Metabolic Brain Disease (2019) 34:875–887
https://doi.org/10.1007/s11011-018-0370-8

ORIGINAL ARTICLE

Enriched environment modulates behavior, myelination and augments molecules governing the plasticity in the forebrain region of rats exposed to chronic immobilization stress

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Received: 15 September 2018 / Accepted: 11 December 2018 / Published online: 2 January 2019

Abstract

Recently, several reports on chronic stress have shown that prolonged exposure to stress contributes to psychological and neurological complications. However, the impact of stress-induced alterations in myelination remains to be unexplored. Therefore, in the current study, the rats were subjected to immobilization stress (IS) followed by enriched environment (EE) and the behavioral, neurochemical changes pertaining to neuronal survival pathway, in addition, to the ultrastructural changes in myelin in forebrain (FB) region of rats were analyzed. Immobilization stress-exposed rats (4 h/day IS, for 28 days) exhibited increased anhedonia, anxiety, immobility, and reduced social interaction, which could be reflected in increased levels of corticosterone. In contrast, exposure to EE (4 h IS+2 h EE/day, for 28 days) was found to minimize anhedonic state, supress the depressive-like features, enhance social interaction and also reduce the levels of corticosterone. The ultrastructural changes in the FB region of the brain revealed that IS group showed enhanced g-ratio indicating decreased myelin thickness, while EE group exhibited reduced g-ratio manifesting increased myelination. Further, the study revealed that IS exposed group showed decreased levels of NGF, TrkA, PI3K, AKT, ERK, CREB, and MBP in FB regions whereas EE group could preserve normal protein and *mRNA* levels of these neuronal survival molecules. The results from this study suggest that EE exerts a positive impact by improving myelination in rats exposed to chronic immobilization stress.

Keywords Myelination · Corticosterone · Neurotrophic growth factor · Myelin basic protein · Enriched environment · Immobilization stress · Behavioral plasticity

Introduction

Globally stress is one of the most common disorders disturbing the quality of life of millions of people (Sin et al. 2015). Chronic stress can hinder normal homeostasis by disturbing the structural integrity and neuronal conduction (Newcomer et al. 1999) (Sarabdjitsingh et al. 2017). Chronic exposure to stress has been shown to induce symptoms of anxiety, depressive-like phenotypes and cognitive impairment in rodent models (Beauquis et al. 2010). Chronic immobilization stress (IS) is the most validated model to induce both psychological and physiological stress in rats (Bhagya et al. 2011). Thus, it was proven that the prolonged exposure to stress has negative impact on the animal behavior (Willner 2017), HPA axis (Martin et al. 2011) and brain plasticity (Radley et al. 2015).

Myelin, which is produced by oligodendrocytes in the Central Nervous System (CNS), wraps the axons of neurons. Myelination has the primary function of faster conduction of electrical impulses along the axon, which are necessary for signal coordination across long distances (Freeman et al. 2016; Hartline and Colman 2007). At the beginning of this decade, a report showed that adult rat brain function could be attained only after myelination was complete and this highlights the importance of myelin in physiology (Kolb and Gibb 2011) and in the functioning of neural signaling. Very recently, many works have been focused on the importance of

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myelin and its conduction (Bellesi et al. 2017; Miyata et al. 2016). Reports from both human (Scholz et al. 2009) and rodent (Blumenfeld-Katzir et al. 2011; Sampaio-Baptista et al. 2013), as well as mouse knockout models (McKenzie et al. 2014), and neuroimaging studies revealed that white matter has a dynamic and experience-dependent plasticity contributing to learning and cognitive function in the adult brain. Also, evidence from human samples strongly suggests that reduced white matter content is seen in patients with major depressive disorders (Yang et al. 2015).

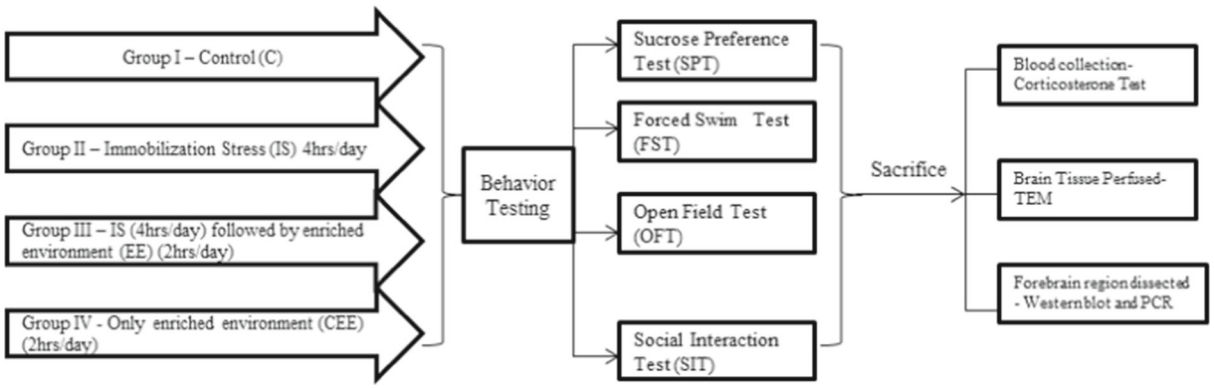
The neurotrophins play a vital role in regulating the adult neurogenesis and neuronal functioning (Banerjee et al. 2012). The nerve growth factor (NGF) is critical for the survival and maintenance of sympathetic and sensory neurons. It supports proliferation and differentiation of oligodendroglial progenitors and regeneration of oligodendrocytes (Althaus et al. 1984). It activates the TrkA receptor and functions as a signaling molecule (McAllister 2001). It has been suggested that activated Trk receptors at the cell surface promote cell survival signaling by activating the PI3K/AKT pathways while Trk receptors signaling from the internalized endosomes help in differentiating the cell by activation of the MEK/ERK pathways and regulating CREB (Zhang and Guan 2000).

Enriched environment (EE) is a powerful environmental factor that contributes to enhancement of the cognitive functioning and neurogenesis in brain (Speisman et al. 2013). EE favors the enhanced brain plasticity in epileptic rats (Fares et al. 2013), improves cognition in animal models of neurodegenerative diseases (Salmin et al. 2017; Mora 2013; Schloesser et al. 2010). Despite wider investigations, the protective and myelinating effects of EE on stress-related disorders remains elusive and little is known about the mechanistic aspects of EE on exposure to stress-induced alterations in the rat brain. Therefore, we hypothesized that enriched environment could modulate the behavioral plasticity in forebrain region of rats by governing the myelination and signaling pattern of molecules involved in chronic immobilization stress. The present study attempts to investigate the effect of EE in mitigating the severity of stress by assessing depressive-like features, especially the anhedonic state, anxiety and social interaction in rats and to determine whether the ultrastructural changes in myelin and key signaling molecules related to myelination could be involved in the behavioral alterations.

