Evaluation of metformin, sertraline and their combination in the co-occurring condition of type-2 diabetes mellitus (T2DM) and post-traumatic stress disorder (PTSD)

Introduction

In the earlier objective, we have evaluated the effect of combination of drugs in co-occurring condition of stress and T2DM. We found that the combination of drugs showed better pharmacological effect than their individual use. PTSD is a clinically defined stress disorder according to DSM-V. Now, we evaluate the effect of combination of anti-PTSD drug SER and anti-diabetic drug MET in co-occurring condition of T2DM and PTSD.

Diabetes mellitus (DM) is associated with several psychiatric disorders such as depression, anxiety and post-traumatic stress disorder (PTSD; Farr et al., 2015; Farr et al., 2014; Rao et al., 2014). The pathophysiology of T2DM causes dysfunction in several organs including brain (Uysal et al., 2005; Gürpınar et al., 2012). Moreover, clinical studies demonstrate that the prevalence of anxiety symptoms is considerably higher in T2DM patients (Ball et al., 2002; Collins et al., 2009; Sharma et al., 2010; Kahl et al., 2015; Browne et al., 2015).It has also been suggested that PTSDcould be a marker of neuroendocrine and metabolic dysregulation which may cause T2DM (Vaccarino et al., 2014). However, there is no report on the effect of PTSD on insulin resistance and related mitochondria-linked PI3K/Akt/GLUT-4 signaling after the induction of T2DM.

Mitochondria dysfunction and IR are the cardinal features of the pathophysiology of T2DM and neuropsychiatric disorders (Jurysta et al., 2013; Bazotte et al., 2014; Petersen et al., 2003; Lowell and Shulman, 2005; Kim et al., 2008; Johannsen and Ravussin, 2009). It has been well suggested that there exist a relationship between mitochondria function and phopstidylionositol-3 kinase/Akt/glucose transporter-4 (PI3K/Akt/GLUT-4) signaling in several tissues including brain (Furtado et al., 2002; Watson et al., 2004; Benomar et al., 2006; Leney et

al., 2009;Jurysta et al., 2013; Blázquez et al., 2014). Substantial literature review suggests that $GABA_A$ receptor activation facilitates the PI3K/Akt/GLUT-4 signaling through calcium-dependent mechanism (Soltani et al., 2011). Moreover, 5-HT2A receptor activation inhibits the phosphorylation of Akt and thereby attenuates the PI3K/Akt/GLUT-4 signaling (Li et al., 2013).

Metformin exerts anxiolytic activity in addition to anti-diabetic activity (Garabadu and Krishnamurthy, 2014). Sertraline attenuates the PTSD-like manifestations and also mitigates the PTSD-induced IR (Panahi et al., 2011; Wilson et al., 2014; Zhang et al., 2015; Erenmemisoglu et al., 1999; Khanam and Pillai, 2006; Mahmood et al., 2010). Moreover, both of these drugs have substantial effect on the PI3K/Akt/GLUT-4 signaling in experimental animal models (Garabadu and Krishnamurthy, 2014; Panahi et al., 2011; Wilson et al., 2014). Hence, it can be assumed that both of these drugs perhaps facilitate the tissue level insulin sensitivity. However, there is no report of their combination on the mitochondria-linked PI3K/Akt/GLUT-4 signaling in the co-occurring condition of T2DM and PTSD.

Therefore, the present study evaluated the therapeutic effectiveness of metformin, sertraline and their combination on mitochondria-linked PI3K/Akt/GLUT-4 signaling in parallal to IR and PTSD-like behavioral manifestations in animals.Further, the efficacy of these drugs was proposed to evaluate on $GABA_A$ and $5\text{-}HT_{2A}$-mediated PI3K/Akt/GLUT-4 signaling in these animals.

Materials and methods

Animals

Male adult Charles Foster rats (200 ± 20 g) were procured from the Central Animal House, Institute of Medical Sciences, Banaras Hindu University (BHU) and housed in polypropylene cages under controlled environmental conditions with a 12 hr light/dark cycle. The experimental

animals had free access to food and water *ad libitum*. The experiment was conducted in accordance with the principles of laboratory animal care (2011) guidelines. Experiments on animals were approved by the Institutional Animal Ethics Committee of BHU, Varanasi, India (Protocol No: Dean/11-12/CAEC/328).

Chemicals and reagents

Sertraline and metformin were purchased from Sigma (St. Louis, MO, USA). Antibodies such as 5-HT_{2A}, $GABA_A$, phosphor-Akt^{ser473} (p-Akt), total Akt, GLUT-4 and beta-actin were purchased from Abcam Plc., Cambridge, USA. All other chemicals and reagents were available commercially from local suppliers and were of analytical grade.

The co-occurring T2DM and PTSD (DMP) paradigm

Briefly, the T2DM was induced in overnight fasted rats by a single injection of streptozotocin (45mg/kg, i.p.), 15 min after nicotinamide (110mg/kg, i.p.) administration. Streptozotocin was dissolved in 0.1M citrate buffer (pH4.5) and nicotinamide was dissolved in physiological saline (Masiello et al., 1998). Further, rats were individually exposed to 2 hr restraint in an animal holder, followed by 20 min forced swimming (25 °C) and halothane (0.8 ml of 4% halothane) anesthesia until the loss of consciousness on D-7 of streptozotocin injection. The rats were "re-stressed" by exposure to the forced swimming procedure for 20 min on D-13, D-19, D-25, D-31 and D-37 of the experimental protocol (Krishnamurthy et al., 2013). The immobility period was evaluated during last re-stress session as a measure of depression-like symptom. Immobility was defined as sustained immobility except for respiratory movements.

Experimental protocol

The whole experimental designs consisted of three sub sets of experiment and were scheduled for 37 days. Briefly, animals were divided into six groups with six each and named control, DMP, DMP+MET, DMP+SER and DMP+MET+SER. On Day-1 (D-1), all animals were

exposed to trial session of elevated plus maze (EPM). Except control group animals, all other group rats were exposed to modified SRS paradigm. Metformin (25.0 mg/kg, p.o.; Garabadu and Krishnamurthy, 2014), sertraline (10.0 mg/kg, p.o.; Wilson et al., 2014) and their combination were administered orally through oral gavage 1 hr after re-stress session to DMP+MET, DMP+SER and DMP+MET+SER group animals from D-13 to D-37 respectively. In experiment-1 and 2, all animals were subjected to oral glucose and insulin tolerance tests on D-37. In experiment-3, on D-37, after 2 hr of re-stress session the animals were subjected to EPM test followed by Y-maze test. There was a lag of 5 min between the behavioral tests. All the behavioral observations were recorded and quantified with ANY-maze^{TM} (Version-3.72; USA) video tracking system. Thereafter animals were killed and then plasma was collected and stored at -80 °C for corticosterone estimation. The brain was microdissected (Palkovits and Brownstein, 1988) into hippocampus (HIP), hypothalamus (HYP), pre-frontal cortex (PFC) and amygdala (AMY) and the brain parts were stored at -80 °C for further analysis.

Oral glucose tolerance test (OGTT)

Oral glucose tolerance test (OGTT) is considered as a classical and model-based estimate of beta-cell function (Rijkelijkhuizen et al., 2009). The OGTT was performed on overnight fasted rats on 13th day of the experimental schedule. Drug or vehicle was given 60 min prior to glucose administration (2 g/kg, i.g.). The blood samples were collected through retro-orbital puncture just before glucose load (0 min) and at 30, 60 and 120 min after glucose administration. Plasma glucose concentrations were determined with glucose GOD PAP kit (Priman Instrument Pvt. Ltd., India) based on glucose oxidase method (Wang et al., 2013).

Insulin tolerance test (ITT)

Insulin tolerance test (ITT) is a simple and reliable method of estimating insulin sensitivity (Duseja et al., 2007; Muniyappa et al., 2008). The ITT was performed on overnight fasted rats on 13^{th} day of the experimental schedule. Drug or vehicle was given 60 min prior to insulin administration (0.4 IU/kg, s.c.). The blood samples were collected through retro-orbital puncture just before insulin load (0 min) and at 30, 60 and 90 min after insulin administration. Plasma glucose concentrations were determined with glucose GOD PAP kit (Priman Instrument Pvt. Ltd., India) based on glucose oxidase method (Wang et al., 2013).

Insulin resistance and β-cell function

Homoeostasis model assessment (HOMA) of IR (HOMA-IR)and HOMA of β-cell function (HOMA-B) were calculated by the HOMA method (Matthews et al., 1985) using the following equations: IR (HOMA-IR) = ¼ (fasting glucose (mmol/l) × fasting insulin(μIU/ml))/22·5, and β-cell function (HOMA-B) = ¼ (20 × fasting insulin (μIU/ml))/(fasting glucose (mmol/l) – 3·5).

