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Environmental enrichment ameliorates chronic immobilisation stress-induced spatial learning deficits and restores the expression of BDNF, VEGF, GFAP and glucocorticoid receptors

Shilpa BM [a,1], Bhagya V [a,1], Harish G [b], Srinivas Bharath MM [b], Shankaranarayana Rao BS [a,*]

[a] Department of Neurophysiology, National Institute of Mental Health and Neuro Sciences, Hosur Road, Bengaluru 560 029, India
[b] Department of Neurochemistry, National Institute of Mental Health and Neuro Sciences, Hosur Road, Bengaluru 560 029, India

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ABSTRACT

Severe and prolonged stress is the main environmental factor that precipitates depression, anxiety and cognitive dysfunctions. On the other hand, exposure to environmental enrichment (EE) has been shown to induce progressive plasticity in the brain and improve learning and memory in various neurological and psychiatric disorders. It is not known whether exposure to enriched environment could ameliorate chronic immobilisation stress-induced cognitive deficits and altered molecular markers. Hence, in the present study we aimed to evaluate the effect of enriched environment on chronic immobilisation stress (CIS) associated changes in spatial learning and memory, behavioural measures of anxiety, depression and molecular markers as well as structural alterations. Male Wistar rats were subjected to chronic immobilisation stress for 2 h/day/10 days followed by 2 weeks of exposure to EE. CIS resulted in weight loss, anhedonia, increased immobility, spatial learning and memory impairment, enhanced anxiety, and reduced expression of BDNF, VEGF, GFAP and glucocorticoid receptors (GR) in discrete brain regions. Interestingly, stressed rats exposed to enrichment ameliorated behavioural depression, spatial learning and memory impairment and reduced anxiety behaviour. In addition, EE restored BDNF, VEGF, GFAP and GR expression and normalized hypotrophy of dentate gyrus and hippocampus in CIS rats. In contrast, EE did not restore hypertrophy of the amygdalar complex. Thus, EE ameliorates stress-induced cognitive deficits by modulating the neurotrophic factors, astrocytes and glucocorticoid receptors in the hippocampus, frontal cortex and amygdala.

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1. Introduction

Severe and prolonged stress exacerbates psychiatric illnesses like depression and anxiety (Vyas et al., 2002, 2004; Dinan, 2005; Mitra et al., 2006; Veena et al., 2009b; Bhagya et al., 2016a, 2016b) and cognitive impairment (Srikumar et al., 2007; Veena et al., 2009a; Bhagya et al., 2016a). These symptoms are often exhibited by major depressive patients (Jaeger et al., 2006; Naismith et al., 2007). Both animal and human studies show that mood disorders like depression is a consequence of hypothalamo-pituitary-adrenal (HPA) axis dysfunction and impaired glucocorticoid signalling (Kendler et al., 1999; Frodl and O'Keane, 2013). Glucocorticoid receptors (GR) regulate HPA axis by negative feedback mechanisms (Yehuda et al., 2012) and it has been shown that chronic stress results in decreased GR expression in the hippocampus (Sapolsky et al., 1984; Park et al., 2015).

Chronic immobilisation stress, a putative animal model of exogenous depression includes both physical and psychological stress components (Vyas et al., 2002, 2004; Govindarajan et al., 2006; Lakshminarasimhan and Chattarji, 2012). Prolonged stress has been shown to induce depressive and anxious phenotypes (Vyas and Chattarji, 2004; Mitra et al., 2005; Kim and Han, 2006; Veena et al., 2009b; Bhagya et al., 2016a, 2016b) and cognitive impairment (Radecki et al., 2005; Srikumar et al., 2007; Veena et al., 2009a; Ramkumar et al., 2008; Bhagya et al., 2016a, 2016b). Morphologically, CIS resulted in hippocampal atrophy and amygdalar hypertrophy (Vyas et al., 2002, 2004). Moreover, CIS rats show cognitive deficits and anxiety-like behaviour with reduced expression of BDNF in the hippocampus (Govindarajan et al., 2006; Lakshminarasimhan and Chattarji, 2012).

Brain derived neurotrophic factor (BDNF) is a molecular marker of neuronal plasticity. Adverse events like severe stress results structural alterations and functional impairment in the brain by reducing BDNF expression (Duman, 2009) and previous studies show that stress

* Corresponding author at: Department of Neurophysiology, National Institute of Mental Health and Neuro Sciences (NIMHANS), Hosur Road, PB # 2900, Bengaluru 560 029, India.
E-mail addresses: bssrao.nimhans@gmail.com, bssrao@nimhans.ac.in (B.S. Shankaranarayana Rao).
[1] Both authors contributed equally.

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decreased BDNF expression in the hippocampus (Govindarajan et al., 2006; Duman, 2009) and in cortical and sub-cortical regions (Pizarro et al., 2004). Also, diminished BDNF levels were correlated with reduced neurogenesis (Schmidt and Duman, 2007) and depressive symptoms (Taliaz et al., 2010). In addition, BDNF alters activity of HPA axis in the chronic stress (Givalois et al., 2004; Tapia-Arancibia et al., 2004; Naert et al., 2010). It has been reported that low levels of BDNF increases the desensitisation of GR, enhances susceptibility to stress (Arango-Lievano et al., 2015) and induces phosphorylation of glucocorticoid receptors (Lambert et al., 2013).

Vascular endothelial growth factor (VEGF), an endothelial cell mitogen factor is vital for angiogenesis, vascular function and produces neuroprotective effects (Storkebaum et al., 2004). In addition, VEGF plays a major role in neurogenesis (Jin et al., 2002) and synaptic transmission (McCloskey et al., 2005). Earlier studies illustrated altered VEGF expression in chronic stress and psychiatric diseases (Heine et al., 2005; Fournier and Duman, 2012). Previous studies have showed differential expression of VEGF in various stress conditions. VEGF levels in the hippocampus increased in acute foot shock, whereas chronic mild stress decreased its expression (Bergström et al., 2008; Uysal et al., 2012). Further, Kiuchi et al. revealed regular exercise induce antidepressant-like activity by modulating VEGF signalling (2012). In addition, down regulation of neurotrophic factors like BDNF, VEGF and IGF, and decreased astrocytes were restored by EE exposure (Huang et al., 2012; Malik and Chattarji, 2012; Beauquis et al., 2013; Pang and Hannan, 2013). Accordingly, the current study was designed to assess the effect of enriched environment on chronic immobilisation stress-induced alterations in the expression of BDNF and VEGF in different regions of the brain.

Glial fibrillary acidic protein (GFAP), a structural protein expressed by astrocytes stabilises cytoskeleton (Sloan and Barres, 2014) and altered GFAP expression results in abnormal synaptic plasticity. Decreased GFAP levels in the dorsolateral prefrontal cortex (Cotter et al., 2002), orbitofrontal, anterior cingulate cortex (Benes et al., 2001; Chana et al., 2003) and hippocampus (Stockmeier and Rajkowska, 2004) were associated with depressive symptoms. Also, both preclinical and clinical data suggests reduced levels of GFAP in the cortical and limbic structures in depression (Miguel-Hidalgo et al., 2000; Gosselin et al., 2009). Several volumetric studies showed atrophy of the hippocampal and fronto-cortical areas (Sheline et al., 1996; Sheline, 2000; Mahati et al., 2016), and hypertrophy of amygdalar complex (von Gunten et al., 2000; Frodl et al., 2002; Karl et al., 2006; Mahati et al., 2016) in depressed patients as well as in the animal models of depression.

Positive environment is known to be critical for brain development, and can elicit significant structural and functional changes in different brain regions. Certain neuropsychiatric rehabilitation regimes like social support are known to play a major role in enhancing quality of patient's life (Li et al., 2013; Roohafza et al., 2014). In the laboratory animals, positive stimuli like enriched environment (EE) provides an opportunity for structural and functional changes. Hence, several studies have implicated the beneficiary role of EE as an alternative approach to reverse cognitive deficits in animal models of neurodegenerative diseases (Dhanushkodi and Shetty, 2008; Veena et al., 2009a, 2009b; Mahati et al., 2016).

EE regulates brain plasticity at multiple levels of neural organization. EE reverses stress-induced hippocampal atrophy, reduced neurogenesis and deficits in learning and memory (Veena et al., 2009a; Pang and Hannan, 2013). In amblyopic (monocular blindness, partial or complete blindness in one eye) animals, EE could enhance the vision by increasing trophic support and epigenetic modifications in the visual cortex (Baroncelli et al., 2010). It was also reported that EE modulate glucocorticoid levels (Xu et al., 2009) and exhibit anxiolytic effect (Ravenelle et al., 2014). Enrichment ameliorated stress-induced hyper-activate HPA-axis by reducing the hippocampal mineralocorticoid receptor (MR)/GR mRNA levels (Zhang et al., 2011), which helps in coping anxiety-like behaviour (Ravenelle et al., 2014). Further, EE favours anxiolytic effect in animal models of PTSD (Hendriksen et al., 2010) and ameliorates depressive-like behaviour (Jha et al., 2011; Richter et al., 2013).

