Evaluation of anti-PTSD effect of risperidone against stress re-stress model in rats

6.1 Introduction

Risperidone (RIS) is an atypical antipsychotic drug found to be effective in the treatment of PTSD in clinical studies. Our previous studies on RIS in an animal model of PTSD showed it to possess significant anti-PTSD effects(Krishnamurthy et al., 2013; Monnelly et al., 2003). It efficiently modulated factors like the plasma corticosterone and brain monoamines disrupted due to PTSD. At the cellular level, RIS also alleviated PTSD-induced abnormalities in the mitochondria and cell death processes (Garabadu et al., 2015). Further, the drug also mitigated the behavioural abnormalities related to anxiety and memory caused by PTSD (Krishnamurthy et al., 2013). However, many studies have shown that the therapeutic effect of RIS also involves the modulation of cell signalling pathways that assist in neuronal resilience. It is reported to explicitly enhance neurotrophic proteins like the Brain-derived neurotrophic factor (BDNF), cyclic AMP response element-binding (CREB) protein, extracellular signal-regulated kinase (ERK) and also minimizes stress-induced cell degenerative factors like caspases which induce apoptosis (Gassó et al., 2012; Rogóż et al., 2017).In PTSD, there is the persistence of fear memories and also hyper anxiety leading to disruptive behaviour (Chhatwal et al., 2006). In the brain, the amygdala (AMY) acts as a center for fearful memories and anxiety response (Gale et al., 2004; Ressler, 2010). While, the prefrontal cortex (PFC) is involved in fear processing and has an inhibitory effect on AMY(Quirk et al., 2003). Hence, the therapeutic interventions in these two regions could lead to effective treatment of PTSD(Koenigs et al., 2009).

BDNF is found to be involved in the consolidation and extinction of fear memories in PFC and AMY(Chhatwal et al., 2006; Rosas-Vidal et al., 2014). Besides, it also has a regulatory role in anxiety and neurogenesis(Frielingsdorf et al., 2010; Zhang et al., 2011). CREB,

another neuronal protein, helps to regulate anxiety, memory formation and also prevents neurodegeneration (Wallace et al., 2004). Previous studies have found a significant downregulation of CREB protein in patients with PTSD (Martini et al., 2013). Both BDNF and CREB are regulated by the ERK signalling cascade. ERK is found to regulate anxiety and memory through activation of CREB. And, it also mediates the effects of BDNF on memory formation (Alonso et al., 2002; Zhang et al., 2016a).Hence, these three proteins BDNF, CREB and ERK, are involved in the regulation of memory, anxiety and also neuronal survival and could be contributing to the pathogenesis of PTSD. Caspases are the set of enzymes involved in apoptotic cell death and are found to play an essential role in PTSD (Zhang et al., 2016b).

In a rat model of single-prolonged stress-induced PTSD, the expression of caspase-9 and caspase-3 was significantly elevated (Li et al., 2010). Further, these caspases disrupt the ERK cascade, thereby affecting the BDNF and CREB meditated role in the alleviation of PTSD symptoms (McKay et al., 2007).

In the current experiment, we have selected the stress re-stress (SRS) model as it is the most appropriate model of PTSD (Liberzon et al., 1997). In this paradigm, animals are subjected to initial traumatic stress and then subsequent “reminder episodes” as contextual triggers for the development of PTSD. This reminder leads to the development of a stable anxiety state and other characteristics similar to PTSD in humans. Clinically, PTSD treatment involves long-term drug administration for an effective outcome (Bartzokis et al., 2005; Rothbaum et al., 2008). So, an animal model which induces PTSD symptoms chronically would be helpful in drug discovery. With these facts in consideration, a slightly modified version of the SRS model was used for the long-term evaluation of PTSD-related behavioural and physiological changes.

Therefore, in this study, we evaluated the effect of repeated doses of RIS and PAX on BDNF, ERK, CREB and caspase-3 in the modified stress restress model of PTSD in rats. Apart from this, memory and anxiety-like behavioural symptoms were evaluated to ascertain the potential improvements in symptoms of PTSD due to the treatment.

6.2 Materials and methods

6.2.1 Animals

All the experiments were performed following the principles of laboratory animal care (National Research Council US Committee for the Update of the Guide for the Care and Use of Laboratory Animals 2011) guidelines. Before initiation of the experiments, approval from the animal ethical committee was obtained (Protocol No: Ref No. Dean/10-11/148). Adult male Charles Foster strain albino rats weighing between 220-260gmswere procured from the Central Animal House, Institute of Medical Sciences, Banaras Hindu University. They were housed in polypropylene cages and allowed to acclimatize for a week at 25±1^{o}C with a 12-hr light-dark cycle with free access to food and water. Every possible effort was taken to minimize the suffering and the number of animals used for the experimentation.

6.2.2 Drugs

Pure samples of RIS and Paroxetine (PAX) were generous gift samples from Ranbaxy Laboratories, India. Both the drugs were suspended in 0.5% carboxymethylcellulose (CMC) solution separately. Antibodies used for the western blot studies were procured from Santa Cruz Biotechnology Inc (Santa Cruz, California, USA). All other chemicals and reagents used in the experiment were of analytical grade and obtained from local suppliers.

6.2.3 Animal Treatment

The experimental protocol was carried for 32 days. Animals were randomly assigned into six groups with 6 in each (n=6) as control (group-1), PTSD control (group-2), RIS doses as 0.1mg/kg, 1.0 mg/kg and 10 mg/kg (group-3, 4, and 5 respectively) and PAX-10mg/kg (group-6). All the animals except those of control were subjected to modified stress restress paradigm. After one hour of restress session, all the animals were administered with the corresponding dosing orally from Day-8 to D-32. Control and PTSD groups received 0.5% CMC suspension. While groups 3, 4 and 5 received 0.1, 1.0 and 10mg/kg doses of RIS suspension. And the group-6 received PAX-10mg/kg suspension. On D-32, after 2 hours of restress session, animals were subjected to behavioural assessments for anxiety-like symptoms and memory abnormalities using EPM and Y-maze, respectively. There was a lag of 5minbetween the behavioural tests. After that, animals were decapitated, and their brains were microdissected into the PFC and AMY (Palkovits et al., 1988). These isolated brain parts were stored at -80^{o}C until further experimentation.