Assessment of behavioral performance

Evaluation of anxiolytic activity in EPM

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

Evaluation of spatial recognition memory in Y-maze test

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

Estimation of intracellular calcium

Briefly, tissues wereharvested andagitatedin an ice-cold Hank' balanced salt solution (HBSS), containing 200 UI/mL collagenase (Type IA, Sigma), albumin (0.5 mg/mL) and 2.7 mM $CaCl_2$, for 60–120 min at 37 °C, in a water-saturated atmosphere of 95% air and 5% CO_2 for the collagenase withdrawal. Thereafter, the cells were loaded with Krebs/Hepes buffer solution (mM: 143.3 Na^+; 4.7 K^+; 2.5 Ca^{2+}; 1.3 Mg^{2+}; 125.6 Cl^-; 25 HCO^{3-}; 1.2 H_2PO^{4-}; 1.2 SO_4^{2-}; 11.7 glucose and 10 HEPES; pH 7.4) followed by 10 µM acetoxymethylester fura-2 (AM form, dissolved in dimethylsulfoxide; Molecular Probes, USA) and 5 µl (10% in DMSO) pluronic acid, and agitated to attain a complete deesterification of the probe. The cell number was adjusted to 0.8 million cells/mL. The alteration in the fluorescence intensity was monitored in Hitachi fluorescence spectrophotometer (model F-2500) by alternative excitation at 340/380 nm. The emission was estimated at 510 nm and the ratio of the emitted light from the 2 wavelengths (R) was used as a measurement of $[Ca^{2+}]_i$ (Grynkiewicz et al., 1985). The intensity of fluorescence was calculated automatically. The Rmax and Rmin values were determined by addition of digitonin (50 µM) and Mn^+ (2 mM) + EGTA (5 mM), respectively. The $[Ca^{2+}]_i$ levels were expressed as nM and calculated by using the following formula: $[Ca^{2+}]_i = 224 \times [(R - Rmin)/(Rmax - R)]$.

Assessment of mitochondrial integrity, function and oxidative stress

Isolation of mitochondria from discrete rat brain tissues

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

Estimation of mitochondrial bioenergetics

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

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

The activity of NADH dehydrogenase (complex-I) was measured by catalytic oxidation of NADH with potassium ferricyanide as an artificial electron acceptor at excitation and emission wavelengths for NADH were 350 nm and 470 nm, respectively (Shapiro et al., 1979). Activity of NADH dehydrogenase was expressed as nmole NADH oxidised/min/mg protein. The mitochondrial succinate dehydrogenase (SDH; complex-II) was determined by the progressive reduction of nitro blue tetrazolium (NBT) to an insoluble colored compound, diformazan at 570 nm (Sally et al., 1989). The SDH activity was expressed as micromole formazan

produced/min/mg protein. The activity of cytochrome oxidase (complex-IV) was measured in mitochondrial fraction in presence of reduced cytochrome c at 550 nm for 3 min (Storrie and Madden, 1990). Results were expressed as nmole cytochrome c oxidized/min/mg protein (ε_{550} = 19.6 $mmol^{-1}cm^{-1}$). The F1-F0 synthase (complex-V) was measured by incubating mitochondrial suspension in ATPase buffer (Griffiths and Houghton, 1974) and the phosphate content was measured (Fiske and Subbarao, 1925). Results were expressed as nmole ATP hydrolyzed/min/mg protein.

Evaluation of MMP in discrete brain regions

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

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

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

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

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

Western blot analysis

The preparation of cellular membrane fraction was performed as described previously (Nishiumi and Ashida, 2007). In brief, tissues were lysed with buffer A [Tris, pH 8.0, 50 mM; dithiothreitol, 0.5 mM; NP-40, 0.1% (v/v); protease inhibitors (phenylmethylsulphonyl fluoride, 1 mM; leupeptin, 5 $mg \cdot mL^{-1}$; and aprotinin, 5 $mg \cdot mL^{-1}$) and phosphatase inhibitors (NaF, 10 mM and Na_3VO_4, 1 mM)] and centrifuged at 1000 × *g* for 10 min at 4 °C. Pellets were re-suspended in NP-40 free buffer A in ice for another 10 min and re-centrifuged at 1000 × *g* for 10 min at 4 °C. The pellets were re-suspended in buffer A and placed in ice for 1 h and centrifuged at 16 000 × *g* for 20 min at 4 °C. The supernatant was collected as the plasma membrane fraction and stored at -80 °C until use. The supernatants from the first and second spins at 1000 × *g* were combined and centrifuged at 16 000 × *g* for 20 min at 4 °C. The resultant supernatant was collected and used as the cytosol fraction. Protein concentrations were determined according to Bradford (1976) in each fraction. A standard plot was generated using bovine serum albumin. An aliquot of each sample were electrophoresed in 10% SDS-PAGE gels for Akt, p-Akt and GLUT-4proteins, transferred to polyvinylidene fluoride membranes and probed with specific antibodies. The membrane was incubated overnight with rabbit anti-Akt (Abcam Plc., Cambridge, USA), anti-p-Akt (Abcam Plc., Cambridge, USA) and anti-GLUT-4 (Abcam Plc., Cambridge, USA) polyclonal primary antibody at a dilution of 1:1000. After detection with the desired antibodies against the proteins of interest the membrane was stripped with stripping buffer (25 mM Glycine pH 2.0, 2 % SDS for 30 min at room temperature) and re-probed overnight with rabbit anti β-actin (Santa Cruz Biotechnology Inc.; Santa Cruz, California, USA) polyclonal primary antibody at a dilution of 1:500 to confirm equal loading of protein. Further, membrane was probed with corresponding secondary antibodies. Immunoreactive band of proteins were detected by

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

Data analysis

All the data were mean ± standard error of the mean (S.E.M). The plasma glucose level at different time points in OGTT and ITT, and the data of total arm entries in trial-1 and 2, and percentage entries into known and novel arm in trial-2 in Y-maze in experiment-3 were evaluated by using repeated measure two-way analysis of variance (ANOVA) with Bonferroni Post-hoc test. Two-way ANOVA followed by Bonferroni Post-hoc test was performed to measure arm discrimination behavior between known and novel arm in Y-maze paradigm for each group. All other statistical analysis of data was done using one-way ANOVA with Newman-Keuls Post-hoc analysis to monitor significance among groups. $P<0.05$ were considered as significant.

Results

Effect of MET, SER and their combination on DMP-induced changes in the plasma glucose level during OGTT and ITT paradigm

Table-37 and 38 depicts the effect of MET, SER and their combination on DMP-induced changes in the plasma glucose level of rats at different time points after administration of glucose and insulin respectively. Repeated measures of two-way ANOVA revealed that there were significant differences for plasma glucose level in OGTT and ITT among groups ([$F(4, 100) = 196.2$; $p<0.05$] and [$F(4, 100) = 293.1$; $p<0.05$] respectively), time ([$F(3, 100) = 0.2$; $p<0.05$] and [$F(3, 100) = 2.1$; $p<0.05$] respectively) and there was a significant interaction between

group and time ([F (12, 100) = 0.8; p<0.05] and [F (12, 100) = 0.2; p<0.05] respectively). Post-hoc analysis showed that before loading of either glucose or insulin to the animals (0 min), DMP paradigm caused a significant increase in the level of plasma glucose compared to vehicle treated rats. Only, the combination of MET and SER treatment caused a significant decrease in the plasma glucose level in DMP exposed animals at 0 min of both OGTT and ITT paradigms. This effect of the combination on DMP-induced increase in the plasma glucose level in animals was persistent even after either glucose or insulin administration.

Effect of MET, SER and their combination on DMP-induced changes in depressive-like behavior during FST paradigm

Fig-49 depicts the effect of MET, SER and their combination on DMP-induced changes in the depressive-like behavior in terms of immobility period in FST paradigm. Statistical analysis revealed that there were significant differences in immobility period [F (4, 25) = 45.2, p<0.05] among groups. Post-hoc test showed that DMP paradigm increased the depressive-like behavior in rats compared to control animals. Monotherapy of MET and SER, and their combination significantly decreased the DMP-induced increase in the depressive-like behavior in rats. Moreover, their combination further significantly decreased the DMP-induced increase in the depressive-like behavior compared to their monotherapy.

