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Co-treatment of piracetam with risperidone rescued extinction deficits in experimental paradigms of post-traumatic stress disorder by restoring the physiological alterations in cortex and hippocampus

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ABSTRACT

Pharmacotherapy and cognitive behavioral therapy, both fail to treat post-traumatic stress disorder (PTSD) in a considerable number of populations. The persistence of traumatic memories and deficit in extinction contributes to the failure of exposure therapy in PTSD.

With the objective to enhance the outcomes of exposure therapy by targeting the extinction window using pharmacological agents in PTSD, the present study was aimed to explore the effect of piracetam, risperidone and their combinations in experimentally-induced PTSD-like phenotype in rats. Male SD rats were exposed to single prolonged stress model (SPS) for induction of PTSD-like behavioral changes. Piracetam, risperidone and their combination were used as therapeutic interventions while sertraline was used as a standard treatment for 14 days along with extinction training. Induction of PTSD-like behaviors were assessed in behavioral tests such as fear conditioning, elevated plus maze, social interaction test, and the marble burying test. Neurotransmitters (dopamine and serotonin and their metabolites), BDNF, proinflammatory cytokines (TNF-α, IL-6), caspase-3, and markers for oxidative stress were assessed in the hippocampus and cortex while corticosterone and nitrite levels were estimated in plasma. Our result indicated that the SPS paradigm efficiently induced PTSD-like phenotype in rats. Risperidone and piracetam were found to be effective alone, while their high dose combination, produced potentiating effect in reversing the extinction deficit, behavioral alterations, altered cortical and hippocampal BDNF, IL-6, TNF-α, caspase-3, oxidative stress markers, and neurotransmitter levels. Plasma corticosterone and nitrite levels were also found to be reversed in the combination treated groups. Our preliminary study suggests that piracetam, risperidone and their combination restored the physiological cascades in cortex and hippocampus along with successful suppression of fear memory and a symptom cluster of PTSD-like phenotype in rats. Hence they could be used as an effective adjunct to enhance the outcome of exposure therapy for the management of PTSD.

1. Introduction

PTSD is a psychopathological response that comes into the picture as a result of exposure to an extremely traumatic event. Its prevalence ranges from 0.5% to 14.5% worldwide (Kessler et al., 2017). Diagnostic and Statistical Manual of Mental Disorder's (DSM-5) fifth edition recommends the diagnostic standard of PTSD as "an individual presenting symptom cluster such as intrusive memory or re-experiencing the trauma, hyperarousal or heightened anxiety, avoidance of trauma associated stimuli and negative mood alteration after experiencing the trauma" (DSM 5, 2013). Current treatments for PTSD include both pharmacotherapy and psychotherapy such as exposure therapy or cognitive behavioral therapy (Abdallah et al., 2019; Asmundson et al., 2019; Kar, 2011). The selective serotonin reuptake inhibitors (SSRI) hold the first-line therapy for PTSD but these drugs are partially effective against this disorder (Mendlowicz, 2010). Exposure therapies are the gold standard for anxiety disorders but the higher possibility of failure has been reported with PTSD patient (Kar, 2011; Steenkamp et al., 2015). Exposure-based therapy mainly aims at replacing the trauma conditioned stimulus associations with new and more appropriate associations. Generalized extinction deficit in PTSD patients makes them resistant towards exposure-based therapies (Davis et al., 2006; Milad et al., 2008, 2006). Thus, regardless of the glorious therapeutic efficacy of exposure therapies the high non-response and dropout rates were reported with PTSD (Schottenbauer et al., 2008).

The traumatic event associated fear memories have a significant contribution to the development and progression of PTSD. PTSD patient feels frightened in a safe environment as the memories of the traumatic event are revisited. Renewal of fear memory activates the hippocampal neurons which are projecting towards the amygdala and provides contextual information (Jin and Maren, 2015) whereas, the cortical neurons regulates the output response of amygdala by top-down regulation (Bukalo et al., 2015). Hence it could be concluded that cortical and hippocampal neuronal input drives the amygdala to form, proceed

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and behave with regard to fear memory input and healthy interaction between cortico-hippocampal amygdala circuitry is essential for a successful extinction and recall of fear memory (Hobin et al., 2006; Milad et al., 2007; Rosas-Vidal et al., 2018, 2014). The amygdala which critically contributes in the processing of emotions and modulation of fear response seems to be highly reactive in patients with PTSD, as medial prefrontal cortex which exhibits inhibitory control over the stress and emotional response of the amygdala seems to be less responsive in PTSD individuals (Morgan et al., 1993).

This neurocircuitry involved in the normal processing of fear memories gets disturbed in PTSD thus making fear memory to remain dominate even after years of trauma. The disruption in multiple domains of emotional memory processing enhances the susceptibility to develop PTSD and further maintenance of symptoms (Blechert et al., 2007; Fani et al., 2012; McGlinchey et al., 2014). The extinction deficit and neuropsychological aberration in PTSD patients are further evident by structural and functional impairments in various brain regions majorly the prefrontal cortex (PFC), hippocampus, and amygdala (Pitman et al., 2012; Rauch et al., 2006). The low hippocampal volume leading to the disrupted functioning, impaired activation of the PFC resulting in impaired top-down control and hyperactive amygdala, are associated with symptomatic severity in patients with PTSD (Pitman et al., 2012; Rauch et al., 2006).

The emotional response associated with psychological trauma can have long-term cognitive effects and altered cognition is one of the hallmarks of PTSD (Hayes et al., 2012). Piracetam is a nootropic that enhances glutamatergic transmission by acting on neuronal AMPA receptors (Ahmed and Oswald, 2010). The recent preclinical report had also demonstrated that piracetam suppresses neuroinflammation and improve cognitive function (Tripathi et al., 2017). Besides other symptoms, there are coexisting psychotic symptoms like hallucinations, delusions, violent thoughts and behaviors in PTSD patients, hence atypical antipsychotics are gradually gaining more importance in terms of adjunctive utilization (Adetunji et al., 2005). Risperidone which is an atypical antipsychotic agent, a 5-HT_{2A} antagonist found to suppress the symptoms of PTSD pre-clinically as well as clinically up to an extent although it did not provide a significant therapeutic relief (Krystal et al., 2011). Thus, we hypothesized, that targeting the extinction window during the exposure therapy, along with suppressing the symptoms of PTSD could provide a better therapeutic platform for the management of PTSD. This combination has been successfully used in a certain population with autism spectrum disorder thus, representing the high translational value of the regimen (Akhondzadeh et al., 2008). Hence, the present study was undertaken to target the cognitive system to enhance extinction and simultaneously managing the symptoms of PTSD, with piracetam and risperidone combined with extinction therapy in single-prolonged stress-induced rat PTSD model.

2. Material and methods

2.1. Animals

Male Sprague-Dawley rats, weighing 200–250 g, were procured from Central Animal House of Panjab University Chandigarh, India. All the rats were housed in a room with 12 h alternate light and dark periods and food, as well as water ad libitum, was provided. Whole experimental protocols were conducted between 9 am and 5 pm. Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA) guidelines were followed to conduct the experiments. The study was approved by the Institutional Animal Ethical Committee, Panjab University, Chandigarh approval number (PU/45/99/CPCSE/IAEC/2017/11).

2.2. Drugs

Sertraline, risperidone, and piracetam were procured from Sigma-Aldrich (St. Louis, MO, United States). 5% dimethyl sulfoxide (DMSO) and water for injection was used to dissolve sertraline HCl, which was then administered intraperitoneally (10 mg/kg). Normal saline (0.9% NaCl) was used to dissolve piracetam and given i.p. in doses of 150 mg/kg and 300 mg/kg. Normal saline was also used to dissolve risperidone, followed by incorporation of a minimal amount of acetic acid and the pH was maintained to 6.5–7.0 by addition of sodium hydroxide which was then injected i.p. at doses of 0.5 mg/kg and 1 mg/kg. All the interventions were given for a duration of 14 days. Piracetam and risperidone doses were decided according to previously reported studies (Abdel-Salam et al., 2011; Baptista et al., 2002; Sun et al., 2010).

2.3. Experimental design

The whole experimental-protocol lasted for 31 days from the induction of PTSD-like phenotype to the day of sacrifice. The induction of PTSD-like phenotype by the SPS model was performed on day zero followed by 7 days of the undisturbed period. At the end of the seventh day of the undisturbed period, a brief re-stress was given to animals. On 8th and 9th, day, the fear conditioning paradigm was performed in order to install fear and further 14 days of extinction training (with or without treatment) was given. On 25th day, elevated plus maze test was performed and the social interaction test and marble burying test was done on 28th and 30th day, respectively. Animals were sacrificed on the 31st day, blood was collected, and brain areas (cortex and hippocampus) were dissected and used for further biochemical and ELISA analysis. The experimental design is shown in Fig. 1 and the timeline of the protocol in Fig. 2. A total number of 66 rats were used in this study, out of which 56 rats underwent SPS for induction of PTSD-like phenotype. Whereas, the results of 3 animals were removed from the study as they fit the exclusion criterion which was performance in any task analysis ≥ 2 S.D. away from the mean. We randomly assigned rats into groups as control, PTSD and treatments, comprising of 7 animals (n = 7) in each group. Normal saline (0.9% NaCl) was given to the control group. PTSD group was exposed to SPS paradigm thus representing the diseased symptomatic and pathological clusters, suitable for comparison with other groups. PTSD induced rats received sertraline (10 mg/kg) which served as a standard group while piracetam (150 and 300 mg/kg) and risperidone (0.5 and 1 mg/kg) served as test groups. The combination of piracetam and risperidone were given in two different groups with low doses [piracetam (150 mg/kg) + risperidone (0.5 mg/kg)] and high dose [piracetam (300 mg/kg) + risperidone (1 mg/kg)].

2.4. Single prolonged stress (SPS)-induced rat model of PTSD-like phenotype

The SPS paradigm to induce PTSD-like behavioral phenotype in rats was carried out according to previous studies (Liberzon et al., 1997a, 1997b). In this model, the plastic cone is used to restrain the rats for 2 h. Immediately after restraining, animals were subjected to forced swim test for 20 min in a plexiglass container (22.0-inch diameter) filled with 2/3rd water (maintained at 20 °C) followed by a recuperation period of 15 min. Further, diethyl ether vapor was used in desiccators containing rats, to make the animals fall unconscious. After completion of the whole procedure, animals were then kept to their respective cages, which were then kept undisturbed for one week. On the last day of the undisturbed period, rats were briefly restrained for 10 min in order to expose them to re-stress.

