Chapter 4

Determine the effect of orexin antagonist on mitochondrial dynamics in rats

4.1 Introduction

Post-traumatic stress disorder (PTSD) is a neuropsychological disorder caused due to exposure to traumatic events (Koenigs et al., 2008). The clusters of events such as exposure of the traumatic event, re-experience of traumatic event, avoidance, hyperarousal and cognitive disturbances are mainly used to define PTSD (Battle, 2013). The pathophysiology of PTSD includes abnormalities in HPA-axis function and the monoaminergic system (Harvey et al., 2006). Typically psychotherapy and medication with SSRIs such as sertraline and paroxetine are prescribed for treatment purposes (Frommberger et al., 2004). However, these drugs are useful only for 30 percent of PTSD patients (Fani et al., 2009). Therefore, there is a need to ascertain a new pathology for a better treatment strategy for PTSD.

Evidence suggested that mitochondrial dysfunction is one of the pathological causes for PTSD (Garabadu et al., 2015, Zhang et al., 2015). Several reports indicate that mitochondrial dynamic plays an essential role in regulating energy balance, mitochondrial biogenesis, and homeostasis during a stressful condition (Perez-Ternero et al., 2017, Zemirli et al., 2018). However, there is a limited information on the alteration of mitochondrial dynamics in the experimental model of PTSD. Therefore, the assessment of mitochondrial dynamics in an animal model of PTSD would be a novel and important pathology of PTSD.

Mitochondrial dynamics are responsible for energy production in cells and are vital for cell survival, and mitochondrial dysfunction is a critical mechanism for the progression of a neurological disorder (de la Monte and Wands, 2006). Mitochondrial dynamics includes the process of fission and fusion, which is regulated by different proteins such as dynamin-related protein-1 (Drp-1), mitochondrial fission protein-1 (Fis-1), optic atrophy-1 (Opa-1), and mitofusin-1 and -2 (Mfn-1, Mfn-2) (Flippo and Strack, 2017). Drp-1 and

Fis-1 mediate the mitochondrial fission process, whereas Mfn-1, Mfn-2, and Opa-1 control fusion (Chen and Chan, 2009, Zemirli et al., 2018). The modulation of any one of the protein can cause an imbalance in mitochondrial dynamics and energy production (Chen and Chan, 2009). The dysfunction of mitochondrial dynamics directly impairs mitophagy, which plays a crucial role in the maintenance of healthy mitochondria. Hence, evaluation of mitophagy impairment through mitochondrial dynamics could be a valuable strategy in controlling mitochondrial homeostasis during PTSD conditions (Bartolomé et al., 2017). The mitochondrial dynamics is directly regulated by the mTORC-1 pathway (Morita et al., 2017). The mTORC-1 is a serine/threonine kinase that acts as a node for downstream signalling to regulate mitochondrial dynamics and protein translation in the body (Klann et al., 2004). Reports have suggested that the m-TOR facilitates mitochondrial fission by enhancing Drp-1 activity and reduces mitochondrial fusion (Lerner et al., 2013, Morita et al., 2017). The mechanism underlies the phosphorylation of the MTFP-1, a downstream substrate responsible for the activation of mitochondrial fission protein, i.e., Drp-1 (Morita et al., 2017). Dysregulation of the mTORC-1 pathway has been observed in neurological disorders such as stress, anxiety, and depression (Abelaira et al., 2014). Recent data suggests that the m-TOR is an important in the formation of fear memories by enhancing the phosphorylation of the downstream cascades such as p70S6K in the hippocampus and AMY (Parsons et al., 2006). The use of rapamycin significantly inhibits the acquisition and consolidation of associative fear memories (Blundell et al., 2008). Further, the m-TOR pathway is directly regulated by the orexinergic system and chronic stress conditions (Wang et al., 2014). Orexin-A aggravates cytotoxicity and mitochondrial impairment in SH-SY5Y cells via MAPK/m-TOR pathway (Li et al., 2020).

Orexin-A has been shown to regulate mitochondrial function and dynamics (Lassiter et al., 2015). We have reported the anti-PTSD-like effect of non-selective orexin antagonist, i.e., suvorexant. Suvorexant attenuated the dysregulation of hypothalamic HPA-axis, extrahypothalmic CRH system and serotonergic abnormities in the SRS model of PTSD (Prajapati and Krishnamurthy, 2020). However, the effect of suvorexant on mitochondrial dynamics and function in the pathology of PTSD has not been investigated. Therefore, it would be interesting to know whether orexin's pharmacological antagonism can affect mitochondrial dynamics in rats exhibiting PTSD-like symptoms.

This study investigated the role of mitochondrial dynamics in the SRS model of PTSD by assessing proteins and mRNA expression of fission (Drp-1and Fis-1) and fusion (Mfn-2, Opa-1) markers in AMY. Immunofluorescene assays were performed to confirm mitochondrial fission and fusion markers' localization in terms of fluorescence intensity ratio in AMY. Transmission Electron microscopy (TEM) analysis was performed to assess swollen mitochondria as a measure of mitophagy. mTORC-1 and MTFP1 expression along with plasma and CSF orexin-A were measured to relate the orexinergic system and mitochondrial dynamics in PTSD. Mitochondrial function was assessed through mitochondrial membrane potential, complex activity, and respiratory control rate (RCR). The effect of suvorexant and rapamycin on PTSD-like phenotypes such as cognitive inflexibility, fear response and anxiety-like behaviour were measured by using attentional set-shifting, contextual fear response, and EPM tests respectively. Further, the effect of rapamycin and suvorexant on HPA axis and the serotonergic system was measured by evaluating plasma corticosterone and serotonin level in the AMY.

4.2 Hypothesis

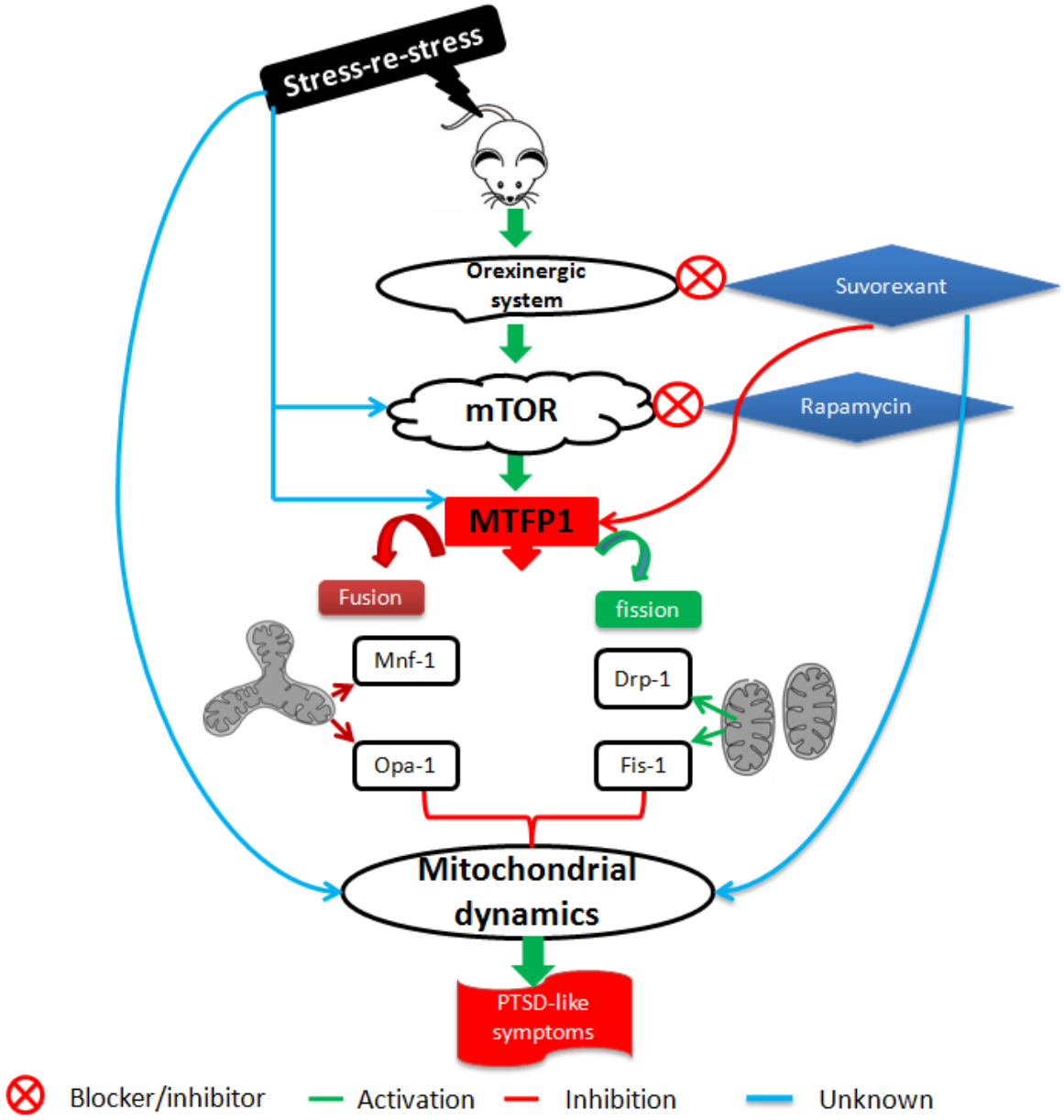

Figure 4.1 The proposed hypothesis of the effect of orexin antagonist on mitochondrial dynamics in rats

In our previous study, we have shown that orexin regulates the HPA-axis and monoaminergic function both of which are involved in the pathophysiology of PTSD. However, the downstream mitochondrial pathways of orexin signalling are not clearly understood. Evidence has suggested that orexin plays an important role in regulating mitochondrial dynamics and energy homeostasis by activating various downstream pathways such as m-TOR. Mitochondrial dysfunction is experimentally observed in the

pathology of PTSD. The activation of m-TOR phosphorylates the downstream signalling MTFP-1, a downstream substrate for activation of mitochondrial fission protein (Drp-1). Reports have suggested that the mTORC-1 facilitates mitochondrial fission by enhancing Drp-1 activity and reduces mitochondrial fusion (Opa-1 and Mfn-2) activity. However, no studies as of now have correlated mitochondrial dynamics to PTSD. Therefore, the assessment of mitochondrial dynamics in an animal model of PTSD would give new insight into PTSD pathology and be a pharmacological target for PTSD. Further, the assessment of mitochondrial mitophagy could help to know the effect of SRS exposure on mitochondrial morphology and whether orexin antagonist could affect mitophagy for the clearance of unhealthy mitochondria.

4.3 Materials and methods

4.3.1 Animals

Male Wistar rats (200 ± 20g) were obtained from Central animal house, IMS-BHU. Experiments on animals were approved by the Institutional Animal Ethical Committee, BHU, Varanasi, India. Rats were housed in a temperature-controlled room (25 ± 1ºC, the humidity of 45-55 %) with the light/dark cycle of 12 h. Water and feed are available at ad libitum during the experiment.

4.3.2 Experimental Design

We follow the modified SRS paradigm. In which the experiment was conducted for 32 days; the animals were divided into six groups (n=12/group), which include Control, SRS, Rapamycin (RAPA) (0.5 mg/kg), Suvorexant (SUVO) (10 mg/kg), Rapamycin+Suvorexant (RAPA+SUVO) and Sertraline (SERT) (10 mg/kg) through randomization. On Day-1 (D-1), all animals were exposed to a trial session of EPM. The procedure for SRS was similarly as performed on chapter 2 and 3 (Prajapati and Krishnamurthy, 2020).

