ORIGINAL RESEARCH

Immobilised Stress Induces Structural Plasticity in pyramidal Neurons of Hippocampus in Female Wistar Rats

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ABSTRACT

Introduction: Anything which threatens homeostasis is called stress. Stress can be physical, psychological or emotional. Changes in brain is called neuronal plasticity which can be structural or functional. **Aim:** To study the dendritic branches and their lengths of pyramidal neurons in Hippocampus of wistar rats after immobilized stress. **Materials and Methods:** This experimental study was performed at College of medicine, NAIHS, Kathmandu, Nepal from August 2021 to February 2022. Sixteen female Wistar rats of different age group consisting both of control and experimental were chosen. They underwentimmobilized stress. At the end of stress protocol, rats were sacrificed and hippocampus was taken out and staining procedure were performed. Confocal microscope was used to obtain images of neurons. IMARIS software was for neuronal reconstruction. The different parameters of apical and basal dendrites of both control and experimental rats were compared using unpaired t-test. The data were compared with Microsoft Excel worksheet (Microsoft office professional plus 2013 version). **Results:** It was found that all the apical dendritic parameters (principle dendrite's length, branch points & branch length) in group A were shrunk and the changes were significant (<0.000217, <0.027854 &<0.001). It was also found in group A that all the basal dendritic parameters (number, branch length & branch point) were diminished too but the changes were not significant (<0.842873,<0.156947 &<0.180474).In group B, all apical dendritic parameters (principal dendrite's length, branch point & branch length) were shrunk too and the shrinkage was also significant (<0.007705, <0.000423 &<0.007811). All the basal parameters in group B have decreased except branch lengths but the differences were not significant (<0.743144,<0.364572 &<0.507876). **Conclusion:** Immobilized stress causes significant atrophy in apical dendrite parameters of pyramidal neurons while the changes of basal dendritic parameters were not significant in hippocampus of rats aged between 3 to 11 weeks.

Key Words: Stress, Hippocampus, Pyramidal Neurons, Structural Plasticity, Apical Dendrites, Basal Dendrites

INTRODUCTION

Stress can be defined as anything which threatens homeostasis. Stress are the actual or perceived threats to an animal. "Stress response" is the response to stressors by the animal.[1]The stressful experiences elicit a cascade of behavioral and physiological changes for the contribution of optimal coping by the animal with the situation.[2] Stress response include the release of stress hormones which are produced by sympathetic nervous system and hypothalamus-pituitary-adrenal cortex system (HPA axis).[1] The ability of neural circuits in the different parts of brain to alter through development and rearrangement is termed as Neuroplasticity. It is grouped into two broad categories i.e. structural and functional. The expansion or retraction of the synaptic area is structural plasticity which is achieved through the remodeling of spines, dendrites and axons. The regulation of the strength or efficiency of synaptic transmission and the reorganization of synaptic components and receptors are functional plasticity.[3]

Hippocampus, amygdala and prefrontal cortex are most plastic areas of brain.[4] The hippocampus is considered as the vulnerable region of brain because of its involvement with stress hormones.[5] The hippocampal formation includes three distinct areas; the dentate gyrus, hippocampus (hippocampal proper) which is further subdivided into 3 regions (CA1, CA2

& CA3) and the subiculum.[6] Pyramidal cells are the dominant neurons in the hippocampal proper and the subiculum. The size and density of pyramidal neurons are variable in the different regions of hippocampus proper.[7] Pyramidal neurons bears a long thick apical dendrite and many basal dendrites which emerge from the apex and base of the teardrop-shaped cell body. The orientation of apical and basal dendrite are in opposite directions.[8] In this study, principal dendrite is the longest and thickest apical dendrite and branches from it are considered as branch points. Any branches from basal dendrites are also considered as branch points. The Wistar rats are used because these rats cab be easily kept and bred. They respond to changes in environment and can be trained easily.[9] The principal aim of this research is to find out structural plasticity induced due to Immobilised stress.

MATERIALS AND METHODS

This experimental study was conducted at College of Medicine, NAIHS, Kathmandu, Nepal. Institutional Review Committee (IRC) clearance was obtained from the institution. (IRC Reg. No. 425 April 2021) This study was carried out from August 2021 to February 2022. Sixteen female Wistar rats of various age groups were procured from Department of Plant Resources, Thapathali, Kathmandu. These rats were grounded into 2 groups according to their age.