2.5. Behavioral tests

2.5.1. Fear conditioning paradigm

Fear memory expression and dominance were evaluated by a fear conditioning paradigm as it is an effective technique for the same (Curzon et al., 2009). The procedures were taken from previously

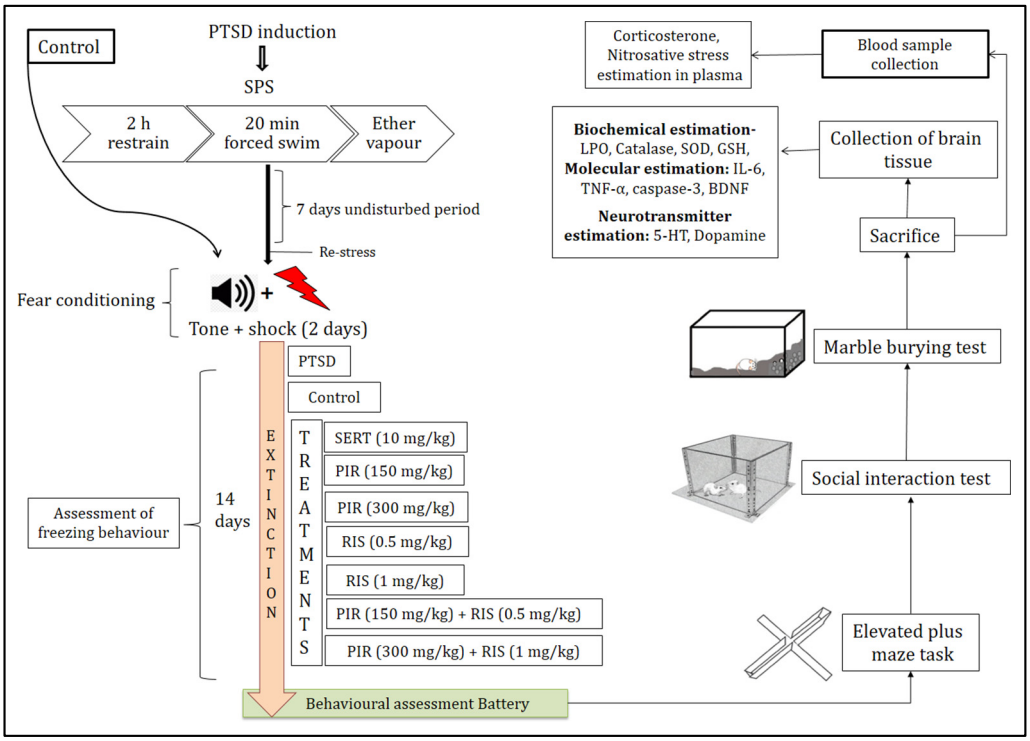

Fig. 1. Study design. PTSD - PTSD-like phenotype, PIR - Piracetam, RIS - Risperidone, SERT - Sertraline.

published work with some minimal modifications (Noble et al., 2019, 2017). In the fear conditioning paradigm, the "ABB" model was used: conditioning was done in context A (plexiglass chamber with black rare wall, electric grid floor, and overhead red illuminating light) and extinction task was performed in context B (transparent plexiglass chamber, overhead white illuminating light and peppermint oil added for distinct odor). On the 1st day of fear conditioning, rats were individually put into the plexiglass chamber to get acclimatize for 2 min. After acclimatization, rats were exposed to 4 pre-tones (70 dB) lasting for 30 s without unconditioned stimulus (foot shock) and baseline readings were measured by scoring the freezing behavior. Immediately after the pre-tones, rats were exposed to 8-tones i.e. conditioned stimulus (CS) coupled with a foot shock (1 s, 0.4 mA). A random interval was chosen between the tone presentation ranging between 60 and 120 s. During the last 20 s of each 30 s tone, the foot shocks were paired. After this fear installation process, animals were put back to the respective cages. After 24 h, rats again underwent fear conditioning paradigm but without exposure to pre-tones, the eight tones and shock were merged in the same way as on the previous day.

2.5.2. Extinction training

The extinction training was performed for 14 days in context B after 24 h of fear conditioning paradigm. During the extinction days, only CS was presented as 4 tones every day without the presentation of any reinforcement (US) and this training period lasted for 14 days. Along with extinction training, the interventions were given to see the effect of pharmacological agents on extinction. Administration of each intervention was done 1 h before extinction training. This extended extinction training period is very similar to a clinical presentation of a multiple day's exposure therapies for PTSD patients. The percent freezing duration in each 30 s tone presentation was recorded and scored. Time spent on presenting freezing behavior was used as a measure of fear response during that 30 s tone presentations. The freezing was considered as the cessation of movement apart from breathing. Scoring was done in a blind fashioned manner by 2 observers. The remission of fear was considered as the freezing time below 10% of the total time of tone presentation (120 s) (Peña et al., 2013)

2.5.3. Elevated plus maze

Elevated plus maze was performed for assessment of anxiety-related behavior in rats. The whole procedure was performed as previously described (Cruz et al., 1994; Pellow et al., 1985). The apparatus comprised of two arms. One is an open arm and another one is a closed arm of dimensions, 10 cm wide and 50 cm long. On the other hand, the closed arms were enclosed by 30 cm wall, while 2 open arms were kept with no walls. The whole apparatus was kept at 60 cm of height from the floor. Animals were put one by one in the center area facing towards the open arm. The trial duration lasted for 5 min and the sessions were videotaped for further analysis. The scoring was done for 3 parameters separately: as a number of entries in closed arms and open arms as well time duration stayed in the open arms. The arm entries were considered as rat put all four paws in a particular arm. All parameters were scored in a blinded fashioned manner.

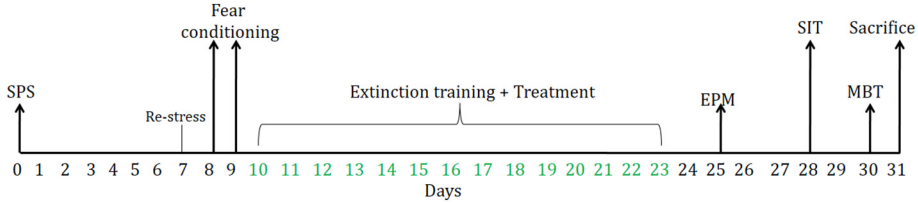

Fig. 2. Timeline of the protocol. SPS - Single prolonged stress, EPM - Elevated plus maze, SIT - Social interaction test, MBT - Marble burying test.

2.5.4. Social interaction test

Social interaction test was done to assess social withdrawal (File, 1978). A black square shaped wooden box arena (40 × 40 × 35 cm) was used. Two days before the trial, the weight of each experimental rat was matched to another new target rat (not from this study). The rats were acclimatized in arena for 10 min a day before testing. On testing day, the rats were put one by one inside the arena for 10 min, and social interaction session (10 min) began by introducing the target weight matched rat into the arena. All rats were used a single time for the study. After each session, 70% ethanol was used to clean the arena. The social interaction sessions were videotaped and the scoring was performed. The time in which the experimental rats were found to be actively engaged in social interaction with target rats was measured. The active social interaction time was measured by summing up the time spent on behaviors such as contact behavior (physical contact: genital exploration, sniffing along with direct body touch), following, boxing, sniffing, crawling and grooming with the target male rat. Whereas, zero contact time was considered as non-social interaction time and counted as such. The percentage of social interaction time was calculated.

Percentage social interaction time

= (Time spent on active social interaction/total time) × 100

2.5.5. Marble burying test

Defensive burying behavior is used as a parameter to assess avoidance-like behavior (De Boer and Koolhaas, 2003). Rats undergone fear conditioning were observed individually for this avoidance-like behavior. Marble burying test is considered to be sensitive for anxiolytic agents (Njung'e and Handley, 1991). Hence, the marble burying test was used to measure anxiety and avoidance like behaviors in rats. In this test, rats were acclimatized for wood chips bedding in their home cages. On the day of the assessment, the rats were individually placed in a square-shaped transparent acrylic chamber, with same wood chip bedding filled up to 10 cm height and having fifteen identical black shiny marbles (1.5 cm diameter) placed in three rows. After completion of 30 min, the rats were taken out of the chamber and the number of marbles buried was counted. The marbles covered with wood chip bedding > 2/3rd were considered as buried. The novel object avoidance was measured by calculating the percentage of marble buried.

Percentage marbles buried

= (Number of marbles buried/Number of marbles unburied) × 100.

2.6. Molecular and biochemical estimations

2.6.1. Sample collection and isolation

After the completion of behavioral assessments, the anesthesia was induced in rats using ketamine (70 mg/kg) and xylazine (10 mg/kg). Blood was isolated through retro-orbital plexus and kept in EDTA containing centrifugation tubes. Plasma was separated by centrifugation process performed at 10,000 rpm for 10 min. Further, plasma was stored at −80 °C for corticosterone and nitrite assay. Rats were sacrificed, the brain was separated from which cortex and hippocampus was dissected. The isolated tissue were homogenized in 10% (w/v) homogenization buffer (consisting of 10 mM Tris-HCl, 150 mM MgCl2, 1 mM EDTA, 1% Triton X 100, pH −7.4). Further, at 10,000 rpm the homogenate was centrifuged at 4 °C for 20 min. The supernatant was pipette out and kept at a storage temperature of −80 °C for the enzymatic activity of antioxidants assay and ELISA assay.

2.6.2. Estimation of plasma corticosterone

The corticosterone estimation was done following a previously described study (Bartos and Pesez, 1979). The reagent A (0.10% p-nitroso-*N*,*N*-dimethylaniline in ethanol), reagent B (0.10% phenol in ethanol) and reagent C (1% aqueous solution of potassium ferricyanide, freshly prepared just before use) were prepared. 1 mL of sample was added with an equal volume of ethanol, further 1 mL reagent A was included and then the resulting solution was put for 5 min in ice water. After that 0.5 mL of 0.10 M freshly prepared NaOH was added followed by incubation for 5 h, protecting from light at 0 °C. Further, the 2 mL of freshly prepared buffer (0.20 M boric acid, 0.20 M KCl and 0.20 M KOH, pH 9.4) was added into the above solution followed by the addition of reagent B and reagent C. This final mixture was kept for 10 min in a water bath (20 ± 2 °C). Reading was taken at 650 nm (UV–VIS Spectrophotometer, Perkin Elmer, Lambda 20).

2.6.3. Estimation of plasma nitrite

The assay for nitrite was done as observed by the nitrite production indicated by Griess reagent (1:1 solution of 1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylamine diamine dihydrochloric acid in water). The 500 µL of Greiss reagent was added to 100 µL of a plasma sample. Absorbance readings were taken at 546 nm (Perkin Elmer UV–VIS Spectrophotometer, Lambda 20) (Green et al., 1982). For calculating the plasma nitrite, an already established standard curve of sodium nitrite was used and results were expressed as µg/mL.

2.6.4. Estimation of protein

The Lowry method was used for determining the protein concentration of each sample (Lowry et al., 1951). Bovine serum albumin (BSA) is used as a standard. The working reagent was prepared by addition of 2 mL mixture of an equal volume of 1.56% copper sulfate solution and 2.37% sodium potassium tartrate with 100 mL of a mixture of equal volumes of 2% sodium carbonate and 0.1 N NaOH and then 10 min incubation was done. Folin-Phenol reagent (0.5 mL) was mixed and incubated for 30 min. BSA concentrations from 0.05 to 1 mg/mL were prepared for the standard plot. The intensity of color was observed at 660 nm (UV–VIS Spectrophotometer, Perkin Elmer, Lambda 20) and the protein content of the sample was found out from the graph obtained by the standard plot.

2.6.5. Estimation of reduced glutathione

The procedure for the assay of reduced glutathione was followed based on a previous study (Jollow et al., 1974). In this method, precipitation of 1.0 mL of the supernatant of tissue homogenate in a concentration of 10% w/v was done with 1.0 mL of sulphosalicylic acid (4%). Further, these processed samples were incubated for 1 h at 4 °C followed by centrifugation at 1200*g* at 4 °C for 15 min. Further to this, 2.7 mL of 0.1 M phosphate buffer (pH adjusted to 7.4) and 0.2 mL of 5,5, dithiobis-2-nitro benzoic acid (DTNB) was added. The yellow color was produced whose absorbance was taken immediately at 412 nm (UV–VIS Spectrophotometer, Perkin Elmer, Lambda 20). Calculated values were depicted as µmoles per mg protein.