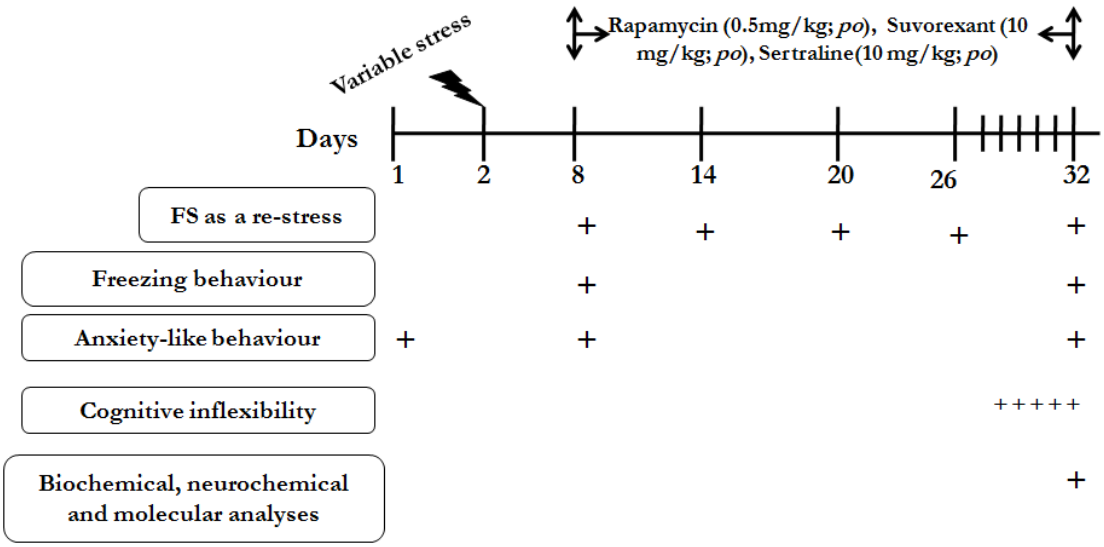

Figure 4.2 represents the experimental design of the study, which includes exposure of variable stress to all the groups except the control on D-2. Then the FS as a re-stress was given on D-8, 14, 20, 26, and 32. During the behavioural assessment, the animals were first subjected to EPM. Freezing behaviour was measured 6 h post-FS as re-stress cue. Test for cognitive inflexibility was performed for five days (D-28 to D-32). The biochemical and molecular analysis were performed on the last day (D-32) of the experiment.

4.3.3 Behavioural experiments

4.3.3.1 Assessment of contextual fear using FS

FS of 2mA for 10 sec was given in a plexiglass chamber (20×10×10 cm) of Inco Instrument and Chemicals Pvt. Ltd. This test was performed 6 h after the foot shock in stress and re-stress session on D-2, 8, and 32 (Prajapati and Krishnamurthy, 2020). Rats were placed in the foot shock apparatus for 5 min, and the freezing response was recorded as a measure of contextual fear.

4.3.3.2 Assessment of anxiolytic activity in EPM

EPM consist of four arms forming a cross or plus shape, out of which two arms are open (50×10 cm), and the other two are closed (50×10×40cm) respectively, at the height of 50cm from the ground. It was placed in a dimly lighted room where the experiment was performed. The number of open arm entries and the time spent were estimated as the intimation of anxiety-like behaviours. The test was started by placing the animals at the one end of the open arm facing away from the centre. EPM was performed on D-1, 2, 8 and D-32 of the experimental design (Prajapati and Krishnamurthy, 2020).

4.3.3.3 Assessment of attention set-shifting task

Attentional set-shifting is a widely used method for measuring cognitive flexibility (Mikics et al., 2008). The set-shifting box was fabricated rectangular shape with the dimension of 40x71x20 cm. During the testing session, the bowl with a digging medium was placed in each section. Each bowl was defined by a pair of cues along two stimulus dimensions, the digging medium, and odour. In all discrimination trials, a small quantity of powdered Cheerio was sprinkled onto the digging medium in the unbaited pot to eliminate the possibility that the rat may locate the bait by smell rather than by learning the discrimination. The procedure took five days (D-28 to D-32) for each rat.

Day 28-30, *Habituation:* On Day-1, rats were trained to dig in the ceramic pot for the food reward. Two unscented cups, both baited and covered with sawdust, were positioned in the home cage. Once the rat started digging, they were introduced to the test arena and given three trials for 5 min each to retrieve food from the sawdust covered-baited cups.

Day 31, *Training:* Rats were trained for simple discrimination (SD) tasks until it reached the six consecutive correct trials. In these trials, rats first have to learn to condition odour cues with the food reward. In this, both the pots were filled with sawdust, but only one was baited, and the rat has to learn to associate food with the odour cue.

Day 32, *Testing:* On this day, rats were tested for a series of discriminations with increasing difficulties. Testing at each stage continued until the criterion of six consecutive correct trials was reached. The test started with the SD stage, involving only one dimension discrimination (for example-odour, and digging medium). For CD, discrimination in two dimensions was introduced, but correct and incorrect exemplars remain constant. For the reversals (Rev.1, Rev.2, Rev.3), the exemplars of relevant dimensions remain unchanged, and the rat had to learn that the previous correct stimuli were now incorrect. For both ID and ED shift, there were a new set of examples introduced in both the dimensions, and also the relevant dimension become irrelevant and vice-versa in ED shift (Mikics et al., 2008).

4.3.4 Biochemical estimations

4.3.4.1 Corticosterone estimation in plasma

Quantification of plasma corticosterone was done with the help of HPLC-UV (Waters, USA), using dexamethasone as an internal standard. The mobile phase consists of methanol: water (70:30) at a flow rate of 1ml/min and CORT was detected at 250 nm using a UV detector (Model 2849, Waters, USA) (Krishnamurthy et al., 2011). Twenty

microliters of the plasma sample was injected in to HPLC system for separation of quantification of corticosterone.

4.3.4.2 Estimation of Plasma and CSF orexin-A level

Plasma and CSF orexin-A was quantified by Orexin-A ELISA kit (#E-EL-R0693, Elabscience, USA). The enzyme-substrate reaction was observed in terms of colour change and measured spectrophotometrically at 450±2 nm. Each sample was measured in triplicates. A standard curve was recognized using orexin-A standards between 0 and 2000 pg/ml. The minimal detectable concentration was 100 pg/ml. The concentration of orexin-A in the sample was determined with respect to the standard curve.

4.3.5 Assessment of mitochondrial function and dynamics

4.3.5.1 Isolation of Mitochondria

The mitochondrial fraction was isolated as per standard protocol. The amygdalar tissue was isolated and homogenised in isolation buffer (IB) containing 75Mm sucrose, 215mM mannitol, 20mM HEPES buffer, 0.1% BSA, and 1mM EGTA in distilled water, pH 7.2 and then centrifuged at 1300g for 5 min at 4ºC. The supernatant (S1) was collected while the pellet was dissolved in isolation buffer and again centrifuged at 1300g for 3min. Then the supernatant (S2) was collected, and the pellet was discarded. The supernatants S1 and S2 were taken in a single test tube and centrifuged at 14000g for 10min at 4ºC. Top off the supernatant and the pellet was re-suspended in IB without EGTA and again centrifuged at 14000g for 10 min. The mitochondrial pellet was collected, and protein was measured by Bradford assay.

4.3.5.2 Assessment of mitochondrial respiratory control ratio (RCR)

Mitochondrial functions were assessed using an oxytherm (Hansatech Instruments, UK). 20 µl of the mitochondrial sample was introduced to an oxytherm chamber containing respiration buffer (20mM HEPES, 0.1% BSA, 125mM KCl, 2.5mM KH_2PO_4, 2mM

$MgCl_2$, and pH 7.2) at 37ºC. Different mitochondrial states (S-2, S-3, S-4, S-5 complex-1, and S-5 complex- 2) were determined. RCR was estimated as a ratio of oxygen consumption during S-3 to S-4 (Samaiya et al., 2017).

4.3.5.3 Measurement of mitochondrial complex I, II, IV, and V activity

The electron transfer activity of complex-I (NADH dehydrogenase) was measured by catalytic oxidation of NADH at an excitation and emission wavelength of 350 and 470 nm, respectively. The reaction mixture consists of 10 mM potassium ferricyanide, 1 mM NADH in 2 mM potassium phosphate buffer at pH 8.5 was incubated for 5 min. The activity of complex-I was expressed as nanomole NADH oxidised/min/mg protein (Shapiro et al., 1982). The complex-II (succinate dehydrogenase, SDH) activity was estimated through the reduction of nitro blue tetrazolium (NBT) into formazan, at 570 nm. The complex-II activity was expressed as micromole formazan produced/min/mg protein (Old and Johnson, 1989).

The oxidation of ferrocytochrome-C determined the complex-IV (cytochrome-C oxidase) activity in assay buffer containing 50 mM phosphate buffer, 60 μM ferrocytochrome c at pH 7.4. The decrease in the rate of absorbance of ferrocytochrome-c at 550 nm was considered as an activity of cytochrome-c oxidase. The Complex IV enzyme activity was expressed as the oxidation rate of ferrocytochrome-c (nmol ferrocytochrome-c oxidized/min/mg protein) (Old and Johnson, 1989).

Complex-V activity was determined by measuring inorganic phosphate content in the mitochondrial fraction, incubated with ATPase buffer. The results were expressed as nanomole (nM) ATP hydrolysed/min/mg of protein.

4.3.5.4 Measurement ROS (Reactive oxygen species)

The mitochondrial pellet was collected and re-suspended in PBS. iROS was estimated by incubating with 100μl of 20mM dichloro-fluorescein-diacetate (DCFH-DA) for 45 min at

37ºC in the dark. After incubating, fluorescence was measured at excitation wavelength: 490nm and emission wavelength: 515nm. Values were normalised with the fluorescence intensity of normal cells.

4.3.6 Neurotransmitter measurement

4.3.6.1 Measurement of serotonin (5-HT) and its metabolite in AMY

The rats were sacrificed by decapitation, and the brains were removed and microdissected to obtain AMY (Krishnamurthy et al., 2013). The levels of 5-HT and its metabolites were quantified using HPLC/ECD similarly, as described earlier (Krishnamurthy et al., 2013).

4.3.7 Molecular analysis

4.3.7.1 mRNA expression of dynamic proteins

Total RNA was extracted from brain cortex using TRIzol solution (Invitrogen, USA) according to the manufacturer instructions. Reverse transcription was performed using Prime Script RT master mix. The specific primers for Opa-1, Mfn-2, Drp-1, Fis-1, and β-actin (an internal control) were as follows:

Opa-1, 5’ -CGCATAAAACTTAAAACTTTAC-3’(forward),
5’-CTTTGCGTAGTTGTATATAGC-3’ (reverse)

Mfn-2, 5’ -AGCCTGGTGAGTGTGATGTG-3’ (forward),
5’ -CTCCGTGGTGACATCGATCC-3’ (reverse)

Drp-1, 5’ -CCAGGAATGACCAAGGTCCC-3’ (forward),
5’ -CCTCGTCCATCAGGTCCAAC-3’ (reverse)
Fis-1, 5’ -TTTGAATACGCCTGGTGCCT-3’ (forward),
5’ -TACCTTTGGGCAACAGCTCC-3’ (reverse)

β-actin, 5’ -CACGGCATTGTCACCAACTG-3’ (forward),
5’ -AACACAGCCTGGATGGCTAC3’ (reverse).

RT-PCR was performed at 95◦C for 2 min, followed by 40 cycles at 95◦C for 3 s and 60◦C for 30 s. The relative mRNA expression levels of the particular genes were analysed using the $2^{-\Delta\Delta Ct}$ method normalized to mRNA levels for β-actin (Li et al., 2017).

4.3.7.2 Western blot expression of dynamic proteins

The aliquot of amygdalar protein samples were electrophoresed in 10 % SDS-PAGE gel for dynamic proteins (Opa-1, Mfn-2, Drp-1, Fis-1) and blotted onto PVDF membrane (Bio-Rad, CA, USA), which was was blocked with 3% bovine serum albumin for 2 h. The blocked membrane was washed thrice with 0.05% Tween-20 containing tris-buffered saline and incubated with primary anti-Opa-1 (rabbit) antibody (E-AB-63977, Elabscience, USA), Mfn-2 (rabbit) antibody (E-AB-32025, Elabscience, USA), Drp-1(rabbit) antibody (E-AB-31239, Elabscience, USA) and Fis-1(rabbit) antibody (E-AB-11241, Elabscience, USA), overnight at 4ºC. The treated membrane was subsequently incubated with HRP-conjugated anti-rabbit IgG antibody (E-AB-1003, Elabsciences USA) for 1 h. The dynamic proteins expression was visualised using ECL reagents in chemiluminescence detector (Fusion FX vilber lourmat) using A and B (1:1) and further quantified by densitometric analysis.

