numerous gastric ulcers which were not observed in 5 days restraint stressed group.

4. DISCUSSION

In life of an animal and man development and growth are almost symphonetic. Studies on rats often begin from before birth i.e. prenatal development and followed to be observed in the postnatal life. Altogether it makes voluminous literature and at the same time makes it beyond comprehension of the researcher. Therefore this work was limited to postnatal period of albino rats life from day zero to day 21 (3 weeks). It was well recognized for many years that the size of the litter is an important determinant of overall growth of the rats (Gates, 1925). Hughes and Tanner (1970) reported that somatic growth of the laboratory rat is sensitive to litter size. It is directly linked to demand and supply of milk. The more pups in the litter, the lesser milk available for the individual pups, resulting in slower growth rate. In the following year, Park and Nowsieiski (1971) emphasized the importance of genetic pattern and maternal environmental factors. Quality and quantity of food provided to the laboratory animal at any stage has important significance for growth of the animal (Moss, 1954; Morgan, 1982; Lews, 1989). studies, Mukerjee (1987) and Shaligram (1998) they maintained only five pups kept with a healthy dame. It was common to observe that late in third week postnatal the young rat pups a started to nibble the solid food provided to the dame; this can be taken as additional nutrient supplement for the young rat pups. Generally the rats are ominivorous, they prefer fresh food. They instinctively store excess food by covering them under the paddy husk provided on the floor of the cage. However, unless starvations is threatened, rats avoid stale food and water (Lane-Petter, 1976). Chronic exposure to stressors of certain severity cause anorexia and reduce body weight (Marti et al. 1994). Chang J et al., in their study observed that chronic stresses like maternal deprivation, restraint stress and electrical foot shock reduce food intake, increase water consumption and cause reduced physical activity by causing

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longer period of sleep. Rats in the stress group develop fear which is noticed by mere handling before stress presentation. Handling cause more urination and excrement.

A study on Restraint-induced structural changes in the rat brain in which 6 hrs of restraint stress daily for 21 days caused marked morphological alterations in the medial prefrontal cortex (Meng-Yang Zhu1 et al., 2009)

Another study revealed chronic stress impaired hippocampal neurogenesis in mice in terms of cell proliferation; apoptosis; the number and maturation of young neurons; and both the volume and neuronal density in the granular zone (Estela Castilla-Ortega et al 2011)

A study on prefrontal cortex the total number of the neurons and glial cells was significantly reduced (11%a nd 5%, respectively) in stress (distilled water or olive oil) group in comparison to the non-stressed rats (Ali Noorafshan et al., 2014)

Another study observed chronic stress sensitizes midbrain microglia, which enhances microglia activation and exacerbates death of nigral dopaminergic neurons to further inflammatory stimulus. (Rocio et al., 2014)

Acute stress increases the expression of growth factors—substances that stimulate cellular growth and proliferation—which would seem to suggest that stress could enhance neurogenesis (Elizabeth D Kirby et al., 2013)

Babu et al., 2012 reported in their study reduction in body weight gain, tail length gain and body length gain in mice undergone 21 days stress from birth onwards. Eye opening was also delayed in the above age group. Similar results were reported by Pullen A.H. (1977); Drago F et al., (1999); Smagin GN et al., (1999) Nagaraja HS and Jegannathan PS (1999) and Santos J et al., (2000). Retarded body growth may be due to (i) decreased food intake (ii) movement of corticotropin releasing factor (CRF) in the hypothalamic region (Delbende C et al., 1992). CRF receptors in the hypothalamus mediate acute response to stress that can lead to permanent changes in hormonal or metabolic processes that determine the body weight and composition (Smagin GN et al., 1999). Pathways projecting from limbic areas to hypothalamus could stimulate CRF secretion into pituitary adrenal axis. Increased CRF could mediate this stress induced suppression of food intake thereby reducing body weight (Delbende C et al., 1992) and also may reduce other growth parameters. It was reported that growth hormone gene expression in the brain, significantly suppressed by exposure to restraint stress (Yashizato Het al., 1998) which may also cause growth retardation. Circulating growth hormone level is shown to be decreased in animals exposed to stress during neonatal period (Kuhn CM et al., 1978).

