# *Evaluation of anti-stress effect of risperidone against cold restraint stress in rats*

## 3.1 Introduction

Risperidone (RIS), an atypical-antipsychotic, is a highly selective 5-HT2A antagonist. Risperidone was approved for the treatment of schizophrenia, bipolar disorder (Javanbakht 2006) and irritability associated with autism.

Clinical studies show RIS to be successful in therapy for stress disorders (Kozaric et al., 2005; Padala et al., 2006; Meighen et al., 2007). It has also been suggested that these classes of antipsychotics may be effective for the treatment of mood disorders such as anxiety and depressive mood associated with schizophrenia (Ishida- Tokuda et al., 1996). However, a lack of sufficient experimental data provokes the use of RIS. RIS in high doses produces a number of side effects (Olgun et al., 2009; Byrne et al., 2010). 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 (Li, Xia et al. 2012). 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 the treatment of stress disorders. We have shown that repeated RIS treatment had a significant gastroprotective effect in the cold restraint stress (CRS) model by modulating the local gastric parameters (Saxena et al., 2011). Since the demonstration of stress-induced ulceration (Selye 1936). variations of the model have been widely used to study neuroprotectant activity (Garabadu et al., 2011). 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 indoleacetic acid (5-HIAA), and dopamine (DA) and its metabolite; 3, 4 dihydroxy phenylacetic acid (DOPAC) in discrete stress-sensitive brain regions such as HIP, PFC and, striatum (STR). Further, catalepsy was evaluated as an index of an extrapyramidal side (EPS) effect. This study underscores the potential use of the repeated ultra-low dose of RIS (ULD) in stress disorders.

## 3.2 Materials and Methods

### *3.2.1 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 the Institutional animal ethical committee, Banaras Hindu University. Male adult Charles Foster strain albino rats, three 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 a temperature of 25±1$^0$C and 45-55% RH and a 12:12 hr light/dark cycle. The experimental animals had free access to commercial rat feed (Doodh Dhara Pashu Ahar, India) and water *ad libitum* during the experiment. Animals were acclimatized for at least one week before experiments.

### *3.2.2 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.

### *3.2.3 Cold Restraint Stress (CRS) model*

After 18 hr fasting, one stress session was performed during the early phase of the light cycle and consisting of a 2 hr restraint period (rat restrainers were metallic cages of 15 cm long × 6.5 cm wide) at 4-6° C. The cervical dislocation method was used to kill the animals. 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 (Sairam et al., 2003).

### *3.2.4 Experimental protocol*

Animals were randomly divided into four groups of six animals each. Rats received repeated treatment of RIS in a 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 previously published observations (Ishida-Tokuda et al., 1996). In the above-referred study, the repeated dose of RIS (0.1 mg) was more effective in decreasing conditioned fear-induced anxiety than the corresponding higher doses (1mg/kg). In the current experiment, the sham and RIS-treated animals were subjected to a two-hour CRS procedure on day 21 after one hour of vehicle or drug administration. The animals were then transferred to the home cage. After 30 minutes of CRS, animals were subjected to the bar test and were killed immediately, followed by microdissection and estimation of ulcer index.

### *3.2.5 Evaluation of catalepsy behaviour in the bar test*

Catalepsy, defined as the acceptance and retention of abnormal posture, was measured using 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 the 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

the 21st day (Karl et al., 2006).

### *3.2.6 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 (Sairam et al., 2003).

### *3.2.7 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 (Woodward and Emery 1987), with minor modifications using dexamethasone as an internal standard. Briefly, 500µ L of plasma containing a known quantity of dexamethasone was extracted with 5mL of dichloromethane. The dichloromethane extract was evaporated to dryness and dissolved in 100µ L of the mobile phase. Twenty microliters of the extract were injected into the HPLC system for quantification. The 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 a UV detector (Model 2849, Waters, USA). Empower software was used to analyze chromatograms.

