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Original Article

Anatomy Section

Effects of Immobilised Stress on Neuronal Plasticity of Pyramidal Neurons in Prefrontal Cortex of Female Wistar Rats- An Experimental Study

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ABSTRACT

Introduction: Stress is a normal response of an organism to any threat which can be physical, emotional or psychological. Positive stress is called eustress whereas negative stress is distress. Neuronal plasticity is a term which indicates the changes in brain. There can be structural and functional neuroplasticity.

Aim: To study the neuronal plasticity (dendritic branching pattern and their lengths) of pyramidal neurons in Prefrontal Cortex (PFC) of female wistar rats after immobilised stress.

Materials and Methods: It was an experimental study conducted in Nepalese Army Institute of Health Sciences, Kathmandu, Nepal, from May 2021 to July 2022. Based on the age, 24 female rats were divided into three groups; group A more than 12 weeks, group B 8 to 12 weeks and group C 3 to 7 weeks. Each group contained eight rats, of which, four were control and four were experimental. Experimental rats underwent immobilised stress. After stress protocol, rats were sacrificed and PFC was taken out and stained. The images of neurons were taken by confocal microscope. The neuronal reconstruction was done using Interactive Microscopy Image Analysis (IMARIS) software for dendritic lengths. The parameters in apical dendrite category-length of principal dendrite, branch points and lengths of different branches were compared between control and stressed groups. In basal dendrite category-number of basal dendrites, lengths of different basal dendrites and branch points were compared between control and stressed groups using unpaired t-test. The data was analysed with Statistical Package for Social Sciences (SPSS) version 20.0.

Results: It was observed that there was a statistically significant increase in all basal dendrite parameters in experimental category as compared with controls in group A with p-value <0.001, and in group B, significant increase in branch points with a p-value <0.001 in experimental category, and significant increase in branch length of basal dendrites in group C of experimental rats with p-value <0.001. The parameters related to apical dendrites of pyramidal neurons also showed significant shrinkage in experimental rats as compared to control groups. Especially in group C, all the apical dendrite parameters were significantly decreased compared with control group (p-values 0.013, 0.001 and 0.006 for principal dendrite's length, branch points and branch lengths, respectively).

Conclusion: Stress induces neuronal plasticity in pyramidal neurons of PFC of rats. Findings of this study suggest stress induces significant increase in the basal dendrite parameters of pyramidal neurons of PFC of all age group of rats, especially in the rats of more than 12 weeks age and significant shrinkage of apical dendrites was seen in all age group of rats and more so in the rats between 3 to 7 weeks of age.

Keywords: Brain plasticity, Dendrites, Projection neurons

INTRODUCTION

Stress, in the context of biology is a normal response of an organism to any threat which can be physical, emotional or psychological. This ability to stand a stress is vital for survival [1]. The things which cause stress are known as stressors. Stressors can be external (environmental) or internal (illness or induced by different interventions) [2]. All the types of stresses may not have negative effect. It depends upon tolerance of body to stress and overcome laziness to improve performance, hence stress can be healthy, challenging and positive and termed as eustress. Stress becomes negative when the quantity of it exceeds the ability to cope, the body systems get fatigued resulting in behavioural and physical problems. This stress can be termed as distress [2].

Stress has been shown to produce long-term effects in cognitive-emotional response in rats as neonatal experiences. For example, the rats raised by nurturing mothers have higher levels of serotonin activity as compared to rats raised without nurturing mothers [3]. Stress induces series of changes in the cardiovascular, nervous, immune and endocrine system.

Neuronal plasticity is a term which indicates changes in brain. Brain is not "hard wired" with fixed neuronal circuits. Structural neuroplasticity includes synaptic plasticity, synaptogenesis, neurogenesis and changes in different parts of neuron etc., and formation of new neurons is called neurogenesis [4]. Stress has effects on dendritic morphology, spine density and synapse numbers in different brain areas. The major areas of brain affected by stress are hippocampus, amygdala and the PFC etc., [5]. In humans and in primates, neuroscientists use granular and orbital part of frontal cortex as PFC. For rodents, research use the term PFC to refer the same area which is called as Anterior Cingulate Cortex (ACC) in primates [6]. Rats do not have frontal granular cortex. PFC includes areas along the anterior medial wall of cerebral hemisphere [7].

