Enhanced environment alters the myelin composition and neurochemistry of fore and mid brain regions in rats subjected to immobilization stress

AJ Vanisree*, Kirijayini Perumalpillai & Thamizhoviya Gangadharan
Department of Biochemistry, University of Madras, Guindy Campus, Chennai-600 025, India

Received 29 April 2016 revised 12 September 2016

Stress is a major factor contributing to various psychological and neurological disorders and hence needs to be addressed by simple remedial measures to mitigate the severity. This study assesses the impact of enhanced environment (EE) on fore and mid brain regions of rats under stress. We used immobilization restraint stress (IS) strategy (4 h) to impart stress in rats and also exposed the rats to EE (1 h) for 21 days. Behavioural changes in the rats, neurotransmitters, cortisol levels and composition of myelin membrane isolated from forebrain (FB) and midbrain (MB) regions were evaluated. The behavioural assessment revealed an impaired state by immobilisation stress (IS) which, however, improved on EE exposure. The observed levels of serotonin, dopamine, glutamate and GABA emphasized the effectiveness of EE significantly ($P<0.001$). IS had challenged the protein composition of myelin and the activities of membrane bound ATPases, but EE could alter the adverse effect significantly ($P<0.001$). Interestingly, control rats showed a reduction in the activity of acetylcholinesterase, unlike ATPases and 5′nucleotidase in their MB regions. EE had a significant effect on the altered activities of all these enzymes except that of 5′nucleotidase ($P=0.050$). The alterations of lipid profile of myelin also highlighted the impact of EE on IS by maintaining almost normal lipid profile ($P<0.001$). Cortisol was found to be elevated in stress induced group while EE was able to attenuate the levels of cortisol. Our results also suggests that the potential impact of EE on FB was minimal compared to MB. The observed IS+EE–myelin interaction deserves further investigations to validate the significance of myelin in stress biology.

**Keywords:** Enhanced environment, Immobilisation stress, Myelin, Neurotransmitters

Threats to well-being, whether physical or psychological, are components of life-experience of an individual. Individual differs markedly, however, in the frequency with which they experience stressful life events and their vulnerability or resilience to stressful challenges. Stress, although often studied as a psychological construct, may be viewed from a biological perspective. Accordingly, stress responses are composed of the activation of neurobiological systems that help preserve viability through change. Although necessary for survival, the effects of frequent physiological stress responses may increase the risk of future physical and mental health problems.

Resiliency is the ability to recover from the adverse change which comprises psychological and biological processes that allow an individual to avoid or reduce the harmful consequences of extreme stress. Resilient individuals encountering chronic psychosocial stress minimize pathophysiological outcomes, e.g., extended or exaggerated hypothalamic-pituitary-adrenal (HPA) axis activity that can precipitate stress-related diseases such as post-traumatic stress disorder (PTSD), anxiety, and major depression although many studies focus on maladaptive responses to psychological stress, relatively little research has been directed towards the understanding of stress resilience. The staggering prevalence of stress-related disorders emphasizes the need for innovative approaches to treatment. Development of stress resiliency paradigms in animals will facilitate discovery of buffers against the onset of stress-related disorders. Behavioural and physiological responses to stress are influenced by genetic and environmental factors. Enriched Environments (EE), including physical exercise, are powerful environmental factors that mitigate deleterious stress effects on neurobiological systems and endocrine profiles and promote stress adaptability in both humans and rodents. Although the protective effects of EE on stress-related mood disorders are well characterized, little is known about the functional alterations in structure/chemistry of brain that form the basis for resiliency.

The cognitive reserve hypothesis is consistent with several decades of research on environmental
manipulation in rodents. Enrichment may not only influence behaviour of the animals but can also affect physiological parameters\textsuperscript{8}. Investigations in the past had suggested that environmental enrichment may reduce response to stress, yet few studies have directly examined the effects of enrichment on the stress responses, and these studies have reported mixed results\textsuperscript{9}. The present study attempts to find whether an exposure to EE following the stress exposure could exert enduring effects on immobilized rats by focusing on myelin composition of (which is said to get deregulated in various neurological disorders) midbrain and forebrain regions along with neurotransmitters viz serotonin, dopamine, glutamate, and GABA.

Materials and Methods

Animals

Male Wister rats (150-200 g) were used for the investigation. Rats were housed six per cage in polypropylene cages in a controlled environment [temperature (25-28°C), humidity (50–55%) and light-(12 h light–dark cycle)] with access to food and water ad libitum except during the periods of stress exposure. Efforts were made to minimize both the suffering and the number of animals used.