Tail length of the rat which at birth is shorter than the body length, increases rapidly due to addition of the newer vertebra and the intervertebral discs by formation of sclerotomic and notochordal elements. In absence of substantial literature of the development of the vertebrae of albino rat knowledge of the development of vertebrae and the intervertebral discs in humans (Muller and O'Rahilly 1986; O'Rarahilly et al 1990) would explain the sequence of growth in the linear increment of the body size of Wistar rats.

5. CONCLUSION

In conclusion we have observed that restraint stress throughout preweaning age resulted in significant decrease in dendritic intersections in all the concentric circles, dendritic branching points at at 1st (0-20µ), 2nd (20-40µ), 3rd (40-60µ) and 4th (60-80µ) concentric zones. It also induced gastric ulcers and there was increase in weight of suprarenal gland. However, the 5 days of stress during postnatal day 16th to 21 (P_{16}-P_{21}) did not affect any of the parameters, except increase in suprarenal weight.

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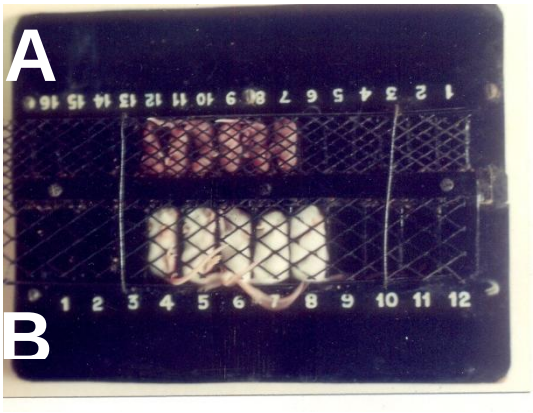

Fig. No:1
Restrainers with mice

A. Restrainer No.1 used to stress mice below 7 days of age
B. Restrainer No.2 used to stress the mice between 8 and 14 days of age

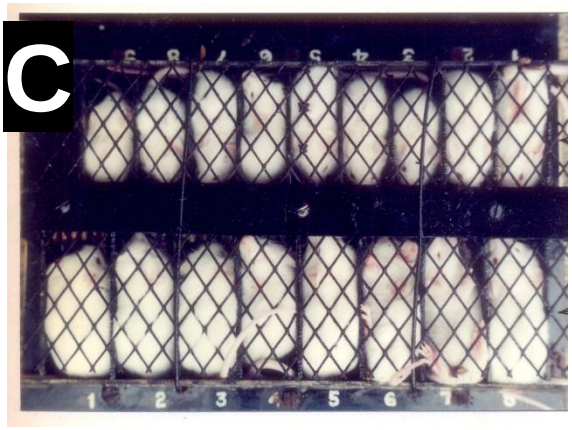

Fig.No 2
Restrainer with mice
C.Restrainer used to stress the mice between 15 and 30 days

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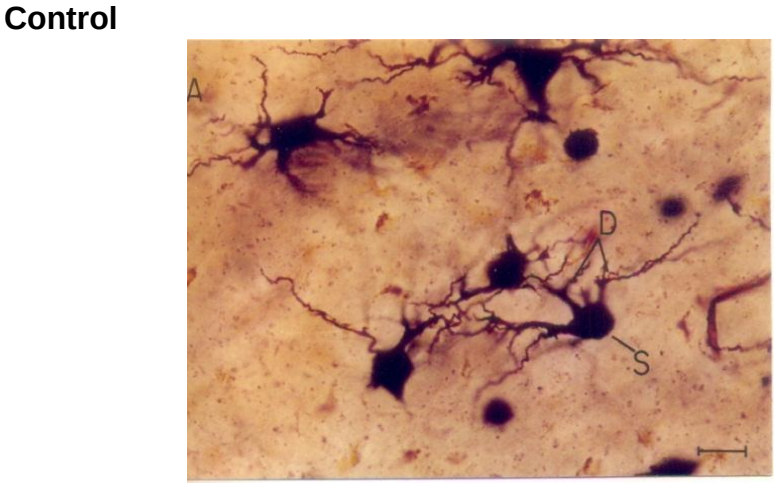

Fig.3: Photomicrographs of substantia nigra neurons 21 days restraint stressed mice (from birth onwards) along with 21 days old control mice. Note significant decrease in the dendritic arborization in stressed mice (B) compared to control mice (A).