In this research, authors have included pyramidal neurons of subregions of PFC, which are medial frontal cortex (mPFC) and Orbitofrontal Cortex (OFC) (orbital and insular areas) [5]. In pyramidal neurons of the cortex, apical dendrites are long which extends from apex of the cell body (soma). The longest and thickest apical dendrite is considered as principal dendrite. The points from where other branches take origin from principal dendrite are called branch points. Other short dendrites extended from cell body (soma) are considered as basal dendrites. The extra branches taking origin from any of the basal dendrite were also considered as branch points [8].

The pyramidal neurons of OFC show increase in dendritic length and spine density due to stress in contrast to changes in mPFC.

The effects of stress on pyramidal neurons of mPFC are different for male and female. Female do not show a general shortening of dendrites as compared to male because of effects of estradiol [7]. Chronic stress decreases lengths of apical dendrites and branch numbers whereas it does not alter basilar dendrite's lengths and branch numbers [5,9]. Due to the above mentioned reasons, the primary focus of this research was to see the stress related changes in pyramidal neurons of PFC of female Wistar rats. The Wistar rats are the best choice for this research because they are easy to keep, breed easily, respond to alterations in environment and can be easily trained. They are also used in neuroscience research because they did not get easily habituated [10]. Hence, the main aim of this study was to find out the effects of immobilised stress on neuronal plasticity of (dendritic branching pattern and their lengths) of pyramidal neurons in PFC of female Wistar rats and to compare with the control group.

MATERIALS AND METHODS

This was an experimental study carried out at Nepalese Army Institute of Health Sciences, Kathmandu, Nepal from May 2021 to July 2022, after obtaining Institutional Review Committee clearance (IRC) from the institution (IRC reg No 425 April 2021). Total 24 female Wistar rats of different age groups were bought from Department of Plant Resources, Thapathali, Kathmandu at different times according to requirement. All 24 rats were divided into three groups as per age of the rats;

- Group A- More than 12 weeks
- Group B- 8-12 weeks
- Group C- 3-7 weeks

Every group was subdivided in to experimental and control rats consisting of four rats each.

Sample size calculation: The sample size was calculated based on degree of freedom (E), with formula (E=Total number of animals-Total number of groups). As per this formula, if the E Value is 20 and above then the sample size is more than adequate. In the present study, the E value is 21 that is (E=total number of animals 24-Total number of groups 3=21) [11].

Separate cages were arranged for control and experimental group rats. Food (Boiled Bengal grams, boiled wheat, lettuce, vitamin A powder) and water provided ad libitum. Day/night cycles of 12 hours were maintained [12]. The rats were kept for 3-4 days for acclimatisation before beginning of experiment [13]. The tails of the rats were coloured with waterproof colour markers. Red colour was used for control rats while blue colour was used for experimental rats.

Inclusion criteria: Healthy active female Wistar rats with different age groups as per the study protocol were included.

Exclusion criteria: Deceased/Died rats were excluded from the study.

Study Procedure

Stress protocol: Restriction of the freedom of locomotion and exploration, better known and referred to as restraint or immobilisation is a kind of deprivation paradigm, is probably the most widespread method of stress induction was used in this study [12].

Experimental rats of different groups were placed individually into transparent acrylic restrainers for 90 minutes everyday for a week followed by daily for two hour over a period of two weeks. In an attempt to avoid habituation caused by repetitive homotypic stress, rats were restrained at random times of day during the light-dark cycle (8 am-8 pm) as shown in [Table/Fig-1]. A corresponding control group was maintained. After stress protocol, rats were anaesthetised with Diethyl Ether $(C_2H_5)_2O$. Thoracic cavity was opened and through the left ventricle initially rats were perfused with 0.9% NaCl (normal saline) and fixed with 100-150 mL of fixative (4% paraformaldehyde) as shown in [Table/Fig-2] [14]. Cervical dislocation technique was used to prevent pre and postsynaptic effects of anaesthesia. A surgical scissor was used to remove head with a cut posterior from the ears. Brains were removed with the olfactory bulbs preserved as shown in [Table/Fig-3]. Olfactory bulbs were used as the landmarks for PFC [15,16].

