Original Article

Exposure to Enriched Environment Restores Altered Passive Avoidance Learning and Ameliorates Hippocampal Injury in Male Albino Wistar Rats Subjected to Chronic Restraint Stress

Abstract

Aims: The aim of the study was to investigate the effects of exposure to enriched environment (EE) on passive avoidance learning and hippocampal cellular morphology in rats exposed to chronic restraint stress. **Materials and Methods:** Adult male albino Wistar rats were assigned into the following groups: normal control (NC) remained undisturbed in their home cages; stressed group (S) subjected to restrained stress (6 h/day) followed by housing in standard housing for 21 days; And stressed + EE (S + EE) subjected to restrained stress followed by housing in EE for 21 days. On 22nd day, six animals from each of the three groups were exposed to passive avoidance test. The remaining animals were sacrificed. Hippocampus was isolated and processed for cellular morphology using cresyl violet staining. **Statistical Analysis Used:** Data were analyzed using one-way analysis of variance followed by Tukey's multiple comparison test (*post hoc*). **Results:** Stressed rats exposed to EE showed significant improvement in passive avoidance learning test compared to NC. Quantification of the surviving neurons in the hippocampal subfields and their cellular morphology revealed significant neuroprotection in S + EE in cornu ammonis-2 (CA2) neurons and CA3 hippocampal neurons. No significant changes were found in CA1 hippocampal subfield. **Conclusions:** The outcome of this study makes us to think the possibilities of adopting EE as an alternative strategy in brain diseases where there is chronic stress and to minimize the impairment in learning and memory.

Keywords: *Chronic-restraint stress, cresyl violet staining, enriched environment, hippocampus, passive avoidance test*

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Introduction

Stress is defined as a perturbation of either physiological or psychological homeostasis.[1] Repeated exposures to stress are known to cause long-term detrimental effects in brain including neuronal atrophy and death of neuron that in turn results in behavioral abnormalities and memory impairments.[1,2] Stress is thought to exacerbate several affective disorders including depression and posttraumatic stress disorder. It causes activation of the hypothalamo–pituitary–adrenocortical (HPA) axis, which results in the secretion of steroid hormones from the adrenal cortex.[3] The hippocampus is one of the crucial brain structures involved in memory processing. It provides negative feedback regulation of the HPA axis and intercepts uncontrolled stress response, which could be maladaptive. In Mammalian brain, hippocampus has been shown to have the presence of highest density of glucocorticoid receptors when compared to any other parts of the brain.[4] Hence, this brain structure receives special emphasis not only because of its role in regulation of the HPA axis but also of its susceptibility to stress-related damage which might lead to this inhibitory control being disrupted. If stress persists for longer duration, the hippocampus begins to falter in its ability to control the release of stress hormones.[2] It has been reported that chronic exposure of rodents to physical stress or exposure of nonhuman primates to psychosocial stress results in atrophy of cornu ammonis-3 (CA3) pyramidal neurons in the hippocampus.[4] Stress markedly impairs hippocampal long-term potentiation, a cellular substrate for learning and memory.[5] The importance of the environmental factors in the regulation of brain, and behavior has long been

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recognized by the researchers.[6] The brain responds to a variety of stimuli that it received from the environment. These include modalities of sensations such as tactile, visual, or auditory stimuli. Enriched environment (EE) is an experimental paradigm, first described in a neuroscientific context by Donald Hebb in early 1947. It refers to special housing conditions, in which the experimental animals could facilitate sensory, cognitive, and motor stimulation in them when compared to the standard housing conditions.[7] Majority of EE experimental paradigms comprise various articles of different colors, shapes, and sizes for visual stimulation, wooden or plastic objects of different textures for sensory stimulation, plastic tunnels of different shapes that guide spatial navigations, ladders, and running wheels to enhance their motor activity. Animals also get a chance for social interactions, when they are exposed to such an environmental condition. Exposure to EE has been shown to induce neural plasticity in the normal rat brain. This includes structural modification, improvements in cognitive function, and mostly favorable alterations in brain chemistry. The neuronal changes on exposure to EE include dendritic pruning,[8] addition of spines on dendrites,[9] and increased involvement of new neurons in the of the brain areas such as the hippocampus and the olfactory bulb.[10] Enhanced neurogenesis was observed in the hippocampus of adult mice that were exposed to EE, primarily in the subgranular zone of the dentate gyrus.[11] It has been reported that short-term EE rescues adult neurogenesis and memory deficits in transgenic mouse model of Alzheimer's disease.[12] Yang *et al*. reported the beneficial effects of using EE in improving the memory of rats that were undergone chronic cerebral hypoperfusion.[13] Although stress-induced impairment in learning and memory has been extensively studied, there are no studies reported in the literature that correlates the effects of exposure of EE on passive avoidance learning and hippocampal cellular morphology in rats that are exposed to chronic-restraint stress. The present study was undertaken to evaluate the effects of EE on passive avoidance learning and hippocampal cellular morphology in adult male albino Wistar rats subjected to chronic-restraint stress.

