Chapter 2

Evaluation of anti-PTSD potential of orexin antagonist in rats

2.1 Introduction

PTSD is a neuropsychiatric disorder that develops following actual or perceived traumatic events and is characterised by hyperarousal, nightmares, and recurrence of aversive thoughts (Diana, 2011). Growing evidence suggested that sleep disturbance is also a common manifestation of PTSD (Mitsushima et al., 2006). The pathophysiology of PTSD includes dysfunction of the HPA-axis and dysregulation of the serotonergic system as the important pathophysiological factors (Davis et al., 1997). Pharmacotherapy of PTSD involves sertraline and paroxetine, but they are effective only against limited symptoms of PTSD (Schwartz and Rothbaum, 2002). Further, long-term use of these drugs may aggravate anxiety in PTSD patients due to permanent desensitization of 5-HT receptors (Mitsushima et al., 2006; Mukherjee et al., 2015). Therefore, it is essential to evaluate the novel targets for the pharmacotherapy of PTSD. Clinical studies have shown modulation of the orexinergic system in PTSD (Strawn et al., 2010). In a clinical study (phase–IV clinical trial), suvorexant, an orexin antagonist, improved the sleep disturbance in PTSD. However, there is a paucity of evidence on the functioning of the orexinergic system in animal models of PTSD. Therefore, evaluating the role of the orexinergic system in an animal model may provide preclinical evidence for the treatment of PTSD.

Orexin is an endogenous neuropeptide that is usually responsible for wakefulness (Winrow et al., 2012). However, the level of orexin neuropeptide is modulated during stressful events (Winrow et al., 2012). The activation of the orexinergic system leads to hyperarousal, sleep disturbance associated with anxiety (Klenowski et al., 2016). Orexin neuropeptide is categorised into two types orexin-A and orexin-B that promote wakefulness. Orexin-A mediates its effect through both OX1-R and OX2-R, while orexin-B selectively acts on OX2-R (Sakurai et al., 1998). Both the receptors are glycoprotein coupled receptor but differ in their function (Bonaventure et al., 2015).

OX1-R is selectively expressed in the AMY, hypothalamus, and PVN and regulates HPA-axis and CRH system (Salehabadi et al., 2020). However, OX2-R predominantly present in LC and HIP and plays a critical role in wakefulness (Wang et al., 2018). Thus, both the receptors are crucial for panic disorders and its antagonism may mitigate core symptoms (fear response and anxiety) of PTSD. Therefore, in the present study, we have used an FDA-approved dual orexin receptor antagonist, i.e., suvorexant, mainly used for management of insomnia (Winrow et al., 2011). Besides, suvorexant also interferes with the synthesis of orexin. Clinical evidence suggested that suvorexant ameliorated sleep disturbance and associated anxiety in psychiatric patients (Flores et al., 2015). Both orexin-A and orexin-B are distributed throughout the peripheral and central nervous system. However, orexin-A is mainly involved in the stimulation of the HPA-axis and regulation of neuroendocrine function. During a stressful situation, orexin-A activates HPA-axis by enhancing the release of corticotropin-releasing factor (CRF) from hypothalamus. Moreover, the intracerebroventricular (ICV) injection of orexin-A caused extra-hypothalamic activation of CRH system and enhanced glucocorticoid activity (So et al., 2018). CRF is released from the extra-hypothalamic region such as central nucleus of amygdala and upregulates the expression of corticotropin-releasing factor receptor-1 (CRF-R1) (Lu et al., 2008). This CRF-R1 mediated mechanism is involved in the stimulation of glucocorticoid response and acquisition of fear response during chronic stress (Krishnamurthy et al., 2013). In addition, the modulation of extra-hypothalamic CRH system and HPA-axis dysfunction is also one of the pathological cause for PTSD (Yehuda et al., 1993; Lu et al., 2008). Therefore, measurement of expression of CRF-R1 in AMY would give a better insight of involvement of extrahypothalamic regions in the etiology of PTSD and its associated fear response and anxiety.

The Orexinergic system induces hyperarousal and anxiety due to the activation of serotonin neurotransmitter in the dorsal raphe nucleus and AMY (Yang et al., 2019). Previously, it has been suggested that there is an enhanced expression of orexin-A in the PFC (Ida et al., 2000). Further, activation of the orexinergic system is also observed in panic anxiety disorder, and thus its reduction can be a useful treatment strategy (Johnson et al., 2010b). Thus, orexin-A has multiple roles, and its dysregulation during traumatic condition may lead to hyperarousal, anxiety and sleep disturbances.

To evaluate the orexinergic system's role as a pharmacological target for the treatment of PTSD, the SRS model was used. This model can be used for chronic evaluation of pharmacological agents (Krishnamurthy et al., 2013; Prajapati et al., 2019b). In addition, the SRS model comprises variable traumatic stresses and contextual triggers that acts as brief "reminder" episodes that are involved in the emergence of PTSD-like phenotypes (Krishnamurthy et al., 2013; Prajapati et al., 2019b). In the present study, the pharmacological effect of suvorexant was studied in the SRS model of PTSD. As part of PTSD-like symptoms, we measured freezing behaviour for the contextual fear response. Further, the anxiety-like behaviour was assessed during elevated plus maze (EPM). Spontaneous locomotor activity was evaluated as a measure of the sedative effect of suvorexant. To correlate the symptoms with the orexinergic system, the levels of orexin-A in plasma and cerebrospinal fluid (CSF) along with plasma corticosterone levels were estimated. Serotonin levels in the AMY were also measured. The CRF-R1 expression in AMY was measured to demonstrate the relationship between CRF-R1 with HPA-axis dysfunction in SRS model of PTSD.

2.2 Hypothesis

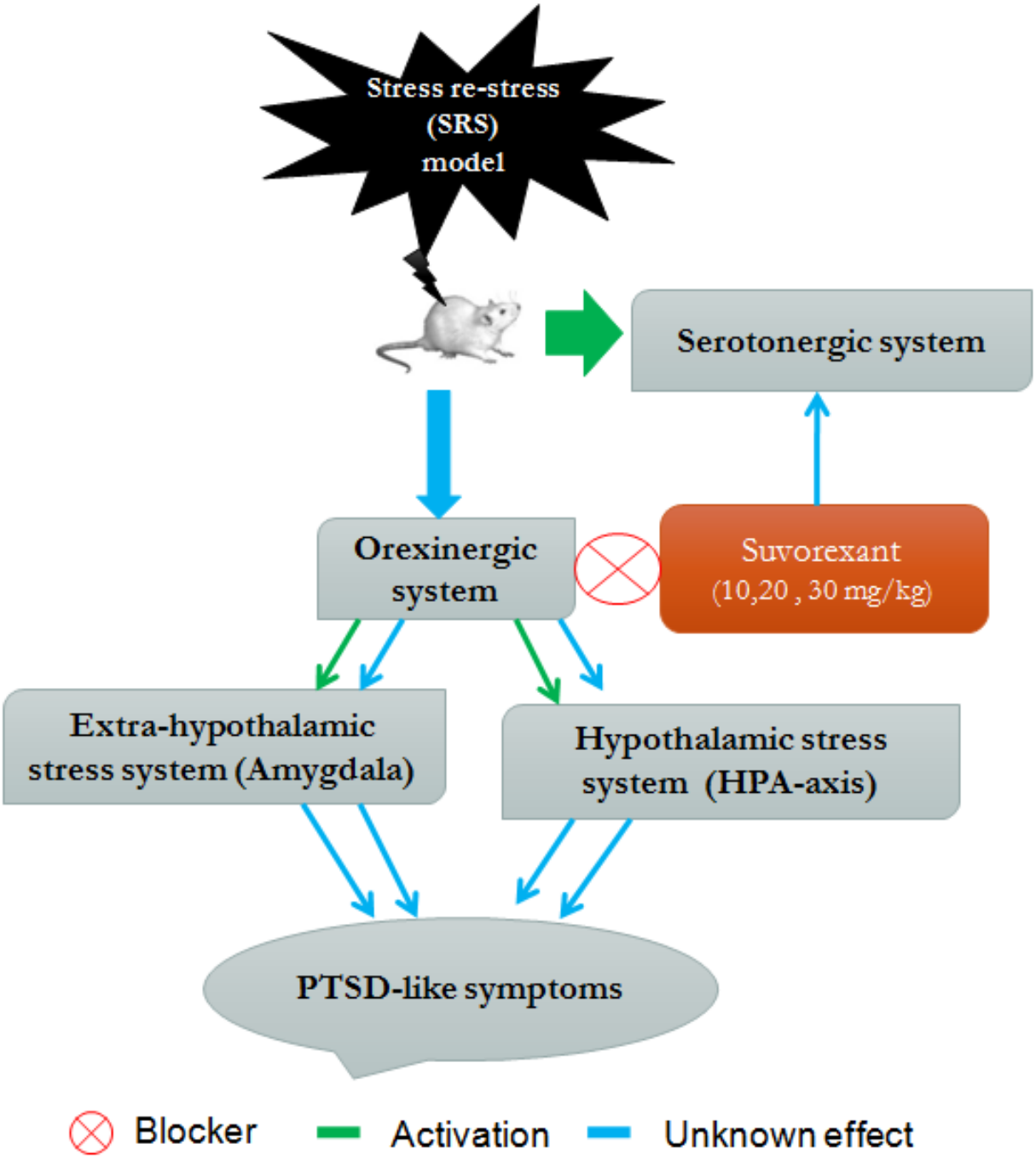

Figure 2.1 proposed hypothesis of evaluation of the anti-PTSD potential of orexin antagonist in rats.

