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Research report

Repeated olanzapine treatment mitigates PTSD like symptoms in rats with changes in cell signaling factors

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ABSTRACT

Post Traumatic Stress Disorder is an anxiety disorder with prolonged distortion of rational behaviour. In this study, we report the preclinical potential of olanzapine (OLZ) in the treatment of PTSD. Since the atypical antipsychotics have modulating effects on cell protective and destructive factors, we tested the effects of OLZ in PTSD regarding these cell modulating factors. Rats, when subjected to stress-restress (SRS) model of PTSD, showed a derangement in cell protective factors like the decline in BDNF, ERK, and CREB. While the adversarial factors like caspase-3 were enhanced. Four weeks treatment with OLZ at doses of 1 and 10 mg/kg significantly mitigated the SRS-induced derangement related to PTSD. OLZ at doses of 1 and 10 mg/kg enhanced BDNF, ERK and CREB levels which were reduced by SRS in PTSD animals. Further, at the fore mentioned doses it also inhibited the elevation of caspase-3 a downstream apoptotic factor. Besides, OLZ also showed mitigation in behavioural alterations related to anxiety and memory brought about by PTSD. These effects of OLZ were comparable to that of paroxetine a clinically approved drug for PTSD in terms of biochemical and behavioural assessments indicating its anti-PTSD potential.

1. Introduction

PTSD is a debilitating psychiatric syndrome that develops in people who have undergone shocking and terrifying experiences in the past (Adetunji et al., 2005; Yehuda et al., 2015). They suffer from persistent behavioural abnormalities like hyperarousal, recurring thoughts, avoid- ance, anxiety and memory disturbances. Besides, PTSD is one of the major contributing factors for the development of heart diseases and stroke (Edmondson et al., 2013; Sumner et al., 2015). Current medical therapy for PTSD involves administration of selective serotonin re- uptake inhibitors (SSRIs) as the first-line drugs. However, due to the moderate response rates and side effects, there is a need for the de- velopment of new medicines (Stein et al., 2000).

Few clinical studies have reported the therapeutic potential of atypical antipsychotics in the treatment of symptoms of PTSD (Adetunji et al., 2005). In particular, clozapine, OLZ, risperidone, and quetiapine were found to be useful in the mitigation of symptoms of PTSD (Hamner, 1996; Petty et al., 2001; Monnelly et al., 2003; Sokolski et al., 2003). However, the experimental studies involving the effect of OLZ in pharmacological animal models of PTSD are lacking. Preclinical data would help in understanding the mechanisms involved and further ascertain the pharmacological claims. The prefrontal cortex (PFC) and amygdala (AMY) are the critical centers responsible for the development and pathophysiology of PTSD (Koenigs and Grafman, 2009). The acquisition and extinction of fear memories occur within the amygdala (Pape and Pare, 2010). While, the PFC tightly regulates the expression of fear (Sotres-Bayon and Quirk, 2010). Therapeutic interventions in these two regions could provide an effective treatment for PTSD (Koenigs and Grafman, 2009). The tendency of PTSD symptoms to persist for long is due to the disruption of the adaptive mechanisms in the brain (Whitaker et al., 2014). These adaptive mechanisms would help to alleviate the responses to perceived fear. This defect in adaptive response is found to be due to the disturbances in the cell signaling
pathways involving BDNF, CREB, ERK, and caspase (Ross, 2009; Andero and Ressler, 2012). Previous studies have found that combat war veterans with PTSD had disturbances in BDNF gene transcription and CREB pathway (Kim et al., 2017; Martini et al., 2013). The removal of BDNF gene in mice leads to impairment of spatial memory and extinction of aversive memories (Heldt et al., 2007). Further, enhancement in phosphorylated ERK is thought to play a crucial role in the development and maintenance of PTSD in rats (Whitaker and Gilpin, 2016). ERK (extracellular signal-regulated kinases) signaling facilitates

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long-term memory and inhibits adaptive short-term memory (Davis and Laroche, 2006). In several studies, OLZ was found to exert neuroprotective effects through enhancement of BDNF and CREB expressions. It elevates the expression of brain BDNF by upregulation of gene transcription which then enhances CREB formation (Park et al., 2013; Reus et al., 2012; Lee et al., 2010). Apart from the promotion of cell survival factors, OLZ also blocked the activation of caspase-3 an apoptotic cell death factor (Wang et al., 2005).

So, with this informational background, we have hypothesized and evaluated the effects of OLZ on rats subjected to PTSD model with specific emphasis on neurotrophic cell signaling pathways involving BDNF, CREB and ERK and also cell destructive pathways involving caspase. Further, we have evaluated the plasma corticosterone and behavioural parameters like freezing, memory and anxiety as the hall- mark symptoms of PTSD.

2. Materials and methods

Drugs & CHEMICALS

OLZ and PAX were provided as gift samples by Ranbaxy Laboratories, India. Both of them were formulated into suspensions in a solution of 0.5% carboxymethylcellulose (CMC) in water. Antibodies used for the western blot studies were purchased from Santa Cruz Biotechnology Inc (Santa Cruz, California, USA). All the other chemicals and reagents of analytical grade were procured from local suppliers.

ANIMALS

Adult male Charles Foster albino rats weighing between 220–260 g were procured from the Central Animal House, Institute of Medical Sciences, Banaras Hindu University. They were housed in polypropylene cages at an ambient temperature of 25 ± 1 °C; and 45–55% Relative Humidity (RH). Food and water were provided *AD libitum*. Approval for the experimentation was obtained from the Institutional animal ethical committee (Ref No. Dean/10–11/148). 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. Every possible effort was taken to minimize the suffering and the number of animals used. All the experiments were conducted between 08:00 and 16:00hrs.

EXPERIMENTAL protocol

The experimental protocol was carried for 29 days as depicted in the Fig. 1. Animals were randomly assigned into six groups with 6 (n = 6) in each as control (group-1), PTSD control (group-2), OLZ doses as 0.1 mg/kg, 1.0 mg/kg and 10 mg/kg (group-3, 4, and 5 respectively) and PAX-10 mg/kg (group-6). Before the start of the experiment, rats were subjected to a baseline test session of behavioural paradigms like freezing, anxiety and memory. After the day of initial stress exposure, rats were given a single dose of treatment daily for the next 28 days.

Group 1 and 2 received 0.5% CMC suspension. The groups 3, 4 and 5 received orally 0.1, 1.0 and 10 mg/kg doses of OLZ suspensions. Group 6 received PAX dose of 10 mg/kg through the same oral route. Behavioral assessments for freezing, anxiety, and memory were conducted on day-1, 7, 14, 21 and 28th day of the drug treatment schedule. Following the 2 h r of the restress procedure, the animals were subjected to freezing analysis, elevated plus maze (EPM) test and the Y-maze test with a lag of 5 min between each consecutive test. On the last day, animals were decapitated; their brain regions like PFC and AMY were isolated and stored at −80 °C until further processing.

Stress-restress (SRS)/ time-dependent SENSITIZATION (TDS)

SRS model was used to induce PTSD in rats wherein they are exposed to prolonged variable stress and then time-dependent stress sensitization paradigm (Liberzon et al., 1997; Krishnamurthy et al., 2013). Initially, animals were subjected to a single session of prolonged stress for 2 h rs in a metallic restrainer on Day-0. Immediately they were subjected to 20 min forced swim test in an 18 cm swim tank at an ambient temperature of 25 °C. They were allowed to recover for 15 min and then promptly exposed to 0.8 ml of 4% halothane vapors (stress) until brief loss of consciousness. After recuperation from anesthesia, they were returned to their home cages. Consequently, starting from D-1, these animals were re-exposed to 20 min forced swim stress (restress) on D-7, 14, 21 and D-28 to enhance the sensitization (Liberzon et al., 1997).

EVALUATION of freezing-like BEHAVIOUR

Freezing-like behaviour was evaluated in all the animals by exposing them to the reminder situation for 5 min on day 1, 7, 14, 21 and 28th day respectively. With modification in the experiment, once the animals were removed from forced swim test, they were placed on an elevated table which is of equivalent height to the cylinder standing just beside it. The animals showed different behavioural patterns like rearing, grooming and freezing-like behaviour (absence of all movements except for breathing). The freezing behaviour was measured for over a period of 5 min using video tracking system. (Krishnamurthy et al., 2013; Kondaurova et al., 2015). The total cumulative time maintained by the animal in freezing posture was measured and scored by ANY-MAZE™ video tracking software.

EVALUATION of ANXIETY

Animals were evaluated for anxiety-like behaviour using EPM on day 1, 7, 14, 21, and 28th after restress procedure in PTSD induced rats (Walf and Frye, 2007). The aversion to heights and open spaces is found to be the cause of anxiety-like behaviour in rats when exposed to the EPM (Carobrez and Bertoglio, 2005). The fabricated maze has two opposite arms 50 × 10 cm, crossed with enclosed arms of the same dimension but having 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 center of the maze,

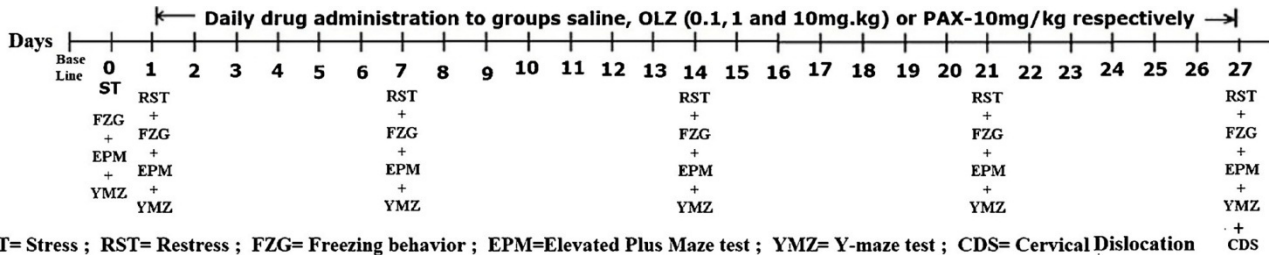

Fig. 1. The schematic representation of the experimental design. Here '+' indicates performed.

facing closed arm. After that number of entries and time spent on both the open and enclosed arm were recorded during the next 5 min using the ANY-MAZE™ video tracking system. An arm entry was defined when all four paws of the rat were in the arm. The behavioural parameters were assessed by a person unaware of the experimental protocol.

Y-MAZE

The behaviours on Y-maze were evaluated on all the days of restress procedure. Y maze is specifically used to assess the spatial recognition memory (Dellu et al., 1992). The novelty seeking tendency of rats is exploited to check the memory traits using Y-maze (Wright and Conrad, 2005). The maze consists of three identical arms starting from a central point (50 cm long, 16 cm wide and 32 cm high) with 120° angles to each other. The floor of the apparatus was covered with soiled animal bed- ding. Colored papers were fixed around the perimeter of the maze as visual cues, and they were changed for each test to maintain novelty to the animals. The arms were designated as starting arm, familiar arm, and a novel arm. In the first trial, entry to novel arm was blocked, and rats were allowed to move within the other two arms for 15 min. Four hours after the first trial, animals were allowed to access all the three arms for 5 min, and the number of entries was were 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™ video tracking software. The behavioural patterns like the total number of entries into all the arms (for the 5 min of trial 1 and the percentage entries in known and novel arms for the 5 min period of trial two were measured. The total number of entries into all the arms (for the 5 min of trial 1 and 2) is indicative of general exploration attitude (curiosity) and the per- centage 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 novel arm to time spent in all arms and in the center of the apparatus during trial 2 is determined as the coping behaviour to the novel arm. Any decrease in the coping behaviour in novel arm is indicative of the increase in anxiety-like behaviour (Poimenova et al., 2010).

ESTIMATION of PLASMA corticosterone by HPLC

Approximately 2 mL of blood sample was collected from rat's trunk immediately after decapitation. The blood was subjected to centrifugation in heparinized tubes to separate the plasma. The plasma corticosterone was measured by using High-performance liquid chromatography (HPLC) connected with a UV detector (Waters, USA) (Krishnamurthy et al., 2011; Woodward and Emery, 1987). An isocratic mobile phase consisting of a mixture of methanol: water in the ratio of 70:30 was run through a HPLC column (Waters: Spherisorb, RP C18, 5-μm particle size, 4.6 mm i.d. and 250 mm at 30 °C). A 500 μL of plasma containing a known quantity of dexamethasone (as internal standard) was extracted with 5 mL of dichloromethane. This dichloromethane extract was evaporated to dryness and then dissolved in 100 μL of the mobile phase. Twenty microliters of the extract were injected into HPLC system for quantification at a flow rate of 1.2 mL/min. The corticos- terone was detected at 250 nm using UV detector (Model 2849, Waters, USA). The chromatogram was recorded and analyzed with Empower software.

Western blot ANALYSIS

Tissue PREPARATION

Rats were decapitated and their skull was cut along the coronal suture and sagittal suture. The PFC and AMY were collected bilaterally and immersed immediately in liquid nitrogen and stored at −80 °C for further protein isolation.

Protein ISOLATION

The brain tissue was homogenized in a lysis buffer supplemented with protease inhibitor cocktail. This tissue sample was then cen- trifuged at 14,000 *g* at 4 °C for 30 min and the resultant protein-con- taining supernatant was stored at −80 °C. The protein concentration was determined by using a standard plot of bovine serum albumin (Bradford, 1976).