Effect of MET, SER and their combination on DMP-induced changes in anxiety-like behaviors during EPM test paradigm

The effect of MET, SER and their combination on DMP-induced alterations in the anxiety-like behaviors in terms of percentage of entries and time spent into open arm is depicted in Fig-50. Statistical analysis revealed that there were significant differences in the percentage of entries [Fig-50 (A); F (4, 25) = 29.4, p<0.05] and time spent [Fig-50 (B); F (4, 25) = 18.3, p<0.05] into

open arm among groups. However, there were no significant differences in the total arm entries [Fig-50 (C); $F(4, 25) = 0.1$, $p>0.05$] during EPM test paradigm. Post-hoc test showed that DMP exposure caused a significant increase in the anxiety-like behaviors in the animals compared to vehicle administered rats. All the treated rats significantly decreased the DMP-induced increase in the anxiety-like behaviors. Further, the attenuation in the DMP-induced increase in the anxiety-like behaviors by the combination of MET and SER was higher compared to their monotherapy.

Effect of MET, SER and their combination on DMP-induced alterations in behaviors during Y-maze test paradigm

Fig-51 illustrates the effect of MET, SER and their combination on DMP-induced alterations in the total arm entries in trial-1 and 2 (curiosity; A), spatial recognition memory (B) and coping behavior to novel arm (anxiety-like behavior; C) in Y-maze test paradigm. Statistical analysis revealed that there were significant differences in curiosity among groups [$F(4, 50) = 30.0$, $p<0.05$] and trials [$F(1, 50) = 229.8$, $p<0.05$], a significant interaction between group and trial [$F(4, 50) = 1.9$, $p<0.05$]. Post-hoc test showed that the curiosity was higher in DMP subjected animals compared to control group rats. All the drug treated rats significantly decreased the DMP-induced increase in the level of curiosity in the rats. Further, the attenuation of the DMP-induced increase in the level of curiosity in the rats by the combination of MET and SER was higher compared to their monotherapy. There were significant differences in arm discrimination behavior between known and novel arm among groups [$F(4, 50) = 0.5$, $p<0.05$] and arms [$F(1, 50) = 23.2$, $p<0.05$], and a significant interaction between group and arm [$F(4, 50) = 63.0$, $p<0.05$]. Post-hoc test showed that the control animals exhibited significantly higher arm discrimination to novel arm as compared to known arm, indicating a gain in spatial recognition

memory. DMP exposed rats exhibited a loss in spatial recognition memory. Only, the combination of MET and SER treated animals showed a significant reversal in the DMP-induced loss in spatial recognition memory. Further, one-way ANOVA revealed that there were significant differences among groups in known [$F(4, 25) = 17.3$, $p<0.05$] and novel [$F(4, 25) = 77.2$, $p<0.05$] arm entries. Post-hoc analysis showed that DMP exposed rats showed significant increase and decrease in the percentage entries into known and novel arm respectively compared to vehicle administered animals. All drug treatment attenuated the DMP-induced increase and decrease in the percentage entries into known and novel arm respectively. Moreover, the combination of MET and SER caused a further attenuation in the DMP-induced increase and decrease in the percentage entries into known and novel arm compared to their monotherapy respectively. Moreover, statistical analysis revealed that there were significant differences in anxiety-like behavior among groups [$F(4, 25) = 26.5$, $p<0.05$]. Post-hoc test showed that DMP exposed animals exhibited significant increase in the anxiety-like behavior compared to vehicle treated rats. All drug treated group rats showed a significant reduction in anxiety-like behavior in DMP exposed animals. Moreover, the combination of MET and SER significantly further attenuated the DMP-induced increase in the anxiety-like behavior compared to their monotherapy.

Effect of MET, SER and their combination on DMP-induced alterations in the levels of plasma glucose, insulin HOMA-IR and HOMA-B

The effect of MET, SER and their combination on DMP-induced alterations in the levels of fasting blood glucose and insulin, and the extent of IR (HOMA-IR), and the function of pancreatic β-cell (HOMA-B) of rats are depicted in Table-39. Statistical analysis revealed that there were significant differences in the level of fasting blood glucose [$F(4, 25) = 77.6$; $p<0.05$],

insulin [F (4, 25) = 6.0; p<0.05], HOMA-IR [F (4, 25) = 48.8; p<0.05] and HOMA-B [F (4, 25) = 40.9; p<0.05] among groups. Post-hoc test showed that the levels of fasting blood glucose, insulin and HOMA-IR were significantly increased and the level of HOMA-B was significantly reduced in DMP exposed rats compared to control group animals. Only, the combination of MET and SER attenuated the DMP-induced changes in these parameters.

Effect of MET, SER and their combination on DMP-induced alterations in the levels of expression of 5-HT_{2A} receptor in discrete brain regions

Fig-52 depicts the effect of MET, SER and their combination on DMP-induced changes in the level of expressions of 5-HT_{2A} receptor in discrete brain regions. Statistical analysis revealed that there were significant differences in the level of expression of 5-HT_{2A} in HIP [F (4, 10) = 26.8; p<0.05], HYP [F (4, 10) = 30.4; p<0.05], PFC [F (4, 10) = 17.4; p<0.05] and AMY [F (4, 10) = 27.0; p<0.05] among groups. Post-hoc test showed that DMP paradigm caused a significant increase in the level of expression of 5-HT_{2A} receptor in all the rat brain regions compared to the vehicle treated animals. All the drug treated animals significantly attenuated the DMP-induced increase in the level of expression of 5-HT_{2A} receptor in all the rat brain regions. Moreover, the combination of these drugs further reduced the level of expression of 5-HT_{2A} receptors in all the DMP exposed rat brain regions compared to their monotherapy.

Effect of MET, SER and their combination on DMP-induced alterations in the level of expression of $GABA_A$ receptor in discrete brain regions

The effect of MET, SER and their combination on DMP-induced changes in the level of expressions of $GABA_A$ receptor in discrete brain regions is depicted inFig-53. Statistical analysis revealed that there were significant differences in the level of expression of $GABA_A$ in HIP [F (4, 10) = 14.3; p<0.05], HYP [F (4, 10) = 15.7; p<0.05], PFC [F (4, 10) = 3.7; p<0.05] and AMY [F

(4, 10) = 12.0; p<0.05] among groups. Post-hoc test showed that DMP paradigm caused a significant decrease in the level of expression of $GABA_A$ receptor in all the rat brain regions compared to the vehicle treated animals. None of the drug treated animals significantly caused any change in the DMP-induced decrease in the level of expression of $GABA_A$ receptor in any rat brain region.

Effect of MET, SER and their combination on DMP-induced alterations in the level of $[Ca^{+2}]_i$ in discrete brain regions

Fig-54 illustrates the effect of MET, SER and their combination on DMP-induced changes in the level of $[Ca^{+2}]_i$ in discrete brain regions. Statistical analysis revealed that there were significant differences in the level of $[Ca^{+2}]_i$ in HIP [F (4, 25) = 18.5; p<0.05], HYP [F (4, 25) = 32.1; p<0.05], PFC [F (4, 25) = 25.9; p<0.05] and AMY [F (4, 25) = 44.2; p<0.05] among groups. Post-hoc test showed that DMP paradigm caused a significant decrease in the level of $[Ca^{+2}]_i$ in all the rat brain regions compared to the vehicle treated animals. None of the drug treatment significantly caused any change in the DMP-induced decrease in the level of $[Ca^{+2}]_i$ in any rat brain region.

Effect of MET, SER and their combination on DMP-induced alterations in the mitochondrial function, efficiency and integrity in discrete rat brain regions

The effect of MET, SER and their combination on DMP-induced changes in the mitochondrial function (A), efficiency (B) and integrity (C) in terms of the level of RCR, ratio of ADP/O and the level of MMP in discrete rat brain regions are illustrated in Fig-55 respectively. Statistical analysis revealed that there were significant differences in the mitochondrial function, efficiency and integrity in HIP ([F (4, 25) = 16.9; p<0.05], [F (4, 25) = 14.5; p<0.05] and [F (4, 25) = 17.0; p<0.05] respectively), HYP ([F (4, 25) = 32.2; p<0.05], [F (4, 25) = 20.8; p<0.05] and [F (4, 25)

= 26.4; p<0.05] respectively), PFC ([F (4, 25) = 38.8; p<0.05], [F (4, 25) = 13.6; p<0.05] and [F (4, 25) = 32.7; p<0.05] respectively) and AMY ([F (4, 25) = 23.6; p<0.05], [F (4, 25) = 29.2; p<0.05] and [F (4, 25) = 42.9; p<0.05] respectively) among groups. Post-hoc test showed that DMP paradigm caused a significant decrease in the mitochondrial function, efficiency and integrity in all the rat brain regions compared to the vehicle treated animals. All the drug treated animals significantly attenuated the DMP-induced decrease in the mitochondrial function, efficiency and integrity in all the rat brain regions. Moreover, the combination of these drugs further increased the mitochondrial function, efficiency and integrity in all the DMP exposed rat brain regions compared to their monotherapy.

