Development, optimization and mitochondrial characterization of experimental animal model of post-traumatic stress disorder (PTSD)

Introduction

Post-traumatic stress disorder (PTSD) is considered as an anxiety disorder which develops following exposure to severe traumatic event(s) such as war, violent personal assault, or following natural disasters (Stanford, 1995). However, according to DSM-V, it is considered as a stress-related disorder. PTSD is characterized by a phenomenological triad incorporating the core symptoms of re-experience, avoidance, and hyperarousal (Stanford, 1995). The prevalence of risk for developing PTSD is about 15% among the population (Skelton et al., 2011). Pharmacotherapy of PTSD comprises of selective serotonin (5-HT) reuptake inhibitors, monoamine oxidase inhibitors, anticonvulsants and other classes of drugs including antipsychotics (Kozaric-Kovacic, 2008). However, controlled clinical trials show that the pharmacotherapy of PTSD is far from satisfactory (Ravindran and Stein, 2009; Stein et al., 2009). Therefore, the disorder lacks the promising strategy in the pharmacotherapy of PTSD. These attributes of this disorder accentuates the establishment of an appropriate animal model of PTSD that can promote our understanding in its pathophysiology which may help to identify its novel and more effective therapeutic strategies.

Recently, we developed an experimental model of PTSD, a modified version of stress re-stress (SRS), which exhibits long-lasting behavioral and neuroendocrinological manifestations in the animals (Krishnamurthy et al., 2013; Garabadu et al., 2015).The SRS model is regarded as the most appropriate experimental model of PTSD (Yehuda and Antelman, 1993; Liberzon et al., 1997). This paradigm is based on use of severe traumatic stress and subsequent presentation of brief “reminder” episodes, which serve as contextual triggers for the development of the pathology. This leads to the formation of a stable anxiety state in rodents in terms of a number of

key behavioral and hormonal characteristics similar to PTSD in humans. The important hallmark of this model is that it can be used for the evaluation of drugs used chronically for the treatment of PTSD as clinical data suggests that long-term use of drugs is essential for treatment of PTSD (Hamner et al., 2003; Bartzokis et al., 2005; Rothbaum et al., 2008). Similar to other authors we also report that SRS paradigm causes a significant decrease in the basal corticosterone level which is the characteristic hallmark of PTSD (Harvey et al., 2006; Krishnamurthy et al., 2013). We further report that the subsequent exposure of the same re-stress session at a regular time interval sustains the PTSD attributes. It has also been reported that the basal steady-state corticosterone level is a predictor of stress susceptibility or resilience to subsequent stress exposures (Kim et al., 2012). Hence, it can be presumed that the re-stress session is an important contributor in the development of aftermath consequences of PTSD. However, there is no report on the interval between the first exposure of re-stress session to the early traumatic event to develop and maintain the severity of PTSD manifestations in the experimental model. Hence, the modified model of PTSD is further optimized to understand the value of re-stress or reminder of the stress event in the pathophysiology of PTSD.

PTSD is characterized by impairments of the endocrine system and its regulation, primarily the dysregulation of hypothalamus-pituitary-adrenocortical (HPA)-axis (Olff et al., 2006) leading to hypocortisolemia (Yehuda et al., 1998). Mineralocorticoid receptor (MR) exhibits high affinity to cortisol, however during stress there is increase in cortisol secretion with a concomitant increase in the level of glucocorticoid receptors (GR; Reul and De Kloet, 1985). As MR and GR activities oppose each other, MR/GR ratio is considered as a marker for stress resilience and vulnerability (Reul et al., 1991). Further, it is also known that the affinity of corticosterone is more to corticotrophin-releasing hormone receptor-1 (CRH-1) than CRH-2

(Elharrar et al., 2013). The over-expression of CRH-1 is a molecular manifestation to anxiety-like states and this is also observed in PTSD condition (Sink et al., 2013). In physiological condition CRH-1 is predominantly found in most of the brain regions while the density of CRH-2 receptor is relatively low in brain tissues (Elharrar et al., 2013). PTSD-induced alteration in corticosteroid receptors activity has been reported in different brain regions including hypothalamus (HYP), hippocampus (HIP), pre-frontal cortex (PFC) and amygdala (AMY; Reul and De Kloet, 1985; Yehuda et al., 1998, Neylan et al., 2005; De Kloet, 2003). In addition to glucocorticoid dysfunction, there is a significant decrease in the Akt phosphorylation in several brain regions during anxiety and depressive-like conditions (Punn et al., 2006). Thus, it is prerequisite to elaborate the molecular basis of CRF/Akt signaling in the pathobiology of the development of PTSD manifestations.

Several neurobiological factors including mitochondrial dysfunction have been implicated in the pathophysiology of PTSD (Hauger et al., 2012; Su et al., 2008; Li et al., 2013; Xing et al., 2013). Recently, we have reported the mitochondrial dysfunction in the pathophysiology of PTSD manifestations (Garabadu et al., 2015). It has also been reported that there is aberrant expression of mitochondrial respiratory chain enzymes in different brain regions in single prolonged stress model of PTSD (Xing et al., 2013). Atypical respiratory enzymes activities could lead to increased reactive oxygen species (ROS) production and may cause a loss in the mitochondrial integrity through decrease in the mitochondrial membrane potential (MMP; Zhang et al., 2006; Xing et al., 2013; Garabadu and Krishnamurthy, 2013; Garabadu and Krishnamurthy, 2014; Geed et al., 2014). Decrease in MMP can cause leakage of the cytochrome-C (Zhang et al., 2006), which activates factors related to intrinsic pathway of apoptosis (Shi, 2001). Several authors have reported apoptosis in HIP, PFC and AMY of PTSD

subjected rodents (Liu et al., 2011; Li et al., 2013; Han et al., 2013). Therefore, mitochondria could be a potential target for the pharmacological interventions in the management of PTSD. Taken into consideration, in the present study, the mitochondrial bioenergetics has been evaluated in the optimized model of PTSD.

Therefore, the present study was proposed to optimize the model in terms of timing of exposure to early re-stress in the development of PTSD manifestations in the first experimental setup. Further, an attempt was made to establish the CRH/Akt signaling mechanism in the development of PTSD symptoms. In the second set of experiment, the mitochondrial bioenergetics was evaluated in the optimized model to further validate the hypothesis of mitochondrial dysfunction.

Materials and methods

Animals

The experiment was conducted in accordance with the Principles of laboratory animal care (NIH, 2011) guidelines. Male adult Charles Foster strain albino rats, 3 months of age (200 ± 20 g) were purchased from the Central Animal House, Institute of Medical Sciences, Banaras Hindu University (BHU). Experiments on animals were approved by the Institutional Animal Ethics Committee of BHU, Varanasi, India (Protocol No: Dean/11-12/CAEC/328). The animals were housed in polypropylene cages under controlled environmental conditions of temperature of 25 ± 1 °C and 45-55% relative humidity and a 12:12 hr light/dark cycle. The experimental animals had free access to commercial rat feed (Doodh dhara Pashu Ahar, India) and water *ad libitum* during the experiment. All the experiments were carried out during 08:00 hr to 16:00 hr. Animals were acclimatized for at least one week before using them for experiments and exposed only once to the experiment.

Chemicals

Antibodies were purchased from Santa Cruz Biotechnology Inc (Santa Cruz, California, USA). All other chemicals and reagents of HPLC and analytical grade were procured from local suppliers.

Experimental design

The whole experimental design was divided into two sub sets. In the first experimental setup, the animals were divided into seven groups of six animals each namely; control, variable stress only (VS), variable stress with re-stress session (SRS-1 to 5). In SRS-1 to 4 group, the re-stress session was introduced in the animals on 8, 14, 20 and 26th day of the experimental schedule respectively. Thereafter, the re-stress session was continued upto the end of the experimental protocol at a regular interval of six days. In SRS-5, the re-stress session was introduced on day-32 of the schedule. On the last day of the schedule, all animals were killed and their brain regions were microdissected (Palkovits and Brownstein, 1988) into hippocampus (HIP), hypothalamus (HYP), pre-frontal cortex (PFC) and amygdale (AMY), and preserved at -80 °C for further studies. In the second set of experiment, the animals were divided into two groups of six animals each namely; control and optimized modified SRS (mSRS). The experiment was conducted for 32 days and all the animals were killed and the brain regions were collected for the analysis of mitochondrial bioenergetics.