2.6.6. Estimation of superoxide dismutase (SOD)

Assay for SOD enzyme was done, in which nitro blue tetrazolium (NBT) was added to the samples and further, the hydroxylamine hydrochloride was added to this mixture (Yasuhisa Kono, 1978). The reaction was observed by taking readings at 560 nm in a spectrophotometer (UV–VIS Spectrophotometer, Perkin Elmer, Lambda 20) (Y Kono, 1978). The SOD units/mg protein was calculated.

2.6.7. Estimation of catalase

The catalase assay was performed as described previously (Lück, 1965). In this, the breakdown of hydrogen peroxides (H_2O_2) was observed during the reaction. Briefly, the H_2O_2 was dissolved in phosphate buffer and to this solution, sample supernatant (0.05 mL) was added. Any change in absorbance for 2 min at 240 nm was observed using UV-spectroscopy (UV–VIS Spectrophotometer, Perkin Elmer, Lambda 20). The amount of H_2O_2 decomposed per mg of protein/min was calculated.

2.6.8. Estimation of lipid peroxidation (LPO)

LPO assay was performed by quantifying malondialdehyde (Wills, 1965). Briefly, the addition of tissue homogenate supernatant (0.5 mL) was done to 0.5 mL of Tris-HCl followed by 2 h incubation at 37 °C. To this, 1 mL of TCA (10%) was added to the above mixture and 10 min centrifugation was performed at 1000*g*. The supernatant (1 mL) from the processed mixture was pipette out and added to 1 mL of thiobarbituric acid (0.67% w/v). The tubes containing mixture were kept for 10 min in hot boiling water followed by cooling and addition of 1 mL of double distilled water (DDW). Reading for absorbance was taken at 532 nm (UV–VIS Spectrophotometer, Perkin Elmer, Lambda 20). The MDA levels were calculated and expression of results was done as the amount of MDA per mg protein.

2.6.9. Estimation of brain-derived neurotrophic factor (BDNF) and caspase-3 by ELISA

BDNF and caspase-3 quantification were done by Elabscience rat BDNF and caspase-3 immunoassay kit, respectively as per manufacturer instructions.

2.6.10. Estimation of interleukin-6 (IL-6) and tumor necrosis factor–alpha (TNF-α) by ELISA

IL-6 and TNF-α quantification was done by Peprotech rat IL-6 and TNF-α immunoassay kit as per manufacturer instructions.

2.6.11. Estimation of serotonin and dopamine neurotransmitters and their metabolite by HPLC

Hippocampus and cortex tissue homogenates were used for estimations of neurotransmitters serotonin (5-HT), 5-hydroxyindoleacetic acid (5-HIAA), a metabolite of serotonin, dopamine (DA) homovanillic acid (HVA), a metabolite of dopamine. Both 5-HT and 5-HIAA estimation was done using UV-detector while photodiode array detector (PDA) was used for DA and HVA. Waters standard system, C18 reverse phase column, acting as autoinjector, having high-pressure isocratic pump was used. 5-HT and DA were given flow rates of 0.8 mL/min and 0.9 mL/min respectively. Injection volume was set to 10 µL and the run time was set for 10 min. Hippocampal and cortical tissues were homogenized in 1% formic acid in 50:50 acetonitrile (ACN)/water solution and centrifugation was done at 14000 rpm at 4 °C for 15 min. The supernatant was separated followed by filtration by 0.22 µm syringe filters before injecting into injection column of HPLC. 1% formic acid in water and 1% formic acid in ACN (ratio for 5-HT, 75:25 and for DA 73:27) was made for the purpose of the mobile phase.

2.7. Statistical analysis

Analysis of data was done using a one-way ANOVA or a two-way ANOVA followed by a Tukey's post hoc test for multiple comparisons or a Bonferroni's post hoc test for multiple comparisons. Statistically significant effects were defined as those with P-values < 0.05. The standard error of the mean was represented by error bars. Prism Graph pad 5.0 (Graphpad Software Inc., Ca, USA) was used to analyze data.

3. Results

3.1. Behavioral tests

3.1.1. Effect of piracetam, risperidone and their combinations on conditioned fear extinction in PTSD-like phenotype rats

A significant effect was observed across the days on freezing behavior after two-way ANOVA analysis [$F_{(13,756)} = 448.8$ ($p < 0.0001$)]. The PTSD-like phenotype rats displayed more freezing behavior on CS presentation as compared to control rats on day 9 ($p < 0.05$) and this effect continued up to day 14 ($p < 0.0001$). PTSD-like phenotype rats did not achieve remission of conditioned fear (< 10% freezing on presentation of CS) during the 14 days of extinction. Control rats showed remission of fear on day 13 and this continued till day 14. Sertraline (10 mg/kg) and piracetam (150 mg/kg) treated group rats demonstrated a significant decreased freezing behavior as compared to PTSD-like phenotype rats, starting from day 11 ($p < 0.05$) and continued to day 14 ($p < 0.0001$). The remission of fear with sertraline (10 mg/kg) and piracetam (150 mg/kg) treatments was achieved on day 14, whereas, piracetam (300 mg/kg) exert a significant decrease in freezing behavior as compared to PTSD-like phenotype rats from day 10 ($p < 0.05$) and continued until the end day. The remission of fear in this group was achieved on day 13 and this continued till day 14. Rats treated with risperidone (0.5 mg/kg) showed significantly decreased freezing as compared to PTSD-like phenotype rats on day 9 ($p < 0.05$) continued up to day 14 ($p < 0.0001$), whereas the fear remission in this group was achieved on day 13 and continued on day 14. The rats treated with risperidone (1 mg/kg) were observed with a significant decrease in freezing behavior as compared to PTSD-like phenotype rats starting from day 8 ($p < 0.05$) and continued up to day 14 ($p < 0.0001$). This group achieved remission of fear on day 13 and the effect was observed to be continued on day 14. Treatment with the combination piracetam (150 mg/kg) + risperidone (0.5 mg/kg) and piracetam (300 mg/kg) + risperidone (1 mg/kg) produced significant decrease in freezing behavior as compared to PTSD-like phenotype rats on day 6 ($p < 0.05$) and day 5 ($p < 0.05$) respectively and continued till day 14 ($p < 0.0001$). The fear remission with low and high dose combination was achieved on day 12 and day 11 respectively and continued across day 14. The high dose combination of piracetam and risperidone suppressed freezing behavior significantly as compared to piracetam (300 mg/kg), risperidone (1 mg/kg) and sertraline (10 mg/kg) on day 7 and 8 (Table 1).

3.1.2. Effect of piracetam, risperidone and their combinations on elevated plus maze test in PTSD-like phenotypic rats

One-way ANOVA followed by Tukey's post hoc test suggested the significant effect across the groups assessed for a number of entries in the open arm [$F_{(8,54)} = 13.87$ ($p < 0.0001$) and time spent in open arms [$F_{(8,54)} = 13.44$ ($p < 0.0001$)]. PTSD-like phenotype rats showed significantly less number of entries into the open arm ($p < 0.0001$) and less time spent in open arms ($p < 0.0001$) as compared to control rats. A significantly increased number of entries and time spent in open arm as compared to PTSD-like phenotype rats was observed with sertraline (10 mg/kg) ($p < 0.001$), piracetam [(150 and 300 mg/kg); ($p < 0.001$, $p < 0.5$; $p < 0.001$) ($p < 0.5$ respectively), risperidone [(0.5 and 1 mg/kg); ($p < 0.001$, $p < 0.01$; $p < 0.0001$, $p < 0.001$ respectively). Treatment with combinations of piracetam (150 mg/kg) + risperidone (0.5 mg/kg) and piracetam (300 mg/kg) + risperidone (1 mg/kg) also significantly increased number of entries ($p < 0.0001$) and time spent ($p < 0.0001$) in open arm as compared to PTSD-like phenotype rats. The high dose combination treatment regimen produces a potent anxiolytic effect than the individual effect of piracetam ($p < 0.05$) and risperidone ($p < 0.05$) and sertraline (10 mg/kg) ($p < 0.05$) (Fig. 3).

3.1.3. Effect of piracetam, risperidone and their combinations on social interaction test in PTSD-like phenotype rats

The social withdrawal was assessed in social interaction behavior. The one-way ANOVA followed Tukey's post hoc test suggested a significant effect across the groups [$F_{(8,54)} = 12.60$ ($p < 0.0001$)]. PTSD-like phenotype rats spent significantly less time in social activities ($p < 0.0001$) as compared to control rats. A significant increase in time spent in social activities as compared to PTSD-like phenotype rats was observed with sertraline (10 mg/kg; $p < 0.01$), piracetam (150 and 300 mg/kg; $p < 0.05$, $p < 0.05$ respectively), risperidone (0.5 and 1 mg/kg; $p < 0.05$, $p < 0.001$ respectively) treated rats. Both combinations of piracetam and risperidone-treated rats also showed significantly increased social interaction ($p < 0.0001$) as compared to PTSD-like phenotype rats High dose combination showed more

Table 1
Effect of piracetam, risperidone and their combination on conditioned fear extinction measured as a percent of time spent freezing.

Day	Control	PTSD	SERT (10 mg/kg)	PIR (150 mg/kg)	PIR (300 mg/kg)	RIS (0.5 mg/kg)	RIS (1 mg/kg)	PIR (150 mg/kg) + RIS (0.5 mg/kg)	PIR (300 mg/kg) + RIS (1 mg/kg)
1	86.24 ± 4.26	88.05 ± 4.66	86.03 ± 4.25	84.98 ± 4.15	86.25 ± 4.27	86.14 ± 4.18	87.09 ± 4.15	89.72 ± 4.46	86.26 ± 4.22
2	80.37 ± 4.37	84.27 ± 3.94	81.58 ± 4.67	83.15 ± 4.13	80.37 ± 4.38	80.09 ± 3.82	82.73 ± 4.21	81.42 ± 4.61	78.66 ± 4.86
3	72.98 ± 5.69	78.55 ± 3.76	76.18 ± 5.89	78.01 ± 4.59	76.98 ± 4.73	74.43 ± 4.38	76.62 ± 4.39	73.67 ± 4.20	69.32 ± 4.97
4	65.20 ± 5.10	75.78 ± 4.72	69.21 ± 2.64	72.68 ± 4.25	68.20 ± 4.49	70.13 ± 4.17	69.25 ± 3.07	66.49 ± 4.46	61.22 ± 4.85
5	61.51 ± 4.93	70.80 ± 3.65	66.11 ± 4.94	68.09 ± 4.06	64.51 ± 4.94	63.59 ± 4.16	60.20 ± 4.79	57.07 ± 4.60	52.44 ± 3.91$^{\#}$
6	54 ± 5.21	66.65 ± 4.04	62.60 ± 4.76	63.09 ± 4.24	58 ± 4.79	54.31 ± 4.05	51.76 ± 3.32	49.07 ± 4.16$^{\#}$	40.21 ± 3.00$^{\#,\alpha,\$}$
7	45.62 ± 4.60	60.56 ± 4.37	58.35 ± 5.26	56.59 ± 4.34	50.63 ± 4.60	49.85 ± 3.52	43.22 ± 3.79	40.48 ± 3.05$^{\#,\$}$	32.22 ± 3.46$^{\#,\alpha,\phi,\$}$
8	39.72 ± 4.02	55.21 ± 4.03	51.83 ± 4.66	48.99 ± 4.52	42.73 ± 3.02	41.07 ± 4.49	36.69 ± 4.29$^{\#}$	33.09 ± 3.63$^{\#,\$}$	24.09 ± 3.48$^{\#,\alpha,\phi,\$}$
9	32.81 ± 4.14	51.25 ± 4.06*	46.26 ± 3.31	42.20 ± 3.23	38.81 ± 3.14	33.04 ± 3.41$^{\#}$	29.3 ± 3.33$^{\#,\$}$	26.54 ± 2.97$^{\#,\$}$	17.61 ± 2.30$^{\#,\alpha,\$}$
10	25.51 ± 3.05	49.09 ± 4.35*	39.09 ± 3.19	37.17 ± 3.25	30.51 ± 3.05$^{\#}$	28.95 ± 3.61$^{\#}$	20.53 ± 2.88$^{\#,\alpha,\$}$	18.03 ± 2.50$^{\#,\alpha,\$}$	10.62 ± 2.96$^{\#,\alpha,\$}$
11	16.13 ± 3.61	48.01 ± 3.98*	31.22 ± 3.10$^{\#}$	28.03 ± 2.35$^{\#}$	20.13 ± 2.61$^{\#}$	20.57 ± 2.49$^{\#}$	18.53 ± 2.88$^{\#}$	13.81 ± 2.16$^{\#,\$}$	8.20 ± 2.51$^{\#,\alpha,\$}$
12	12.97 ± 2.09	45.22 ± 3.23*	26.19 ± 2.19$^{\#}$	19.34 ± 2.00$^{\#}$	13.97 ± 3.09$^{\#}$	15.59 ± 3.19$^{\#}$	14.06 ± 3.42$^{\#}$	9.86 ± 2.63$^{\#,\$}$	5.95 ± 1.60$^{\#,\$}$
13	8.29 ± 2.58	41.35 ± 3.87*	19.13 ± 2.93$^{\#}$	16.30 ± 3.93$^{\#}$	9.29 ± 3.17$^{\#}$	10 ± 2.60$^{\#}$	8.69 ± 2.69$^{\#}$	6.42 ± 2.71$^{\#}$	3.87 ± 2.64$^{\#}$
14	6.92 ± 2.34	39.13 ± 3.49*	8.94 ± 3.51$^{\#}$	9.80 ± 2.40$^{\#}$	7.71 ± 1.92$^{\#}$	9.89 ± 2.63$^{\#}$	6.30 ± 2.33$^{\#}$	5.35 ± 2.99$^{\#}$	3.12 ± 2.94$^{\#}$