However, it is not known whether exposure to enriched environment ameliorates chronic immobilisation stress-induced cognitive deficits and altered expression of neurotrophic factors. Accordingly, the aim of the current study was to evaluate the effects of enriched environment on behavioural depression, anxiety-like behaviour, spatial memory impairment and volumetric changes in the chronically stressed rats and also to determine whether BDNF, VEGF, GFAP and GR could be involved in these behavioural and morphological alterations.

2. Materials and methods

2.1. Animals

Adult male Wistar rats were obtained from Central Animal Research Facility (CARF), NIMHANS, Bengaluru. Rats were housed in polypropylene cages (32 × 24 × 16 cm) with food and water ad libitum. 3–4 rats were housed in a cage and maintained on a 12 h light/dark cycle in a temperature (25 ± 2 °C) and humidity (50–55%) controlled environment. Bedding material used was paddy husk and it was changed on alternate days. All experiments were carried out in accordance with the National Institute of Health Guidelines for the Care and Use of Mammals in Neuroscience and Behavioural Research (The National Academics Press, Washington USA, 2003) and experimental protocols were approved by the institutional animal ethics committee. Maximum efforts were made to reduce the number of animals used and to decrease the suffering to experimental animals.

2.2. Experimental groups

Male Wistar rats (2 to 2.5 months old weighing 200–225 g) were used in the current study. Animals were randomly allocated to four groups; control: animals housed in standard laboratory conditions; chronic immobilisation stress (CIS): animals subjected to immobilisation stress for 2 h/day for 10 days; CIS + EE: stressed animals exposed to enriched environment (EE) for 14 days; EE: Un-stressed animals exposed to EE for 14 days. We have used different cohort of animals for stress indices (body weight, adrenal and spleen weight), anxiety (open field and elevated plus maze), behavioural depression (sucrose preference test and forced swim test), spatial learning and memory (partially baited radial am maze), molecular markers (BDNF, VEGF, GFAP and GR) and volumetric analysis (Fig. 1).

2.3. Stress protocol

The adult rats were subjected to immobilisation stress in rodent immobilisation bags (2 h/day, 10 am - 12 noon) without access to either food or water, for 10 consecutive days (Vyas et al., 2002; Anuradha et al., 2008; Hegde et al., 2008). After the stress protocol, animals were returned back to their home cage with food and water ad libitum.

2.4. Enriched environment (EE)

Animals were exposed to enriched environment in a large cage (108 × 65 × 65 cm) made of wire mesh and wood. The floor of the cage was covered with bedding material on which a variety of objects with different colour, texture and shapes with re-arrangeable tunnels and pipes were placed. Novel objects used in enriched cage were made of wood, plastic and metal. Every day, different cleaned objects were rearranged to maintain novelty in the EE cage. Food and water was available ad libitum during the period of enrichment. The animals were exposed to enrichment for 6 h/day (10 am–4 pm) for 14 days. 10–12 animals were housed in the EE cage and after completion of the assigned time, rats were returned to their home cage (Veena et al., 2009a, 2009b; Bhagya et al., 2016b; Mahati et al., 2016).

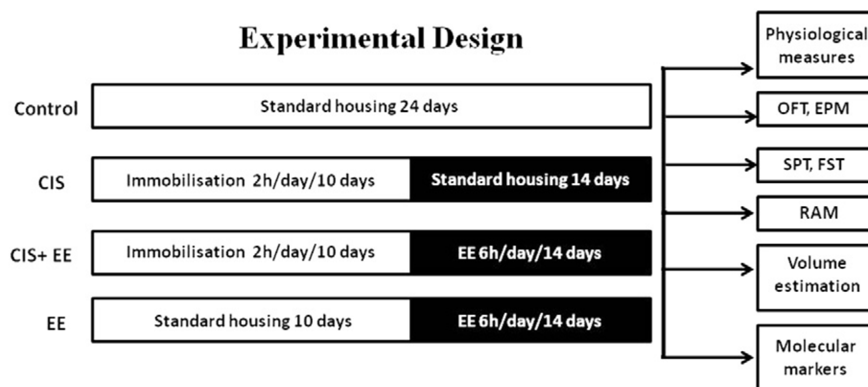

Fig. 1. Illustration of experimental design. Control: animals housed in standard laboratory conditions; chronic immobilisation stress (CIS): animals subjected to immobilisation stress for 2 h/day for 10 days; CIS + EE: stressed animals exposed to enriched environment (EE) for 14 days; EE: Un-stressed animals exposed to EE for 14 days. OF: Open field test; EPM: Elevated plus maze test; SPT: Sucrose preference test; FST: Forced swim test; RAM: Partially baited radial arm maze task.

2.5. Confirmation of stress induction

Stress induction and its severity was assessed by measuring body, adrenal gland and spleen weights using previously standardized protocols (Vyas et al., 2002).

2.6. Evaluation of behavioural depression

2.6.1. Sucrose preference test (SPT)

The sucrose preference test protocol which was previously established in our laboratory was used (Bhagya et al., 2008, 2011, 2015; Veena et al., 2009a; Mahati et al., 2016). Animals were housed individually throughout the habituation and test sessions. Animals were habituated to avoid the isolation induced alteration in the behaviour of animals. Individually housed animals were provided with two bottles; one with 1% sucrose solution and another with tap water. Both bottles were provided throughout the 48 h habituation phase. The volume of water and sucrose water intake was measured every 24 h and the position of two bottles was changed to prevent place preference (Papp et al., 1991). After the habituation session, animals were deprived of food and water for a period of 18 h. Then, test session was conducted for 2 h. The amount of liquid consumed was calculated (sucrose preference = sucrose water consumed/total liquid consumed × 100).

2.6.2. Forced swim test (FST)

This test was performed with the same animals used for SPT. Animals were given a gap of 24 h and the FST was carried out for two days. On the first day, a habituation session was performed. During habituation, rats were put into a cylinder shaped plastic tank (45 cm diameter, 60 cm height) containing about 35 cm of water (25–27 °C), such that the animals could not support themselves by touching the bottom of the cylinder and were allowed to swim in the cylinder for a period of 15 min. After the habituation session, the animal was removed from the water, dried thoroughly and returned to its respective home cage. On the second day, a test session was carried out, where the rat was once again put into the cylinder containing water and allowed to swim for a period of 5 min. Following the test session, the rat was removed from the water, dried and kept warm under a lamp in its home cage. Videos were coded and the experimenter was blind to treatment conditions. Rats were scored as immobile whenever they remained floating passively in the water and only making those movements necessary to keep the nose/head above the water (Porsolt et al., 1978; Bhagya et al., 2008, 2011, 2015; Veena et al., 2009a; Mahati et al., 2016).

2.7. Evaluation of exploratory and anxiety behaviour

2.7.1. Open field test (OFT)

The OFT arena is a square wooden box (100 × 100 × 40 cm) with a black painted floor and inner walls. The floor totally has 25 squares (20 × 20 cm) with sixteen squares in the periphery and nine in the centre in a grid-like fashion. The centre of the field was illuminated, and it was the only direct light in the testing room. Experimental groups were randomly selected and placed in corner facing the centre of the arena. The behaviour of the animal was recorded for 5 min and analysed using the Noldus Ethovision XT tracking software (Noldus, Wageningen, The Netherlands). The arena was cleaned with 70% ethanol after each session and rats were tested only once. Total distance moved in the test period, total zone and latency to enter centre (time taken to first entry to the centre) parameters were analysed (Anuradha et al., 2008; Bhagya et al., 2015, 2016a).

2.7.2. Elevated plus maze (EPM)

The EPM apparatus (Columbus Instruments, USA) consists of a plus (+) shaped platform and raised above floor level (60 cm) which composed of two enclosed arms opposed perpendicularly by two open arms. The EPM was illuminated by an overhead light source (60 W bulb). EPM experiments were performed following OFT after a gap of 24 h. The apparatus was cleaned with 70% alcohol before the each animal was introduced in the maze. Animals were allowed to explore the maze freely for a period of 5 min and the behaviour was video recorded. The videos were stored, coded and offline analysis was performed using the EPM score software (Biotechniques, Compiled version 12.0). Percentage time spent in centre/open/closed arms, number of head dips, defecation, number of vertical rearing, number of zone/arm transitions (centre/open/closed) were the ethological parameters assessed for anxiety-like, exploratory and locomotor behaviour (Vyas et al., 2002, 2004; Anuradha et al., 2008; Bhagya et al., 2016a, 2016b; Mahati et al., 2016).