### *3.2.8 Estimation of Plasma Norepinephrine (NE)*

The plasma NE was quantified in an HPLC with an electrochemical detector (ECD) system (Sastre et al., 2004). Blood samples were collected in a heparinized Eppendorf tube by retro-orbital puncture and were centrifuged for 10 min at 1547*g* (Biofuge Stratos, Heaureas, Germany) at 10°C, and were separated in two aliquots and frozen at −80 $^0$C before analysis. 500 µL 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 ethyl acetate saturated with sodium chloride. The ethyl acetate phase containing NE was evaporated to dryness at 37 $^0$C under a stream of dry nitrogen and frozen at -24$^0$C until

analysis. For NE analysis, the residue was reconstituted in 0.1 ml of mobile phase and twenty microliters were injected via HPLC pump (Model 515, isocratic pump, Waters, Milford, MA, USA) into a column (Spherisorb, RP C18, 5 µm particle size, 4.6mm i.d.×250mm at $30^0$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. The mobile phase consists of 0.1M sodium acetate, 0.02M citric acid, 0.4mM sodium octyl sulfonate, 0.2mM EDTA $Na_2$. The pH of the buffer running solution was adjusted to 4.92, then filtered through a 0.45 µm 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.

### *3.2.9 Estimation of serotonin, dopamine and their metabolites*

The brains were removed after decapitation and microdissected (Palkovits and Brownstein 1988), 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 (Kim et al., 1987). In brief, the brain tissue samples were homogenized in 0.17M perchloric acid by a Polytron homogenizer. Homogenates were then centrifuged at 33,000×*g* (Biofuge Stratos, Heaureas, Germany) at $4^0$C. Twenty microliters of supernatant was injected via HPLC pump (Model 515, isocratic pump, Waters, Milford, MA, USA) into a column (Spherisorb, RP C18, 5 µm particle size, 4.6mm i.d.×250mm at $30^0$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 1.2 mL/min. Quantification was made by comparing the peak heights of the samples to the corresponding standard curve. Two ranges of standard curves, i.e., 10–100

and 100–1000 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., 1951).

### *3.2.10 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 the post-hoc Student Newman-keuls test. $P < 0.05$ was considered to be statistically significant.

## 3.3. Results

### *3.3.1 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-3.1).

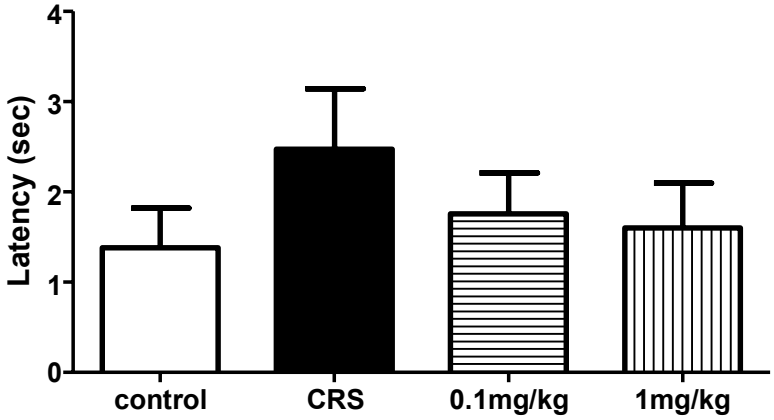


**Fig-3.1.** The effect of repeated treatment of RIS (0.1 and 1.0) on stress-induced changes in catalepsy behaviour of rats in the bar test. All the values are Mean ± SEM with n=5 [One-way ANOVA followed by Student Newman-keuls test].

### *3.3.2. Repeated low dose risperidone decreases Ulcer index due to CRS*

The effect of repeated (0.1 and 1.0 mg/kg) treatment of RIS on stress in terms of ulcer index, as illustrated in Fig-3.2. Statistical analysis by One-way ANOVA revealed that 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. The repeated ultra-low dose of RRIS (0.1 mg/kg) more robustly reduced ulcer index compared with the high dose of RIS (1.0 mg/kg). This shows that there is a dose-specific effect of repeated treatment of RIS in stress.