Experimental design

The animals were randomly divided into four groups: A group of rats designated as Control (C) group of rats neither did undergo restraint stress exposure by immobilization strategy (IS) nor did get exposure to an enriched environment (EE). The rats were not subjected to social isolation. Another group of rats was subjected to immobilization stress (IS) in restrainer for 4 h per day from 10.00 am-12 noon and 4.00 pm-6.00 pm. After the immobilization stress exposure, it was exposed to the enriched environment (EE) for an hour. EE was designed with the accessories which anticipated stimulating neurophysiological and sense of the rats. IS+EE exposed animals had access to objects such as toys, wooden pieces of different shapes and colour, tunnels and pipes which could be realigned and could stimulate their exploratory behaviour and permitted social interaction (6 per cage as opposed to 3 in standard conditions). The objects were rearranged every day, and different objects were placed on alternate days for novelty. In addition to this, another group of rats were subjected to EE alone and was designated as CEE (control + EE). These rats were subjected to the only enriched environment. The experiment was designed for 21 days, and the animals were routinely weighed before the stress exposure. The food and water consumption was monitored at the end of the day. Finally, the animals were sacrificed by cervical decapitation. After weighing the whole brain, the forebrain and midbrain regions were dissected and used for further studies.

Behavioural tests

All rats underwent a series of behavioural evaluations by using Radial Arm Maze test, T Maze test and stride length test. In radial arm maze test, spatial memory was assessed using the eight-arm radial maze, and the rats were made to go to the ends of the arms for food reinforce, as a result they were rewarded pellets. A visit to an arm was scored if the subject traversed three-fourths of the arm's length, if the arm was entered but food not eaten, or if the arm was entered and food was eaten. Re-entries into arms previously visited were counted as mistakes. After the rat had made four choices, it was removed from the maze, put back in its home-cage. Similarly, in the case of T-Maze test the alternating behaviour of rats in searching for food was validated. The task was based on the basis that animals could evolve an optimal strategy to explore their environment and obtain food with a minimum amount of effort. In the case of stride length test, the walking pattern of the animals was assessed. The animal’s hind paws were inked, and footprints were made on the paper covering a narrow runway of 1 m length and 7 cm width. This ensured that the direction of each step was standardized in line. A series of at least five sequential steps were assessed, and the mean values for each stride length were measured between the central pads of two consecutive prints on each side.

Isolation of myelin sheath

About 100 mg of the fore and mid brain tissue was weighed, uniformly homogenized in an ice cold aqueous solution of 0.20 M sucrose containing 1 mM EDTA, 1 mM β mercaptoethanol (β-SH), 0.25 mMphenylmethylanesul phonyl fluoride (PMSF) (1mL medium/ 0.100 g tissue) and the homogenate was used for the isolation of myelin sheath. Myelin sheath was isolated from neuronal axons of forebrain and midbrain tissue by sucrose density gradient centrifugation by the method of Norton and Poduslo\textsuperscript{10}.

Electrophoretic analysis of myelin

Lowry's method\textsuperscript{11} estimated the total protein content from myelin. Myelin proteins were analysed by SDS-PAGE to ascertain the relative protein concentration. Proteins were analysed according to the method of Laemmli\textsuperscript{12}. 
Enzyme profile
The specific activity of membrane bound enzymes in FB and MB were examined in the experimental groups. The method of Bonting estimated Na^+K^+ATPase, Mg^2+ and Ca^2+ ATPases were estimated by Elwood. Acetyl choline esterase (AChE) was estimated by Ellman. The 5′-nucleotidase was determined by Luly et al.

Estimation of neurotransmitters
The dopamine content was estimated by the method of Abdul Barry. Serotonin content was estimated by the method of Subbaraju. Glutamate and GABA were estimated by the method of Raju.

Estimation of cortisol
Retro orbital puncture collected one mL of the blood sample, and it was allowed to clot for 30 min at room temperature. Serum was separated by centrifugation at 2500 rpm for 5 min. The serum samples were then subjected to estimation of serum cortisol by chemiluminescence method using commercially available CLIA kit (Kamiya Biomedical Company, Cat. No. KT-715).

Lipid profile
The lipids were extracted from myelin membrane according to the method of Folch. The cholesterol content of myelin membrane was estimated by the method of Jung and Parekh. The method of Rouser estimated phospholipids in myelin membrane.

Statistical analysis
Statistical analyses were performed (n=6) for investigating the group differences between FB and MB regions using multivariate ANOVA (MANOVA) to protect against higher risk of type one error and then followed up with ANOVA. Fisher’s LSD post hoc tests were performed if the overall MANOVA was found to be significant (P<0.05).