Materials and Methods

Animals

Three-month-old adult male albino Wistar rats (weighing 200–250 g) were obtained from Central Animal Research Facility (CARF) of the university. To minimize the variation in study, all animals were bred in-house. Adult male albino Wistar rats belonging to six different litter sizes were used in this study. They were distributed by random allocation method to each of the experimental groups. Three rats were maintained in each cage to minimize the stress caused by overcrowding. Rats were housed in standard polypropylene cages (dimension 41 cm × 28 cm × 14 cm). All rats were fed with filtered tap water and food except during the stress procedure, with a standard rodent pellet. All animals were handled once daily to reduce the possible anxiety associated with human handling. They were housed in the institutional CARF in a 12:12 h light: dark environment (24°C ± 1°C). All procedures were approved by the Institutional Animal Ethics Committee and were done in accordance with the Guide for Care and Use of Laboratory Animals published by the United States National Institutes of Health (Publication No. 85–23, revised 1985). All efforts were made to minimize the number of animals used in the study.

Study design

Rats were divided into three groups with 12 animals in each group (n = 12).

Normal control (NC) – Remained undisturbed in the home cage throughout the experimental period. Restraint stress (S) – These rats were stressed in wire mesh restrainers, 6 h/day, for 21 days. Stressed rats exposed to EE (S + EE) – subjected to restraint stress for 6 h/day. Following this, they were housed in EE. This procedure continued for 21 days. On 22nd day, six rats from each group were tested individually for the avoidance learning (n = 6 in each group); remaining rats were killed (n = 6 in each group); hippocampus was dissected out from the brain and processed for cresyl violet staining. Cellular morphology of neurons in CA1, CA2, and CA3 hippocampal subfields and quantification of the surviving neurons in these areas were investigated.

Stress procedure

Rats were subjected to restraint stress using a wire mesh restrainer, 6 h/day for 21 days as described in a previous study.[14] Stress procedures were carried out in the Institutional Animal Research Facility (24°C ± 1°C) between 10.00 h and 16.00 h each day. Restrained rats were housed in a separate room, in the Institutional Animal House away from the nonstressed control rats. This was to prevent any possible behavioral change induced by odor or sound between the experimental groups.

Enriched environment

The housing for providing EE was made out of wood 70 cm (L) × 70 cm (B), 45 cm (H) as described by Carughi *et al*.,[15] which contained a variety of objects. The objects include climbing ladders, tunnels, colored balls, and marbles. Every alternate day, these objects were replaced with a new set of objects to avoid behavioral habituation, thereby providing novelty for tactile, visual, and motor stimulation to the animals. At a time, six rats were placed in each of the EE housing. Food and water were made available inside the housing during the period of enrichment. The room temperature was maintained at 24°C ± 1°C.

Passive avoidance testing

Passive avoidance apparatus (Panlab, Barcelona, Spain) was used in this study. It was connected to a computer installed with Shut-avoid software (Panlab, Barcelona Spain) and an inbuilt shock generator. The animal's position in each compartment was detected by the high-sensitive weight transducers incorporated inside the apparatus. The experiment included three parts such as (I) exploration, (II) an aversive stimulation and learning, and (III) retention test. The experimental procedure was carried out as reported earlier by Kumar *et al*.[14]