Materials and methods

Animals and experimental design

Male Wistar albino rats (200–250 g) were procured as per the norms of the IAEC (Institutional Animal Ethical Clearance-205/GO/ReBi/SL/2000/CPCSEA; IAEC No:02/15/2017) from the Kings Institute, Guindy, Chennai. They were maintained at the animal house facility in the University of Madras, Taramani Campus, and acclimatized to animal house conditions, and the animals were fed with commercial rat chow (Hindustan Lever Ltd., Bangalore, India) and water ad libitum. The animals were divided into 4 groups; group I: served as control (C); group II: Immobilization Stress (IS); group III – Immobilization Stress + Enriched Environment (IS+EE) and group IV: Enriched Environment alone (CEE). The animals were subjected to experimental period for 28 days and at the end of the experiment, the animals were subjected to behavioral assessment. After which, the animals were sacrificed by cervical decapitation and the blood was collected for corticosterone assessment and forebrain tissue was dissected and used for further analyses.



Experimental strategy

Immobilization stress (IS) induction

Adult rats were subjected to chronic IS in a restrainer (4 h/day, 10 am – 12 pm and 4 pm – 6 pm) (Wood et al. 2008). After the IS exposure, the rats were returned to their respective home cages with free access to food and water.

Enriched environment (EE)

Rats were exposed to an EE in a large cage (120 cm × 75 cm × 75 cm), where the floor was covered with the husk on which colorful toys of different shapes and objects were kept. Additionally, re-arrangeable tunnels, pipes, and running wheels were also placed in the enriched environment. Each day the objects were changed in order to maintain the novelty (Moncek et al. 2004). EE exposure was given to two groups of rats (group III and group IV) for 2 h per day (12 pm – 1 pm and 6 pm – 7 pm), where group III rats were exposed to the EE after IS, and group IV rats was exposed only to the EE and it served as a positive control group. The rats were returned to their respective home cages after the experimental period.

Behavioral analyses

Sucrose preference test

In sucrose preference test (SPT), the experimental animals ($n = 12$, each group) were put through training and testing sessions. During the training session, the rats were provided with two bottles of water; one with 1% of sucrose and another with normal tap water. The animals had free access to both these bottles for 48 h. The volume of water and sucrose consumption was measured every 24 h duration, and the positions of the two bottles were changed in order to avoid place preference. After the training session, the rats were deprived of food and water for a period of 12 h. The testing session lasted for 2 h. The amount of sucrose liquid consumed was calculated using (Mateus-Pinheiro et al. 2014)

$$\text{sucrose preference} = \text{sucrose water consumed}/\text{total liquid consumed} \times 100\Big)$$

Forced swim test

In forced swim test (FST), the experimental animals ($n = 12$, each group) were initially given training, in which they were placed in a cylindrical tank (50 cm diameter and around 65 cm height) containing water to a height of around 40 cm, and the animals were allowed to swim in the water for 10 mins. After the training session, the animals were dried and returned to their respective home cages. On the next day, the rats were put in the cylinder containing water and allowed to swim for 10 mins. The test session was recorded and the animals were given scores. The parameters analyzed for scoring were the frequency at which the animals were immobile (if the rat floats without any movement except for those necessary for keeping its nose above the water), struggling (if the rat struggles with quick movements by its forelimbs with the front paws breaking the water surface were observed) and swimming (if the movement of forelimbs or hindlimbs were in a paddling fashion was observed).The calculations were based on the activities of the experimental animals during the testing session in water and the graph was plotted (Can et al. 2012).

Open field test

In the open field test (OFT), the experimental animals ($n = 12$, each group) were left on the periphery of an open arena of dimensions 72 cm × 72 cm × 36 cm, which was divided equally into 16 squares, and the animals were allowed to move freely for 15 mins, and the video was recorded in a standard lit room. The parameters assessed included locomotor activity (in centimeters) and anxiety-like behavior in the experimental rats. The results were calculated and graph was plotted using the percentage of total time spent by the experimental rats in the central, peripheral regions and freezing position. (Nandini and Dubhashi 2010).

Social interaction test

In social interaction test (SIT), the experimental animals ($n = 12$, each group) were placed in an open arena of dimensions 72 cm × 72 cm × 36 cm. The animals were subjected to a new cage mate and the duration of time spent by the animal in socially exploring a new cage mate was recorded for a period of 10mins in an open field chamber. (Kaidanovich-Beilin et al. 2011).

Levels of stress assessed by corticosterone

At the end of the experimental period the animals ($n = 6$, each group) were anesthetized and one milliliter of blood sample was collected in EDTA coated vacutainer by a retro-orbital puncture between 9:00 am to 10:30 am. The plasma was separated by centrifugation at 2500 rpm for 5 min. The plasma was used for the estimation of corticosterone by competitive Enzyme Linked Immunosorbent Assay (ELIZA) as per the kit method by the manufacturer's standardized protocol (Code: ITER0859; Batch No: R0859C024). The readings were taken in ELIZA plate reader and the levels of corticosterone were calculated in ng/dl of plasma.

Myelin staining

Three rats from each group were anesthetized and the brain was perfused followed by which the myelin region was

analyzed by Luxol Fast Blue staining (Snodgress et al. 1961). The forebrain sections were hydrated in 95% alcohol and incubated in 0.1% Luxol Fast Blue (LFB) solution overnight at 58 °C. The sections were then differentiated in a lithium carbonate solution (0.05%), alcohol (70%) and mounted in Distyrene Plasticizer Xylene (DPX). The slides were visualized in the light microscope. The percentage of myelination in FB regions of the experimental rats was quantified by ImageJ software.

Transmission Electron microscopy (TEM)

Six rats in each group were anesthetized using ketamine (90 mg/Kg body weight) and xylazine (10 mg/Kg body weight) and underwent thoracotomy, perfused with saline through the ascending aorta, and then the tissue was fixed in 4% paraformaldehyde and 2% glutaraldehyde (Electron Microscopy grade). After perfusion, the brain was dissected and the forebrain region was cut into 1 mm^3 blocks and fixed in TEM grade 2.5% glutaraldehyde, followed by the embedding of the tissue. The embedded tissues were subjected to ultrathin sections of 50 nm thickness and then stained with uranium and lead. The grids were visualized under a transmission electron microscope (Hitachi H-600, Tokyo, Japan), and the myelinated areas in the forebrain regions were photographed. Electron micrographs were subjected to quantification, where a minimum of 20 random axons per field (minimum of 5 fields per group) from FB region was observed. The axons within the image were quantified using ImageJ software. The g-ratio was calculated by measuring the ratio of the axon diameter to the diameter of the myelinated fiber (Chomiak and Hu 2009) and the values of g-ratio were plotted in a graph.