Orexin is a neuropeptide secreted from the hypothalamus and regulates wakefulness through interaction with efferent systems that mediate arousal and energy homeostasis. Evidence has suggested that the emergent role of orexin-A in the regulation of panic anxiety and depression. However, there is limited information on the role of orexin in PTSD. Furthermore, the orexinergic system stimulates hypothalamic and extra-hypothalamic stress system which is essential for the pathophysiology of PTSD. Orexin-A also stimulated the serotonergic system together with activation of HPA-axis, both of which are clinically observed pathology of PTSD. Therefore, the study aims to evaluate the involvement of the orexinergic system in the SRS model of PTSD and whether its pharmacological antagonism could mitigate the PTSD-like symptoms.

2.3 Materials and methods

2.3.1 Animals

Male Wistar rats (200±20g) were procured from the Institutional animal house IMS-BHU, Varanasi, India. All the rats were used in accordance to the Central Animal Ethical Committee of Banaras Hindu University, Varanasi, India (Serial no. Dean/2016/CAEC/324) and the National Institute of Health Guidelines (publication number 85-23, revised 2013), on animal care experimentation. Four animals were accommodated in each polypropylene cage under a controlled temperature of 25±1ºC and relative humidity of 45-55 % with 12/12 h light/dark cycle. The experimental animals had *ad libitum* supply of food (Doodhdharapashuahar, India) and water during the entire experimental protocol.

2.3.2 Drugs

Suvorexant (SUVO) (Cat # 1030377-33-3) was procured, and paroxetine (PAX) (Cat # 61869-08-7) was received as a gift sample (Ranbaxy, India). The other chemicals used in the experiment were procured from SRL Pvt. Ltd., India. All the chemicals used in this study were procured in the year 2019. Suvorexant (10, 20, 30 mg/kg) (Lee et al., 2017) and paroxetine (10 mg/kg) was suspended in 0.5% of sodium carboxymethylcellulose (CMC) (Cat # 9004-32-4) separately and were given by oral route (Krishnamurthy et al., 2013).

2.3.3 Experimental protocols

The sample size for our present study was calculated through A priori G*power analysis using F tests. A randomised allocation was employed to assign animals to the seven experimental groups (n=6, rats/group): CONTROL, SRS, SRS+SUVO (10 mg/kg), SRS+SUVO (20 mg/kg), SRS+SUVO (30 mg/kg), SRS+PAX (10 mg/kg) and CONT+ SUVO (30 mg/kg). The experiment was performed for a period of 32 days. On D-1, all the rats were subjected to trial session of EPM and on D-2 animals were exposed to SRS

except the control groups. The FS as a re-stress was given on D-8, 14, 20, 26, and 32 for all animals except control and *per se* group (Prajapati et al., 2019b). During behavioural assessments, the animals were first subjected to EPM followed by spontaneous locomotor activity with the gap of 30 min. The freezing behaviour was assessed 6 h post FS as the re-stress cue. All the behavioural assessments were performed between 10.30 to 17.00 h on D-8 and D-32 (Reddy and Krishnamurthy, 2018; Prajapati et al., 2019a). All behavioural observations were recorded and analysed by using ANY-MAZE^{TM} video tracking system (version-3.72; USA). All behavioural tests were performed by a blind observer. Dosing was started from D-8 and continued up to D-32 (Figure.2.2). Following decapitation, five animals of each group were used for neurochemical and molecular analysis.

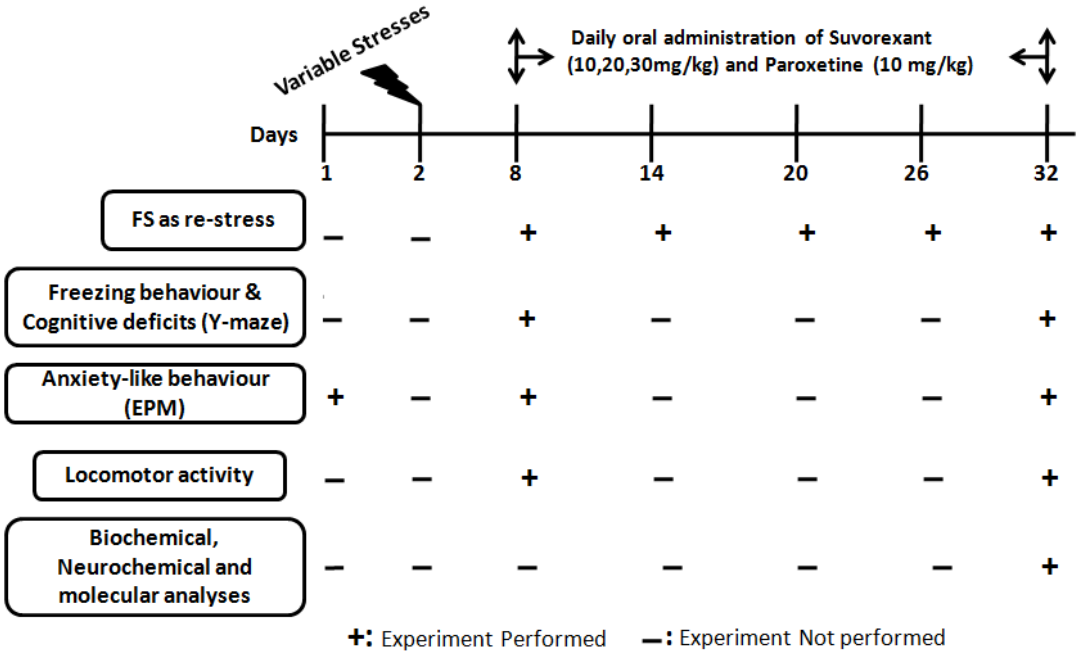

Figure 2.2 experimental design: All animals received variable stresses except for the control on the day (D)-2 and re-stress on D-8, D-14, D-20, and D-32. PTSD-like behavioural assessments were performed on D-8 and D-32 after the re-stress cue procedure for each animal (n=6/group). All the rats were decapitated for neurochemical and molecular analysis (n = 5 animals/group) on D-32. Drugs treatment were started from D-8 and continued till D-32.

2.3.3.1 FS as a stress re-stress

The protocol used for SRS was as described earlier (Krishnamurthy et al., 2013; Prajapati et al., 2019b). Briefly, the rats of all groups except control groups were individually given 'stress' in SRS protocol on D-2 (Krishnamurthy et al., 2013). The variable stress given at about 8.00 h (morning) consisted of 2 h restrain stress followed by FS and exposure to 0.8 ml of 4% halothane. On day 2, after 2 h restraint stress, the rats were given FS of 2 mA for 10 sec in (20×10×10 cm) plexiglass chambers (Inco Instrument and Chemicals Pvt. Ltd., India). Consequently, the rats were exposed to halothane in a transparent container. Then, the rats were shifted to their home cages and left undisturbed. The re-stress procedure was repeated on D-8, 14, 20, 26, and 32 (Prajapati et al., 2019b).

2.3.3.2 Collection of plasma, CSF, and amygdalar tissue

On D-32, after the behavioural tests, animals were anaesthetised with 3% v/v isoflurane inhalation (Cat: R620 veterinary anaesthesia machine, RWD life science, San Diego, USA), approximately for 1-2 min to minimize animal suffering and quickly decapitated. The blood was collected just after decapitation and centrifuged at 3000 rpm for 10 min at 4°C. Plasma was collected and frozen until corticosterone analysis (Krishnamurthy et al., 2013). The CSF was collected from the cisterna magna with a needle (27-gauge). Amygdala was microdissected and stored at −80°C until serotonin and molecular analysis is performed.