2.8. Spatial learning and memory in a partially baited radial arm maze (RAM)

The current study was conducted using a partially baited radial arm maze protocol which was previously standardized in our lab (Srikumar et al., 2007; Bhagya et al., 2008, 2011, 2015, 2016a; Mahati et al., 2016). Prior to habituation, animals were kept on a restricted diet and body weight was maintained at 85% of their free feeding weight. Rats were exposed to the test apparatus for two consecutive days. In order to ensure equal exploration and familiarity with all arms of the apparatus, palatable food bait was placed in all arms. Each trial was started by placing animal at the centre of the octagonal maze and allowed to explore

10 min. Following habituation, acquisition test was carried out for 16 days. Before starting the trial, the maze was thoroughly cleaned with 70% ethanol and four arms (2, 3, 6 and 8) were baited with food pellets (Kellogg's Chocos™ Kelloggs India Pvt. Ltd.). During this phase, rats were subjected to two trials a day with an inter-trial interval of 1 h and animal scoring was recorded manually. The duration of each trial was lasted for five minutes. Total number of entries made, entries into the baited arms, unbaited arms and re-entry into the arms were calculated. All groups were trained for 16 days. After the last day of acquisition, rats were left undisturbed for 10 days. After a gap of ten days, a retention test was carried out to assess the retention of spatial memory in trained rats. Data from 4 trials was averaged and expressed as one block and analysed for percentage correct choice, reference and working memory errors. A reference memory error (RME) is regarded as entry into unbaited arms. The component of short term memory; working memory error (WME) defined as a re-entry into a baited arm (working memory correct, WMEC) and a re-entry into an unbaited arm (working memory error in correct, WMEIC) were calculated and expressed across the trials as blocks.

2.9. Western blotting

Experimental animals from all four groups were sacrificed under halothane anaesthesia. Three anatomical regions; frontal cortex, hippocampus and amygdalar complex were quickly dissected and stored at −80 °C until use. Tissue stored at −80 °C was thawed and homogenized in ice cold 1× PBS buffer containing 10% sucrose, 1 mM EDTA in 20 mM Tris-HCl (pH 7.4) and 1× protease inhibitor cocktail (Sigma-Aldrich, USA) (Bindu et al., 2007) to the minced tissue. The homogenate was then sonicated thrice on ice for 10 s each (QSonica, Sonicators (Model: Q125), New York, USA) and centrifuged at 14,000 rpm for 15 min at 4 °C. The supernatant was collected and stored at −80 °C. Protein estimation was performed using Bradford's method (Chandana et al., 2009; Harish et al., 2011) in ELISA plate reader (TECAN, GmbH, Austria).

30 μg of the protein sample was mixed with equal volume of 2× Laemmlli buffer (loading buffer: 100 mM Tris-HCl pH 6.8; 10% w/v SDS; 20% v/v glycerol; 1% beta mercaptoethanol; dH_2O) and boiled approximately for 10 min. After cooling, samples were centrifuged to pellet insoluble proteins and the supernatant was loaded onto the wells. For the hippocampus and frontal cortex, unpooled samples were loaded as biological duplicates. Amygdalar complex samples were pooled and loaded as duplicates in the corresponding wells (30 μg/lane) along with the molecular weight markers. Samples were resolved by SDS-polyacrylamide gel (10–12% stacking polyacrylamide gel: TEMED; Ammonium per sulphate 10%; SDS 10%; 1.0 M Tris pH 6.8; 1.5 m Tris pH 8.8; Acrylamide mix 30%; Distilled water) (Biorad Laboratories Inc., Hercules, CA, USA), at 100–125 V for 2–2.5 h. Following separation by SDS PAGE, proteins were electrophoretically transferred from the gel to PVDF membranes using a semi-dry transfer apparatus (Sree Maruthi Scientific Works, Bangalore, India). After the transfer, the blots were incubated in blocking solution [Phosphate Buffered Saline Tween-20 (PBST) containing 5% skimmed milk powder (Nandini Milk Products, Bangalore, India)] for 1–1½ h at room temperature or overnight at 4 °C to block non-specific binding. The blot was incubated in primary antibody diluted in PBS containing 5% BSA for 2 h at room temperature (Anti-BDNF 1:500, Anti-VEGF 1:500 and Anti-β-Actin 1:2000 all from Abcam, Cambridge, UK) and GR (Anti-GR 1:500 Santa Cruz Biotechnology). The blot was then washed with PBST (10 min × 4) to remove excess primary antibody and then incubated for 1 h at room temperature with HRP-conjugated secondary antibody (diluted in PBST containing 1:2000 for rabbit and mouse (Bangalore Genei, Bangalore, India). Membranes were washed with PBST and the immune reaction was visualized by developing in 1× PBS containing DAB (1 mg/ml (w/v) and 0.1% H_2O_2 or with SuperSignal™ West Pico chemiluminescent substrate (Pierce Biotechnology, Illinois, USA) detection was done and images were captured by a gel documentation system (SYNGENE, Synoptics Model G: Box Chemi XT4, Cambridge, UK). The image was analysed by densitometry using Image J software (Wayne Rasband, Version 1.47, NIH, USA). Subsequently each protein bands were normalized to β-actin (Chandana et al., 2009; Harish et al., 2011).

2.10. Volumetric analysis of different brain areas

Animals were deeply anesthetized with halothane, transcardially perfused with ice cold saline followed by a 10% solution of formaldehyde and brains were removed, post fixed for 24-48 h. 40 μm thick coronal sections were obtained through the entire anterio-posterior extent of the hippocampus using Vibratome (Leica, Wetzlar, Germany). Prior to stereological estimation, serial brain sections were stained using Nissl (cresyl violet) stain (Veena et al., 2009a; Mahati et al., 2016). The boundaries of the areas of interest were defined in accordance with Paxinos and Watson rat atlas (Paxinos and Watson, 2005). Volume of the dentate gyrus (DG), entire hippocampus and basolateral amygdala (BLA) was estimated as described earlier (Rubinow and Juraska, 2009; Veena et al., 2009a; Mahati et al., 2016) by drawing contours across the aforementioned areas and were calculated using unbiased stereology using Stereo Investigator software (MBF Bioscience, Microbrightfield, Inc., USA). The process was repeated on every 6th section and analysis was done on both the hemispheres separately by adding them together. Summation of left and right hemispheres was expressed as total volume by adopting Cavalieri's principle.

2.11. Statistics

The data was statistically analysed using GraphPad Prism 5 software and was expressed as mean ± S.E.M. Percentage correct choice and reference memory errors of the RAM task data was analysed by Two-way ANOVA with Bonferroni's post-hoc test. The Western blot, anxiety, depression and volumetric data was analysed by One-way ANOVA followed by Tukey's post-hoc test to identify differences between the groups. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Enriched environment reversed stress-induced reduced body weight gain, adrenal and spleen hypertrophy

CIS animals showed reduction in relative body weight gain and this was significantly restored after exposure to enriched environment ($F_{3,56} = 143.4$, $p < 0.001$; Table 1). EE exposure reversed adrenal gland ($F_{3,20} = 120.8$, $p < 0.001$; Table 1) and spleen ($F_{3,20} = 30.00$, $p < 0.001$; Table 1) hypertrophy in CIS animals.

Table 1
Enriched environment restores CIS-induced reduced body weight gain, adrenal gland and spleen hypertrophy.

	Groups	Relative body weight gain (%)	Relative adrenal gland weight (%)	Relative spleen weight (%)
1	Control	9.29 ± 0.55	14.99 ± 0.26	0.46 ± 0.01
2	CIS	−6.60 ± 0.43 ***	23.56 ± 0.57 ***	0.81 ± 0.05 ***
3	CIS + EE	12.73 ± 1.06 ** ###	13.89 ± 0.37 ###	0.45 ± 0.01 ###
4	EE	8.52 ± 0.64	13.63 ± 0.45	0.46 ± 0.01

Data expressed as Mean ± SEM. One-way ANOVA followed by Tukey's post hoc test ($F_{3,56} = 143.4$, $F_{3,20} = 120.8$ and $F_{3,20} = 30.00$, $p < 0.001$) for relative body weight gain, adrenal gland weight and spleen weight, respectively.
** $p < 0.01$ vs. control.
*** $p < 0.001$ vs. control.
$p < 0.001$ vs. CIS.

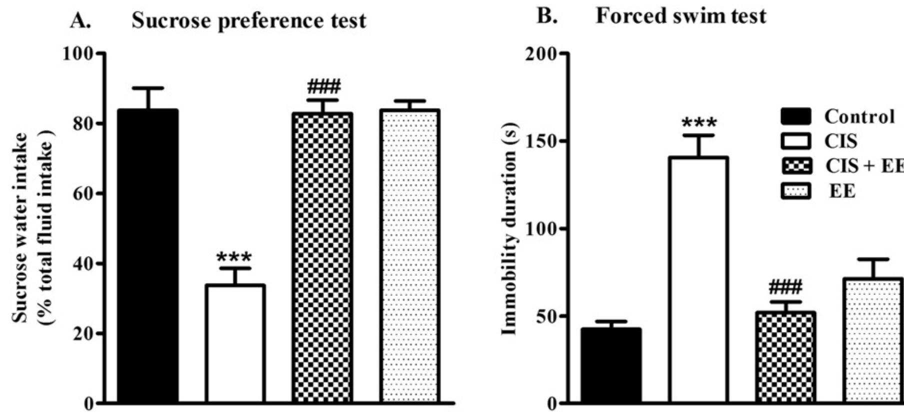

Fig. 2. EE completely reversed behavioural depression in stressed rats. CIS animals showed behavioural depression in SPT and FST. Stressed rats exposed to EE showed more preference to sucrose water in SPT and decreased immobility time in the forced swim test. Data expressed as Mean ± SEM. Control: animals housed in standard laboratory conditions (n = 12); chronic immobilisation stress (CIS): animals subjected to immobilisation stress for 2 h/day for 10 days (n = 12); CIS + EE: stressed animals exposed to enriched environment (EE) for 14 days (n = 10); EE: Un-stressed animals exposed to EE for 14 days (n = 12). One-way ANOVA followed by Tukey's post hoc test; ***p < 0.001 vs. Control. ###p < 0.001 vs. CIS.