6.2.4 Modified stress restress paradigm

Modified stress restress (SRS) model was used to induce PTSD in rats as it is an efficient model (Krishnamurthy et al., 2013). All the animal procedures were conducted from 8.00 to 16.00hrs. On D-2, individual animals were exposed to 2hr restraint stress in metallic cages and then subjected to a forced swim test in an 18cm swim tank at an ambient temperature of 25^{0}C for about 20mins. This was followed by anaesthesia with brief exposure to 0.8ml of 4% halothane vapours (stress) until loss of consciousness. After recuperation, they were returned to their cages. The animals were again exposed to 20min forced swimming “re-stress” on D-8, D-14, D-20, D-26 and D-32. Swim (restress) on of experimentation.

6.2.5 Evaluation of anxiety-like behaviour

Animals were evaluated for anxiety-like behaviour using an elevated plus-maze (Ojha et al., 2010). The fabricated maze has two opposite arms, 50×10 cm, crossed with enclosed arms of the same dimension but having a 40 cm high wall. The arms were connected to a central square, 10×10 cm, giving the apparatus shape of a plus sign. The maze was kept in a dimly lit room and elevated 50 cm above the floor. Naive rats were placed individually in the centre of the maze, facing closed arms. The number of entries and time spent on both the open and enclosed arm were recorded during the next 5 minutes using the ANY-MAZE^{TM} video tracking system. An arm entry was defined when all four paws of the rat were in the arm. The total arm entries were measured as an index of locomotor activity (Itoh et al., 1991). The behavioural parameters were assessed by a person unaware of the experimental protocol.

6.2.6 Evaluation of Memory

The memory defects were evaluated using the Y-maze test. The maze consisted of three identical arms starting from a central point (50 cm long, 16 cm wide and 32 cm high) with 120^0 angles to each other. The floor of the apparatus was covered with soiled animal bedding. The arms were designated as starting arm, another arm, and a novel arm. In the first trial, entry to the novel arm was blocked, and rats were allowed to move within the other two arms for 15 minutes. Four hours after the first trial, animals were allowed to access all three arms for 5 min, and the number of entries was recorded. An arm entry was counted when the head and two front paws were inside the arm. The entire recordings were done using ANY-MAZE^{TM} video tracking software. The total number of entries into all the arms (for the 5 min of trials 1 and 2) is an index of general exploratory behaviour. The percentage entries in known and novel arms for the 5 min period of trial two is an index of spatial recognition memory. Percentage of time spent in the novel arm to time spent in all arms and the center of

the apparatus during trial 2 were estimated as indices of anxiety-like behaviour, respectively. The total number of entries into all the arms (for the 5 min of trials 1 and 2) is indicative of a general exploration attitude (curiosity). The percentage entries in known versus novel arm for the 5 min period of trial two is as a measure of arm discrimination (spatial recognition memory). The percentage of time spent in the novel arm to time spent in all arms indicates anxiety-like behaviour (Dellu et al., 1992; Krishnamurthy et al., 2013).

6.2.7 Estimation of Serotonin and Dopamine by HPLC

The brains were removed after decapitation and microdissected as soon as possible on glass plates over ice into five regions: the prefrontal cortex (PFC) and amygdala (AMY) (Itoh et al., 1991). The levels of 5-HT and DA were estimated using HPLC/ECD (Dellu et al., 1992). In brief, the brain tissue samples were homogenized in 0.17 M perchloric acid by a Polytron homogenizer. Homogenates were then centrifuged at 33,000g (BiofugeStratos, Heaureas, Germany) at 4°C. Twenty microliters of supernatant were injected via HPLC Mobile phase consisted of methanol: water (70:30) pump (Model 515, isocratic pump, Waters, Milford, MA, USA) into a column (Spherisorb, RP C18, 5 lm particle size, 4.6 mm i.d. 9250 mm at 30°C) connected to an ECD (Model 2465, Waters, Milford, MA, USA) at a potential of 0.8V with a glassy carbon working electrode Vs. Ag/AgCl reference electrode. Mobile phase consisted of 32mM citric acid, 12.5mM disodium hydrogen orthophosphate, 1.4mM sodium octyl sulfonate, 0.05mM EDTA and 16% (v/v) methanol (pH 4.2) at a flow rate of 1.2ml/min. Quantification was done by comparing the peak heights of the samples to the corresponding standard curve. Two ranges of standard curves, i.e., 10-100 and 100-1,000ng/ml, were used depending upon the abundance of monoamines in respective brain regions. A constant amount (25ng/ml) of DHBA was added to the tissue samples to calculate recovery. The protein content was estimated and the neurotransmitters were quantified in terms of a fixed weight of protein (Bradford, 1976).