$$g\text{−ratio} = (\text{Axon diameter}/\text{Myelinated fiber})$$

mRNA RT-PCR studies

Total RNA was isolated from 100 mg of brain tissue using TRIsoln (Merck-Genei), and cDNA was converted from *mRNA* using cDNA conversion kit (Takara). Genes for β-Actin, NGF, TrkA, PI3K, AKT, CREB and MBP were amplified from 20 ng of cDNA from each group using the following primers (Sigma): for β-Actin: FP: -5′ -CCCTAAGG CCAACCGTGAAA- 3'and RP: -5′ -TCCGGAGTCCATCA CAATGC-3′ for NGF:FP: -5′ -CATCGCTCTCCTTC ACAGAGTTT- 3′ and RP: -5′ -TGCCTGTACGCCGA TCAAAA- 3′, for TrkA: FP: -5'-CAAAGCCGTGGAAC AGCATC- 3′ and RP: -5'-TCTCATTGAGCACAGAGCCG - 3′, for PI3K: FP: -5'-AGGTCACTGTTTCTCTGCGG-3′ and RP: -5'-TGTCGTGAACGACCTTCCTG-3′, for PI3K: FP: -5'-TGCATAACCTGCAAACACTGC- 3′ and RP; −5' CCAGAAAGGTCCCATCAGCA- 3′, for AKT: FP: -5'-AGGAGGTCATCGTTGCCAAG-3′ and RP: -5'-GTTCTCCAGCTTGAGGTCCC- 3′, for CREB: FP: -5′ -CATGGACTCTGGAGCAGACA- 3′ and RP: -5′ -GGGCTAATGTGGCAATCTGT- 3′, for PACAP: FP:- 5′ – GTCACGCTCCCTCCTAGTTT- 3′ and RP: 5′ – CGCTACACATGGTCATTCGC- 3′ and for MBP: FP: -5′ -TTGGCCACAGCAAGTACCAT- 3′ and RP: -5′ -GTGTGTGAGTCCTTGCCAGA- 3′. PCR conditions were 94 °C for 2 min, 94 °C for 30 s, 52.2 °C for 30 s (34 cycles), 72 °C for 1 min, 72 °C for 3 min (for β-Actin); 94 °C for 2 min, 94 °C for 30 s, 53.3 °C for 30 s (34 cycles), 72 °C for 1 min, 72 °C for 3 min (for NGF); 94 °C for 2 min, 94 °C for 30 s, 53.9 °C for 30 s (34 cycles), 72 °C for 1 min, 72 °C for 3 min (for TrkA); 94 °C for 2 min, 94 °C for 30 s, 53.7 °C for 30 s (34 cycles), 72 °C for 1 min, 72 °C for 3 min (for PI3K); 94 °C for 30 s, 52.6 °C for 30 s (34 cycles), 72 °C for 1 min, 72 °C for 3 min (for AKT); 94 °C for 2 min, 94 °C for 30 s, 54 °C for 30 s (34 cycles), 72 °C for 1 min, 72 °C for 3 min (for CREB); 94 °C for 2 min, 94 °C for 2 min, 94 °C for 30 s, 53.5 °C for 30 s (34 cycles), 72 °C for 1 min, 72 °C for 3 min (for MBP). The products (136 bp for β-Actin, 208 bp for NGF, 101 bp for TrkA, 168 bp for PI3K, 290 bp for AKT, 107 bp for CREB, and 143 bp for MBP) were analyzed in 2% agarose gel. The bands were further quantified using Image J software.

Western blot analysis

100 mg of tissue was homogenized in RIPA buffer (Santa Cruz Biotechnology – Cat. No. sc-24,948), and the homogenates were centrifuged and supernatants were collected. Protein concentrations were estimated by Bradford's method using bovine serum albumin as the standard (Bradford's method, 1976). TrkA, mature NGF protein, PI3K, CREB, MBP and β-actin from 30 μg of total protein homogenate were analyzed by Western blot with primary antibodies: anti-TrkA (140 kDa) (Abcam (1:1000) - Cat. No ab76291), anti-NGF (13 kDa mature NGF) (Santa Cruz Biotechnology (1:1000) – Cat. No. sc-548), anti-PI3K (85 kDa) (Santa Cruz Biotechnology (1:1000)-sc-423), anti-AKT (60 kDa) (Cell Signalling (1:1000)-Cat No.9272), anti-ERK (42 kDa), anti-CREB (40 kDa) (Cloud Clone Corp (1:400)- Cat. No.PAE002Ra01), anti-MBP (14 kDa) (Cloud Clone Corp (1:400)- PAB347Ra01) and anti-β-actin (42 kDa), followed by HRP conjugated secondary antibody (1:1000) for rabbit and mouse(Santa Cruz Biotechnology). Immunocomplexes were detected by 3,3′-diaminobenzidine and normalized with β-actin. The bands were quantified using Image J software.

Statistical analysis Data (expressed as Mean ± SEM) was analyzed using GraphPad Prism 5 software. The data was analyzed by two-way ANOVA followed by Bonferroni's post hoc test and value of $p < 0.05$ was considered statistically significant.

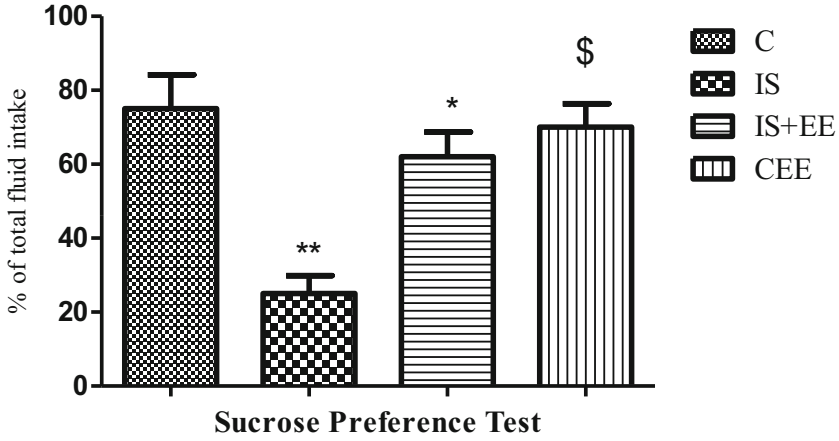


Fig. 1 Anhedonic behavior (expressed in percentage) as exhibited by sucrose preference test in control and experimental groups. [Comparisons were made between C vs IS, IS vs IS+EE and C vs CEE, $**p < 0.01$ vs C, $*p < 0.05$ vs IS, $, non-significant vs C]

Results

The sucrose preference test in the IS group of rats showed that there was significant decrease ($p = 0.006$, F = 14.054) in the preference to sucrose water consumption when compared to control animals. Anhedonic state of decreased sucrose water consumption in IS rats was completely restored to normal on exposure to EE in the IS+EE group of rats ($p = 0.030$, F = 22.37). The CEE group of rats did not show any difference in their sucrose preference (Fig. 1).

The IS exposed group of rats demonstrated longer immobility and struggling time when compared to that of the control group. There was significant decrease ($p = 0.012$, F = 32.123) in the swimming behavior of the IS exposed group of rats when compared to control. IS rats upon exposure to EE showed a decrease in their immobility and struggling, with an increase in their swimming time when compared to those in the IS group. Also, the duration of immobility, struggling and swimming in the CEE group was comparable to those in the control group (Fig. 2).

The group exposed to IS showed a significant decrease in its locomotion and increase in its freezing position when compared to control group as deduced from the number of crossings of the central and peripheral squares ($p = 0.009$, F = 13.865) and the frequency of being immobile in the open field ($p = 0.013$, F = 11.994). The rats in the IS+EE group showed a significant increase in its locomotor activity ($p < 0.05$, F = 40.136) when compared to those of the IS group. No significant variations ($p > 0.05$, F = 1.07) were observed between the rats of CEE group and those of the control (Fig. 3a and b).