Stress paradigm

Briefly, rats were individually exposed to 2 hr restraint in an animal holder, followed by 20 min forced swimming (25 °C) and halothane (0.8 ml of 4% halothane) anesthesia until the loss of consciousness on D-2. The rats were "re-stressed" by exposure to the forced swimming procedure for 20 min on D-8, D-14, D-20, D-26 and D-32 of the experimental protocol

(Krishnamurthy et al., 2013). The immobility period was evaluated during last re-stress session as a measure of depression-like symptom. Immobility was defined as sustained immobility except for respiratory movements.

Assessment of behavioral performance

Evaluation of anxiolytic activity in EPM

The open arm entries and time spent were estimated as indices of anxiety-like behaviors. Total arm entries were measured as an index of locomotor activity using EPM test (Itoh et al., 1991).

Evaluation of spatial recognition memory in Y-maze test

The total number of entries in all arms (for the 5 min of trial 1 and 2), % entries in known versus novel arm for the 5 min period of trial 2 and percentage of time spent in novel arm to time spent in all arms and in the center of the apparatus during trial 2 were estimated as indices of general exploratory behavior, spatial recognition memory and anxiety-like behavior respectively (Dellu et al., 1992; Krishnamurthy et al., 2013).

Estimation of ACTH in plasma

All plasma samples were stored frozen at – 20 °C until the determination of ACTH. ACTH concentration was assayed using a commercially available ELISA kit (Immunodiagnostic Systems Ltd., UK) and was expressed as pg/ml.

Estimation of corticosterone in the plasma

The plasma corticosterone was quantified (Woodward and Emery, 1987; Garabadu et al., 2011) in a HPLC system with ultraviolet detector (Waters, USA).

Assessment of mitochondrial integrity, function and oxidative stress

Isolation of mitochondria from discrete rat brain tissues

Mitochondria were isolated from each tissue by following standard protocol (Pedersen et al., 1978). The mitochondrial protein content was estimated using standard method (Lowry et al., 1951).

Estimation of mitochondrial bioenergetics

Mitochondrial respiration was measured polarographically using a Clark oxygen electrode (Hansatech Instruments Pvt. Ltd., USA). Isolated mitochondria (1 mg/ml) were incubated, at 30 °C, in the respiratory medium containing 125 mM sucrose,65 mM KCl, 2.5 mM $MgCl_2$, 5 mM KH_2PO_4, 5 mM Hepes, pH 7.2. An initial rate of oxygen consumption (state 2 orV_2) was recorded following addition of glutamate plus malate (10 mM/5 mM), and the state 3 rate (V_3) was recorded following the subsequent addition of 250 nmol ofADP. After a measurable state 4 rate (V_4) (i.e., the rate after ADP phosphorylation) was obtained, a second pulse of ADP was added but the phosphorylative cycle was soon inhibited before its completion by adding 1 µg of oligomycin. After a measurable oligomycin oxygen consumption rate (V_{olig}) was obtained, a 1 µM concentration of the uncoupling agent FCCP was added to obtain a rate of oxygen consumption in the absence of coupled oxidative phosphorylation (V_{FCCP}). Further, to elaborate the respiratory complex-II activity, the oxygen consumption was recorded in presence of 15 mM succinate and 2.2 mM rotenone. Respiratory control ratios (RCR), respiratory states, and ADP/O ratios were determined according to the standard protocol (Chance and Williams, 1956).

Estimation of mitochondrial respiratory complex-I, II, IV and V activity

The activity of NADH dehydrogenase (complex-I) was measured by catalytic oxidation of NADH with potassium ferricyanide as an artificial electron acceptor at excitation and emission

wavelengths for NADH were 350 nm and 470 nm, respectively (Shapiro et al., 1979). Activity of NADH dehydrogenase was expressed as nmole NADH oxidised/min/mg protein. The mitochondrial succinate dehydrogenase (SDH; complex-II) was determined by the progressive reduction of nitro blue tetrazolium (NBT) to an insoluble colored compound, diformazan at 570 nm (Sally et al., 1989). The SDH activity was expressed as micromole formazan produced/min/mg protein. The activity of cytochrome oxidase (complex-IV) was measured in mitochondrial fraction in presence of reduced cytochrome c at 550 nm for 3 min (Storrie and Madden, 1990). Results were expressed as nmole cytochrome c oxidized/min/mg protein (ε_{550} = 19.6 $mmol^{-1}cm^{-1}$). The F1-F0 synthase (complex-V) was measured by incubating mitochondrial suspension in ATPase buffer (Griffiths and Houghton, 1974) and the phosphate content was measured (Fiske and Subbarao, 1925). Results were expressed as nmole ATP hydrolyzed/min/mg protein.

Evaluation of MMP in discrete brain regions

The rhodamine dye taken up by mitochondria was measured in spectrofluorometer (Hitachi, F-2500) at an excitation λ of 535 ± 10 nm and emission λ of 580 ± 10 nm (Huang, 2002). The results were expressed as fluorescence intensity/mg protein.

Estimation of lipid peroxidation (LPO) and nitric oxide (NO) level

Mitochondrial malondialdehyde (MDA) content was measured as a marker of LPO at 532 nm (Ohkawa et al., 1979). The extent of LPO was expressed as micromoles of MDA/mg protein. The NO level was estimated as a marker for nitrosative stress (Green et al., 1982) and expressed as nmoles of NO/mg protein.

Assessment of superoxide dismutase (SOD) and catalase (CAT) activity

Superoxide dismutase (SOD) activity was determined by the reduction of NBT in presence of phenazine-methosulphate and NADH at 560 nm using n-butanol as blank (Kakkar et al., 1984). A single unit of the enzyme was expressed as 50% inhibition of NBT reduction/minute/mg protein. Decomposition of hydrogen peroxide in presence of CAT was followed at 240 nm (Beers and Sizer, 1952). The results were expressed as units (U) of CAT activity/min/mg of protein.

Western blot analysis

Briefly, the brain regions were lysed in buffer containing complete protease inhibitor cocktail. Protein concentrations were determined according to Bradford (1976). A standard plot was generated using bovine serum albumin. An aliquot of each sample were electrophoresed in 10% SDS-PAGE gels for CRH-1, CRH-2, Akt, p-Akt, GR and MR proteins, transferred to polyvinylidene fluoride membranes and probed with specific antibodies. The membrane was incubated overnight with rabbit anti-CRH-1 (Abcam Plc., Cambridge, USA), anti-CRH-2 (Abcam Plc., Cambridge, USA), anti-Akt (Abcam Plc., Cambridge, USA) and anti-p-Akt (Abcam Plc., Cambridge, USA), anti-GR (Abcam Plc., Cambridge, USA) and anti-MR (Abcam Plc., Cambridge, USA) polyclonal primary antibody at a dilution of 1:1000, 1:1000, 1:1000, 1:1000, 1:500 and 1:500 respectively. After detection with the desired antibodies against the proteins of interest the membrane was stripped with stripping buffer (25 mM Glycine pH 2.0, 2% SDS for 30 min at room temperature) and re-probed overnight with rabbit anti β-actin (Santa Cruz Biotechnology Inc.; Santa Cruz, California, USA) polyclonal primary antibody at a dilution of 1:500 to confirm equal loading of protein. Further, membrane was probed with corresponding secondary antibodies. Immunoreactive band of proteins were detected by chemiluminescence

using enhanced chemiluminescence (ECL) reagents (Amersham Bioscience, USA). Quantification of the results was performed by densitometric scan of films. The immunoreactive area was determined by densitometric analysis using Biovis gel documentation software.

Data analysis

All the data were mean ± standard error of the mean (S.E.M). In experiment 1, the data total arm entries in trial-1 and 2, and percentage entries into known and novel arm in trial-2 in Y-maze were evaluated by using repeated measure two-way analysis of variance (ANOVA) with Bonferroni Post-hoc test. Two-way ANOVA followed by Bonferroni Post-hoc test was performed to measure arm discrimination behavior between known and novel arm in Y-maze paradigm for each group. All other statistical analysis of data was done using one-way ANOVA with Newman-Keuls Post-hoc analysis to monitor significance among groups. $P<0.05$ were considered as significant. In experiment 2, all the statistical analysis was done using two-tailed Unpaired Student t-test.

Results

The effect of variable stress (VS) and time-dependent exposure of first RS on depressive-like behavior of rats in FST

Fig-27 depicts the effect of VS and time-dependent exposure of first RS on depressive-like behavior in terms of immobility period in FST in rats. Statistical analysis revealed that there were significant differences among groups [$F(6, 28) = 37.2$, $p<0.05$]. Post-hoc test showed that SRS-1 and SRS-2 group animals exhibited significant increase in immobility period during FST compared to all other group rats.