Data was analyzed using two-factor repeated measure ANOVA followed by Bonferroni's multiple comparisons. All values are expressed as a percent of time spent freezing mean ± SEM. PIR - Piracetam, RIS - Risperidone, SERT - Sertraline.

* $p < 0.05$ as compared to control.
$^{\#}$ $p < 0.05$ as compared to PTSD.
$^{\alpha}$ $p < 0.05$ as compared to PIR (300 mg/kg).
$^{\phi}$ $p < 0.05$ as compared to RIS (1 mg/kg).
$^{\$}$ $p < 0.05$ as compared to SERT (10 mg/kg).

significant results as compared to the individual effect of piracetam ($p < 0.001$) and risperidone ($p < 0.05$). Moreover, this combination was significantly more effective than sertraline (10 mg/kg) treatment ($p < 0.01$) (Fig. 4).

3.1.4. Effect of piracetam, risperidone and their combinations on avoidance-like behavior in marble burying test in PTSD-like phenotype rats

The avoidance-like behavior was tested in the marble burying test. A one-way ANOVA followed by Tukey's post hoc test indicated the significant effect across the groups [$F_{(8,54)} = 16.11$, $p < 0.001$]. PTSD-like phenotype rats buried a significantly more number of marbles ($p < 0.001$) as compared to control rats. A significant decrease in number of marble buried as compared to PTSD-like phenotype rats was observed in rats treated with sertraline (10 mg/kg, $p < 0.01$), piracetam (150 and 300 mg/kg; $p < 0.05$, $p < 0.05$ respectively), risperidone (0.5 and 1 mg/kg; $p < 0.05$, $p < 0.01$). The piracetam and risperidone combinations decreased avoidance-like behavior ($p < 0.001$) as compared to PTSD-like phenotype rats. Whereas, high dose combination of piracetam and risperidone produced significant more effect as compared to the individual effect of piracetam ($p < 0.01$), risperidone ($p < 0.05$) and sertraline ($p < 0.01$) (Fig. 5).

3.2. Molecular and biochemical estimations

3.2.1. Effect of piracetam, risperidone and their combinations on plasma corticosterone levels in PTSD rats

A significant effect was demonstrated across the groups after analysis by one-way ANOVA followed by Tukey's post hoc test on serum corticosterone levels [$F_{(8,54)} = 17.28$, $p < 0.001$]. The serum corticosterone levels were significantly decreased in PTSD-like phenotype rats as compared to control rats ($p < 0.001$). A significant increase in serum corticosterone levels as compared to PTSD-like phenotype rats was observed with sertraline (10 mg/kg, $p < 0.01$), piracetam (150 and 300 mg/kg; $p < 0.05$, $p < 0.05$), risperidone (0.5 and 1 mg/kg; $p < 0.001$, $p < 0.001$). Treatment with combinations also increased corticosterone levels in PTSD-like phenotype rats ($p < 0.001$). The high dose combination treatment produced significantly more effect than the individual effect of piracetam ($p < 0.05$) and risperidone ($p < 0.05$) (Fig. 6).

3.2.2. Effect of piracetam, risperidone and their combinations on hippocampal and cortical antioxidant enzymes in PTSD-like phenotype rats

A significant effect across the groups was observed on antioxidant enzyme activity of GSH, SOD, catalase in hippocampus after one-way ANOVA followed by Tukey's post hoc test [($F_{(8,27)} = 20.67$; $p < 0.0001$), ($F_{(8,27)} = 25.25$; $p < 0.0001$) and ($F_{(8,27)} = 15.62$; $p < 0.0001$), respectively], and also in cortex [($F_{(8,27)} = 23.18$; $p < 0.0001$)], ($F_{(8,27)} = 20.13$; $p < 0.0001$), and [$F_{(8,27)} = 25.78$ ($p < 0.0001$), respectively]. The antioxidant enzyme activity of GSH, SOD, and catalase were found to be significantly decreased in the hippocampal and cortical region of PTSD-like phenotype rats brain as compared to control rats ($p < 0.0001$). A significance increase in enzymatic activity of GSH, SOD and catalase was observed in hippocampus and cortex on treatment with sertraline (10 mg/kg; $p < 0.05$, $p < 0.01$ respectively), piracetam (150 mg/kg; $p < 0.05$, $p < 0.05$ respectively), piracetam (300 mg/kg; $p < 0.05$, $p < 0.01$ respectively), risperidone (0.5 mg/kg; $p < 0.01$, $p < 0.01$) and risperidone (1 mg/kg; $p < 0.001$, $p < 0.001$). Both combinations significantly restored the enzymatic activity of GSH, SOD, and catalase in hippocampus and cortex as compared to PTSD-like phenotype rats ($p < 0.0001$). The high dose combination treatment produced potentiating effect which was significantly increased in hippocampus and cortex than individual dose of piracetam ($p < 0.01$, $p < 0.001$ respectively), risperidone ($p < 0.01$, $p < 0.05$ respectively) and sertraline (10 mg/kg; $p < 0.01$, $p < 0.001$ respectively) (Fig. 7).

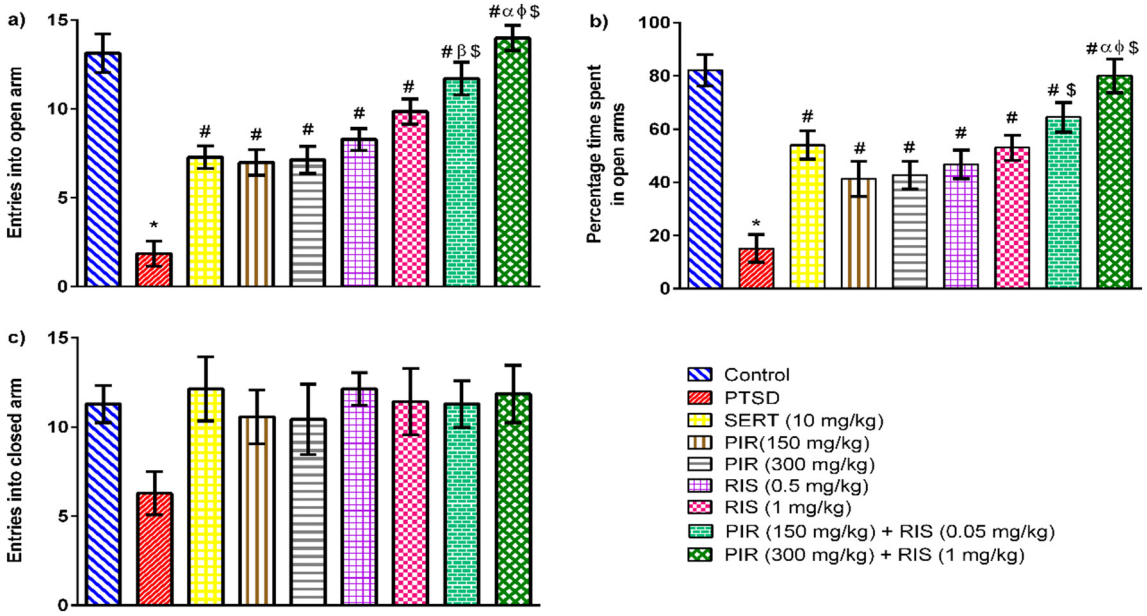

Fig. 3. Effect of piracetam, risperidone and their combinations on anxiety-like behavior in PTSD-like phenotypic rat: a) entries into open arms b) percentage time spent in open arms c) entries into closed arms. Data was analyzed using one-way ANOVA followed by Tukey's post hoc analysis. All values are expressed as mean ± SEM. $^{*}p < 0.05$ as compared to control, $^{\#}p < 0.05$ as compared to PTSD, $^{\beta}p < 0.05$ as compared to PIR (150 mg/kg), $^{\alpha}p < 0.05$ as compared to PIR (300 mg/kg), $^{\phi}p < 0.05$ as compared to RIS (1 mg/kg) and $^{\$}p < 0.05$ as compared to SERT (10 mg/kg).PIR - Piracetam, RIS - Risperidone, SERT - Sertraline.

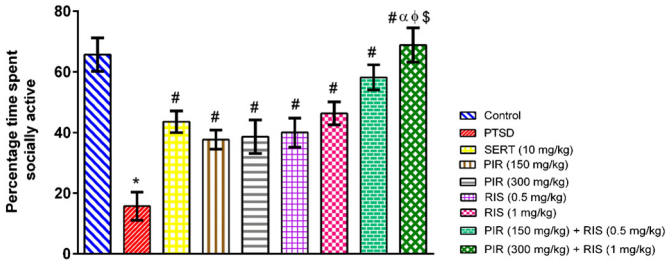

Fig. 4. Effect of piracetam, risperidone and their combinations on social interaction test in PTSD-like phenotype rats. Data was analyzed using one-way ANOVA followed by Tukey's post hoc analysis. All values are expressed as mean ± SEM. $^{*}p < 0.05$ as compared to control, $^{\#}p < 0.05$ as compared to PTSD, $^{\phi}p < 0.05$ as compared to RIS (1 mg/kg) and $^{\$}p < 0.05$ as compared to SERT (10 mg/kg). PIR - Piracetam, RIS - Risperidone, SERT - Sertraline.