3.2. Enrichment reverses chronic stress-induced behavioural depression

3.2.1. Sucrose preference test (SPT)

CIS animals show decreased preference to sucrose water compared to control animals. Anhedonia exhibited by stressed rats was completely restored following environmental enrichment ($F_{3,42} = 28.72$, $p < 0.001$; Fig. 2A). Subjecting naïve animals to EE did not alter sucrose preference.

3.2.2. Forced swimming test (FST)

Stressed animals show longer immobility time compared to control group. CIS animals upon exposure to EE show reduction in immobility time ($F_{3,42} = 22.04$, $p < 0.001$; Fig. 2B) in FST. Duration of immobility in EE group was comparable to controls.

3.3. Unaffected locomotor and exploratory behaviours after enriched environment

The total distance travelled by CIS animals (Fig. 3B) was lower compared to control (Fig. 3A), indicating decreased exploratory drive. Enrichment did not alter the path length in CIS + EE group, as seen in video tracks (Fig. 3C) and exploratory behaviour remained unchanged ($F_{3,52} = 14.31$, $p < 0.001$; Fig. 3E). EE exposure had no influence on number of zone transitions ($F_{3,52} = 10.88$, $p < 0.001$;

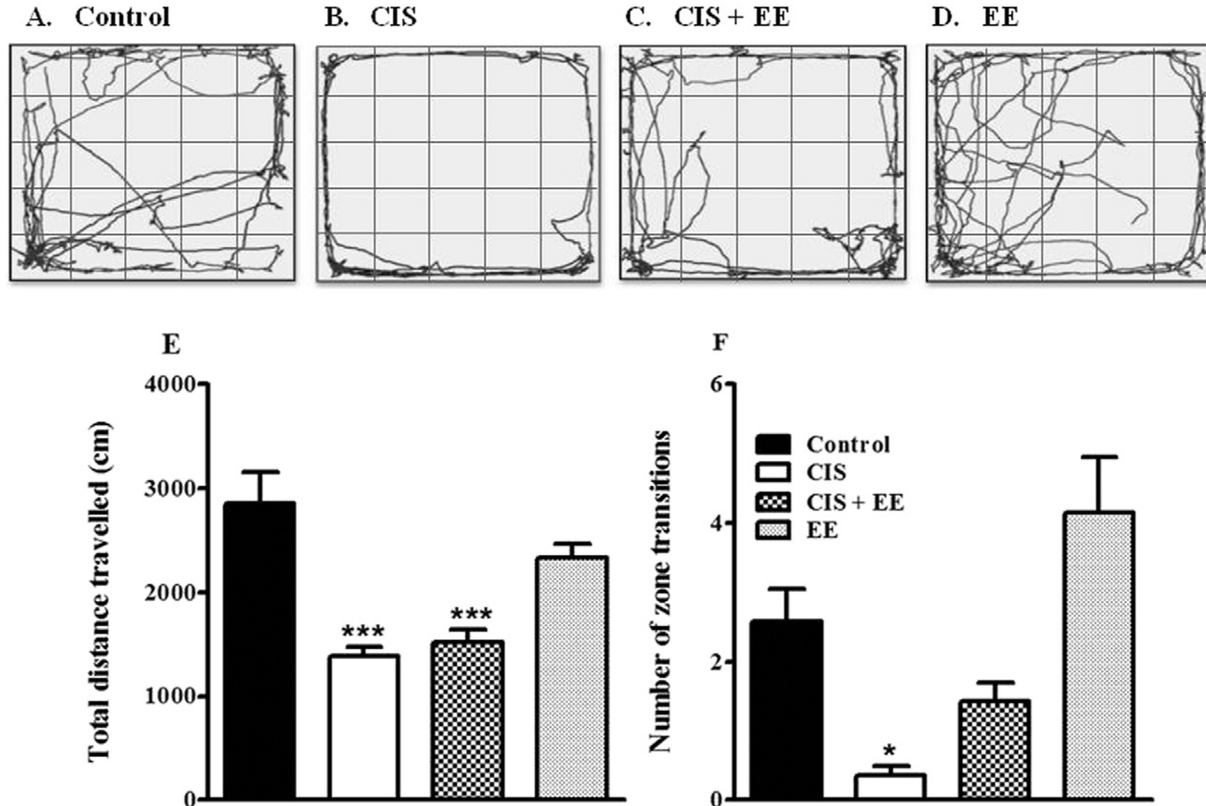

Fig. 3. Representative path length tracks in open field. Image showing OFT tracks of different groups: (A) Control: animals housed in standard laboratory conditions (n = 14); (B) CIS: animals subjected to immobilisation stress for 2 h/day for 10 days (n = 14); (C) CIS + EE: stressed animals exposed to enriched environment (EE) for 14 days (n = 14); (D) EE: Un-stressed animals exposed to EE for 14 days (n = 14). Enrichment did not affect locomotor and exploratory behaviours in stressed animals. Total distance moved (cm) (E) and number of zone transitions (F). Data expressed as Mean ± SEM. One-way ANOVA followed by Tukey's post hoc test; *p < 0.05, ***p < 0.001 vs. Control.

Fig. 3F) in stressed animals. EE animals showed similar exploratory behaviour as that of control group (Fig. 3D). Hence, environmental enrichment had no effect on CIS-induced decreased exploratory behaviour in the OFT.

3.4. Enriched environment exerts anxiolytic-like effect

In the elevated plus maze, CIS animals spent less time in open arms ($F_{3,52} = 33.12$, $p < 0.001$; Fig. 4A) and more time in closed arms ($F_{3,52} = 13.54$, $p < 0.001$; Fig. 4B) compared to control group. Also, these animals showed less number of entries in open arms ($F_{3,52} = 10.15$, $p < 0.001$; Fig. 4C). On the other hand, exposure to enriched environment resulted in little longer duration and more number of entries in open arms compared to stress group ($F_{3,52} = 33.12$, $p < 0.001$; Fig. 4A: $F_{3,52} = 10.15$, $p < 0.001$; Fig. 4C).

The number of faecal pellets was increased in stress condition compared to control group, which was restored by EE ($F_{3,52} = 8.65$, $p < 0.001$; Fig. 4D). Vertical rearing behaviour was partially re-established by enrichment ($F_{3,52} = 5.28$, $p < 0.01$; Fig. 4E). CIS animals subjected to enriched environment showed partial recovery in the number of head dips compared to CIS group ($F_{3,52} = 16.91$, $p < 0.001$; Fig. 4F).