Results
Figure 1 denotes the growth pattern of animals, in which, we have reported the monitored BMI and food and water consumption of control and experimental animals. The control rats showed a standard growth curve during the period of study whereas the stress induced rats showed a significant loss of body weight (P<0.01). This could be understood from the food and water consumption analysis (Fig. 1) in which IS rats exhibited significant reduction (P=0.003) in food and water consumption when compared to the control ones. Interestingly, IS+EE rats consumed more (P=0.038) food pellets and water than IS rats thus the treated rats showed normal growth curve when compared to IS. CEE rats showed no significant change in BMI as well as food and water consumption when compared to control rats. The data reported in Fig. 2, (P<0.01) suggest that the behaviour of rat was affected by repeated restraint stress. IS exposed rats showed a significant decrease in its activity while IS+EE were active in its reward finding.

Fig. 3 depicts wet weight of myelin membrane obtained by density gradient centrifugation. In stress induced group, significant reduction of myelin content was observed in both FB region (P=0.002) and MB region (P<0.001); the reduced myelin yield was also reflected in their respective protein content; stress induced group showed a reduction both in FB (P=0.002) and MB (P<0.001) regions when compared...
to that of control rats. IS+EE rats showed an improved myelin content (wet weight) \( (P=0.009) \) as well as the protein levels \( (P=0.028) \) when compared to those of IS rats. However, EE was found to improve the contents only in the MB regions, and interestingly EE could not exert an effect on the myelin in FB regions for the unknown reasons. In the case of CEE rats, there was no significant change in myelin content and protein levels when compared to control group.

Fig. 4 represents the activities of membrane bound ATPases depicting a reduction in the activities of Na\(^+/K^+\), Ca\(^{2+}\), and Mg\(^{2+}\) ATPase in both FB \( (P=0.003, 0.001 \text{ and } 0.001) \) and MB \( (P<0.001) \) regions of stress induced group of rats when compared with those of control groups. IS + EE exhibited enhanced activities of Na\(^+/K^+\), Ca\(^{2+}\), and Mg\(^{2+}\) ATPase in both FB \( (P=0.002, 0.001 \text{ and } 0.003) \) and MB \( (P=0.002, 0.001 \text{ and } 0.003) \) regions when compared to IS group. CEE rats showed no significant changes in their Na\(^+/K^+\)ATPase activity as compared to control rats. It was clearly understood from Fig. 5 that the immobilized stress has affected the activities of both AChE and 5′-nucleotidase; but EE did not influence the stress modified activities especially AChE in both MB and FB regions. EE was also inept
to modify the stress-induced reduction in the activity of 5'-nucleotidase in MB region though it can modify that of FB region \((P=0.001)\). CEE rats showed no significant changes in the activities of AChE and 5'-Nucleotidase compared with control rats.

The assessment of neurotransmitters such as serotonin, dopamine, glutamate and GABA (Fig. 6) has revealed that the stress induction had culminated in the reduced contents of serotonin, dopamine and GABA in both FB \((P=0.001, 0.003 and 0.001)\) and MB \((P=0.001, 0.004 and 0.001)\) regions when compared to control. IS+EE showed increased levels of serotonin, dopamine, and GABA when compared to stress induced group. However, EE significantly improved the levels of GABA only in the MB regions \((P=0.007)\) but could not show any effect on the FB regions. On the other hand, elevated levels of glutamate in IS group of rats both in FB \((P=0.001)\) and MB \((P=0.046)\) regions (when compared with control group) were significantly decreased both in FB \((P=0.001)\) and MB \((P=0.004)\) regions on EE exposure (when compared with IS). CEE rats showed no significant changes in the levels of serotonin, dopamine, glutamate and GABA when compared with control rats. Fig. 7 depicts the levels of cortisol, in which IS group showed an increase \((P<0.001)\) in the level of cortisol when compared with that of the control group. IS+EE rats showed a significant reduction \((P=0.030)\) in the levels of cortisol in comparison to IS rats and CEE exhibited non-significant results when compared to control rats.

Fig. 8 shows the levels of cholesterol and phospholipids; IS rats showed significant reduction in...
the levels of cholesterol and PL both in FB ($P=0.003, 0.001$) and MB ($P=0.002, 0.001$) regions when compared to control group. IS+EE rats showed increased levels of cholesterol and PL when compared with IS. Although there were increased levels of cholesterol and phospholipid, significant improvement was witnessed in PL contents both in FB ($P=0.003$) and MB ($P=0.004$) regions, but EE could not have its impact on the levels of cholesterol.