Staining procedure: FD Rapid Golgi Stain™ Kit (Small) was used as the staining solution.

It consists of:

a. Solution A-125 mL
b. Solution B-125 mL
c. Solution C-125×2=250 mL
d. Solution D-125 mL
e. Solution E-125 mL

Forty-eight hours prior to sacrifice of rats, same volumes of solutions A and B were mixed and left without stirring in the dark chamber (impregnation solution). A 5 mL of impregnation solution per cubic cm of tissue was used. After brain removal, the PFC tissue was rinsed with doubled distilled water to remove blood stain. A 15 mL conical tube were labelled and filled with 5 mL of impregnation solution each. The brain tissues of all control and experimental rats of particular group were transferred to the conical tubes and left for two weeks at room temperature in the dark chamber. After two weeks, the tissues were transferred into solution C (same amount as impregnation solution) and left in dark at 4°C for one week, then the tissues were transferred into the new vial filled with water. The tissues were washed under running water over night and placed onto histo-cassette and labelled with unique identification number.

After this the tissues were processed in automatic tissue processor which included process of dehydration, clearing, infiltration and embedding, a support matrix was provided by processing and embedding the tissue in wax. The tissues were dehydrated using

[Table/Fig-1]: Rat inside the restrainer (immobilised stress). **[Table/Fig-2]:** Rat perfusion. **[Table/Fig-3]:** Rat brain after removal. (Images from left to right)

a series of progressive more concentrated ethanol bath. The following solutions were kept in sequence; 50%, 70%, 80%, and 90% and absolute alcohol (ethanol) twice. The tissues were kept in solutions for one hour each. For clearing, xylene was used. After different concentrations of ethanol, next containers included xylene. The tissues remained in both xylene container for one hour each. Next two container included molten paraffin wax and tissue remained for one hour in each. This process was performed by using automatic "electra" tissue processor (microprocessor based) manufactured by York Scientific Industries Pvt. Ltd.

After the tissues were thoroughly infiltered in wax, blocks were prepared using L-moulds. All the blocks were labelled and sectioned (coronal) at 50 µm on a semi-automatic rotatory microtome. The sections were made to float in distilled water at 10^{0}C and then the sections were picked up on Superfrost plus microscope slides. The slides were left in hot air oven at 60°C over night to improve adhesion. Sections were deparaffinised in xylene for three times and it remained in solutions for five minutes each. The sections were hydrated in decreasing concentrations of ethanol, 100%, 95%, 70% and 50% of ethanol twice, five minutes each. Sections were rinsed in distilled water and were kept in double distilled water two times, two minutes each. A solution consisting of one part of solution D, one part solution E and two parts of double distilled water was prepared. Sections were placed in this staining solution for 10 minutes. Sections were kept in double distilled water two times for four minutes each followed by this section and were dehydrated in progressively higher ethanol concentrations, 50%, 75% and 90% for four minutes each. The sections were continued for dehydration in 100% ethanol four times, four minutes each. Sections were cleared in xylene and mounted with Dibutylphthalate Polystyrene Xylene (DPX). The slides were dried at room temperature for 24-48 hours. Slides were protected from light [17].

The images of coronal section of rat brain after staining with Golgi stain were taken using Leica sp8 confocal microscope with 10x and 40x as shown in [Table/Fig-4-6]. These neurons had apical and basal dendrites [Table/Fig-7,8]. Photomultiplier Tubes (PMT) were used and sample was illuminated using a specific laser wavelength. Z-stacks were obtained so as to capture the whole length of the stained neuron. One neuron per image was ensured while imaging the samples. The image acquisition from confocal microscope and neuronal reconstruction from IMARIS software as mentioned below was performed in Indian Institute of Science Education and Research (IISER-PUNE).