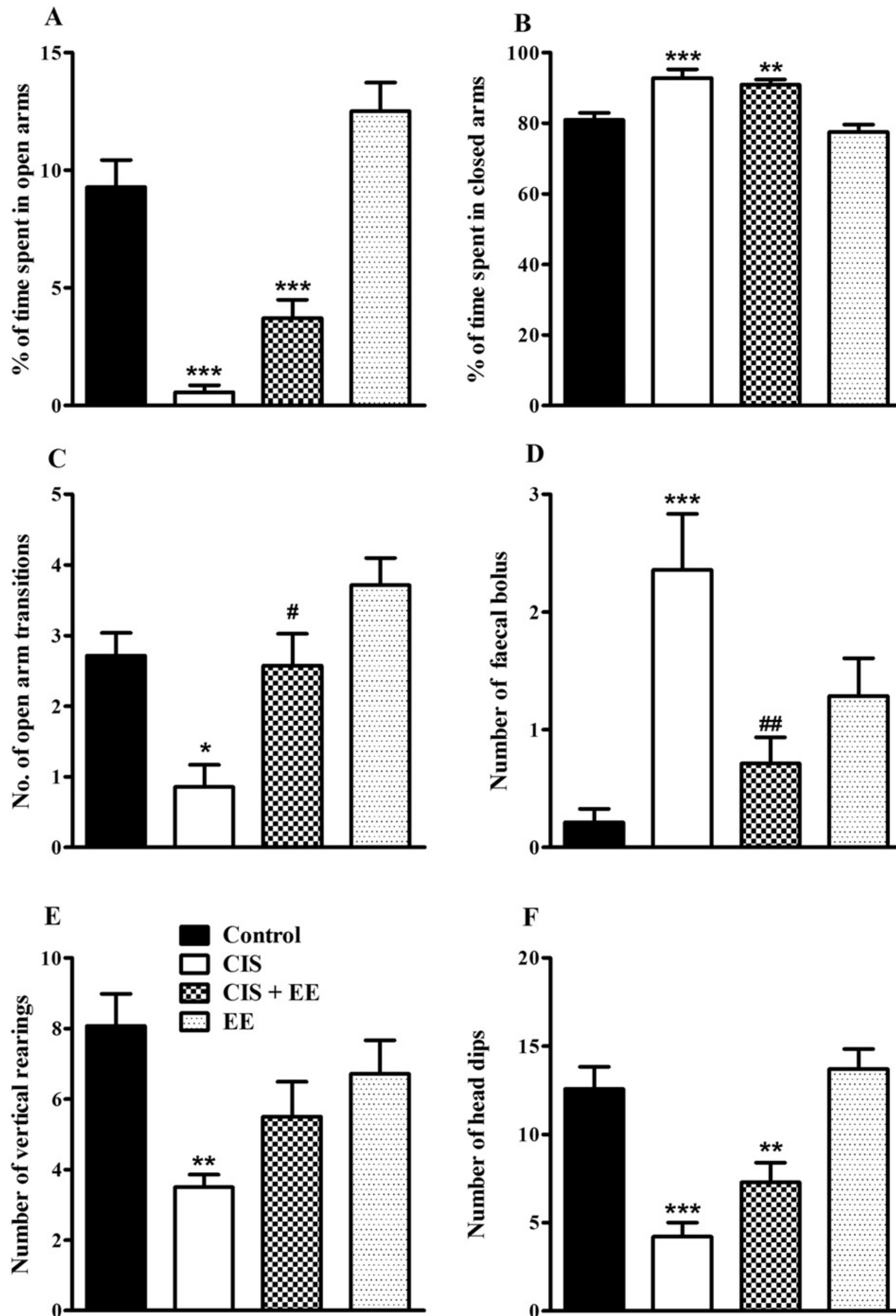

Fig. 4. Enriched environment exerted partial anxiolytic-like effect. % of time spent in open arms (A), % of time spent in closed arms (B), number of open arm transitions (C), number of faecal bolus (D), number of vertical rearings (E) and number of head dips (F). Data expressed as Mean ± SEM. Control: animals housed in standard laboratory conditions (n = 14); CIS: animals subjected to immobilisation stress for 2 h/day for 10 days (n = 14); CIS + EE: stressed animals exposed to enriched environment (EE) for 14 days (n = 14); EE: Unstressed animals exposed to EE for 14 days (n = 14). One-way ANOVA followed by Tukey's post hoc test; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. Control. #$p < 0.05$, ##$p < 0.01$ vs. CIS.

3.5. Enriched environment improved spatial learning and memory performance

To investigate whether exposure to enriched environment affects spatial learning and memory in stressed animals, the rats were evaluated in the partially baited radial arm maze task. During acquisition phase, both CIS and control groups initially showed a similar level of accuracy but control group's performance significantly enhanced from 4th block compared to CIS group. Enrichment restored stress-induced impairment of spatial learning in the RAM task ($F_{21,364} = 3.36$, $p < 0.001$: interaction effect and $F_{3,364} = 15.29$, $p < 0.001$: group effect; Fig. 5A). This was evident from the 7th and 8th block ($F_{3,52} = 11.70$, $p < 0.001$ and $F_{3,52} = 26.38$, $p < 0.001$; Fig. 5C). The performance of EE animals was comparable to controls ($p < 0.001$).

Number of RMEs in stressed rats gradually diminished across trials after EE exposure. CIS + EE showed a steady decline from the 8th day and were almost similar to control on 16th day ($F_{21,364} = 2.34$, $p < 0.001$: interaction effect and $F_{3,364} = 13.09$, $p < 0.001$: group effect; Fig. 5B). This was seen in the 7th and 8th blocks ($F_{3,52} = 9.10$ and $F_{3,52} = 15.89$, $p < 0.001$; Fig. 5D). Number of RMEs in EE animals was similar to controls ($p > 0.05$). Chronically stressed rats did not exhibit working memory deficits in RAM task. Neither stress nor EE affected working memory correct ($p > 0.05$) or working memory error incorrect ($p > 0.05$; Data not shown).

CIS rats demonstrated poor performance in the retention test with decreased % correct choice and greater number of RMEs indicating persistent learning impairment. On the other hand, chronically stressed animals exposed to EE performed better than CIS group in the retention test with increased % correct choice ($F_{3,52} = 8.51$, $p < 0.001$; Fig. 6A). Additionally, RMEs were also reduced in CIS + EE animals ($F_{3,52} = 9.43$, $p < 0.001$; Fig. 6B).

3.6. EE restores BDNF, VEGF, GFAP and GR expression in the hippocampus and frontal cortex

Since BDNF and VEGF play neurotrophic and angiogenic roles in the brain and their function declines in chronic stress, we evaluated the effects of enrichment on the hippocampal and frontal cortical BDNF and VEGF levels in CIS rats. Western blotting analysis revealed that, compared to control rats, CIS rats showed reduced levels of BDNF in the hippocampus ($p < 0.05$, Fig. 7A; $F_{3,16} = 9.71$). The level of BDNF in the frontal cortex also showed tendency to be lower in stressed group than in the control group but the difference between the two groups was insignificant ($p > 0.05$, Fig. 7B; $F_{3,16} = 13.73$). The VEGF level was lower in the hippocampus ($p < 0.001$, Fig. 8A; $F_{3,16} = 12.77$) and frontal cortex of stressed group ($p < 0.05$, Fig. 8B; $F_{3,16} = 5.00$). Interestingly, exposure to enrichment significantly up-regulated the expression of both BDNF and VEGF levels in the hippocampus ($p < 0.05$, Fig. 7A; $F_{3,16} = 9.71$ and $p < 0.05$, Fig. 8A; $F_{3,16} = 12.77$) and frontal cortex (Fig. 7B; $F_{3,16} = 13.73$ and Fig. 8B; $F_{3,16} = 5.00$), respectively in the CIS + EE group.

We next evaluated the effects of EE on the expression of GFAP, an astrocytic marker in the hippocampus and frontal cortex in stressed condition. Western blot data indicates that CIS rats had significantly reduced levels of GFAP in the hippocampus ($p < 0.001$, Fig. 9A; $F_{3,16} = 12.61$) and frontal cortex ($p < 0.01$, Fig. 9B; $F_{3,16} = 7.78$). Interestingly, stressed rats subjected to enrichment showed significantly

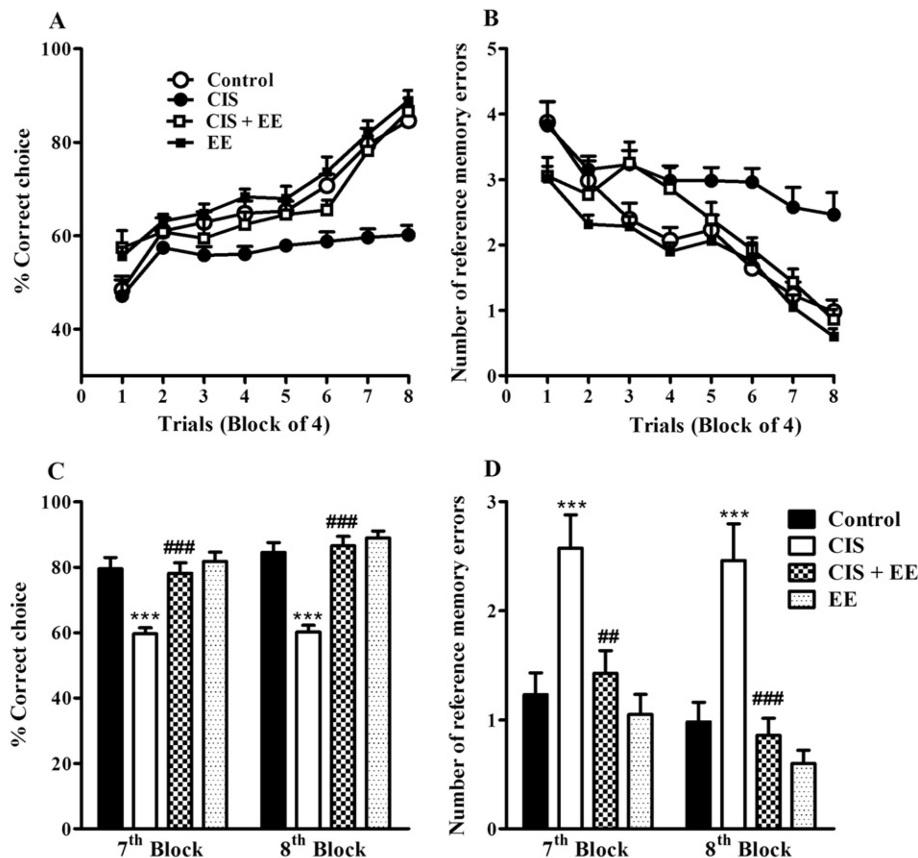

Fig. 5. Enrichment ameliorated spatial learning impairment in stressed animals in the partially baited radial arm maze. (A) % correct choice and (B) Number of reference memory errors in the acquisition of the RAM task across trials. (C) % correct choice and (D) Number of reference memory errors in the block 7 and 8. Control: animals housed in standard laboratory conditions (n = 14); CIS: animals subjected to immobilisation stress for 2 h/day for 10 days (n = 13); CIS + EE: stressed animals exposed to enriched environment (EE) for 14 days (n = 14); EE: Un-stressed animals exposed to EE for 14 days (n = 15). Data expressed as Mean ± SEM. Two-way repeated measure ANOVA followed by Bonferroni's post hoc test and One-way ANOVA followed by Tukey's post hoc test; ***$p < 0.001$, 0.05 vs. Control; ##$p < 0.01$, ###$p < 0.001$ vs. CIS.