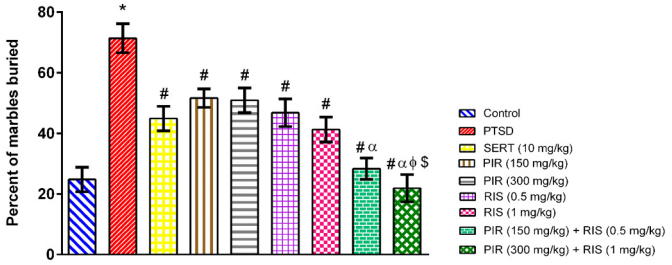

Fig. 5. Effect of piracetam, risperidone and their combinations on avoidance-like behavior in marble burying test in PTSD-like phenotype rats. Data was analyzed using one-way ANOVA followed by Tukey's post hoc analysis. All values are expressed as mean ± SEM. $^{*}p < 0.05$ as compared to control, $^{\#}p < 0.05$ as compared to PTSD, $^{\alpha}p < 0.05$ as compared to PIR (300 mg/kg), $^{\phi}p < 0.05$ as compared to RIS (1 mg/kg) and $^{\$}p < 0.05$ as compared to SERT (10 mg/kg). PIR - Piracetam, RIS - Risperidone, SERT - Sertraline.

3.2.3. Effect of piracetam, risperidone and their combinations on hippocampal and cortical lipid peroxidation and plasma nitrite levels in PTSD-like phenotype rats

The significant effect was seen on lipid peroxidation (LPO) in hippocampus [$F_{(8,27)} = 29.57$ ($p < 0.001$)] and cortex [$F_{(8,27)} = 28.38$ ($p < 0.001$)], and plasma nitrite (NO) [$F_{(8,27)} = 22.88$ ($p < 0.0001$)]. LPO levels were significantly higher in the hippocampus ($p < 0.0001$) and cortex ($p < 0.0001$) along with significantly elevated NO levels in plasma ($p < 0.0001$) of PTSD-like phenotype rats as compared to control rats. The LPO levels were suppressed in hippocampus and cortex of rats administered with sertraline (10 mg/kg; $p < 0.01$, $p < 0.05$ respectively), piracetam (150 mg/kg; $p < 0.01$, $p < 0.05$), piracetam (300 mg/kg; $p < 0.001$, $p < 0.01$ respectively), risperidone (0.5 mg/kg; $p < 0.001$, $p < 0.001$) and risperidone (1 mg/kg; $p < 0.001$, $p < 0.001$). The plasma NO levels were also decreased with sertraline (10 mg/kg; $p < 0.05$), piracetam (150,300 mg/kg; $p < 0.05$; $p < 0.05$ respectively), risperidone (0.5 and 1 mg/kg; $p < 0.01$, 0.001 respectively) as compared to PTSD-like phenotype rats. Both combinations significantly decreased LPO levels in the hippocampus as well as in cortex, and also the plasma NO levels as compared to PTSD-like phenotype rats ($p < 0.0001$). The high dose combination significantly reversed the SPS induced LPO and NO levels, this effect was significant as compared to the individual effect of piracetam ($p < 0.001$), risperidone ($p < 0.05$) and sertraline (10 mg/kg)

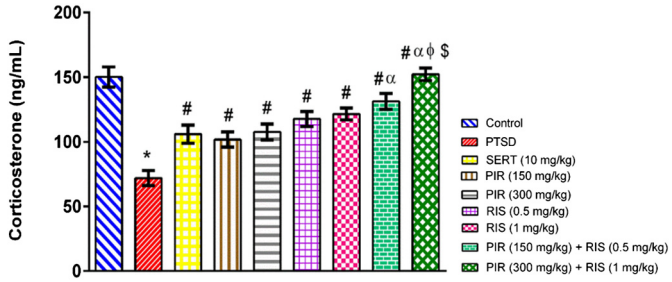

Fig. 6. Effect of piracetam, risperidone and their combinations on plasma corticosterone levels in PTSD-like phenotype rats. Data was analyzed using one-way ANOVA followed by Tukey's post hoc analysis. All values are expressed as mean ± SEM. $^{*}p < 0.05$ as compared to control, $^{\#}p < 0.05$ as compared to PTSD, $^{\alpha}p < 0.05$ as compared to PIR (300 mg/kg), $^{\phi}p < 0.05$ as compared to RIS (1 mg/kg) and $^{\$}p < 0.05$ as compared to SERT (10 mg/kg). PIR - piracetam, RIS - risperidone, SERT - Sertraline.

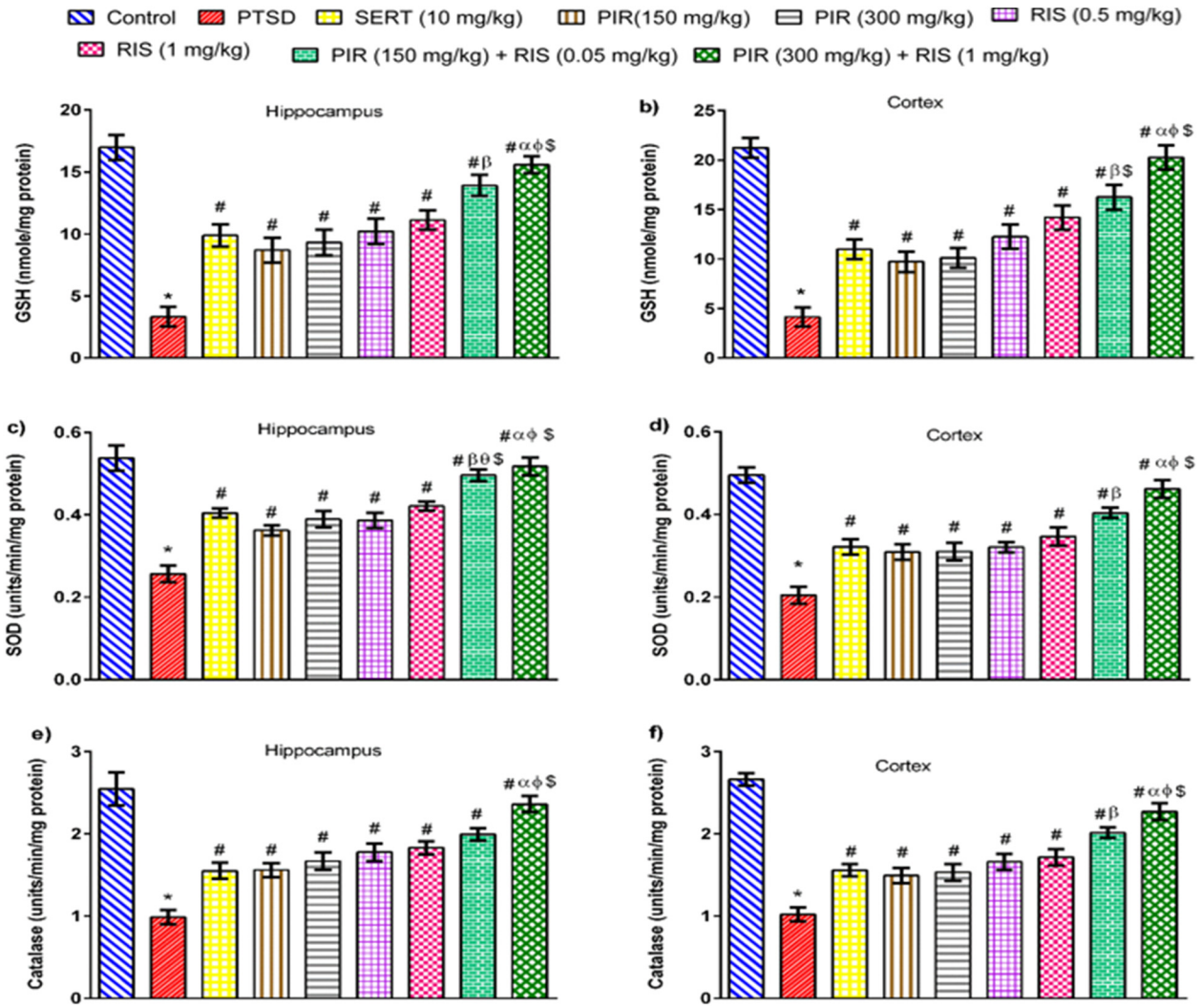

Fig. 7. Effect of piracetam, risperidone and their combinations on hippocampal and cortical antioxidant enzymes in PTSD-like phenotype rats. GSH level in a) hippocampus b) cortex, SOD level in c) hippocampus d) cortex, catalase level in e) hippocampus and f) cortex. Data was analyzed using one-way ANOVA followed by Tukey's post hoc analysis. All values are expressed as mean ± SEM. $^{*}p < 0.05$ as compared to control, $^{\#}p < 0.05$ as compared to PTSD, $^{\beta}p < 0.05$ as compared to PIR (150 mg/kg), $^{\alpha}p < 0.05$ as compared to PIR (300 mg/kg), $^{\theta}p < 0.05$ as compared to RIS (0.5 mg/kg), $^{\phi}p < 0.05$ as compared to RIS (1 mg/kg) and $^{\$}p < 0.05$ as compared to SERT (10 mg/kg). PIR - Piracetam, RIS - Risperidone, SERT - Sertraline.

($p < 0.001$) (Fig. 8).

3.2.4. Effect of piracetam, risperidone and their combinations on hippocampal and cortical BDNF levels in PTSD-like phenotype rats

A significant decreased hippocampal [$F_{(8,18)} = 17.21$ ($p < 0.001$)] and cortical [$F_{(8,18)} = 12.70$ ($p < 0.001$)] levels of BDNF across the groups were observed in one-way ANOVA followed by Tukey's post hoc test. The BDNF levels were reduced significantly in both hippocampal and cortical regions of PTSD-like phenotype rats as compared to control rats ($p < 0.001$ for both). BDNF levels were significantly increased in hippocampus and cortex of rats treated with sertraline (10 mg/kg; $p < 0.05$ for both regions), piracetam (150 mg/kg; $p < 0.05$ for both regions), piracetam (300 mg/kg; $p < 0.01$ for both regions) and risperidone (0.5 and 1 mg/kg; $p < 0.01$ for both regions) as compared to PTSD-like phenotype rats. Rats treated with both combinations were also found to have significantly increased BDNF levels in both regions as compared to PTSD-like phenotype rats ($p < 0.001$). The high dose combination showed the significant potentiating effect on BDNF levels as compared to an individual dose of piracetam ($p < 0.05$) and risperidone ($p < 0.05$), and sertraline (10 mg/kg; $p < 0.05$) (Fig. 9).

3.2.5. Effect of piracetam, risperidone and their combinations on cortical and hippocampal neuroinflammation in PTSD-like phenotype rats

One-way ANOVA followed by Tukey's post hoc test demonstrated significant effect on hippocampal [$F_{(8,18)} = 18.31$ ($p < 0.001$)] and cortical [$F_{(8,18)} = 16.58$ ($p < 0.001$)] levels of IL-6 and hippocampal [$F_{(8,18)} = 24.44$ ($p < 0.0001$)] and cortical [$F_{(8,18)} = 21.90$ ($p < 0.0001$)] TNF-α levels across the groups. The cortical and hippocampal IL-6 and TNF-α levels were significantly increased in PTSD-like phenotype rats as compared to control rats ($p < 0.001$, $p < 0.0001$ respectively) A significantly decreased IL-6 level in hippocampus and cortex was observed with sertraline (10 mg/kg) ($p < 0.05$, $p < 0.01$ respectively), piracetam (150 mg/kg; $p < 0.01$ for both regions), piracetam (300 mg/kg; $p < 0.01$ for both regions), risperidone (0.5 mg/kg; $p < 0.05$, $p < 0.01$ respectively), and risperidone (1 mg/kg; $p < 0.01$ for both regions) treatments as compared to PTSD rats. Both the combinations significantly reduced the levels of IL-6 in both hippocampal and cortical regions as compared to PTSD-like phenotype rats ($p < 0.001$). The high dose combination reduced the IL-6 in both hippocampus and cortex significantly as compared to piracetam (300 mg/kg; $p < 0.05$ for both regions), risperidone (1 mg/kg; $p < 0.05$ for both regions) and sertraline (10 mg/kg; $p < 0.05$ for both regions).