Western blotting

A standard plot was generated using bovine serum albumin. An aliquot of each sample was electrophoresed in 10% SDS-PAGE gels for BDNF, pERK, ERK, CREB and caspase-3 proteins, transferred to poly- vinylidene 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 de- tection 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. 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 secondary antibodies, anti rabbit Cy5 (1:200, ab97051; Abcam plc., India). Immunoreactive band of proteins was detected by chemiluminescence detector (Fusion FX vilber lourmat) using enhanced chemiluminescence (ECL) reagents (Amersham Bioscience, USA). The densitometric scan of images was performed for the quantification of results. The immunoreactive area was determined by densitometric analysis using Biovis gel doc- umentation software.

STATISTICAL ANALYSIS

The results obtained were analyzed statistically using GraphPad Prism version-5 software. Behavioural data of the freezing activity, EPM, the total entries and the arm discrimination behaviour between known and novel arm in Y-maze was measured by using repeated measures two-way analysis of variance (ANOVA) with Bonferroni post hoc test. The data of plasma corticosterone and western blot were analyzed with one-way ANOVA with Newman-keuls post hoc test. All the data are represented as the mean ± standard error of the mean (S.E.M). $P < 0.05$ was considered as statistically significant.

3. Results

OLZ ALLEVIATES SRS-induced rise in the freezing BEHAVIOUR

Fig. 2 shows the effect of repeated treatment of OLZ (0.1, 1.0 and 10 mg/kg) and PAX-10.0 mg/kg on SRS-induced changes in freezing behaviour. Statistical analysis by repeated measures two way ANOVA revealed that there was significant difference among groups [$F(5, 180) - 139.4, p < 0.05$], time [$F(5, 180) - 137.3, p < 0.05$] and interaction between group and time [$F(25, 180) - 26.54, p < 0.05$]. Post-hoc analysis showed that there was no change in freezing behaviour among groups on baseline test day and D-1. SRS significantly increased the freezing behaviour from D-7 up to D-28 compared to control.

Repeated treatment with OLZ at doses of 1.0 and 10 mg/kg significantly alleviated the SRS-induced enhancement in freezing behaviour on D-21 and D-28. However, PAX-10 mg/kg showed a significant decline in the freezing behaviour from D-14 and was maintained till D-28. There was no significant difference between OLX (1&10 mg/kg) and PAX-10 mg/kg on D-28.

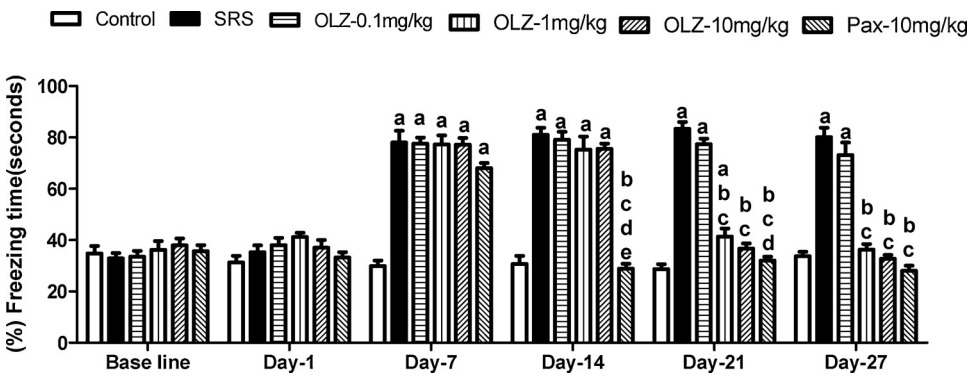

Fig. 2. The effect of OLZ (0.1, 1 and 10 mg/kg) and PAX on SRS-induced changes in freezing behaviour. All the values are Mean ± SEM (n = 6). [a]P < 0.05 compared to control, [b]P < 0.05 compared to SRS control (SRS), [c]P < 0.05 compared to OLZ (0.1 mg/kg), [d]P < 0.05 compared to OLZ (1 mg/kg) and [e]P < 0.05 compared to OLZ (10 mg/kg).
[Repeated measure two-way ANOVA followed by Bonferroni test].

Table 1
Effect of OLZ (0.1, 1 and 10 mg/kg) and PAX on SRS exposed rats in terms of percentage number of entries and time spent in open arm, fecal pellets, immobility time and total arm entries depicted in (A), (B), (C), (D) and (E) respectively.

Day	Control	SRS	OLZ-0.1	OLZ-1	OLZ-10	PAX
(A) Open arm entries (in percent)						
Base line	30.12 ± 1.790	25.40 ± 1.30	27.67 ± 1.12	28.51 ± 1.78	25.91 ± 1.450	29.70 ± 1.11
1	27.0 ± 1.10	26.80 ± 2.30	28.60 ± 1.26	27.82 ± 2.21	26.65 ± 1.17	27.70 ± 0.97
7	28.0 ± 1.34	07.30 ± 1.07[a]	08.31 ± 0.5[a]	09.10 ± 0.7[a]	10.04 ± 0.20[a]	9.2 0 ± 0.67[a]
14	27.4 ± 1.50	10.60 ± 0.90[a]	11.20 ± 0.8[a]	12.07 ± 0.8[a]	13.80 ± 0.46[a]	22.80 ± 1.96[b,c,d,e]
21	31.1 ± 1.31	14.22 ± 0.73[a]	15.02 ± 1.0[a]	17.54 ± 1.90[a]	27.92 ± 0.75[b,c,d]	29.04 ± 1.10[b,c,d]
28	30.46 ± 1.28	15.40 ± 0.61[a]	16.87 ± 1.0[a]	18.30 ± 2.23[a]	28.30 ± 1.5075[b,c,d]	28.40 ± 1.01[b,c,d]
(B) Open arm time spent (in percent)						
Base line	9.21 ± 0.78	8.31 ± 1.01	8.60 ± 0.57	9.01 ± 0.86	8.21 ± 0.830	8.31 ± 0.59
1	8.13 ± 0.41	7.81 ± 0.38	7.95 ± 0.66	8.18 ± 0.51	7.95 ± 0.720	8.50 ± 0.67
7	9.4 ± 0.52	3.20 ± 0.41[a]	3.92 ± 0.18[a]	3.20 ± 0.70[a]	3.89 ± 0.620[a]	4.36 ± 0.70[a]
14	8.7 ± 0.74	4.20 ± 0.46[a]	4.30 ± 0.43[a]	4.10 ± 0.53[a]	4.84 ± 0.310[a]	7.10 ± 0.50[b,c,d]
21	9.7 ± 0.56	3.60 ± 0.63[a]	4.00 ± 0.82[a]	4.84 ± 0.37[a]	8.00 ± 1.010[b,c,d]	9.0 ± 0.48[b,c,d]
28	9.3 ± 1.30	3.50 ± 0.70[a]	5.00 ± 1.00[a]	5.29 ± 0.50[a]	8.40 ± 0.640[b,c,d]	9.23 ± 0.38[b,c,d]
(C) Fecal pellets (in numbers)						
Base line	3.94 ± 0.450	4.41 ± 0.39	4.12 ± 0.68	3.99 ± 0.42	3.67 ± 0.460	4.10 ± 0.54
1	4.02 ± 0.22	4.50 ± 0.30	4.48 ± 0.72	4.48 ± 0.31	4.13 ± 0.620	4.36 ± 0.70
7	3.67 ± 0.40	6.10 ± 0.64[a]	5.90 ± 0.53[a]	6.30 ± 0.41[a]	6.10 ± 0.430[a]	6.05 ± 0.67[a]
14	3.80 ± 0.34	5.80 ± 0.71[a]	6.30 ± 0.46[a]	5.90 ± 0.50[a]	5.80 ± 0.290[a]	3.90 ± 0.84[b,c,d,e]
21	3.64 ± 0.70	5.59 ± 0.43[a]	5.60 ± 0.31[a]	4.40 ± 0.96[b,c]	4.02 ± 0.370[b,c]	3.30 ± 0.47[b,c,d]
28	3.40 ± 0.24	6.06 ± 0.37[a]	5.40 ± 0.28[a]	3.60 ± 0.39[b,c]	3.53 ± 0.260[b,c]	3.43 ± 0.35[b,c]
(D) Immobility (in seconds)						
Base line	9.71 ± 0.460	9.5 ± 0.89	10.1 ± 0.84	10.2 ± 1.49	9.93 ± 1.030	10.4 ± 0.77
1	9.5 ± 0.30	9.4 ± 0.20	9.7 ± 0.24[a]	9.1 ± 0.31	9.0 ± 0.272	9.5 ± 0.37
7	10.4 ± 0.54	34.7 ± 1.70[a]	36.0 ± 2.86[a]	37.0 ± 3.09[a]	38.0 ± 1.302[a]	37.5 ± 1.78[a]
14	8.90 ± 0.34	36.0 ± 2.92[a]	35.6 ± 3.04[a]	34.0 ± 2.21[a]	32.0 ± 1.472[a]	13.0 ± 1.24[b,c,d,e]
21	11.3 ± 1.74	37.0 ± 3.52[a]	34.6 ± 1.39[a]	25.2 ± 2.01[b,c]	15.0 ± 1.720[b,c,d]	12.0 ± 1.33[b,c,d]
28	10.6 ± 0.45	35.0 ± 2.36[a]	35.9 ± 1.27[a]	16.2 ± 1.92[b,c]	14.0 ± 1.192[b,c]	12.7 ± 1.07[b,c]
(E) Total arm entries (in numbers)						
Base line	7.78 ± 0.67	9.01 ± 1.10	9.00 ± 0.780	8.9 ± 0.820	8.7 ± 0.900	9.1 ± 0.850
1	8.10 ± 0.88	8.9 ± 0.90	9.15 ± 0.80	9.5 ± 1.23	7.9 ± 0.672	8.5 ± 1.37
7	7.97 ± 1.01	9.3 ± 0.93	8.60 ± 0.84	9.2 ± 0.77	8.9 ± 1.272	9.1 ± 0.88
14	8.70 ± 0.73	8.3 ± 0.80	9.12 ± 0.82	8.7 ± 0.39	9.4 ± 0.572	9.4 ± 0.70
21	9.50 ± 0.90	9.9 ± 1.79	9.20 ± 2.21	8.7 ± 1.31	9.2 ± 0.720	8.8 ± 1.37
28	8.20 ± 1.30	8.9 ± 1.54	8.69 ± 1.32	9.7 ± 1.80	8.9 ± 1.720	8.4 ± 1.50

All the values represent Mean ± SEM (n = 6). Repeated measures two-way ANOVA analysis with Bonferroni Post hoc test.
[a] P < 0.05 compared to control.
[b] P < 0.05 compared to SRS control (SRS).
[c] P < 0.05 compared to OLZ (0.1 mg/kg).
[d] P < 0.05 compared to OLZ (1 mg/kg).
[e] P < 0.05 compared to OLZ (10 mg/kg).

Effect of OLZ in EPM ANXIETY BEHAVIOUR in EPM

The effect of repeated treatment with OLZ (0.1, 1, 10 mg/kg) and PAX (10 mg/kg) on changes induced by SRS in open arm entries, time spent, the number of fecal pellets, immobility period and the number of total arm entries in EPM were depicted in Table 1(A)–(E) respectively.

Analysis by repeated measures two-way ANOVA showed that there were significant differences in percentage in open arm entries and time spent, number of fecal pellets and immobility period among groups ([F (5, 180) - 77.56; P < 0.05], [F (5, 180) - 30.49; P < 0.05], [F(5,180) - 57.29; P < 0.05], [F(5,180) - 95.26; P < 0.05] respectively), time ([F (5,180) - 139.8; P < 0.05], [F(5, 180) - 29.88; P < 0.05], [F(5,180) -

59.37; P < 0.05], [F (5, 180) - 182.3; P < 0.05] respectively) and an interaction between group and time ([F (25, 180) – 10.76; P < 0.05], [F (25,180) - 4.190; P < 0.05], [F (25,180)- 10.11; P < 0.05], [F (25, 180) - 19.94; P < 0.05] respectively) in the EPM paradigm. However, no significant differences were observed in the number of total entries among groups [F (5, 180) - 1.956; P > 0.05], time [F (5, 180) - 1.028; P > 0.05], and there was no significant interaction between group and time [F (25, 180) - 1.048; P > 0.05]. Post-hoc analysis revealed that there were no significant differences among groups on baseline test day and D-1. But there was a significant difference among groups in the percentage of open arm entries and time spent; the number of fecal pellets and immobility. The percentage of open arm entries and time spent were decreased due to stress from D-7 to D-28 compared to control rats. Stress also increased the number of fecal pellets and im- mobility period compared to control rats. Treatment with OLZ 10 mg/ kg significantly enhanced the SRS-induced decrease in the percentage of open arm entries and time spent on D-21 and D-28. Further, OLZ 10 mg/kg significantly reduced the SRS instigated a rise in the number of fecal pellet droppings and immobility period on D-21 and D-28. PAX (10 mg/kg) treatment also alleviated the SRS-induced decline in the percentage of open arm entries, time spent and increase in fecal drop- pings and immobility time on D-14 and this effect continued up to D-28. There was no significant difference between OLZ 10 mg/kg and PAX on D-28 on all the paradigms of EPM. However, OLZ-1 mg/kg did not show any changes regarding open arm entries and time spent on all the days tested. However, it showed a significant reduction in fecal droppings and immobility period compared to SRS group on D-21 and D-28.