6.2.8 Western blot analysis

Briefly, the brain tissues were lysed in a buffer containing a complete protease inhibitor cocktail. Protein concentrations were determined according to Bradford (1976) (Bradford, 1976). A standard plot was generated using bovine serum albumin. An aliquot of each sample was electrophoresed in 10% SDS-PAGE gels for pERK, ERK, BDNF, CREB and caspase-3 proteins, transferred to polyvinylidene fluoride membranes and probed with specific antibodies. The membrane was incubated overnight with rabbit ERK 1/2 (1:500, 41 kDa; ab196883; Abcam plc., India), rabbit pERK (1:500, 44 kDa; ab214362; Abcam plc., India), sheep anti-BDNF (1:500, 28 kDa; ab24491; Abcam plc., India), rabbit CREB (1:500, 43 kDa; ab5803; Abcam plc., India) and rabbit active anti-caspase-3 (1:1000, 32 kDa; ab90437; Abcam plc., India) polyclonal primary antibodies. After detection of 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. Then it was re-probed overnight with rabbit anti-β-actin (1:500, 42 kDa; ab93027; Abcam plc., India) polyclonal primary antibody to confirm equal loading of protein. Further, the membrane was probed with corresponding secondary antibodies. An immunoreactive band of proteins was detected by chemiluminescence using enhanced chemiluminescence (ECL) reagents (Amersham Bioscience, USA). Quantification of the results was performed by the densitometric scan of films. The immunoreactive area was determined by densitometric analysis using Biovis gel documentation software (Krishnamurthy et al., 2013).

6.2.9 Statistical analysis: All the data were subjected to statistical analysis using graph pad prism software version-5. The western blot data were analyzed using one-way ANOVA followed by Neumann kuelsTest. The data of total arm entries in trials 1 and 2 and percentage entries into the 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. The arm discrimination behaviour between known and novel arms in the Y-maze paradigm was measured by two-way ANOVA followed by Bonferroni post hoc test for each group. The data was a mean±standard error of the mean (SEM) and $P<0.05$ was considered as significant.

6.3 Results

6.3.1 RIS Reduced SRS-Induced Anxiety-Like Symptoms in EPM Test

The effect of repeated treatment with RIS (0.01, 0.1, and 1.0 mg/kg) and PAX (10.0 mg/kg) on SRS-induced anxiety-like symptoms is represented in Table-6.1. The SRS decreased the percentage of open arm entries [Table 1A; $F5, 30=22.24$, $p<0.05$] and time spent [Table 1B; $F5, 30=15.52$, $p<0.05$] in rats as compared to normal animals. Repeated RIS (0.1 mg/kg) and PAX (10.0 mg/kg) treatment significantly alleviated the SRS-induced anxiety-like behaviour. However, there were no significant differences in the total arm entries [Table 1C; $F5, 30=2.278$, $p>0.05$] among the groups tested.

Table-6.1: Effect of RIS on SRS-induced anxiety-like symptoms in the EPM test.

Sl.No	Behaviour	Control	SRS	RIS-0.1	RIS-1	RIS-10	PAX
A	Open arm entries (%)	27 ± 1.10	6.89 ± 2.30[a]	16.60±1.26[a,b]	8.82± 2.21[a,c]	9.65±1.172a,c	17.5±0.97[a,b,d,e]
B	Open arm time spent (%)	8.13±0.41	2.67± 0.38[a]	5.53± 0.66[a,b]	2.43± 0.51[a,c]	2.95±0.720[a,c]	5.64±0.67[a,b,d,e]
C	Total arm Entries (in numbers)	8.10±0.88	8.9± 0.90	9.15 ± 0.80	9.5± 1.23	7.9 ± 0.672	8.5 ± 1.37

The effect of RIS (0. 1, 1.0, and 10.mg/kg) and PAX on modified SRS-induced changes in percentage in open arm entries (a) and time spent (b) and the number of total arm entries (c) in elevated plus-maze test paradigm. All values are mean±SEM (n=6). [a]$P<0.05$ compared to

control, [b]P<0.05 compared to SRS, [c]P<0.05 compared to RIS (0.1 mg/kg), [d]P<0.05 compared to RIS (1.0 mg/kg) and [e]P<0.05 compared to RIS (10mg/kg). [one-way ANOVA followed by Student-Newman–Keuls post hoc test]

6.3.2 RIS improved SRS-induced loss in spatial recognition memory in Y-maze Test

Table 6.2: RIS effect on SRS-induced loss in spatial recognition memory in Y-maze Test

A	**% arm entries Spatial recognition memory**						
	Known	29.6 ±1.51	45.40± 1.78[a]	30.4±2.54 [b]	45.70±2.04[a,c]	43.90±1.95[a,c]	31.6± 2.01[b,d,e]
	Novel arm	45.5±3.71	23.70±2.93 [a]	42.8±3.34 [b]	25.70±3.22[a,c]	22.70±2.99[a,c]	44.4±3.49[b,d,e]
B	**Total arm entries trial-II (in numbers) Curiosity**						
	Trial-I	7.70± 0.94	2.50± 0.89 [a]	6.83± 0.62 [b]	2.70±0.95[a,c]	3.1± 0.64[a,c]	6.87 ± 1.02[b,d,e]
	Trial-II	16.40±1.30	7.57±1.11 [a]	11.40±0.74[a,b]	7.37±1.05[a,c]	8.1±0.88[a,c]	11.60±0.79[a,b,d,e]
C	**Time spent in the novel: time spent in all arm*100 Coping behaviour to novel arm**						
		27.7±2.10	19.89±1.10 [a]	26.4±1.60 [b]	19.40±2.05[a,c]	20.7±1.87[a,c]	28.50±1.61[b,d,e]

SRS-induced alterations in the spatial recognition memory (A), total arm entries in trials 1 and 2 (curiosity; B) and coping behaviour to novel arm (anxiety-like behaviour; C) in the Y-maze test paradigm. All values are mean±SEM (n=6). [a]P<0.05 compared to control, [b] P<0.05 compared to SRS, [c] P<0.05 compared to RIS (0. 1 mg/kg), [d]P<0.05 compared to RIS (1.0 mg/kg), and [e]P<0.05 compared to RIS (10.0 mg/kg) [repeated measure two-way ANOVA followed by Bonferroni test for curiosity analysis and percentage entries into the known and novel arm. One-way ANOVA followed by Student–Newman–Keuls test was performed for the analysis of anxiety-like behaviour]. *P<0.05 compared to known arm entries [two-way ANOVA followed by Bonferroni test].