The effect of VS and time-dependent exposure of first RS on anxiety-like behaviors of rats in EPM test paradigm

The effect of VS and time-dependent exposure of first RS on percentage of open arm entries (A), time spent (B), and total arm entries (C) in rats are illustrated in Fig-28. Statistical analysis revealed that there were significant differences in anxiety-like behaviors in terms of percentage of entries [$F(6, 28) = 40.4$, $p<0.05$] and time spent [$F(6, 28) = 18.5$, $p<0.05$] into open arm in EPM test paradigm among groups. However, there were no significant differences among groups in total arm entries [$F(6, 28) = 0.07$, $p>0.05$]. Post-hoc test showed that SRS-1 and SRS-2 group animals exhibited significant increase in both percentage of open arm entries and time spent during EPM compared to all other group rats.

The effect of VS and time-dependent exposure of first RS on the curiosity, spatial recognition memory and anxiety-like behaviors in Y-maze test paradigm

Fig-29 depicts the effect of VS and time-dependent exposure of first RS on the total arm entries in trial-1 and 2 (curiosity; A), spatial recognition memory (B) and coping behavior to novel arm (anxiety-like behavior; C) in Y-maze test paradigm. Statistical analysis revealed that there were significant differences for curiosity among groups [$F(6, 56) = 24.0$, $p<0.05$] and trials [$F(1, 56) = 245.2$, $p<0.05$], a significant interaction between group and trial [$F(6, 56) = 1.4$, $p<0.05$]. Post-hoc test showed that the curiosity was higher in SRS-1 and 2 group animals compared to all other group rats. There were significant differences for arm discrimination behavior between known and novel arm among groups [$F(6, 56) = 0.6$, $p<0.05$] and arms [$F(1, 56) = 19.3$, $p<0.05$], and a significant interaction between group and arm [$F(6, 56) = 52.5$, $p<0.05$]. Post-hoc test showed that the control animals exhibited significantly higher arm discrimination to novel arm as compared to known arm, indicating gain of spatial recognition memory. SRS-1 and 2

group rats exhibited loss in spatial recognition memory. However, all other stress treated group animals did not show any loss in spatial recognition memory. Further, one-way ANOVA revealed that there were significant differences among groups in known [$F(6, 28) = 17.2$, $p<0.05$] and novel [$F(6, 28) = 43.6$, $p<0.05$] arm entries. Post-hoc analysis showed that SRS-1 and 2 group rats showed significant increase and decrease in the percentage entries into known and novel arm compared to all other group animals respectively. Further, the percentage arm entries into known and novel arm for SRS-1 group rats were significantly higher and lower compared to SRS-2 group animals respectively. Moreover, statistical analysis revealed that there were significant differences in anxiety-like behavior among groups [$F(6, 28) = 9.9$, $p<0.05$]. Post-hoc test showed that SRS-1 and SRS-2 group animals exhibited significant increase in anxiety-like behavior compared to all other group rats.

The effect of VS and time-dependent exposure of first RS on the levels of plasma corticosterone and ACTH

The effect of VS and time-dependent exposure of first RS on the levels of plasma corticosterone (A) and ACTH (B) are depicted in Fig-30. Statistical analysis revealed that there were significant differences among groups in plasma levels of corticosterone [$F(6, 28) = 23.9$, $p<0.05$] and ACTH [$F(6, 28) = 21.3$, $p<0.05$]. Post-hoc test showed that VS did not cause any change in the levels of plasma corticosterone and ACTH in the rats compared to control animals. But, both plasma corticosterone and ACTH levels were significantly decreased in SRS-1 and 2 group animals compared all other group animals. However, these levels were significantly higher in SRS-3, 4 and 5 group rats compared to all other group rats.

The effect of VS and time-dependent exposure of first RS on the levels of expression of CRH-1 and 2 receptors in discrete brain regions

Fig-31 depicts the effect of VS and time-dependent exposure of first RS on the levels of expression of CRH-1 (A) and CRH-2 (B) receptors in discrete brain regions. Statistical analysis revealed that there were significant differences among groups in the levels of expression of CRH-1 and 2 in HIP ([F (6, 20) = 27.8, $p<0.05$] and [F (6, 20) = 18.7, $p<0.05$] respectively), HYP ([F (6, 20) = 24.7, $p<0.05$] and [F (6, 20) = 5.6, $p<0.05$] respectively), PFC ([F (6, 20) = 12.1, $p<0.05$] and [F (6, 20) = 32.6, $p<0.05$] respectively) and AMY ([F (6, 20) = 13.1, $p<0.05$] and [F (6, 20) = 13.3, $p<0.05$] respectively). Post-hoc analysis showed that SRS-1 and 2 group animals showed significant increase and decrease in the level of expression of CRH-1 and 2 respectively in all the brain regions compared to all other group rats respectively.

The effect of VS and time-dependent exposure of first RS on the extent of phosphorylation of Akt in discrete brain regions

The effect of VS and time-dependent exposure of first RS on the levels of expression of Akt (A) and p-Akt (C) in HIP, HYP, PFC and AMY is illustrated in Fig-32. Statistical analysis showed that there were significant differences in the level of expression of p-Akt (D) and the ratio of p-Akt/Akt (E) in HIP ([F (6, 20) = 6.0; $p<0.05$] and [F (6, 20) = 15.3; $p<0.05$] respectively), HYP ([F (6, 20) = 5.6; $p<0.05$] and [F (6, 20) = 5.6; $p<0.05$] respectively), PFC ([F (6, 20) = 2.7; $p<0.05$] and [F (6, 20) = 10.7; $p<0.05$] respectively) and AMY ([F (6, 20) = 3.3; $p<0.05$] and [F (6, 20) = 13.1; $p<0.05$] respectively) among groups. However, there were no significant differences in the level of expression of Akt (B) in HIP [F (6, 20) = 0.3; $p>0.05$], HYP [F (6, 20) = 0.1; $p>0.05$], PFC [F (6, 20) = 0.6; $p>0.05$] and AMY [F (6, 20) = 0.4; $p>0.05$] among groups. Post-hoc test showed that SRS-1 and 2 group rats exhibited significant decrease in the level of p-

Akt and ratio of p-Akt/Akt in all the brain regions compared to all other group animals, indicating a significant decrease in the extent of phosphorylation of Akt with the exposure of both SRS-1 and SRS-2. It is interesting to note that SRS-1 group rats showed a further decrease in the extent of phosphorylation of Akt in HIP compared to SRS-2 group animals.

The effect of VS and time-dependent exposure of first RS on the levels of MR, GR and the ratio of MR/GR in discrete brain regions

The effect of VS and time-dependent exposure of first RS on the levels of expression of MR (A) and GR (C) in HIP, HYP, PFC and AMY is illustrated in Fig-33. Statistical analysis showed that there were significant differences in the level of expression of MR (B),GR (D) and the ratio of MR/GR (E) in HIP ([$F(6, 20) = 52.1$; $p<0.05$], [$F(6, 20) = 8.3$; $p<0.05$] and [$F(6, 20) = 57.5$; $p<0.05$] respectively), HYP ([$F(6, 20) = 22.4$; $p<0.05$], [$F(6, 20) = 5.6$; $p<0.05$] and [$F(6, 20) = 35.0$; $p<0.05$] respectively), PFC ([$F(6, 20) = 43.0$; $p<0.05$], [$F(6, 20) = 10.7$; $p<0.05$] and [$F(6, 20) = 67.2$; $p<0.05$] respectively) and AMY ([$F(6, 20) = 52.3$; $p<0.05$], [$F(6, 20) = 13.1$; $p<0.05$] and [$F(6, 20) = 81.7$; $p<0.05$] respectively) among groups. Post-hoc test showed that SRS-1 and 2 group rats exhibited significant decrease in the levels of MR and GR, and the ratio of MR/GR in all the brain regions compared to all other group animals.