OLZ MODULATES SRS-induced BEHAVIOURAL ALTERATIONS in the Y-MAZE test

The effect of repeated OLZ (0. 1, 1.0 and 10 mg/kg) and PAX- 10 mg/kg treatment on SRS-induced changes in exploratory behaviour (curiosity) in trial-1 and trial-2, and coping time are presented in Table 2(A) and (B) respectively. Analysis with repeated measure two-way ANOVA showed significant differences of curiosity in both trial-1 and trial-2 among groups ([F (5, 180) - 90.51; P < 0.05] and [F (5, 180) - 19.8 16.053; P < 0.05] respectively), time ([F (5,180) - 161.7; P < 0.05] and [F (5, 180) - 32.54; P < 0.05] respectively) and an interaction between group and time ([F (25, 180) - 21.82; P < 0.05] and [F (25, 180) - 3.742; P < 0.05] respectively). Further, Post-hoc analysis revealed that the stress paradigm showed a significant decrease in curiosity behaviour in comparison to the control rats from D-7 to D-28 but not on baseline test day and D-1. OLZ in the doses of 1 and 10 mg mg/kg significantly mitigated the SRS-induced decrease in curi- osity behaviour on D-21 and D-28. However, the treatment with PAX- 10 mg/kg reversed this SRS-induced decrease in curiosity on D-14, D-21 and D-28 persistently. Also, no significant differences were observed between OLZ (1 and 10 mg/kg) and PAX on D-28, regarding curiosity behaviour in Y-maze paradigm. The data of percentage time spent (coping behaviour) in the novel arm is presented in Table 2(B). Repeated measures two-way ANOVA showed significant differences of percentage of time spent in the novel arm among groups ([F (5, 180)- 13.65; P < 0.05], time ([F (5,180) - 14.84; P < 0.05] and an interaction between group and time [F (25, 180) - 2.537; P < 0.05] respectively). SRS significantly decreased the percentage of time spent in novel arm compared to control from D-7 till D-28. Repeated treatment with OLZ-1 and 10 mg/kg showed a significant increase in the time spent in the novel arm that was decreased by SRS from D-21 to D-28. PAX-10 mg/kg showed the reversal in an SRS-induced decrease of novel arm time spent from D-14 till D-28. Also, there were no significant differences in effects in percentage time spent in the novel arm in between OLZ (1 and 10 mg/kg) and PAX-10 mg/kg on D-21 and D-28.

SPATIAL recognition memory

Fig. 3 depicts the effect of OLZ (0.1, 1 and 10 mg/kg) and PAX-10 mg.kg on SRS-induced changes in spatial recognition memory on D-1, D-7, D-14, D-21, and D-28. Fig. 3A depicts the entry of rats into

Table 2
Effect of OLZ (0.1, 1 and 10 mg/kg) and PAX on SRS exposed rats in terms of changes in total arm entries during trial-1 and trial-2 indicating curiosity behaviour (A) and coping behaviour (B).

Day	Control	SRS	OLZ-0.1	OLZ-1	OLZ-10	PAX
(A) Total arm entries trial-I (in numbers)						
Base line	7.1 ± 0.73	7.5 ± 075	7.4 ± 0.62	7.33 ± 0.54	7.20 ± 0.55	7.3 ± 0.57
1	7.0 ± 0.62	7.7 ± 1.01	7.6 ± 0.54	7.50 ± 0.43	7.3 ± 0.58	7.7 ± 0.86
7	7.4 ± 1.10	3.0 ± 0.30[a]	3.0 ± 0.26[a]	3.50 ± 0.21[a]	3.9 ± 0.97[a]	3.8 ± 0.47[a]
14	7.3 ± 0.81	3.6 ± 0.73[a]	4.0 ± 0.66[a]	3.80 ± 0.34[a]	4.1 ± 0.62[a]	6.9 ± 0.59[b,c,d,e]
21	7.2 ± 0.41	3.2 ± 0.46[a]	3.7 ± 0.37[a]	6.70 ± 0.5[b,c]	7.1 ± 0.52[b,c]	7.1 ± 0.72[b,c]
28	7.6 ± 0.21	3.8 ± 0.55[a]	3.6 ± 0.42[a]	7.09 ± 0.7[b,c]	7.27 ± 0.47[b,c]	7.5 ± 0.69[b,c]
(B) Total arm entries trial-II (in numbers)						
Base line	14.2 ± 0.84	14.7 ± 1.04	15.1 ± 0.98	14.98 ± 0.79	14.56 ± 1.20	15.2 ± 0.85
1	14.0 ± 0.70	15.0 ± 1.10	14.5 ± 0.60	15.80 ± 0.91	14.8 ± 1.50	15.80 ± 1.31
7	15.4 ± 0.76	7.9 ± 0.70[a]	9.0 ± 0.90[a]	8.30 ± 0.79[a]	7.9 ± 0.90[a]	8.02 ± 0.67[a]
14	15.6 ± 0.91	8.3 ± 1.60[a]	8.7 ± 1.80[a]	8.80 ± 0.82[a]	9.1 ± 0.57[a]	12.70 ± 0.45[b,c,d]
21	14.7 ± 0.60	8.1 ± 0.34[a]	9.5 ± 0.82[a]	11.76 ± 0.61[b]	13.0 ± 0.54[b]	13.50 ± 0.76[b,c]
28	15.2 ± 1.01	8.4 ± 0.93[a]	10.0 ± 0.76[a]	13.90 ± 1.50[b,c]	14.2 ± 1.21[b,c]	14.60 ± 0.99[b,c]
(C) [(Time spent in Novel arm/(time spent in all the arms and center))*100]						
Base line	25.12 ± 1.93	24.45 ± 1.69	24.58 ± 0.97	26.02 ± 1.16	25.30 ± 1.45	24.88 ± 1.24
1	24.61 ± 1.68	25.90 ± 1.64	25.48 ± 1.10	26.78 ± 1.26	25.80 ± 2.11	26.60 ± 1.80
7	26.70 ± 1.13	17.89 ± 0.62[a]	17.71 ± 1.09[a]	17.96 ± 1.10[a]	18.70 ± 1.80[a]	17.90 ± 2.41[a]
14	27.90 ± 2.21	15.95 ± 1.56[a]	18.60 ± 1.91[a]	19.95 ± 1.63[a]	19.63 ± 2.04[a]	25.70 ± 1.22[b,c,d,e]
21	26.04 ± 1.76	17.14 ± 1.86[a]	19.12 ± 1.56[a]	22.76 ± 0.97[b]	22.90 ± 1.2[b]	24.98 ± 1.86[b,c]
28	25.33 ± 1.24	16.40 ± 0.85[a]	19.43 ± 1.34[a]	24.48 ± 1.05[b]	24.67 ± 1.1[b]	25.14 ± 1.58[b,c]

All the values represent Mean ± SEM (n = 6). Repeated measures two-way ANOVA analysis with Bonferroni Post hoc test.
[a] P < 0.05 compared to control.
[b] P < 0.05 compared to SRS control (SRS).
[c] P < 0.05 compared to OLZ (0.1 mg/kg).
[d] P < 0.05 compared to OLZ (1 mg/kg).
[e] P < 0.05 compared to OLZ (10 mg/kg).

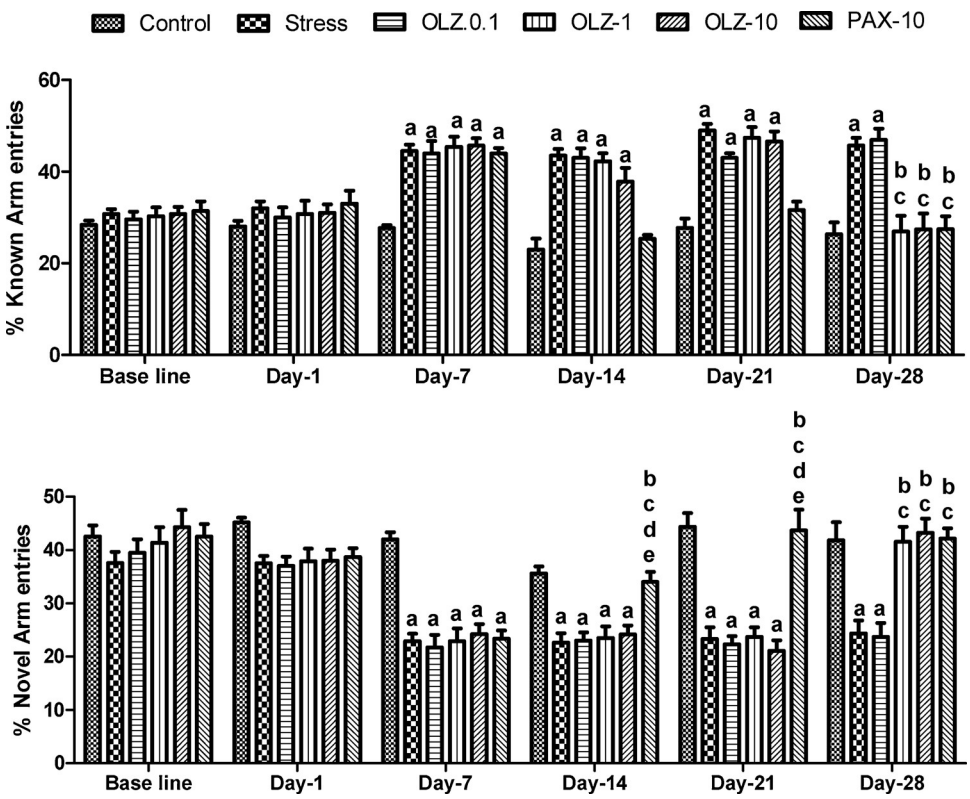

Fig. 3. The effect of OLZ (0.1, 1 and 10 mg/kg) and PAX on SRS-induced changes in Y-maze arm discrimination in term of % known arm entries (A) and % novel arm entries (B) on D-1, D-7, D-14, D-21 and D-28 of the treatment schedule. All the values are Mean ± SEM (n = 6). [a]P < 0.05 compared to control, [b]P < 0.05 compared to SRS, [c]P < 0.05 compared to OLZ (0.1 mg/kg), [d]P < 0.05 compared to OLZ (1 mg/kg) and [e]P < 0.05 compared to OLZ (10 mg/kg). [Two-way ANOVA followed by Bonferroni test].

known arm and Fig. 3B depicts entry into the Novel arm. Two-way ANOVA of data of Fig. 3A showed significant differences among groups [F (5, 180) - 37.15, p < 0.05], time [F (5, 180) - 34.22, p < 0.05] and
interaction between group and time [F (25, 180) - 7.264, p < 0.05]. Post-hoc analysis of the data showed that there were no changes in entries into the known arm on baseline day and D-1. However, SRS significantly increased the entries into known arm from D-7 up to D-28 compared to control. OLZ at doses of 1 and 10 mg/kg significantly decreased the entries into the known arm on D-28. PAX-10 significantly alleviates the SRS-induced increase in entries into known arm from D- 14 to D-28. Further, there was no significant difference of entries into known arm between doses of OLZ-1 and 10 mg/kg and PAX-10 mg/kg on D-28. Two-way ANOVA analysis of data in Novel arm entries is shown in Fig. 3B showed significant differences among groups [F (5, 180) - 37.17, p < 0.05], time [F (5, 180) - 50.65, p < 0.05] and in-
teraction between group and time [F (25, 180) - 5.810, p < 0.05]. On analysis of data by post-hoc analysis, there were no changes of entries into the Novel arm on D-1. However, SRS significantly decreased the entries into Novel arm from D-7 up to D-28 in comparison to control. OLZ at doses of 1 and 10 mg/kg significantly enhanced the entries into the known arm on D-28. On the other hand, PAX significantly increased the entries into known arm from D-14 to D-28. Further, there were no significant differences of entries into the Novel arm on D-28 between OLZ (1 and 10 mg/kg) and PAX-10 mg/kg.

OLZ ALLEVIATES the SRS-induced decline in PLASMA corticosterone

The effect of OLZ (0.1, 1.0 and 10 mg/kg) and PAX (10 mg/kg) on SRS-induced alterations in plasma corticosterone is shown in the Fig. 4. One-way ANOVA analysis of the data showed that there were sig- nificant differences of plasma corticosterone [F (5,30) - 66.71; P < 0.05] among the groups on day 28. Post hoc analysis showed that SRS significantly decreased the plasma corticosterone levels compared to control animals. Repeated OLZ (1.0 and 10 mg/kg) treatment in- creased the SRS-induced decline in plasma corticosterone levels. However, OLZ 0.1 mg and PAX-10 mg showed no effect on SRS-induced

changes in plasma corticosterone levels.

OLZ induces the expression of BDNF

Fig. 5 illustrates the effects of repeated OLZ (0.1, 1.0 and 10 mg/kg/) and PAX-10 mg/kg treatment on the expression of BDNF in different brain regions. Statistical analysis showed that there was significant difference among groups in the level of expression of BDNF in PFC [F (5,12) - 40.5, P < 0.05] and AMY [F (5,12) - 40.5, P < 0.05]. Post-hoc analysis revealed that SRS significantly decreased the expression of the protein BDNF in both PFC and AMY compared to control. Repeated treatment with OLZ in the dose of 1 and 10 mg/kg and PAX-10 mg/kg significantly reversed this SRS-induced decline in the expression of BDNF in both the brain regions.

Effect of OLZ TREATMENT on the expression of pERK/ERK in PFC

Fig. 6 illustrates the effects of repeated treatment with OLZ (0.1, 1.0 and 10 mg/kg) and PAX-10 mg/kg in the level of expression of pERK and ERK and their ratio in the PFC brain region. Statistical analysis showed that there was a significant difference in the expression of pERK/ERK among groups [F (5,12) - 26.1, P < 0.05]. Post-hoc analysis revealed that modified SRS significantly increased the expression of the pERK/ERK in PFC compared to control. Repeated treatment of OLZ in the dose of 1 and 10 mg/kg and PAX-10 mg/kg significantly alleviated the SRS-induced enhancement in the expression of pERK/ERK in the PFC while OLZ-0.1 mg showed no change.