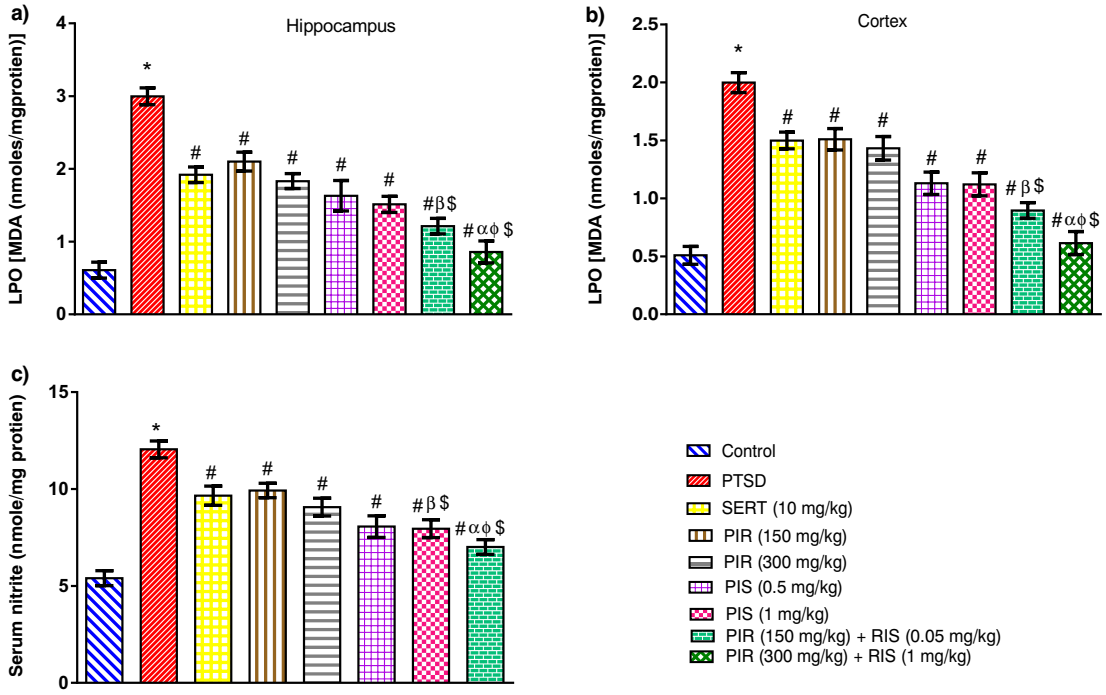

Fig. 8. Effect of piracetam, risperidone and their combinations on a) hippocampal and b) cortical LPO level and c) plasma nitrosative stress in PTSD-like phenotype rats. Data was analyzed using one-way ANOVA followed by Tukey's post hoc analysis. All values are expressed as mean ± SEM. $^{*}p < 0.05$ as compared to control, $^{\#}p < 0.05$ as compared to PTSD, $^{\beta}p < 0.05$ as compared to PIR (150 mg/kg), $^{\alpha}p < 0.05$ as compared to PIR (300 mg/kg), $^{\Phi}p < 0.05$ as compared to RIS (1 mg/kg) and $^{\$}p < 0.05$ as compared to SERT (10 mg/kg). PIR - Piracetam, RIS - Risperidone, SERT - Sertraline.

A significant reduction in SPS induced TNF-α levels was observed in hippocampus and cortex of rats treated with sertraline (10 mg/kg; $p < 0.01$ for both regions), piracetam (150 mg/kg; $p < 0.05$ for both regions), piracetam (300 mg/kg; $p < 0.05$ for both regions), risperidone (0.5 mg/kg; $p < 0.001$ for both regions) and risperidone (1 mg/kg; $p < 0.000$, $p < 0.001$ respectively) as compared PTSD-like phenotype rats. Treatment with both the combinations of piracetam and risperidone, also significantly reduced the TNF-α levels in the hippocampus and cortex region as compared the PTSD-like phenotype rats ($p < 0.0001$). The high dose combination reversed the TNF-α level and this was significantly more effective as compared to the individual effect of piracetam ($p < 0.001$), risperidone ($p < 0.05$) and sertraline (10 mg/kg; $p < 0.001$) (Fig. 10).

3.2.6. *Effect of piracetam, risperidone and their combinations on cortical and hippocampal caspase-3 activity in PTSD-like phenotype rats*

A significant effect on hippocampal [$F_{(8,18)} = 27.82$ ($p < 0.001$)] and cortical [$F_{(8,18)} = 29.70$ ($p < 0.001$)] levels of caspase-3 across

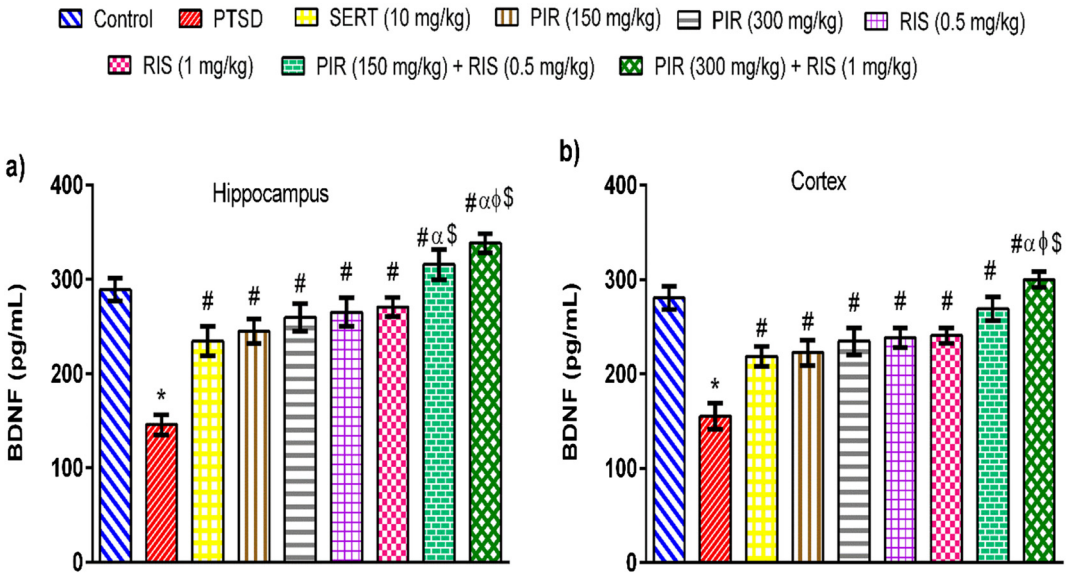

Fig. 9. Effect of piracetam, risperidone and their combinations on hippocampal and cortical BDNF levels in (a) Hippocampus (b) Cortex in PTSD-like phenotype rats. Data was analyzed using one-way ANOVA followed by Tukey's post hoc analysis. All values are expressed as mean ± SEM. $^{*}p < 0.05$ as compared to control, $^{\#}p < 0.05$ as compared to PTSD, $^{\alpha}p < 0.05$ as compared to PIR (300 mg/kg), $^{\Phi}p < 0.05$ as compared to RIS (1 mg/kg) and $^{\$}p < 0.05$ as compared to SERT (10 mg/kg). PIR - Piracetam, RIS - Risperidone, SERT - Sertraline.

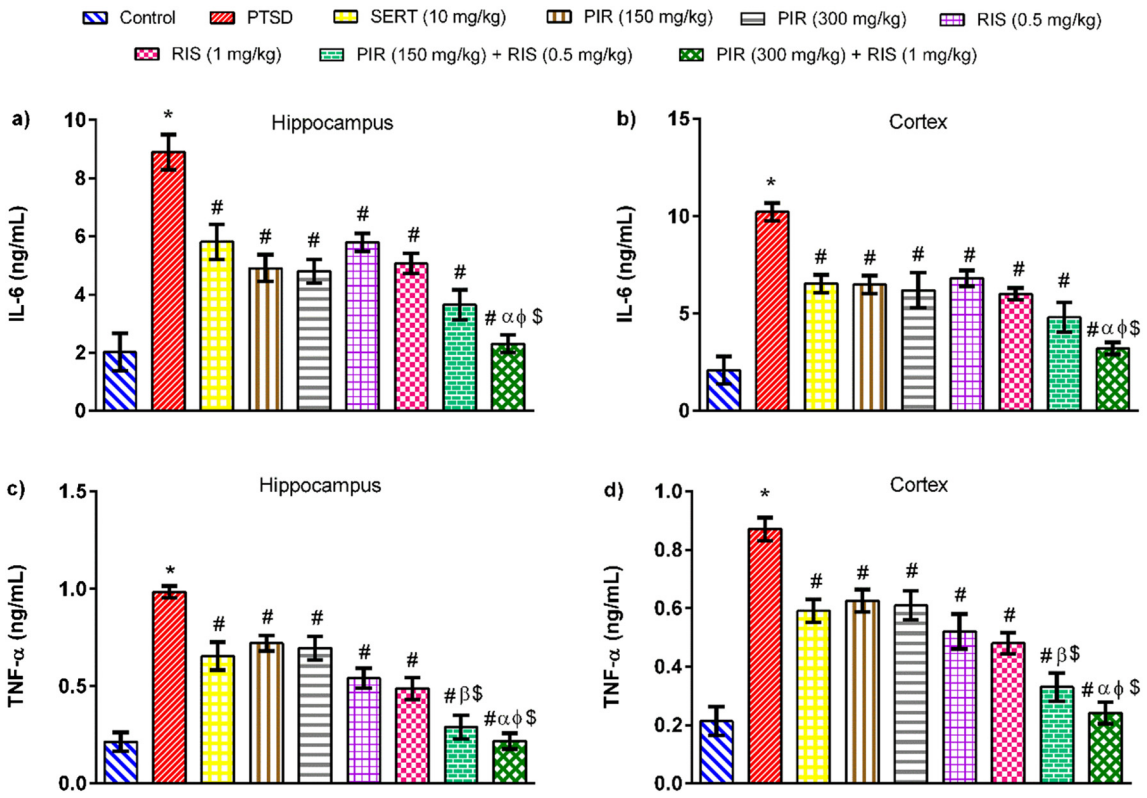

Fig. 10. Effect of piracetam, risperidone and their combinations on cortico-hippocampal neuroinflammation in PTSD-like phenotype rats. IL-6 level in a) hippocampus b) cortex and TNF-α levels in c) hippocampus d) cortex. Data was analyzed using one-way ANOVA followed by Tukey's post hoc analysis. All values are expressed as mean ± SEM. $^{*}p < 0.05$ as compared to control, $^{\#}p < 0.05$ as compared to PTSD, $^{\beta}p < 0.05$ as compared to PIR (150 mg/kg), $^{\alpha}p < 0.05$ as compared to PIR (300 mg/kg), $^{\phi}p < 0.05$ as compared to RIS (1 mg/kg) and $^{\$}p < 0.05$ as compared to SERT (10 mg/kg). PIR - Piracetam, RIS - Risperidone, SERT - Sertraline.