Neuronal Reconstruction

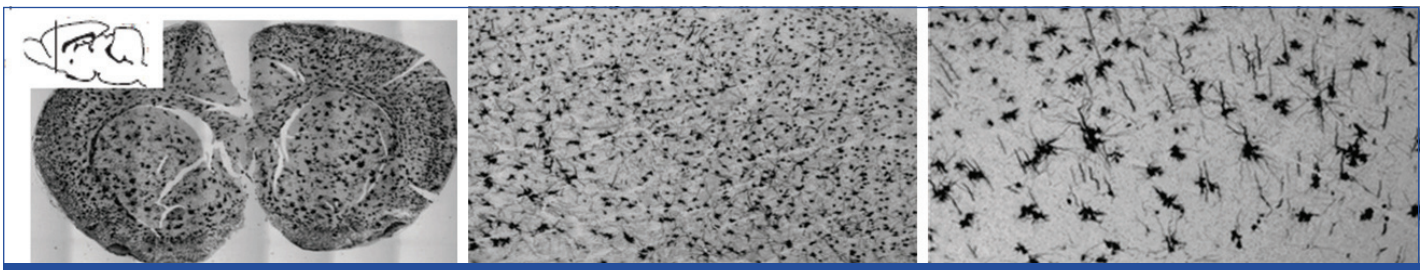
The Z-stacks obtained were opened using IMAGE J software. The Z-projects were selected and images were intensified by using maximum intensity. Then the images were imported to IMARIS software. Neurons were traced with the filament tracing wizard in IMARIS semi-automatically as shown in [Table/Fig-9]. From each group 32 (16 experimental and 16 control) neurons were reconstructed, so for three groups, total 96 neurons (48 experimental and 48 control) were reconstructed and apical and basal dendrite parameters were measured. The dendritic length was selected and obtained an excel file with branch names with their respective length in µm.

STATISTICAL ANALYSIS

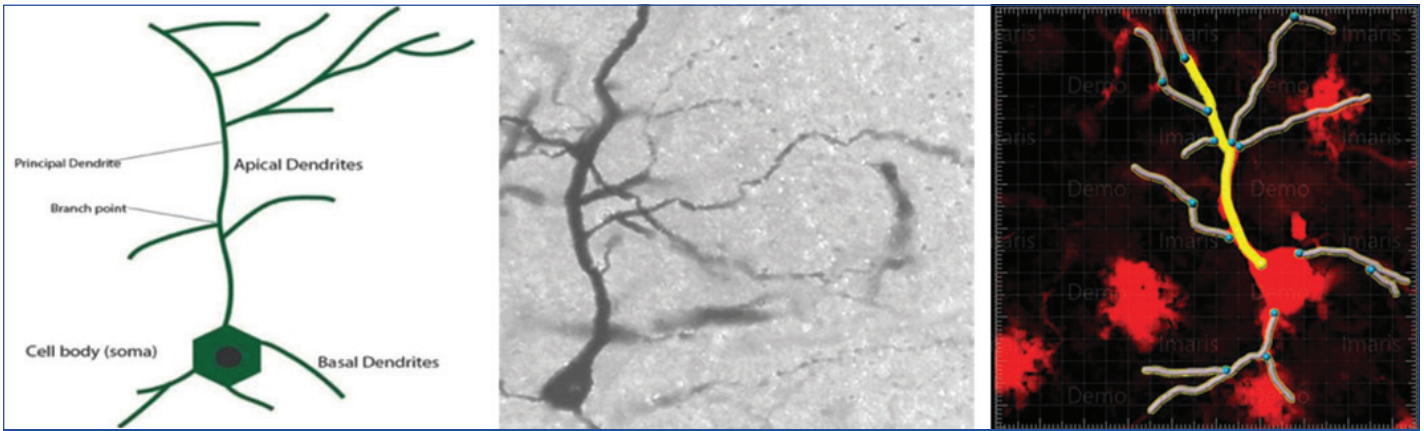
Unpaired t-test was used to compare the parameters of pyramidal neurons between experimental and control group of rats. In apical dendrite category, length of principal dendrite, branch points and lengths of different branches were compared between control and stressed groups. In basal dendrite category, number of basal dendrites, lengths of different basal dendrites and branch points were compared between control and stressed groups. The data was analysed with SPSS version 20.0. The p-value less than 0.05 was considered as statistically significant.