Histological analysis of hippocampus

Cresyl violet is a basic dye, which is used for the staining of nucleoproteins and Nissl substance.[16] It is used to identify the functional regions of brain and to obtain a detailed view of the cell bodies. At the end of 21 days, the set of animals assigned for histological study were perfused transcardially with 100 ml ice-cold phosphate-buffered saline [PBS] 0.1 M pH 7.4) followed by 4% paraformaldehyde in cold PBS (0.1 M pH 7.4). Brains were removed quickly and postfixed with same fixative solution for 48 h. The tissue was then blocked in paraffin using an L block over the tissue embedder. Care was taken to orient the tissue properly. Coronal section (5 μm thick) of brain through hippocampus was cut using a rotary microtome (Leica; model: RM2155, Germany). The sections were then spread in water bath at 50°C. Twenty sections from each animal were selected and mounted on gelatinized slides. After draining water from the sections, they were fixed to the slide by gentle warming on Leica hot plate at 50°C for 1–2 min. Hundred milligrams of cresyl violet (Sigma Chemicals, USA) was dissolved in 100 ml of distilled water. To this, 0.5 ml of 10% acetic acid was added to give a pH of 3.5–3–8. The stain was filtered and then used. After staining, slides were coded for subsequent blind analysis. Neurons were quantified by direct visual counting for viable neurons using a light microscope (Leica, Germany) at a magnification of ×400. Cresyl violet-stained sections were observed for any morphological changes such as cell shrinkage, cell size, cell number, Nissl substance distribution, and nuclear size and position. Twelve sections from each rat were chosen for quantification analysis. The sections were distributed evenly along the septotemporal axis of the hippocampal formation. Pyramidal neurons in the CA1, CA2, and CA3 subfields of dorsal hippocampus were counted over 125 μm length and were expressed as the number of cells per unit length of the cell field (cells/125 μm) as reported earlier by Wood *et al*.[17]

Statistical analysis

Data were analyzed using one-way analysis of variance followed by Tukey's multiple comparison test (*post hoc*). All results were expressed in mean ± standard error of the mean; the significance level was fixed at $P < 0.05$. GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA) (California, USA), statistical software package, was used for the analysis.

Results

Passive avoidance learning

Figure 1 shows that retention test performed after 24 h and 48 h, respectively, showing the latency to enter the dark compartment of passive avoidance apparatus. Retention test performed after 24 h indicated a decreased latency time taken by the stressed group (7.66 ± 2.5 s) to enter the dark compartment in comparison with NC (60.25 ± 1.7 s) group ($P < 0.001$). S + EE could significantly increase the time latency (39 ± 2.21 s) when compared to the stressed group ($P < 0.001$) [Figure 1].

Retention test performed after 48 h: stressed group showed a shorter latency (3.20 ± 2 s) to enter dark compartment when compared to NC group (20.48 ± 2.59 s) ($P < 0.001$). Exposure to EE significantly enhanced the time latency in S + EE (14.67 ± 1.39 s) ($P < 0.001$) compared to the stressed group [Figure 1].

Hippocampal cellular quantification

Quantification of viable neurons in the CA1 hippocampal subfield revealed no significant differences between any of the three groups [Table 1]. Upon evaluating CA2 and CA3 hippocampal subfields among the three groups, we could identify the presence of increased surviving neurons in CA2 hippocampal subfields of S + EE group in comparison with stressed group ($P < 0.01$). On evaluating the CA3 hippocampal subfields, we could identify more viable neurons in S + EE group ($P < 0.001$) compared to the stressed group. These results indicate that the exposure to EE could significantly protect the hippocampal neuron survival in CA2 and CA3 hippocampal subfields nearing to NC.

Hippocampal cellular morphology

Figure 2 shows the photomicrograph of CA1 region in hippocampal subfield. The CA1 neurons in the stressed

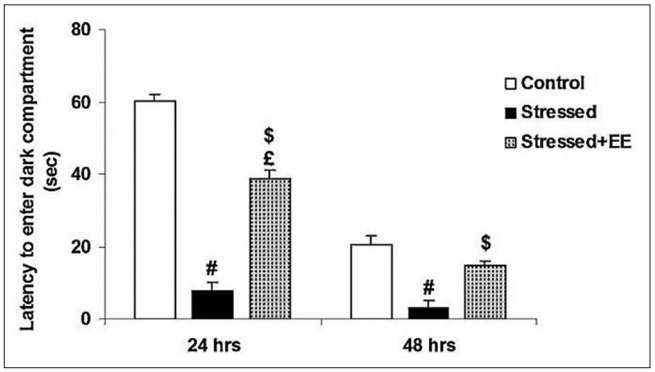

Figure 1: Retention test performed after 24 h and 48 h respectively showing the latency to enter the dark compartment of passive avoidance apparatus. NC versus S, #$P < 0.001$; NC versus S + enriched environment, £$P < 0.001$; S versus S + enriched environment, \$$P < 0.001$ after 24 h. NC versus S, #$P < 0.001$; S versus S + enriched environment, \$$P < 0.001$ after 48 h