Group A: (7-11 weeks)

Group B: (3-6 weeks)

Both the groups were subdivided into stressed and control rats. (Four in each subgroups)

Sample Size Calculation: This method suggested the E value (degree of freedom) must be between 10 1nd 20 based on ANOVA calculations.

E = Total numbers of animals – total number of groups

= 16-2 = 14,if E is between 10-20 then sample size is considered enough. [10]

The stressed and control group of rats were kept in different cages. Food such as boiled wheat & Bengal grams, cabbage, lettuce and water provided ad libitum. Twelve hours of day/night cycles were maintained.[11] For acclimatization, rats were kept for 3-4 days prior to the commencement of stress protocol.[12] For identification, rat tails were marked.

Inclusion/Exclusion Criteria: Healthy female Wistar rats were included whereas dead of diseased rats were excluded.

Stress paradigm: In this study, Immobilised stress was used as restriction of the freedom of the locomotion and exploration worked as stress for rats.[11]

Stressed rats of both the groups were kept into transparent acrylic restrainer individually for 90 minutes at same time of day for 7 days followed by 2 hours daily at different times of day for 14 days (Fig 1). Control groups were also maintained.

Diethyl ether was used to anaesthetize the rats after the completion of stress protocol. Once the thoracic cavity was opened, the rats were perfused with 0.9% Nacl (fig 2) through left ventricle and fixed with 4% paraformaldehyde .[13] A cut posterior from the ears was performed to remove the head and brain was taken out (Fig 3).