The social interaction test was performed to study the effect of the EE on IS-exposed rats. The IS group of rats showed a significant reduction in social interaction ($p = 0.013$, F = 14.385) when compared to control, while the IS+EE group of rats showed a significant increase ($p = 0.05$, F = 26.742) in its social interaction to a new animal when compared to stressed rats. The CEE group showed no significant changes ($p > 0.05$, F = 3.506) in its social interaction behavior when compared to the control group (Fig. 4).

Corticosterone is the main factor that reflects the levels of stress in rats. Figure 5 represents the levels of corticosterone,

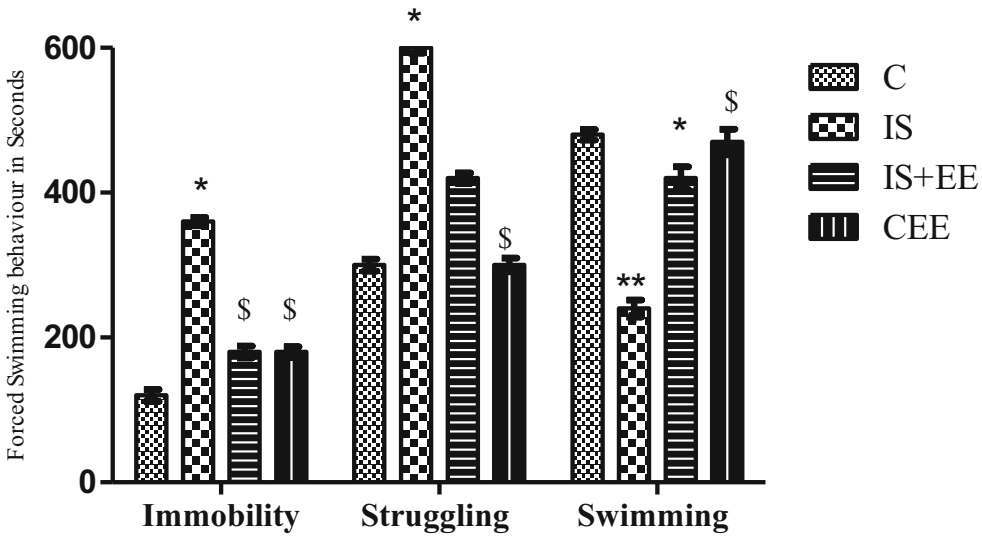


Fig. 2 Depression-like behavior (seconds) assessed by forced swim test in control and experimental groups. [Comparisons were made between C vs IS, IS vs IS+EE and C vs CEE, $*p < 0.05$ vs C, $**p < 0.01$ vs C, $, non-significant vs C]

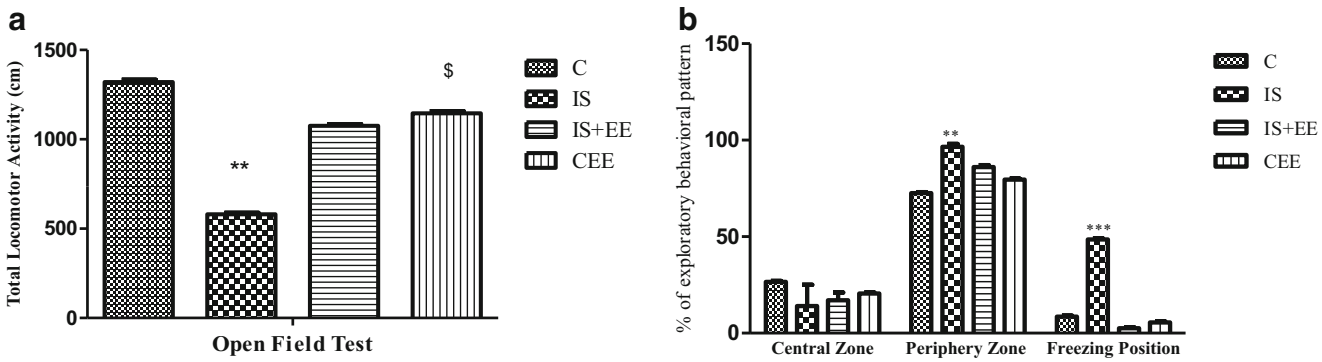


Fig. 3 **a** Total Locomotor activity (cm) in the control and experimental groups of rats. **b** Exploratory behavioral pattern (%) by open field test [Comparisons were made between ***$p < 0.001$ vs C, *$p < 0.05$ vs C, $, non-significant vs C]

in which the IS group showed a significant increase ($p = 0.006$, F = 11.782) in the levels of corticosterone when compared with those of the control group. IS+EE rats showed a marked reduction in the levels of corticosterone when compared to IS group of rats and CEE exhibited non-significant results when compared to control rats.

Luxol Fast Blue staining (Fig. 6a) shows myelination staining pattern as seen in the control and experimental groups, wherein, the chronic stress-exposed IS group shows a significant reduction ($p < 0.05$, F = 43.217) in the myelination when compared to the control group as evidenced in the graph (Fig. 6b). Rats in the IS+EE group have shown increased staining of myelination when compared to the IS group of rats. The CEE group did not show any significant differences when compared with control.

Using transmission electron microscopic (TEM) analysis, the thickness of the myelin sheath (Fig. 7a) and quantification of myelin thickness relative to its axonal diameter (g-Ratio) (Fig. 7b) in the control and experimental groups were analyzed. IS-exposed group of rats exhibited pathological changes in the myelin sheath ranging from abnormal structural features, which included distortion, disintegration or lysis in myelin architecture, to a reduction in myelination. IS+EE group of rats exhibited a compact myelin sheath and increased myelination when compared to the IS group. The g-ratio was determined to demonstrate the specific relationship between the axon diameter and the myelin sheath thickness. It could be evident from the Fig. 7b that g-ratio among the 4 groups was found to be significantly varied. The IS-exposed group of rats showed significantly higher g-ratio ($p = 0.001$, F = 28.229) than the control group of rats. The g-ratios in IS+EE group of rats showed significantly reduced g-ratio ($p < 0.05$, F = 47.503). G-ratios in the CEE group, however, did not show any significant changes in the myelin thickness. The TEM results clearly show that the higher the g-ratio the lesser the myelin sheath thickness and thus, it is apparent that the IS+EE group of rats have increased myelin thickness when compared to the IS group of rats.

The protein and *mRNA* levels of neurotrophic ligand, NGF and its TrkA receptor (Figs. 8a, b and 9), further related neuronal survival and differentiation molecules, including PI3K, AKT, ERK, (respectively, Figs. 8c, d and 9), were significantly reduced ($p < 0.001$, F = 19.83) on exposure to immobilization stress, whereas in the IS+EE group they showed significantly