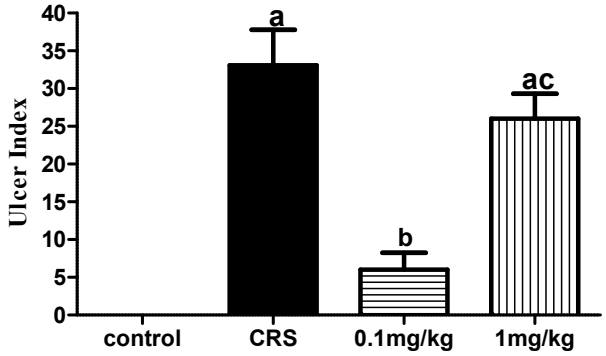


**Fig. 3.2.** The effect of repeated (0.1 and 1.0 mg/kg) treatment of RIS on the ulcer index. All values are Mean ± SEM. $^{a}p<0.05$ compared to control, $^{b}p<0.05$ compared to stress, $^{c}p<0.05$ compared to RIS (0.1 mg/kg) [One-way ANOVA followed by Student Newman-keuls test].

### *3.3.3. 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-3.2 (A) and NE in Fig-2 (B). Statistical analysis showed that there was a 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 the Student-Newman-keuls test revealed that stress significantly increased plasma CORT and NE levels. Repeated RIS treatment (RRIS; 0.1 mg/kg and 1.0 mg/kg) significantly reversed plasma CORT and NE levels compared to

stress. Comparison of dose showed that RRIS; 0.1 mg/kg more significantly reversed the 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.

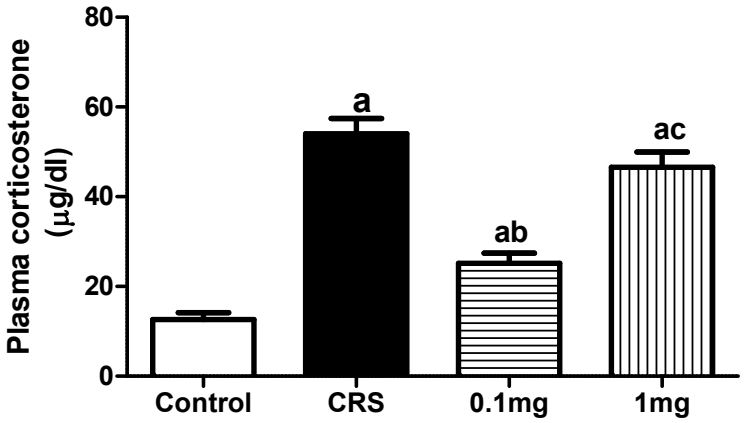


**Fig-3.3.** The effect of repeated (0.1 mg/kg and 1.0 mg/kg) treatment of RIS on plasma corticosterone. All values are Mean ± SEM. [a]p<0.05 compared to control, [b]p<0.05 compared to stress, [c]p<0.05 compared to RIS (0.1 mg/kg). [One-way ANOVA followed by Student Newman-keuls test].

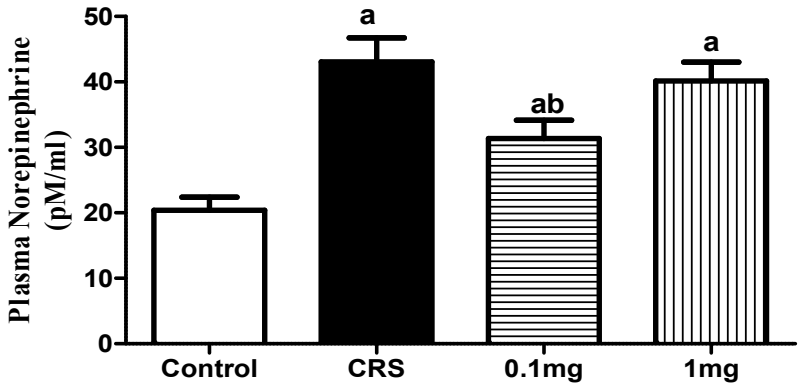


**Fig-3.4.** The effect of repeated (0.1 mg/kg and 1.0 mg/kg) treatment of RIS on norepinephrine levels. All values are Mean ± SEM. [a]p<0.05 compared to control, [b]p<0.05 compared to stress. [One-way ANOVA followed by Student Newman-keuls test].