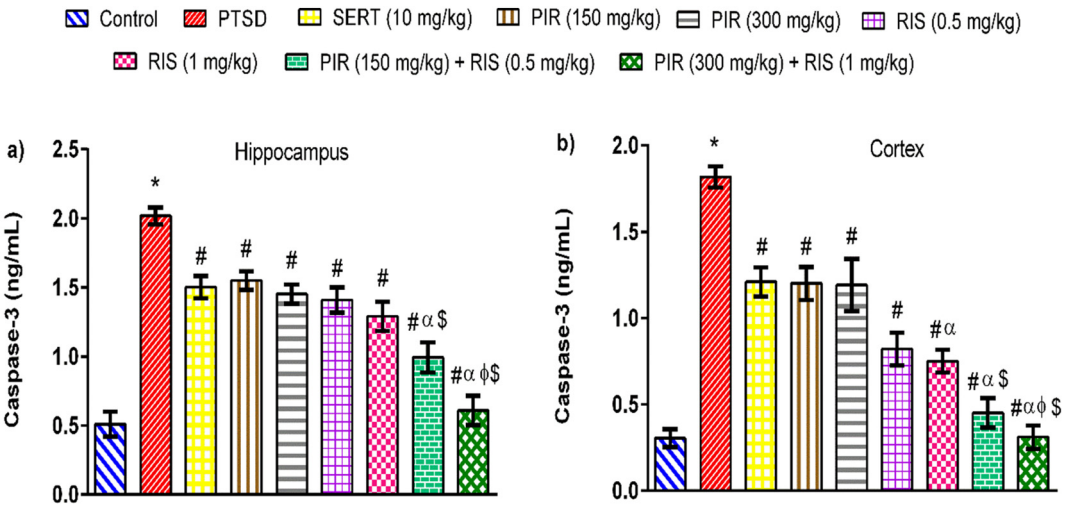

Fig. 11. Effect of piracetam, risperidone and their combinations on hippocampal and cortical Caspase-3 activity in a) hippocampus b) cortex in PTSD-like phenotype rats. Data was analyzed using one-way ANOVA followed by Tukey's post hoc analysis. All values are expressed as mean ± SEM. $^{*}p < 0.05$ as compared to control, $^{\#}p < 0.05$ as compared to PTSD, $^{\alpha}p < 0.05$ as compared to PIR (300 mg/kg), $^{\phi}p < 0.05$ as compared to RIS (1 mg/kg) and $^{\$}p < 0.05$ as compared to SERT (10 mg/kg). PIR - Piracetam, RIS - Risperidone, SERT - Sertraline.

the groups was seen in one-way ANOVA followed by Tukey's post hoc analysis. Caspase-3 levels were increased in brain hippocampal and cortical regions of PTSD-like phenotype rats as compared to control rats ($p < 0.001$). The hippocampus and cortex caspase-3 levels were reduced significantly by treatment with sertraline (10 mg/kg; $p < 0.05$, $p < 0.01$ resp), piracetam (150 mg/kg; $p < 0.05$, $p < 0.01$ respectively), piracetam (300 mg/kg; $p < 0.01$ for both regions), risperidone (0.5 mg/kg; $p < 0.01$, $p < 0.001$ respectively) and risperidone (1 mg/kg; $p < 0.01$, $p < 0.001$ respectively) as compared to PTSD-like phenotype rats. Both combination treatments also reduced caspase-3 levels in hippocampal and cortical regions as compared to PTSD-like phenotype rats ($p < 0.001$). The high dose combination reversed the SPS induced elevated caspase-3 levels and this effect was significant as compared to an individual dose of piracetam ($p < 0.001$), risperidone

($p < 0.05$), and sertraline (10 mg/kg; $p < 0.001$) (Fig. 11).

3.2.7. Effect of piracetam, risperidone and their combination on cortical and hippocampal serotonergic neurotransmitter level in PTSD-like phenotype rats

A significant effect on 5-HT and 5-HIAA levels in hippocampus [($F_{(8,18)} = 42.38$ ($p < 0.0001$)), ($F_{(8,18)} = 17.99$ ($p < 0.0001$) respectively), and cortex [($F_{(8,18)} = 14.36$ ($p < 0.0001$), ($F_{(8,18)} = 13.88$ ($p < 0.0001$) respectively) across the groups was observed in one-way ANOVA followed by Tukey's post hoc analysis. The 5-HT and 5-HIAA levels were decreased in the hippocampus ($p < 0.0001$) and cortex ($p < 0.01$) of PTSD-like phenotype rats as compared to control rats. A significantly improved 5-HT levels were found in hippocampus and cortex of rats treated with piracetam (300 mg/kg; $p < 0.001$, $p < 0.05$) respectively, risperidone (0.5 mg/kg; $p < 0.0001$, $p < 0.01$ respectively), risperidone (1 mg/kg; $p < 0.0001$; $p < 0.01$ respectively) and sertraline (10 mg/kg: $p < 0.0001$, $p < 0.05$ respectively) as compared to PTSD-like phenotype rats. Piracetam (150 mg/kg; $p < 0.01$) treatment significantly increased the 5-HT level in the hippocampus only as compared to PTSD-like phenotype rats. Whereas, 5-HIAA levels were significantly increased in cortex and hippocampus by piracetam (300 mg/kg; $p < 0.001$, $p < 0.01$), risperidone (1 mg/kg; $p < 0.001$, $p < 0.01$ respectively) and sertraline (10 mg/kg; $p < 0.0001$, $p < 0.05$ respectively). Treatment with both the combinations also increased 5-HT levels in both hippocampus ($p < 0.0001$) and cortex ($p < 0.0001$) of PTSD-like phenotype rats and this effect was significantly more than the individual dose of piracetam ($p < 0.05$), risperidone ($p < 0.05$) and sertraline (10 mg/kg; $p < 0.05$). 5-HIAA levels were also reversed by both the combinations in the hippocampus ($p < 0.0001$) and cortex ($p < 0.01$) (Fig. 12).

3.2.8. Effect of piracetam, risperidone and their combination on cortical and hippocampal dopaminergic neurotransmitter levels in PTSD-like phenotype rats

One-way ANOVA followed by Tukey's post hoc test demonstrated A significant effect on DA and HVA levels in hippocampus [($F_{(8,18)} = 20.82$ ($p < 0.0001$)), ($F_{(8,18)} = 32.52$ ($p < 0.0001$)) respectively] and cortex [($F_{(8,18)} = 23.88$ ($p < 0.0001$)), ($F_{(8,18)} = 48.90$ ($p < 0.0001$)) respectively] The DA and HVA levels were decreased in PTSD-like phenotype rats as compared to control rats ($p < 0.0001$). Piracetam (150 mg/kg), risperidone (0.5 mg/kg) significantly increased cortical DA levels ($p < 0.0001$) and HVA levels in both the hippocampal ($p < 0.0001$) and cortical regions ($p < 0.0001$). Whereas, treatments with piracetam (300 mg/kg) and risperidone (1 mg/kg) significantly improved DA and HVA levels as compared to PTSD rats in hippocampus [($p < 0.05$) ($p < 0.0001$)] and cortex [($p < 0.0001$) ($p < 0.0001$) respectively]. Treatment with both the combinations increased DA and HVA levels significantly as compared to PTSD-like phenotype rats in both hippocampus ($p < 0.001$) and cortex ($p < 0.0001$). The low dose combination produced a significant rise in DA levels in the hippocampal region as compared to PTSD-like phenotype rats and this effect was significant as compared to their individual effects. While the high dose combination increased DA levels in hippocampus and cortex significantly more than the individual effect of piracetam ($p < 0.05$) and risperidone ($p < 0.05$) (Fig. 13).

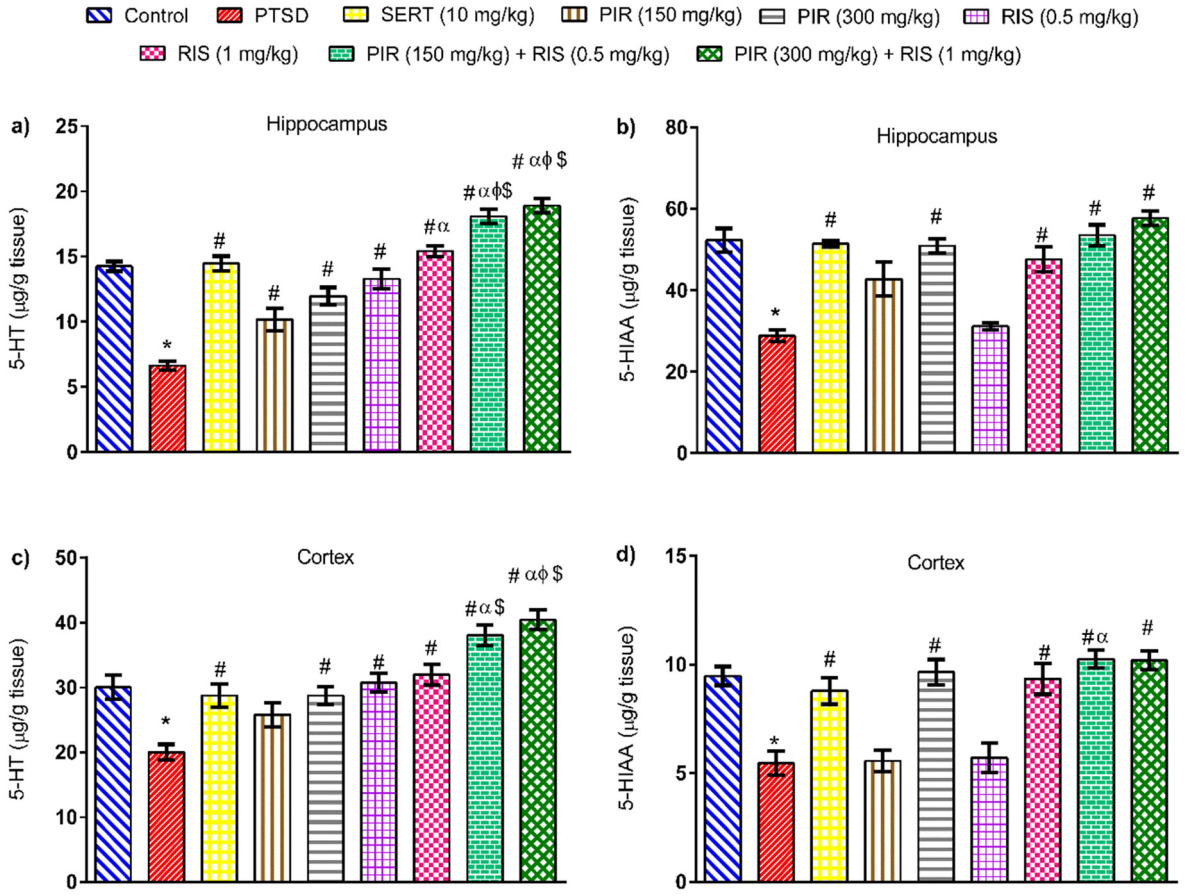

Fig. 12. Effect of piracetam, risperidone and their combinations on hippocampus a) 5-HT levels b) 5-HIAA levels; and c) Cortex c) 5-HT levels and d) 5-HIAA levels in PTSD-like phenotype rats. Data was analyzed using one-way ANOVA followed by Tukey's post hoc analysis. All values are expressed as mean ± SEM. $^{*}p < 0.05$ as compared to control, $^{\#}p < 0.05$ as compared to PTSD, $^{\alpha}p < 0.05$ as compared to PIR (300 mg/kg), $^{\phi}p < 0.05$ as compared to RIS (1 mg/kg) and $^{\$}p < 0.05$ as compared to SERT (10 mg/kg). PIR - Piracetam, RIS - Risperidone, SERT - Sertraline.