Effect of modified stress-restress (mSRS) paradigm on mitochondrial bioenergetics of discrete rat brain regions

Fig-34 depicts the representative patterns of mitochondrial respiration in HIP (A), HYP (B), PFC (C) and AMY (D) of both control and mSRS group animals. Table-25 illustrates the effect of mSRS on mitochondrial bioenergetics of discrete brain regions of the animals. Statistical analysis showed that there were significant differences in the extent of oxygen consumption in S_2, S_3, S_4, S_{FCCP} and S_{Succ} levels of RCR and the ratio of ADP/O in HIP ($t_8 = 5.0$, $p<0.05$, $t_8 =$

10.3, $p<0.05$, $t_8 = 9.2$, $p<0.05$, $t_8 = 4.6$, $p<0.05$, $t_8 = 3.1$, $p<0.05$, $t_8 = 10.1$, $p<0.05$ and $t_8 = 5.8$, $p<0.05$ respectively) HYP ($t_8 = 5.4$, $p<0.05$, $t_8 = 13.5$, $p<0.05$, $t_8 = 7.1$, $p<0.05$, $t_8 = 4.6$, $p<0.05$, $t_8 = 5.2$, $p<0.05$, $t_8 = 8.3$, $p<0.05$ and $t_8 = 12.0$, $p<0.05$ respectively), PFC ($t_8 = 3.2$, $p<0.05$, $t_8 = 12.9$, $p<0.05$, $t_8 = 8.5$, $p<0.05$, $t_8 = 5.1$, $p<0.05$, $t_8 = 4.1$, $p<0.05$, $t_8 = 8.0$, $p<0.05$ and $t_8 = 5.0$, $p<0.05$ respectively) and AMY($t_8 = 6.2$, $p<0.05$, $t_8 = 9.9$, $p<0.05$, $t_8 = 5.3$, $p<0.05$, $t_8 = 5.2$, $p<0.05$, $t_8 = 2.2$, $p<0.05$, $t_8 = 8.9$, $p<0.05$ and $t_8 = 6.3$, $p<0.05$ respectively) among groups. However, there were no significant differences in the extent of oxygen consumption in S_{Olig} in HIP ($t_8 = 1.3$, $p<0.05$), HYP ($t_8 = 0.9$, $p<0.05$), PFC ($t_8 = 0.4$, $p<0.05$) and AMY ($t_8 = 0.9$, $p<0.05$) among groups. Post-hoc test revealed that the extent of oxygen consumption in all the states except S_{Olig} was significantly increased in all the brain regions of mSRS treated rats compared to control group animals. Further, mSRS caused a significant decrease in the level of RCR and the ratio of ADP/O in all the brain regions compared to control animals.

Effect of mSRS on mitochondrial function of discrete brain regions

Table-26 illustrates the effect of mSRS on mitochondrial function in terms of changes in the activities of respiratory complex enzymes in discrete brain regions of the animals. Statistical analysis showed that there were significant differences in the activity of mitochondrial complex-I, II, IV and V, and the extent of MMP in HIP ($t_8 = 8.7$, $p<0.05$, $t_8 = 21.2$, $p<0.05$, $t_8 = 21.2$, $p<0.05$, $t_8 = 4.7$, $p<0.05$ and $t_8 = 6.2$, $p<0.05$ respectively) HYP ($t_8 = 14.7$, $p<0.05$, $t_8 = 7.1$, $p<0.05$, $t_8 = 10.5$, $p<0.05$, $t_8 = 6.7$, $p<0.05$ and $t_8 = 9.7$, $p<0.05$ respectively), PFC ($t_8 = 6.2$, $p<0.05$, $t_8 = 16.4$, $p<0.05$, $t_8 = 7.8$, $p<0.05$, $t_8 = 7.0$, $p<0.05$ and $t_8 = 6.7$, $p<0.05$ respectively) and AMY ($t_8 = 5.6$, $p<0.05$, $t_8 = 3.1$, $p<0.05$, $t_8 = 7.8$, $p<0.05$, $t_8 = 7.4$, $p<0.05$ and $t_8 = 4.2$, $p<0.05$ respectively) among groups. Post-hoc test revealed that the activity of all the mitochondrial

complex enzymes and the level of MMP were significantly increased and decreased in all the brain regions of mSRS exposed rats compared to control group animals respectively.

Effect of mSRS on mitochondrial oxidative stress in discrete brain regions

Table-27 depicts the effect of mSRS on mitochondrial oxidative stress in terms of changes in the extent of LPO, level of NO and, activities of SOD and CAT in discrete brain regions of the animals. Statistical analysis showed that there were significant differences in the extent of mitochondrial LPO, level of NO and, the activities of SOD and CAT in HIP ($t_8 = 3.7$, $p<0.05$, $t_8 = 14.1$, $p<0.05$, $t_8 = 8.9$, $p<0.05$ and $t_8 = 3.5$, $p<0.05$ respectively) HYP ($t_8 = 8.3$, $p<0.05$, $t_8 = 20.6$, $p<0.05$, $t_8 = 17.9$, $p<0.05$ and $t_8 = 5.5$, $p<0.05$ respectively), PFC ($t_8 = 5.2$, $p<0.05$, $t_8 = 21.2$, $p<0.05$, $t_8 = 11.7$, $p<0.05$ and $t_8 = 6.1$, $p<0.05$ respectively) and AMY ($t_8 = 4.0$, $p<0.05$, $t_8 = 22.0$, $p<0.05$, $t_8 = 14.6$, $p<0.05$ and $t_8 = 5.0$, $p<0.05$ respectively) among groups. Post-hoc test revealed that the extent of LPO and the level of NO were significantly increased in all the brain regions of mSRS treated rats compared to control group animals. Moreover, the activity of SOD and CAT were significantly lower in all the brain regions of mSRS treated animals compared to the control rats.

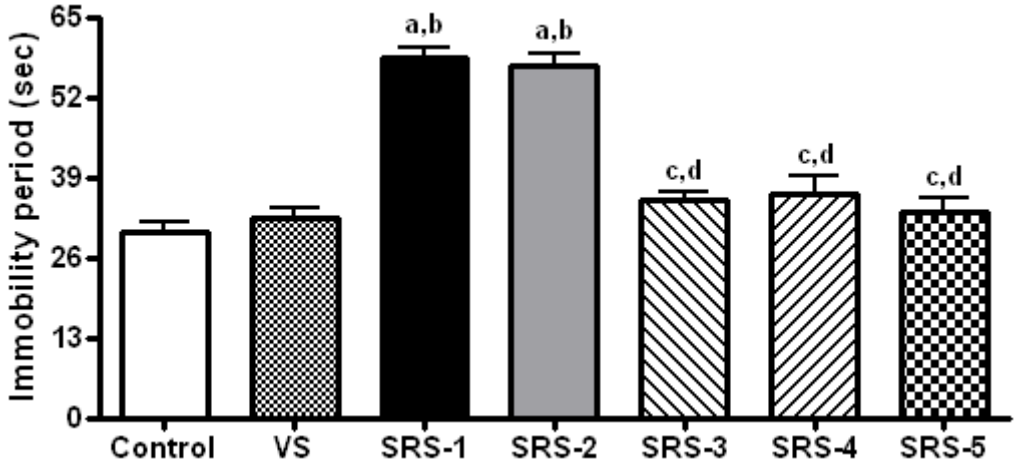

Figure 27: The effect of VS and time-dependent exposure of first RS on immobility period in FST in rats. All values are mean ± SEM (n = 5). [a]P<0.05 compared to control, [b]P<0.05 compared to VS, [c]P<0.05 compared to SRS-1 and [d]P<0.05 compared to SRS-2 (One-way ANOVA followed by Student Newmann Keuls post-hoc test).