2.3.4 Behavioural assessments

2.3.4.1 Fear extinction behaviour

The fear extinction behaviour was examined in SRS-exposed rats as it is one of the major symptoms of PTSD (Halonen et al., 2016). The freezing behaviour is a reliable measure of extinction fear response elicited by contextual stimuli and was observed after FS. On D-2, rats were given FS as a part of variable stress and as a re-stress on D-8, 14, 20, 26, and 32 using plexiglass chambers (20×10×10 cm) equipped with a metal grid

floor (Inco Instrument and Chemicals Pvt. Ltd., India). The contextual fear response was measured on D-8 and D-32 by placing the rats in an identical chamber without applying any shock for 5 min, 6 h post FS application (Prajapati et al., 2019b). The entire duration of freezing behaviour was counted as an index of contextual fear.

2.3.4.2 Elevated plus-maze test (EPM)

Elevated plus-maze is a renowned method for the measurement of anxiety-like behaviour (Krishnamurthy et al., 2013). The fabricated EPM comprises two closed arms (50x10x40 cm) and two open arms (50x10 cm) at the height of 50 cm from the ground. In order to habituate the rats to the laboratory condition, they were shifted to the experimental room 30 min before the commencement of the experiment. The rats were positioned at the edge of an open arm facing away from the centre. The parameters like percentage open arm entries and the time spent in the open arm were recorded for 5 min on D-1, D-8 and D-32 (Prajapati et al., 2019a).

2.3.4.3 Spontaneous locomotor activity as a sedative parameter

The locomotion behaviour was performed using actophotometer (Ikon Instrument), and a decrease in spontaneous locomotor motor behaviour was considered a measure of the sedative effect (Rampin et al., 1991). Initially, the rats were acclimatized for 5 min on actophotometer. The movement of the animal cuts off a beam of light which was recorded and displaced digitally on D-8 and D-31. The spontaneous locomotor behaviour is expressed in terms of total photo beams counts/5 min per animal (Bonaventure et al., 2015).

2.3.5 Biochemical and neurochemical estimations

2.3.5.1 Plasma corticosterone level

The plasma CORT level was assessed using HPLC with attached UV detector system (Waters, USA), as performed earlier by Krishnamurthy et al. (Krishnamurthy et al., 2013).

2.3.5.2 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.

2.3.5.3 Measurement of serotonin (5-HT) and its metabolite in the amygdala

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/electrochemical detector (ECD) similarly, as described earlier (Krishnamurthy et al., 2013).

2.3.6 Molecular analysis

2.3.6.1 Tissue preparation and protein isolation

All the rats were sacrificed and AMY was collected after behavioural assessment on the last day of the experiment. The AMY (n=5) was homogenised using a RIPA buffer containing 1% protease inhibitor phenyl methane sulfonyl fluoride (PMSF). The above sample was centrifuge at 4ºC for 10 min (14000 g), and then the supernatant was pulled out and stored at −80ºC for the next step.

2.3.6.2 CRF-R1 western blotting

The aliquot of amygdalar protein samples was electrophoresed in 10 % SDS-PAGE gel for CRF-R1 protein and blotted onto PVDF membrane (Cat # 88585, 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-CRF (rabbit) antibody diluted 1:200 (RRID: SC-10718, Santa Cruz Biotechnology Inc, California, USA) overnight at 4°C. The treated membrane was subsequently incubated with horseradish peroxidase-conjugated anti-rabbit IgG antibody (E-AB-1003, Elabsciences USA) for 1 h The CRF-R1 expression was visualised using ECL reagents (# 1705061, Bio-Rad, CA, USA), in chemiluminescence detector (Fusion FX vilber lourmat) using A and B (1:1) and further quantified by densitometric analysis.

2.4 Data analysis

The sample size for our present study was calculated through A priori G*power analysis using F tests. Data of biochemical, neurochemical, and molecular studies were assessed by one-way ANOVA followed by Newman-Keuls Multiple Comparison Test. Two-way ANOVA, followed by Bonferroni post-hoc test, was used to evaluate the behavioural data. In the event of significance ($\alpha = 0.05$), post-hoc tests were performed and Pearson's correlation was used to analyse the correlation among selected parameters. All statistical analyses were performed on Prism 5.0 for Windows (Graph Pad prism, RRID: SCR-002798 Software, San Diego, California). All the data are presented as mean±SD and $p<0.05$ was considered significant.

2.5 Results

2.5.1 Suvorexant reduces SRS-induced freezing behaviour

The fear response is one of the characteristic features of PTSD, in which animals show freezing behaviour in response to traumatic stress (Gonzalez and Martinez, 2014). Freezing behaviour in the animal is similar to hyperarousal in humans, which is a learned behaviour in response to a traumatic event (Gonzalez and Martinez, 2014). In the present study, repeated measure two-way ANOVA showed a significant increase in freezing behaviour among the group [$F_{6,70}$ = 82.3, $p<0.05$] following FS as a re-stress cue from D-8 to D-32 [$F_{1,70}$ = 93.86, $p<0.05$]. There was a significant interaction between the groups and time [$F_{6,70}$ = 14.64, $p<0.05$]. Post hoc analysis revealed that, all the doses (10, 20 and 30 mg/kg) of suvorexant and paroxetine (10 mg/kg) significantly decreased the FS-induced increase in freezing behaviour. However, the effect of suvorexant at the doses of 20 and 30 mg/kg and paroxetine was more significant compared to suvorexant 10 mg/kg. Further, suvorexant *per se* did not modulate freezing behaviour in control rats as shown in Figure 2.3.

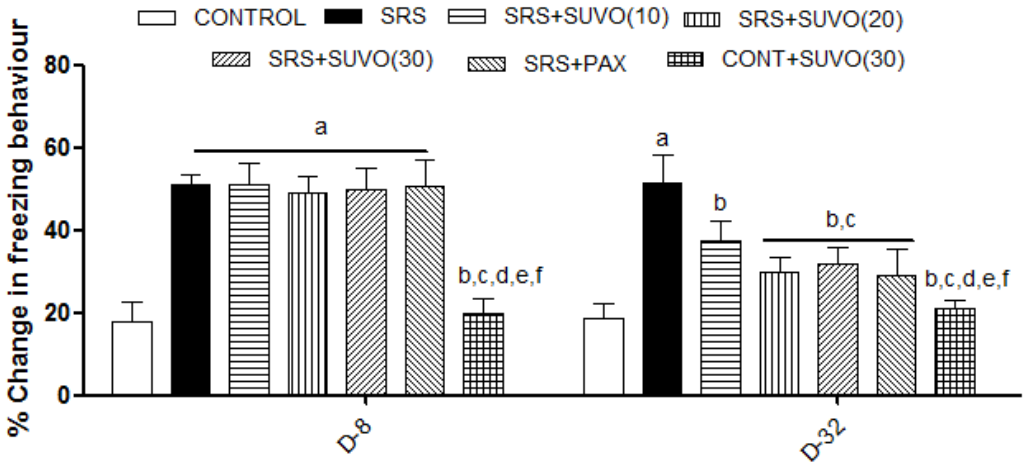

Figure 2.3 the effect of suvorexant (10, 20, 30 mg/kg) and paroxetine (10 mg/kg) on SRS-induced alteration on extinction of fear response. All values are Mean±SD (n= 6; number of rats). [a]p< 0.05 compared to control, [b]p< 0.05 compared to SRS, [c]p< 0.05 compared to SRS+SUVO (10 mg/kg), [d]p< 0.05 compared to SRS+SUVO (20 mg/kg), [e]p< 0.05 compared to SRS+SUVO (30 mg/kg) and [f]p< 0.05 compared to PAX (10 mg/kg) [Repeated measure two-way ANOVA followed by Bonferroni test].

2.5.2 Suvorexant decreases SRS-induced anxiety-like behaviour during EPM

Anxiety-like behaviour is one of the core symptoms observed among PTSD patients (Kok et al., 2002). In the EPM paradigm, the percentage open arm entries and time spent were decreased with SRS ([$F_{6,105}$ = 32.26, $p<0.05$] and [$F_{6,105}$ = 48.25, $p<0.05$]) respectively. These changes were observed on D-8 to D-32 ([$F_{2,105}$ = 140.5, $p<0.05$] and [$F_{2,105}$ = 136.8, $p<0.05$]) respectively (Figure.2.4). Further, there was a significant interaction between groups and time ([$F_{12,105}$ = 12.69, $p<0.05$] and [$F_{12,105}$ = 18.15, $p<0.05$] respectively in the EPM paradigm. Post hoc analysis represented that, all the doses of suvorexant (10, 20, and 30 mg/kg) and paroxetine (10 mg/kg) ameliorated SRS-induced decrease in percentage open arm entries and time spent on D-32. Further, the effect of suvorexant at the dose of 20 and 30 mg/kg and paroxetine was more significant compared to suvorexant 10 mg/kg. No per se effect was observed with suvorexant, as shown in Figure 2.4.