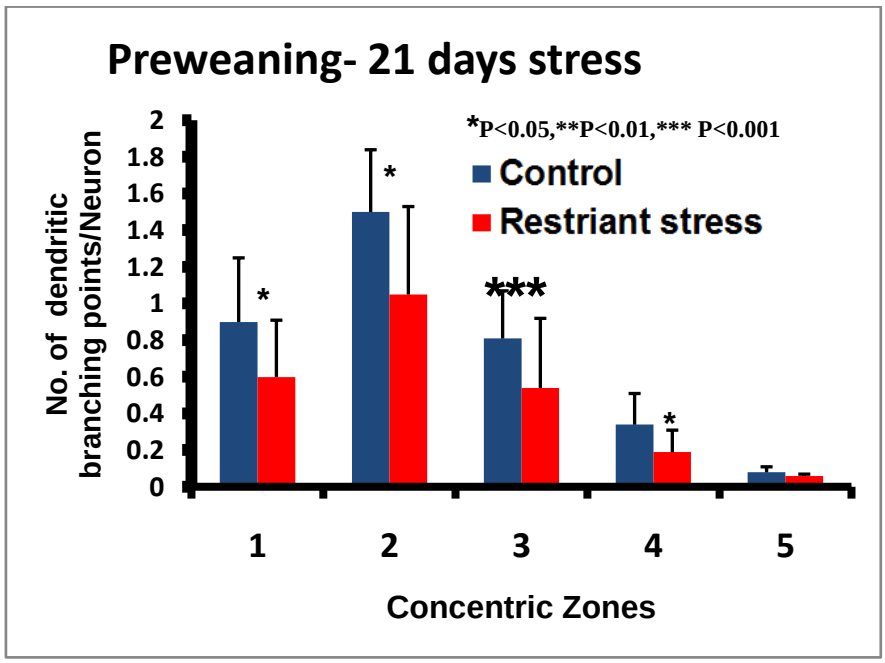

Fig.4: Dendritic branching points of substantia nigra neurons in mice stressed for 21 days (from birth onwards-Postnatal day (0)-P0-P21). Note significant decrease in branching points in mice stressed for 21 days at 0-20µ,20-40µ,40-60µ,60-80µ concentric zones. C-Control (n=6), RS-restraint stressed (n =6)