4.3.7.3 Immunofluorescence of dynamic proteins

The brain were perfused with cold 0.9 % NaCl followed by chilled 4 % paraformaldehyde in 0.01 M phosphate-buffered saline (PBS). The Coronal sections were made through the amygdala (2-3 mm posterior from bregma). The immunofluorescence assay was performed by keeping section with sodium citrate at 700ºC for 30 min followed by washing PBS. Then the sections were treated with methanol and incubated with blocking buffer (10% BSA) for 1h. Thereafter, the sections were incubated with primary antibodies anti-Mfn-2 (rabbit) antibody (E-AB-32025, Elabscience, USA) and anti-Drp-1(rabbit) antibody (E-AB-31239, Elabscience, USA) overnight at 40ºC and then mounted by using DAPI. The fluorescence image captured and quantified by Olympus DS-52 fluorescent microscopy.

4.3.7.4 Transmission Electron microscopy (TEM)

Midbrain sections of the perfused rats were identified and isolated according to rats brain atlas of Franklin and Paxinos-3rd Edition- 2008. Sections were cut in 1 mm thickness and kept in Karnovsky solution. Further samples were post-fixed in 1% osmium tetroxide (OsO4) for 1 hour at 4°C. Sections were dehydrated in acetone and cut with an ultramicrotome (Leica Ultracut UC7, Austria). Sections were further stained with alcoholic uranyl acetate and alkaline lead citrate for 10 minutes in each and viewed under Tecnai G2 20 high-resolution transmission electron microscope (Morgagni 268 D, Fei Company, The Netherlands). Images were digitally acquired by a CCD camera (Megaview III, Fei Company) using TIA software attached to the microscope for observing mitochondrial morphology.

4.4 Data analysis

Data of biochemical, neurochemical, and molecular studies were assessed by one-way ANOVA followed by Newman-Keuls Multiple Comparison Test, whereas two-way ANOVA followed by Bonferroni post-hoc test was used to evaluate the behavioural data. All the data are presented as mean±SD and $p<0.05$ was considered significant.

4.5 Results

4.5.1 Suvorexant and rapamycin reduces the contextual fear response

The contextual fear response was measured as indicative of hyperarousal and depicted in Figure 4.3. Two-way ANOVA indicated that, there was significant difference of contextual fear response among the groups [$F_{5,198}$ = 197.8, $p<0.05$] with duration [$F_{2,198}$ = 112.6, $p<0.05$] and their interaction [$F_{10,198}$ = 15.85, $p<0.05$]. There was no significant difference in contextual fear response was observed on D-1. Post-hoc test represented that there was a significant increase in contextual fear response following SRS exposure compared to control. Treatment with suvorexant and rapamycin significantly decreased the contextual fear response on D-32. However, combination (suvorexant + rapamycin) and sertraline induced reduction in contextual fear response was significant compared to individual treatments on D-32.

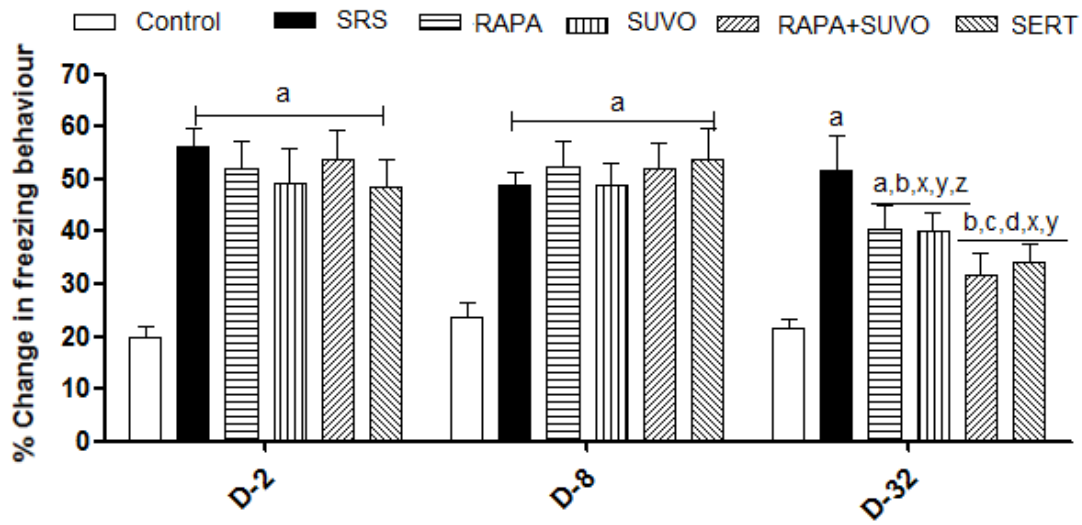

Figure 4.3 the effect of suvorexant and rapamycin on SRS-induced changes in contextual fear response on different days. All values are in mean±SD (n=12; number of rats). [a]$p<0.05$ compared to control, [b]$p<0.05$ compared to SRS, [c]$p<0.05$ compared to RAPA, [d]$p<0.05$ compared to SUVO, [x]$p<0.05$ compared to D-2 and [y]$p<0.05$ compared to D-8 [Repeated measure two-way ANOVA followed by Bonferroni test].

4.5.2 Suvorexant and rapamycin reduces the anxiety-like behavior during EPM

EPM was mostly used to measure the anxiolytic activity of drugs. In the EPM paradigm, the percentage open arm entries and time spent were decreased with SRS ([$F_{5,264}$ = 75.91,

$p<0.05$] and [$F_{5,264} = 147.7$, $p<0.05$]) respectively, with time ([$F_{3,264} = 406.5$, $p<0.05$] and [$F_{2,264} = 437.8$, $p<0.05$]) respectively. Further, there was a significant interaction between groups and time ([$F_{15,264} = 19.97$, $p<0.05$] and [$F_{15,264} = 37.1$, $p<0.05$] respectively in the EPM paradigm. Post-hoc analysis demonstrated that there was a significant decrease in open arm entries following SRS exposure compared to control. Suvorexant and rapamycin significantly increased the open arms entries during EPM on D-32. However, suvorexant+rapamycin and setraline induced increase in open arm entries was more significant compared to individual treatment and SRS group (Figure 4.4).

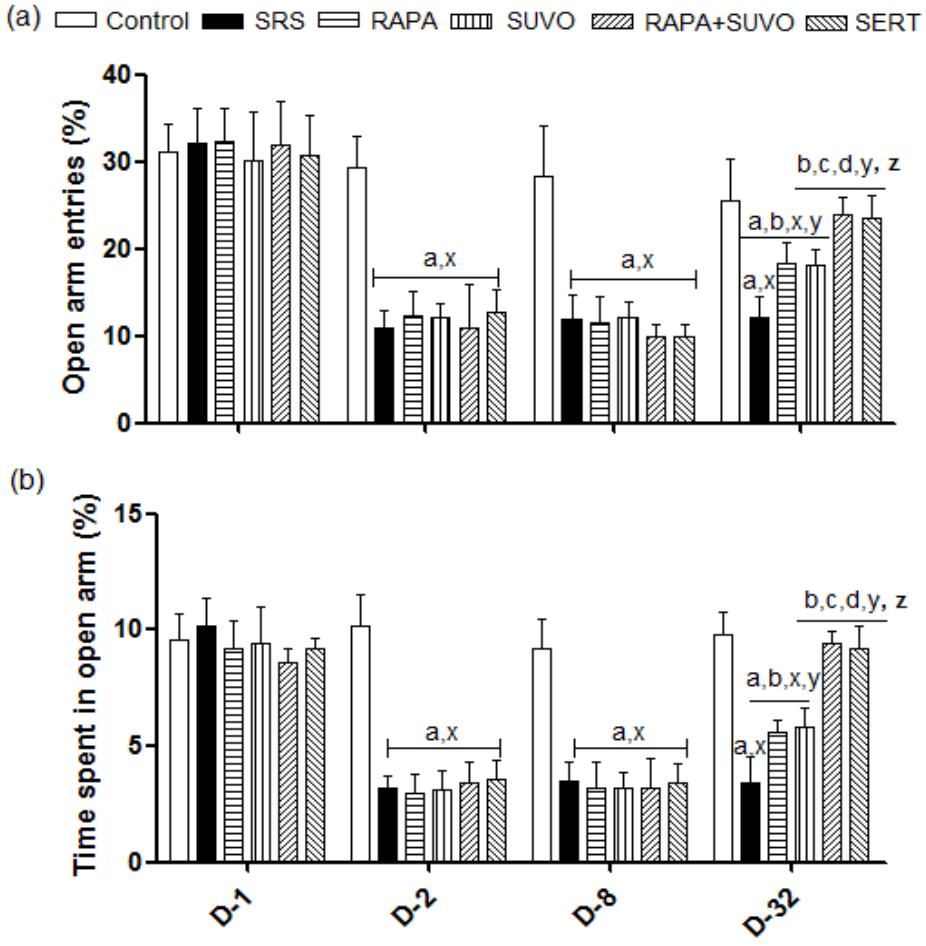

Figure 4.4 the effect of suvorexant and rapamycin on SRS-induced changes in (a) open arm entries and (b) time spent in the open arm during EPM on different days. All values are in mean±SD (n=12; number of rats). [a]$p<0.05$ compared to control, [b]$p<0.05$ compared to SRS, [c]$p<0.05$ compared to SRS+RAPA, [d]$p<0.05$ compared to SRS+SUVO, [x]$p<0.05$ compared to D-1, [y]$p<0.05$ compared to D-2 and [z]$p<0.05$ compared to D-8 [Repeated measure two-way ANOVA followed by Bonferroni test].

4.5.3 Suvorexant and rapamycin ameliorates the SRS-induced cognitive inflexibility in rats

Cognitive inflexibility was measured by attention set-shifting behaviour. The attention set-shifting or discrimination trial in SD, CD, ID, ED, and reversal learning in Rev-1, Rev-2 and Rev-3 shown in Figure 4.5 (a). ANOVA showed a difference in the discrimination trial of criterion among groups ($F_{5,264}$ = 105.5, $p<0.05$), treatment ($F_{3,264}$ = 18.6, $p<0.05$) and their interaction ($F_{15,264}$ = 5.2, $p<0.05$). Post-hoc analysis revealed that exposure of SRS caused an increase in criterion trial of ID, ED, and its reversal learning compared to control. Treatment with rapamycin and suvorexant significantly decreased the SRS-induced increase in the trial criterion of ID, ED, and Rev-2 and Rev-3. However, sertraline decreased only SRS-induced increase in trial criterion of ID and Rev-2 but did not affect ED and Rev-3.

Further, the error criterion was measured in the mean of delay in retrieval of memory and is shown in Figure 4.5 (b). ANOVA showed that, there was a significant increase in error criterion in ID, Rev-2, ED and Rev-3 following SRS exposure ($F_{5,264}$ = 136, $p<0.05$), treatment ($F_{3,264}$ = 27.6, $p<0.05$) and their interaction ($F_{15,264}$ = 4.6, $p<0.05$). Post-hoc analysis showed that rapamycin and suvorexant individual and in combination significantly decreased the error criterion during ID, ED and its reversal learning (Rev-2 and Rev-3), however, sertraline caused a decrease in ID and Rev-2 but not in ED and Rev-3.