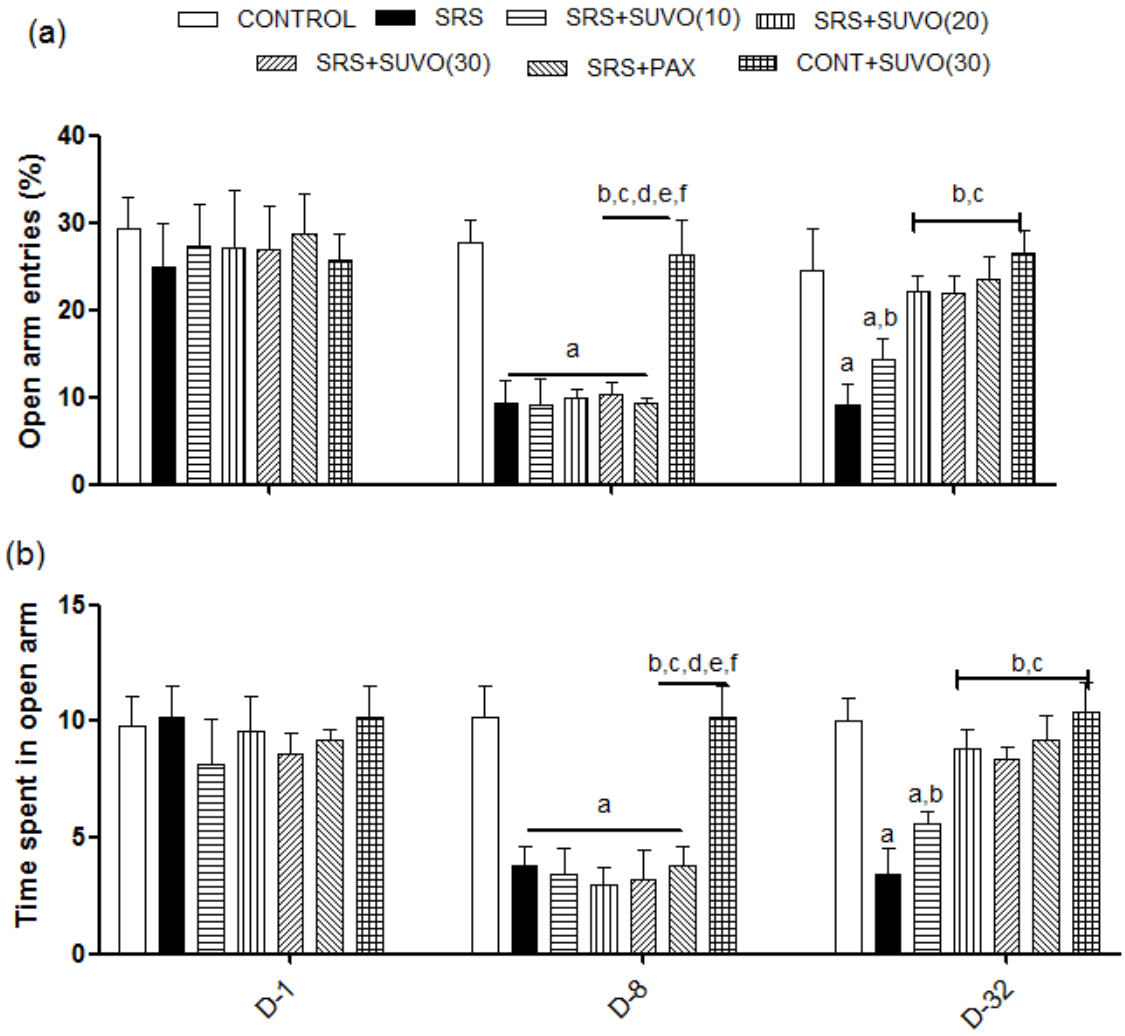

Figure 2.4 the effect of suvorexant (10., 20., 30. mg/kg) and paroxetine on SRS-induced changes in open arms entries (a) and time spent in open arms (b) during EPM on different days. All values are in mean±SD (n=6; number of rats). [a]p<0.05 compared to control, [b]p<0.05 compared to SRS, [c]p<0.05 compared to SRS+SUVO (10 mg/kg), [d]p<0.05 compared to SRS+SUVO (20 mg/kg), [e]p<0.05 compared to SRS+SUVO (30 mg/kg) and [f]p<0.05 compared to paroxetine (10.0 mg/kg) [Repeated measure two-way ANOVA followed by Bonferroni test].

2.5.3 Suvorexant mitigates SRS-induced alteration on spontaneous locomotor activity

The spontaneous locomotor activity was performed in actophotometer to measure the sedative effect of suvorexant in rats. Repeated measure two-way ANOVA represented significant alteration between the groups [$F_{6,70}$ = 133.7, p<0.05], time [$F_{1,70}$ = 44.33, p<0.05] and their interaction [$F_{6,70}$ = 29.11, p<0.05], respectively. Post-hoc analysis showed that exposure of SRS decreased the total beam crossing on D-8 to D-32, showing a sedative-like effect. Suvorexant (10 and 20 mg/kg) treatment increased SRS-induced

decrease in total beam crossing in actophotometer on D-32 (Figure 2.5). However, suvorexant at the highest dose (30 mg/kg) and paroxetine did not modulate spontaneous locomotor activity in SRS exposed rats but caused a decrease in locomotion in *per se* rodents.

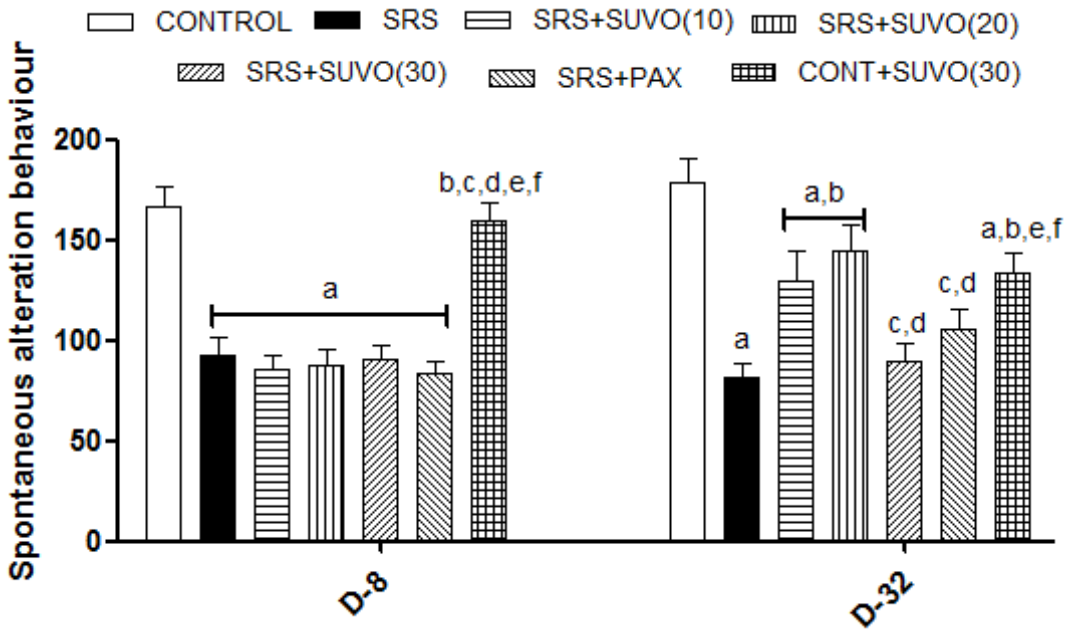

Figure 2.5 the effect of suvorexant (10, 20, 30 mg/kg) and paroxetine on SRS-induced spontaneous alteration on locomotion. All values are in mean±SD (n=6; number of rats). [a]p<0.05 compared to control, [b]p<0.05 compared to SRS, [c]p<0.05 compared to SRS+SUVO (10 mg/kg), [d]p<0.05 compared to SRS+SUVO (20 mg/kg), [e]p<0.05 compared to SRS+SUVO (30 mg/kg) and [f]p<0.05 compared to paroxetine (10 mg/kg) [Repeated measure two-way ANOVA followed by Bonferroni test].