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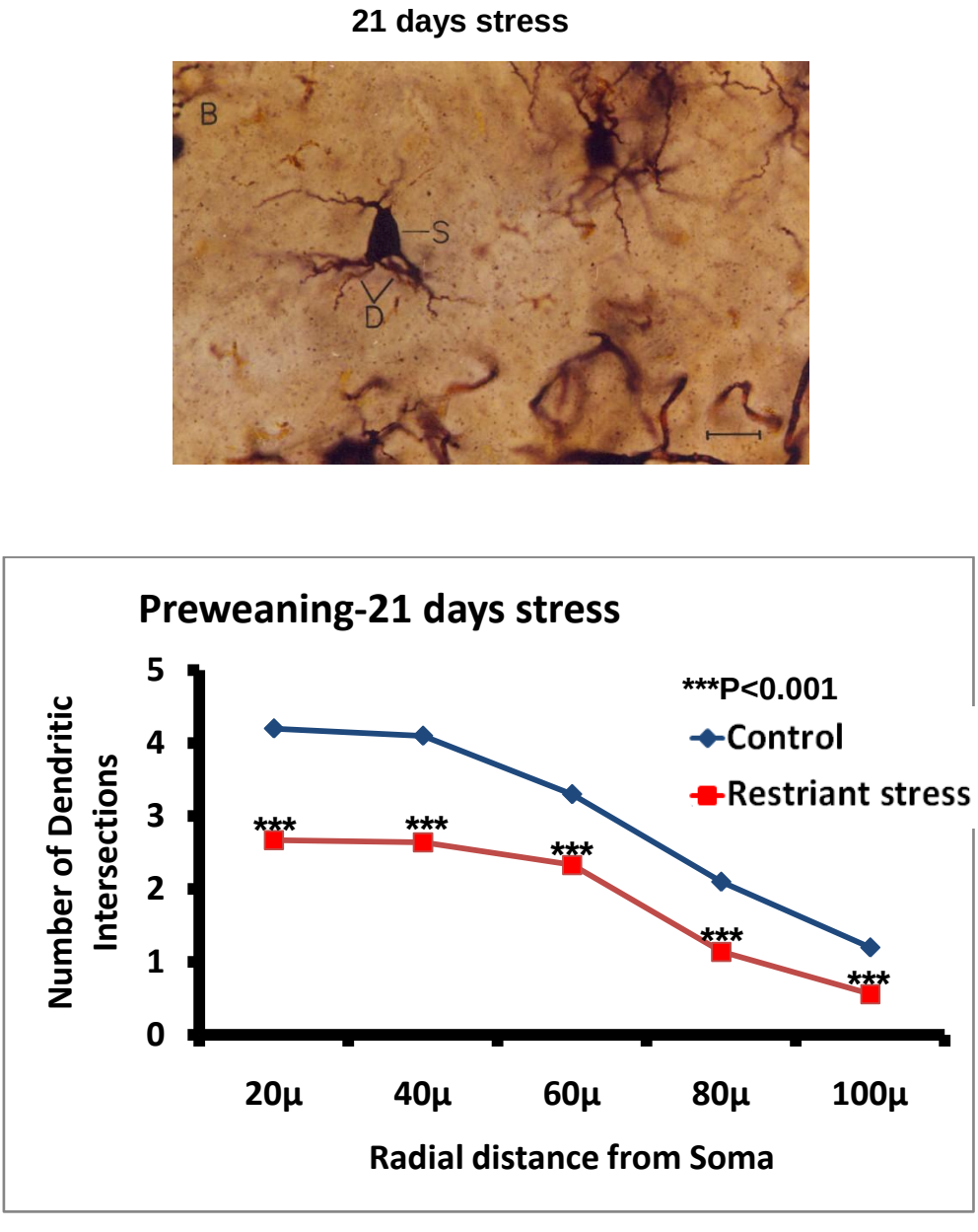

Fig.5: No. of dendritic intersections of substantia nigra neurons in mice stressed for 21 days (from birth onwards-- Postnatal day (P) $-P_0$-P_{21}). Note significant decrease in dendritic intersections in stressed mice at 20µ,40µ,60µ,80µ and 100µ concentric circles. C-control (n=6), RS-restraint stressed (n=6).

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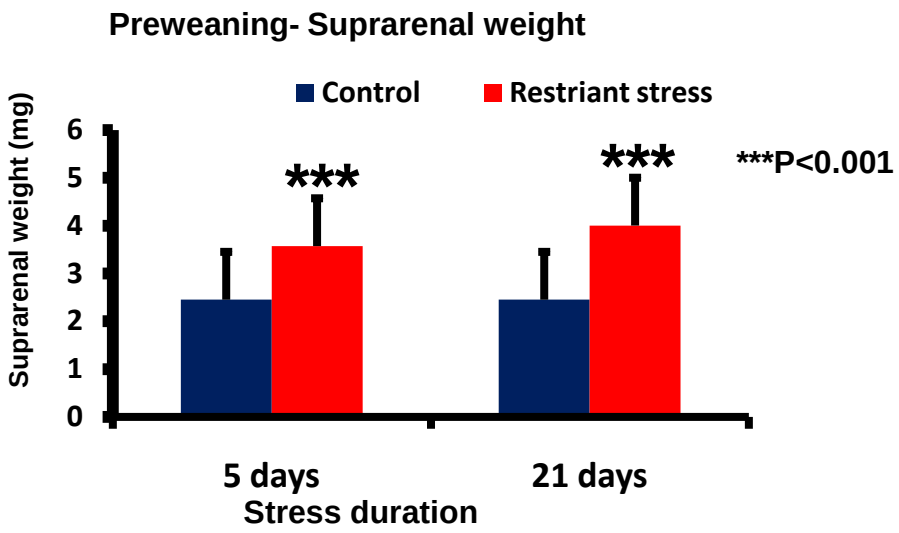