### *3.3.4 RIS selectively alters the level of 5-HT and its metabolite in the hippocampus, prefrontal cortex, and striatum*

The effect of repeated administration of RIS (0.1 and 1.0 mg/kg) on the 5-HT level in different brain regions in stress is illustrated in Fig-3.5 (A). 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 Newman-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 the HIP, PFC and STR compared to stress. However, there was no significant change with high dose (RRIS; 1.0 mg/kg) on the hippocampal 5-HT level compared to stress.

The effect on the 5-HIAA level in different brain regions of repeatedly administered RIS (0.1 and 1.0 mg/kg) rats is depicted in Fig-3.5 (B). 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 the 5-HIAA level in the HIP and increased in PFC, but there was no significant change in STR. Repeated treatment (RRIS; 0.1 mg/kg and 1.0 mg/kg) further augmented the 5-HIAA level in the HIP, PFC and STR compared to stress. Similar to the effect on the 5-HT level, there were dose differences in the 5-HIAA level between repeatedly treated animals. 5-HIAA levels in the HIP, PFC and STR were statistically lower in repeated high dose compared to low dose treatment. It is interesting to note that the profile of the 5-HIAA level in stress and treatment mimics the changes observed in the 5-HT level.

Fig-3.5 (C) 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, $p<0.05$], 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 the 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 treatment (0.1 mg/kg) significantly increased striatal 5-HIAA/5-HT ratios compared to stress.