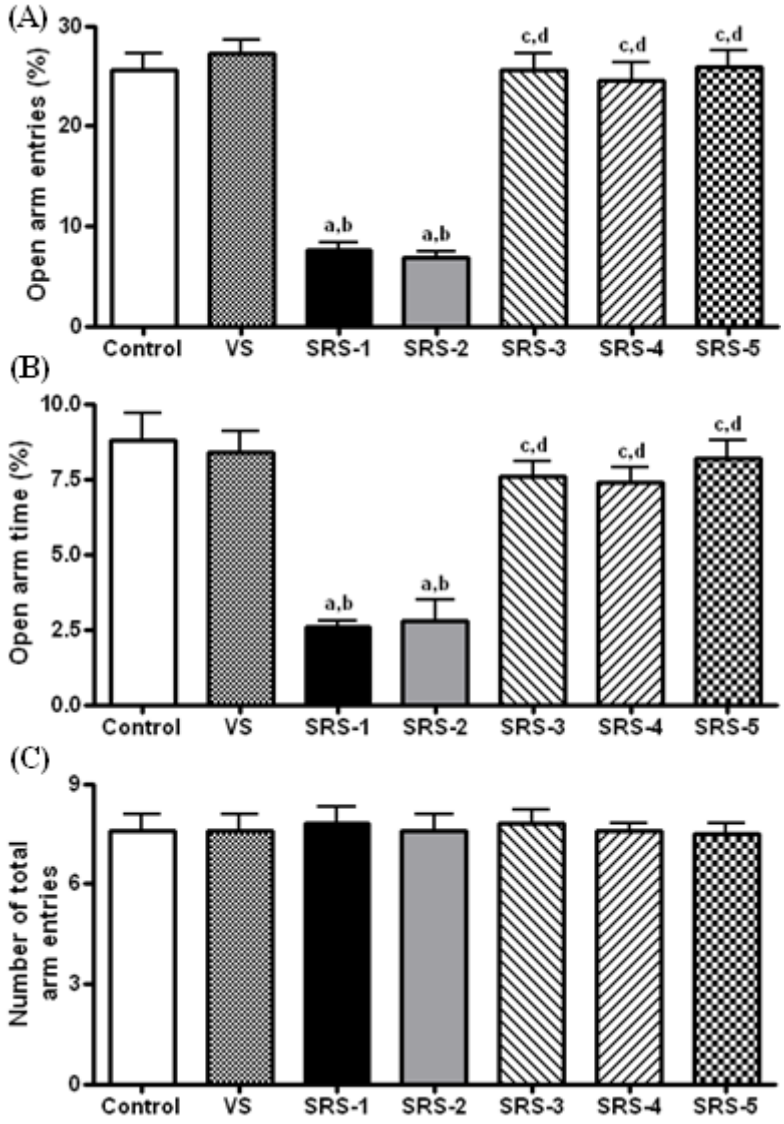

Figure 28: The effect of VS and time-dependent exposure of first RS on percentage of open arm entries (A), time spent (B), and total arm entries (C) in rats. All values are mean ± SEM (n = 5). [a]P<0.05 compared to control, [b]P<0.05 compared to VS, [c]P<0.05 compared to SRS-1 and [d]P<0.05 compared to SRS-2 (One-way ANOVA followed by Student Newmann Keuls post-hoc test).

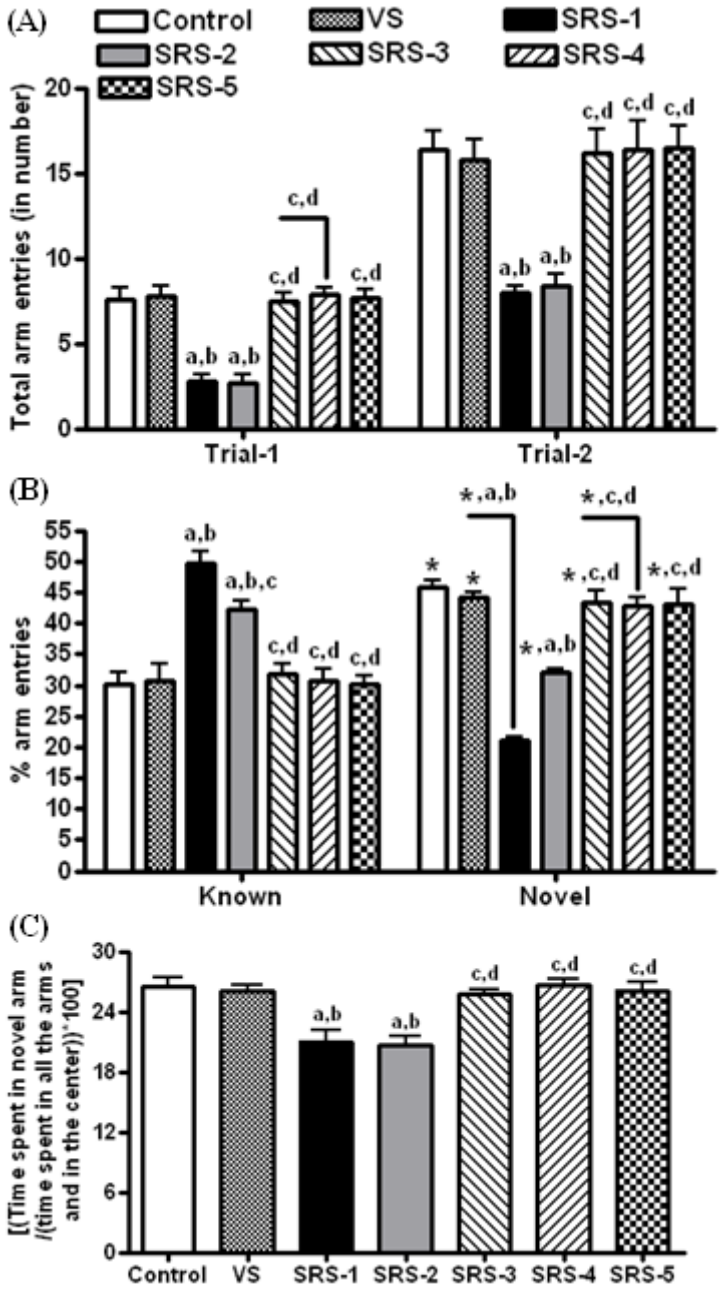

Figure 29: The effect of VS and time-dependent exposure of first RS on the total arm entries in trial-1 and 2 (curiosity; A), spatial recognition memory (B) and coping behavior to novel arm (anxiety-like behavior; C) in Y-maze test paradigm. All values are mean ± SEM (n=5). [a]P<0.05 compared to control, [b]P<0.05 compared to VS, [c]P<0.05 compared to SRS-1 and [d]P<0.05 compared to SRS-2 [Repeated measure two-way ANOVA followed by Bonferroni test for curiosity analysis and, percentage entries into known and novel arm. One-way ANOVA followed by Student Newmann-Keuls test was performed for the analysis of anxiety-like behavior]. [*]P<0.05 compared to known arm entries [Two-way ANOVA followed by Bonferroni Post-hoc test].

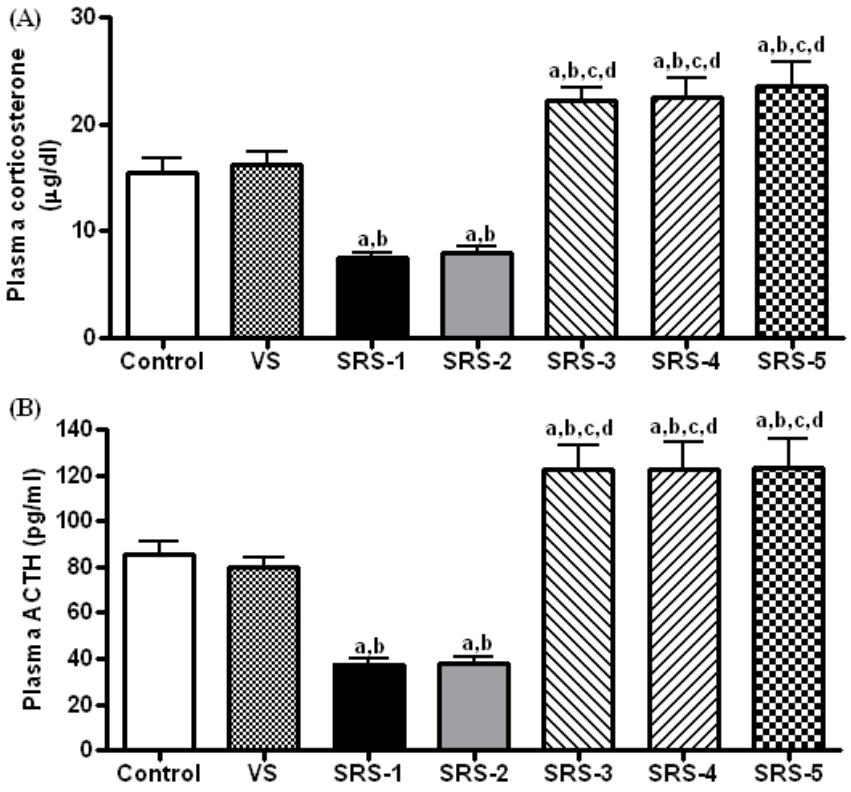

Figure 30: The effect of VS and time-dependent exposure of first RS on the plasma level of ACTH and corticosterone in rats. All values are mean ± SEM (n = 5). $^{a}P<0.05$ compared to control, $^{b}P<0.05$ compared to VS, $^{c}P<0.05$ compared to SRS-1 and $^{d}P<0.05$ compared to SRS-2 (One-way ANOVA followed by Student Newmann Keuls post-hoc test).