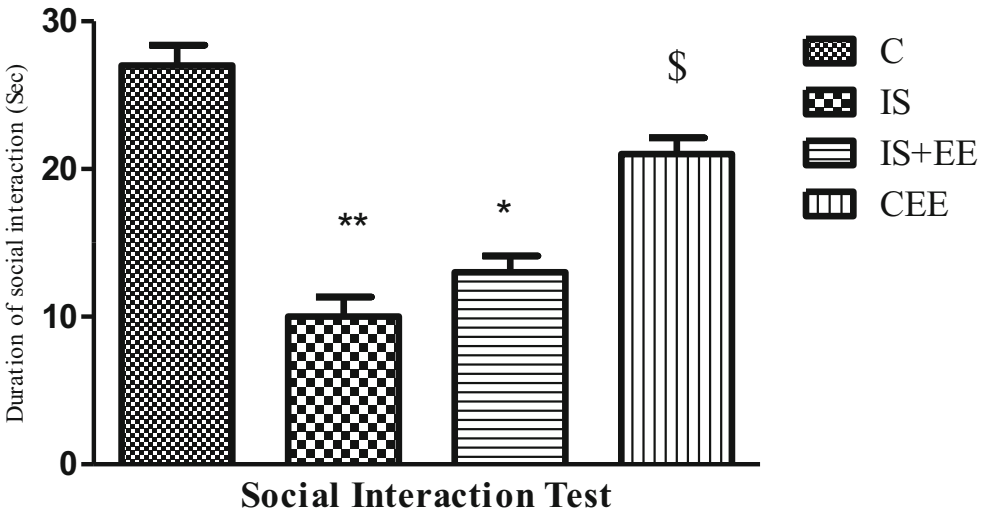


Fig. 4 Social interaction (expressed in seconds) in control and experimental groups. [Comparisons were made between C vs IS, IS vs IS+EE and C vs CEE, **$p < 0.01$ vs C, *$p < 0.05$ vs IS, $, non-significant vs C]

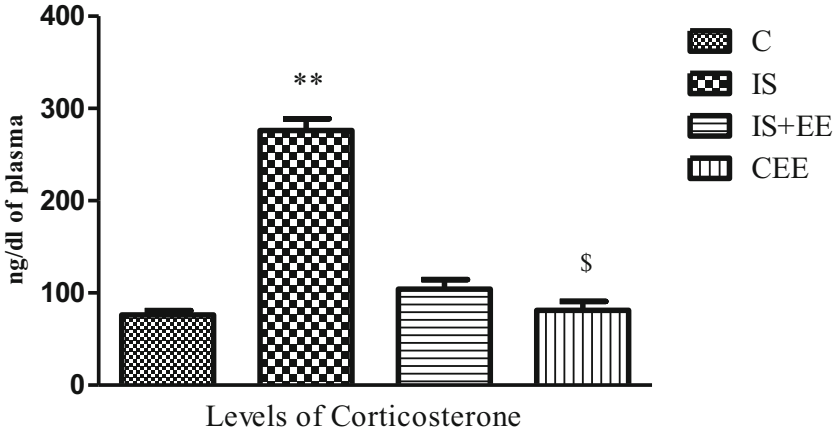


Fig. 5 Levels of corticosterone (ng/dl of plasma) in control and experimental groups ($n = 6$) [Comparisons made between *$p <$ 0.05 vs C, $, non-significant vs C]

higher ($p < 0.05$, F = 26.54) levels when compared to those in the IS group. CEE group did not show any significant difference when compared to control ($p > 0.05$, F = 1.59).

The IS group of rats exposed to chronic stress presented significantly decreased levels of both protein and *mRNA* expression of CREB and MBP ($p < 0.001$, F = 18.13) when compared to those of the control group. IS+EE group, however, showed an increase in the levels of CREB and MBP, which regulates the myelination. The CEE group did not show any significant changes in the protein and *mRNA* levels of CREB and MBP when compared to control (Figs. 8e, f and 9, $p > 0.05$, F = 1.42).

Discussion

The results of the current study demonstrate that the rats subjected to chronic IS exhibit enhanced depressive-like features as evidenced from the FST (Fig. 2) similar to the observation by various authors (Schloesser et al. 2010; Vyas et al. 2002; Anuradha et al. 2008; Bhagya et al. 2016). Increased anhedonia as shown by the SPT (Fig. 1), reduced locomotory, exploratory activity as well as elevated anxiety-like features assessed through the OFT (Fig. 3), similar to that observed by Vyas et al. (2004), and avoidance of social interaction (Fig. 4), all of which are typical indicators of stress in rats were also encountered in our study. On the other hand, our data on EE exposure suggests that it augments the behavioral plasticity in chronic IS in rats by the characteristic improvement in locomotory and exploratory behavior, reduced levels of anxiety, depressive-like features, and anhedonia with active participation in social interaction with new animals. This suggests that EE has a distinctive role in improving the behavioral plasticity in stress exposed group of rats. Earlier, we have reported our observation of decreased learning and cognitive function in the chronic stress exposed group of rats, while on exposure to EE, there was an increased learning and cognitive ability in rats (Vanisree et al. 2016) which are said to be associated with the behavioral plasticity, supporting our current data. Behavioral plasticity refers to a change in an organism's behavior that results from exposure to stimuli, such as in changing environmental conditions (Oliveira 2009). Furthermore, our experimental results support the idea that NGF expression might be involved in behavioral activation.

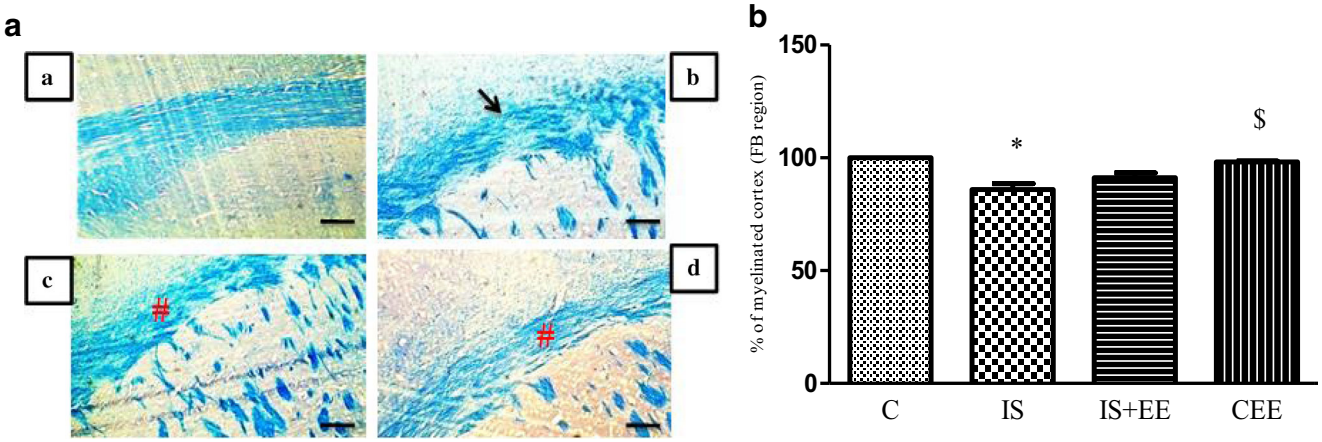


Fig. 6 **a** Microscopic appearance of myelin in forebrain region by Luxol Fast Blue staining, a- Control, b-IS, c-IS+EE, d-CEE. Arrows (→) indicate emaciated lining of white matter and the symbol, (#) denotes normal architecture of white matter. [Scale bar = 40X]. **b** Bar graph representing the percentage of myelinated cortex in the FB region. [Comparisons made between *$p < 0.05$ vs C, $, non-significant vs C]