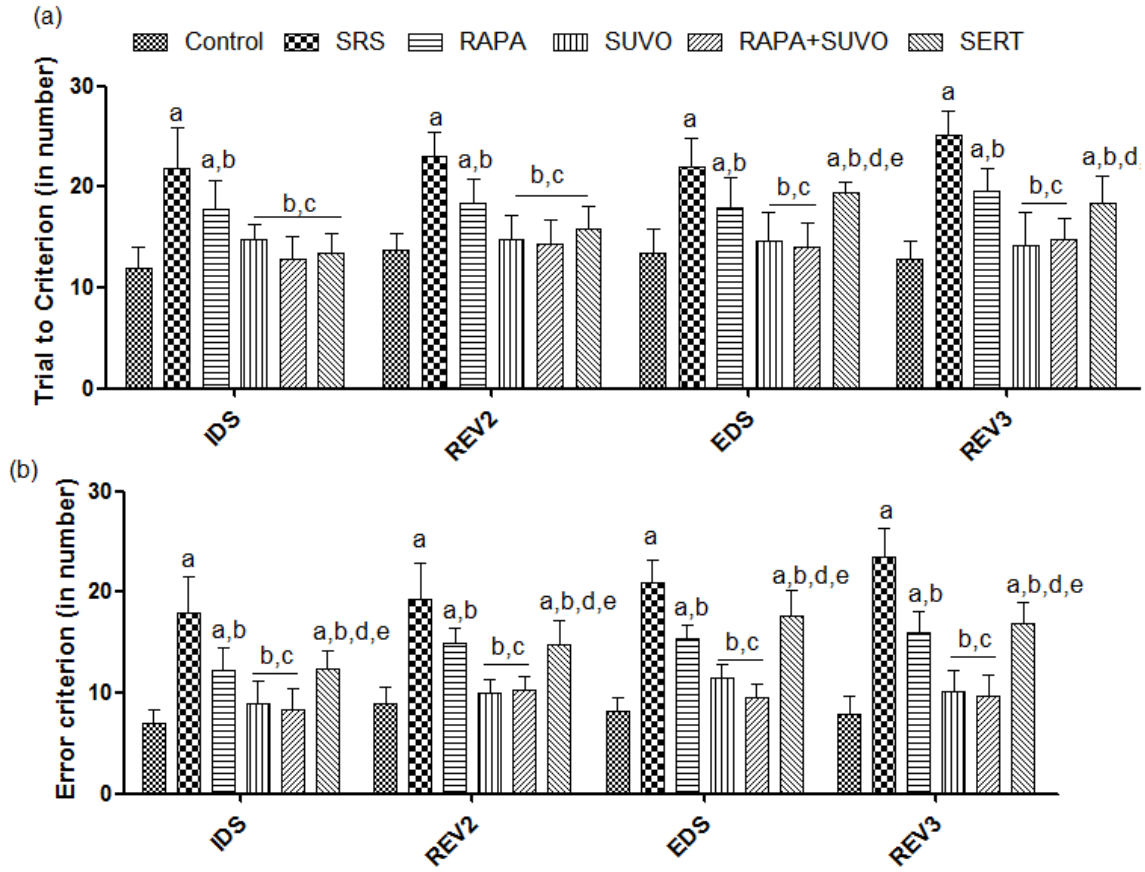

Figure 4.5 the effect of suvorexant and rapamycin on SRS-induced alteration in trial to criterion (a) and error criterion (b) in rats. All values are Mean±SD (n=12). [a]p<0.05 compared to control, [b]p<0.05 compared to SRS and [c]p<0.05 compared to RAPA, [d]p<0.05 compared to SUVO, and [e]p<0.05 compared to RAPA+SUVO [Repeated measure two-way ANOVA followed by Bonferroni test].

4.5.4 Suvorexant and rapamycin attenuated the plasma corticosterone level in SRS-exposed rats

Statistical analysis revealed that there was significant difference in plasma corticosterone level among the groups [$F_{5,71}$ = 62.69, p<0.05]. Post-hoc analysis represented that, there was significant decrease in plasma corticosterone following SRS exposure compared to control. Suvorexant and rapamycin significantly increased the plasma corticosterone on D-32. Suvorexant+rapamycin and setraline induced increase in plasma corticosterone was more significant compared to individual treatment and SRS group (Figure 4.6).

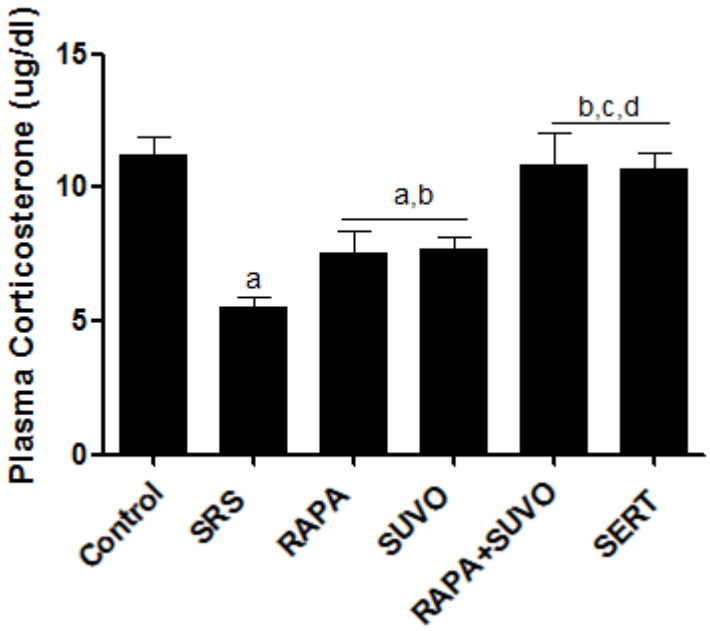

Figure 4.6 the effect of suvorexant and rapamycin on SRS-induced changes on plasma corticosterone level. All values are in mean±SD (n=12; number of rats). [a]$p<0.05$ compared to control, [b]$p<0.05$ compared to SRS, [c]$p<0.05$ compared to RAPA, [d]$p<0.05$ compared to SUVO, and [e]$p<0.05$ compared to RAPA+SUVO [one-way ANOVA followed by Newman-Keuls Multiple Comparison Test].

4.5.5 Suvorexant and rapamycin reduced the SRS-induced increase in plasma and CSF orexin-A level

Plasma and CSF orexin-A were measured to correlate the peripheral and central alteration in the orexinergic system in SRS-exposed rats (Figure 4.7). One-way ANOVA revealed a difference in plasma [$F_{5,71}$= 77.17, $p<0.05$] and CSF $F_{5,71}$ = 126.2, $p<0.05$] orexin-A level among the groups. Post-hoc test showed a significant increase in plasma and CSF orexin-A level following exposure of SRS compared to the control rats. Treatment with suvorexant significantly decreased the SRS-induced increased in plasma and CSF orexin-A level. However, rapamycin and sertraline did not cause any change on plasma and CSF orexin-A level in SRS-exposed rats.

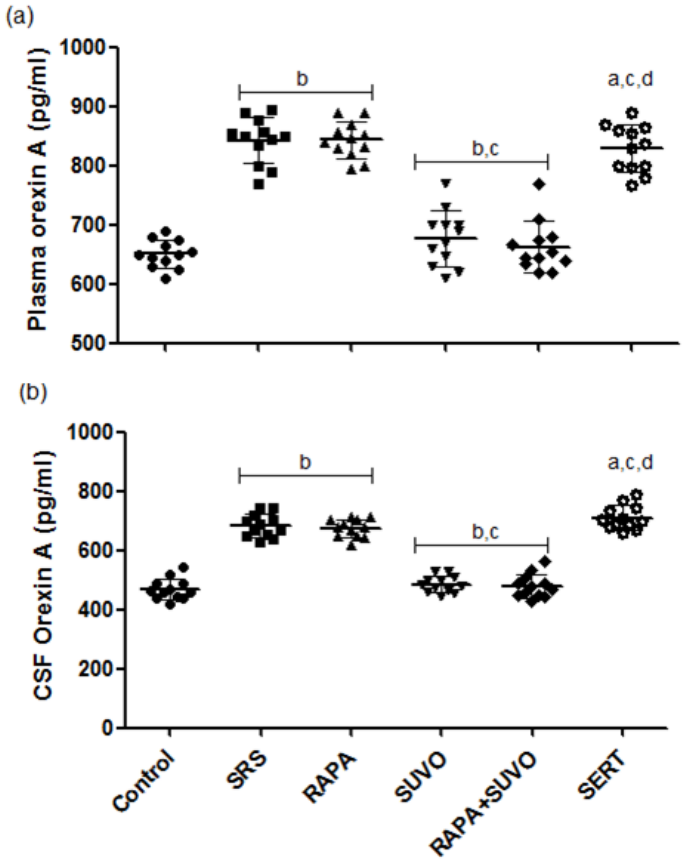

Figure 4.7 the effect of suvorexant and rapamycin on SRS-induced changes on plasma (a) and CSF (b) orexin-A level. All values are in mean±SD (n=12; number of rats). [a]p<0.05 compared to control, [b]p<0.05 compared to SRS, [c]p<0.05 compared to RAPA, [d]p<0.05 compared to SUVO, and [e]p<0.05 compared to RAPA+SUVO [one-way ANOVA followed by Newman-Keuls Multiple Comparison Test].

4.5.6 Suvorexant and rapamycin attenuates SRS-induced alterations in mitochondrial complex activity

Data analysis using one-way ANOVA showed significant difference in complex I [$F_{5,23}$ = 20.16; p<0.05], II [$F_{5,23}$ = 34.12, p<0.05], IV[$F_{5,23}$ = 39.49, p<0.05] and V [$F_{5,23}$ = 98.49, p<0.05] activities among groups. Post-hoc analysis using Student-Neumann-Keuls test showed a significant decrease in complex-I, II, IV, and V activities in SRS exposed rats as compared to control rats. Treatment with suvorexant and rapamycin individually and in combination significantly improved the SRS-induced reduction of mitochondrial complex activities. There was no significant effect of sertraline treatment on any complex activity (table 4.1).

Groups	Complex-I activity (nmol NADH oxidized/min/mg/protein)	Complex-II activity (µmol formazan/min/mg protein)	Complex-IV activity (nmol cyt C oxidized/min/mg protein)	Complex-V activity nmol ATP hydrolyzed/min/mg protein
Control	44.23±3.3	21.5±1.5	1.6±0.05	2.4±0.11
SRS	23.4±2.2 [a]	6.3±0.52 [a]	0.32±0.04 [a]	0.41±0.02 [a]
RAPA	30.21±2.5 [a,b]	9.01±1.4 [a,b]	0.40±0.06 [a,b]	0.63±0.4 [a]
SUVO	32.3±3.7 [a,b]	10.55±1.2 [a,b]	0.46±0.09 [a,b]	0.67±0.08 [b,c]
RAPA+SUVO	40.48±3.4 [a,b,c,d]	16.8±2.3 [a,b,c,d]	1.2±0.03 [a,b,c,d]	1.8±0.06 [b,c,d]
SERT	35.5±1.6 [a,b,e]	11.3±2.1 [a,b,e]	0.42±0.02 [a,b,e]	0.65 ± 0.1[a,b,e]

Table 4.1 the effect of suvorexant and rapamycin on SRS-induced alterations in the mitochondrial complex activity. All values are in mean±SD (n=4; number of rats). [a]$p<0.05$ compared to control, [b]$p<0.05$ compared to SRS, [c]$p<0.05$ compared to RAPA, [d]$p<0.05$ compared to SUVO, and [e]$p<0.05$ compared to RAPA+SUVO [one-way ANOVA followed by Newman-Keuls Multiple Comparison Test].

4.5.7 Suvorexant and rapamycin enhances the mitochondrial bioenergetics and RCR

One-way ANOVA examined oxygen consumption rate during various respiratory states. There was a significant difference on state-I [$F_{5,23} = 134.3$, $p<0.05$], state-II [$F_{5,23} = 109.2$, $p<0.05$], state-III [$F_{5,23} = 64.9$, $p<0.05$], state-V with FCCP [$F_{5,23} = 43.4$, $p<0.05$] and state-V with succinate [$F_{5,23} = 65.84$, $p<0.05$]. Post-hoc test revealed a marked decrease in oxygen consumption rate during all respiratory states in the SRS-exposed group. Rapamycin+suvorexant treated rats showed significantly higher oxygen consumption rates as compared to the SRS group during all respiratory states. Moreover, rapamycin+suvorexant treatment increased state-II respiration as compared to SRS subjected rats. Sertraline treatment also significantly increases oxygen consumption rates during all the respiratory states (Figure 4.8). One-way ANOVA revealed a statistically significant difference among groups [$F_{5,23} = 126.8$, $p<0.05$] in

terms of RCR. Post hoc analysis revealed a significant decrease in RCR in the SRS-subjected group as compared to control rats. There was a significant increase in RCR in combination treated group as compared to SRS group.