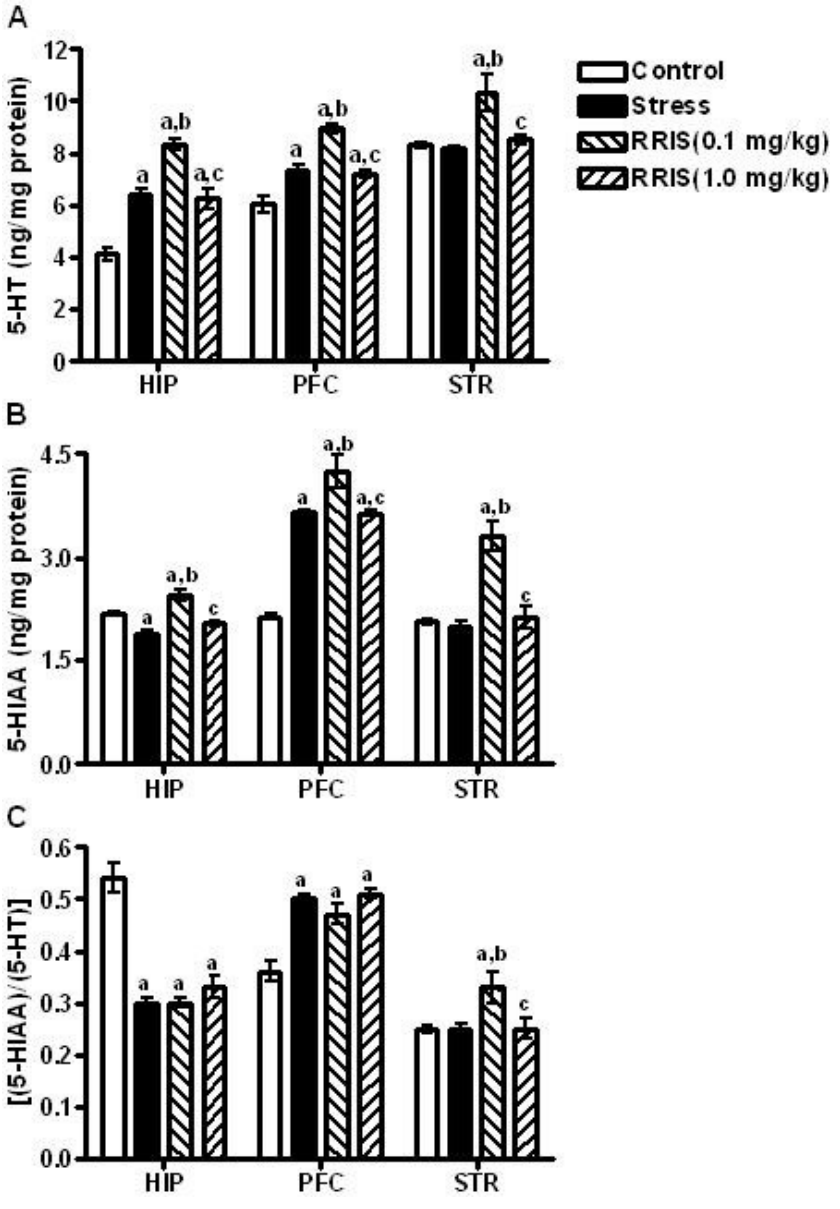


**Fig-3.5.** Depicts the effect in 5-HT (**A**), 5-HIAA (**B**) level and 5-HIAA/5-HT (**C**) ratios in the HIP, PFC and STR after stress and treatment with repeated (0.1 mg/kg and 1.0 mg/kg) doses of RIS in rats. All values are Mean ± SEM. [a] $p<0.05$ compared to control, [b] $p<0.05$ compared to the stress and [c] $p<0.05$ compared to RIS (0.1 mg/kg) [One-way ANOVA followed by Student Newman-keuls test].

### *3.3.5 RIS selectively alters the level of DA and its metabolite in the hippocampus, prefrontal cortex, and striatum*

The effect of repeated treatment of RIS (0.1 and 1.0 mg/kg) on DA levels in different brain regions in stress is depicted in Fig-3.6 (A). 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 the HIP, PFC and STR compared to control. Repeatedly RIS (RRIS; 0.1 mg/kg and 1.0 mg/kg) reversed the 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 doses. The effect on the DOPAC level in different brain regions of repeatedly administered RIS (0.1 and 1.0 mg/kg) in stress is depicted in Fig-3.6 (B). 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 the DOPAC level in the HIP but increased in PFC and decreased in STR. However, repeated dose (RRIS; 0.1 mg/kg 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 doses compared to repeated high doses, 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-3.6 (C). Effect of RIS on hippocampal and prefrontal cortical DOPAC/DA ratios are shown in panel A and striatal DOPAC/DA ratios are 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 the HIP, PFC, and STR. Stress-induced hippocampal DOPAC/DA ratios were not altered by repeated low doses (RRIS; 0.1 mg/kg and 1.0 mg/kg) of RIS. However, in PFC and STR, repeated doses (RRIS; 0.1 mg/kg 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.