RESULTS

The apical and basal dendritic parameters after manual tracing in IMARIS software are presented in [Table/Fig-10-12] as per the groups A, B, C, respectively. In each table, control and stressed (experimental) groups were compared. The percentage changes were also calculated. From the [Table/Fig-10-12], it was observed that for basal dendrite parameters in group A there was statistically highly significant increase in all basal dendrite parameters in experimental category as compared with control group with p-value <0.001, in group B also there was significant increase in



[Table/Fig-4]: Coronal section of rat Prefrontal Cortex (PFC). **[Table/Fig-5]:** Prefrontal Cortex (PFC) neurons at low magnification (10x). **[Table/Fig-6]:** Prefrontal Cortex (PFC) neurons at high magnification (40×). (Images from left to right)



[Table/Fig-7]: Schematic-Pyramidal neuron with apical and basal dendrites. **[Table/Fig-8]:** Pyramidal neuron. **[Table/Fig-9]:** IMMARIS software pyramidal neuron dendritic length, branch points, branch lengths. (Images from left to right)

branch points (p-value <0.001) dendritic length (p-value 0.003) and numbers (p-value 0.014) of basal dendrites. However in group C, there was a significant increase in numbers of basal dendrite in experimental rats with (p-value <0.001) and branch points (p-value 0.025) except dendritic length which was not significant. It was also observed that there was shrinkage of apical dendrite parameters as compared with control group.

Pyramidal neurons	Control n=16	Stressed n=16	% Change	p-value
Apical dendrites				
Principal dendrite's length	117.83±11.57	102.58±4.88	-15	0.234
Branch points	3.06±0.37	2.93±0.30	-4	0.794
Branch lengths	35.55±2.76	24.83±2.01	-43	0.025
Basal dendrites				
Numbers	2.68±0.17	4.25±0.19	+37	<0.001
Dendritic lengths	19.05±1.22	33.84±2.45	+44	<0.001
Branch points	0.56±0.43	3.81±0.41	+85	<0.001

[Table/Fig-10]: Effects of immobilised stress on dendritic lengths (µm) and number of branch points on pyramidal neurons of Prefrontal Cortex (PFC) of group A rats.
p-value <0.05 considered significant

Pyramidal neurons	Control n=16	Stressed n=16	% Change	p-value
Apical dendrites				
Principal dendrite's length	113.62±9.46	99.76±5.74	-14	0.220
Branch points	4.62±0.44	2.62±0.39	-76	0.002
Branch lengths	41.82±3.15	31.23±3.61	-34	0.035
Basal dendrites				
Numbers	2.81±0.34	4.06±0.33	+44	0.014
Dendritic lengths	23.64±2.42	35.75±2.88	+51	0.003
Branch points	0.68±0.21	2.56±0.34	+276	<0.001

[Table/Fig-11]: Effects of immobilised stress on dendritic lengths (µm) and number of branch points on pyramidal neurons of Prefrontal Cortex (PFC) of group B rats.
p-value <0.05 considered significant

Pyramidal neurons	Control n=16	Stressed n=16	% Change	p-value
Apical dendrites				
Principal dendrite's length	111.37±4.54	92.70±5.38	-20	0.013
Branch points	3.87±0.48	1.62±0.32	-139	0.001
Branch lengths	36.85±2.75	23.33±3.64	-58	0.006
Basal dendrites				
Numbers	2.56±0.24	3.93±0.21	+54	<0.001
Dendritic lengths	27.46±3.07	33.26±2.09	+21	0.109
Branch points	1.25±0.39	2.81±0.53	+125	0.025

[Table/Fig-12]: Effects of immobilised stress on dendritic lengths (µm) and number of branch points on pyramidal neurons of Prefrontal Cortex (PFC) of group C rats.
p-value <0.05 considered significant

In group C all the apical dendrite parameters were significantly decreased compared with control group with (p-values 0.013, 0.001 and 0.006 for principal dendrite's length, branch points and branch lengths, respectively). In group B also apical branch points and branch length significantly decreased with p-values 0.002 and 0.035, respectively. In group A only, apical dendrite branch length was significantly decreased with p-value 0.025. Overall there was a significant increase in the all basal dendrite parameters in the rats of more than 12 (group A) weeks of age and significant decrease in all the apical dendrite parameters in the rats between the age group of 3 to 7 (group C) weeks.

DISCUSSION

The present study explored the effects of stress on pyramidal neurons (basal and apical dendrites) of PFC in female Wistar rats. Findings of the present study suggests that stress induces significant increase in the basal dendrite parameters of pyramidal neurons of PFC of all age group of rats, especially in the rats of more than 12 weeks age and significant shrinkage of apical dendrites was seen in all age group of rats and more so in the rats between 3 to 7 weeks of age. In this study, Wistar rats were restrained for 21 days. Same type of stress protocol was also used by McEwen BS and Gianaros PJ [18].