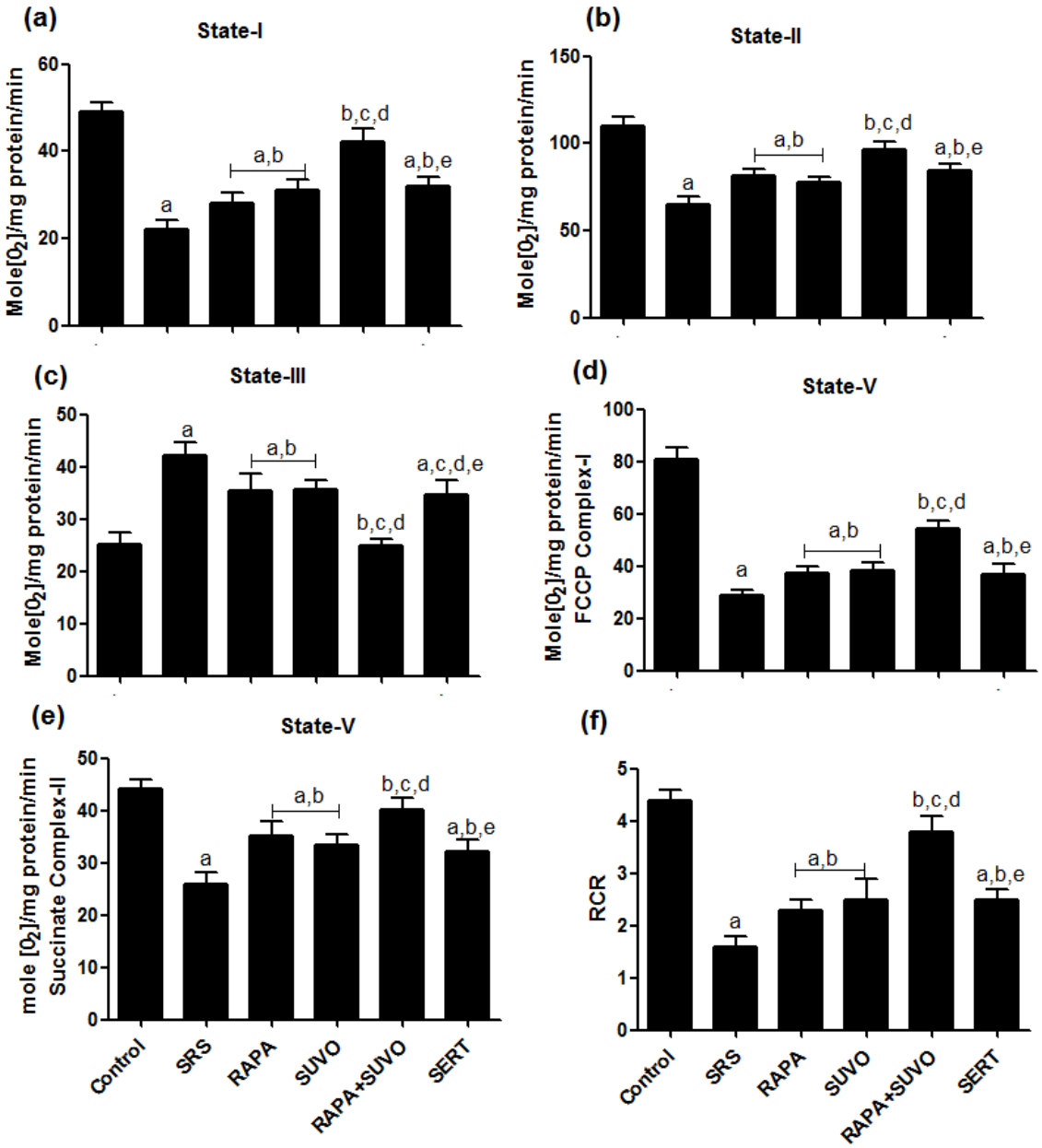

Figure 4.8 the effect of suvorexant and rapamycin on SRS-induced changes in mitochondrial respiration state-I (a), state-II (b), state-III (c), state-V with FCCP (d), state-V with succinate (e) and RCR (f) in AMY. All values are in mean±SD (n=4; number of rats). [a]p<0.05 compared to control, [b]p<0.05 compared to SRS, [c]p<0.05 compared to RAPA, [d]p<0.05 compared to SUVO, and [e]p<0.05 compared to RAPA+SUVO [one-way ANOVA followed by Newman-Keuls Multiple Comparison Test].

4.5.8 Suvorexant and rapamycin reduces the mTORC-1 activity in SRS-subjected rats

The mTORC-1 and MTFP-1 protein expression is depicted in Figure 4.9. One-way ANOVA revealed that there was significant difference in mTORC-1 expression [$F_{5,23}$ = 65.76, $p<0.05$] and MTFP-1 expression [$F_{5,23}$ = 68.13, $p<0.05$] among the group. Post-hoc analysis revealed that there was significant increased in the expression of mTORC-1 and MTFP-1 following SRS exposure. Sub-chronic treatment with suvorexant and rapamycin but not sertraline significantly decreased mTORC-1 and MTFP-1 expression in SRS exposed rats. Moreover, rapamycin+suvorexant induced decrease in mTORC-1 and MTFP-1 expression was more significant compared to individual treatment.

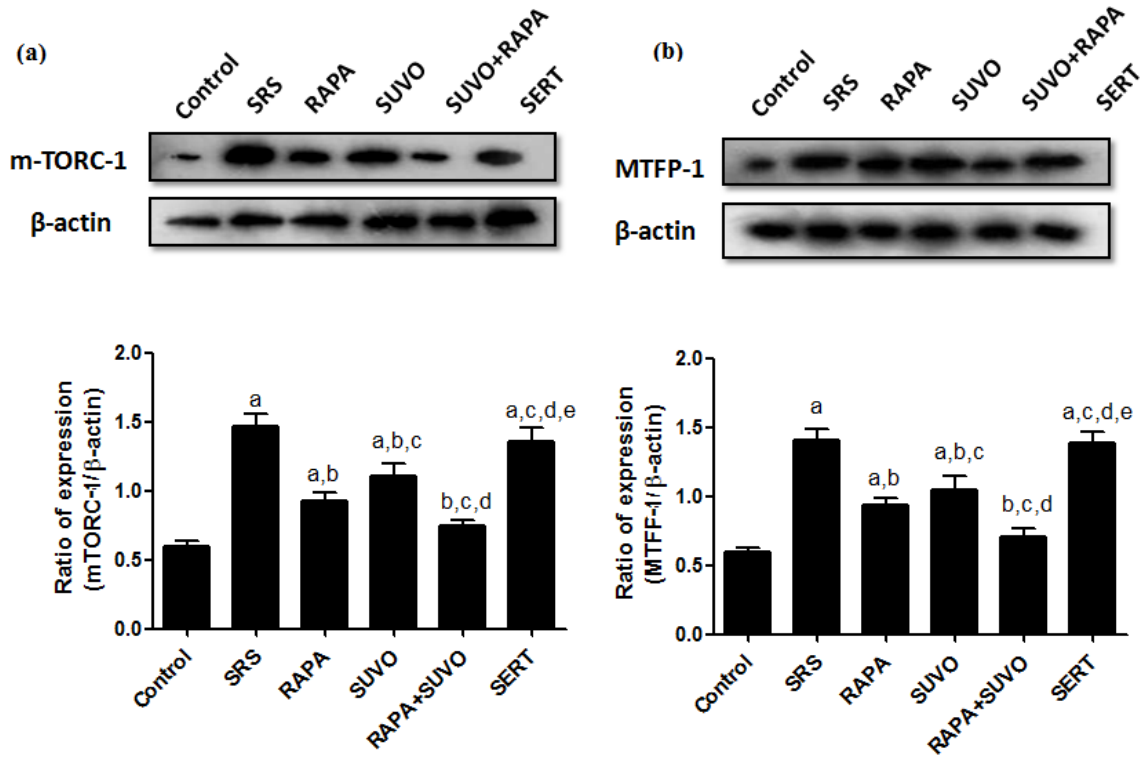

Figure 4.9 the effect of suvorexant and rapamycin on SRS-induced changes on mTORC-1 (a) and MTFP-1 (b) expression. All values are in mean±SD (n=4; number of rats). [a]$p<0.05$ compared to control, [b]$p<0.05$ compared to SRS, [c]$p<0.05$ compared to RAPA, [d]$p<0.05$ compared to SUVO, and [e]$p<0.05$ compared to RAPA+SUVO [one-way ANOVA followed by Newman-Keuls Multiple Comparison Test].

4.5.9 Suvorexant and rapamycin facilitates the mitochondrial dynamics in SRS-subjected rats

The mitochondrial fission protein (Drp-1 and Fis-1) expression is depicted in Figure 4.10 (a) and (b) respectively. One-way ANOVA followed by post-hoc test revealed that there

was a significant increase in Drp-1 [$F_{5,23}$ = 28.48 $p<0.05$] and Fis-1 [$F_{5,23}$ = 30.1 $p<0.05$] protein expression following SRS exposure in the amygdala as compared to control. Treatment with suvorexant and rapamycin significantly decreased the Drp-1 and Fis-1 protein expression in SRS exposed rats. Further, the combination (suvorexant and rapamycin) induced reduction in fission protein expression was more significant than individual treatment. There was no effect observed in sertraline treated group.

Further, the expression of mitochondrial fusion protein such as Opa-1 and Mfn-2 is represented in Figure 4.10 (c) and (d) respectively. Data analysis showed there was a significant difference in Opa-1 [$F_{5,23}$ = 88.96, $p<0.05$] and Mfn-2 [$F_{5,23}$ = 71.84, $p<0.05$] in amygda among the groups. Post-hoc test revealed a significant decrease in Opa-1 and Mfn-2 protein expression in SRS exposed rats. Sub-chronic treatment with suvorexant and rapamycin significantly increased the Opa-1 and Mfn-2 protein expression in SRS subjected rats. Further, suvorexant+rapamycin induced increase in fusion protein expression was more significant compared to their individual treatment. Moreover, sertraline did not produce any significant effect on mitochondrial fusion protein expression.