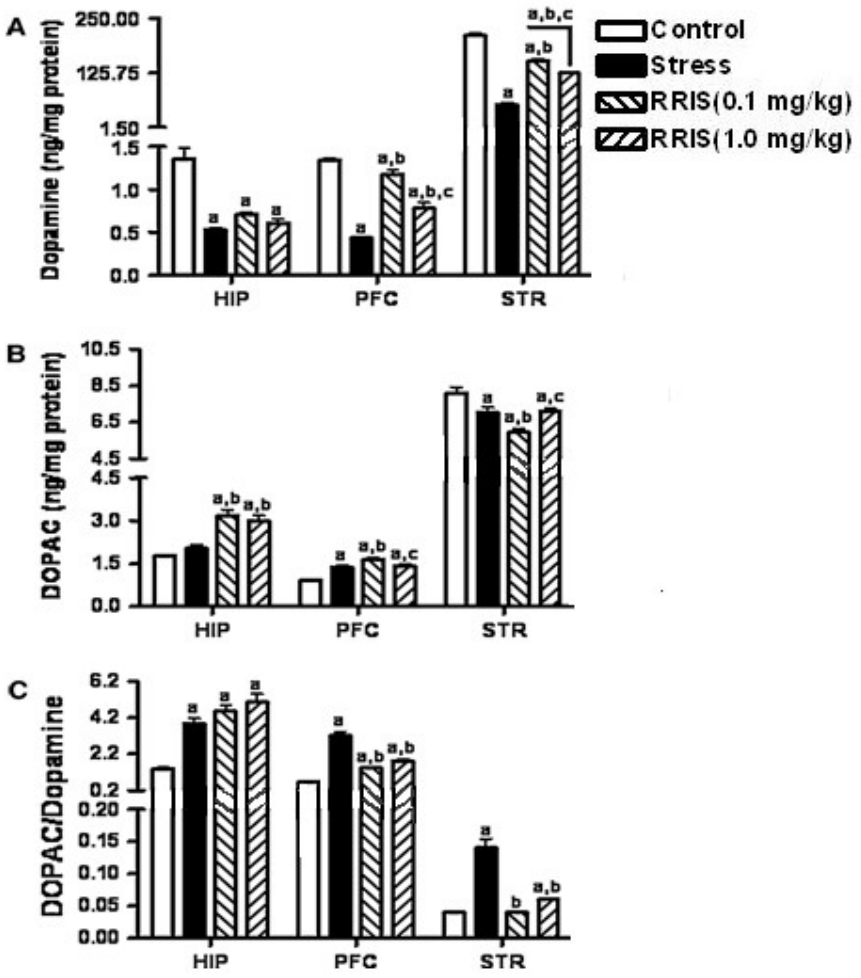


**Fig-3.6** The effect in Dopamine (A), DOPAC (B) level and DOPAC/Dopamine ratio (C) in the HIP, PFC and STR after stress and treatment with repeated (0.1 mg/kg and 1.0 mg/kg) doses of RIS in rats. All values are Mean ± SEM. $^{a}p<0.05$ compared to control, $^{b}p<0.05$ compared to the stress and $^{c}p<0.05$ compared to RRIS (0.1 mg/kg) [One-way ANOVA followed by Student Newman-keuls test].

### 3.4 Discussion

The current study was undertaken to investigate 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 the 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 antipsychotic dose. Further, both doses of RIS showed no catalepsy behaviour, indicating the absence of extrapyramidal side effects. Therefore, the present observations point to the potential use of ULD in stress disorders.

RIS significantly decreased the number and severity of ulcers, as reported earlier (Saxena et al., 2011). 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 (Bhargava et al., 1980; Soll 1990). The regulatory role of the central nervous system in various functions of the gastrointestinal tract has been well documented. The two crucial pathways involved in stress response to maintain physiological homeostasis are the HPA axis and the SNS, both of which are markedly activated by stressors (Kvetnansky et al., 1995). The activation of HPA and SNS leads to an increase in plasma CORT and NE levels, respectively. As per previously published results, CRS increased plasma CORT and NE levels (Kvetnansky et al., 1993; Retana-Marquez et al., 1996), 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 (Tsapatsaris and Breslin 1989). Although NE does not cross the blood-brain barrier, the peripheral actions of these catecholamines cause an increase in brain NE through the activation of the locus coeruleus. Further, it has been reported that locus coeruleus has a direct influence on the hypothalamus, finally secreting CORT (Morilak et al., 2005). 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 (Koob 1999). It has been reported that stress ulcers could be induced by activation of the pituitary-adrenal axis leading to increases in levels of serum CORT (Bhargava et al., 1980). Inhibition of serum CORT levels may consequently reduce the incidence of stress ulcers (Xu et al., 1995). The repeated ultra-low dose of RIS decreased the 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 SNS and the HPA axis. 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 the HPA axis to stress are regulated by higher centres such as HIP and PFC through several transmitter systems (Herman and Cullinan 1997; Sheikh et al., 2007). 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 (Bhargava et al., 1980). It is reported that simultaneous cold-exposure and restraint stress procedure mobilizes monoaminergic systems in areas of the brain connected with behavioral responses to aversive stimuli (Tache et al., 2001).