Effect of OLZ TREATMENT on the expression of pERK/ERK in AMY

Fig. 7 illustrates the effects of repeated OLZ (0.1, 1.0 and 10 mg/kg/) and PAX-10 mg/kg in the level of expression of pERK, ERK and their ratio in AMY region. As per the statistical analysis, there was a sig- nificant difference among groups in the level of expression of pERK/ ERK in AMY [F (5,12) - 25.4, P < 0.05]. Post-hoc analysis revealed that SRS significantly enhanced the expression of the pERK/ERK in

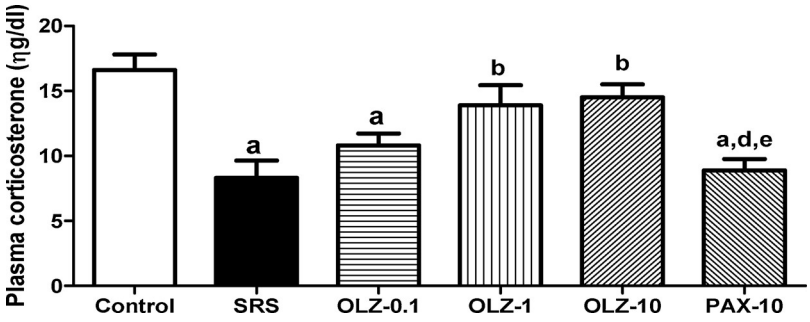

Fig. 4. The effect of OLZ (0.1, 1 and 10 mg/kg) and PAX on the levels of plasma corticosterone in SRS subjected rats. All the va- lues are Mean ± SEM (n = 6). $^{a}P < 0.05$ compared to control, $^{b}P < 0.05$ compared to SRS control (SRS), $^{c}P < 0.05$ compared to OLZ (0.1 mg/kg), $^{d}P < 0.05$ compared to OLZ (1 mg/kg) and $^{e}P < 0.05$ compared to OLZ (10 mg/kg). [One-way ANOVA fol- lowed by Student NewmanneKeulstest].

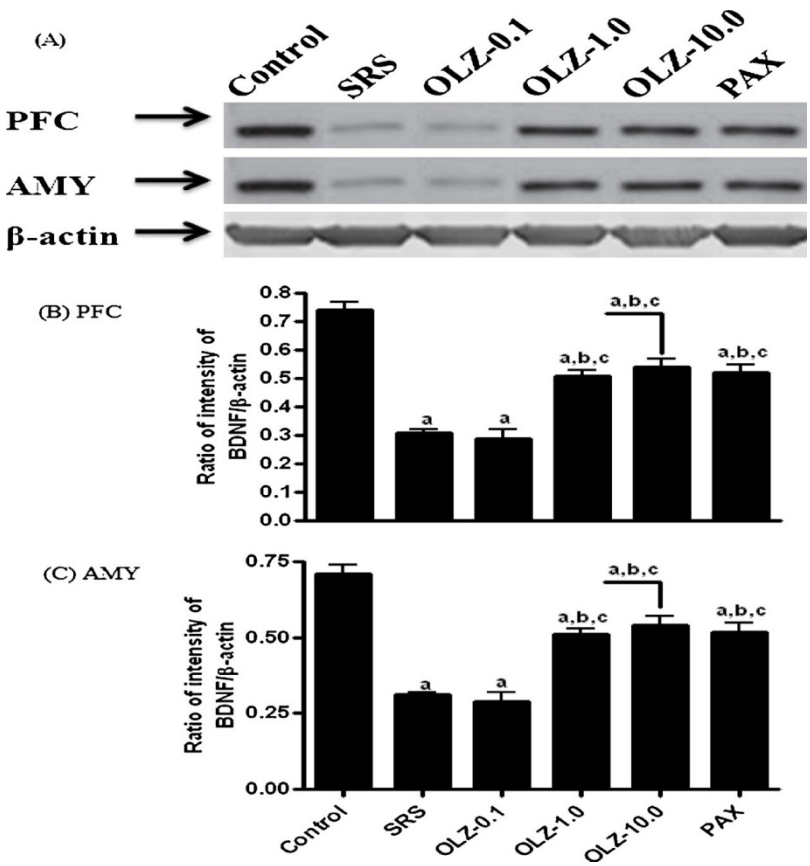

Fig. 5. The effect of OLZ (0.1, 1 and 10 mg/kg) and PAX on SRS- induced changes in the expression of BDNF in PFC and AMY. (A) Indicates of the blot representations of BDNF in PFC and AMY. (B) and (C) are the histogram data expressed as the ratio of the re- lative intensity of levels of BDNF to β-Actin in PFC and AMY re- spectively. 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 OLZ (0.1 mg/kg), $^{d}P < 0.05$ compared to OLZ (1 mg/kg) and $^{e}P < 0.05$ compared to OLZ (10 mg/kg).
[One-way ANOVA followed by Student NewmanneKeuls test].

AMY compared to control. Repeated treatment with OLZ at the doses of 1 and 10 mg/kg and PAX-10 mg/kg significantly mitigated this SRS- induced increase in expression of pERK/ERK in the AMY region.

OLZ promotes the expression of CREB

The effects of repeated OLZ (0.1, 1.0 and 10 mg/kg) and PAX-10 mg/kg in the level of expression of CREB in the two brain regions viz. PFC and AMY were depicted in Fig. 8. Statistical analysis showed significant differences among groups in the degree of expression of CREB in PFC [F (5,12) - 36.3, P < 0.05] and AMY [F (5,12) - 35.5,
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. Repeated treatment of OLZ in the dose of 1 and 10 mg/kg and PAX-10 mg/kg significantly reversed the modified SRS-induced decrease in the expression of CREB in both the brain regions.

OLZ inhibits the expression of CASPASE-3

The effects of repeated OLZ (0.1, 1.0 and 10 mg/kg) and PAX-

10 mg/kg on the levels of expression of caspase-3 in different brain regions as illustrated in the Fig. 9. Statistical analysis of data showed significant differences among groups in the level of expression of cas- pase-3 in PFC [F (5,12) - 44.6, P < 0.05] and AMY [F (5,12) - 30.7,
P < 0.05]. Post-hoc analysis revealed that SRS significantly increased the expression of the enzyme caspase-3 in both PFC and AMY compared to control. Repeated treatment of OLZ in the dose of 1 and 10 mg/kg and PAX-10 mg/kg significantly reduced the increase in the expression of caspase-3 in both the brain regions compared to SRS group.

4. Discussion

The salient finding of the study is the anti-PTSD potential of OLZ in the animal model. To the best of our knowledge, this is the first report showing the preclinical anti-PTSD outcome of OLZ. This study also indicates the imperativeness of regulation of the cell signaling factors in the treatment of PTSD. The repeated treatment with OLZ mitigated the modified SRS-induced PTSD like symptoms. There was an alleviation of the behavioural disturbances brought about by PTSD regarding anxiety and memory. PTSD also disrupts the expression pattern of cell signaling

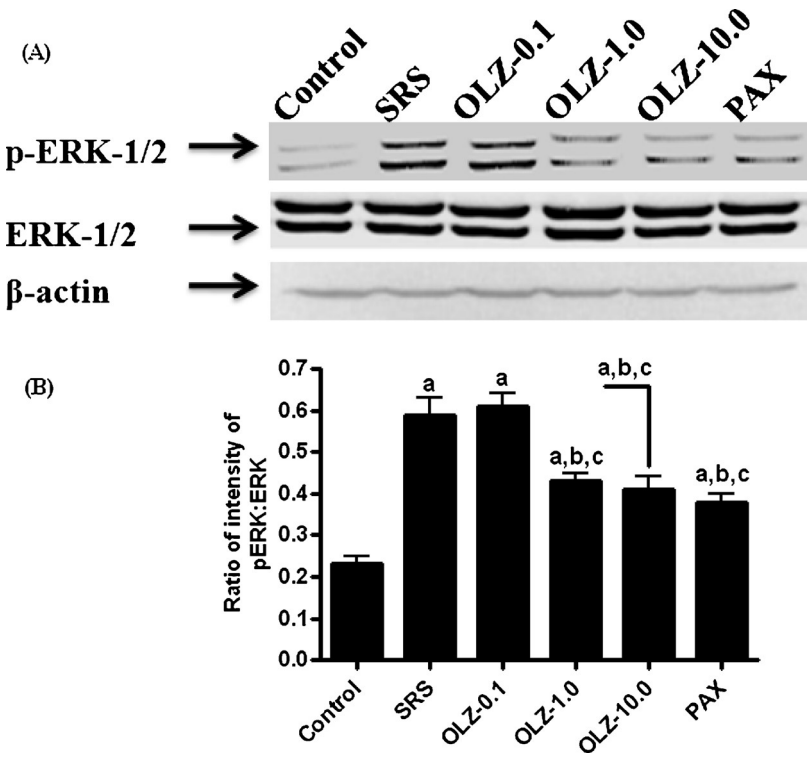

Fig. 6. The effect of OLZ (0.1, 1 and 10 mg/kg) and PAX on SRS- induced changes in the expression of ERK in PFC. (A) Consists if the blots representations of levels of pERK and ERK in PFC. The
(B) is the histogram data expressed as the ratio of the relative intensity of levels of pERK/ERK in PFC. All 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 OLZ (0.1 mg/kg), [d]P < 0.05 compared to OLZ (1 mg/kg) and [e]P < 0.05 compared to OLZ (10 mg/kg). [One-way ANOVA followed by Student NewmanneKeulstest].

proteins like caspase-3, BDNF, CREB, and ERK. This disruption was also significantly alleviated by repeated OLZ treatment for 28 days.

Animals with PTSD perceive most stimuli as threatening and respond immediately through behavioural and physiological action, such as freezing. Freezing is a defensive response with symptoms like re- duction in physical movements and the heart rate (Fragkaki et al., 2017). This behavioural pattern is thought to be the reason for the sus- tenance of the disease as it inhibits the adaptive risk assessment of stimuli leading to persistent maladaptive defensive responses. In this study, animals subjected to SRS showed a longer duration of freezing- like response. Repeated drug treatment with OLZ at doses of 1 and 10 mg/kg showed a decrease in the duration of freezing on D-21 and D-28. This reduction in freezing due to treatment indicates an enhancement of adaptive risk assessment which could contribute to the remission of PTSD signs. In another study, OLZ at the doses of 5 and 10 mg/kg moderated contextual freezing behaviour in response to shock in ovariectomized rats (Frye and Seliga, 2003). Besides, PAX also miti- gated freezing behaviour from D-14 to D-28. This observation was also consistent with other studies wherein chronic administration of PAX showed a decrease in freezing behaviour in rats subjected to single prolonged stress. However, PAX did not show this effect in acute treatment (Takahashi et al., 2006).

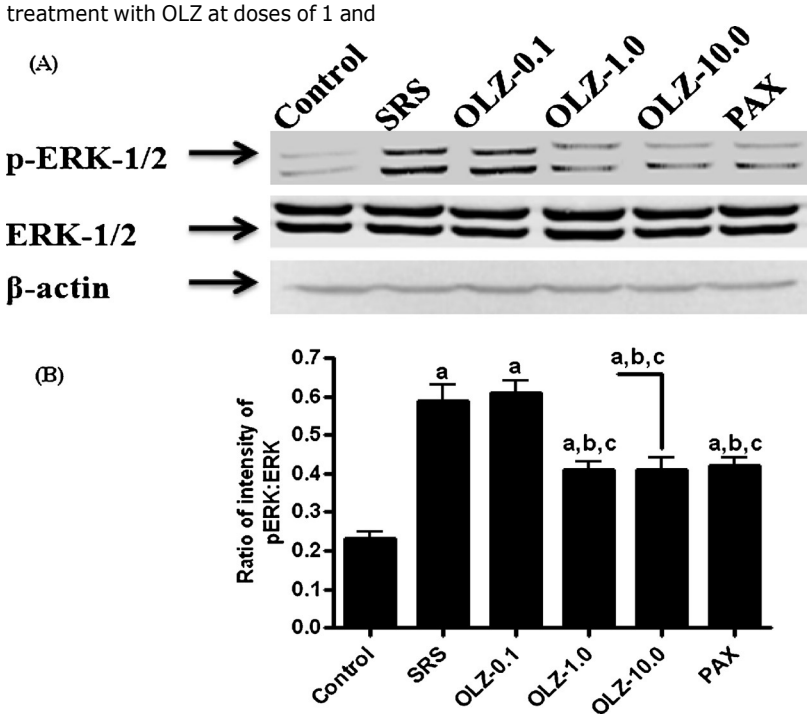

Fig. 7. The effect of OLZ (0.1, 1 and 10 mg/kg) and PAX on SRS- induced changes in the expression of ERK in AMY. (A) consists of the blots which are representations of the levels of pERK and ERK in AMY. (B) Is the histogram data expressed as the ratio of the relative intensity of levels of pERK/ ERK in AMY. 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 OLZ (0.1 mg/kg), [d]P < 0.05 compared to OLZ (1 mg/kg) and [e]P < 0.05 compared to OLZ (10 mg/kg). [One-way ANOVA followed by Student NewmanneKeulstest].