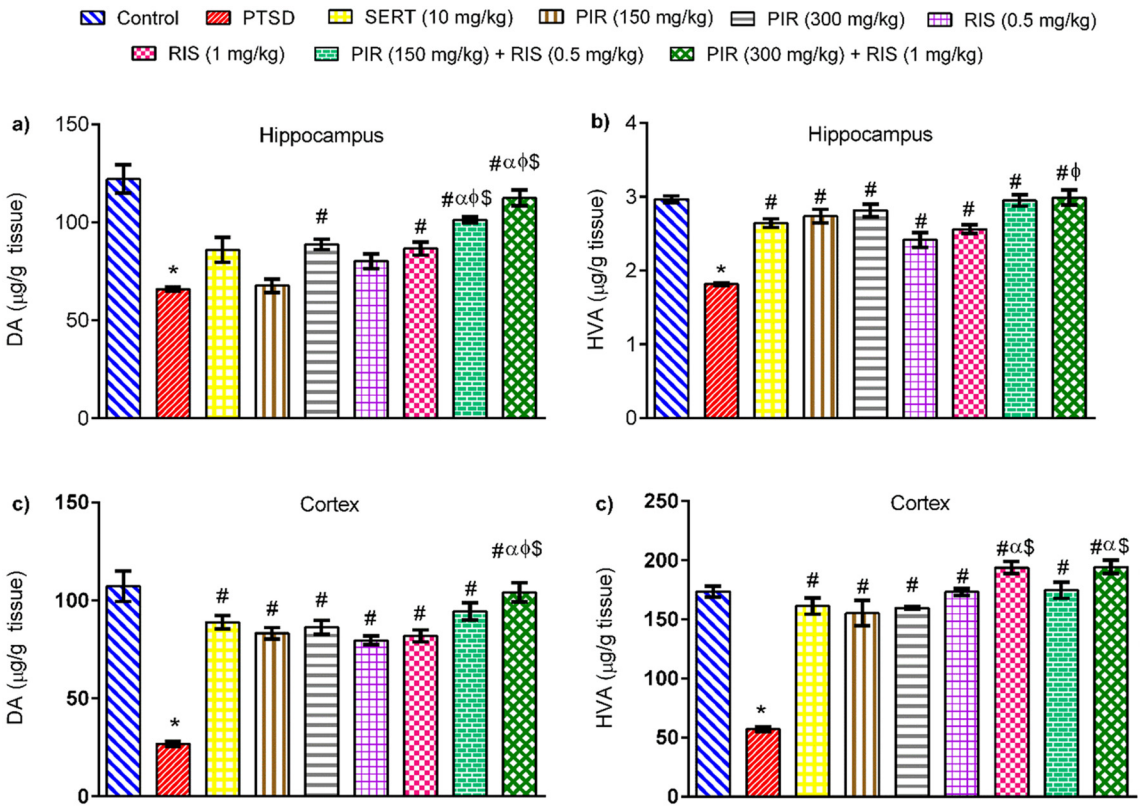

Fig. 13. Effect of piracetam, risperidone and their combinations on hippocampal a) DA b) 5-HVA and cortical c) DA d) HVA levels in PTSD-like phenotype rats: Data was analyzed using one-way ANOVA followed by Tukey's post hoc analysis. All values are expressed as mean ± SEM. $^{*}p < 0.05$ as compared to control, $^{\#}p < 0.05$ as compared to PTSD, $^{\alpha}p < 0.05$ as compared to PIR (300 mg/kg), $^{\phi}p < 0.05$ as compared to RIS (1 mg/kg) and $^{\$}p < 0.05$ as compared to SERT (10 mg/kg). PIR - Piracetam, RIS - Risperidone, SERT - Sertraline.

4. Discussion

The impaired extinction in PTSD is responsible for the dominance and persistence of fear memory and development of anxiety-like symptoms. In the current study we had used the pharmacological targeting of extinction which gives a relevance to the clinical implication of these agents with exposure therapy. Furthermore, this work has demonstrated the common molecular mechanisms of piracetam and risperidone that contribute to the significant suppression of PTSD pathophysiology. In the present study, rats exposed to SPS paradigm for 7 days earlier to the fear conditioning protocol, showed impaired extinction of the fear responses. In the presence of trauma associated reminders (CS), they re-experience the trauma which was evident by increased freezing behavior and untouched fear remission margin. This finding is consistent with previous observations of extinction deficit rats in the SPS paradigm (Noble et al., 2017; Yamamoto et al., 2008). Anxiety and its ramification (hyperarousal), are associated with PTSD and in the present study the anxiety-like behavior, assessed in EPM revealed that PTSD-like phenotype rats showed heightened anxiety as compared to control rats. Avoidance, which is also a core feature of PTSD, was assessed using marble burying test and PTSD-like phenotype rats showed the increased avoidance which is in line with the previous studies (Noble et al., 2017). Social withdrawal and decreased interest in activities is another symptom cluster of PTSD and rats showed social withdrawal after SPS exposure in the current study. Thus, the SPS model closely represents the PTSD-like phenotype, supporting previous reports regarding the good validity of SPS for induction of PTSD-like phenotype (Yamamoto et al., 2009). In the present study, the piracetam and risperidone were found to decrease the fear response to CS presentation at different doses and combinations. Whereas, the high dose combination achieved the fear remission earliest and hence significantly reversed the conditioned fear by enhancing the extinction phase. The combination of both also successfully managed the symptom cluster of PTSD (anxiety, avoidance-like behavior, social withdrawal).

Cortisol is a compensatory hormone which strengthens the stress combat system. Hypocorticosteronemia, one of the biomarkers for PTSD (Yehuda et al., 2000) is responsible for altered glucocorticoid receptor signaling leading to increased inflammation (Cohen et al., 2012). Thus, the dysregulation of the stress response system occurs in PTSD promoting neuropsychological aberrations (Yehuda, 2006). The stress re-stress paradigms were found to suppress the basal corticosterone levels in rats and our results were in line to these studies (Harvey et al., 2006; Liberzon et al., 1997a, 1997b). In the present study piracetam, risperidone and their combinations maintained the plasma corticosterone levels, while the potentiating effect was seen with high dose combination that successfully reversed the SPS induced decreased basal plasma corticosterone levels.

The oxidative and antioxidant system play a crucial role in maintaining the stress level in the brain and are linked with various harmful neuromodulations such as apoptosis and neuroinflammation (Garabadu et al., 2015a) which are also associated with PTSD (Wilson et al., 2013). SPS impaired antioxidant system (GSH, SOD and catalase levels), increased oxidative stress (LPO levels), and plasma nitrosative stress in PTSD-like phenotype group, which is in accordance to a previous report (Garabadu et al., 2015a). Piracetam, risperidone and their combinations successfully improved the antioxidant enzymatic activity of GSH, SOD, and catalase whereas, reduced the oxido-nitrosative stress. The additional stress compensating the effect of risperidone is supported by our study (Garabadu et al., 2015b). This reversal of oxido-nitrosative stress could be attributed to strengthened stress response system and homeostasis.

Synaptic plasticity is critical for the formation of new memories and

consolidation process. Brain-derived neurotrophic factor (BDNF) has been implicated in the neurobiological mechanisms of memory formation, synaptic plasticity and has a close correlation with clinical manifestations of PTSD (Heldt et al., 2007; Kozlovsky et al., 2007; Martinotti et al., 2015). In the present study, SPS-induced PTSD rats showed decreased hippocampal as well as cortical BDNF levels, while treatment with piracetam, risperidone, and their combinations increased BDNF levels in both the regions which are in line to the previous studies (Chen and Huang, 2011; Chen et al., 2017; Pandey and Garabadu, 2017; Rogóż et al., 2017). The extinction itself is a learning process, thus the increase in BDNF levels after treatment gives a representation of the piracetam and risperidone induced synaptic plasticity which could have contributed in extinction enhancement.

Neuroinflammation plays an important role in the symptomatic progression of fear and anxiety based symptom cluster in PTSD (Furtado and Katzman, 2015; Mendoza et al., 2016; Michopoulos et al., 2017). In the present study, the elevated hippocampal and cortical IL-6 and TNF-α levels were observed in SPS-induced PTSD-like phenotype rats, similar to previous findings (Lee et al., 2018; Liu et al., 2016). Piracetam and risperidone high dose combination significantly suppressed the neuroinflammation in the PTSD-like phenotype rats. Thus, our study supports the anti-inflammatory properties of piracetam and risperidone which is in line with previous studies (MacDowell et al., 2016; Tripathi et al., 2017).

PTSD-like phenotype rats in the study showed apoptosis in hippocampal and cortical regions which was evidenced by increased levels of caspase-3, supporting the previous studies (Li et al., 2010, 2013). The SPS induced elevation in hippocampal and cortical caspase-3 levels were attenuated by piracetam, risperidone, and their combination. This finding supports the anti-apoptotic effect of risperidone (Garabadu et al., 2015a; Kang et al., 2011) and also gives an insight, that high dose combination of piracetam and risperidone reverses the increased caspase-3 levels in the cortical and hippocampal brain areas.

Brain serotonergic and dopaminergic neurotransmission contributes to the extinction of aversive memories (Raczka et al., 2011; Wellman et al., 2007). Moreover, the serotonergic system was found to be impaired in PTSD patients (Maes et al., 1999). Antipsychotics such as risperidone, having a higher affinity to block the $5HT_{2A}$ receptors than D_2 receptors were found to increase the extracellular dopamine level (Kuroki et al., 1999). In the current study, the increase in cortical and hippocampal serotonin and dopamine levels were observed with piracetam, risperidone and combination treated PTSD-like phenotype rats. Brain serotonergic, as well as the dopaminergic system, is found to be directly linked with behavioral changes especially related to anxiety and negative mood alterations (Albert et al., 2014; Grace, 2016).

In a nutshell, SPS model exhibited PTSD-like symptom cluster and similar molecular mechanism involved in pathophysiology as seen in humans. SPS-induced hypocorticosteronemia increased oxido-nitrosative stress and neuroinflammation in cortical and hippocampal brain regions, which further initiated apoptotic cascades. Fall in levels of BDNF, 5-HT, and dopamine in cortex and hippocampus leads to a symptomatic progression of PTSD. Piracetam, a positive allosteric modulator of AMPA receptor and risperidone, a $5HT_{2A}$ antagonist combined treatment showed a protective effect against SPS-induced PTSD by increasing BDNF, 5HT and dopamine levels at cortex and hippocampus and strengthening extinction process and suppression of the aversive memories and a symptom cluster of PTSD. Furthermore, anti-inflammatory and anti-apoptotic effect also attenuated the progression of the disease and lead to the functional restoration of the cortex and hippocampus, promoting the therapeutic efficacy of regimen.

5. Conclusion

The present study concludes that the single prolonged stress paradigm is an efficient animal model for PTSD and ensures various symptomatology as well as biomarker representations which closely resemble that seen in PTSD patients. Piracetam and risperidone alone have limited efficacy to suppress the symptoms and pathophysiology of PTSD, while their combination successfully reversed cortical and hippocampal functioning, strengthens the stress response system and suppressed the symptoms of PTSD. Thus, in order to enhance the therapeutic outcome of exposure therapy, piracetam and risperidone combined treatment based multidimensional targeting can be used as an effective adjunct with exposure therapy.

Declaration of competing interest

The authors declare that there is no conflict of interest.

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