A study conducted by Brown SM et al., demonstrates pronounced changes in the dendritic morphology of pyramidal neurons in medial PFC as a result of a short duration, mild and chronic stress resulted in a selective remodelling of apical dendrites, with atrophy of upto 31% occurring in more distal branches and relative sparing of proximal branches and also mentioned longer-term, more severe stress produced a pronounced decrease in both branch number and branch length in the apical dendrites of pyramidal neurons in medial PFC [19]. In the present study, authors have also witnessed atrophy of apical dendrites and it was significant. Cook SC and Wellman CL, in their study mentioned three hours of daily restraint, resulting in average peak corticosterone titters of ~74 µg/dL, produced more pronounced decreases in distal apical arbor [9]. Cook SC and Wellman CL and Hauger RL et al., mentioned daily corticosterone administration, which typically produces average peak corticosterone titers of ~95 µg/dL produced similarly pronounced decreases in distal dendritic material accompanied by increases in dendritic material proximal to the soma [9,20]. So the changes which are taking place in the pyramidal neurons may be related to the corticosterone level which increase due to stress. Depending on the level of stress the corticosterone level increases and changes may occur in pyramidal neurons needs to be established.

In this study, authors did not check the corticosterone level in both experimental rats and control rats but it would be interesting to see correlation between level stress with corticosterone levels in the blood and the amount of changes in the pyramidal neurons. Cook SC and Wellman CL, in their study of chronic stress and its effect on dendritic morphology mentioned that, sholl analysis demonstrated a significant alteration of apical dendrites in stressed animals and overall, the number and length of apical dendritic branches was reduced by 18% and 32%, respectively. The reduction in apical dendritic arbor was restricted to distal and higher order branches, and may reflect atrophy of terminal branches: terminal branch number and length were also reduced by 19% and 35%. On the other hand, basilar dendrites were not affected [9]. Liston C et al., also mentioned repeated immobilised stress showed contrasting effects on apical dendrites and basal dendrites in PFC. It had been found that apical dendritic material (p-value=0.007) and branching (p-value=0.05) of medial frontal cortex were decreased by 20% and 11%, respectively whereas basal dendritic materials were not affected (p-value=0.69) [21]. The apical dendritic branches' length decreases by 20% and the decrement is more visible in distal apical dendritic branches after stress while basal dendritic lengths remain unchanged [9,22,23].

But in this study, apart from atrophy of apical dendrite, significant increase in the basal dendrites were also observed. Sex differences in the effects of stress on mPFC pyramidal neurons was observed by Garrett JE and and Wellman CL and they mentioned pyramidal neurons of mPFC were drawn in three dimensions, and morphology of apical and basilar arbors was quantified and concluded that in males, stress decreased apical dendritic branch number and length, whereas in females, stress increased apical dendritic length. So, finally they concluded stress induced increase in apical dendrites in female rats is oestradiol dependent [24]. Contrast to that in this study there was significant decrease in apical dendrites and significant increase in basal dendrites.

Pyramidal cell neuronal plasticity in the PFC was observed due to stress. It needs to be correlated with stress hormones in the blood. Also, the amount of stress correlation with level of stress hormones needs to be seen. Apart from level of stress, stress hormones and its effects on neurons (neuronal plasticity) the decision making behaviour needs to studied for better understanding.

Limitation(s)

As, the research was performed on female rats, the effects of stress on neuronal plasticity need to be established in male Wistar rats as well. The PFC was studied as a whole but not in specific areas of it.

CONCLUSION(S)

Stress induces neuronal plasticity in pyramidal neurons of PFC of female Wistar rats. Findings of this study suggest immobilised chronic stress induces significant increase in the basal dendrite parameters of pyramidal neurons of PFC of all age group of rats, especially in the rats of more than 12 weeks age and significant shrinkage of apical dendrites was seen in all age group of rats and more so in the rats between 3 to 7 weeks of age. The changes in pyramidal neurons need to be correlated with corticosterone levels in the stressed rats in further future studies.

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