The data in Table-6.2 depicts the effect of repeated treatment with RIS (0.01, 0.1, and 1.0 mg/kg) and PAX (10.0 mg/kg) on SRS-induced changes in the spatial recognition memory (A), total arm entries in trials 1 and 2 (curiosity; B), and coping behaviour to novel arm (anxiety-like behaviour; C) in Y-maze test. Upon analysis, there were significant differences of arm discrimination patterns between known and novel arms among groups ($F_{5, 60}= 19.37$, $p<0.05$) and arms ($F_{1, 60}= 103.2$, $p<0.05$) and a significant interaction between group and trial ($F_{5, 60}= 1.480$, $p<0.05$). SRS group rats showed significantly lower arm discrimination to novel arm as compared to known arm, indicating a loss of spatial recognition memory than Control rats. RIS (0.1 mg/kg) and PAX (10.0 mg/kg) treatment attenuated the SRS-induced loss in the spatial recognition memory. Further, one-way ANOVA revealed that there were significant differences among groups in terms of percentage entries into known ($F_{5, 30}=95.73$, $p<0.05$) and novel ($F_{5, 30}=68.73$, $p<0.05$) arms. SRS decreased total arm entries in both trials as compared to control rats. There was an increase in entries into the known arm and a decrease in the entries into the novel arm, respectively. Repeated treatment with RIS (0.1 mg/kg) and PAX (10.0 mg/kg) enhanced the curiosity index by enhancing novel arm entries and decreasing known arm entries. In regards to the coping behaviour, SRS-induced a significant increase in anxiety-like behaviour as seen by the decrease in the coping pattern in the novel arm ($F_{5, 30}= 5.736$, $p<0.05$). However, RIS (0.1 mg/kg) and PAX (10.0 mg/kg) treatment increased the SRS-induced decrease in coping behaviour in the novel arm in comparison to control animals.

6.3.3 Effect of RIS (0.1, 1.0 and 10mg/kg) on SRS-induced alteration on 5HT level in PFC and AMY

Repeated treatment with RIS (0.1, 1.0 and 10 mg/kg) on 5-HT levels among brain regions is shown in table-6.3. One-way ANOVA shows that, there was not any significant differences

among groups in the 5-HT levels in PFC [F (5, 24) = 0.08, p>0.05], but it was significant in AMY [F (5, 24) = 20.71, p<0.05]. Post-hoc analysis showed that SRS significantly increased 5-HT levels in AMY regions compared to control. Repeated doses of RIS did not show any decline in levels of 5-HT at the doses tested. But PAX showed to alleviate the changes in 5HT in the AMY region induced by SRS.

Table-6.3: RIS effect on 5HT in SRS rats brain

	Control	**SRS**	**0.1 mg/kg**	**1 mg/kg**	**10 mg/kg**	**PAX**
PFC	1.457 ± 0.18	1.58 ± 0.08	1.65 ± 0.11	1.75 ± 0.17	1.81 ± 0.16	1.61 ± 0.19
AMY	5.23 ± 0.64	9.21 ± 0.81[a]	9.57 ± 0.58[a]	10.1 ± 0.67[a]	9.723 ± 1.01[a]	6.5 ± 0.89,[b,c,d,e]

The effect of repeated treatment of RIS (0.1, 1.0 and 10mg/kg) on brain 5HT levels in regions like PFC and AMY. All values are Mean ± SEM with n=5. [a]p<0.05 compared to control, [b]p<0.05 compared to SRS, [c]p<0.05 compared to RIS (0.1 mg/kg), [d]p<0.05 compared to RIS (1 mg/kg), [e]p<0.05 compared to RIS (10 mg/kg) [One-way ANOVA followed by Student Newman-keuls test].

6.3.4 Effect of RIS (0.1, 1.0 and 10mg/kg) on SRS-induced alteration on dopamine level in PFC and AMY

The effect of repeated treatment of RIS (0.1, 1.0 and 10 mg/kg) on DA levels in PFC and AMY is depicted in table-6.4. Analysis of data by One-way ANOVA showed that there was not any significant differences of DA levels in PFC [F (5, 24) = 0.21, p>0.05] and AMY [F (5, 24) = 0.85, p>0.05].

Table-6.4: RIS effect on DA in SRS induced rat brain

	Control	SRS	0.1 mg/kg	1 mg/kg	10 mg/kg	PAX
PFC	2.23±0.13	2.61±0.12	2.11 ± 0.3	2.46 ± 0.12	2.32 ± 0.41	2.62 ± 0.21
AMY	1.53±0.18	2.5±0.17	2.43±0.41	2.11±0.52	2.67±0.41	2.48±0.61

The effect of repeated treatment of RIS (0.1, 1.0 and 10mg/kg) on brain DA levels in PFC and AMY. All values are Mean ± SEM with n=5. [One-way ANOVA followed by Student Newman-keuls test].

6.3.5 RIS treatment enhances BDNF formation

Fig.6.1 illustrates the effects of repeated RIS (0.1, 1.0 and 10mg/kg) and PAX-10mg/kg treatment on the expression of caspase-3 in different brain regions. Statistical analysis by one-way ANOVA showed that there was a significant difference among groups in the level of expression of BDNF in PFC [$F(5, 12) - 42.4$, $P < 0.05$] and AMY [$F(5, 12) - 43.7$, $P < 0.05$]. Post-hoc analysis revealed that modified SRS significantly decreased the expression of the BDNF in both PFC and AMY compared to control. Repeated treatment of RIS in the dose of 0.1 mg/kg and PAX-10mg/kg significantly alleviated the modified SRS-induced decline in the expression of BDNF in both brain regions.