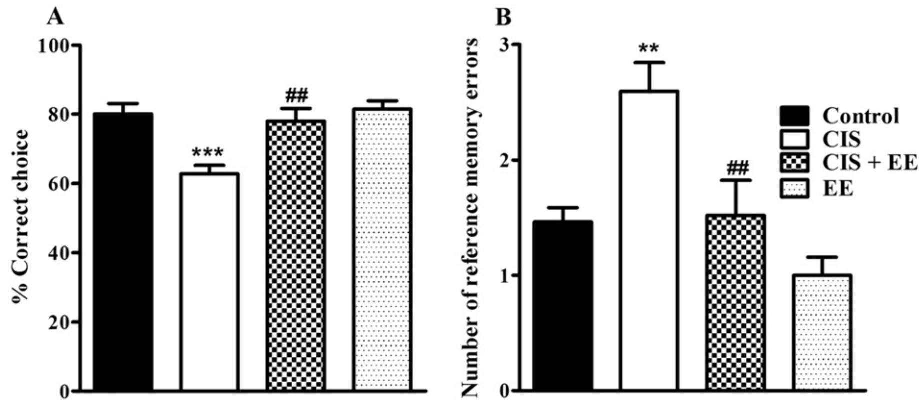

Fig. 6. Enrichment restored memory impairment in the retention test. (A) % correct choice and (B) Number of reference memory errors in the retention test. Control: animals housed in standard laboratory conditions (n = 14); CIS: animals subjected to immobilisation stress for 2 h/day for 10 days (n = 13); CIS + EE: stressed animals exposed to enriched environment (EE) for 14 days (n = 14); EE: Un-stressed animals exposed to EE for 14 days (n = 15). Data expressed as Mean ± SEM. One-way ANOVA followed by Tukey's post hoc test; $^{**}p < 0.01$, $^{***}p < 0.001$ vs. Control. $^{\#\#}p < 0.01$ vs. CIS.

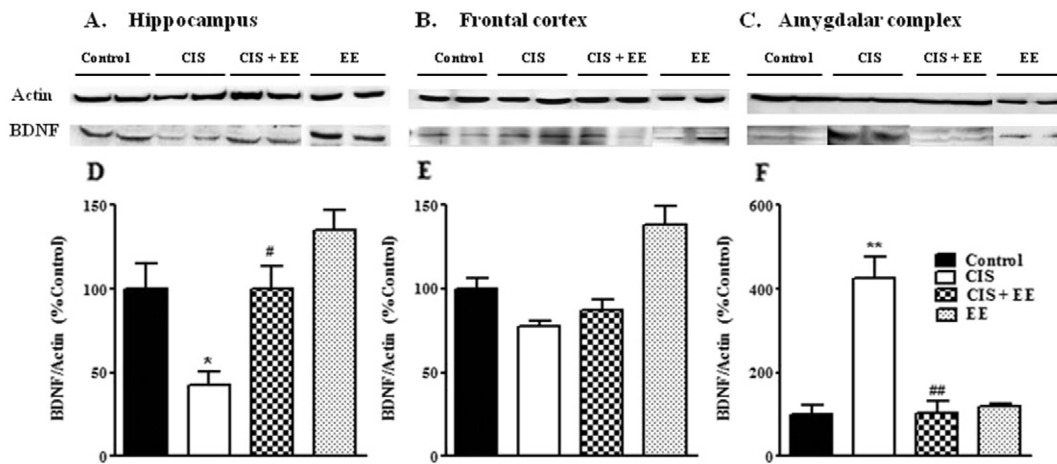

Fig. 7. EE normalized stress-induced altered expression of BDNF in the hippocampus and amygdala. Representative immunoblots of BDNF and β-actin from the Hippocampus (A), Frontal cortex (B) and Amygdalar complex (C). EE exposure restores CIS-induced down-regulation of BDNF levels in the hippocampus (D). Frontal cortical BDNF expression was not significantly altered in all groups (E). Up-regulated amygdalar BDNF was restored in CIS+ EE group (F). Control: animals housed in standard laboratory conditions (n = 5); CIS: animals subjected to immobilisation stress for 2 h/day for 10 days (n = 5); CIS + EE: stressed animals exposed to enriched environment (EE) for 14 days (n = 5); EE: Un-stressed animals exposed to EE for 14 days (n = 5). Data expressed as Mean ± SEM. One-way ANOVA followed by Tukey's post hoc test; $^{*}p < 0.05$, $^{**}p < 0.01$ vs. Control. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. CIS. Values were normalized to β-Actin and compared with controls.

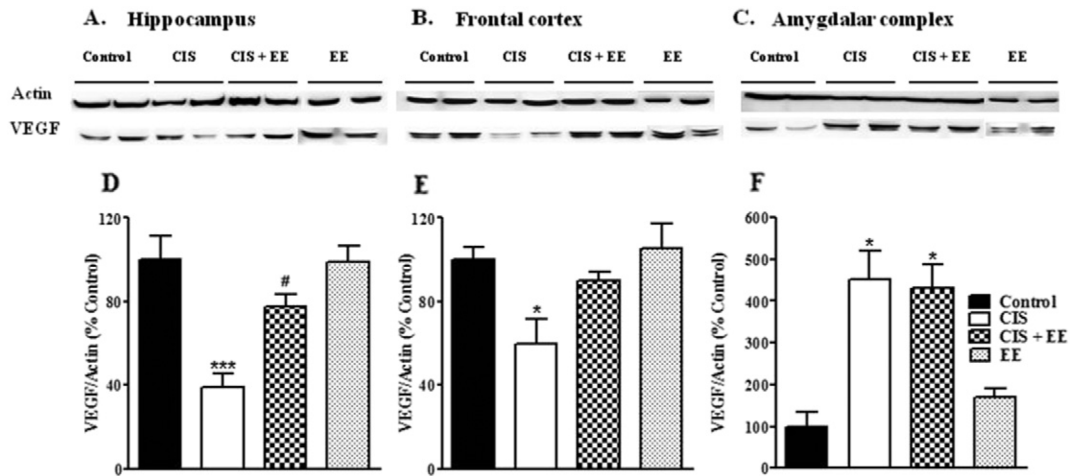

Fig. 8. Enrichment restored expression of VEGF in the hippocampus and frontal cortex. Representative immunoblots of VEGF and β-actin from the Hippocampus (A), Frontal cortex (B) and Amygdalar complex (C). EE exposure restores CIS-induced down-regulation of VEGF levels in the hippocampus (D). Enrichment partially restores frontal cortical VEGF expression in CIS group (E). Up-regulated amygdalar VEGF was not restored in CIS+ EE group (F). Control: animals housed in standard laboratory conditions (n = 5); CIS: animals subjected to immobilisation stress for 2 h/day for 10 days (n = 5); CIS + EE: stressed animals exposed to enriched environment (EE) for 14 days (n = 5); EE: Un-stressed animals exposed to EE for 14 days (n = 5). Data expressed as Mean ± SEM. One-way ANOVA followed by Tukey's post hoc test; $^{*}p < 0.05$, $^{***}p < 0.001$ vs. Control. $^{\#}p < 0.05$ vs. CIS. Values were normalized to β-Actin and compared with controls.

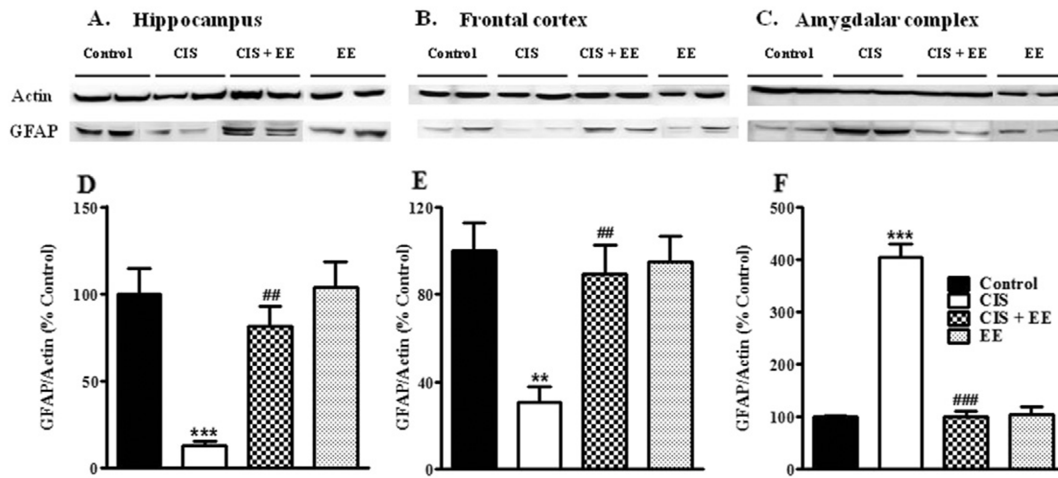

Fig. 9. EE reversed altered expression of GFAP levels in the hippocampus, frontal cortex and amygdala. Representative immunoblots of GFAP and β-actin from the Hippocampus (A), Frontal cortex (B) and Amygdalar complex (C). Enrichment restores chronic stress-induced down-regulation of GFAP levels in the hippocampus (D) and frontal cortex (E). EE also restores up-regulated amygdalar GFAP (F). Control: animals housed in standard laboratory conditions (n = 5); CIS: animals subjected to immobilisation stress for 2 h/day for 10 days (n = 5); CIS + EE: stressed animals exposed to enriched environment (EE) for 14 days (n = 5); EE: Un-stressed animals exposed to EE for 14 days (n = 5). Data expressed as Mean ± SEM. One-way ANOVA followed by Tukey's post hoc test; $^{**}p < 0.01$, $^{***}p < 0.001$ vs. Control. $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. CIS. Values were normalized to β-Actin and compared with controls.

higher levels of GFAP in the hippocampus ($p < 0.01$, Fig. 9A; $F_{3,16} = 12.61$) and frontal cortex ($p < 0.05$, Fig. 9B; $F_{3,16} = 7.78$).