2.5.4 Suvorexant enhances SRS-induced alteration in plasma CORT level

There was a significant effect of FS as a re-stress exposure on serum corticosterone among the groups [$F_{6,35}$ = 28.67; p<0.05] on D-32. Post-hoc analysis revealed that exposure to SRS significantly reduced corticosterone compared to control. Treatment with different doses (10, 20 and 30 mg/kg) of suvorexant and paroxetine significantly mitigated the plasma corticosterone compared to SRS exposed rats. Further, the effect of suvorexant at the dose of 20 and 30 mg/kg and paroxetine was more significant compared

to suvorexant 10 mg/kg. However, there was no *per se* effect observed with suvorexant (Figure 2.6).

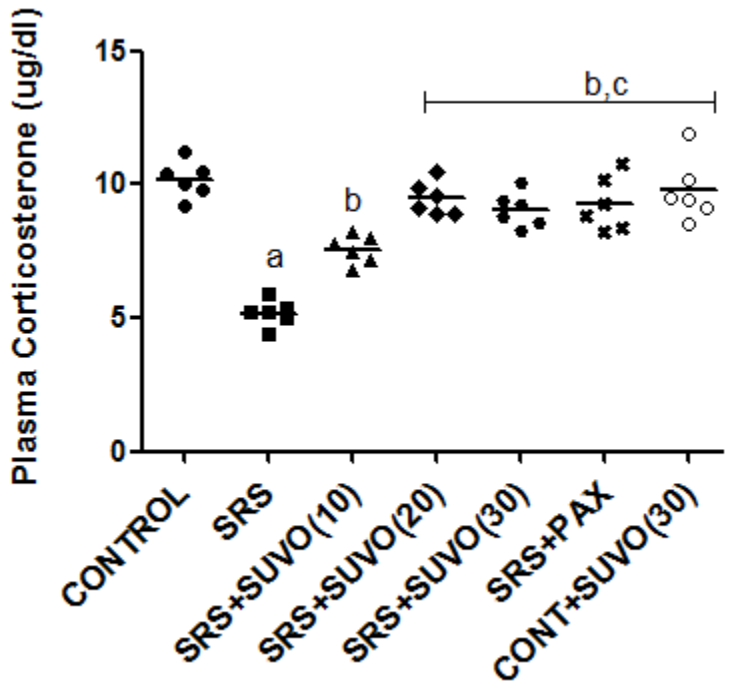

Figure 2.6 the effect of suvorexant (10, 20, 30 mg/kg) and paroxetine on SRS-induced alteration on plasma corticosterone level. All values are in Mean±SD (n=6; number of rats), [a]p<0.05 compared to control, [b]p<0.05 compared to SRS and [c]p<0.05 compared to SRS+SUVO (10 mg/kg) [One-way ANOVA followed by Newman-Keuls Multiple Comparison Test].

2.5.5 Suvorexant reduces SRS-induced alteration on 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. One-way ANOVA revealed a difference in plasma and CSF orexin-A level [$F_{6,35} = 54.17$, $p<0.05$] and [$F_{6,35} = 71.63$, $p<0.05$] 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 different doses of suvorexant significantly decreased the SRS-induced increased in plasma and CSF orexin-A level. However, paroxetine and suvorexant *per se* did not affect the plasma, and CSF orexin-A level shows in Figure 2.7.

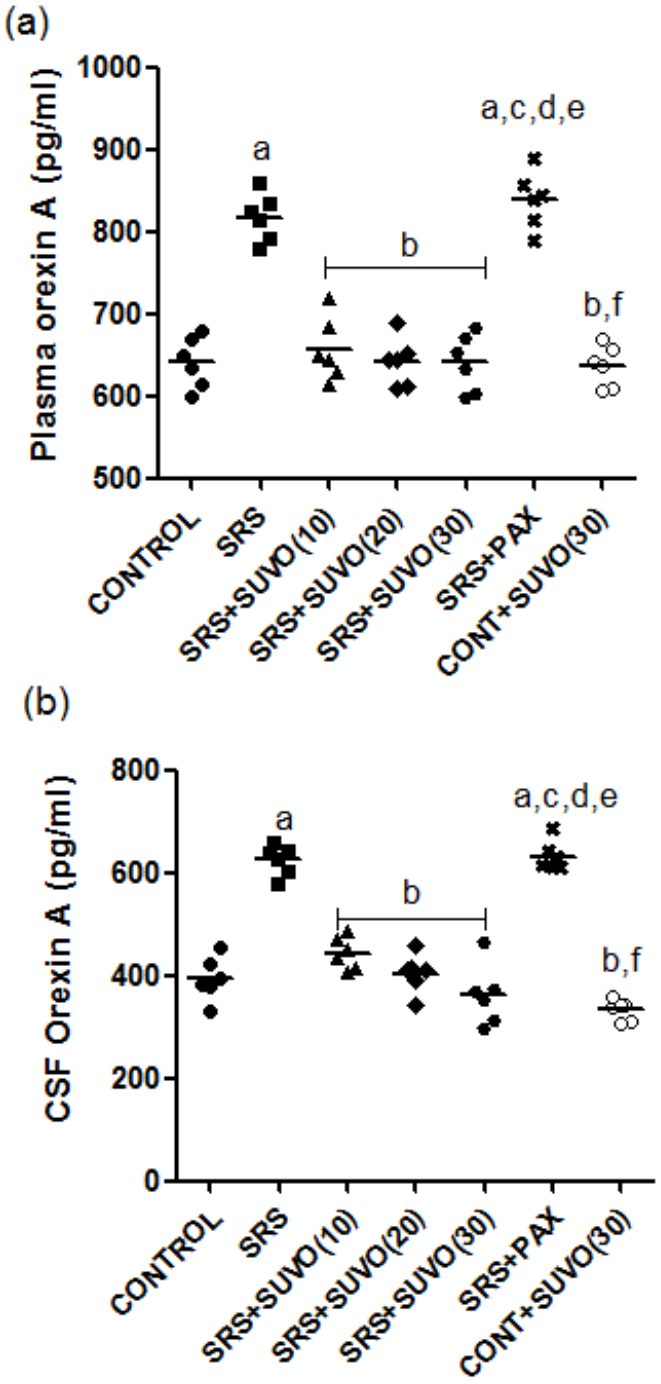

Figure 2.7 the effect of suvorexant (10, 20, 30 mg/kg) and paroxetine on SRS-induced alteration on plasma (a) and CSF (b) orexin-A levels. All values are in Mean±SD (n=6; number of rats), [a]p< 0.05 compared to control, [b]p< 0.05 compared to SRS, [c]p< 0.05 compared to SRS+SUVO (10 mg/kg), [d]p<0.05 compared to SRS+SUVO (20 mg/kg), [e]p< 0.05 compared to SRS+SUVO (30 mg/kg) and [f]p<0.05 compared to SRS+PAX (10 mg/kg) [One-way ANOVA followed by Newman-Keuls Multiple Comparison Test].

2.5.6 SRS-induced alteration on CRF-R1 expression in the AMY

In the present study, we have measured the CRF-R1 expression. Its overexpression is associated with fear response (Bale and Vale, 2004). Statistical analysis revealed that there was a significant increase in the expression of CRF-R1/β-actin ratio [$F_{6,28}$ = 101.5, $p<0.05$] among the groups. The post-hoc test showed an increase in CRF-R1 expression following exposure of FS as a re-stress. Treatment with different dose of suvorexant and paroxetine caused a significant decrease in the protein levels compared to control rats as shown in Figure 2.8. Further, there was no *per se* effect observed after treatment with different doses of suvorexant.