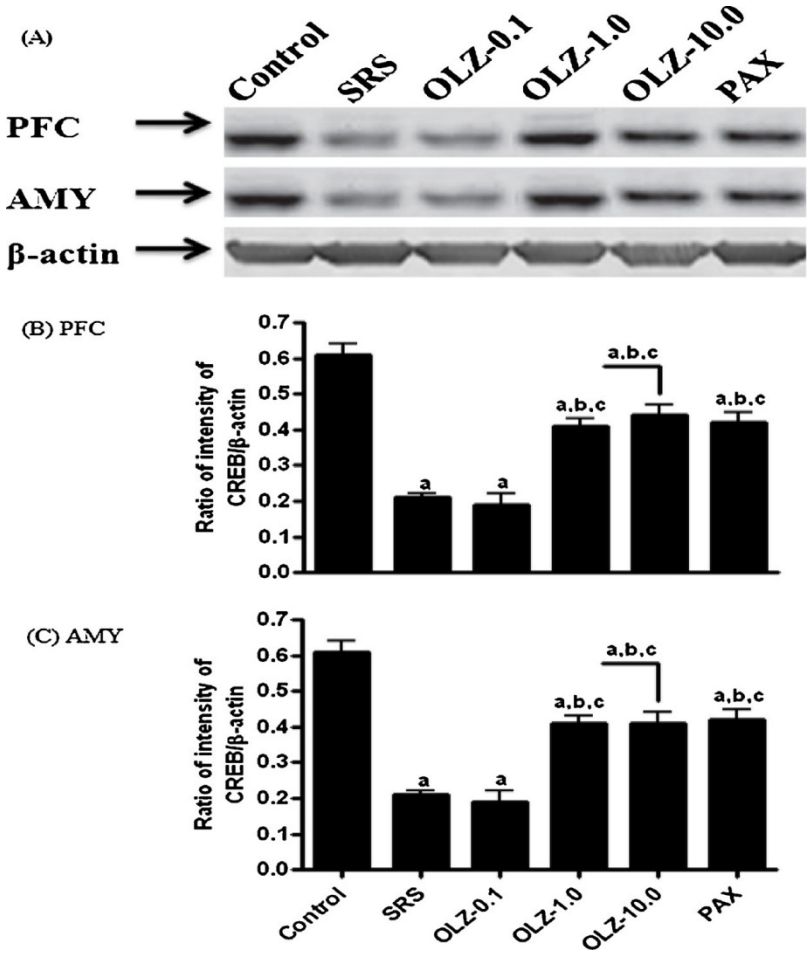

Fig. 8. The effect of OLZ (0.1, 1 and 10 mg/kg) and PAX on SRS-induced changes in the expression of CREB in PFC and AMY. (A) Indicates the blots representing the CREB in PFC and AMY. (B), (C) are histograms which express the ratio of the relative intensity of levels of CREB to β-Actin in PFC, AMY respectively. 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 OLZ (0.1 mg/kg), [d]P < 0.05 compared to OLZ (1 mg/kg) and [e]P < 0.05 compared to OLZ (10 mg/kg). [One-way ANOVA followed by Student NewmanneKeuls test].

Hyperarousal or anxiety is the primary behavioural symptom ob- served in patients with PTSD (Ahman and Stalnacke, 2008). PTSD rats, when exposed to EPM, showed a decline in the number of entries and time spent on the open arm but had enhancement of fecal droppings and immobility period which are indications of anxiety (Krishnamurthy et al., 2013).

In this study, there was no change in behavioural parameters im- mediately after variable stress on D-1, but there were significant changes in between groups from D-7. Similarly, in a previous study, rats subjected to acute stress showed significant changes in EPM within one hour of acute stress. However, those rats subjected to restress showed changes in the EPM only from D-7 and not immediately after one hour of restress (Harvey et al., 2006). Whereas, in the previous experiment involving the evaluation of risperidone in PTSD, the exposure to SRS showed changes in EPM immediately after variable stress even before induction of restress (Krishnamurthy et al., 2013). However, these be- havioral variations seem to be correlated with the changes in the cor- ticosterone levels in the brain in response to a different stressor (Harvey et al., 2006).

Repeated treatment with OLZ 10 mg/kg and PAX-10 mg/kg showed to alleviate anxiety related behaviour regarding the entries and time spent in the open arms, the number of fecal droppings and also im- mobility time. Treatment with OLZ-10 mg/kg increased the number of entries and time spent on the open arms and also diminished the number of fecal droppings and immobility periods starting from D-21 to D-28. However, OLZ at a dose of 1 mg/kg showed only decrease in fecal droppings from on D-28; but had no effect on open arm entries and time spent. Similar variations in effects were also observed in previous studies wherein at 1 mg/kg dose OLZ had mitigating effects on fecal droppings in a stress-induced model of rats (Locchi et al., 2008; Sun et al., 2010). However, at the doses of 10 mg/kg, it significantly enhanced the entries and time spent in open arm indicating dose-dependent effects (Frye and Seliga, 2003). These differences in the behavioural effects on EPM could be due to the superior potential of atypical antipsychotics in alleviating fear-related anxiety responses like defecations besides the ability to mitigate intrinsic anxiety (Mead et al., 2008). On the other hand, PAX enhanced the number of entries and time spent in the open arm beside decreasing the fecal dropping and immobility period from D-14 up to D-28. Anxiolytic properties of paroxetine were also reported earlier for EPM and social fear (Drapier et al., 2007; Toth et al., 2018). However, there were no differences in the total number of entries in between the groups on all the days tested. This is because; the rats in EPM showed enhanced entries into the closed arms despite the decreased entries into open arms leading to a lack of significant change in the total number of entries between groups. Further, there was no development of tolerance to open arm exposures in control group animals due to repeated trials on EPM. This could be due to the time interval gap of 1 week in between exposures. Concurrently in another study, rats exposed to EPM for 18 consecutive days did not show any signs of development of tolerance to the open arms (Treit et al., 1993).

Another distinctive symptom of PTSD is the change in the cognitive processes like memory, attention, planning and problem-solving (Hayes

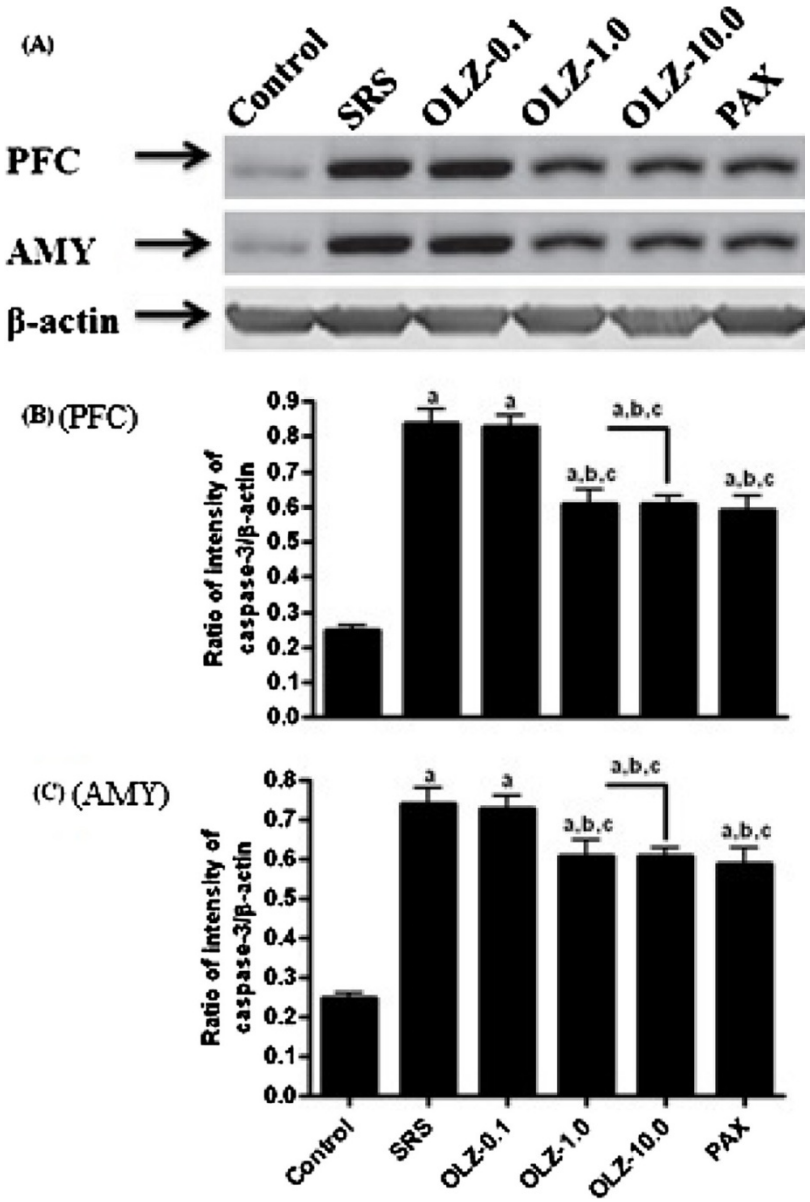

Fig. 9. The effect of OLZ (0.1, 1 and 10 mg/kg) and PAX on SRS-induced changes in the expression of caspase-3 in PFC and AMY. (A) Consists of the blots as representations of caspase-3 in PFC and AMY. (B) And (C) are the histograms data expressed as the ratio of the relative intensity of levels of caspase-3 to β-Actin in PFC and AMY respectively. 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 OLZ (0.1 mg/kg), $^{d}P < 0.05$ compared to OLZ (1 mg/kg) and $^{e}P < 0.05$ compared to OLZ (10 mg/kg). [One-way ANOVA followed by Student NewmanneKeuls test].

et al., 2012). There is predominance to threat detection and interpretation of offensive stimuli as threatening, thereby constricting at- tention focus at the expense of other cognitive performances (Hayes et al., 2012). In the Y-maze exploration test, SRS caused a gradual loss of exploration behaviour, spatial memory and enhanced anxiety-like behaviour in the form of decrease coping (time spent) from D-7 up to D-28. These disruptive changes in behaviour were mitigated by OLZ (1.0 and 10 mg/kg) and PAX from D-14 and D-21 respectively. In previous studies, OLZ showed to improve spatial learning function and relieve cognitive deficits at doses of 0.1 and 5 mg/kg body weights (Hou et al., 2006; Wolff and Leander, 2003). Even PAX at a dose of 10 mg/kg re- stored the impaired spatial learning and memory in depressed rats (Han et al., 2015). It indicates that both PAX and OLZ (1 and 10 mg/kg) improved memory deficits in PTSD which is critical for the develop- ment of optimal adaptive behaviour. Another intriguing observation in the study is that there was a decrease in the total number of entries in the Y-maze test. This was because the rats subjected to SRS spent more time in the familiar arm in the direction of the cue with decreased overall movement. Further, the rats in control group did not show any signs of development of tolerance to the Y maze arms after repeated exposure due to the novelty presented in terms of change in cues for each test.

Research into previous experiments indicates a wide variation in the doses of OLZ tested. At the lower dose range of 0.5 to 2 mg/kg body weight, OLZ was found to be effective in anxiety and memory like mood-related behaviours (Locchi et al., 2008; Sun et al., 2010; Hou et al., 2006). However, others found OLZ to be effective in anxiety and memory in the dose ranges of 5 to 10 mg/kg body weight in rats. At 10 mg/kg it showed to have anxiolytic effects in EPM and open field tests, and at a dose of 5 mg/kg had effects on memory-related behaviour (Frye and Seliga, 2003; Wolff and Leander, 2003). However, in contrast to this, few studies also indicated sedative-like effects of OLZ at a dose

higher than 2 mg/kg (Ahnaou et al., 2003). These dose-dependent variations of effects could result from OLZ's ability to bind to multiple receptors. At regular doses, it binds to 43–80% of dopamine and near saturation of serotonin receptors. Moreover, at the doses of 5 to 10 mg, OLZ is found to enhance the allopregnanolone (3α-hydroxy-5α- pregnan-20-one) in the brain which acts as a positive modulator of GABA leading to sedative-like effects (Marx et al., 2000). However, in PTSD there is a pattern of hyperarousal which is the cause of the an- xiety, memory and sleep disturbances. In this study, OLZ at a dose of 10 mg/kg had positive implications on anxiety and memory behaviours without any significant sedative effects indicating insufficient dose. Apart from this, the repeated exposure of rats to forced swim tests (PTSD sensitization) and other behavioural tests performed on the same day could also have hindered OLZ's sedative properties.

The hypothalmopitutary adrenal axis (HPA) is perturbed in PTSD with increased activity of negative feedback loop of HPA-axis leading to hypocorticosteronemia (de Kloet et al., 2006; Yehuda, 2001). SRS significantly reduced the levels of plasma corticosterone and the repeated treatment with OLZ (1 and 10 mg/kg) showed an increase in plasma corticosterone levels. However, repeated administration of PAX did not show any mitigating effects on the SRS-induced decline in plasma corticosterone levels. This inability of PAX is ascertained to be due to the lack of HPA-axis modulation (Krishnamurthy et al., 2013; Philbert et al., 2012).

This indicates OLZ's modulating effects on plasma corticosterone through the activity on HPA-axis. Inconsistent with our observations, in another study, OLZ and clozapine showed to enhance plasma corti- costerone levels in normal rats (Assie et al., 2008). Interestingly, the repeated administration of PAX did not show any mitigating effects on the SRS-induced decline in plasma corticosterone levels. However, in a rat line selectively bred for high anxiety-related behaviour (HAB) emo- tionality, PAX at a dose of 5 mg/kg normalized the alterations in plasma corticosterone levels brought about by disturbances in HPA (Keck et al., 2003). However, this difference could be due to pathology condition wherein the previous study was based on depression while in this study it is a PTSD model.