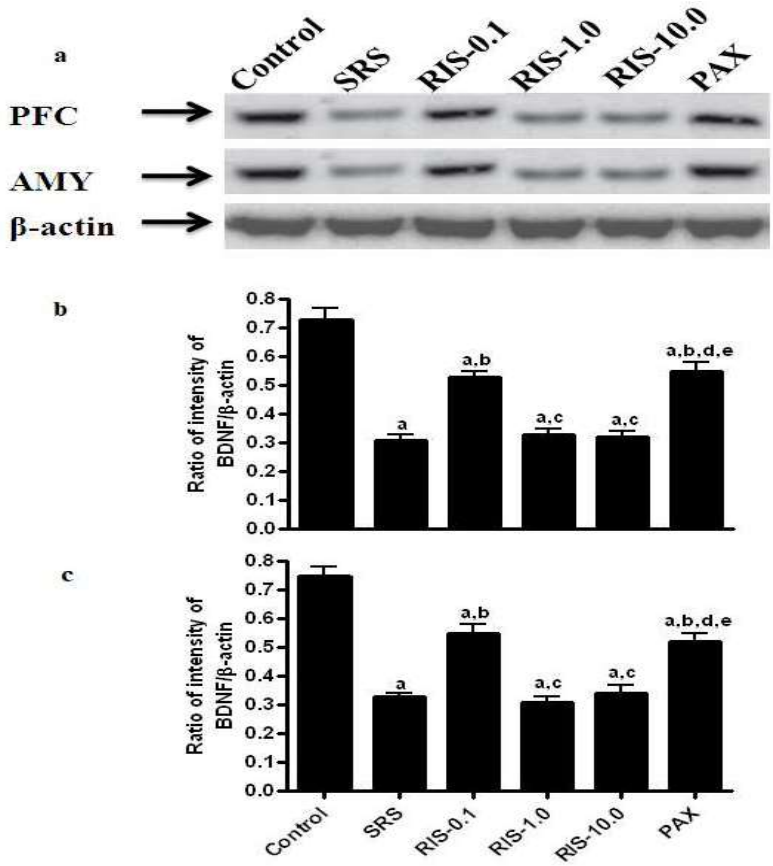

Fig. 6.1: The effect of RIS (0.1, 1 and 10 mg/kg) and PAX on SRS-induced changes in the expression of BDNF in PFC and AMY. The blots are representations of BDNF in PFC and AMY. The histogram data are expressed as the ratio of the relative intensity of levels of BDNF to β-Actin. All values are expressed as Mean±SEM (n=3). $^{a}P<0.05$ compared to control, $^{b}P< 0.05$ compared to SRS, $^{c}P< 0.05$ compared to RIS (0.1 mg/kg), $^{d}P< 0.05$ compared to RIS (1 mg/kg) and $^{e}P< 0.05$ compared to RIS (10mg/kg). [One-way ANOVA followed by Student Newman-Keuls test].

6.3.6. RIS decreases the pERK expression in PFC

Fig.6.2 illustrates the effects of repeated treatment with RIS (0.1, 1.0 and 10mg/kg) and PAX-10mg/kg on the level of expression of ERK and pERK in the PFC brain region. One way ANOVA analysis showed that there was a significant difference of treatment among groups [F (5, 12) 34.3, P < 0.05]. Post-hoc analysis revealed that there was a significant increase in the expression of the pERK in PFC compared to control due to modified SRS.

Repeated treatment with RIS in the dose of 0.1mg/kg and PAX-10mg/kg significantly reversed the modified SRS-induced increase in expression of pERK in the PFC regions.

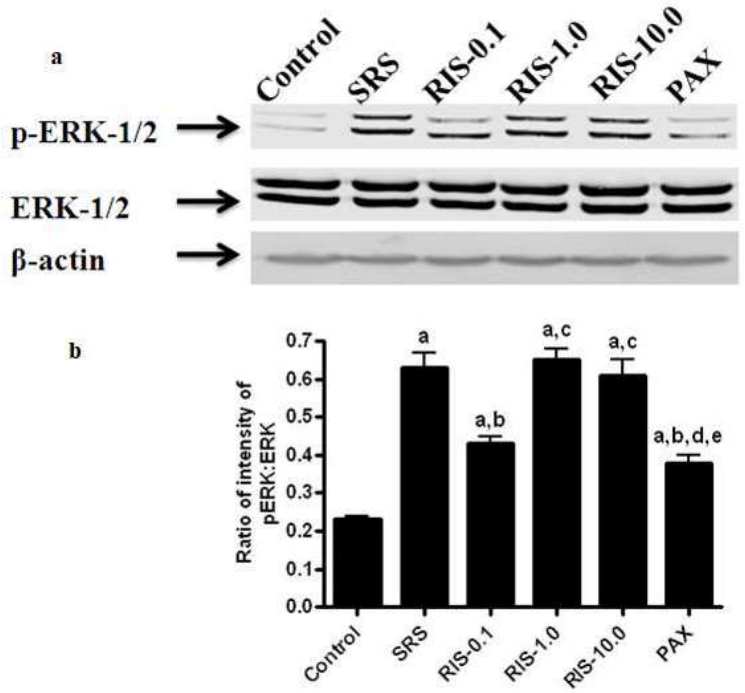

Fig.6.2. The effect of RIS (0.1, 1 and 10 mg/kg) and PAX on SRS-induced changes in the expression of pERK in PFC. The blots are representations of levels of pERK and ERK in PFC. The histogram data are expressed as the ratio of the relative intensity of levels of pERK to ERK. All values are Mean±SEM (n=3). $^{a}P< 0.05$ compared to control, $^{b}P< 0.05$ compared to SRS, $^{c}P<0.05$ compared to RIS (0.1 mg/kg), $^{d}P< 0.05$ compared to RIS (1 mg/kg) and $^{e}P<0.05$ compared to RIS (10mg/kg). [One-way ANOVA followed by Student Newman-keuls test].