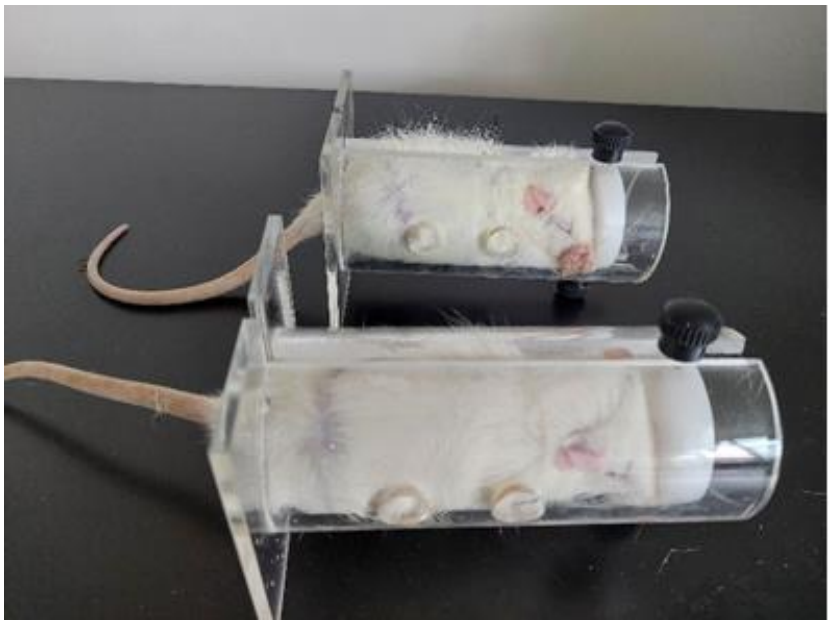

Figure 1: Rats in restrainer

Figure 1: Perfusion of rats

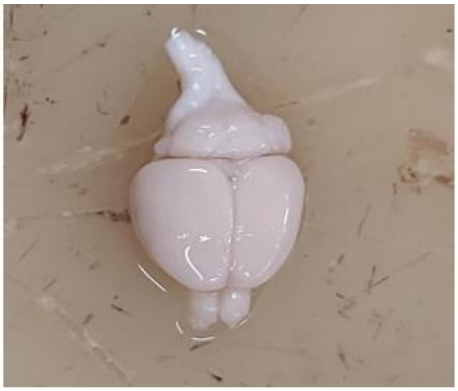

Figure 3: The Rat brain

STAINING PROCEDURE

The staining solution (FD Rapid Golgi Stain^{Tm} Kit) consisting of solution A (125 ML), solution B (125 ML), solution C (250 ML), solution D (125 ML) and solution E (125 ML) was used. Equal volumes of solution A & B were mixed 48 hours before the dissection of rats and left in dark chamber (impregnation solution). Five ML of impregnation solution per cubic cm of tissue was used. For removing the blood stain, the brain tissue were rinsed with distilled water. Eight 15 ML conical tubes were labelled and each tubes was filled with 5 ML of impregnation solution. The brain tissues of all stressed and control groups rats were kept into the above tubes and left in dark chamber for 2 weeks. At the end of 2 weeks, the brain tissue were kept into solution C and left in dark at 4^0 C for next 1 week. After processing in solution A, B & C, the tissues were kept under running water over night. The tissues were transferred into histo-cassette and unique identification number was labelled. Automatic tissue processor was used for processing the brain tissues which included dehydration, clearing, infiltration and embedding. A series of progressive concentrated ethanol (50%, 70%, 80%, 90% and absolute alcohol twice) were used to dehydrate the tissue. Xylene was used to clearing. Paraffin wax was used for embedding. The automatic "electra" tissue processor (microprocessor based) manufactured by York scientific industries Pvt. Ltd. was used. Blocked were prepared and labelled. Fifty µm sections (coronal) were cut on a semi-automatic rotatory microtome. The sections were made to float in distilled water at 10^0C lower than the melting point of wax and picked up on super-frost plus microscope slides. To improve the adhesion, the slides were kept in hot air oven at 60^0C overnight. Deparaffinization with xylene was done for three times. Hydration of the sections were done in decreasing concentrations of ethanol (100%, 95%, 70% and 50%) twice. The sections were rinsed in distilled water. A solution consisting of two parts of double distilled water, one part of solution D and one part of solution E was prepared. Sections were kept in the above solution for 10 minutes. The sections were kept in double distilled water again. Then the sections were dehydrated in increasing concentrations of ethanol (50%, 75%, 90% and 100%). Sections were cleared in xylene thrice and mounted with Dibutylphthalate polystyrene xylene (DPX). At room temperature, the slides were dried for 24-48 hours but protected from light.[14]

Leica Sp8 confocal microscope was used to take the images at 10x and 40x(Fig 4,5 &6). PMT(photomultiplier tubes) were used and samples were illuminated by a specific laser wavelength. Whole length of the stained neuron were captured by obtaining Z-stacks. While imaging the samples, one neuron per image was ensured (Fig 7). The image acquisition from confocal microscope and neuronal reconstruction by IMARIS software as mentioned below was performed in Indian Institute of Science Education and Research (IISER-PUNE).