Table 1: Number of viable hippocampal neurons counted from Cornu Ammonis-1, Cornu Ammonis-2, and Cornu Ammonis-3 hippocampal subfields of rat brain

Groups	CA1 neurons	CA2 neurons	CA3 neurons
Normal control (NC)	27.92±0.3	16.75±0.27	11.25±0.8
Stressed (S)	25.92±0.8	8.4±0.39$^{\#}$	4.75±0.32*
Stressed + Enriched environment (S+EE)	26.50±0.35	10.58±0.8$^{\$,¥}$	10.92±0.55**

Viable neurons counted from the CA1 hippocampal subfield showed no significant difference between groups. CA2 neurons: NC versus S, $^{\#}P$<0.001; NC versus S+EE, $^{\$}P$<0.001; S versus S+EE, $^{¥}P$<0.01. CA3 neurons: NC versus S, *P<0.001; S versus S+EE, **P<0.001. n=6/group and the data is expressed as Mean±SE. CA1: Cornu Ammonis-1; CA2: Cornu Ammonis-2; CA3: Cornu Ammonis-3; NC: Normal control; S: Stressed ; EE: Enriched environment; SE: Standard error of mean

group are more widely dispersed and show the presence of more darkly stained degenerated neurons as indicated by the black arrows [Figure 2]. Note the intact neuronal arrangement of CA1 subfield in the NC and S + EE groups. The presence of minimum degenerating neurons can be noted in S + EE.

Figure 3 shows the photomicrograph of CA2 region in the hippocampal subfield. Note the minimum degenerated neurons in S + EE group compared to S [Figure 3]. The cells are more widely dispersed and show the presence of darkly stained degenerated neurons (indicated by black arrows) in S. Exposure to EE enhanced the cell survival rate of the CA2 neurons as indicated in Table 1 when compared to the stressed group (P < 0.01).

Figure 4 shows the photomicrograph of CA3 region in the hippocampal subfield. Quantification of CA3 neurons revealed a significant loss of neurons [Table 1] in the S compared to NC (P < 0.001). Loss of neurons was minimized [Figure 4] in S + EE group (P < 0.001). Note the presence of more darkly stained degenerated neurons (indicated by black arrows) in S.

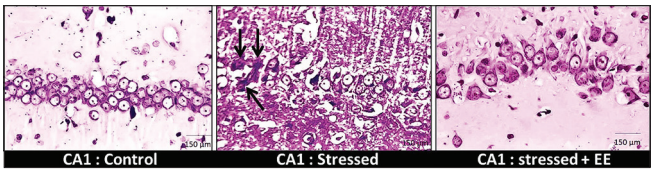

Figure 2: Photomicrograph of cornu ammonis-1 region of the hippocampal subfield (×400, Scale bar: 20 µm)

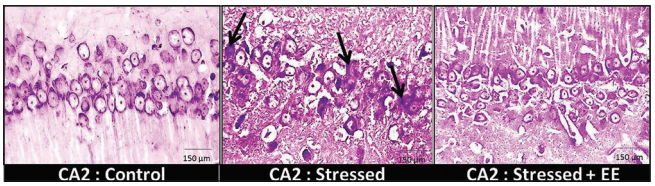

Figure 3: Photomicrograph of cornu ammonis-2 region of the hippocampal subfield (×400, Scale bar: 20 µm)

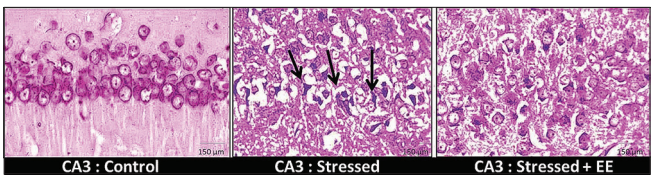

Figure 4: Photomicrograph of cornu ammonis-3 region of the hippocampal subfield (×400, Scale bar: 20 µm)