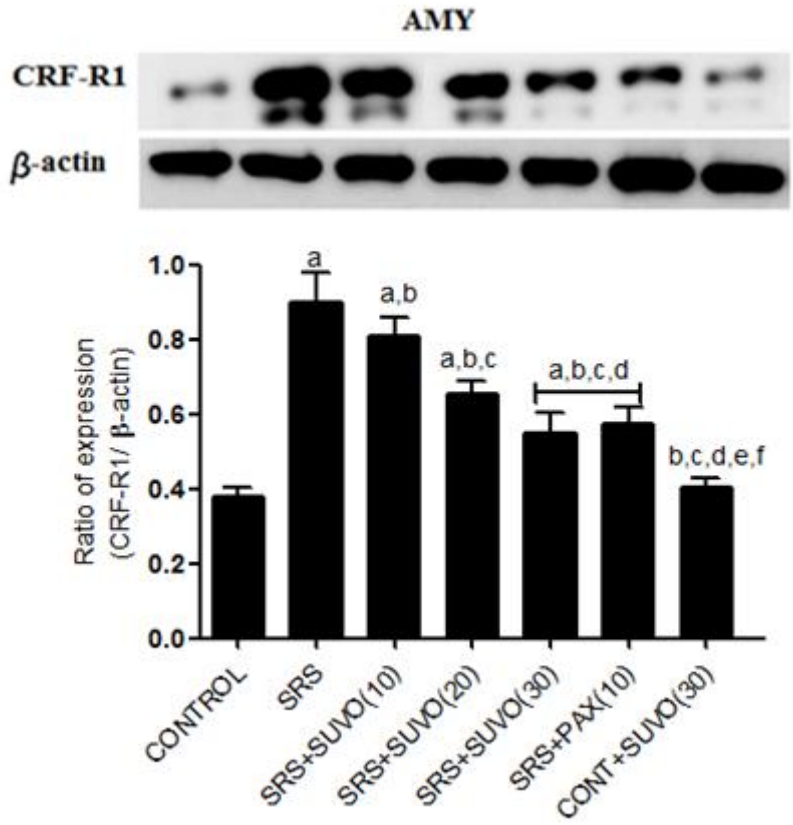

Figure 2.8 the effect of suvorexant (10, 20, 30 mg/kg) and paroxetine on SRS-induced alteration on amygdalar CRF-R1 expression. All values are Mean±SD (n=5; number of rats). [a]$p<0.05$ compared to control, [b]$p<0.05$ compared to SRS, [c]$p<0.05$ compared to SRS+SUVO (10 mg/kg), and [d]$p<0.05$ compared to SRS+SUVO (20 mg/kg), [e]$p<0.05$ compared to SRS+SUVO (30 mg/kg) and [f]$p<0.05$ compared to SRS+PAX (10 mg/kg) [One-way ANOVA followed by Newman-Keuls Multiple Comparison Test].

2.5.7 Suvorexant attenuates SRS-induced alteration on serotonin levels in the AMY

To investigate the effect of suvorexant (10, 20, and 30 mg/kg) on SRS-induced alteration on the serotonergic system, we examined 5-HT, 5-HIAA level, and their turnover in the amygdala. In the present study, one-way ANOVA showed that, there was increase in 5-HT [$F_{6,28}$ = 45.55, $p<0.05$], 5-HIAA [$F_{6,28}$ = 147.5, $p<0.05$] level and its turnover (5-HIAA/5-HT) [$F_{6,28}$ = 8.79, $p<0.05$] following SRS exposure. Treatment with suvorexant 20 and 30 mg/kg significantly decreased the SRS-induced alteration in 5-HT, 5-HIAA and its turnover. However, paroxetine and suvorexant *per se* did not show any changes in 5-HT, 5-HIAA and their turnover (Figure 2.9).

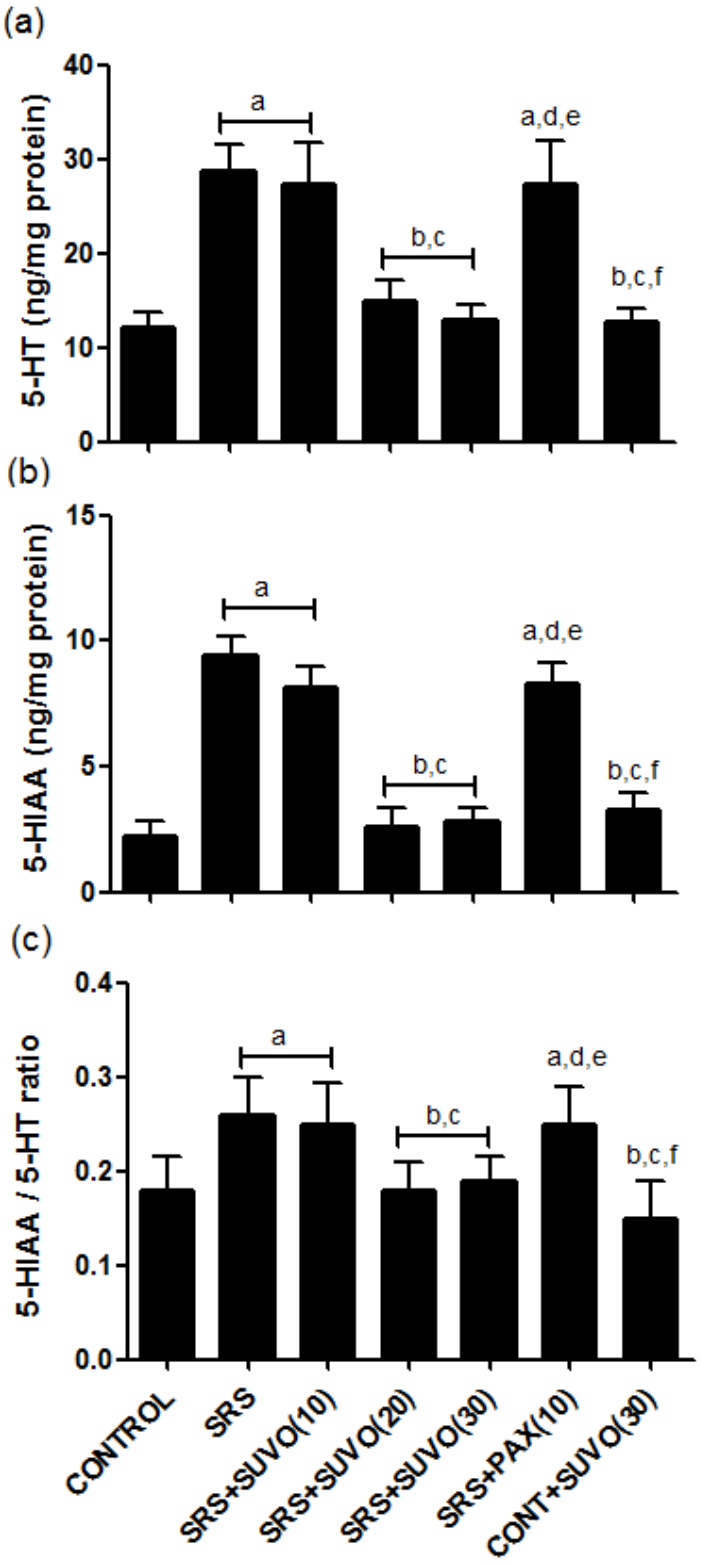

Figure 2.9 the effect of suvorexant (10, 20 and 30 mg/kg) and paroxetine on SRS-induced alterations on serotonin levels in amygdala. All values are Mean±SD (n=5), [a]p<0.05 compared to control, [b]p<0.05 compared to SRS, [c]p<0.05 compared to SRS+SUVO (10 mg/kg), [d]p<0.05 compared to SRS+SUVO (20 mg/kg), [e]p<0.05 compared to SRS+SUVO (30 mg/kg) and [f]p< 0.05 compared to SRS+PAX [One-way ANOVA followed by Newman-Keuls Multiple Comparison Test].

2.5.8 Correlation analysis of orexin-A with HPA axis marker and behaviour parameter

A correlation between the plasma and CSF orexin-A level with SRS-induced phenotypic changes, corticosterone, and serotonin level is illustrated in Figure 2.10. Plasma orexin-A level was significantly and positively correlated with freezing behaviour (Pearson's $r = 0.53$, $p<0.05$), but not correlated with open arm entries (Pearson's $r = -0.34$, $p>0.05$) and time spent in open arm (Pearson's $r = -0.37$, $p>0.05$). Further, a significant negative correlation was observed in corticosterone level with plasma orexin-A (Pearson's $r = -0.57$, $p<0.05$) and CSF orexin-A (Pearson's $r = -0.56$, $p<0.05$). Moreover, the hyperactivity of serotonergic system significantly and positively correlated with plasma orexin-A (Pearson's $r = 0.72$, $p<0.05$) and CSF orexin-A (Pearson's $r = 0.76$, $p<0.05$) level. Similarly, the SRS-induced increase in expression of CRH-R1 was positively correlated with plasma orexin-A (Pearson's $r = 0.52$, $p<0.05$) level but not with CSF orexin-A level (Pearson's $r = 0.41$, $p>0.05$). Furthermore, CRH-R1 expression negatively correlated with corticosterone, (Pearson's $r = -0.74$, $p<0.05$).