Repeated OLZ treatment significantly alleviated the PTSD induced perturbations of cell signaling factors. The pathology of PTSD also involves the disturbance in the cell signaling pathways involving BDNF, CREB, ERK, and caspase-3 as observed in both clinical studies and experimental models (Ross, 2009; Andero and Ressler, 2012). OLZ at the doses of 1 and 10 mg/kg and PAX-10 mg/kg enhanced BDNF levels in the PFC and AMY regions. BDNF is a nerve growth factor which is crucial in modulating resilience and vulnerability to stress (Kim et al., 2017). In a stress re-stress model of PTSD in rats, it was shown that repeated administration of OLZ reversed the BDNF and Bcl-2 levels to normal (Luo et al., 2004). This ability of OLZ to enhance BDNF is through its ability to increase basal BDNF gene promoter activity in a dose-dependent manner and also CREB meditated transcription via PKA, PI3K, PKC, and CaMKII signaling pathways (Lee et al., 2010).

SRS significantly enhanced the formation of phosphorylated ERK (pERK) over ERK. pERK is predominantly formed under the conditions of stress. A previous study reported that rats subjected to forced swim stress had enhanced pERK levels in the PFC and AMY regions (Shen et al., 2004). The inhibition of ERK phosphorylation leads to decrease in avoidance behaviour in rats, one of the critical symptoms of PTSD (Whitaker and Gilpin, 2016). Further, increased signaling of ERK reg- ulates gene expression, CREB formation, neuronal plasticity and memory (Davis and Laroche, 2006; Shen et al., 2004; Sweatt, 2001). The increased formation of pERK would activate proapoptotic tran- scription factor CHOP gene (C/EBP-homologous protein) (Harding et al., 2001). It implies that enhancement of ERK formation would help in correction of neuronal plasticity and memory deficits. On the other hand minimization of pERK, formation prevents the activation of apoptotic factors. In this study repeated treatment by OLZ (1and 10 mg/kg) and PAX-10 mg/kg alleviated the formation of pERK.

CREB is a transcription factor involved in the neuronal process of learning, neuronal plasticity, and modulation of stress responses. Patients with PTSD were found to have lower levels of total CREB protein in their blood samples (Martini et al., 2013). In this experiment, repeated administration of OLZ (1 and 10 mg/kg) and PAX-10 mg/kg showed a significant up-regulation of CREB expression which was de- creased due to SRS in both the PFC and AMY. This result is similar to another study involving the assessment of mood stabilizing effects of OLZ. Four weeks administration of lithium and OLZ showed a sig- nificant up-regulation of CREB in rats (Hammonds and Shim, 2009). Also, PAX at a dose 0f 10 mg/kg was found to up-regulate CREB ex- pression in a rat model of depression (Han et al., 2015). It indicates that the repeated drug administration could have modulated PTSD induced derangements through enhancement of CREB.

Apoptotic factors like Caspase-3, 9, and cytochrome C oxidase act as the contributing factors for the development of PTSD (Han et al., 2013; Garabadu et al., 2015). Caspase-3 is one of the critical enzymes in- volved in the downstream pathway of apoptotic cell death. SRS sig- nificantly increased caspase-3 in both PFC and AMY regions of the brain. In a model of PTSD involving single prolonged stress, there was increased expression of caspase-3 and 9 (Zhang et al., 2016). Repeated treatment with OLZ (1 and 10 mg/kg) and PAX (10 mg/kg) significantly diminished the increase in expression of caspase-3 in both the regions. Previous studies also support the modulating effect of OLZ on apoptosis (Wang et al., 2005). Another antipsychotic risperidone also showed neuroprotective effects by inhibiting the activation of caspases (Garabadu et al., 2015; Ukai et al., 2004). Similarly, PAX at a dose of 10 mg/kg reduced the hippocampal expression of caspase-3 in a rat model of chronic mild stress (Khedr et al., 2015).

From the above discussion, it can be inferred that BDNF enhance-

ment leads to the extinction of fear and memory consolidation in PTSD through the stimulation of ERK signaling cascade (Andero and Ressler, 2012; Ji et al., 2016). This ERK, in turn, translocates to the nucleus and phosphorylates the CREB to mediate downstream transcriptional acti- vation which further modulates the memory consolidation (Sgambato et al., 1998; Kida et al., 2002). On the other side, the decreased ex- pression of pERK prevents the activation of apoptotic factors like the caspase-3 resulting in promotion of cell survival pathways (McKay and Morrison, 2007). Thus the OLZ treatment could have the potential to minimize cell death, enhance plasticity; mitigate anxiety and memory disturbances in PTSD rats.

However, the classical theory holds that atypical antipsychotics like OLZ produce their therapeutic effects through their action on the ser- otonergic system. Hence, its therapeutic potential through the cell signaling systems can be contrasting. Still, studies have found that there exists a synergistic mechanism in between serotonin and BDNF systems in affective behaviours (Martinowich and Lu, 2008). Serotonergic cells have been found to enhance BDNF function and pretreatment with a 5- HT2A receptor antagonist can prevent the stress-induced decrease in BDNF (Madhav et al., 2001; Vaidya et al., 1997). Also, SSRIs which enhance synaptic serotonin levels reverse the stress-induced down- regulation of BDNF gene expression (Gonul et al., 2005; Autry and Monteggia, 2012). Hence, these drugs acting through the serotonergic pathway could yet be involved in activation of BDNF related pathway (Martinowich and Lu, 2008).

Also, the requirement of prolonged drug treatment in clinic and their ability to enhance neurotrophic factors indicate that these drugs have a notable effect on cell survival than the monoamine pathway alone.

Thus from the above study, it can be concluded that OLZ has po- tential anti-PTSD properties comparable to PAX in terms of both the behavioural improvements and also neurotrophic enhancement. However, the effect of PAX seems to start earlier as seen by the im- provement in behavioural parameters on D-14 while that of OLZ's starts from D-21. Therefore, the study shows the preclinical potential of OLZ in the treatment of PTSD.

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ORIGINAL PAPER

Risperidone in Ultra Low Dose Protects Against Stress in the Rodent Cold Restraint Model by Modulating Stress Pathways

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Abstract The present investigation evaluates the anti-stress activity of risperidone (RIS) in the cold restraint stress (CRS) model and related stress pathways. Rats were pretreated with RIS (0.1 and 1.0 mg/kg) for 21 days before subjecting to CRS. Ultra low dose of RIS (ULD; 0.1 mg/kg) in contrast to higher dose (1.0 mg/kg) significantly reduced stress in terms of ulcer index. ULD also reversed stress-induced increase in plasma corticosterone and norepinephrine levels used as markers for the function of hypothalamo-pituitary-adrenal (HPA) axis and sympathetic nervous system (SNS) respectively. ULD caused dose and brain region (hippocampus, prefrontal cortex and striatum) specific changes to stress-induced perturbations of serotonin, dopamine and its metabolites indicating modulation of brain monoaminergic system (BMS). ULD did not show any extrapyramidal side effects. Thus, the anti-stress effect ULD is probably mediated through the HPA axis, SNS and BMS. The study indicates a potential use of ULD in stress disorders.

Keywords Risperidone · Anti-stress · Monoamines · Hippocampus · Prefrontal cortex · Striatum

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Introduction

Stress is reported to play an important role in the genesis and pathophysiology of different psychological disorders like depression, anxiety and psychosis [1]. Physiologically stress refers to the non-specific response by the body to maintain homeostasis, while psychologically it is development of adaptive response to defend stability of internal environment and ensure survival of organism. The stress-vulnerability model of schizophrenia [2] is widely accepted although its clinical implications have not yet been fully realized. Stress responses are mainly through two distinct but interrelated systems viz: the hypothalamic-pituitary-adrenocortical (HPA) system [3] and the sympathetic nervous system (SNS) system [4]. Hippocampus (HIP) regulates the HPA axis during the stress responses [5] and there exists a reciprocal modulation between serotonin (5-HT) and HPA axis [6]. The 5-HT_{1A} and 5-HT_{2A} receptor subtypes play an important role in epidemiology of stress [7] and are predominant in HIP and prefrontal cortex (PFC [8, 9]). Risperidone (RIS), an atypical-antipsychotic is a highly selective 5-HT_{2A} antagonist. Hence, we presume that RIS acting through this serotonergic pathway may modulate neurochemical perturbations induced by stress.

Clinical studies show RIS to be successful in therapy for stress disorders [10–12]. It has also been suggested that these classes of antipsychotics may be effective for the treatment of mood disturbances such as anxiety and depressive mood associated with schizophrenia [13]. However, there are very few experimental data to support its use. RIS in high doses produces a number of side effects [14, 15]. Therefore, a study evaluating the optimal dose has concluded that the ultra low doses (≤2 mg/day) is ineffective compared to low doses (≥2–4 mg/day) mg/day), standard-low (≥4–6 mg/day), standard-higher doses

(▯6–10 mg/day) and high dose (▯10 mg/day) in schizophrenia [16]. Hence, it is imminent that there are dose differences in the pharmacological effects of RIS and it is important that this is further investigated to help in choosing dosing schedules for treatment of stress disorders. We have shown that repeated RIS treatment had significant gastroportective effect in cold restraint stress (CRS) model by modulating the local gastric parameters [17]. Since the demonstration of stress-induced ulceration by Selye [18], variations of the model have been widely used to study neuroprotectant activity [19]. In the present experiment we explore the central mechanisms involved in the anti-stress effect of RIS.

The present study investigates the anti-stress effect of repeated RIS treatment and related dose differences in a well validated CRS model in rats. Gastric ulcer, plasma corticosterone (CORT) and norepinephrine (NE) levels were measured as indices of stress, the HPA axis and the SNS respectively. The effect of the monoaminergic system was measured by estimation of 5-HT and its metabolite 5-hydroxy indolacetic acid (5-HIAA), and dopamine (DA) and its metabolite; 3, 4 dihydroxy phenyl acetic acid (DOPAC) in discrete stress sensitive brain regions such as HIP, PFC and striatum (STR). Further, catalepsy was evaluated as an index of extra pyramidal side (EPS) effect. This study underscores the potential use of repeated ultra low dose of RIS (ULD) in stress disorders.

Materials and Methods

Animals

All experiments were conducted in accordance with the Principles of laboratory animal care (NIH publication number 85–23, revised 1985) guidelines. The experimental procedures were approved by Institutional animal ethical committee, Banaras Hindu University. 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. The animals were housed in polypropylene cages under controlled environmental conditions of temperature of 25 ± 1°C and 45–55% RH and a 12:12 h 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. Animals were acclimatized for at least 1 week before using them for experiments.

Drugs

Risperidone (RIS) was procured from Sigma (St. Louis, MO, USA). RIS was suspended in distilled water using 0.5% of sodium carboxymethylcellulose (CMC). All other chemicals and reagents of HPLC and analytical grade were procured from local suppliers.

Cold Restraint Stress (CRS) Model

After 18 h fasting, one stress session was performed during the early phase of the light cycle and consisting of a 2 h restraint period (rat restrainers were metallic cages of 15 cm long 9 6.5 cm width) at 4–6°C. The animals were then killed by cervical dislocation. The stomach was taken out and cut open along the greater curvature and ulcers were scored by a person unaware of the experimental protocol in the glandular portion of the stomach [20].

Experimental Protocol

Animals were randomly divided into four groups of six animals each. Rats received repeated treatment of RIS in dose of 0.1 and 1.0 mg/kg orally through oral gavage using a ball-ended feeding needle for 21 days. The doses were selected based on previous published observations [13, 17]. In the above referred study [13] repeated dose of RIS (0.1 mg) was more effective in decreasing conditioned fear-induced anxiety than the corresponding lower and higher higher doses (1 mg/kg). Similarly, repeated treatment of RIS for 21 days showed significant gastroprotective effect in dose of 0.1 mg/kg compared to the corresponding lower and higher doses [17]. In the current experiment the sham and RIS treated animals were subjected to 2 h CRS procedure on day 21 after 1 h of vehicle or drug administration. The animals were then transferred to home cage. After 30 min of CRS, animals were subjected to the bar test and were killed immediately. All animals were killed within 60 min after CRS procedure followed by microdissection and estimation of ulcer index.

Evaluation of Catalepsy Behaviour in Bar Test

Catalepsy, defined as the acceptance and retention of abnormal posture, was measured by means of the bar test. The bar test was carried out by gently removing rats from their home cage and placing their forepaws on a horizontal bar, fixed at a height of 10 cm above the working surface. The length of time during which the animal retained in this position was recorded by measuring the time from the placement of the rat until removal of one of its forepaws (mean of three consecutive trials; cut-off time =60 s). All the groups were tested on 21st day [21].

Estimation of Ulcer Index

The stomach was cut through greater curvature and the ulcer index was calculated by following standard protocol by a blind observer [20].

Estimation of Plasma Corticosterone (CORT)

The plasma corticosterone was quantified in a High Performance Liquid Chromatography (HPLC) with Ultraviolet (UV) detector system (Waters, USA), according to Woodward and Emery ([22]) with minor modifications using dexamethasone as an internal standard. Briefly, 500 lL of plasma containing known quantity of dexamethasone was extracted with 5 mL of dichloromethane. The dichloromethane extract was evaporated to dryness and dissolved in 100 lL of mobile phase. Twenty microliter of extract was injected into HPLC system for quantification. Mobile phase consisted of methanol:water (70:30) at a flow rate of 1.2 mL/min and CORT was detected at 250 nm using UV detector (Model 2849, Waters, USA). The chromatogram was recorded and analyzed with Empower software.