Fig.6: Suprarenal gland weight in preweaning group stressed for 5 and 21
Note the significant increase in the suprarenal gland weight in both 5 and 21days stressed mice.C–Control (n=6), RS-restraint stressed (n=6).

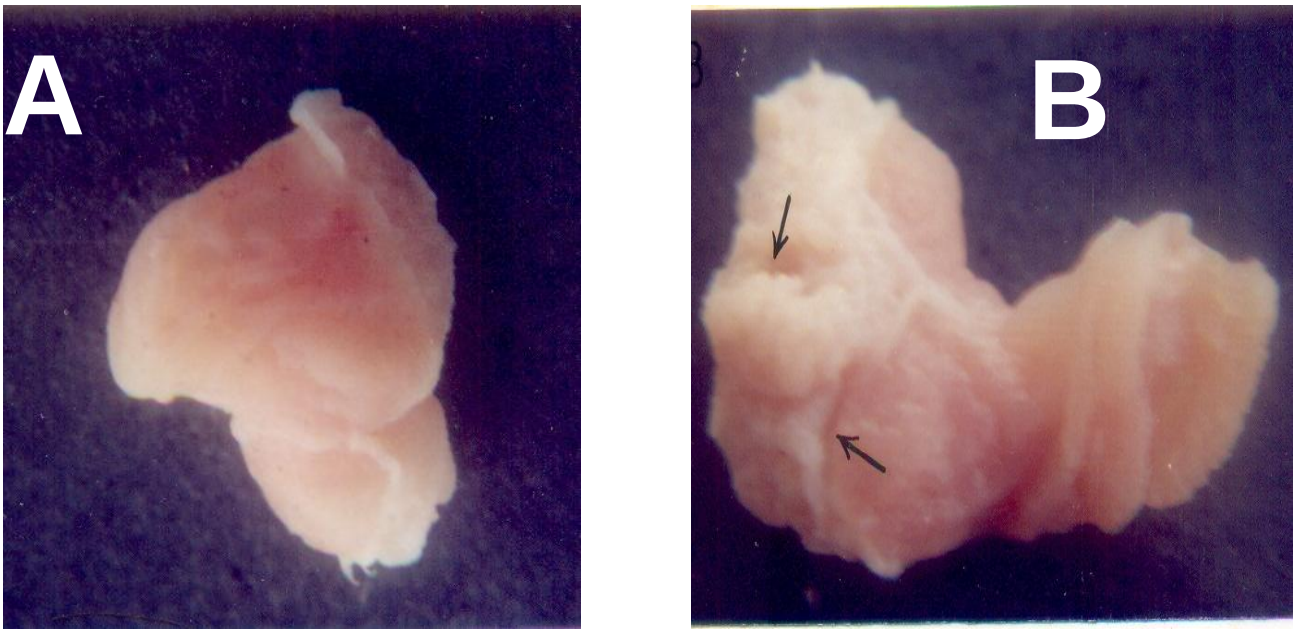

Fig.7: Photomicrographs of gastric mucosa of 21 days old control (A) and 21 days restraint stressed (from birth onwards, B) mice. Note the presence of gastric ulcers (arrows) in B.