Effect of MET, SER and their combination on DMP-induced alterations in the extent of phosphorylation of Akt and translocation of GLUT-4 in discrete rat brain regions

Fig-56 depicts the effect of MET, SER and their combination on DMP-induced alterations in the extent of phosphorylation of Akt in terms of the level of expression of Akt (A) and p-Akt (C) in discrete rat brain regions. Statistical analysis showed that there were significant differences in the level of expression of p-Akt (D) and the ratio of p-Akt/Akt (E) in HIP ([F (4, 10) = 31.8; p<0.05] and [F (4, 10) = 39.1; p<0.05] respectively), HYP ([F (4, 10) = 14.0; p<0.05] and [F (4, 10) = 7.6; p<0.05] respectively), PFC ([F (4, 10) = 31.8; p<0.05] and [F (4, 10) = 39.1; p<0.05] respectively) and AMY ([F (4, 10) = 46.4; p<0.05] and [F (4, 10) = 47.4; p<0.05] respectively) among groups. However, there were no significant differences in the level of expression of Akt (B) in HIP [F (4, 10) = 0.1; p>0.05], HYP [F (4, 10) = 0.2; p>0.05], PFC [F (4, 10) = 0.1; p>0.05] and AMY [F (4, 10) = 0.7; p>0.05] among groups. Post-hoc analysis revealed that the DMP paradigm caused a significant decrease in the level of expression of p-Akt and the ratio of p-Akt/Akt in all the brain regions of the animals compared to control rats, indicating a loss in the

extent of phosphorylation of Akt in all the brain regions with DMP exposure. All the drug treated animals significantly attenuated the DMP-induced decrease in the extent of phosphorylation of Akt in all the rat brain regions. Moreover, the combination of these drugs further amplified the extent of phosphorylation of Akt in all the DMP exposed rat brain regions compared to their monotherapy.

The effect of MET, SER and their combination on DMP-induced alterations in the extent of translocation of GLUT-4 in terms of the levels of expression of GLUT-4 in membranous (A) and cytoplasmic (C) fraction of discrete rat brain regions is depicted in Fig-57. Statistical analysis revealed that there were significant differences in the levels of membranous (B) and cytoplasmic (D) GLUT-4, and their ratio (E; membranous/cytoplasmic) in HIP ([F (4, 10) = 23.7; $p<0.05$], [F (4, 10) = 81.8; $p<0.05$] and [F (4, 10) = 15.2; $p<0.05$] respectively), HYP ([F (4, 10) = 26.6; $p<0.05$], [F (4, 10) = 36.0; $p<0.05$] and [F (4, 10) = 8.3; $p<0.05$] respectively), PFC ([F (4, 10) = 32.1; $p<0.05$], [F (4, 10) = 80.8; $p<0.05$] and [F (4, 10) = 15.2; $p<0.05$] respectively) and AMY ([F (4, 10) = 79.5; $p<0.05$], [F (4, 10) = 25.0; $p<0.05$] and [F (4, 10) = 18.4; $p<0.05$] respectively) among groups. Post-hoc test showed that DMP paradigm significantly decreased the extent of translocation of GLUT-4 to membrane from cytoplasm in all the rat brain regions compared to vehicle administered animals. All the drug treatment group animals showed a significant reduction in the DMP-induced decrease in the extent of translocation of GLUT-4 in all the rat brain regions. Moreover, the combination of these drugs further augmented the extent of translocation of GLUT-4 in all the DMP exposed rat brain regions compared to their monotherapy.

Table-37: Effect of MET, SER and their combination on the DMP-induced increase in the plasma glucose level of rats in OGTT.

	Plasma glucose level (mmol/L)			
Groups	0 min	30 min	60 min	120 min
Control	3.6 ± 0.14	6.6 ± 0.06	7.4 ± 0.08	5.4 ± 0.11
DMP	24.3 ± 1.32 [a]	23.8 ± 1.13 [a]	24.2 ± 1.23 [a]	24.4 ± 1.13 [a]
DMP+MET	13.9 ± 1.05 [a]	12.3 ± 1.13 [a]	12.1 ± 1.33 [a]	12.3 ± 1.23 [a]
DMP+SER	12.5 ± 1.19 [a]	11.1 ± 1.32 [a]	11.9 ± 1.13 [a]	11.7 ± 1.14 [a]
DMP+MET+SER	5.3 ± 1.07 [b, c, d]	5.7 ± 1.22 [b, c, d]	6.1 ± 1.21 [b, c, d]	6.4 ± 1.27 [b, c, d]

All values are mean ± SEM (n = 6). [a]p<0.05 compared to control, [b]p<0.05 compared to DMP, [c]p<0.05 compared to DMP+MET and [d]p<0.05 compared to DMP+SER (repeated measures of two-way ANOVA followed by Bonferroni post hoc test).

Table-38: Effect of MET, SER and their combination on the DMP-induced increase in the plasma glucose level of rats in ITT.

	Plasma glucose level (mmol/L)			
Groups	0 min	30 min	60 min	90 min
Control	4.4 ± 0.16	2.2 ± 0.22	2.6 ± 0.33	2.8 ± 0.25
DMP	25.1 ± 1.23 [a]	24.7 ± 1.19 [a]	23.9 ± 1.14 [a]	23.1 ± 1.23 [a]
DMP+MET	15.3 ± 1.25 [a]	14.5 ± 1.21 [a]	14.3 ± 1.14 [a]	13.9 ± 1.18 [a]
DMP+SER	14.7 ± 1.09 [a]	14.1 ± 1.11 [a]	14.9 ± 1.04 [a]	14.1 ± 1.03 [a]
DMP+MET+SER	5.3 ± 1.13 [b, c, d]	3.2 ± 1.13 [b, c, d]	3.8 ± 1.02 [b, c, d]	3.1 ± 1.09 [b, c, d]

All values are mean ± SEM (n = 6). [a]p<0.05 compared to control, [b]p<0.05 compared to DMP, [c]p<0.05 compared to DMP+MET and [d]p<0.05 compared to DMP+SER (repeated measures of two-way ANOVA followed by Bonferroni post hoc test).

Table-39: Effect of MET, SER and their combination on the DMP-induced changes in fasting blood glucose and insulin level, HOMA-IR and HOMA-B indices of rats.

Groups	Fasting blood Glucose (mmol/L)	Fasting blood Insulin (pmol/L)	HOMA-IR	HOMA-B
Control	4.1 ± 0.21	74.2 ± 2.42	2.3 ± 0.53	163.5 ± 12.13
DMP	27.5 ± 1.39 [a]	124.4 ± 9.42 [a]	22.3 ± 1.49 [a]	16.7 ± 1.76 [a]
DMP+MET	15.3 ± 1.17 [a]	93.4 ± 8.73 [a]	10.8 ± 0.53 [a]	95.3 ± 1.15 [a]
DMP+SER	15.5 ± 1.08 [a]	94.3 ± 9.85 [a]	10.5 ± 1.37 [a]	94.9 ± 1.11 [a]
DMP+MET+SER	6.1 ± 1.03 [b, c, d]	74.8 ± 8.89 [b, c, d]	3.7 ± 1.33 [b, c, d]	143.9 ± 15.53 [a, b, c, d]

All values are mean ± SEM (n = 6). [a]p<0.05 compared to control, [b]p<0.05 compared to DMP, [c]p<0.05 compared to DMP+MET and [d]p<0.05 compared to DMP+SER (one-way ANOVA followed by Student–Newman–Keuls test).

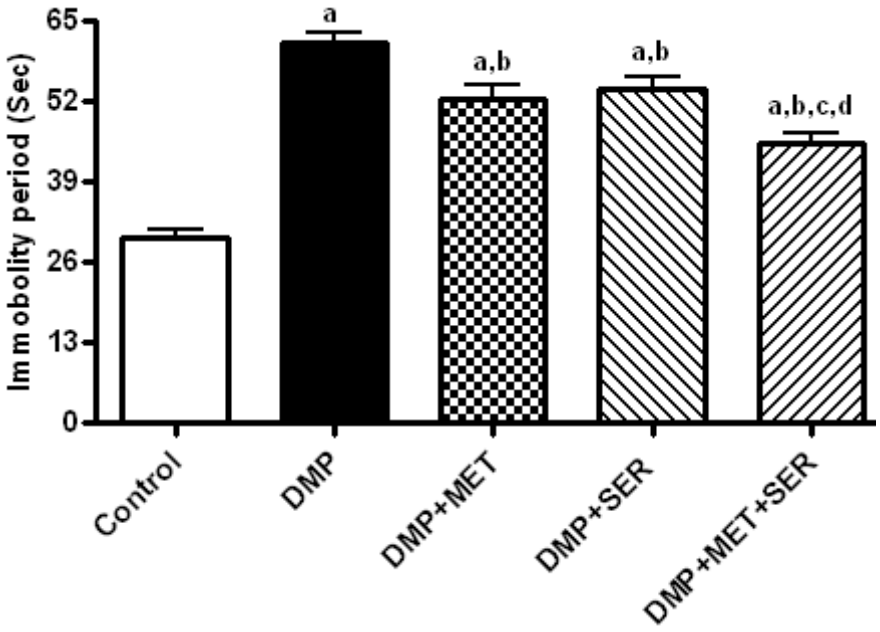

Figure 49: The effect of metformine, sertraline and their combination on SRS-induced changes in the immobility period in FST. All values are mean ± SEM (n = 6). [a]p<0.05 compared to control, [b]p<0.05 compared to DMP, [c]p<0.05 compared to DMP+MET and [d]p<0.05 compared to DMP+SER (one-way ANOVA followed by Student–Newman–Keuls test).