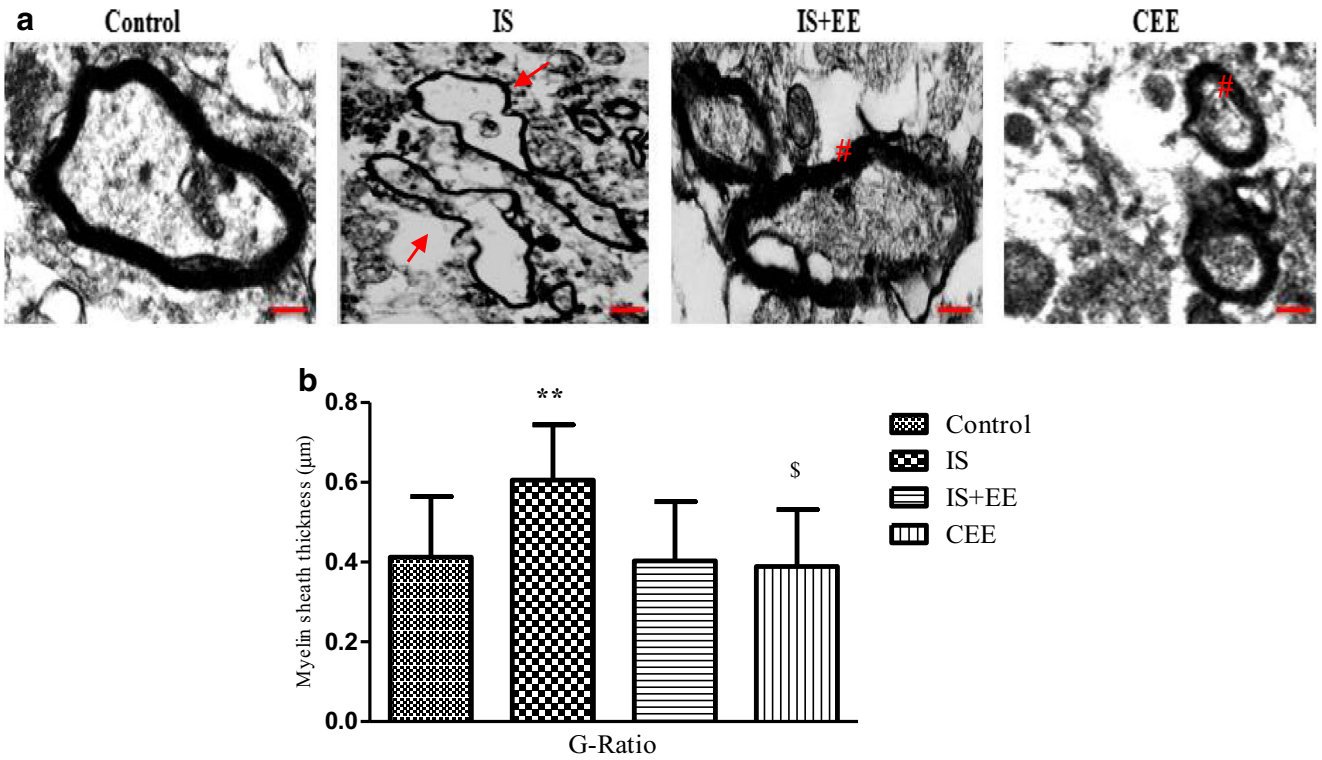


Fig. 7 **a** Ultrastructural imaging of perfused rat brain in control and experimental groups of rats. Arrows represent varied architecture of myelin and # indicates normal myelination (Scale bar-1 μm). **b** Graphical representation of G-Ratio in the control and experimental group of rats. [Comparison made between $^{**}p < 0.01$ vs C, $, non-significant vs C]

NGF was shown to play a critical role in stress-mediated changes in behavioral responses, in which social stimuli suddenly become relevant and produce longlasting behavioral alterations in adult rats (Isaev et al. 2017). Spillantini et al. (1989) had revealed that increased NGF *mRNA* and protein levels in hypothalamus, especially in the paraventricular nucleus exhibits aggressive behavior in rats.

Varied levels of NGF are linked withchanges in structural architecture, such as dendritic spine formation or collateral sprouting, leading to alterations in the neural connections in the mature brain (Alleva and Santucci 2001). Wion et al. (1985) hypothesized that NGF could affect the levels of other peptides or hormones present in the hypothalamus (Wion et al. 1985). From these evidences it is obvious that hypothalamic NGF levels could be responsive, or modified by the stimuli of a psychological nature, which is most likely related with anxiety and fear. Since hypothalamus is concerned in the maintenance of physiological homeostasis, it could be possibly the role of hypothalamic NGF that affects, or cooperates with, hormones and/or neurotransmitters eventually integrating the behavioral and neuroendocrine responses (Alleva and Aloe 1989).

Likewise, the experience-dependent white matter plasticity requires mechanisms through which activity along an axon can alter the structural properties of that axon. These alterations in structure would, in turn, be related to the changes in the functional properties of the axon, thus giving rise to alterations in behavior. Therefore, behavioral plasticity is closely associated with the structural and functional properties of the myelinating axon. This is in agreement with the studies by Fields (2015) implying that the alterations in the structural properties of the axon, such as myelin, axon diameter, or internode length, give rise to changes in physiological properties such as conduction speed, which has an impact on rodent behavior. Similarly, in our studies also, we see that myelination and behavioral plasticity are intertwined. In the case of the IS group, we showed that there was reduced myelination as well altered behavior; on the other hand, the EE augmented myelination and behavioral plasticity. This result is in accordance with the studies by Zhao et al. (2012) who had reported that when the adult rats were subjected to an EE for 4 months, there was a higher volume of myelinated fibers and myelin sheaths in the corpus callosum of 14-month-old rats. This clearly highlights that social and physical environments play a major role in the organization of white matter structure in adult rats. Interestingly, in support of our data, a study by Makinodan et al. (2012) suggested that social experience play a vital role in the development of the prefrontal cortex of mice during their developmental stage.

We have reported an elevated levels of corticosterone (Fig. 5) in IS exposed rats and this evidence is in accordance with the previous other investigations (Koehl et al. 2002; Morley-Fletcher et al. 2003). Interestingly, our reports on the anxiety-like features and enhanced secretion of corticosterone