Discussion

Hippocampus is a brain structure located in temporal lobe which plays a key role in spatial and episodic memory.[18] It is also reported to be involved in avoidance learning, anxiety,[19] and contextual fear conditioning.[20] This brain structure is also known to undergo structural plasticity modulated by a variety of stimulus.[21]

Chronic-restraint stress model has been extensively used in investigating hippocampal-dependent behaviors such as spatial memory.[22] It has been reported that male Sprague-Dawley rats exposed to chronic-restraint stress (6 h/day for 21 days) resulted in spatial memory deficits and hippocampal damage.[22] In the present study, cresyl violet staining of hippocampus revealed significant damage at CA3 and CA2 neurons in stressed rats without having noticeable effects on the CA1 neurons. Our findings are in line with a previous report which showed extensive damage to CA3 neurons in primates after repeated glucocorticoid exposure.[23] Magariños *et al*.[24] reported that daily injections of corticosterone for 3 weeks in adult rats induced atrophy of apical dendrites in CA3 pyramidal neurons and did not have any effect on CA1 or granule cells. The selective susceptibility of CA3 hippocampal area to damage in the above research is comparable with our present study. We could observe selective damages in CA3 hippocampal area without having significant cell damage on CA1 area. The cause for this selective vulnerability of CA3 hippocampal neurons is believed to be due to the lack of calcium-binding proteins, namely calbindin-D28K and parvalbumin.[25] The presence of an oxidative imbalance in brain regions such as hippocampus has been highlighted as one of the possible components of stress-mediated neurodegeneration.[26] This could be one of the reasons liable for the observed memory loss in stressed animals.

The interactions between an organism and its environment are known to influence and evoke neurobehavioral changes. Researchers have been trying to identify the use of EE to induce these changes in both intact and in injured central nervous systems. Zhang *et al*.[27] reported the beneficial effects of using EE in rats having temporal lobe epilepsy.

Their study revealed enhanced hippocampal neurogenesis, improved cognitive impairments, and decreased long-term seizure activity in epileptic rats after exposure to EE. In the present study, exposure of stressed rats to EE effectively minimized the behavioral deficits in passive avoidance task and could provide significant neuroprotection to the hippocampal neurons. Avoidance learning behavior in passive avoidance task was positively enhanced in stressed rats after exposure to EE. N-methyl-D-aspartate receptor (NMDA) receptors are believed to play a major role in hippocampal synaptic plasticity. Interestingly, one of the mechanisms involved in chronic stress-induced hippocampal dendritic retraction is proposed to be mediated through CA3 NMDA receptors.[28] Exposure to EE is also reported to increase the functional response of presynaptic NMDA receptors in rodent hippocampus.[29] One study suggests that exposure to EE could effectively prevent the alterations in hippocampal brain-derived neurotropic factor (BDNF), which is an essential molecule required for learning and memory.[30]

All above-mentioned suggested factors could be considered as the possible mechanisms that could have helped in improving the avoidance learning and hippocampal neuroprotection in stressed animals that were exposed to EE. With the existing data, it is difficult to pinpoint the exact neural mechanism for the improved avoidance learning in stressed rats after exposure to EE. However, when we correlate the results of avoidance learning and hippocampal morphology in rats that were exposure to EE, definitely, it gives us some valuable clues regarding the usefulness of EE in chronic stress. We assume that the mechanisms responsible for improved memory observed in our experiment could be of multifactorial origin. Involvement of oxidative stress pathways, NMDA receptor modulation, alterations in the hippocampal BDNF expression, variations in nerve growth factors, and hippocampal neurogenesis are some aspects that need to be investigated. Therefore, further investigation of this project is warranted.

Conclusion

The findings of this study demonstrates the ameliorative effects of environmental enrichment on stress induced alterations in learning and hippocampal morphology in rats. It also believed to throw light and pave way for developing alternative strategies like environmental enrichment in learning and memory impairments caused by chronic stress.

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Conflicts of interest

There are no conflicts of interest.

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