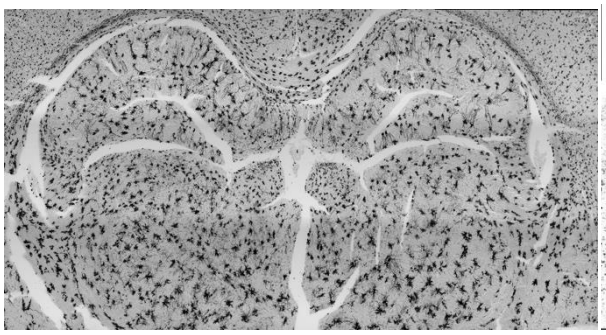

Figure 4: Hippocampus as a whole

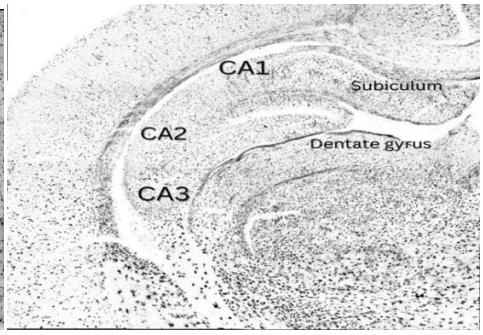

Figure 5: Parts of hippocampus

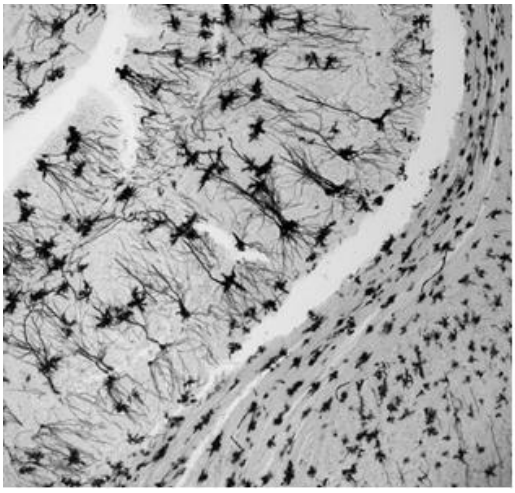

Figure 6: Neurons at 40X

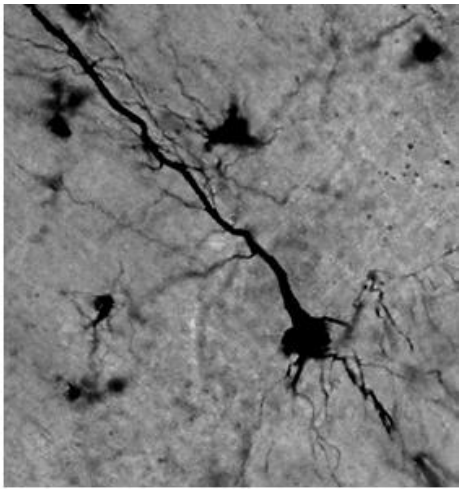

Figure 7: Pyramidal neuron

Neuronal Reconstruction: IMAGE J software was used to open the Z-stacks. In IMAGE J software, Z-projects were chosen and intensified by maximum intensity.

Images were imported to IMARIS software. Filament tracing wizard was used to trace neurons semi-automatically (Fig 8). For each rat (stressed or control), 5 neurons were reconstructed so total 80 neurons were reconstructed. The apical and basal dendritic parameters were measured. The dendritic length was selected and Excel file was obtained with branch names with their respective lengths in µm.

Unpaired t-test was used to compare the different parameters of pyramidal neurons. The data was analyzed with Microsoft Excel worksheet (Microsoft office professional plus 2013 version). The p-value less than 0.05 was considered as significant.

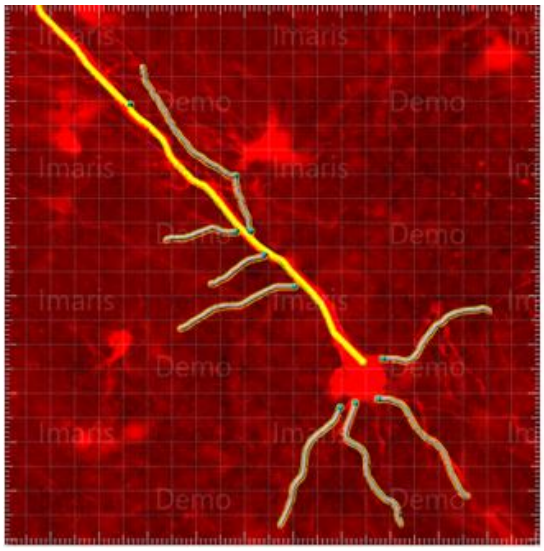

Figure 8: Neuronal reconstruction

RESULTS

The measurements of both apical and basal dendritic parameters after semi-automatic tracing in IMARIS software are presented in table (1&2) as per the groups A & B respectively. Comparison between control and stressed groups was done and percentage change was calculated too. In pyramidal neurons of group A rats, the % changes in principal dendrite lengths, branch points from the principal lengths, branch lengths, number of basal dendrites, lengths of basal dendrites and branch points of basal dendrites were -42, -51, -68, -3, -17 and -69 observed respectively.It was observed that all the apical dendritic parameters (principle dendrite's length, branch points & branch length) in group A were shrunk and the changes were significant (<0.000217, <0.027854 &<0.001). It was also found in group A that all the basal dendritic parameters (number, branch length & branch point) were diminished too but the changes were not significant (<0.842873,<0.156947 &<0.180474).In pyramidal neurons of group B rats, the % changes in principal dendrite lengths, branch points from the principal lengths, branch lengths, number of basal dendrites, lengths of basal dendrites and branch points of basal dendrites were -25, -76, -41, -6, +12 and -88 observed respectively. In group

B, all apical dendritic parameters (principal dendrite's length, branch point & branch length) were shrunk too and the shrinkage was also significant (<0.007705, <0.000423 &<0.007811). All the basal parameters in group B have decreased except branch lengths but the changes were not significant (<0.743144,<0.364572 &<0.507876).

Table 1: Comparison between control and stressed dendritic lengths (um) and number of branch points on pyramidal neurons of Hippocampus in group A rats.