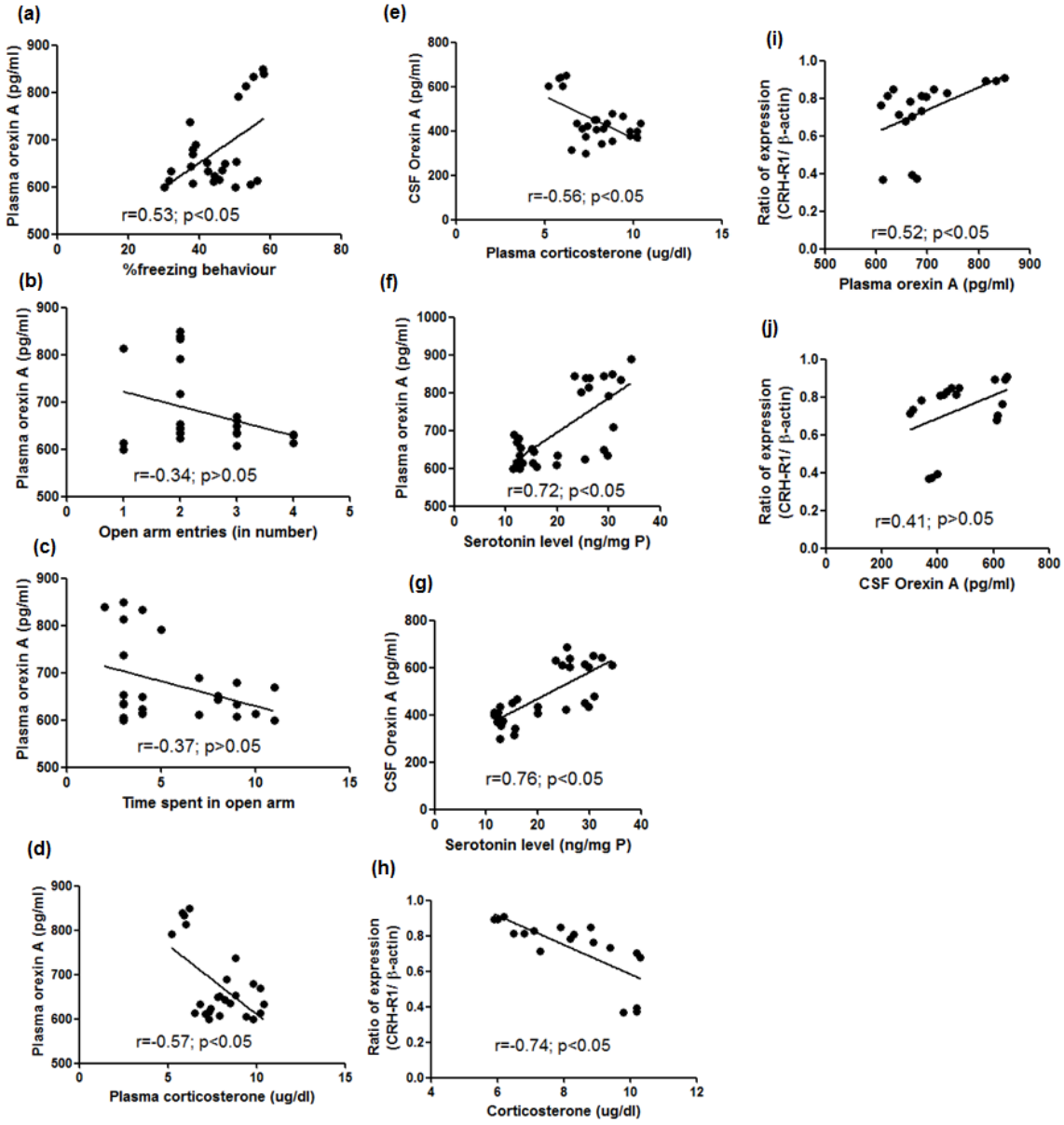

Figure 2.10 the correlation analysis of plasma orexin-A with SRS-induced alterations on (a) freezing behaviour, (b) open arm entry and (c) time spent in open arm. Similarly, the correlation of plasma corticosterone with the plasma and CSF orexin-A levels depicted in Figure 2.10 (d) and (e) respectively. Further, the correlation of plasma and CSF orexin-A with SRS-induced alterations on serotonin levels shown in Figure 2.10 (f) and (g) respectively. Moreover, the correlation of CRH-R1expression with corticosterone, plasma and CSF orexin-A level represented in Figure 2.10 (h), (i) and (J) respectively, [Pearson's correlation analysis at $p<0.05$].

2.6 Discussion

The salient findings of our study are the relationship between hyperactivity of the orexinergic system and PTSD-like symptoms in rats. To our knowledge, this is the first report exhibiting the preclinical anti-PTSD effect of suvorexant, a dual orexin receptor antagonist. Interestingly, exposure to SRS caused activation of the extra hypothalamic stress system, which was significantly attenuated by suvorexant.

Dysregulation of fear response contributes to a number of neuropsychiatric disorders, including PTSD and phobias associated with panic disorders (Holmes and Singewald, 2013). In the current study, SRS subjected rats showed an exaggerated fear response with a persistent increase in freezing behaviour. Freezing is a defensive phenomenon with manifestations like decreased physical activity and reduced heart rate (Fragkaki et al., 2017). However, this behavioural pattern is believed to be the main cause for maladaptive defensive responses as it inhibits the adaptive risk assessment of traumatic cues (Gonzalez and Martinez, 2014). Treatment with suvorexant (10, 20, and 30 mg/kg) has reduced freezing response in SRS exposed rats without showing *per se* effect. In support of our observation, Flores et al. reported that orexin antagonist reduced fear-based startle learning in stressed rats (Flores et al., 2014). This reduction in freezing response indicates the development of risk adaptive behaviour, which can lead to a reduction in acquisition of fear response. Besides, paroxetine also alleviated freezing behaviour on D-32. Even in previous study, paroxetine ameliorated the fear response by reducing freezing behaviour in single prolonged stressed rats (Takahashi et al., 2006).

Moreover, traumatic stress caused the acquisition of fear and exacerbated the anxiety in PTSD patients (Millan et al., 2012). The anxiety-like behaviour was observed in present study as the exposure of SRS decreased the percentage open arm entries and time spent in open arms. Sub-chronic treatment with various doses of suvorexant (10, 20, and 30

mg/kg) and paroxetine mitigated the SRS-induced anxiety-like behaviour on D-32. This indicates the potential of suvorexant in alleviating fear-related anxiety responses. Moreover, the PTSD-like symptoms such as fear response and anxiety are associated with HPA-axis dysfunction. Clinically HPA-axis dysfunction is observed as hypocortisolism during PTSD condition (de Kloet, 2003). In the current study, exposure of SRS caused a reduction in plasma corticosterone level, indicating the HPA-axis dysfunction (Krishnamurthy et al., 2013). Suvorexant significantly increased plasma corticosterone in SRS subjected rats indicating the role of the orexinergic system in the regulation of HPA-axis. Orexin-A is reported to regulate the sensitisation of HPA-axis through hypothalamus and extra-hypothalamus-dependent pathways (Sharko et al., 2017). Orexin-A binds to glycoprotein coupled receptor OX1-R and OX2-R (Gs subtype) and stimulates adenyl cyclase that ultimately activates downstream cascade pathways of cAMP (Kukkonen, 2013). That includes cAMP-dependent phosphorylation of hormone-sensitive lipase that stimulates CRH system followed by glucocorticoid synthesis (Kukkonen, 2013). In the present study, exposure of SRS significantly increased the plasma and CSF orexin-A level in rats indicating hyperactivity of peripheral and central orexinergic system. The increased CSF orexin-A level is also observed in patients suffering from panic anxiety (Salomon et al., 2003; Johnson et al., 2010a). Sub-chronic treatment with suvorexant *per se* did not show any significant effect on the orexin-A levels in plasma and CSF, but significantly amelioration was observed in the SRS-exposed rats. Therefore, suvorexant acts only during the malfunctioning of orexinergic system by antagonising the orexin receptors and also by inhibiting the release of orexin. In this regard, apart from dual orexin receptor antagonism, suvorexant also modulates orexin release in neuropsychiatric disorders (Yeoh et al., 2014). The Orexin-A regulates HPA-axis function by regulating extra-hypothalamic CRH system during the chronic stress (Lu et al., 2008). The

activation of the extra-hypothalamic CRF system was observed in the SRS model where there was up-regulation of CRF-R1 in the amygdala following SRS exposure. Suvorexant did not alter amygdalar CRF-R1 expression in *per se* rats, but dose-dependent attenuation was observed in SRS-exposed rats indicating its direct effect on dysfunctional HPA-axis that is responsible for stress vulnerability (Nakamura and Nagamine, 2017). This finding is further affirmed by previous report on central administration of orexin-A that caused sensitization of the HPA axis through CRH mediated mechanism (Samson et al., 2002). Paroxetine also reduced SRS-induced increase in CRF-R1 expression in the amygdala. Thus, both the drugs alleviated the HPA dysfunction and CRH-R1 expression and its associated symptoms like acquisition of fear and anxiety-like behaviour in SRS-exposed rats.