Estimation of Plasma Norepinephrine (NE)

The plasma NE was quantified in an HPLC with Electrochemical detector (ECD) system (Waters, USA; [23]). Blood samples were collected in heparinized eppendorf tube by retro-orbital puncture and were centrifuged for 10 min at 1,5479*g* (Biofuge Stratos, Heaureas, Germany) at 10°C, and were separated in two aliquots and frozen at −70°C before analysis. 500 lL of plasma sample was first washed by hexane to remove lipids. Proteins were precipitated with sulfosalicylic acid (10 g/100 mL) and 0.1 mL of internal standard 3, 4-dihydroxybenzylamine (DHBA) was added. After centrifugation, the supernatant was washed with ethylacetate saturated by sodium chloride. The ethylacetate phase containing NE was evaporated to dryness at 37°C under a stream of dry nitrogen and frozen at −24°C until analysis. For NE analysis, the residue was reconstituted in 0.1 mL of mobile phase and twenty microliter was injected via HPLC pump (Model 515, isocratic pump, Waters, Milford, MA, USA) into a column (Spherisorb, RP C18, 5 lm particle size, 4.6 mm i.d. 9 250 mm at 30°C) connected to a ECD (Model 2465, Waters, Milford, MA, USA) at a potential of ?0.8 V with glassy carbon working electrode Vs Ag/AgCl reference electrode. The mobile phase consists of 0.1 M sodium acetate, 0.02 M citric acid, 0.4 mM sodium octyl sulfonate, 0.2 mM EDTA Na_2. The pH of the buffer running solution was adjusted to 4.92 then filtered through a 0.45 lm filter (Millipore, Bedford, MA, USA). Methanol was added to give a final composition of 4.5% methanol (v/v). A flow rate of 0.8 mL/min was used. The chromatogram was recorded and analyzed with Empower software.

Estimation of Serotonin, Dopamine and their Metabolites

The brains were removed after decapitation and microdissected according to Palkovits and Brownstein [24] as soon as possible on glass plates over ice into three regions: the total hippocampus (HIP), prefrontal cortex (PFC) and striatum (STR). The level of 5-HT, DA and their metabolites were estimated using HPLC/ECD as described by Kim et al. ([25]). In brief, the brain tissue samples were homogenized in 0.17 M perchloric acid by Polytron homogenizer. Homogenates were then centrifuged at 33,0009*g* (Biofuge Stratos, Heaureas, Germany) at 4°C. Twenty microliter of supernatant was injected via HPLC pump (Model 515, isocratic pump, Waters, Milford, MA, USA) into a column (Spherisorb, RP C18, 5 lm particle size, 4.6 mm i.d. 9 250 mm at 30°C) connected to a ECD (Model 2465, Waters, Milford, MA, USA) at a potential of ?0.8 V with glassy carbon working electrode Vs Ag/AgCl reference electrode. Mobile phase consisted of 32 mM citric acid, 12.5 mM disodium hydrogen orthophosphate, 1.4 mM sodium octyl sulfonate, 0.05 mM EDTA and 16% (v/v) methanol (pH 4.2) at a flow rate of 1.2 mL/min. Quantification was made by comparing peak heights of the samples to the corresponding standard curve. Two ranges of standard curves, i.e. 10–100 and 100–1,000 ng/mL were used depending upon the abundance of monoamines in respective brain regions. The constant amount (25 ng/mL) of DHBA added to the tissue samples was used to calculate recovery. The protein content was estimated using the method of Lowry et al. [26].

Statistical Analysis

The results are expressed as mean ± S.E.M. The statistical significance was determined by One-Way Analysis of Variance (ANOVA) followed by *Post-hoc* Student–Newman–Keuls test. $p < 0.05$ was considered to be statistically significant.

Results

Repeated Low Dose Risperidone Decreases Stress Induced by Cold-Restraint

The effect of repeated (0.1 and 1.0 mg/kg) treatment of RIS on stress in terms of ulcer index is illustrated in Fig. 1. Statistical analysis by One-way ANOVA revealed that

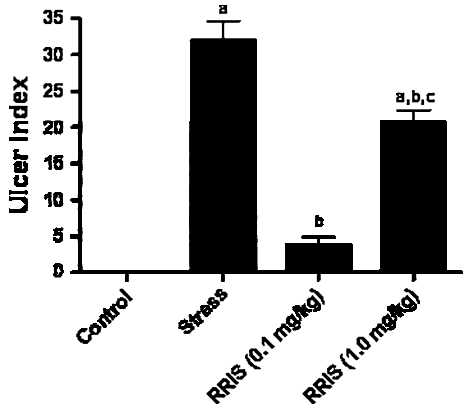

Fig. 1 The effect of repeated treatment of RIS (0.1 and 1.0 mg/kg) on ulcer index. All values are Mean ± SEM. *a* $p < 0.05$ compared to control, *b* $p < 0.05$ compared to stress, *c* $p < 0.05$ compared to RRIS (0.1 mg/kg) [One-way ANOVA followed by Student Newmann keuls test]

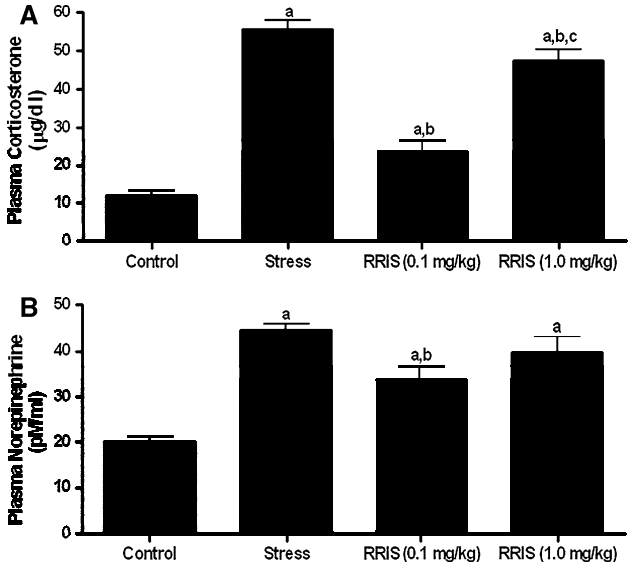

Fig. 2 The effect of repeated treatment of RIS (0.1 mg/kg and 1.0 mg/kg) on plasma corticosterone (a) and norepinephrine (b) levels. All values are Mean ± SEM. *a* $p < 0.05$ compared to control, *b* $p < 0.05$ compared to stress, *c* $p < 0.05$ compared to RRIS (0.1 mg/kg). [One-way ANOVA followed by Student Newmann keuls test]

there was significant interaction among groups [$F(3, 20) = 81.72$, $p < 0.05$]. *Post-hoc* analysis showed that stress significantly increased the ulcer index compared to control. Repeated ultra low dose of RRIS (0.1 mg/kg) more robustly reduced ulcer index compared with the high dose of RRIS (1.0 mg/kg). This shows that there is a dose-specific effect of repeated treatment of RIS in stress.

Risperidone Alters Plasma CORT and NE in Stressed Animals

The effect of RIS (0.1 mg/kg and 1.0 mg/kg) on plasma CORT is depicted in Fig. 2a and NE in Fig. 2b. Statistical analysis showed that there was significant difference in the concentration of plasma CORT [F (3, 20) = 68.26, $p < 0.05$] and NE [F (3, 20) = 20.76, $p < 0.05$] among groups. *Post-hoc* analysis by Student-Newmann keuls test revealed that stress significantly increased plasma CORT and NE levels. Repeated RIS treatment (RRIS; 0.1 and 1.0 mg/kg) significantly reversed plasma CORT and NE levels compared to stress. Comparison of doses showed that RRIS; 0.1 mg/kg more significantly reversed stress-induced increase in plasma CORT than repeated high dose RRIS; 1.0 mg/kg. However, in contrast to the low dose, RRIS; 1.0 mg/kg did not show any effect on stress-induced changes in plasma NE levels.

RIS Selectively Alters Level of 5-HT and its Metabolite in Hippocampus, Prefrontal Cortex and Striatum

The effect of repeated administration of RIS (0.1 and 1.0 mg/kg) on 5-HT level in different brain regions in stress is illustrated in Fig. 3a. Analysis by one-way ANOVA showed that there was significant differences among groups in the 5-HT level in HIP [F (3, 20) = 38.72, $p < 0.05$], PFC [F (3, 20) = 32.28, $p < 0.05$] and STR [F (3, 20) = 7.009, $p < 0.05$]. *Post-hoc* analysis by Student Newmann keuls test showed that stress significantly increased 5-HT level in HIP and PFC, but not in STR compared to control. Repeated low dose RIS (RRIS; 0.1 mg/kg) further augmented the 5-HT level in HIP, PFC and STR compared to stress. However, there was no significant change with high dose (RRIS; 1.0 mg/kg) on hippocampal 5-HT level compared to stress.

The effect on 5-HIAA level in different brain regions of repeatedly administered RIS (0.1 and 1.0 mg/kg) rats is depicted in Fig. 3b. One-way ANOVA revealed that among groups, there was significant differences in the 5-HIAA level in HIP [F (3, 20) = 17.84, $p < 0.05$], PFC [$F(3, 20) = 56.52$, $p < 0.05$] and STR [$F(3, 20) = 19.72$, $p < 0.05$]. *Post-hoc* analysis showed that stress decreased 5-HIAA level in HIP and increased in PFC, but however there was no significant change in STR. Repeated treatment (RRIS; 0.1 and 1.0 mg/kg) further augmented the 5-HIAA level in HIP, PFC and STR compared to stress. Similar to the effect on 5-HT level, there were dose differences in 5-HIAA level between repeatedly treated animals. 5-HIAA level in HIP, PFC and STR was statistically lower in repeated high dose compared to low dose treatment. It is interesting to note that the profile of 5-HIAA level in stress and treatment mimic the changes observed in 5-HT level.

Figure 3c represents the effect of repeated treated of RIS (0.1 and 1.0 mg/kg) on 5-HIAA/5-HT ratios in different brain regions. Significant interaction among groups with respect to 5-HIAA/5-HT ratios in HIP [F (3, 20) = 35.80,

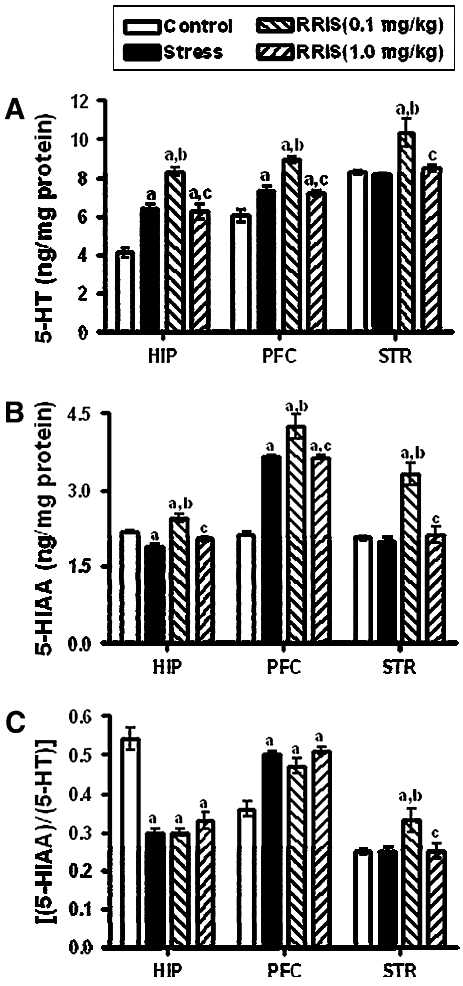

Fig. 3 The effect of repeated treatment of RIS (0.1 mg/kg and 1.0 mg/kg) on levels of 5-HT (a) and 5-HIAA (b), and 5-HIAA/5-HT ratios (c) in HIP, PFC and STR. All values are Mean ± SEM. *a* $p < 0.05$ compared to control, *b* $p < 0.05$ compared to stress and *c* $p < 0.05$ compared to RRIS (0.1 mg/kg) [One-way ANOVA followed by Student Newmann keuls test]

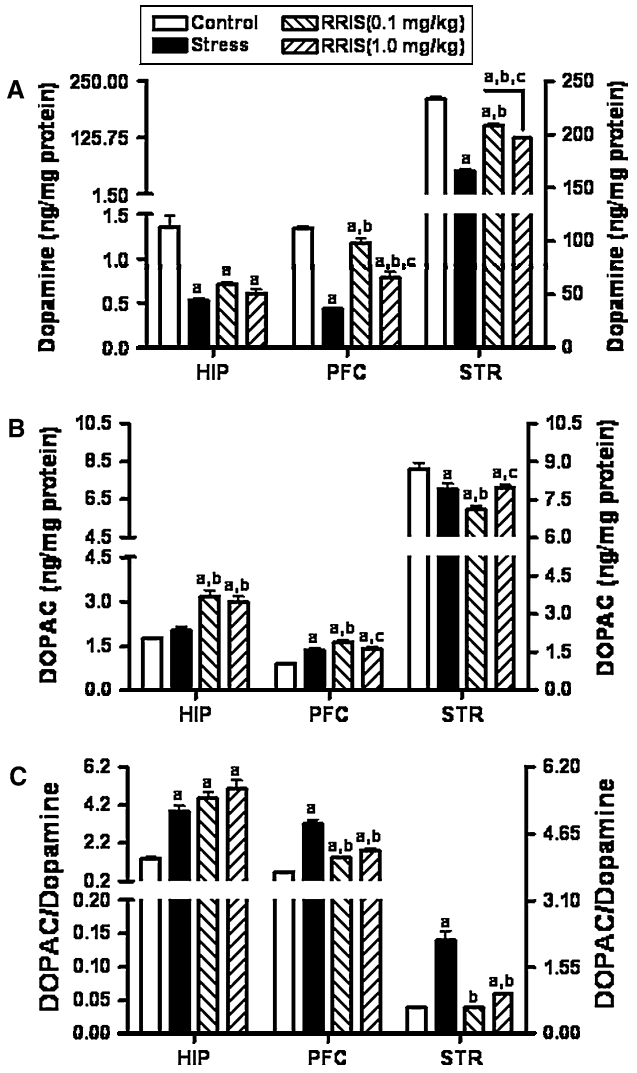

Fig. 4 The effect of repeated treatment of RIS (0.1 mg/kg and 1.0 mg/kg) on Dopamine (a), and DOPAC (b) levels, and DOPAC/Dopamine ratios (c) in HIP, PFC and STR. All values are Mean ± SEM. *a* $p < 0.05$ compared to control, *b* $p < 0.05$ compared to stress and *c* $p < 0.05$ compared to RRIS (0.1 mg/kg) [One-way ANOVA followed by Student Newmann keuls test]

$p < 0.05$], PFC [F (3, 20) = 18.93, $p < 0.05$] and STR [F (3, 20) = 4.354, $p < 0.05$] were observed by statistical analysis. Further, *Post-hoc* analysis showed that the 5-HIAA/5-HT ratios decreased in HIP, increased in PFC and no statistical change was observed in STR with CRS compared to control. Repeated drug treatment did not significantly alter stress-induced 5-HIAA/5-HT ratios in HIP and PFC. However, repeated low dose (0.1 mg/kg) significantly increased striatal 5-HIAA/5-HT ratios compared to stress.