Chronic immobilisation stress resulted in down regulation of GR in the hippocampus and EE partially restored and the reversal was not statistically significant ($p < 0.05$, Fig. 10A; $F_{3,12} = 4.05$). In the frontal cortex, GR level expression was not altered in stressed condition compared to control ($p > 0.05$, Fig. 10B; $F_{3,12} = 1.72$).

3.7. Enrichment showed differential effect on expression of BDNF, VEGF, GFAP and GR in the amygdalar complex

CIS resulted in up-regulation of BDNF and VEGF levels in the amygdala. EE restored BDNF levels in the amygdala of CIS group ($p < 0.01$, Fig. 7C; $F_{3,4} = 27.50$). On the other hand, enrichment failed to reverse the up-regulated expression of VEGF in the amygdala ($p > 0.05$, Fig. 8C; $F_{3,4} = 13.93$) in stressed rats. Up-regulated GFAP expression in the amygdala was restored in CIS + EE group ($p < 0.001$, Fig. 9C; $F_{3,4} = 94.46$). GR receptor expression was increased in CIS group compared to control group in the amygdala. Exposure to enrichment did not restore GR expression in the amygdala ($p > 0.05$, Fig. 10C; $F_{3,4} = 33.20$).

3.8. Enriched environment restored dentate gyrus (DG) and hippocampal hypotrophy without any effect on amygdala hypertrophy

Stereological analysis demonstrated a stress-induced reduction in the volumes of the DG and hippocampus. Stressed animals exposed to EE for 14 days showed complete restoration of DG ($F_{3,20} = 41.29$, $p < 0.001$; Fig. 11A) and hippocampal volumes ($F_{3,20} = 12.22$, $p < 0.01$; Fig. 11B). Strikingly, compared to the hippocampus, the amygdala showed hypertrophy (40%) in stressed animals and exposure to EE could not restore ($F_{3,20} = 16.51$, $p > 0.05$; Fig. 11C).

4. Discussion

The main findings of this study is that exposure to enriched environment ameliorates spatial memory deficits, decreases depressive–like behaviour, partially reduces anxiety and completely restores

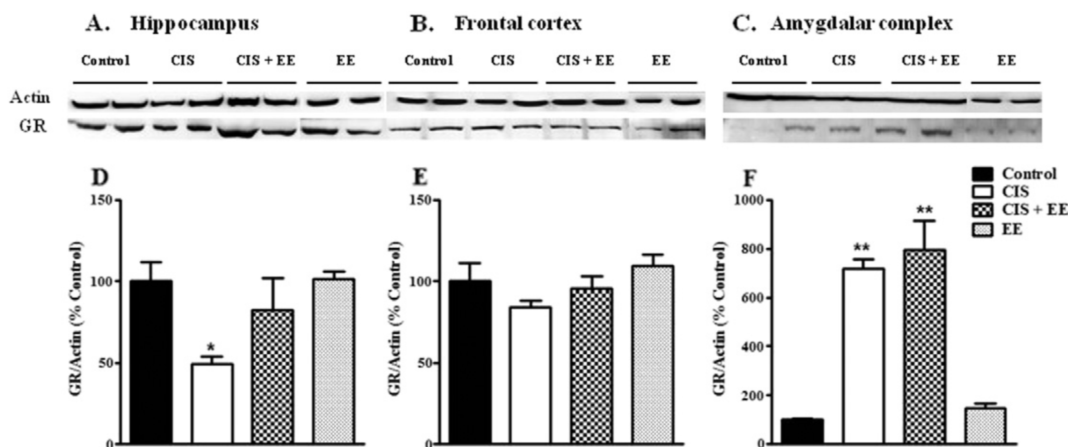

Fig. 10. Enrichment partially restored GR expression in the hippocampus. Representative immunoblots of GR receptor and β-actin from the Hippocampus (A), Frontal cortex (B) and Amygdalar complex (C). Enrichment partially restores chronic stress-induced down-regulation of GR receptor levels in the hippocampus (D). Frontal cortical GR expression was unaltered in all groups (E). Up-regulated GR in the amygdala was not restored by EE (F). Control: animals housed in standard laboratory conditions (n = 4); CIS: animals subjected to immobilisation stress for 2 h/day for 10 days (n = 4); CIS + EE: stressed animals exposed to enriched environment (EE) for 14 days (n = 4); EE: Un-stressed animals exposed to EE for 14 days (n = 4). Data expressed as Mean ± SEM. One-way ANOVA followed by Tukey's post hoc test; $^{*}p < 0.05$, $^{**}p < 0.01$ vs. Control. Values were normalized to β-Actin and compared with controls.

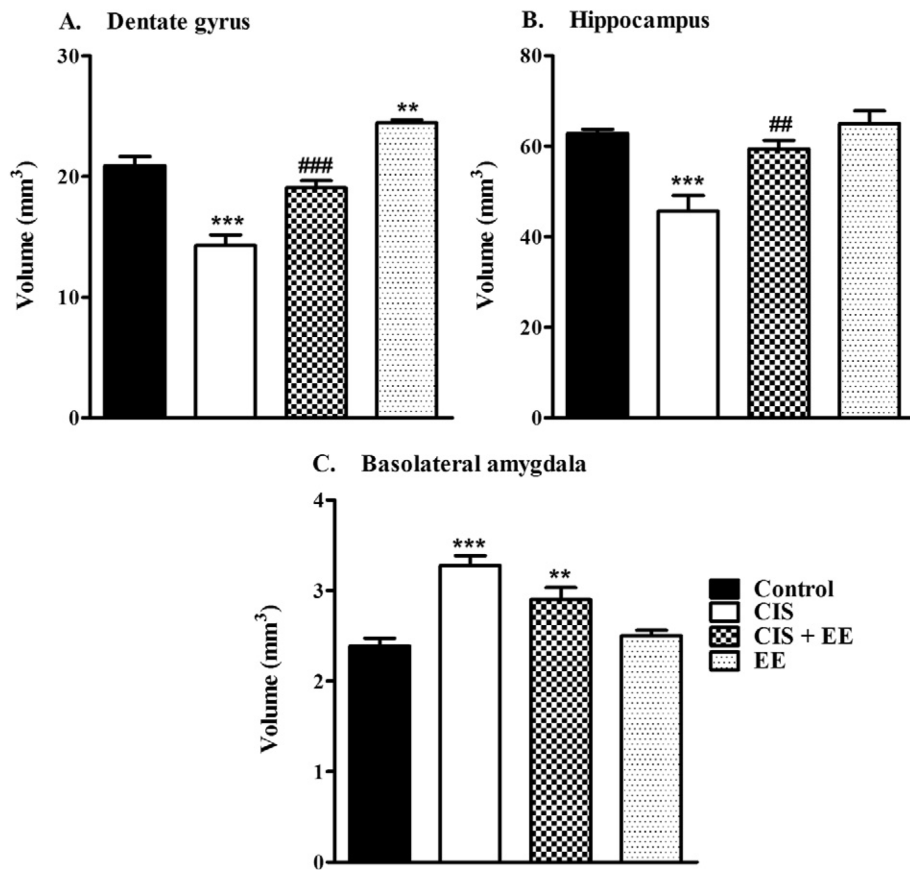

Fig. 11. Enriched environment restores hypotrophy of dentate gyrus and hippocampus without altering amygdalar hypertrophy. Dentate gyrus (A), Hippocampus (B), and Basolateral amygdala (C). Control: animals housed in standard laboratory conditions (n = 5); CIS: animals subjected to immobilisation stress for 2 h/day for 10 days (n = 5); CIS + EE: stressed animals exposed to enriched environment (EE) for 14 days (n = 5); EE: Un-stressed animals exposed to EE for 14 days (n = 5). Data expressed as Mean ± SEM. One-way ANOVA followed by Tukey's post hoc test; $^{**}p < 0.01$, $^{***}p < 0.001$ vs. Control. $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. CIS.

hippocampal hypotrophy in chronically stressed rats. Our data suggests that enriched environment restores chronic stress-induced cognitive deficits by modulating BDNF, VEGF, GFAP and GR signalling in the brain.