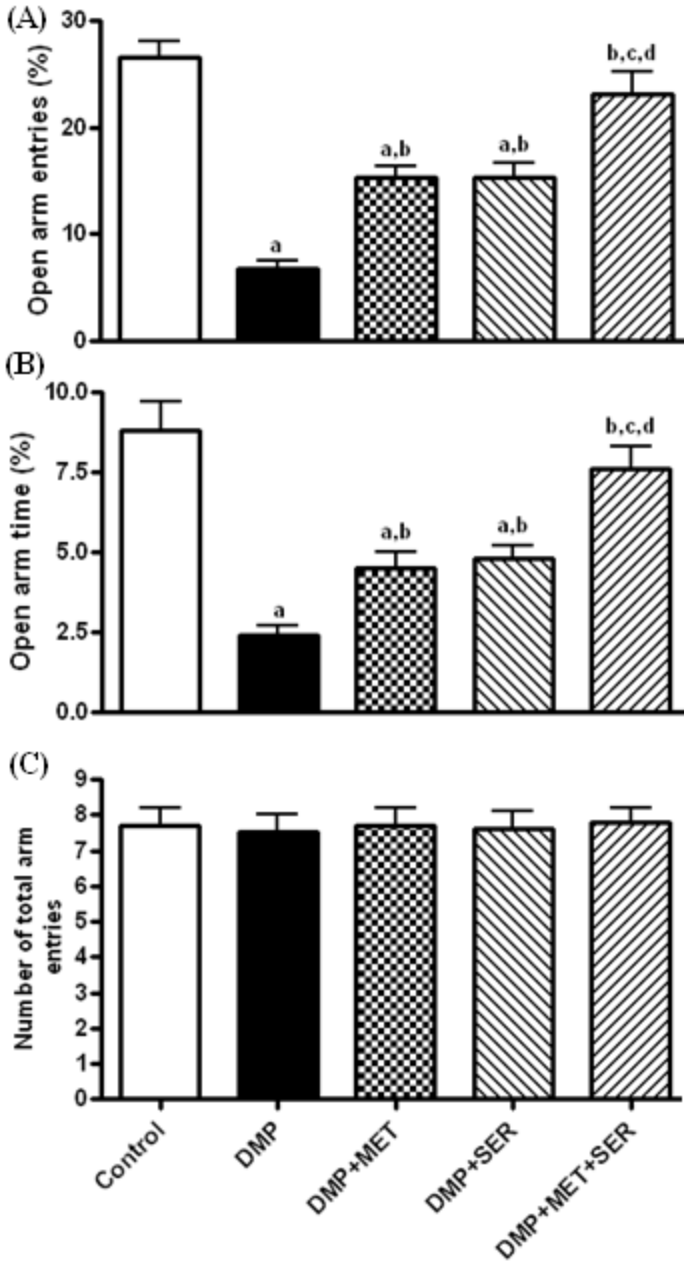

Figure 50: The effect of metformin, sertraline and their combination on SRS-induced changes in the percentage open arm entries (A), time spent (B) and total arm entries (C) in EPM. All values are mean ± SEM (n = 6). [a]$p<0.05$ compared to control, [b]$p<0.05$ compared to DMP, [c]$p<0.05$ compared to DMP+MET and [d]$p<0.05$ compared to DMP+SER (one-way ANOVA followed by Student–Newman–Keuls test).

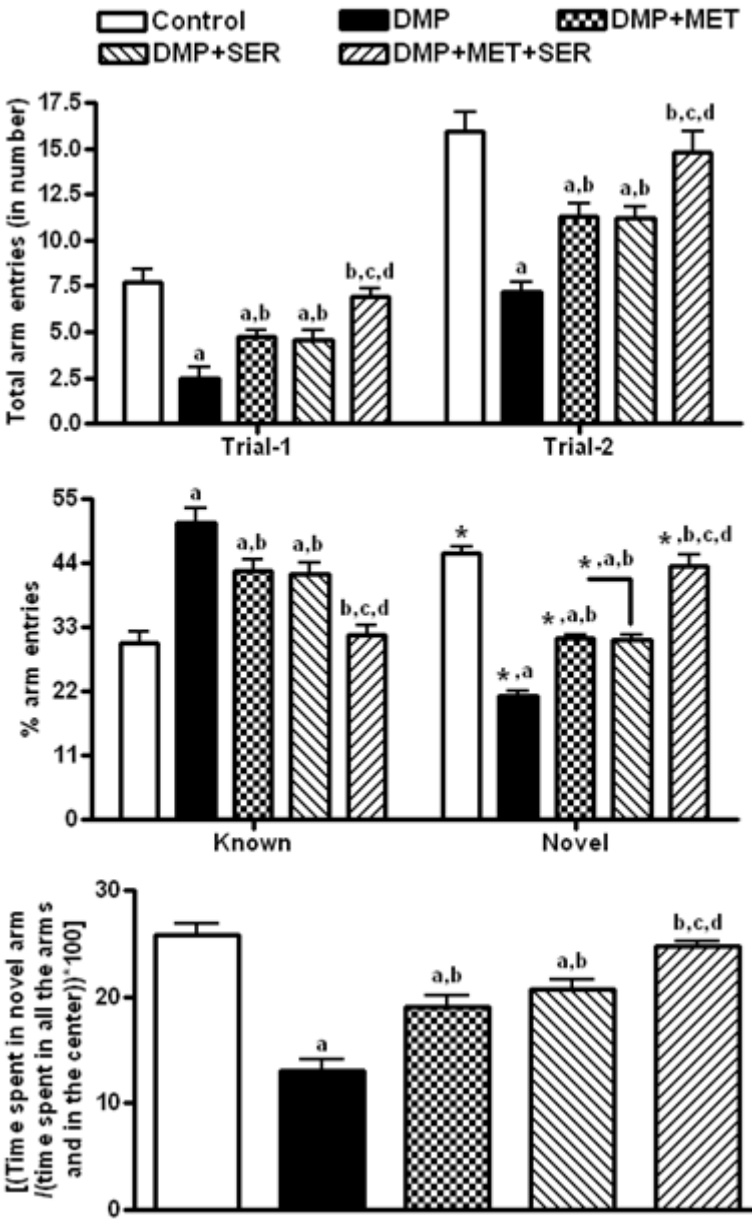

Figure 51: The effect of MET, SER and their combination on DMP-induced changes in the total arm entries in trial-1 and 2 (curiosity; A), spatial recognition memory (B) and coping behavior to novel arm (anxiety-like behavior; C) in Y-maze test paradigm. All values are mean ± SEM (n=5). $^{a}P<0.05$ compared to control, $^{b}P<0.05$ compared to DMP, $^{c}P<0.05$ compared to DMP+MET and $^{d}P<0.05$ compared to DMP+SER [Repeated measure two-way ANOVA followed by Bonferroni test for curiosity analysis and, percentage entries into known and novel arm. One-way ANOVA followed by Student Newmann-Keuls test was performed for the analysis of anxiety-like behavior]. $^{*}P<0.05$ compared to known arm entries [Two-way ANOVA followed by Bonferroni test].