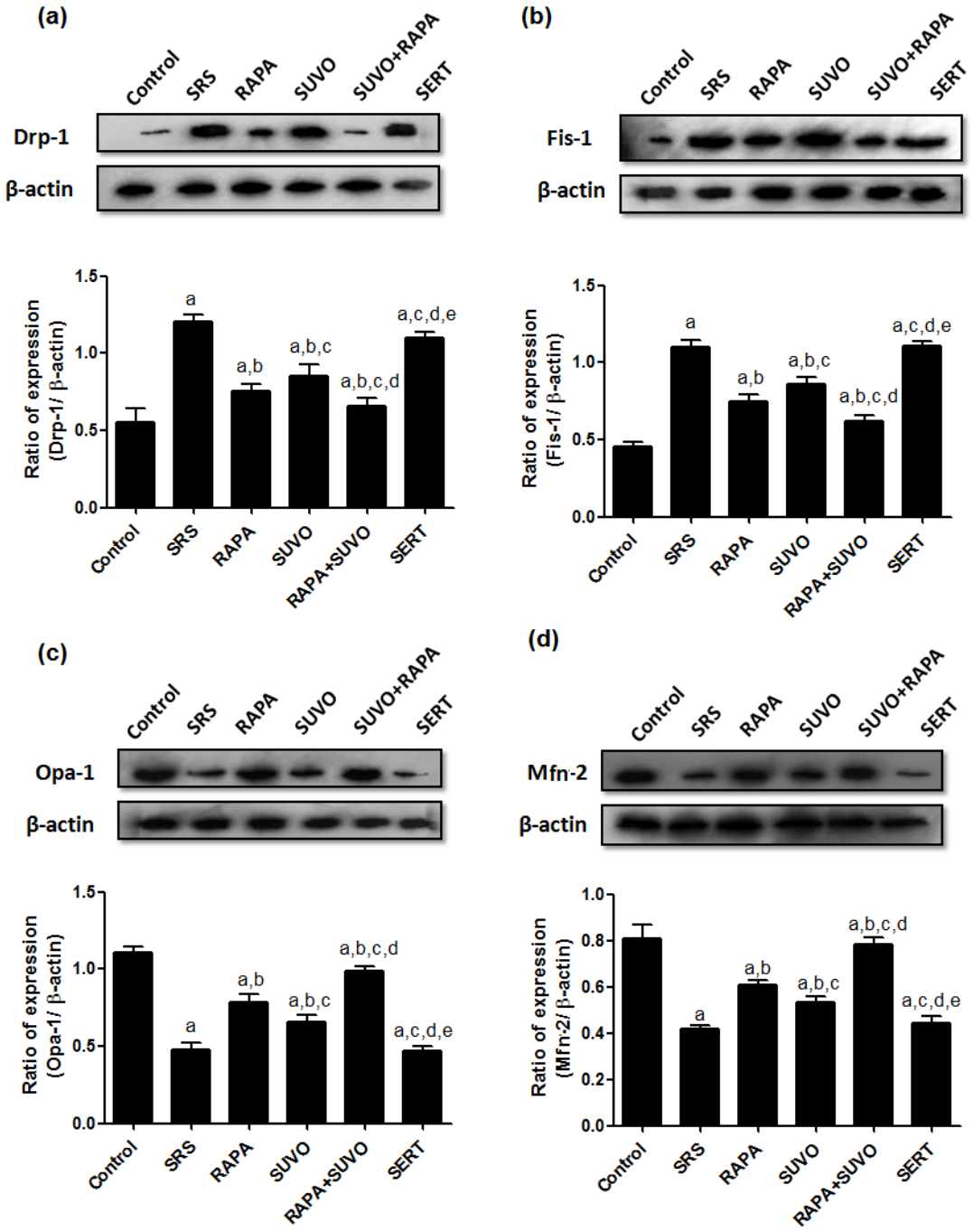

Figure 4.10 the effect of suvorexant and rapamycin on SRS-induced changes in mitochondrial fission protein such as Drp-1 (a), Fis-1 (b) and fusion protein such as Opa-1 (c) and Mfn-2 (d) expression. All values are in mean±SD (n=4; number of rats).). [a]p<0.05 compared to control, [b]p<0.05 compared to SRS, [c]p<0.05 compared to RAPA, [d]p<0.05 compared to SUVO, and [e]p< 0.05 compared to RAPA+SUVO [one-way ANOVA followed by Newman-Keuls Multiple Comparison Test].

4.5.10 Suvorexant and rapamycin attenuates the mRNA expression of mitochondrial dynamicsmarkers in SRS-subjected rats

The mRNA expression of fission protein marker such as Drp-1 and Fis-1 is depicted in Figure 4.11(a) and (b) respectively. One-way ANOVA followed by post-hoc test revealed that there was a significant increase in Drp-1[$F_{5,23}$ = 104.6, $p<0.05$] and Fis-1[$F_{5,23}$ = 138.4, $p<0.05$] mRNA expression following SRS exposure in amygdala as compared to control. Treatment with suvorexant and rapamycin significantly decreased the Drp-1, and Fis-1 mRNA expression in SRS exposed rats. However, rapamycin-induced decrease in Drp-1 and Fis-1 protein expression was significant compared to suvorexant. Further, suvorexant+rapamycin induced reduction in fission protein expression was more significant compared to their individual treatment. There was no effect observed in sertraline treated group.

Further, the mRNA expression of mitochondrial fusion protein such as Opa-1 and Mfn-2 is represented in Figure 4.11 (c) and (d) respectively. Data analysis demonstrated significant decrease in Opa-1[$F_{5,23}$ = 289.5, $p<0.05$] and Mfn-2 [$F_{5,23}$ = 171.3, $p<0.05$] in amygdala as compared to control in SRS exposed rats. Treatment with suvorexant and rapamycin significantly increased the Opa-1 and Mfn-2 protein expression in SRS exposed rats but rapamycin-induced increase the Opa-1 and Mfn-2 protein expression was more significant compared to suvorexant. Further, suvorexant+rapamycin induced increase in fusion protein expression was more significant compared to individual treatment. Moreover, sertraline did not produce any significant effect on mitochondrial fusion protein expression.

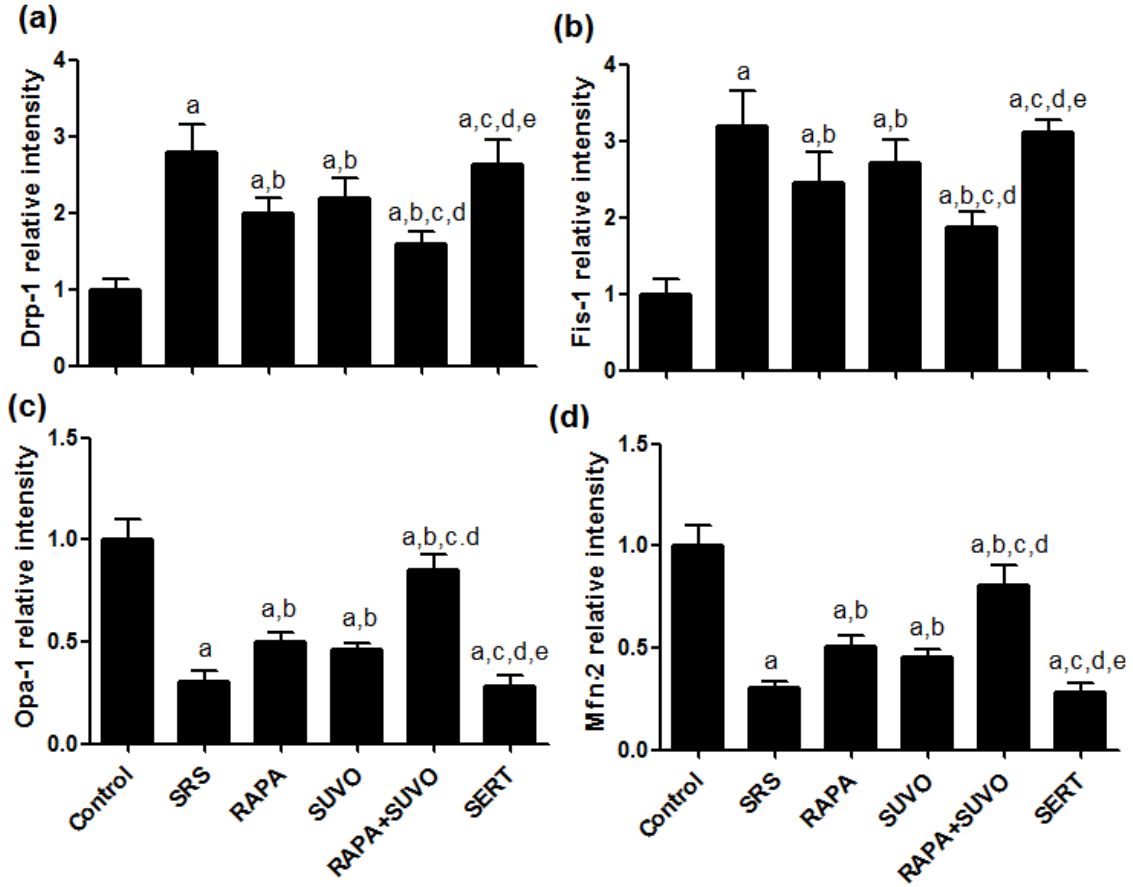

Figure 4.11 the effect of suvorexant and rapamycin on SRS-induced changes in m-RNA expression of mitochondrial fission protein such as Drp-1 (a), Fis-1 (b) and fusion protein such as Opa-1 (c) and Mfn-2 (d). All values are in mean±SD (n=4; number of rats). $^{a}p<0.05$ compared to control, $^{b}p<0.05$ compared to SRS, $^{c}p<0.05$ compared to RAPA, $^{d}p<0.05$ compared to SUVO, and $^{e}p<0.05$ compared to RAPA+SUVO [one-way ANOVA followed by Newman-Keuls Multiple Comparison Test].

4.5.11 Suvorexant and rapamycin ameliorated the intensity of Drp-1in SRS-subjected rats

The SRS-induced increase in mitochondrial fission conformed by immunofluorescene assay depicted in Figure 4.12. The intensity ratio of Drp-1was used as a measure of mitochondrial fission and fusion respectively. DAPI was used for counterstaining of the nucleus. One-way ANOVA revealed there was a significant difference in intensity ratio Drp-1[$F_{5,23}$ = 136.4, $p<0.05$] among the groups. Post-hoc test represented an increase in intensity ration of Drp-1 in AMY following SRS exposure. Treatment with suvorexant and rapamycin significantly attenuated the SRS-induced alteration in intensity ratio of Drp-1 in rats. However, the combination induced attenuation was more significant

compared to individual treatment. Further, there was no effect observed following sertraline treatment.

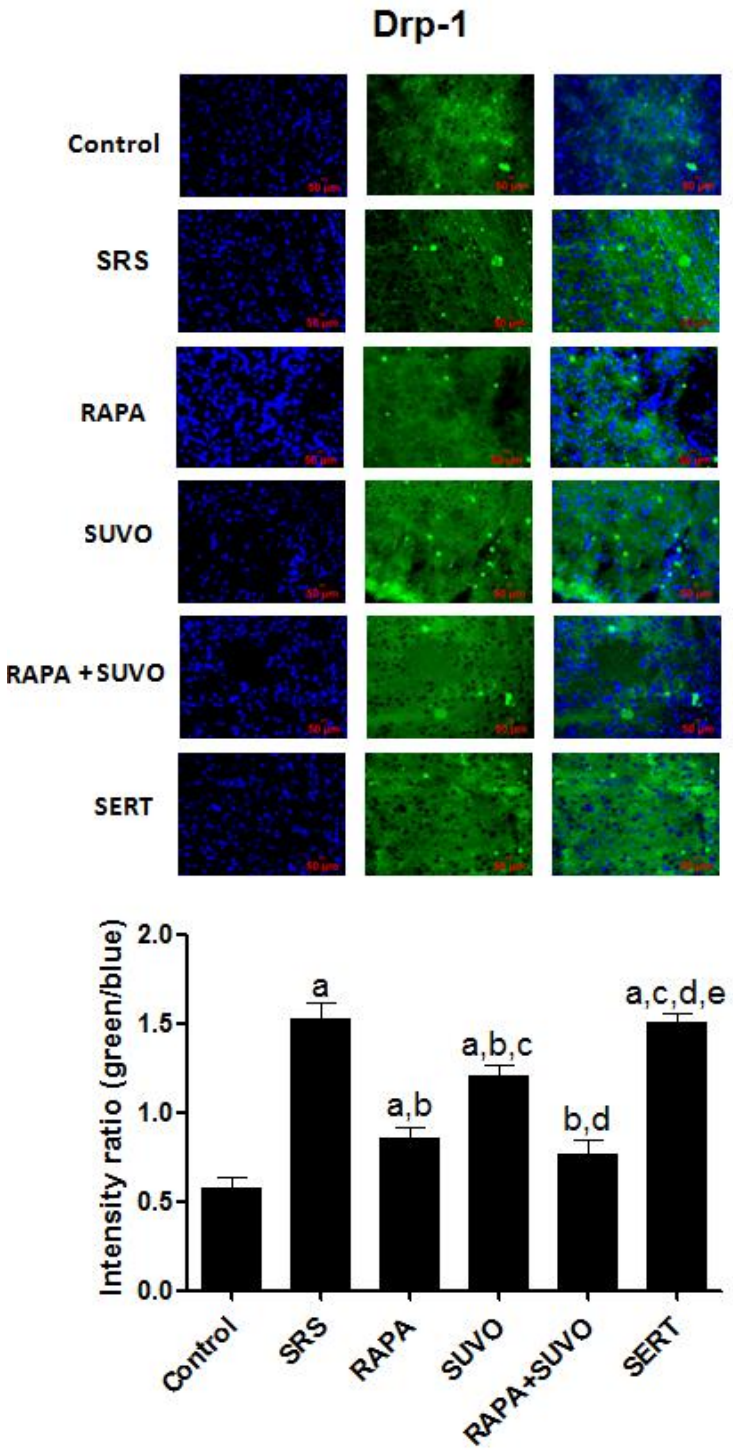

Figure 4.12 the effect of suvorexant and rapamycin on SRS-induced changes on the intensity of Drp-1. All values are in mean±SD (n=4; number of rats). [a]p<0.05 compared to control, [b]p<0.05 compared to SRS, [c]p<0.05 compared to RAPA, [d]p<0.05 compared to SUVO, and [e]p<0.05 compared to RAPA+SUVO [one-way ANOVA followed by Newman-Keuls Multiple Comparison Test].