RIS Selectively Alters Level of DA and its Metabolite in Hippocampus, Prefrontal Cortex and Striatum

The effect of repeated treatment of RIS (0.1 and 1.0 mg/kg) on DA level in different brain regions in stress is depicted in Fig. 4a. Analysis of data by One-way ANOVA showed that there was significant differences in the DA level in HIP [F (3, 20) = 28.29, $p < 0.05$], PFC [F (3, 20) = 79.85, $p < 0.05$] and STR [F (3, 20) = 204.1, $p < 0.05$] among groups. Further, *post-hoc* analysis showed that stress significantly decreased DA level in HIP, PFC and STR compared to control. Repeatedly RIS (RRIS; 0.1 and 1.0 mg/kg) reversed stress-induced decrease in DA level in PFC and STR. However, the increase in DA level in PFC and STR with repeated low dose (RRIS; 0.1 mg/kg) was significantly higher compared to repeated high dose.

The effect on DOPAC level in different brain regions of repeatedly administered RIS (0.1 and 1.0 mg/kg) in stress is depicted in Fig. 4b. One-way ANOVA revealed that

there were significant differences among groups in the DOPAC level in HIP [$F(3, 20) = 20.37, p < 0.05$], PFC [$F(3, 20) = 17.38, p < 0.05$] and STR [$F(3, 20) = 12.73, p < 0.05$]. *Post-hoc* analysis showed that stress did not change DOPAC level in HIP, but increased in PFC and decreased in STR. However, repeated dose (RRIS; 0.1 and 1.0 mg/kg) treatments altered hippocampal DOPAC level compared to control and stress, but there was no significant difference between treatment schedules. Repeated low dose treatment (RRIS; 0.1 mg/kg) further augmented and decreased DOPAC level in PFC and STR respectively compared to stress. The DOPAC level in PFC and STR was significantly higher and lower in repeated low dose compared to repeated high dose respectively.

The effect of repeated treatment of RIS (0.1 and 1.0 mg/kg) on DOPAC/DA ratios in different brain regions in stress is illustrated in Fig. 4c. Effect of RIS on hippocampal and prefrontal cortical DOPAC/DA ratios are shown in panel A and striatal DOPAC/DA ratios is shown in panel B. Statistical analysis by One-way ANOVA showed that there was a significant interaction of treatment with respect to DOPAC/DA ratios in HIP [$F(3, 20) = 23.01, p < 0.05$], PFC [$F(3, 20) = 52.59, p < 0.05$] and STR [$F(3, 20) = 38.26, p < 0.05$] among groups. *Post-hoc* analysis test indicated that stress significantly increased DOPAC/DA ratios in HIP, PFC and STR. Stress-induced hippocampal DOPAC/DA ratios were not altered by repeated low doses (RRIS; 0.1 and 1.0 mg/kg) of RIS. However, in PFC and STR, repeated doses (RRIS; 0.1 and 1.0 mg/kg) of RIS significantly decreased DOPAC/DA ratios compared to stress. Furthermore, there were no dose differences in DOPAC/DA ratios between repeatedly administered animals.

Effect of Risperidone on Catalepsy Behaviour in Bar Test

Statistical analysis by One-way ANOVA revealed that there was no significant difference in cataleptic behaviour among groups [$F(3, 20) = 2.15, p < 0.05$] treatment. Hence, stress and RRIS (0.1 and 1.0 mg/kg) treatment did not significantly alter the catalepsy parameter compared to control (Fig. 5).

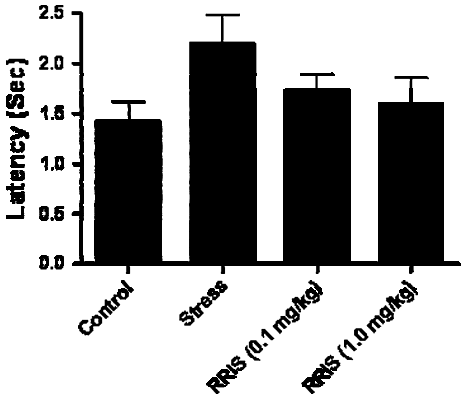

Fig. 5 The effect of stress and repeated treatment of RIS (0.1 mg/kg and 1.0 mg/kg) on catalepsy behaviour of rats in the bar test. All values are Mean ± SEM. [One-way ANOVA]

Discussion

The objective of the present investigation was to evaluate the anti-stress activity of RIS in the CRS model and associated stress pathways. Interestingly, the repeated ultra low dose RIS (0.1 mg/kg) treatment modulated the stress-induced changes in HPA axis and the SNS compared to the corresponding high dose (1.0 mg/kg). The effective anti-stress dose of RIS in the current study is several times lower than its anti-psychotic dose. Further, both doses of RIS showed no catalepsy behaviour, indicating absence of extra pyramidal side effect. Therefore, the present observations point to potential use of ULD in stress disorders.

RIS significantly decreased the number and severity of ulcers as reported earlier [17]. It has been previously demonstrated that simultaneous cold-exposure and restraint stress causes pronounced gastric ulceration suggesting the involvement of stress in the etiology of gastric ulcer formation [27, 28]. The regulatory role of the central nervous system in various functions of the gastrointestinal tract has been well documented. The two important pathways involved in stress response to maintain physiological homeostasis are the HPA axis and the SNS, both of which are markedly activated by stressors [29]. The activation of HPA and SNS leads to increase in plasma CORT and NE levels respectively. As per previous published results, CRS increased plasma CORT and NE levels [30, 31] indicating activation of the HPA axis and the SNS. NE binds to various adrenoreceptors in multiple target organs and thus plays multiple roles in fight/flight reactions [32]. Although NE does not cross the blood brain barrier, the peripheral actions of these catecholamines cause increase in brain NE through the activation of the locus coeruleus. Further, it has been reported that locus coeruleus has direct influence on hypothalamus, finally secreting CORT [33]. On the other hand CRH can activate NE release at different levels forming a feed forward cycle. This feed forward cycle is activated in response to biological stressors and derangement in its function would lead to the collapse of the stress response [34]. It has been reported that stress ulcer could be induced by activation of pituitary adrenal axis leading to increase in levels of serum CORT [27]. Inhibition of serum CORT levels may consequently reduce the incidence of stress ulcer [35]. Repeated ultra low dose of RIS decreased

stress-induced increase in plasma CORT as well as NE indicating its modulating effect on both the HPA and SNS systems during stress. Hence, it can be assumed that RIS by decreasing CORT and NE can break the vicious cycle of stress-induced feed-forward interactions between HPA axis and the SNS. However, this has to be verified by further studies on the effect of RIS on the different components of the HPA axis.

The responses of HPA axis to stress are regulated by higher centers such as HIP and PFC through several transmitter systems [36, 37]. A variety of neurotransmitter systems appear to be involved in the pathogenesis of stress-induced gastric mucosal injury, including DA, NE and 5-HT systems [27]. It is reported that simultaneous cold-exposure and restraint stress procedure mobilizes monoaminergic systems in areas of brain connected with behavioural responses to aversive stimuli [38].

One of the major neurotransmitters regulating HPA axis in stress is the serotonergic system [39]. CRS caused region specific changes in 5-HT. It significantly increased 5-HT level in the HIP and PFC, but not in striatum (STR). Brain 5-HIAA decreases in CRS [40] and several stress stimuli lead to increase in brain 5-HT turnover [41]. In the present study, 5-HIAA level decreased in HIP while an increase in 5-HT level was observed. This translated into decrease in the ratio of 5-HIAA/5-HT indicating decreased turnover of 5-HT. However, in the PFC there was increase in 5-HIAA level with concomitant increase in 5-HT level. These changes led to increase in 5-HT turnover as observed from the increase 5-HIAA/5-HT ratio. However, in the STR there was no change in 5-HT and 5-HIAA. The 5-HIAA levels are probably a measure after metabolism by MAO and clearance from the brain across the blood–brain barrier by acid metabolite carriers [42]. RRIS (0.1 mg/kg) treatment further augmented the 5-HT levels in HIP, PFC and STR in contrast to the higher dose. Low dose RIS had no effect on 5-HT turnover except in the STR. The anti-stress effect may be due to augmentation of 5-HT levels as the antiserotoninergic agent methysergide caused gastric erosions in cold-restraint stress exposed rats [43]. Further, ketanserin, a 5-HT_{2A} antagonist dose-dependently attenuated the psychological stress gastric lesion formation [44]. Furthermore, the exact localization of 5-HT_{2A} receptors in HIP and PFC, and their interaction with RIS may further elucidate the appropriate mechanism behind the anti-stress effect of RIS in the CRS model. Interestingly, the changes in 5-HT with RIS treatment was observed only with ULD but the higher dose did not change the 5-HT levels in any of the three brain regions studied. Similarly, high dose did not reverse stress-induced increase in NE level. However, both ULD and high dose significantly reversed stress-induced increase in the plasma CORT level. This observation perhaps shows that the anti-stress effect by RIS in low dose is modulated by more than one stress response systems compared with the high dose. This probably accounts for more significant anti-stress effect with ULD compared to the high dose. However, this contention has to be supported by further experiments involving individual stress pathways.

Such region specific changes are reported not only for 5-HT, but also for DA in response to different stressors [41]. EPS effects are considered to be due to impaired neurotransmission in the nigrostriatal system [45]. Hence, most of the experiments related to EPS have been focused on the STR as it has profuse dopaminergic innervations and is also involved in motor coordination [46, 47]. It has been reported that stress induces the biggest turnover of DA in the PFC [48]. In stressed animals there was decrease in DA level in all the brain regions except in the HIP. The DOPAC level of stressed animals increased in PFC and decreased in STR and no change was observed in HIP. Although, there were differences in DA and DOPAC response to stress in different brain regions, there was a general increase in DA turnover in all the regions. These reports are similar to an earlier study, where DA turnover in stressed animals increased in all brain regions under investigation. CRS produced a decrease in DA and increase in DOPAC level in the PFC and decreased DA level in the STR [49, 50]. In the present experiment, RIS reversed the decreased DA levels in the PFC and STR of stressed animals, but DA levels were within the limits of control animals. Further, the stress-induced increase in DA turnover was reversed by RIS in PFC and STR and had no effect on HIP. We can assume this may be reason for absence of EPS in low doses of RIS [51]. According to Ichikawa et al. [52], subcutaneous treatment of RIS in doses of 0.01, 0.03, 0.1 and 1.0 mg/kg increased DA level in STR. Hence, there are regional differences in mechanisms leading to RIS-induced DA release.

Another important observation from this study is dose differences in the anti-stress effect of RIS. It is previously reported that RIS in median doses (0.1 and 0.3 mg/kg daily p.o. for 14 days) effectively reduced freezing behaviour, while lower and higher doses of 0.03 and 1.0 mg/kg respectively did not show any effect [13]. The effective dose in schizophrenia is considered to more than 2 mg/day [16]. RIS at the dose level of 0.25–3 mg/kg has more affinity for the 5-HT_{2A} receptor than D_2 receptor. This preferential occupation of 5-HT_{2A} over D_2 receptor becomes narrow as the dose is increased [51]. Low doses of RIS (0.3–1.0 mg/kg) occupy 50–80% of D_2 receptor, while doses more than 2.0 mg/kg occupy more than 80% but can also produce catalepsy [53]. Preclinical study also reveal that RIS (2 mg/kg) showed significant neutropenia compared to RIS (1 mg/kg) and hyperprolactinaemia dose-dependently in mice neonates [54]. Therefore, it can be

assumed that doses about 1.0 mg/kg of RIS in rats may correspond to the clinically comparable dose range in patients. Both children and adults are at the risk to develop common dose-dependent adverse effects such as EPS and hyperprolactinemia [55]. In the current study, both the doses of RRIS did not show catalepsy behaviour. In that respect using low doses of RIS may have some therapeutic advantages with respect to EPS and other metabolic disorders related to dopaminergic transmission. Hence, ULD may offer safer therapeutic options with RIS in the treatment of stress-related disorders.

In summary, repeated administration of repeated ultra low dose RIS (0.1 mg/kg) for 21 days significantly mitigated stress in the CRS model. Repeated ultra low dose modulated stress-induced changes in HPA axis, SNS and, brain serotonergic and dopaminergic systems. Hence, the anti-stress effect of RIS may involve the modulation of the above stress pathways. Further, the ULD of RIS did not show any catalepsy behaviour, indicating absence of EPS. If the current experimental evidence of anti-stress effect of RIS in ULD is successfully translated into clinical practice, it may provide a safe and effective option to treat stress disorders.

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