Additionally, exposure of SRS to rats caused elevation of 5-HT and 5-HIAA levels as well as its turnover in the amygdala, indicating the activation of the serotonergic system. The hyperactivity of the amygdalar serotonergic system is another pathological cause of PTSD (Krystal and Neumeister, 2009). The activation of the serotonergic system is responsible for the augmentation of PTSD-like symptoms such as fear response and anxiety-like behaviour in rats (Krishnamurthy et al., 2013). Previous studies have demonstrated that orexinergic neuron directly activates the hypothalamic serotonergic neurons and facilitates arousal behaviour (Saito et al., 2018). Suvorexant significantly attenuated the SRS-induced activation of the serotonergic system and associated metabolites. This indicates the involvement of orexinergic system in the regulation of the serotonergic system in the SRS model. In contrast, paroxetine did not modulate the serotonergic system in the amygdala but ameliorated the same in the hypothalamus and prefrontal cortex as reported earlier (Krishnamurthy et al., 2013). This indicates the region-specific effect of paroxetine on the serotonergic system. This is further confirmed

by the correlation analysis in which increased plasma, and CSF orexin-A levels are positively correlated with serotonin level in the amygdala. In support of the present finding, an earlier report revealed that the central administration of orexin-A directly activates the serotonergic system in the amygdala (Feng et al., 2008). Further, Pearson's analysis indicated that increased plasma and CSF orexin-A level is positively correlated with PTSD-like phenotypes such as fear response and anxiety-like behaviour. Therefore, orexin-A can also be a useful biomarker for PTSD, which is strongly supported by clinical reports on the pathological cause of PTSD (Strawn et al., 2010).

Suvorexant is safe and well-tolerated in patients, and the recommended dose is 10 to 20 mg once at night (Kishi et al., 2019). However, high doses (>20 mg/kg) of suvorexant may induce a sedative effect by reducing the threshold to transition into sleep (Merlo-Pich and Melotto, 2014). Therefore, the decrease in spontaneous locomotor behaviour was used to assess the sedative-like effect (Volke et al., 1997). The decrease in spontaneous locomotor behaviour is also observed in SRS exposed rats. The SRS-induced reduction in spontaneous may be due to an increase in freezing response. Earlier it is demonstrated that an intense stressful situation such as 2 h immobilisation stress might induce paradoxical sleep by altering locomotive behaviour with slight changes in sleep-wake state (Rampin et al., 1991). Sub-chronic treatment with low and median doses (10 and 20 mg/kg) but not higher dose (30 mg/kg) of suvorexant attenuated the stress-induced decrease in spontaneous locomotor behaviour. Further, suvorexant caused a reduction in spontaneous locomotor behaviour in *per se* rats. This decrease in locomotion may be due to the sleep-inducing effect of suvorexant as suggested previously (Kukkonen, 2013). Further, this dose-specific effect of suvorexant may also be due to differences in receptor occupancy. Suvorexant at low doses (10 and 20 mg/kg) occupies 50–60% of OX2-R (orexin receptor) and produces an anxiolytic effect, while doses more

than 20 mg/kg occupy more than 80% and can also produce sedation (Gotter et al., 2013; Dubey et al., 2015; Nakamura and Nagamine, 2017). Therefore, the dose of 30 mg/kg of suvorexant in rats corresponds to the clinically comparable dose to develop common dose-dependent adverse effects such as sedation (Dubey et al., 2015). Hence, non-sedative doses of suvorexant can be used for the treatment of PTSD without the occurrence of common side effects. Paroxetine did not modulate spontaneous locomotor activity in SRS-exposed rats similarly as suggested clinically (Dickens et al., 2000).

2.7 Summary

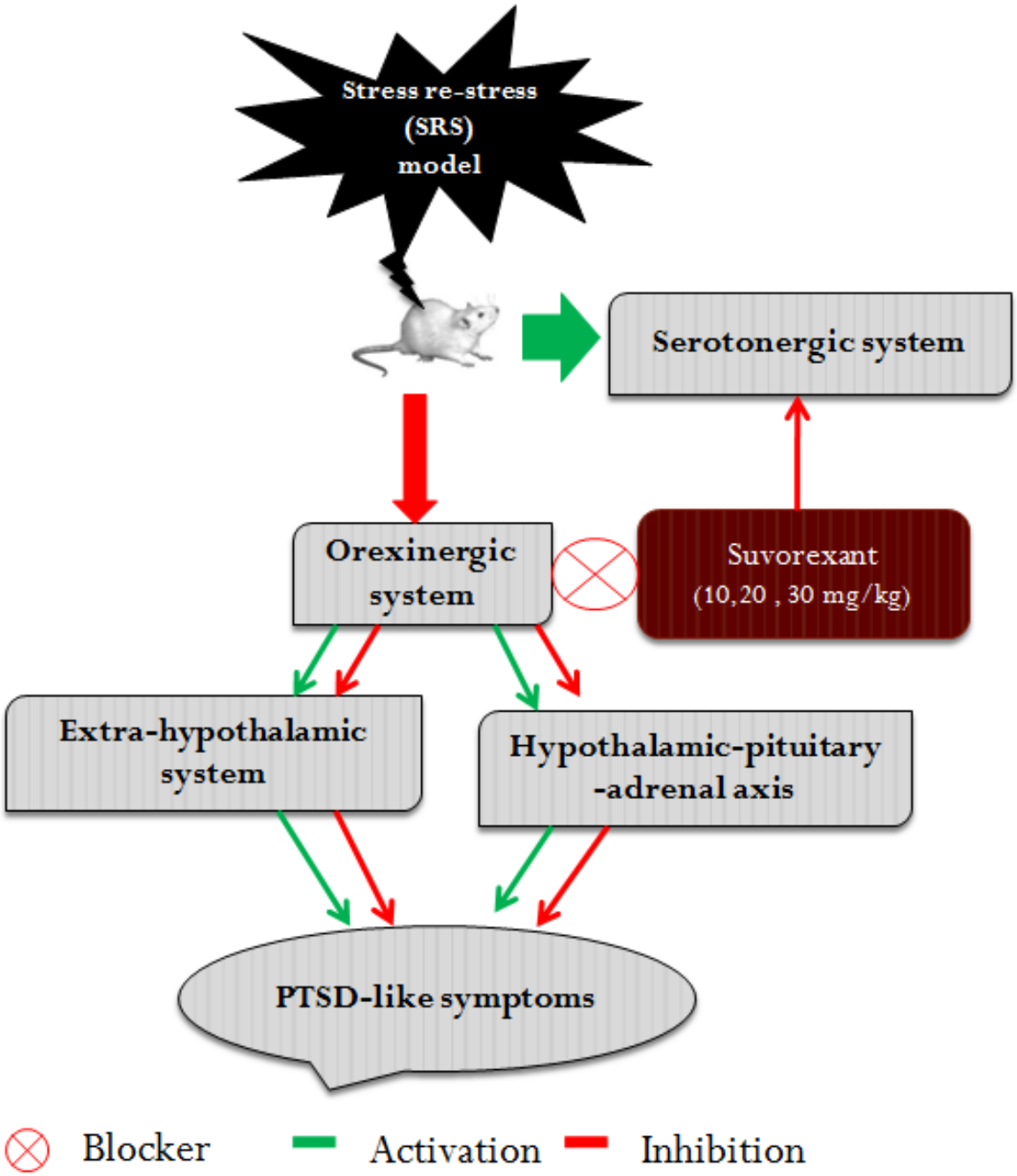

Figure 2.11 Summary of the study, in which encircled red-cross symbol represents blocker and green and the red colour represents activation and inhibition respectively.

The current findings indicate the hyperactivity of peripheral and central orexinergic systems as the plasma and CSF orexin-A levels respectively were increased in PTSD. There was disruption of the HPA-axis which is manifested as a decrease in corticosterone level. Further, the activation of the extra-hypothalamic CRF system was observed where there was up-regulation of CRF-R1 in the amygdala. Moreover, the amygdalar serotonergic system was hyperactive in rats. Sub-chronic treatment with suvorexant counteracted the hyperactivity of orexinergic system by antagonising orexinergic receptor non-selectively. Further, suvorexant improved the HPA-axis function and CRH system. Suvorexant also alleviated the serotonergic hyperactivity in the SRS model and attenuated PTSD-like symptoms. Moreover, the suvorexant-induced anti-PTSD-like effect was comparable to paroxetine. Suvorexant at 10 and 20 mg/kg attenuated SRS-induced alteration in locomotion and produced a sedative effect in its highest dose (30 mg/kg) on unstressed rats. Therefore, suvorexant may be an ideal treatment strategy for SSRIs resistant PTSD patients. Further, targeting of the orexinergic system can be a potential treatment strategy for patients with sleep-related disturbances in PTSD, but however this has to be further verified.