Fig. 9 denotes the SDS-PAGE pattern of myelin proteins for the experimental group of rats. IS group showed less intense bands in FB and MB when compared to those of C, IS+EE, and CEE which exhibited a similar higher intense banding pattern. In our study, though we could not figure out a significant difference in the susceptibility of FB and MB to IS, it was intriguing that EE could influence myelin of MB but not the myelin of FB significantly.

Discussion

Prolonged stress is a recognized risk factor in the development of depression\textsuperscript{22}, anxiety\textsuperscript{23}, drug addiction\textsuperscript{24} and other psychosomatic disorders. Furthermore, different animal models demonstrate that chronic stressful experiences such as prolonged immobilization, housing in dominance hierarchies or early maternal separation can remodel hippocampal neurons\textsuperscript{25}. In escapable stress, commonly called restraint stress or immobilization stress (IS) is an easy and convenient method to induce both psychological (escape reaction) and physical stress (muscle work), resulting in restricted motility and aggression and is believed to be the most severe type of stress in rodent models that is comparable to humans\textsuperscript{26,27}. So, the current study is designed to reveal the impact of immobilization stress and the effect of enriched environment on the stress induced animals. When
analysing the growth pattern of the animals, it could be understood that IS might suppress the intake of food and water, probably due to the release of corticotrophin releasing hormone (CRH) from the hypothalamus which is a mediator of stress-related suppression of food intake. Our observation is well supported by reports of Britton et al. which provided an impression about CRH and loss of appetite; Britton et al. had demonstrated that CRH causes (i) decrease in the number of approaches to the food pellets and (ii) decrease of food eaten per approach. The observation of lesser consumption by IS group of rats and a comparative more consumption by IS+EE group of rats could be due to the modulating effect on CRH induced appetite-suppressing action. The observed varied behavioural pattern of IS exposed rats, when compared to the control ones, reveal the IS mediated memory loss, anxiety, and even the motor functions. The rats exposed to EE (IS+EE group), did not register the same variations as those of the IS group implying that an abnormal behaviour of an animal under stress could be counteracted by an enriched environment.

According to the physical stress theory, the nerve will undergo predictable physiological and structural modifications proportional to the levels of reduced stress and the duration of immobilization. Immobilization induces cell biological changes in axons and axon terminals. The structural changes of myelin and nerve connective tissue layers could modify the ability of nerves to tolerate subsequent physical stress. Myelin is a very special membrane with a unique molecular composition and architecture, with very few protein components. Earlier, researchers believed that the myelin was a simple and inert membrane which was devoid of any significant biochemical functions. Later, it was found to contain enzymes which were essential for various functions. The primary enzyme in myelin membrane is Na\(^+\)-K\(^+\) ATPase, and it is responsible for active transport of sodium and potassium ions in the nervous system, maintaining and re-establishing, after each depolarization, the electrochemical gradient necessary for neuronal excitability and regulation of neuronal cell volume. Erecinska et al. reported that Na\(^+\)-K\(^+\) ATPase are present in high concentrations in brain cellular membranes and it consumes about 40-50% of the ATP generated in this tissue. Furthermore, Hokin et al. observed that the pathophysiology of some psychiatric disorders was believed to be related with some perturbation of ion homeostasis, and they showed that Na\(^+\)-K\(^+\) ATPase activity was decreased in patients with depression and other psychiatric disorders. Gamaro et al. reported that there was decreased activity of Na\(^+\)-K\(^+\) ATPase in the hippocampus of animals subjected to chronic stress. Hoyer et al. claimed that the impairing effects of chronic stress could result from a disruption of brain energy metabolism and this energy deficit could, therefore, affect the activity of Na\(^+\)-K\(^+\) ATPase. In the current study also, we report that the activity of ATPases was decreased in the stress induction; however, the enriched environment could mitigate the IS induced ionic disturbances as evident from the improved activities of ATPases. Thus, it was presumed that the disrupted energy metabolism owing to IS could be challenged by an exposure to an enriched environment. The decreased activity of 5′-nucleotidase observed in IS group of rats did highlight that the nucleoside mediated actions via adenosine A\(_{2A}\) receptor (Elizabetetetal. 2010) could be altered by stress. Surprisingly, EE could not alter the activity of 5′-nucleotidase in MB region but could increase that of FB well beyond the control values. We interpret that such observations might have implications in purinergic based glial-neural cell interactions suggested by Alan North and also in the adenosine influenced GABAergic pathways as reported by Saransaari et al. which however do require more detailed investigations.