Pyramidal Neurons	Control n = 20	Stressed n = 20	% Change	P value
Apical Dendrites				
Principal dendrite's length	151.75 ± 7.27	106.92 ± 8.20	- 42	<0.000217
Branch points	4.3 ± 0.61	2.85 ± 0.16	- 51	<0.027854
Branch lengths	56.31± 3.90	33.49 ± 3.33	- 68	<0.001
Basal Dendrites				
Numbers	3.7 ± 0.41	3.8 ± 0.28	- 3	<0.842873
Dendritic lengths	38.63 ± 3.02	33.04 ± 2.51	- 17	<0.156947
Branch points	1.35 ± 0.35	0.8 ± 0.18	- 69	<0.180474

Values are mean ± standard error of mean; percentage values were calculated relative to control mean values.

Table 2: Comparison between control and stressed dendritic lengths (um) and number of branch points on pyramidal neurons of Hippocampus in group B rats

Pyramidal Neurons	Control n = 20	Stressed n = 20	% Change	P value
Apical Dendrites				
Principal dendrite's length	138.52± 5.21	110.76 ± 8.36	- 25	<0.007705
Branch points	4.3 ± 0.32	2.45 ± 0.35	- 76	<0.000423
Branch lengths	47.24 ± 3.15	33.60 ± 3.83	- 41	<0.007811
Basal Dendrites				
Numbers	3.65 ± 0.41	3.45 ± 0.44	- 6	<0.743144
Dendritic lengths	31.30 ± 3.03	35.41 ± 3.36	+ 12	<0.364572
Branch points	1.55 ± 0.27	1.3 ± 0.25	- 88	<0.507876

Values are mean ± standard error of mean; percentage values were calculated relative to control mean values.

DISCUSSION

In this study, the immobilized stress was conducted for 21 days. This type of restraining stress was also used in past by McEwen BS and Gianaros PJ.(4) This research was performed to find out the effects of immobilized stress on pyramidal neurons of hippocampus in female Wistar rats. The present study indicates that restraining the Wistar rats for 21 days induces significant decrease in the apical dendrite parameters of pyramidal neurons in hippocampus of both the groups of rats and even more in rats between 7-11 weeks. The basal dendrite parameters also shows shrinkage but they are not significant.

A study conducted by Watanabe Y et al; showed significant decrease in both the total dendritic length ($p < 0.05$) and in number of branch points ($p < 0.05$) in apical dendritic trees in CA3 pyramidal cells whereas no significant change in total length of dendrite ($p > 0.1$) and number of branch points ($p > 0.1$) as a result of 6 hours of daily restrain stress for 3 weeks.[15]

In present study, we have also witnessed the same result as significant atrophy in apical dendritic parameter and no significant shrinkage in basal dendritic parameters.

Magarinos A.M. et al mentioned repeated restrain stress for 21 days induced shrinkage of apical dendrite of CA3 pyramidal neurons in hippocampus and administration of steroid synthesis inhibitor cynoketone peripherally to chronically stressed rats blocked the stress induced atrophy of apical dendrite in CA3 pyramidal neurons.[16]

Vyas A et al used chronic unpredictable stress (CUS) and chronic immobilization stress (CIS) protocol. CIS induced significant shrinkage in dendritic length ($p < 0.01$) and number of branch points ($p < 0.01$) in CA3 pyramidal neurons of hippocampus as compared with neurons in control rats. The atrophy in total dendritic length and number of branch points was present in both apical and basal dendrite of CA3 pyramidal neurons. The shrinkage in basal dendrite was not as significant as in apical dendrites. This study is also in line with our present study. CUS did not cause shrinkage in dendritic arbor of CA3 pyramidal neurons.[17]

It was also found that the daily restrain stress or corticosterone injections for 3 weeks induced shrinkage of apical dendrites of CA3 pyramidal neurons and this effect of chronic stress on dendritic morphology can also occur outside CA3 such as dentate gyrus & area CA1.[18]

CA3 pyramidal neurons show a more vulnerability to chronic stress. Apical dendrite of CA3 pyramidal neurons can also shrunk by psychosocial stress.[5]
Limitations: This study was conducted on female Wistar rats so the effects of immobilized stress on structural plasticity of pyramidal neurons in hippocampus need to be established in male Wistar rats. The hippocampus was researched as a whole but not in particular regions of it.

CONCLUSION

Immobilized stress induces structural plasticity in pyramidal neurons of hippocampus of female Wistar rats. The results of this study indicate immobilized stress causes significant atrophy in apical dendrite parameters of pyramidal neurons in hippocampus of rats aged between 3 to 11 weeks. The findings also suggest that the chronic immobilized stress induce atrophy in basal dendrite parameters but these changes are not significant. The relationship between the shrinkage in pyramidal neurons and corticosterone levels in stressed rats need to be established in further future researches.

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