6.3.7 RIS decreases the pERK expression in AMY

Fig.6.3 illustrates the effects of repeated RIS (0.1, 1.0 and 10mg/kg) and PAX-10mg/kg in the level of expression of ERK and pERK in the AMY region. Statistical analysis by one-way ANOVA showed that there was a significant difference among groups in the level of expression of pERK in AMY [F (5, 12) - 35.6, P < 0.05]. Post-hoc analysis revealed that modified SRS significantly enhanced the expression of the pERK in AMY compared to control. Repeated treatment with RIS at the doses of 0.1mg/kg and PAX-10mg/kg significantly reversed this modified SRS-induced increase in expression of pERK in the AMY region.

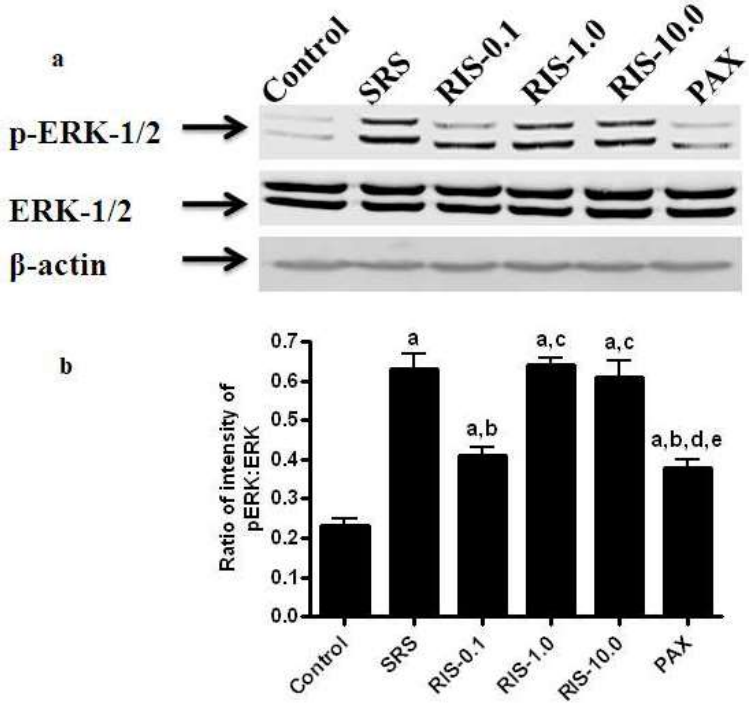

Fig.6.3 The effect of RIS (0.1, 1 and 10 mg/kg) and PAX on SRS-induced changes in the expression of pERK in AMY. The blots are representations of levels of pERK and ERK in AMY. The histogram data are expressed as the ratio of the relative intensity of levels of pERK to ERK. All values are Mean±SEM (n=3). $^{a}P< 0.05$ compared to control, $^{b}P< 0.05$ compared to SRS, $^{c}P< 0.05$ compared to RIS (0.1 mg/kg), $^{d}P< 0.05$ compared to RIS (1 mg/kg), $^{e}P< 0.05$ compared to RIS (10mg/kg).[One-way ANOVA followed by Student Newman-keuls test].

6.3.8 RIS enhances the expression of CREB

Fig.6.4 illustrates the effects of repeated administration of RIS (0.1, 1.0 and 10mg/kg) and PAX-10mg/kg in the level of expression of CREB in the two brain regions like PFC and AMY. Statistical analysis by one way ANOVA analysis of data showed that there was a significant difference among groups in the degree of expression of CREB in PFC [$F(5, 12) - 51.4$, $P < 0.05$] and AMY [$F(5, 12) - 48.9$, $P < 0.05$]. Post-hoc analysis revealed that modified SRS significantly decreased the expression of the CREB in both PFC and AMY compared to control groups. Repeated treatment with RIS in the dose of 0.1mg/kg and PAX-10mg/kg significantly reversed the modified SRS-induced decrease in the expression of CREB in both the brain regions.