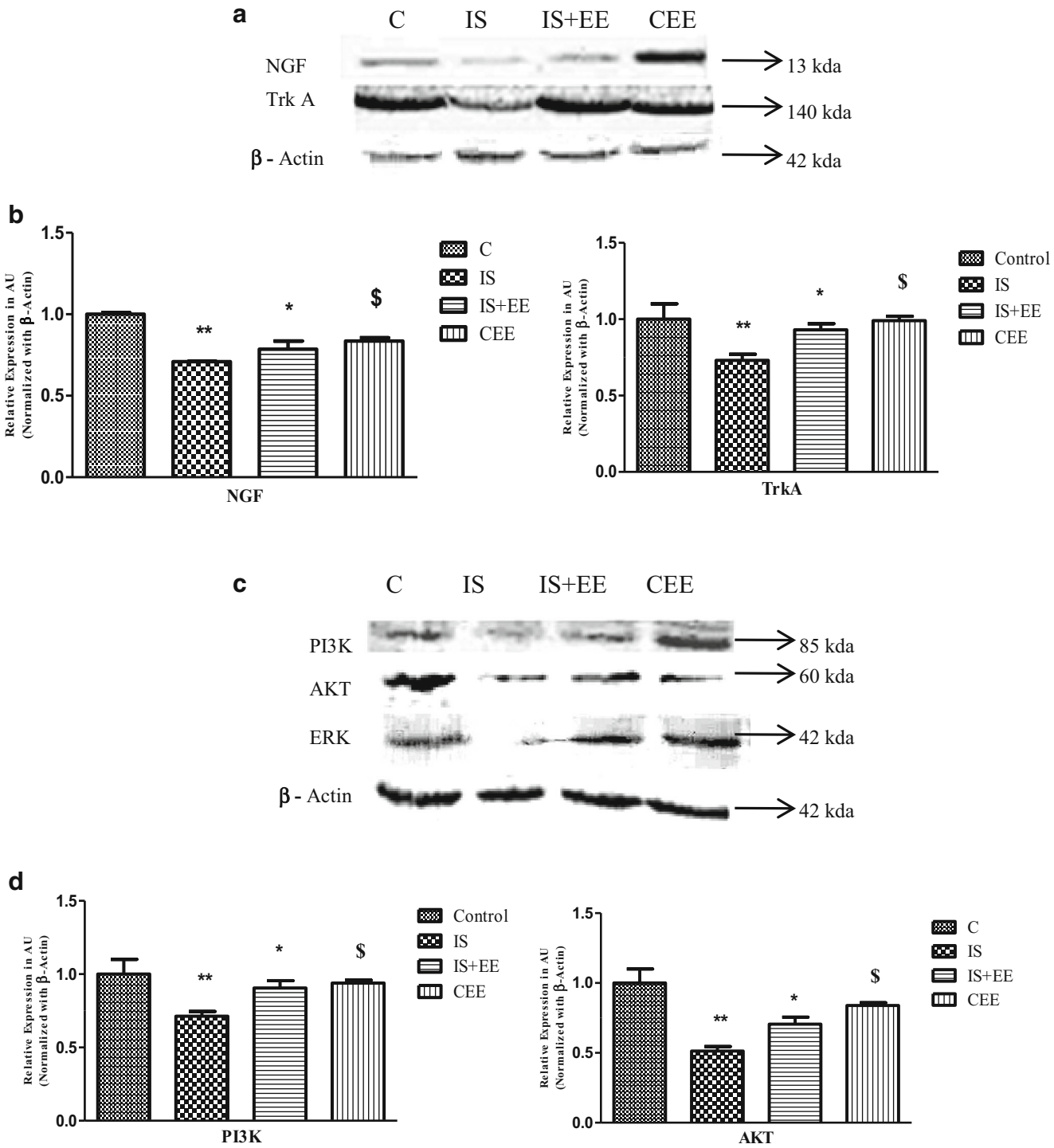


Fig. 8 **a**, **c**, **e** Immunoblots of NGF, TrkA, PI3K, AKT, CREB and MBP in control and experimental groups of rats. **b**, **d**, **f** Densitometric data on the levels of protein as arbitrary units and the values were normalized with β–Actin. [Comparisons were made between C vs IS, IS vs IS+EE and C vs CEE, $^{**}p < 0.01$ vs C, $^{*}p < 0.05$ vs IS, $, non-significant vs C]

might be due to chronic exposure to immobilization stress and this could be brought to normalcy by EE, thus, highlighting the importance of EE.

The myelination was evaluated by Luxol Fast Blue staining (Fig. 6) and g-ratio assessment from TEM analysis (Fig. 7). The levels of the myelin basic protein and the structural integrity of myelination were found to be affected in the stress exposed group of rats, an observation that is similar to that of Liu et al. (2012) who did show that myelination could decrease in socially isolated rats (for 8 wks). Interestingly, in

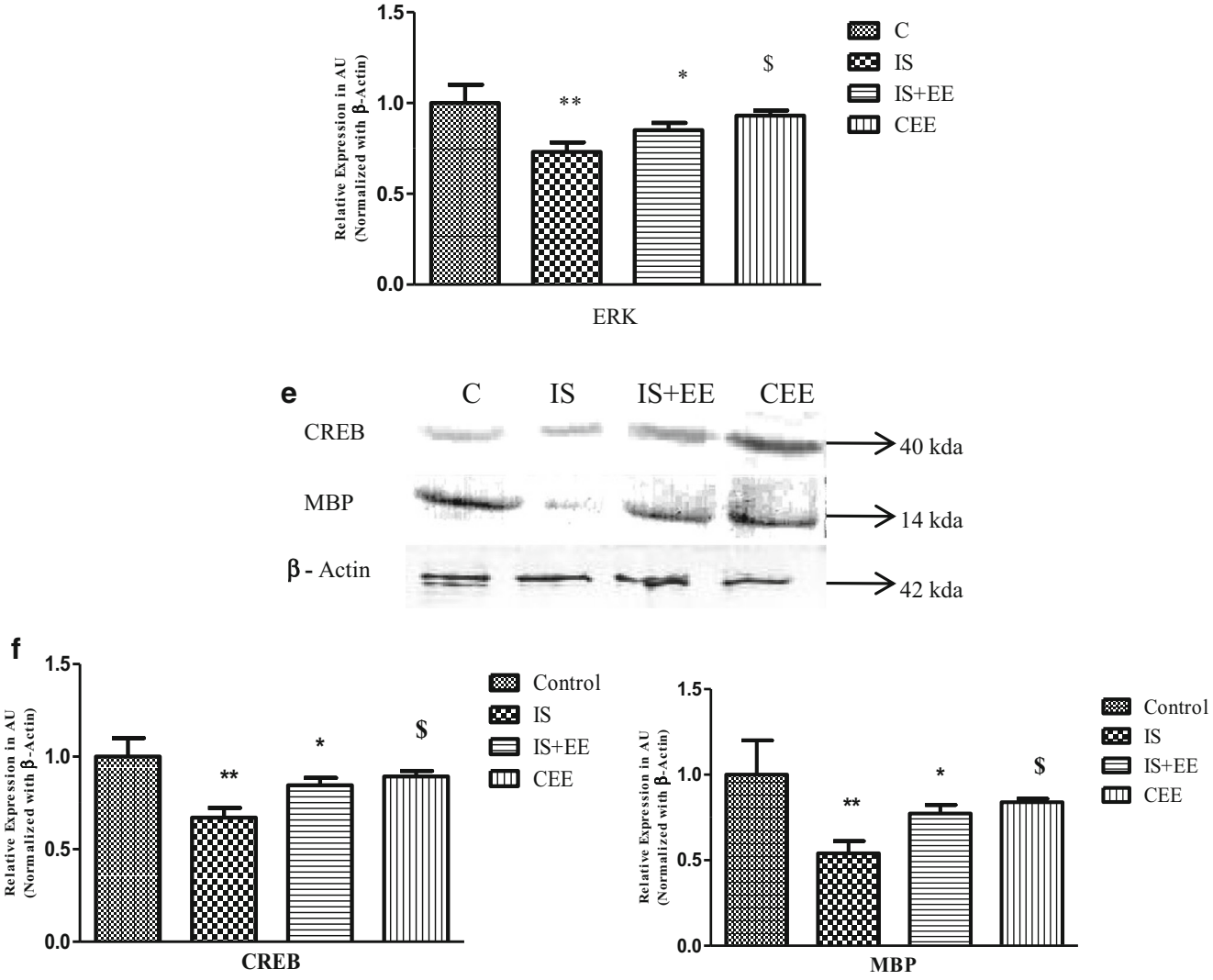


Fig. 8 (continued)