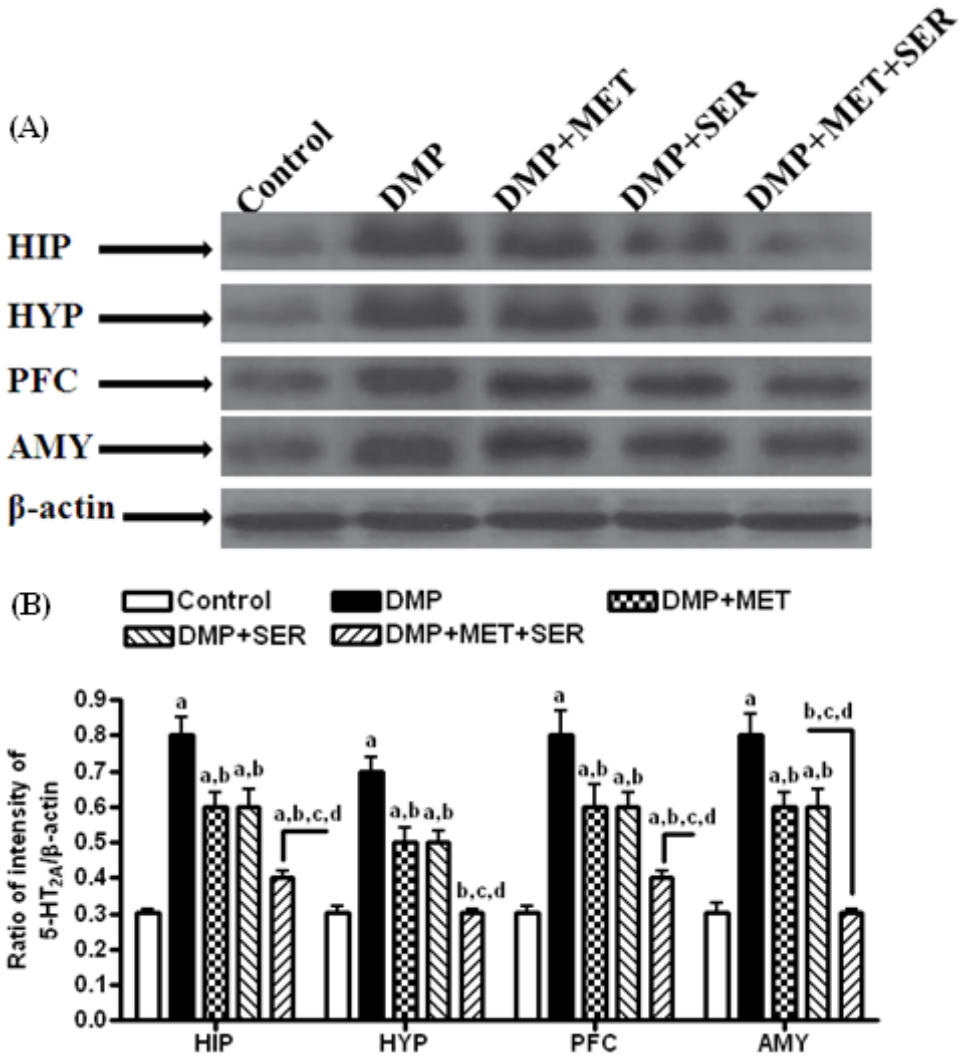

Figure 52: The effect of MET, SER and their combination on DMP-induced changes in the level of expression of 5-HT_{2A} (B) in HIP, HYP, PFC and AMY of rats. The blots are representative of 5-HT_{2A} (A) in HIP, HYP, PFC and AMY. The results in the histogram are expressed as the ratio of relative intensity of levels of expression of 5-HT_{2A} to β-actin. All values are mean ± SEM of three separate sets of independent experiments. [a]$p<0.05$ compared to control, [b]$p<0.05$ compared to DMP, [c]$p<0.05$ compared to DMP+MET and [d]$p<0.05$ compared to DMP+SER [One-way ANOVA followed by Student Newmann-Keuls Post-hoc test].

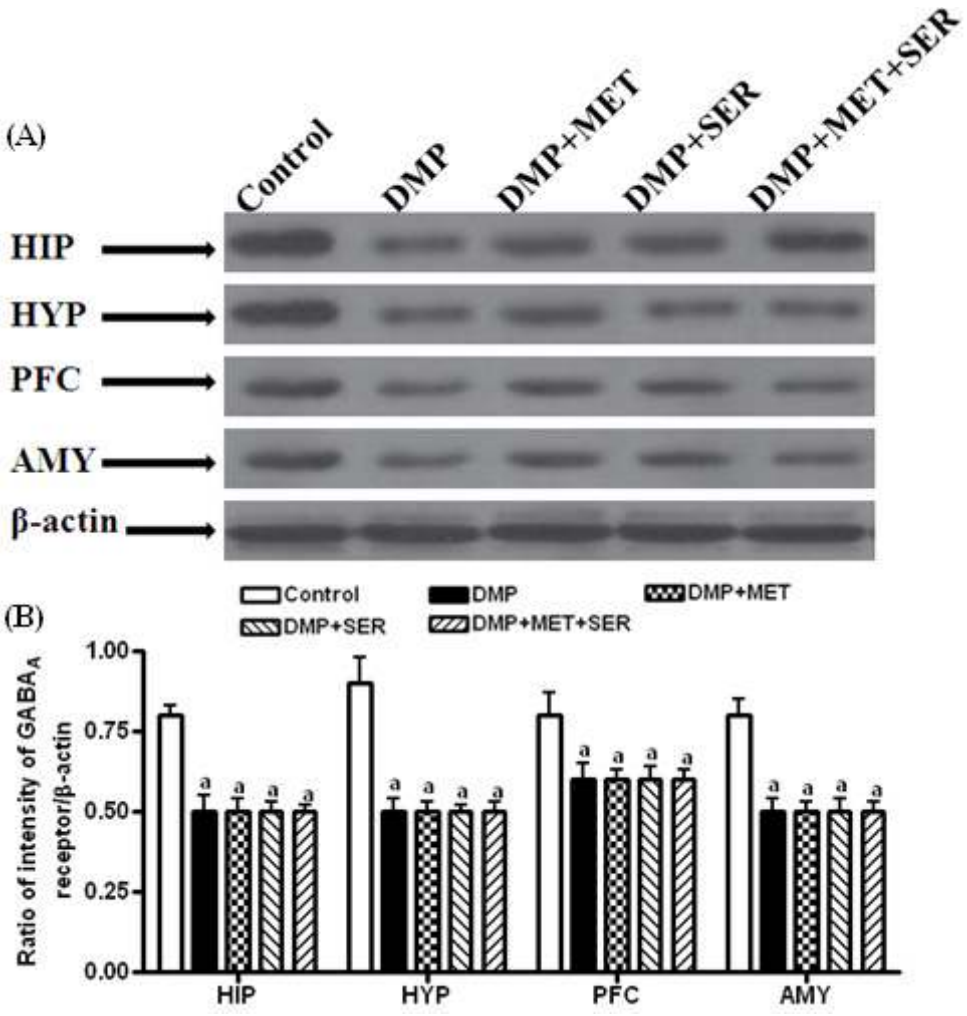

Figure 53: The effect of MET, SER and their combination on DMP-induced changes in the level of expression of $GABA_A$ (B) in HIP, HYP, PFC and AMY of rats. The blots are representative of $GABA_A$ (A) in HIP, HYP, PFC and AMY. The results in the histogram are expressed as the ratio of relative intensity of levels of expression of $GABA_A$ to β-actin. All values are mean ± SEM of three separate sets of independent experiments. $^{a}p<0.05$ compared to control, $^{b}p<0.05$ compared to DMP, $^{c}p<0.05$ compared to DMP+MET and $^{d}p<0.05$ compared to DMP+SER [One-way ANOVA followed by Student Newmann-Keuls Post-hoc test].

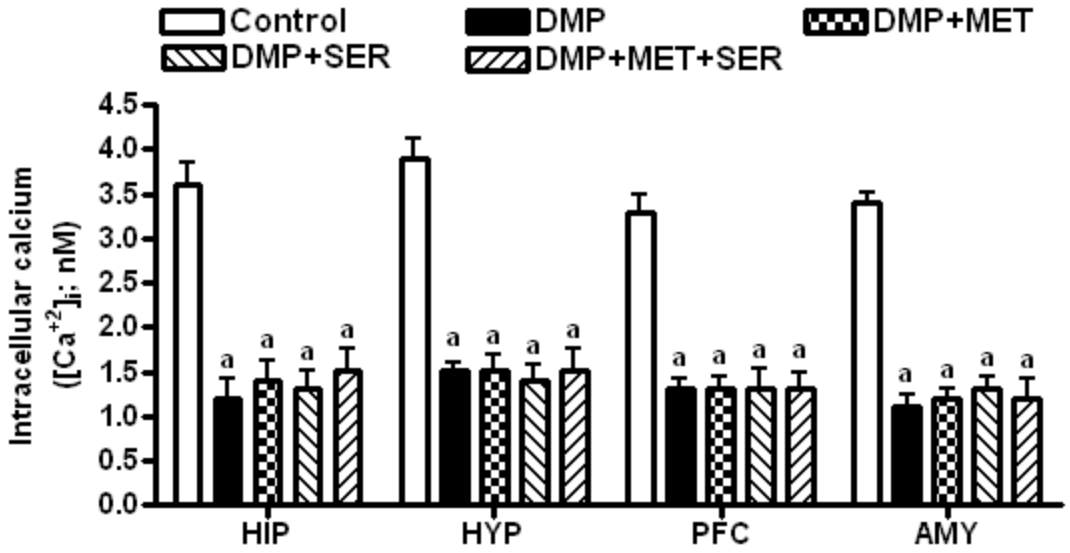

Figure 54: The effect of MET, SER and their combination on DMP-induced changes in the intracellular Ca^{+2} in discrete brain regions. All values are mean ± SEM (n = 6). $^{a}p<0.05$ compared to control, $^{b}p<0.05$ compared to DMP, $^{c}p<0.05$ compared to DMP+MET and $^{d}p<0.05$ compared to DMP+SER (one-way ANOVA followed by Student–Newman–Keuls test).