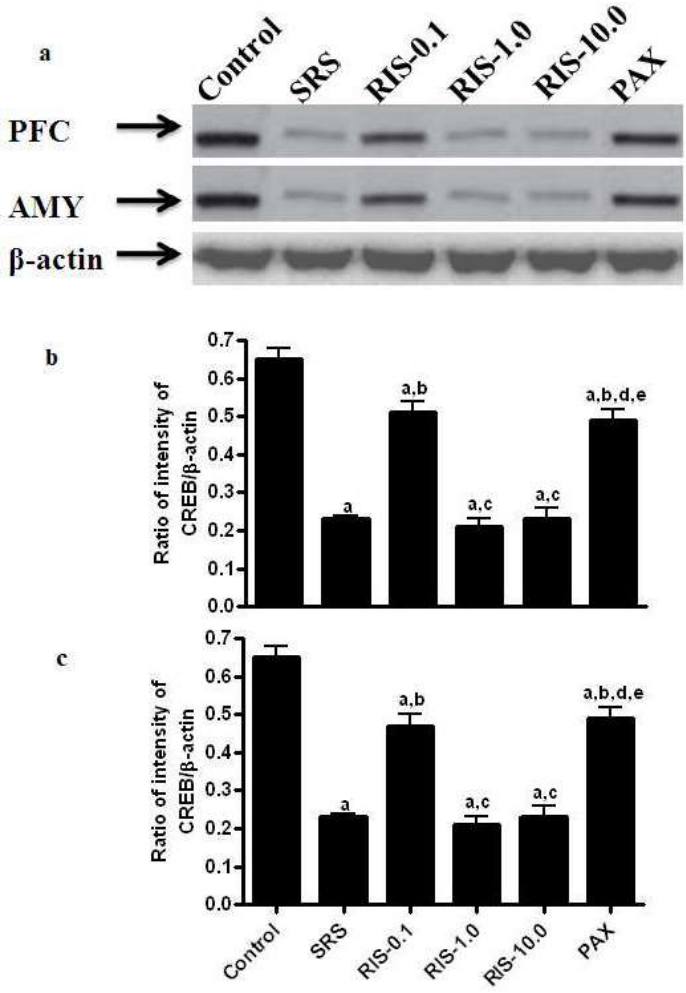

Fig.6.4. The effect of RIS (0.1, 1 and 10 mg/kg) and PAX on SRS-induced changes in the expression of CREB in PFC and AMY. The blots are representations of CREB in PFC and AMY. The histogram data are expressed as the ratio of the relative intensity of levels of CREB to β-Actin. All values are expressed as Mean±SEM (n=3). $^{a}P< 0.05$ compared to control, $^{b}P< 0.05$ compared to SRS, $^{c}P< 0.05$ compared to RIS (0.1 mg/kg), $^{d}P< 0.05$ compared to RIS (1 mg/kg) and $^{e}P< 0.05$ compared to RIS (10mg/kg). [One-way ANOVA followed by Student Newman–Keuls test].

6.3.9 RIS mitigates the Caspase-3 expression

Fig.6.5 illustrates the effects of repeated RIS (0.1, 1.0 and 10mg/kg) and PAX-10mg/kg administration in the level of expression of caspase-3 in the two brain regions. Statistical analysis showed that there was significant difference among groups in the level of expression of caspase-3 in PFC [F (5, 12) - 39.3, $P < 0.05$] and AMY [F (5, 12) - 37.1, $P < 0.05$]. Post-hoc analysis revealed that modified SRS significantly increased the expression of the protein caspase-3 in both PFC and AMY compared to control. Repeated treatment of RIS in the dose of 0.1mg/kg and PAX-10mg/kg significantly reduced the enhanced expression of caspase-3 in both the brain regions compared to the SRS group.

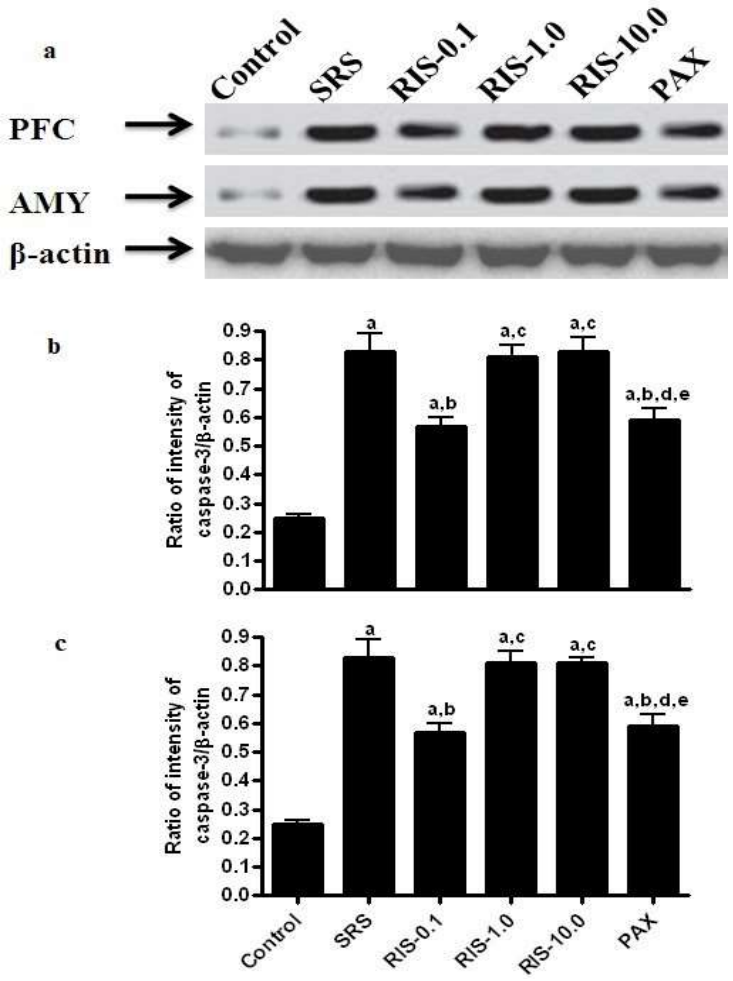

Fig 6.5: The effect of RIS (0.1, 1 and 10 mg/kg) and PAX on SRS-induced changes in the expression of caspase-3 in PFC and AMY. The blots are representations of caspase-3 in PFC and AMY. The histogram data are expressed as the ratio of the relative intensity of levels of caspase-3 to β-Actin. All values are expressed as Mean±SEM (n=3). $^{a}P< 0.05$ compared to control, $^{b}P< 0.05$ compared to SRS, $^{c}P< 0.05$ compared to RIS (0.1 mg/kg), $^{d}P< 0.05$ compared to RIS (1 mg/kg) and $^{e}P< 0.05$ compared to RIS (10mg/kg). [One-way ANOVA followed by Student Newman-Keuls test].

6.4 Discussion

Repeated treatment with RIS at the dose of 0.1mg/kg showed a significant enhancement in the expression of neurotrophic factors, which aid cell survival in the brain. This is the first study showing the role of these factors in the alleviation of PTSD in animals. The treatment specifically enhanced the expression of BDNF, CREB and mitigated the phosphorylation of ERK besides inhibiting the caspase-3 activity at 0.1mg/kg dose. Further, the treatment also showed improvements in the anxiety-like symptoms and memory deficiencies induced by PTSD.