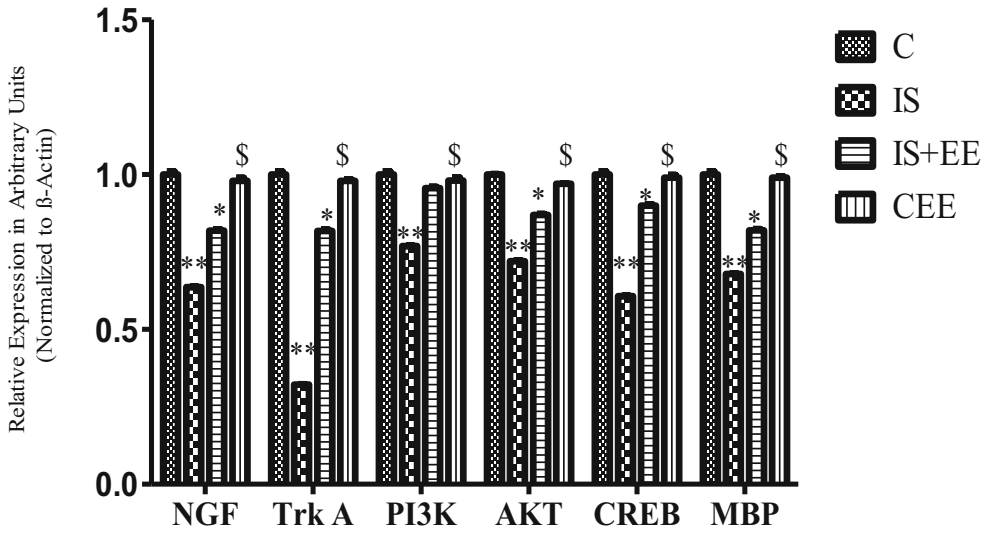


Fig. 9 Densitometric data on the levels of *mRNA* as arbitrary units and the values were normalized with β–Actin. [Comparisons were made between C vs IS, IS vs IS+EE and C vs CEE, $^{**}p < 0.01$ vs C, $^{*}p < 0.05$ vs IS, $, non-significant vs C]

our IS+EE group, we were able to encounter decreased levels of g-ratio that was in turn reflected in enhanced myelination. From this, we deduce that EE promotes myelination in adult rats. Recently, there have been reports on oligodendrocyte plasticity and preexisting oligodendrocytes that remodel the myelination in adult rat brain, supporting our evidence. Gibson et al. (2014) did neuronal stimulation studies and reported that behavior and myelin thickness alternations are related to socially isolated animals. Rivers et al. (2008) critically analyzed and reported that there are more than 20% of myelinating oligodendrocytes present in the adult brain. From these reports and our results, it could be manifested that enhanced environment mediates myelination in adult rat brain. A recent study also claimed that oligodendrocyte plasticity can enhance white matter in response to the experience gained by the animal in complex wheel running (Xiao et al. 2016). Thus, it could be inferred that myelination and behavioral interactions are inter-related.

EE is crucial in regulating the neuronal circuits as it stimulates sensory, motor and cognitive stimuli that are vital for normal brain development (Porsolt et al. 1978). Our preliminary reports on EE also showed that IS+EE has a positive impact on myelin contents (Vanisree et al. 2016), which may improve cognitive functioning in adult rats. In our current investigation, we targeted the neuronal survival and differentiation molecules of NGF signaling in the brain and it was evident that EE could restore the levels of NGF and its downstream signaling molecules (Figs. 8 and 9) including TrkA, PI3K, AKT and ERK where, PI3K/AKT and ERK are responsible for neuronal survival and differentiation, respectively. The binding of NGF to TrkA receptor leads to activation of tyrosine kinase activity and the activated TrkA triggers signal transduction cascades including MAPK pathway, PI-3 K pathway and PLC pathway (Katz et al. 2007). Reports furnished by investigators revealed that transgenic mice with over activation of PI3K/AKT plays a pivotal role in increased myelin thickness (Flores et al. 2008; Goebbels et al. 2010; Harrington et al. 2010). Similarly, CREB is implicated in myelination via the neurosurvival pathway. Afshari et al. (2001) suggested that CREB might be a mediator of neuroligand and growth factor signals which activates multiple signal transduction pathways via cAMP as a secondary messenger in stimulating the maturation of oligodendrocytes and myelination in in vitro model.

Previous reports have elucidated that PI3K activation couples with CREB signaling, leading to the upregulation of myelin-specific molecules in oligodendrocytes (Jana et al. 2018). Myelin basic protein (MBP) is the most abundant protein found in the myelin membrane, which contains about 30% of all the myelin proteins in the CNS (Braun 1984). It has been reported that MBP determines the structural integrity of the myelin sheath and it is called the "executive" molecule, which is responsible for many signaling interactions. In the current study, the levels of MBP in IS+EE exposed group was found to be increased when compared to that of stressed group of rats. Also the upregulation of CREB results in enhanced myelination activity in the EE exposed rats (Figs. 8 and 9). The stress exposed group of rats exhibited significant reduction ($p < 0.05$) in the levels of NGF, TrkA, PI3K, AKT, ERK, CREB and MBP when compared to those of control. Our observation is supported by a report showing the predominant role of ERK1/ERK2 signaling in vivo in promoting rapid myelin growth and increased myelination (Ishii et al. 2012). Reports furnished by Jeffries et al. (2016) have also shown that conditional upregulation of the cellular signaling pathway in adult mice triggered the elevated myelin thickness, which ensures faster nerve conduction and increased hippocampal-dependent emotional learning.

It could be perceived from our results that the downregulation of NGF and TrkA could regulate the PI3K/AKT pathway, including the levels of CREB thus downregulating the expression of myelin specific molecule (MBP) in stress exposed group of rats. From this, it is conceivable that induction of psychological stress impacts the signaling cascade, in which the NGF plays a vital role in psychological stress induced 'heterostasis', the means by which an individual tries to remove a stressor and regain the condition of 'homeostasis' that existed prior to the exposure to the stressor (Li et al. 2016). EE could mitigate the severity of stress perhaps by restoring the homeostasis, which could be mediated via exosomes, as previously reported by Pusic and Kraig (2014). However, future investigations are needed to dissect the combating strategy by EE nurturing remyelination in adult rats. Thus, by demonstrating the modulation in NGF levels along with its downstream signaling molecules, we could strongly reiterate that EE promotes myelination and augments the behavioral pattern in rats exposed to chronic IS.

Conclusion

The results of this study provide an additional clue on how EE can improve behavioral plasticity and myelination in forebrain regions and mitigates the chronic stress-induced variations by maintaining the neuroendocrine milieu in rats. The study warrants further scrutiny on the behavioral impact, by EE, pertaining to myelination.

Acknowledgments The authors thank the University Grants Commission-Special Assistance Programme, New Delhi, India, for the necessary financial support in the form of Fellowship.

Compliance with ethical standards

Disclosure statement The authors indicate that there is no potential conflict of interest in this study.

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