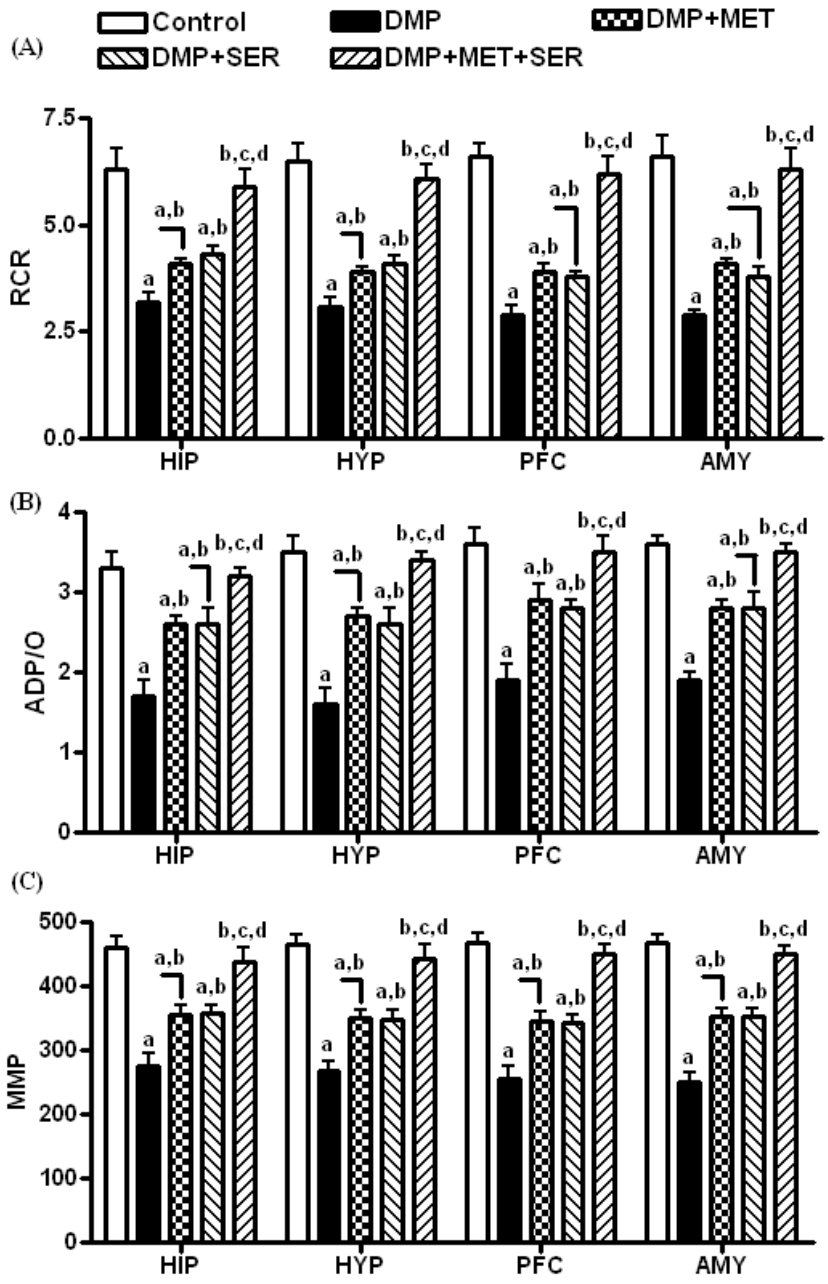

Figure 55: The effect of MET, SER and their combination on DMP-induced changes in the RCR (A), ADP/O (B) and MMP (C) in discrete brain regions. All values are mean ± SEM (n = 6). [a]p<0.05 compared to control, [b]p<0.05 compared to DMP, [c]p<0.05 compared to DMP+MET and [d]p<0.05 compared to DMP+SER (one-way ANOVA followed by Student–Newman–Keuls test).

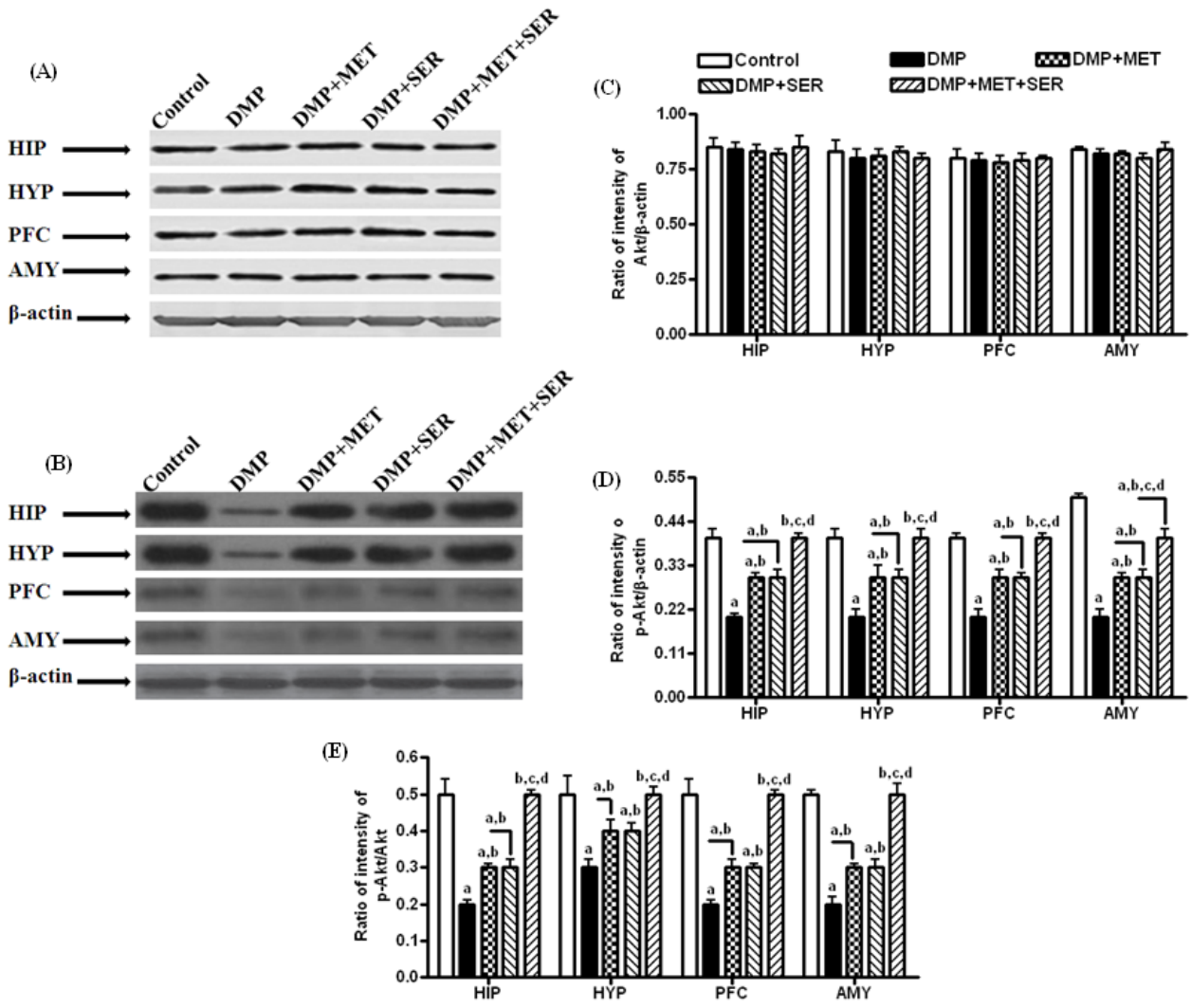

Figure 56: The effect of MET, SER and their combination on the level of expression of Akt (C) and p-Akt (D), and ratio of p-Akt to Akt (E) in HIP, HYP, PFC and AMY of control and T2DM rats. The blots are representative of Akt (A) and p-Akt (B) in HIP, HYP, PFC and AMY. The results in the histogram are expressed as the ratio of relative intensity of levels of expression of Akt or p-Akt to β-actin. All values are mean ± SEM of three separate sets of independent experiments. [a]$p<0.05$ compared to control, [b]$p<0.05$ compared to DMP and [c]$p<0.05$ compared to DMP+MET and [d]$p<0.05$ compared to DMP+SER [One-way ANOVA followed by Student Newmann-Keuls Post-hoc test].