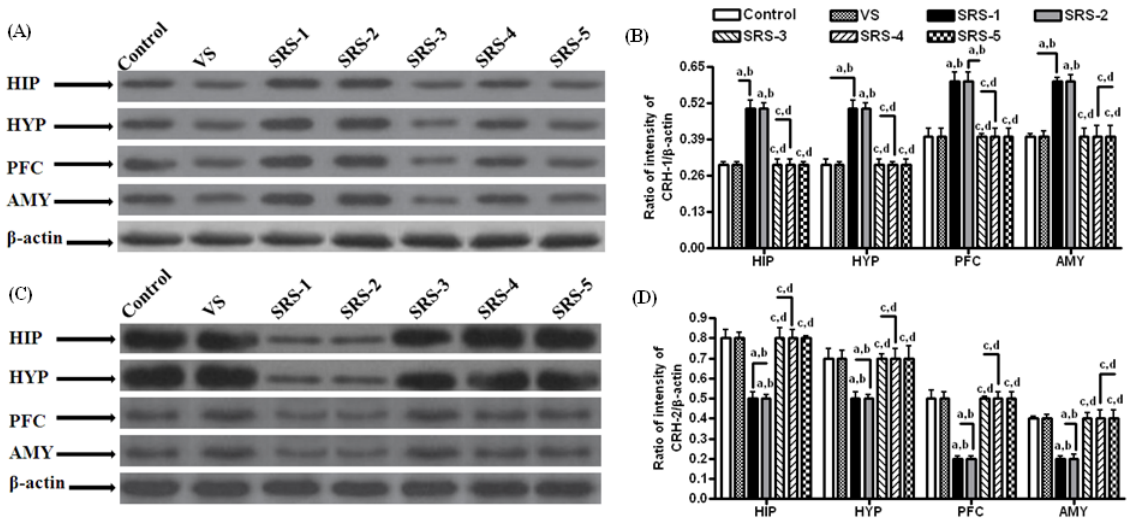

Figure 31: The effect of VS and time-dependent exposure of first RS on the level of expression of CRH-1(B) and CRH-2 (D) in HIP, HYP, PFC and AMY of rats. The blots are representative of CRH-1 (A) and CRH-2 (B) in HIP, HYP, PFC and AMY. The results in the histogram are expressed as the ratio of relative intensity of levels of expression of CRH-1 or CRH-2 to β-actin. All values are mean ± SEM (n = 5). [a]P<0.05 compared to control, [b]P<0.05 compared to VS, [c]P<0.05 compared to SRS-1 and [d]P<0.05 compared to SRS-2 (One-way ANOVA followed by Student Newmann Keuls post-hoc test).

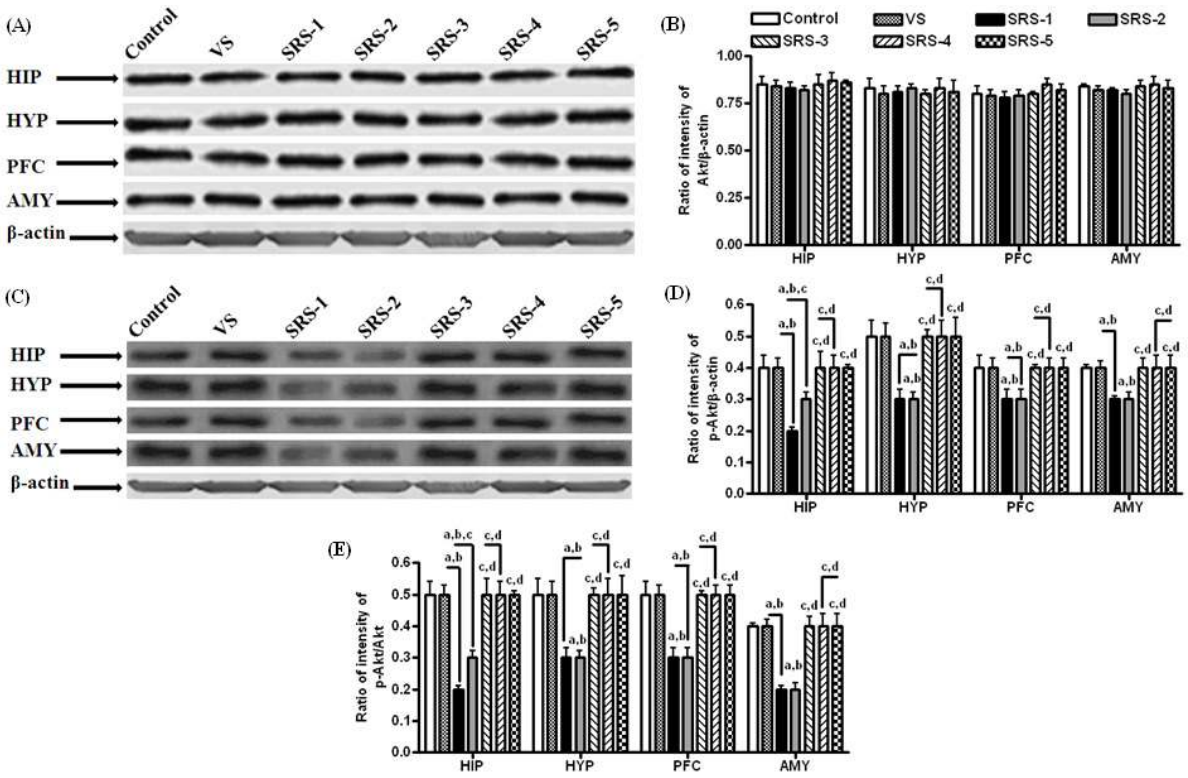

Figure 32: The effect of VS and time-dependent exposure of first RS on the level of expression of Akt (B) and p-Akt (D), and ratio of p-Akt to Akt (E) in HIP, HYP, PFC and AMY of rats. The blots are representative of Akt (A) and p-Akt (C) in HIP, HYP, PFC and AMY. The results in the histogram are expressed as the ratio of relative intensity of levels of expression of Akt or p-Akt to β-actin. All values are mean ± SEM (n = 5). $^{a}P<0.05$ compared to control, $^{b}P<0.05$ compared to VS, $^{c}P<0.05$ compared to SRS-1 and $^{d}P<0.05$ compared to SRS-2 (One-way ANOVA followed by Student Newmann Keuls post-hoc test).