Anxiety is a predominant symptom of PTSD and rats subjected to SRS showed a significant increase in anxiety-like behaviour on D-32. There was a decrease in the open arm entries and time spent during the EPM exploration (Xiang et al., 2017). Repeated drug treatment with RIS at the doses of 0.1mg/kg and PAX-10mg/kg significantly enhanced the number of entries and time spent in the open arms indicating alleviation of PTSD-induced anxiety-like behaviour. Previous studies have also reported the anxiolytic effect of RIS at 0.1mg/kg and PAX in EPM tests (Karanges et al., 2011; Rogoz et al., 2011).

Memory disturbances are another presentation of PTSD with re-experiencing symptoms (Samuelson, 2011). In this study, SRS altered the spatial memory, general exploration attitude (curiosity) and coping behaviour to a novel environment in the Y-maze test. RIS-0.1mg/kg and PAX-10mg/kg showed a significant improvement in spatial memory, exploration attitude and also coping behaviour. Previous studies also showed memory improvement properties of RIS in PTSD and MK-801-induced rats (Celikyurt et al., 2011; Krishnamurthy et al., 2013). Similarly, PAX at 10mg/kg also showed improvement in spatial memory in a rat model of depression (Han et al., 2015).

SRS enhanced the 5HT levels in the brain AMY region. This was mitigated by PAX at a dose of 10mg/kg while repeated administration of RIS showed no significant variations in levels

of 5HT. There is no dose-dependent alteration of monoamine levels in the study. Previous studies involving antipsychotics in PTSD models in rats did not find any dose-dependent effects with respect to serotonin and dopamine (Krishnamurthy et al., 2013). The effect of stress can lead to changes in the extracellular neurotransmitter levels. These extracellular neurotransmitters can be measured precisely using microdialysis technique (Plock and Kloft, 2005). In this study, both extracellular and intracellular monoamines were measured. Further, the dose dependent effects are seen when there is a sole target or agent causing the disease. The PTSD is a complex neuropsychiatric disorder with diverse pathophysiology involving, neurotransmitter, coricoids, oxidative stress, neuroinflammation, disruption of nerve growth factors.

BDNF, CREB and ERK play an important role in anxiety, memory and neuronal survival in PTSD (Hauger et al., 2012; Li et al., 2016). THE modified SRS model significantly decreases the expression of BDNF in both PFC and AMY regions. Treatment with RIS-0.1mg/kg and PAX- 10mg/kg reversed this decrease in expression of BDNF. Consistent with this, RIS at sub-therapeutic doses of 0.25mg/kg was showed to enhance the expression of BDNF in the prefrontal cortex and hippocampus in normal rats (Yu et al., 2015).PTSD is characterized by impairment in the extinction of fear memories. BDNF is thought to enhance this extinction of fear memories in PTSD. In the AMY, BDNF is essential for both fear acquisition and also extinction (Rattiner et al., 2004). While in PFC, BDNF is required in the prelimbic cortex for fear acquisition and in the infralimbic cortex for fear extinction (Sierra-Mercado et al., 2011). This is further ascertained by an experiment wherein deletion of BDNF in the brain region impaired fear extinction. However, administration of an endogenous BDNF agonist rescued this effect (Andero et al., 2012)

CREB is another protein that has a prominent role in memory and anxiety (Pandey et al., 2005; Silva et al., 1998). RIS enhanced the CREB levels, which were decreased due to SRS

at the dose of 0.1mg/kg in both PFC and AMY. PAX also showed this reversal of the decline in CREB due to SRS in both brain regions. It was found that decreased CREB signalling in the amygdala is thought to be the cause of enhanced anxiety in alcohol-preferring rats (Pandey et al., 2005).RIS enhanced CREB levels in the rat hippocampus. Apart from this, activation of BDNF and ERK pathways leads to stimulation of CREB expression (Yi et al., 2014).ERK protein plays a critical role in memory and anxiety through its effects on CREB (Weeber et al., 2002). Variable stress enhanced the ratio of pERK: ERK in both PFC and AMY. RIS 0.1mg/kg and PAX-10mg/kg decreased this pERK: ERK ratio.In a rat model of anxiety-like behaviour induced by conditioned fear training, inhibition of pERK in medial PFC showed an anxiolytic effect. Further, ERK, through activation of CREB, helps in memory formation. The rise in pERK expression leads to activation of proapoptotic factors, which can lead to cell death (Harding et al., 2001). Hence, inhibition of its expression would decrease the chances of neuronal degeneration. Apart from this, BDNF and CREB are also found to inhibit neurodegeneration by inhibiting pro-apoptotic factors (Almeida et al., 2005; Balogh et al., 2014).

Patients with PTSD have enhanced amygdala activity due to the decreased PFC inhibitory effect. This is thought to be due to neuronal degeneration in prefrontal cortex volumes observed through magnetic resonance imaging in PTSD patients (Morey et al., 2016; Shin et al., 2004).

Further, a study using a single prolonged stress model of PTSD in rats reported the enhanced apoptotic enzymes caspase 9 and 3 in the amygdala (Li et al., 2010). The previous experiment also showed the presence of apoptosis in PTSD rats (Garabadu et al., 2015).SRS-induced enhancement in caspase-3 levels was inhibited by RIS 0.1mg/kg and PAX-10mg/kg.RIS at 1mg/kg showed an anti-apoptotic effect of methamphetamine in PFC (Abekawa et al., 2008).

Thus from the above discussion, we can conclude that SRS-induced disruption of BDNF, CREB and ERK functionality is potentially leading to anxiety-like and memory-related behavioural problems. RIS and PAX alleviated this disruption of neuronal factors leading to a moderation of PTSD behavioural symptoms. Further, RIS and PAX also attenuated the elevated levels of apoptotic factors, which could prevent neuronal degeneration. Therefore, RIS seems to have potential anti-PTSD effects through the enhancement of the neurotrophic factors in the brain and also through neural survival mechanisms.