4.5.12 Suvorexant and rapamycin ameliorated the intensity of Mfn-2 in SRS-subjected rats

The SRS-induced decrease in fusion is conformed by immunofluorescene assay depicted in Figure 4.13. The intensity ratio of Mfn-2 was used as a measure of mitochondrial fission and fusion respectively. DAPI was used for counterstaining of the nucleus. One-way ANOVA revealed there was a significant difference in Mfn-2 [$F_{5,23} = 163.2$, $p<0.05$] intensity among the groups. Post-hoc test represented an decreased intensity ration of Mfn-2 in AMY following SRS exposure. Treatment with suvorexant and rapamycin significantly attenuated the SRS-induced alteration in intensity ratio of Mfn-2 in rats. However, the combination induced attenuation was more significant compared to individual treatment. Further, there was no effect observed following sertraline treatment.

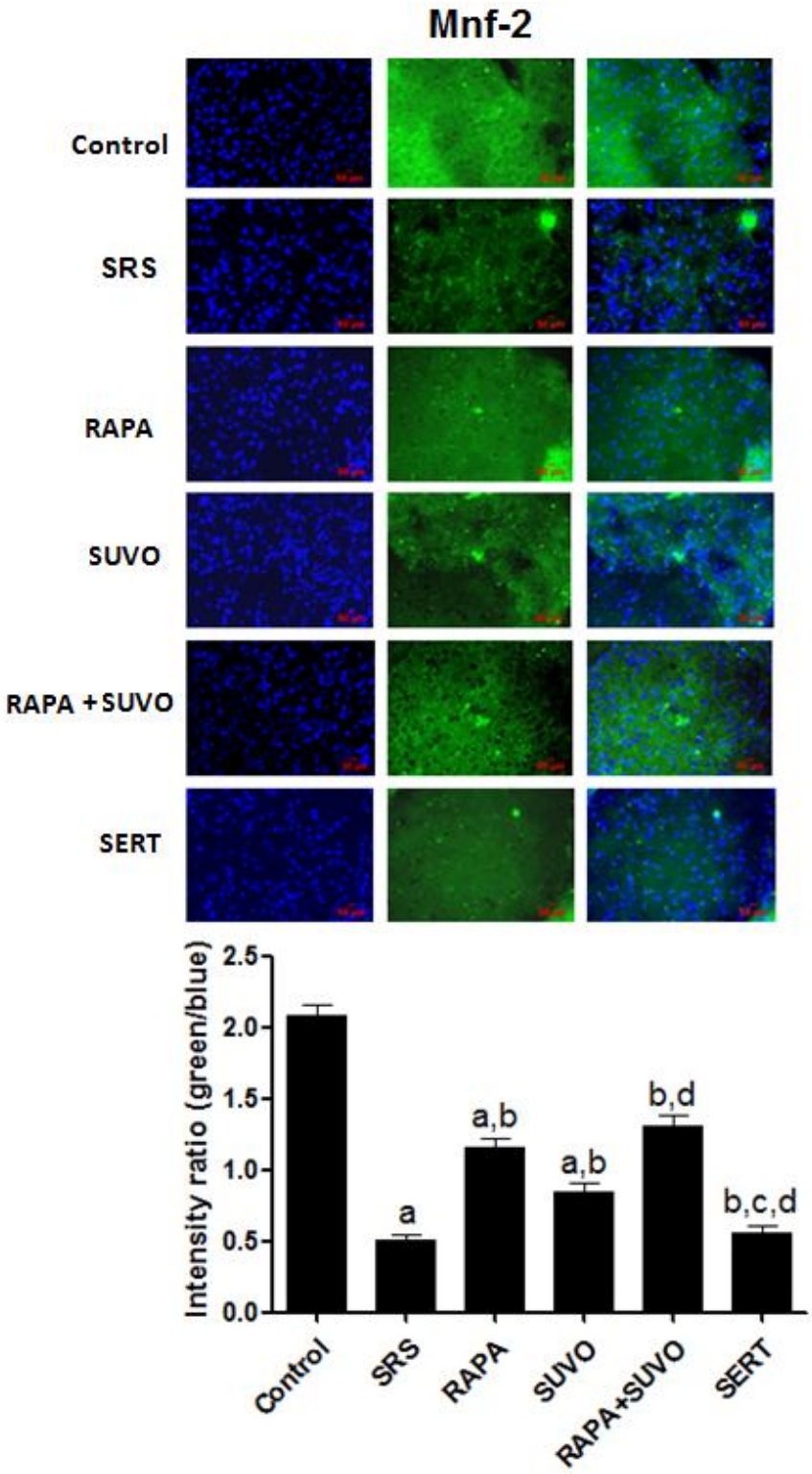

Figure 4.13 the effect of suvorexant and rapamycin on SRS-induced changes on the intensity of Mfn-2. All values are in mean±SD (n=4; number of rats). [a]p<0.05 compared to control, [b]p<0.05 compared to SRS, [c]p<0.05 compared to RAPA, [d]p<0.05 compared to SUVO, and [e]p<0.05 compared to RAPA+SUVO [one-way ANOVA followed by Newman-Keuls Multiple Comparison Test].

4.5.12 Suvorexant and rapamycin attenuated the mitophagy in SRS-subjected rats

TEM analysis was performed to analysed the SRS-induced alteration in mitochondrial morphology (Figure 4.14). The SRS-exposed group showed fewer mitophagy vacuoles with more swollen and damaged mitochondria. Suvorexant and rapamycin-treated group showed autophagosomes formation in the cytoplasm where damaged mitochondria can be seen residing inside them for clearance. There was no effect observed on SRS-induced reduction in mitophagy following sertraline treatment.

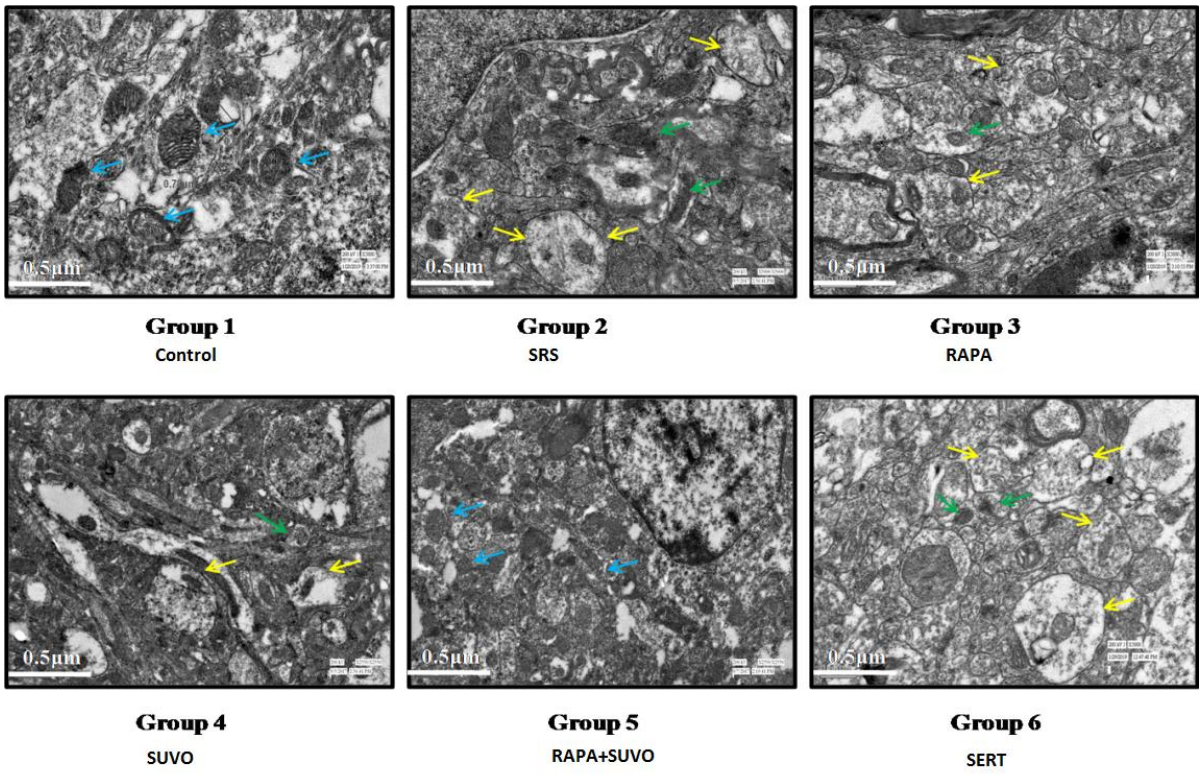

Figure 4.13 TEM analysis to analyse the morphology of mitochondria in amygdalar region.

Group1: Normal Control-blue arrow represents mitochondrial ultrastructure. Here the mitochondrion has round morphology with well-distributed cristae and intact morphology.
Group 2: Disease group: Green arrow represents damage and swollen mitochondria. Here the mitochondria are more fragmented with complete loss in the morphology. Yellow arrow represents autophagic vacuoles where damaged mitochondria can be seen for recycling.
Group 3: Green arrow represents damaged mitochondria with loss of cristae.Yellow arrow represents mitophagic vascoules
Group 4: Green arrow represents damage mitochondria with loss of cristae. Yellow arrow represents mitophagic vascoules with damaged mitochondria for clearance.
Group 5: Blue arrow represents healthy mitochondria with the treatment of drug with restore morphology and no mitophagic vasoules are seen.
Group 6: Green arrows represent damaged mitochondria which loss in membrane potential. Yellow arrow represents mitophagic vacuoles when damaged mitochondria can be seen for clearance.

4.6 Discussion

In the present study, we, for the first time report that the SRS paradigm modulates mitochondrial dynamics by enhancing fission regulating protein (Drp-1 and Fis-1) and reducing fusion regulating protein (Opa-1 and Mfn-2) in rats. The modulation in mitochondrial dynamics is mediated by the activation m-TOR pathway and translation of MTFP-1. Suvorexant individually and in combination with rapamycin significantly attenuated PTSD-like phenotype and improved mitochondrial dynamics in SRS-exposed rats.

Attention deficit is one of the key characteristic observed during PTSD and measured as cognitive inflexibility (Fani et al., 2009). Earlier, we have reported the development of cognitive inflexibility in the rodent model of PTSD. Cognitive inflexibility is the difficulty in the differentiation of ID and ED shift (Tait et al., 2018). ID shift is a mild perceptual attention set that requires mild attention for recognition such as colour or shape of the visual object (Birrell and Brown, 2000, Jin et al., 2014). ED shift is associated with higher perceptual attention related to current intelligence quotient and intellect for recognition of such as odour and texture (Birrell and Brown, 2000). In the present study, SRS-exposed rats showed deficits in ID and ED shifts and its reversal learning. Reduction in set-shifting behaviour is similar to clinically observed task difficulty discrimination behaviour in PTSD patients (Herrmann et al., 2011). Treatment with suvorexant and rapamycin attenuated the SRS-induced impairment in ID, ED shifting and their reversal learning. However, the rapamycin+suvorexant-induced amelioration is more significant compared to individual treatments. Sertraline was only able to modulate SRS-induced impairment in ID shift but not ED shifting. Thus, inclusion of m-TOR inhibitor potentiate the effect of suvorexant, indicating that re-stress induced deficits in ID and ED shifts are m-TOR-dependent. Of note, studies have demonstrated

the benefits of rapamycin treatment on cognition deficit by preventing neuronal loss and Aβ-deposition in transgenic mice (Richardson et al., 2015).