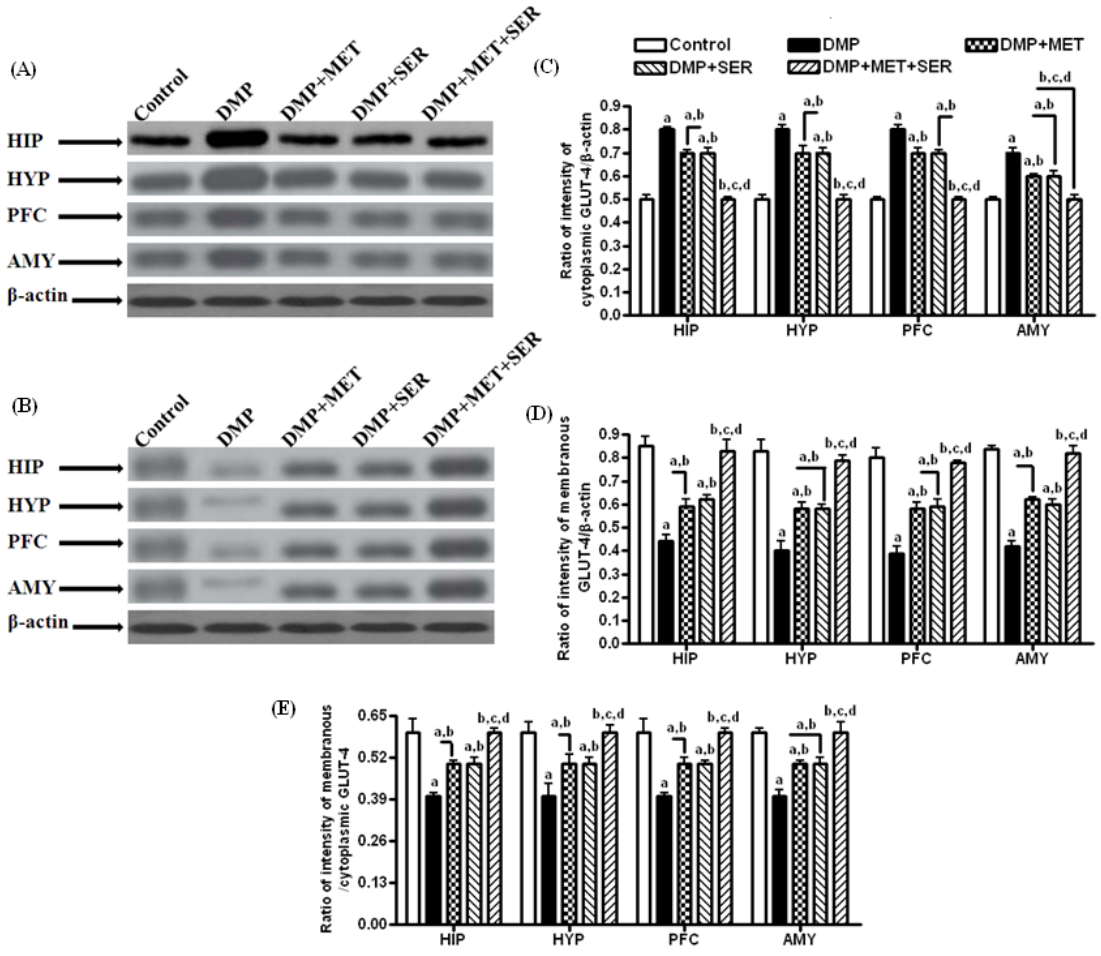

Figure 57: The effect of MET, SER and their combination on DMP-induced changes in the level of expression of cytoplasmic (C) and membranous (D), and ratio of membranous to cytoplasmic (E) GLUT-4 in HIP, HYP, PFC and AMY of rats. The blots are representative of cytoplasmic (A) and membranous (B) GLUT-4 in HIP, HYP, PFC and AMY. The results in the histogram are expressed as the ratio of relative intensity of levels of expression of cytoplasmic or membranous GLUT-4 to β-actin. All values are mean ± SEM of three separate sets of independent experiments. [a]$p<0.05$ compared to control, [b]$p<0.05$ compared to DMP, [c]$p<0.05$ compared to DMP+MET and [d]$p<0.05$ compared to DMP+SER [One-way ANOVA followed by Student Newmann-Keuls Post-hoc test].

Discussion

The present study clearly demonstrates for the first time that the combination of metformin and sertraline was better in terms of reducing IR and PTSD-like behavioral manifestations than their monotherapy in the co-occurring condition of T2DM and PTSD (DMP). Further, the combination significantly attenuated the DMP-induced altered mitochondria-linked PI3K/Akt/GLUT-4 signaling in addition to 5-HT_{2A}-mediated mechanism than their monotherapy in all the brain regions. These results emphasize the fact that the combination of metformin and sertraline could be a promising candidate in the management of such co-occurring condition.

In the present study, there was significant existence of depression, anxiety and cognitive impairment in the co-occurring condition of DMP. Further, DMP induced significant hyperglycemia, hyperinsulinemia, insulin resistance and beta-cell dysfunction in these animals. Earlier reports suggest the presence of T2DM characteristics in PTSD animals (Cohen et al., 2009). However, this is the first co-occurring model that exhibits both the characteristics of T2DM and PTSD. The anxiolytic and anti-hyperglycemic activities are well established for both metformin and sertraline in several experimental animal models (Erenmemisoglu et al., 1999; Khanam and Pillai, 2006; Mahmood et al., 2010; Panahi et al., 2011; Garabadu and Krishnamurthy, 2014, Wilson et al., 2014; Zhang et al., 2015). It is interesting to note that the combination of these two drugs showed better therapeutic efficacy in this model, suggesting the effectiveness of the combination than their monotherapy in such condition.

The mitochondria dysfunction and the impairment in PI3K/Akt/GLUT-4 signaling are well documented in the pathophysiology of T2DM and PTSD (Garabadu and Krishnamurthy, 2014; Garabadu et al., 2015). Here, the DMP paradigm caused significant mitochondria dysfunction and the impairment in PI3K/Akt/GLUT-4 signaling in all the brain regions.

Metformin and sertraline individually improved the mitochondrial function and PI3K/Akt/GLUT-4 signaling in several experimental animal models. The combination of these drugs attenuated the DMP-induced alterations in the mitochondria function and PI3K/Akt/GLUT-4 signaling in all the brain regions than their monotherapy. These observations suggest the fact that the combination may exerts its therapeutic effect against IR and behavioral manifestations in such co-occurring condition possible through the synergistic effect of the individual constituent.

In the present study, DMP paradigm significantly increased and decreased the level of expression of 5-HT_{2A} and $GABA_A$ receptors in all the brain regions respectively. Neither metformin nor sertraline caused any change in the DMP-induced alterations in the expression of $GABA_A$ receptor and intracellular calcium in any brain regions of these rats. However, they individually attenuated the DMP-induced increase in the expression of 5-HT_{2A} receptor in all the rat brain regions. Interestingly, their combination further attenuated the DMP-induced increase in the expression of 5-HT_{2A} receptors in all the brain regions. These results indicate that the combination exerts the therapeutic activity through 5-HT_{2A}-mediated mechanism in addition to mitochondria-linked PI3K/Akt/GLUT-4 signaling in such co-occurring condition.

In conclusion, the combination of metformin and sertraline exhibited better pharmacological effect in terms of mitigating behavioral manifestations and IR in the co-occurring condition of T2DM and PTSD. Further, the combination attenuated the DMP-induced altered mitochondria-linked PI3K/Akt/GLUT-4 signaling and 5-HT_{2A} receptor density in all brain regions. Hence, it can be speculated that the combination of metformin and sertraline perhaps exerts synergistic therapeutic effect in terms of acting through mitochondria-linked PI3K/Akt/GLUT-4 signaling in such condition. Moreover, the combination may synergistically

disrupt the 5-HT_{2A}-mediated deleterious effect in such co-occurring condition. Thus, it would be a promising pharmacological option in the management of such co-occurring condition.