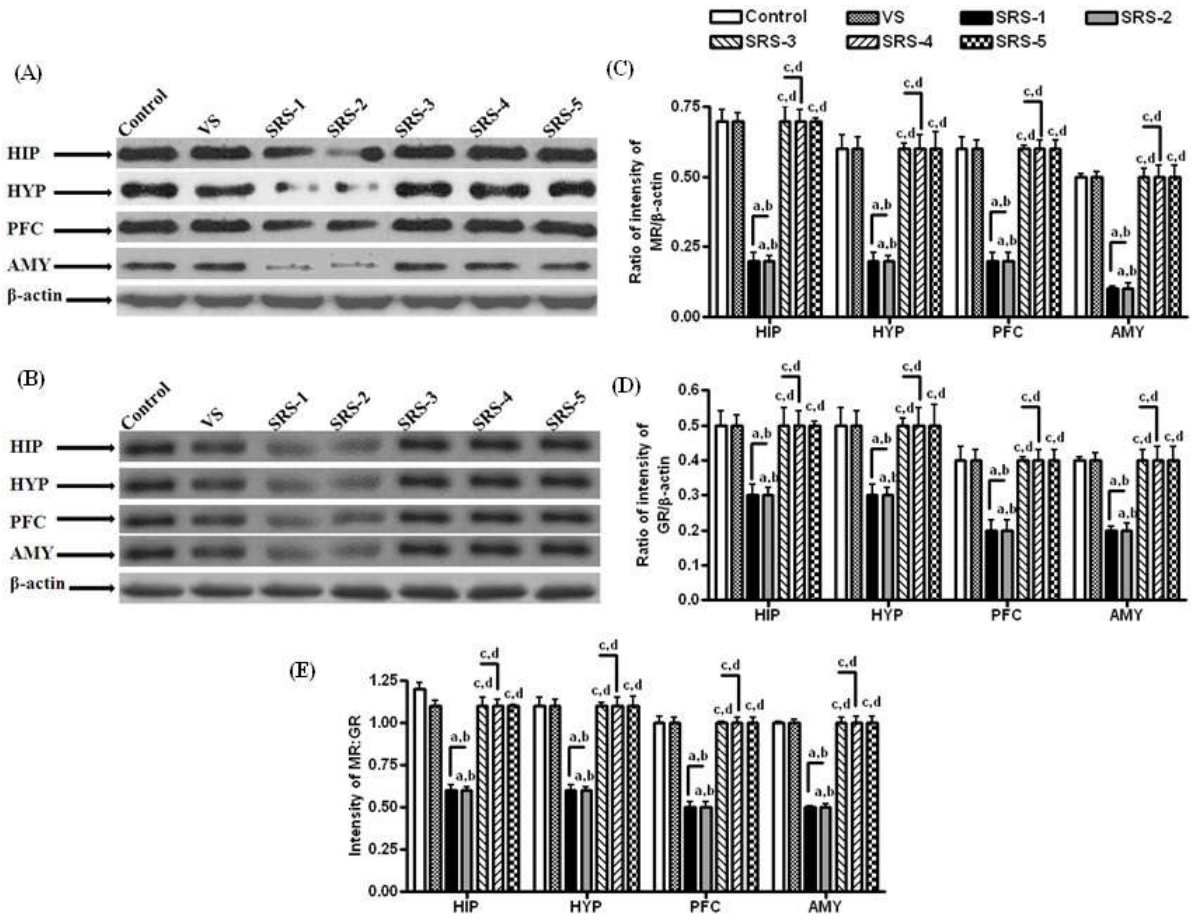

Figure 33: The effect of VS and time-dependent exposure of first RS on the level of expression of MR (B) and GR (D), and ratio of MR to GR (E) in HIP, HYP, PFC and AMY of rats. The blots are representative of MR (A) and GR (C) in HIP, HYP, PFC and AMY. The results in the histogram are expressed as the ratio of relative intensity of levels of expression of MR or GR to β-actin. All values are mean ± SEM (n = 5). [a]P<0.05 compared to control, [b]P<0.05 compared to VS, [c]P<0.05 compared to SRS-1 and [d]P<0.05 compared to SRS-2 (One-way ANOVA followed by Student Newmann Keuls post-hoc test).

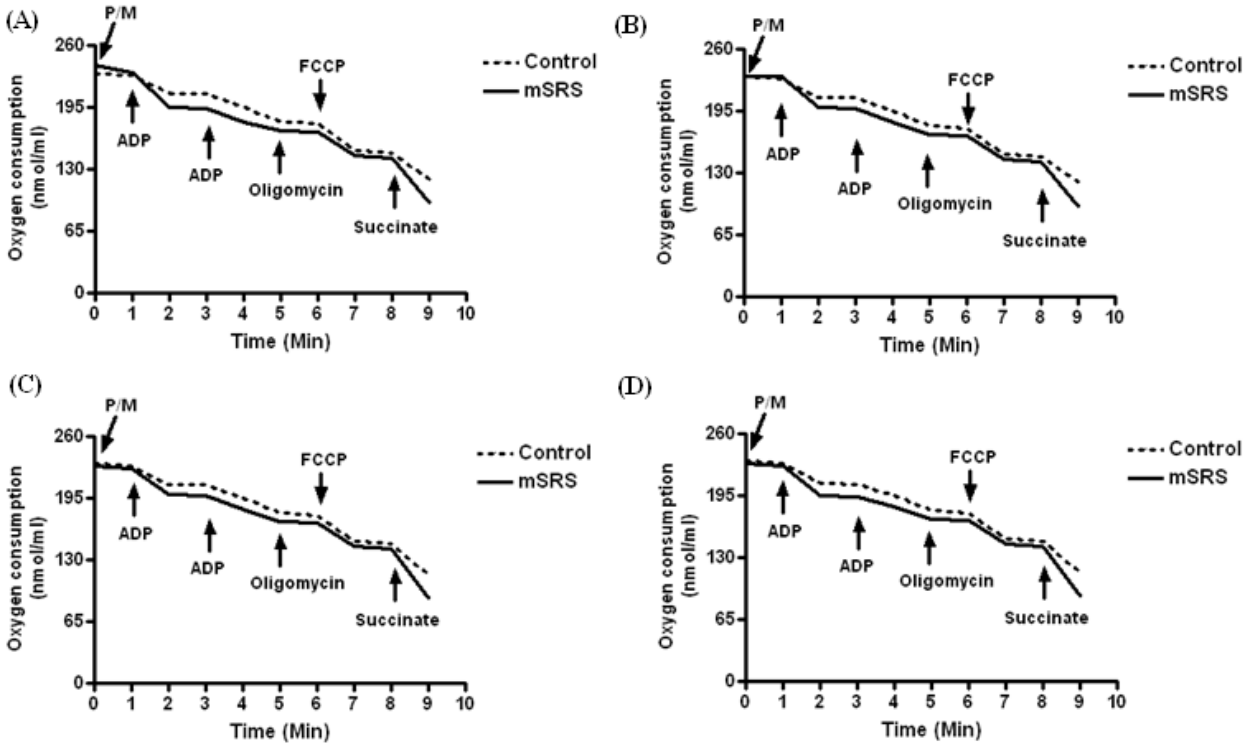

Figure 34: Representative figures for mitochondrial respiration pattern in HIP (A), HYP (B), PFC (C) and AMY (D) of control and PTSD-like rats.

Table-25: Effect of mSRS on mitochondrial bioenergetics in HIP, HYP, PFC and AMY of rats.

Groups	S_2	S_3	S_4	S_{Olig}	S_{FCCP}	S_{Succ}	RCR	ADP/O
HIP								
Control	5.3±0.2	30.5±1.2	4.9±0.1	3.1±0.1	23.5±1.3	35.2±2.4	6.3±0.3	3.1±0.2
mSRS	7.1±0.3*	59.2±2.5*	15.1±1.1*	2.8±0.2	37.4±2.7*	47.3±3.1*	3.1±0.1*	1.8±0.1*
HYP								
Control	4.9±0.3	28.7±1.3	5.1±0.2	3.3±0.1	23.8±1.5	34.3±1.7	6.5±0.4	3.3±0.1
mSRS	7.6±0.4*	63.3±2.2*	14.5±1.3*	3.1±0.2	35.7±2.1*	49.2±2.3*	2.8±0.2*	1.6±0.1*
PFC								
Control	5.6±0.5	29.4±1.4	4.5±0.1	2.9±0.1	22.7±1.5	35.3±2.5	6.1±0.3	3.5±0.3
mSRS	8.1±0.6*	66.4±2.5*	13.9±1.1*	2.8±0.2	36.6±2.3*	48.7±2.1*	2.7±0.3*	1.7±0.2*
AMY								
Control	5.2±0.3	32.3±2.1	4.1±0.2	2.9±0.2	23.1±2.1	36.2±3.1	6.7±0.4	2.9±0.2
mSRS	8.3±0.4*	69.3±3.1*	15.3±2.1*	2.7±0.1	39.4±2.3*	47.9±4.2*	2.7±0.2*	1.5±0.1*

All values are mean ± SEM (n = 5). * $p<0.05$ compared to control (Two-tailed Unpaired Student-t test).

Table-26: Effect of mSRS on mitochondrial function and integrity in HIP, HYP, PFC and AMY of rats.

Groups	HIP	HYP	PFC	AMY
Complex-I(nmol NADHoxidized/min/mg protein)				
Control	5.1 ± 0.5	5.5 ± 0.2	4.8 ± 0.7	6.1 ± 0.3
mSRS	10.2 ± 0.3*	10.8 ± 0.3*	9.5 ± 0.3*	10.9 ± 0.8*
Complex-II(µmolformazan produced/min/mg protein)				
Control	0.4 ± 0.02	0.4 ± 0.01	0.5 ± 0.03	0.4 ± 0.04
mSRS	1.0 ± 0.02*	0.5 ± 0.01*	1.6 ± 0.06*	0.6 ± 0.05*
Complex-IV(nmol cytochrome coxidized/min/mg protein)				
Control	0.9 ± 0.03	1.2 ± 0.05	1.5 ± 0.02	1.4 ± 0.04
mSRS	1.8 ± 0.03*	2.1 ± 0.07*	3.0 ± 0.19*	2.1 ± 0.08*
Complex-V(nmol ATPhydrolyzed/mg protein)				
Control	7.5± 0.4	16.8 ± 0.6	12.5 ± 0.7	12.3 ± 0.6
mSRS	16.6 ± 1.9*	37.2 ± 3.0*	29.4 ± 2.3*	30.5 ± 2.4*
MMP				
Control	463.7±6.0	457.8±10.0	478.5±16.8	456.4±16.5
mSRS	314.6±23.4*	332.4±8.2*	316.8±17.1*	325.8±26.4*

All values are mean ± SEM (n = 5). * $p<0.05$ compared to control (Two-tailed Unpaired Student-t test).