Exposure of SRS exaggerated fear response and associated anxiety-like behaviour similalr to our previous study and closely resembles the hyper-arousal observed in PTSD patients (Gonzalez and Martinez, 2014). Treatment with suvorexant reduced contextual fear response in SRS subjected rats similar to the previous study (Prajapati and Krishnamurthy, 2020). Rapamycin attenuated the SRS-induced fear response and anxiety-like behaviour, indicating re-stress induced fear response and associated anxiety-like behaviour are m-TOR-dependent. This finding is supported by the reports on rapamycin significantly inhibits acquisition and consolidation of associative fear memories (Blundell et al., 2008) by preventing the mTORC-1 dependent phosphorylating the downstream cascades like p70S6K in the hippocampus and in the amygdala (Parsons et al., 2006). Interestingly, the combination of suvorexant and rapamycin induced amelioration of acquisition of fear and associated anxiety was more significant. This finding is further supported by the report that mTORC-1 is important in the formation of the fear response and acquired anxiety and via phosphorylation of the downstream target of mTORC-1 in AMY (Kim et al., 2010, Abelaira et al., 2014). Further, this reduction in fear response is due to the development of adaptive behaviour in context to fearful stimuli. Besides, sertraline also alleviated contextual fear response and anxiety-like behaviour on D-32, similar to the previous report (Takahashi et al., 2006).

Apart from phenotypic anomalies, suvorexant attenuated the SRS-induced hypocorticosteronemia and hyperactivity of the orexinergic system similar to the earlier study (Prajapati and Krishnamurthy, 2020). The HPA-axis function is directly regulated by the orexinergic system through hypothalamic and extra-hypothalamic corticotrophin releasing hormone dependent pathways in this model (Prajapati and Krishnamurthy,

2020). Rapamycin mitigated the SRS-induced reduction in plasma corticosterone level without producing any effect on the orexin-A level. Interestingly, the combination of suvorexant and rapamycin-induced alleviation in hypocorticosteronemia was more significant. Thus, suvorexant also mediating it effect through mTORC-1 dependent pathway.

Evidence has demonstrated that the HPA-axis dysfunction also contributes to mitochondrial dysfunction in the pathophysiology of PTSD. Orexin-A has been shown to regulate mitochondrial function and dynamics in brain-related disorders (Lassiter et al., 2015). In our study, mitochondrial complex (I, II, IV, and V) activities are significantly decreased in SRS exposed rats, which were similar to results from the previous study (Garabadu et al., 2015). Suvorexant and rapamycin significantly improved mitochondrial complex activities in the SRS paradigm. However, the suvorexant+rapamycin induced improvement in mitochondrial complex activities was more significant compared to individual treatments. This suggests potential role of orexin-A in the regulation of mitochondrial complex activity probably through m-TOR dependent pathway. This finding is supported by previous report where orexin is reported to be essential for the regulation of mitochondrial homeostasis by activating various downstream pathways such as m-TOR and PI3K/Akt in humans (Li et al., 2014). Further, there was a significant decrease in RCR in SRS subjected rats, which suggests that coupling was not perfect, and thereby, ATP synthesis was limited. Treatment with suvorexant and rapamycin treatment caused prominent drop in generation of reactive oxygen species and improvement in the different states of mitochondrial respiration (S-3, S-4, RCR, S-5 via complex-I and S-5 complex II). This may be due to improvement in oxidative phosphorylation complex I, III, and V and mitochondrial ribosomal subunit proteins (Gandin et al., 2016; Larsson et al., 2012; Morita et al., 2013). The respiratory control rate (RCR) is the ratio of state III

and state IV, which measures mitochondrial integrity. It also indicates the tightness of coupling between respiration and phosphorylation. Sertraline also attenuated the mitochondrial respiration by improving different states of mitochondria.

Together, the present findings indicate that activation of the orexinergic system may contribute to mitochondrial dysfunction in mTORC-1 dependent pathway in the SRS model. Moreover, SRS exposure caused upregulation of mTORC-1 regulated MTFP-1 protein expression, which is an integral protein of the mitochondrial inner membrane whose loss results in a hyperfused mitochondrial, whereas its overexpression engenders fragmentation (Tondera et al., 2004, 2005; Wai and Langer, 2016). Treatment with suvorexant and rapamycin significantly mitigated the SRS-induced upregulation of MTFP-1. However, the combination-induced attenuation was more significant than individual treatment. Sertraline did not cause any effect on SRS-induced activation of mTORC-1 and MTFP-1. Thus, suvorexant and sertraline act through different mechanisms to mitigate the mitochondrial dysfunction in this model.

The phosphorylation of MTFP-1 is responsible for activation of mitochondrial dynamin-like protein (DLP-1) (Morita et al., 2017). In our study, exposure of SRS caused increase in mRNA and protein expression of mitochondrial fission marker such as Drp-1 and Fis-1 and decrease in fusion marker such as Opa-1 and Mfn-2 in rats. Therefore, mitochondrial dynamics has a critical role in the pathophysiology of PTSD. This finding is quite similar to the report on dysregulation of mitochondrial dynamics observed in anxiety and depression (Gormanns et al., 2011). Suvorexant and rapamycin significantly attenuated the mitochondrial fission by reducing Drp-1 and Fis-1 protein and fusion by up-regulating Opa-1 and Mfn-2 in SRS exposed rats, but the combination induced attenuation was more significant. The SRS-induced alteration in mitochondrial dynamics is further confirmed by immunofluorescene assay as observed by an increase in the intensity of Drp-1 and a

decrease in the intensity of Mfn-2 co-localisation in the amygdala. Suvorexant and rapamycin significantly attenuated the SRS-induced alteration in the intensity ratio of Drp-1 and Mfn-2 in rats; however, the combination induced attenuation was more significant. The overall finding suggested that mitochondrial dynamics can be a pharmacological target for PTSD. Suvorexant improved mitochondrial dynamics through m-TOR dependent pathway. Therefore, orexin-A regulated mitochondrial dynamics potentially contributes to the pathophysiology of PTSD and its pharmacological manipulation significantly attenuated these consequences.

The present finding is further supported by TEM analysis for the AMY region of the rat's brain. Exposure to SRS caused less autophagic vacuoles with more swollen and damaged mitochondria. Rapamycin and suvorexant treated group showed autophagosomes formation in the cytoplasm where damaged mitochondria can be seen residing inside them for clearance. However, in the presence of m-TOR inhibitor potentiates the activity of suvorexant which thereby enhances mitophagic machinery for the clearance of non-functional mitochondria. Taken together, results from experiments were sufficient to prove that suvorexant promotes the elimination of depolarized mitochondria dynamics through m-TOR dependent pathway and promotes autophagosome formation for final degradation. Further, the ROS level was also a marked reduced following combination treatment. This effect may be due to the formation of new healthy mitochondria through mitochondrial biogenesis. The findings helped in drawing the conclusion that combination of suvorexant and rapamycin might remove excess ROS generation via mitophagy.

4.7 Summary

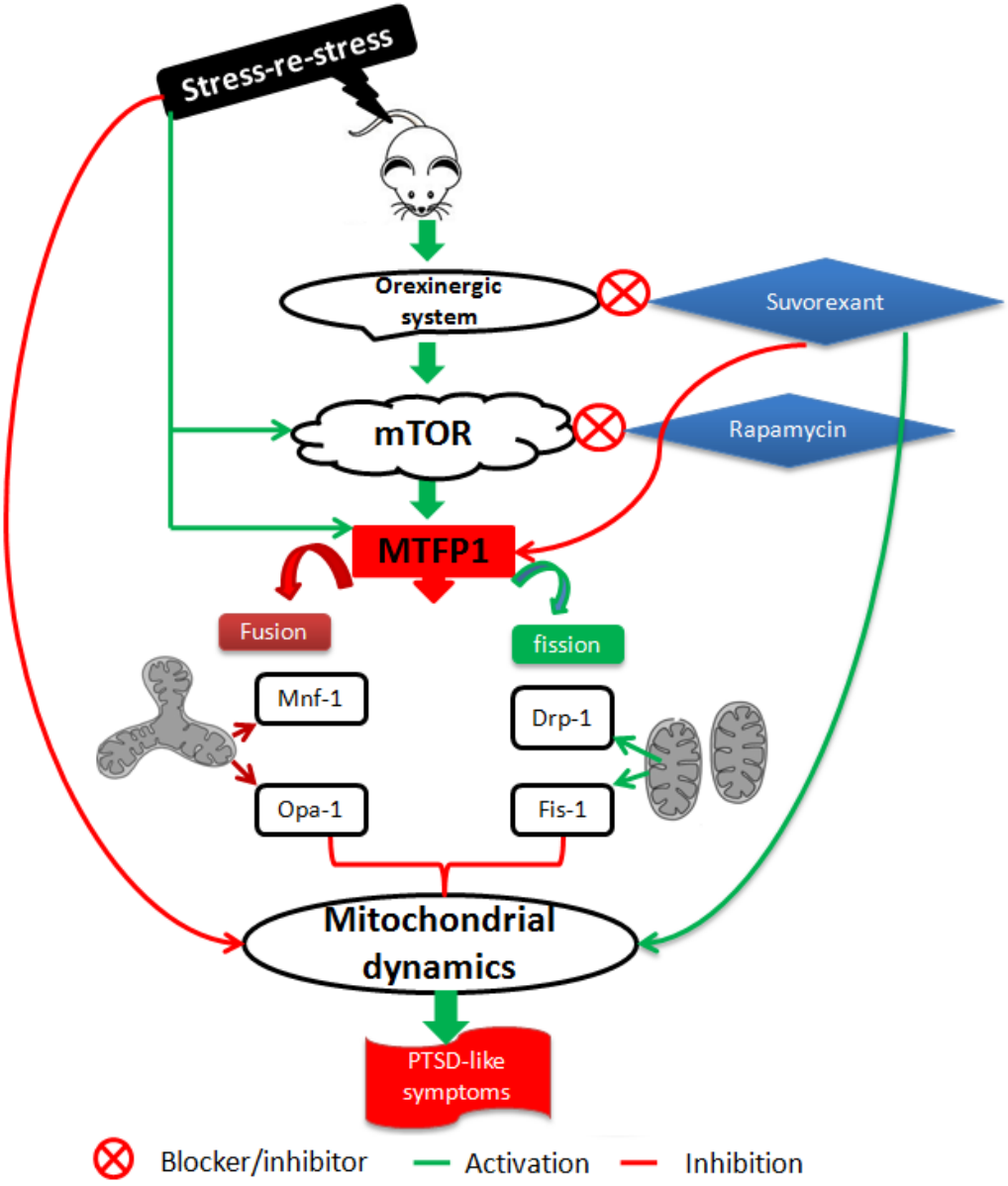

Figure.4.14 summary of the proposed hypothesis

The salient finding of the present study is the disparity in mitochondrial dynamics as observed by enhanced fission regulating protein (Drp-1 and Fis-1) and reduced fusion regulating protein (Opa-1 and Mfn-2) following exposure of SRS. The modulation in mitochondrial dynamics is mediated by orexin-A dependent activation of the mTORC-1 pathway and translation of MTFP-1. There was an increase in Drp-1 and a decrease in Mfn-2 co-localisation intensity in the AMY. Further, SRS exposure caused less mitophagic vacuoles with more swollen and damaged mitochondria observed during TEM analysis. Treatment with suvorexant and rapamycin significantly improved mitochondrial dynamics together with PTSD-like phenotypes in rats. The combination of suvorexant and rapamycin synergistically mitigated the mTORC-1 activation and the elimination of non-functional mitochondria and promoted mitophagy for final degradation. Therefore, the mitochondrial function could be the essential factor for an anti-PTSD drug for better management of PTSD.