Torres et al. had reported that chronic restraint stress reduces the levels of dopamine and serotonin transmission in the hypothalamus. Similarly, we also speculate that could lead to disturbances in the serotonergic and dopaminergic pathway as evident from the levels of serotonin and dopamine (Fig 6A). A report by Kusek et al. had demonstrated that repeated stress resulted in the modification of glutamatergic transmission in the frontal cortex. Similarly, Alok et al. had shown that the GABA levels could be decreased, however, with an significant increase in the glutamate levels in hypothalamus, thalamus, and cortex of the brain of the stressed rats. These reports are in agreement with the present study in which IS group of rats had depicted increased levels of glutamate and attenuated levels of GABA when compared to those of the controls. IS+EE group of rats were found to be devoid of such intensified stress-mediated effects indicating the effectiveness of EE. The only observation with lesser clarity at present is that the GABA levels could not be changed in FB by EE. But, EE exposure did result in a comparative reduction in
the levels of cortisol, which were higher in IS group, a key observation implying that there exists a prolonged activation of hypothalamic pituitary adrenocortical (HPA) axis in this group but not in the former. The present results concord with the investigations from Liu et al.\(^1\), in which they reported that serum corticosterone levels in mice exposed to chronic restraint stress for 21 days (242.55 ng/mL) increased significantly compared with those in unstressed control mice (114.45 ng/mL). Similarly, Bhat et al.\(^2\) reported plasma cortisol levels were significantly increased in rats subjected to chronic repeated restraint stress for 14 days. Also, Yin et al.\(^3\) reported that rats exposed to unpredictable stress for 21 days similarly showed increased levels of cortisol.

Myelin has high levels of lipid components (70–75% of its dry weight) which are unusually high when compared to other eukaryotic plasma membranes. The molar ratio of lipid content of myelin (2:2:1:1 for cholesterol/phospholipid/galactolipid/plasminogen) discriminates myelin from other membranes.\(^4\) Enforced immobilization causes physical and emotional stress and could induce the release of free radicals. Subsequent exposure of lipids in the membrane to free radicals stimulates the process of lipid peroxidation (LPO). The cascade of (LPO) further produces reactive oxygen species which could lead to extensive damage to the membranes and organelle of the cell\(^5\) thus to the function of tissue. In brain tissue, about 70% of the lipids are concentrated in myelin and cholesterol:phospholipid:galactolipid:plasminogen ratios vary and contribute to the function of myelin.\(^6\) Changes in the levels of cholesterol which constitutes about 27% of myelin lipids would be reflected in altered membrane fluidity. Stress induced group of rats both in FB and MB regions exhibited reduced cholesterol and phospholipid content which suggests that stress could have resulted in the impairment of salutary conduction and slow nerve impulse transmission. IS+EE group showed increased levels of phospholipids (both in FB and MB) when compared to those of stress induced rats (Fig. 8). But interestingly we had observed that EE could not exert a positive effect on IS induced variations in the levels of cholesterol. Thus, it was presumed that the enhanced environment effect could protect one of the major components of myelin lipid (PL).

Many studies in brain regions such as the hippocampus,\(^7\) the prefrontal cortex (PFC),\(^8\) and the amygdala\(^9\) have well established that prolonged exposure to stress triggers deleterious effects on the brain structure and function. The implications of these stress-induced alterations are multiple, with an impact on learning and memory\(^10\) decision making\(^11\) and emotional responses.\(^12\) In our study, IS+EE group did provide an impression that thermic energy balance could be restored by EE along with retrieval of disturbed neurotransmission as evident from NT and enzyme profiles. Thus, it could be summarized that the structural and functional variations on IS could be altered by exposure to EE.

**Conclusion**

The results of this study provide additional evidence on how restraint stress could affect the physiology of the organism and emphasize the importance of biochemical, hormonal and behavioural adaptations; it becomes obvious from the study that IS+EE has a positive impact on myelin contents. We showed that IS+EE could impart changes in the forebrain (that is primarily cognitive in function) and more significantly in midbrain regions thereby opening up avenues for further tuning of future investigations on valid targets such as myelin of different regions of the brain in stress biology.

**References**

3. Sapolsky RM, Glucocorticoids and hippocampal atrophy in neuropsychiatric disorders. *Arch Gen Psychiatry*, 57 (2000) 925.
32 Marcianak M, Morphometric ultrastructural evaluation of the axonal endings in the neuromuscular junctions of pigeons after long lasting limitation of movement ExpPathol, 23 (1983)27.
33 Baranski S & Marcianak M, Stereological ultrastructural analysis of the axonal endings in the neuromuscular junction of rats after a flight on Biotopunik 782, AviatSpaceEnvironMed, 50 (1979)14.