Table-27: Effect of mSRS on mitochondrial oxidative stress parameters in HIP, HYP, PFC and AMY of rats.

Groups	HIP	HYP	PFC	AMY
LPO level(µM MDA/mg protein)				
Control	0.5 ± 0.02	0.6 ± 0.03	0.7 ± 0.03	0.6 ± 0.03
mSRS	0.7 ± 0.05*	0.9 ± 0.02*	1.1 ± 0.07*	0.8 ± 0.04*
Nitrite level (nM NO/mg protein)				
Control	1.2 ± 0.05	0.9 ± 0.05	1.2 ± 0.04	1.1 ± 0.03
mSRS	2.2 ± 0.05*	2.1 ± 0.03*	2.4 ± 0.04*	2.2 ± 0.04*
SOD activity (Units/min/mg protein)				
Control	0.4 ± 0.01	0.4 ± 0.002	0.4 ± 0.002	0.6 ± 0.014
mSRS	0.2 ± 0.02*	0.2 ± 0.011*	0.2 ± 0.017*	0.3 ± 0.015*
CAT activity(Units/min/mg protein)				
Control	2.2 ± 0.11	3.8 ± 0.06	2.6 ± 0.05	2.7 ± 0.12
mSRS	1.6 ± 0.13*	2.8 ± 0.17*	1.7 ± 0.14*	2.0 ± 0.07*

All values are mean ± SEM (n = 5). * $p<0.05$ compared to control (Two-tailed Unpaired Student-t test).

Discussion

In the present study, we for the first time demonstrate that the maximum lag time for the exposure of initial and subsequent re-stress session to induce PTSD-like manifestations after traumatic events i.e., VS was two week. It is interesting to note that the first week re-stress session was more precarious than the second week experience in terms of exhibiting PTSD-like manifestations. In the molecular study, it was also observed that the dysfunction of glucocorticoid signaling pathway was observed in the course of PTSD pathophysiology. However, the first week re-stress session caused severe loss in the phosphorylation of Akt in HIP selectively. Thus, the first week re-stress session exposure was considered as the best optimized model to induce PTSD-like manifestations. Furthermore, the optimized model exhibited significant alterations in the mitochondrial bioenergetics, function and oxidative stress in all the brain regions. These observations suggest the fact that this optimized animal model of PTSD exhibits significant mitochondrial impairment in all the brain regions. Thus, it can be presumed that mitochondrial targeted drugs may be evaluated in this model of PTSD.

The validity of an animal model is typically constitutes: phenomenological similarity (face validity), corresponding theoretical explanatory frameworks (construct validity), and the ability to predict that a pharmacological agent with efficacy demonstrated in animal studies to have a subsequent therapeutic effect in humans (predictive validity). Till date, no specific animal model is being validated as a model for PTSD, which is cause of debate in research for this disorder to evaluate the effectiveness of anti-PTSD agents. Stress-re-stress (SRS) paradigm is considered, as one the best among several experimental models, to induce the cardinal features of PTSD in animals (Yehuda and Antelman, 1993; Liberzon et al., 1997). This recurrent stress causes continuous reminder of trauma which leads to the development of pathophysiology and

its characteristic symptoms. Stress is developed with the help of forced swim test (FST) due to inability of the animal to escape from the water cylinder. As PTSD is a chronic disorder and drugs prescribed for it are mostly associated with long-lasting dosage regimen, there is a need to develop a model which chronically exhibits PTSD-like behavioral and neuroendocrinological manifestations. Recently, our group has modified the SRS model to evaluate the efficacy of chronic administered drugs in animals (Krishnamurthy et al., 2013). In the present study, the re-stress session exposure on first and second week after traumatic event caused a significant execution of PTSD-like behavioral manifestations and HPA-axis dysfunction. However, when the re-stress session was introduced after second week i.e., third week to traumatic event it did not elicit any PTSD-like behavioral symptoms. Moreover, the re-stress session exposure after second week caused a significant increase in the level of plasma corticosterone. It has been already reported that stress cause a significant increase in the plasma level of corticosterone in the animals (Garabadu et al., 2011). Thus, it can be presumed that the re-stress session after second week to traumatic events acts as a stressor to induced stress-related manifestations rather than PTSD-like symptoms.

In earlier studies it has been suggested that the glucocorticoid system derails in the pathogenesis of PTSD (Krishnamurthy et al., 2013; Garabadu et al., 2015). It is interesting to note that in the present study there was a significant increase in the level of expression of CRH-1 in all the brain regions in both first and second week re-stress exposure groups. Further, the levels of CRH-2 were significantly decreased in all the brain regions in these two groups exhibiting PTSD-like manifestations. It is well known that CRH-1 activation causes exaggeration of stress consequences and the stress actions are terminated due to the stimulation of CRH-2 in the several brain regions (Elharrar et al., 2013; Sink et al., 2013).Additionally, the level of GR

and MR were significantly decreased in all the brain regions when the re-stress session was introduced either in first or second week to traumatic events. In our earlier studies it has been reported that the levels of GR and MR, and their ratio (MR/GR) decreased to a significant extent to cause PTSD-like manifestations (Krishnamurthy et al., 2013). Hence, in a nutshell it can be assumed that in addition to the changes in the cascade pathway of GR and MR, the increase and decrease in CRH-1 and 2 may be responsible to induce PTSD-like manifestations. Moreover, there was a significant decrease in the extent of phosphorylation of Akt in all the brain regions in these two groups those exhibiting PTSD-like symptoms. It is well characterized that the decrease in the extent of phosphorylation of Akt exhibits anxiety and depression-like attributes in several animal models (Punn et al., 2006). It is interesting to note that the first week re-stress session exposure caused a higher decrease in the extent of phosphorylation of Akt than the second week re-stress session. Hence, it can be assumed that in addition to glucocorticoid derangement, Akt signaling pathway may also be responsible for the pathogenesis of PTSD characteristics.

The concept of mitochondrial dysfunction in the pathogenesis of PTSD-like manifestation has already been reported (Garabadu et al., 2015). Similar to earlier study, mSRS paradigm caused an elevation in the function of mitochondria in terms of increased respiratory complex activities in several brain regions of PTSD-like rats (Garabadu et al., 2015). Further, there was a loss in mitochondrial integrity in terms of decrease in the MMP in all these rat brain regions similar to earlier finding (Garabadu et al., 2015). However, there was no report on the mitochondrial bioenergetics to support the hypothesis of mitochondrial dysfunction in the pathogenesis of PTSD. In the present study, there was a significant increase in the level of consumption of oxygen in all the states except oligomycine-induced state in all the brain regions. Further, there was a significant decrease in the level of RCR and the ratio of ADP/O in all the

brain regions of SRS exposed animals, suggesting the fact that mSRS paradigm caused a significant loss in mitochondrial function and efficiency in the animals. In previous reports it has been reported that the decrease in the level of RCR and the ratio of ADP/O are characteristic properties for the mitochondrial dysfunction (Saleh et al., 2013; Soto-Urquieta et al., 2014). These observations further validate the concept of mitochondrial dysfunction in all the brain regions to induce PTSD-like manifestations.

In conclusion, the lag time for the exposure of initial and subsequent re-stress session to induce PTSD-like manifestations after traumatic events is two week. Interestingly, the first week re-stress session is more precarious than the second week experience in terms of exhibiting PTSD-like manifestations. In the molecular study, the PTSD-like symptoms were parallel to the dysfunction of glucocorticoid signaling pathway. However, the first week re-stress session caused severe loss in the phosphorylation of Akt in HIP selectively. Thus, the first week re-stress session exposure was considered as the best model to induce PTSD-like manifestations. Furthermore, the optimized model exhibited significant mitochondrial dysfunction in terms of mitochondrial bioenergetics, function and oxidative stress in all the brain regions. These observations suggest the fact that this optimized animal model of PTSD is also characterized with significant mitochondrial impairment in all the brain regions. Thus, it can be speculated that mitochondrial targeted drugs can be evaluated in this experimental model of PTSD.