The CIS resulted in behavioural depression, reduced exploratory activity, heightened anxiety in the open field and elevated plus maze (EPM) tasks, respectively. CIS rats exhibited impaired spatial learning and memory in radial arm maze test. These findings are consistent with earlier studies that chronically stressed rats show behavioural depression (Veena et al., 2009a,b), reduced exploratory behaviour (Vyas et al., 2004), enhanced anxiety (Vyas et al., 2002; Anuradha et al., 2008; Bhagya et al., 2016a,b) and cognitive deficits (Srikumar et al., 2007; Ramkumar et al., 2008; Veena et al., 2009a; Bhagya et al., 2016a,b). Previously it was shown that chronic stress rats exhibited cognitive deficits and anxiety-like behaviour with reduced expression of BDNF in the hippocampus (Lakshminarasimhan and Chattarji, 2012).

In the current study, enrichment significantly ameliorated spatial learning and memory deficits with an antidepressant and anxiolytic actions in stressed rats. These beneficial effects were associated with restoration of BDNF, VEGF, GFAP and GR levels in the hippocampus and frontal cortex. Also, EE reversed BDNF, GFAP levels but not VEGF and GR levels in the amygdala. Stereological study showed that EE reversed hypotrophy of dentate gyrus and hippocampus; however, it failed to restore hypertrophy of the amygdala.

Enrichment play a key role in optimizing and modifying the neuronal circuitry by stimulating sensory, motor and cognitive stimuli, which is critically required for normal brain development (Veena et al., 2009a,b; Novkovic et al., 2015; Bhagya et al., 2016b; Mahati et al., 2016). Earlier findings showed that EE can counteract cognitive deficits (Mahati et al., 2016) and neurogenesis (Veena, 2009a,b) in animal models of depression and also improves object recognition memory performance (Novkovic et al., 2015). In the present study, we report complete restoration of spatial learning and memory impairment by enrichment in stressed rats.

Environmental enrichment showed antidepressant activity by decreasing anhedonia and behavioural despair in CIS rats. This is in accordance with previous studies where EE showed antidepressant effects (Veena et al., 2009a; Hendriksen et al., 2010; Mahati et al., 2016). Heightened anxiety-like behaviour and increased secretion of corticosterone in stress can be reversed by enriched environment (Koehl et al., 2002; Morley-Fletcher et al., 2003). Also, EE exposure has shown to enhance motor and cognitive functions by altering synaptic activity-regulating genes (Cao et al., 2014; Nowakowska et al., 2014), enhancing levels of neurotropic factors (van Praag et al., 1999a,b; Duman, 2005) and inducing progressive neural plasticity (Malik and Chattarji, 2012; Bhagya et al., 2016b; Mahati et al., 2016). BDNF plays a major role in neuronal plasticity and is known to inhibit cell death and enhance cell survival (Yulug et al., 2009). Previous studies showed that chronic stress reduced BDNF expression (Angelucci et al., 2000; Roceri et al., 2002; Govindarajan et al., 2006). Further, both serum and plasma BDNF levels were decreased in major depressive patients (Karege et al., 2002, 2005; Grassi-Oliveira et al., 2008). We hypothesize that reduced BDNF expression in the hippocampus and frontal cortex observed in our findings could be linked to depressive-like behaviour. While, restoration of BDNF levels by enriched environment may be due to its antidepressant effects in CIS model.

In our study, rats subjected to CIS showed decrease in hippocampal and frontal cortical VEGF expression. On contrary, VEGF expression was enhanced in the amygdalar complex following stress. Recent

studies have focused on VEGF which facilitates both angiogenesis and neurogenesis, acts as neuroprotective by inhibiting apoptosis, promotes hippocampal synaptic plasticity and modulates synaptic transmission (Storkebaum et al., 2004; Sun and Guo, 2005) and has trophic influence on glia cells (Nowacka and Obuchowicz, 2012). Previous studies showed that traumatic brain injury and maternal separation associated anxiety correlated with reduced VEGF levels in prefrontal cortex (Baykara et al., 2012). VEGF levels were significantly decreased in the hippocampus and frontal cortex of Flinders sensitive rats (Elfving et al., 2010). Dysregulated VEGF signalling in anxiety and depression has been established by Phenome-transcriptome correlation study (Gormanns et al., 2011). Electroconvulsive therapy resulted in significant increase in serum VEGF levels with improved depressive symptoms (Minelli et al., 2011). Previous studies show that VEGF is a major molecule to be involved in the beneficial effect of enriched environment on cognitive functions (Cao et al., 2004; Warner-Schmidt and Duman, 2007). Also, previous study showed housing animals in enriched environment had no significant effect on VEGF in the amygdala (Kovesdi et al., 2011).

We observed CIS-induced reduction in GFAP expression in the hippocampus and frontal cortex. Low GFAP levels in the dorsolateral prefrontal cortex (Cotter et al., 2002), orbitofrontal and anterior cingulate cortex (Benes et al., 2001; Chana et al., 2003) and hippocampus (Stockmeier and Rajkowska, 2004) were associated with depressive symptoms. Both animal and human studies suggest that depressed conditions are associated with reduced levels of GFAP in cortical and limbic structures (Miguel-Hidalgo et al., 2000; Gosselin et al., 2009). In the current study, GFAP expression was restored to normal levels after EE exposure. In accord with our present data, previous studies have demonstrated an increase in GFAP positive cells after EE exposure (Salmaso et al., 2012; Sampedro-Piquero et al., 2015). In addition, EE enhances generation of astroglia cells in the neocortex (Ehninger and Kempermann, 2003; Alwis and Rajan, 2014). Previous studies have demonstrated that EE exhibits its neuroprotective effect through upregulation of glucocorticoid receptors (GR) against stress (Wislowska-Stanek et al., 2013; Zanca et al., 2015). Enhanced GR levels are associated with improved cognitive functions with reduced anxiety-like behaviours (Reichardt et al., 2000; Fernández-Teruel et al., 2002; Zhang et al., 2013).

Present study shows significant reduction in DG and hippocampal volume and contrasting increase in the volume of the basolateral amygdala in the CIS animals. Environmental enrichment restored both DG and hippocampal volumes in CIS animals, but failed to restore amygdalar hypertrophy. Several structural studies show volumetric reduction in hippocampal and fronto-cortical areas (Sheline et al., 1996; Sheline, 2000; Veena et al., 2009a; Mahati et al., 2016), hypertrophy in amygdalar complex (von Gunten et al., 2000; Frodl et al., 2002; Karl et al., 2006; Mahati et al., 2016) in major depressive patients and in animal models of depression. Studies have shown that exposure to EE result in restoration of DG and hippocampal hypotrophy in animal models of depression (Veena et al., 2009a; Mahati et al., 2016). EE bring about structural plasticity by increasing dendritic complexity and spine density (Beauquis et al., 2010; Bindu et al., 2007) and neurogenesis in the hippocampus (Veena et al., 2009a,b).

Chronic stress not only causes spatial memory deficits, it also enhances emotionality through amygdalar activation (Conrad et al., 1999). Chronic stress differentially regulates hippocampus and amygdalar functions. Hippocampal neurons undergo dendritic atrophy (Ramkumar et al., 2008), whereas amygdala shows dendritic hypertrophy (Vyas et al., 2002, 2004). Basolateral amygdala plays a major role in consolidation of anxiety-like behaviour in the elevated plus maze (Vyas et al., 2004). Also, cessation of stress for 21 days results in complete restoration of hippocampal dendritic atrophy, while amygdala shows dendritic hypertrophy with persistent enhanced anxiety behaviour (Vyas et al., 2004). In addition, CIS resulted in enhanced synaptic activity in the amygdala and results in stronger fear memories (Suvrathan et al., 2013). Also, enhanced BDNF after CIS strengthens synaptic connectivity in the amygdala which may cause heightened anxiety (Lakshminarasimhan and Chattarji, 2012). Earlier studies show that enrichment reduces anxiety-like behaviour in animal models of depression (Bhagya et al., 2016b; Mahati et al., 2016). In contrast, enrichment did not completely reverse chronic immobilisation stress-induced anxiety behaviour and amygdalar hypertrophy in the current study. We speculate that this may be due to partial recovery of VEGF and GR expression in the amygdala.

In conclusion, enriched environment reduces anxiety, improves cognitive functions and has an antidepressant-like effect in chronically stressed animals. These beneficial effects of EE are induced via BDNF, VEGF, GFAP and GR expression in the hippocampus and frontal cortex. Further, enrichment restores hippocampal hypotrophy but not amygdala hypertrophy. Therefore, enriched environment may be considered to be one of the most efficient therapeutic approaches to treat neuropsychiatric diseases. Moreover, the current study indicates the important role of neurotrophins, astrocytes in the mechanism of action of positive enrichment. A more comprehensive understanding of the beneficial effects of environmental enrichment will have implications for the treatment of psychiatric diseases as well as other neurodegenerative disorders.

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