One of the significant neurotransmitters regulating the HPA axis in stress is the serotonergic system (Feldman et al., 1987). CRS caused region-specific changes in 5-HT. It significantly increased the 5-HT level in the HIP and PFC but not in the striatum (STR). Brain 5-HIAA decreases in CRS (Oxenkrug and Requintina 1998), and several stress stimuli lead to an increase in brain 5-HT turnover (Inoue et al., 1994). In the present study, the 5-HIAA level decreased in the HIP while an increase in the 5-HT level was observed. This translated into a decrease in the ratio of 5-HIAA/5-HT, indicating a decreased turnover of 5-

HT. However, in the PFC, there was an increase in the 5-HIAA level with a concomitant increase in the 5-HT level. These changes led to an increase in 5-HT turnover, as observed from the increased 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 (Cumming et al., 1992). RRIS (0.1 mg/kg) treatment further augmented the 5-HT levels in the 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 the augmentation of 5-HT levels as the antiserotoninergic agent methysergide caused gastric erosions in cold-restraint stress-exposed rats (Beattie 1977).

Further, ketanserin, a $5\text{-HT}_{2A}$ antagonist, dose-dependently attenuated the psychological stress gastric lesion formation (Nomura et al., 1994). Also, the knowledge of the exact localization of $5\text{-HT}_{2A}$ 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 were observed only with ULD, but a higher dose did not change the 5-HT levels in any of the three brain regions studied. Similarly, a high dose did not reverse the stress-induced increase in the NE level. However, both ULD and high dose significantly reversed a stress-induced increase in the plasma CORT level. This observation perhaps shows that the anti-stress effect by RIS in a low dose is modulated by more than one stress response system compared with a high dose. This probably accounts for a 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 (Inoue et al., 1994). EPS effects are considered to be due to

impaired neurotransmission in the nigrostriatal system (Arnt 1998). 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 (Andreassen et al., 1998; Mitchell et al., 2002). It was reported that stress induces the biggest turnover of DA in the PFC (Thompson et al., 2004). In stressed animals, there was a decrease in DA levels 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 the 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 an increase in DOPAC level in the PFC and decreased DA level in the STR (Sudha and Pradhan 1995; Ichikawa et al., 2001). 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 the reason for the absence of EPS in low doses of RIS (Matsubara et al., 1993). Also, subcutaneous treatment of RIS in doses of 0.01, 0.03, 0.1 and 1.0 mg/kg increased DA level in STR (Ichikawa et al., 2000). 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 the freezing behaviour, while lower and higher doses of 0.03 and 1.0 mg/kg respectively did not show any effect (Ishida-Tokuda et al., 1996). The effective dose in schizophrenia is considered to be more than 2mg/day (Li et al., 2009). RIS at the dose level of 0.25-3 mg/kg has more affinity for the 5-$HT_{2A}$ receptor than the $D_2$

receptor. This preferential occupation of 5-$HT_{2A}$ over $D_2$ receptor becomes narrow as the dose is increased (Matsubara et al., 1993). Low doses of RIS (0.3-1.0 mg/kg) occupy 50%-80% of the $D_2$ receptor, while doses more than 2.0mg/kg occupy more than 80% but can also produce catalepsy (Kapur et al., 2003). The preclinical study also reveals that RIS (2 mg/kg) showed significant neutropenia compared to RIS (1 mg/kg) and hyperprolactinemia dose-dependently in mice neonates (Mishra and Mohanty 2010). Therefore, it can be assumed that doses of 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 of developing common dose-dependent adverse effects such as EPS and hyperprolactinemia (Tarsy et al., 2002). 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 the absence of EPS. If the current experimental evidence of the 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.