Chapter 3

Validation of cognitive inflexibility in SRS model of PTSD

3.1 Introduction

Cognitive inflexibility is one of the clinically observed major symptom of PTSD together with fear and anxiety (Keith et al., 2015). Cognitive flexibility regulates the core symptoms of PTSD such as fear response or negative emotional state and anxiety (Ramaswamy et al., 2016, Thomas and Stein, 2017). The abnormality in cognitive flexibility causes the inability to shift from negative to positive emotional state (Ramaswamy et al., 2016, Thomas and Stein, 2017). However, cognitive inflexibility has not attracted much interest in animal models of PTSD. Therefore, we evaluated cognitive inflexibility for the first time in the SRS paradigm.

Cognitive flexibility can be categorized into two phases: Intra-dimensional shift (ID) and extra-dimensional shift (ED). ID shift is associated with the mild perceptual attention set and ED is mainly associated with the higher perceptual attentional set which is related to current intelligence quotient (Birrell and Brown, 2000, Jin et al., 2014). An attentional set is formed when a subject learns that a set of rules can be applied to complex stimuli in order to differentiate relevant from irrelevant cues. For example, in the attentional set shifting, animals will learn to pay attention and respond to the relevant cue (i.e., digging medium) and ignore an irrelevant cue (i.e., odour), by pairing a food reward with the medium (Garner et al., 2006). This association is then reinforced in subsequent tasks where the type of digging medium and odor changes, but the paired association between medium and reward remains. This reinforced rule forms a cognitive set (Brigman et al., 2005). Two stages within the attentional set shifting protocol measure aspects of cognitive flexibility: reversal and the ED shift. At the reversal stage, the previously negative stimuli within one dimension (medium in this example) is now positive, which challenges the animal to ignore the positive stimuli from the previous stage (Chase et al., 2012). For example, if felt digging medium was the positive stimuli in the previous stage

and paper was the negative stimuli, now the reverse is true. This challenges the animal's flexibility in that it must maintain the attentional set (i.e., medium is the relevant dimension) while altering the rule learned for stimulus and reward pair within a dimension (S Tait et al., 2014). The formation of an attentional set is challenged at the ED shift stage, when the irrelevant dimension (odor in this example) becomes the relevant dimension. A perseverative response, as indicated by a continued choice using the previously learned rule, at either stage reflects a deficit in cognitive flexibility (Lapiz-Bluhm et al., 2008).

Further, it has been shown that stimulation of cognitive flexibility attenuates anxiety-like symptom in single prolonged stress and chronic restrain stress model (George et al., 2015, Zhao et al., 2017). The mechanism of cognitive inflexibility involves dysfunction of cholinergic system which is responsible for executive behaviour (Herrmann et al., 2011). Acetylcholine (ACh) signals through both muscarinic as well as nicotinic receptors. Further, modulation of alpha-7nicotinic acetylcholine receptor (α-7nAChR) has been observed in the schizophrenic and Alzheimer's patients (Haydar and Dunlop, 2010). In addition to this, studies have shown that, the α-7nAChR is predominantly present in the brain and mainly in PFC and HIP (Takada-Takatori et al., 2008). Moreover, pathophysiological and functional changes are observed in PFC, HIP and amygdalar regions in PTSD patients (Krishnamurthy et al., 2013, Jin et al., 2014). PFC and HIP inhibit, while AMY activates fear-induced memory and anxiety behaviour (Wang et al., 2014). However, there is limited information on the involvement of cholinergic system in animal model of PTSD. Therefore, assessment of cholinergic function in PFC, HIP and AMY would give a better understanding of the pathophysiology of cognitive inflexibility in PTSD.

A preclinical study has examined the impact of a traumatic event on intrusive memory (Qin et al., 2009), but there are limited reports on its effect on cognitive flexibility. PTSD models use different stress paradigms for instance predator stress, SPS and the modified SRS paradigm (Liberzon et al., 1997, Wang et al., 2008, Krishnamurthy et al., 2013). We used SRS model as it shows hypocorticosteronism which is similar to hypocortisolism in PTSD patients (Prajapati et al., 2020). The SRS model consists of variable stress exposure of 2 h restraint, FS, and halothane anaesthesia, develop psychological, physiological and endocrinological stress, respectively in rats (Prajapati et al., 2020). The pathophysiology of PTSD also involves dysfunction of the HPA-axis. In PTSD, the HPA axis is hypersensitive due to sustained activation by lower cortisol levels (Yehuda et al., 1990). Cortisol is required for learning, memory, and cognitive performance. Both low and high levels of cortisol and ACTH can disrupt memory formation. However, the effect of HPA axis on cognitive inflexibility has not been studied much.

Therefore, cognitive inflexibility was assessed as an additional symptom along with its underlying mechanism in the SRS model. Cognitive flexibility was measured using attentional set-shifting and reversal learning behaviour. Freezing behaviour was taken as a measure for the contextual fear response. The alteration in cognitive behaviours such as general exploration and spatial recognition memory was measured using the Y-maze test. Further, the anxiety-like behaviour was assessed by EPM. Further, to evaluate the role of the cholinergic system in PTSD, we have estimated levels of ACh level and AChE activity along with choline acetyltransferase (ChAT) and α-7nAChR expressions in PFC, HIP and AMY. We have also measured 5-HT and NE in the selected brain regions.

3.2 Hypothesis

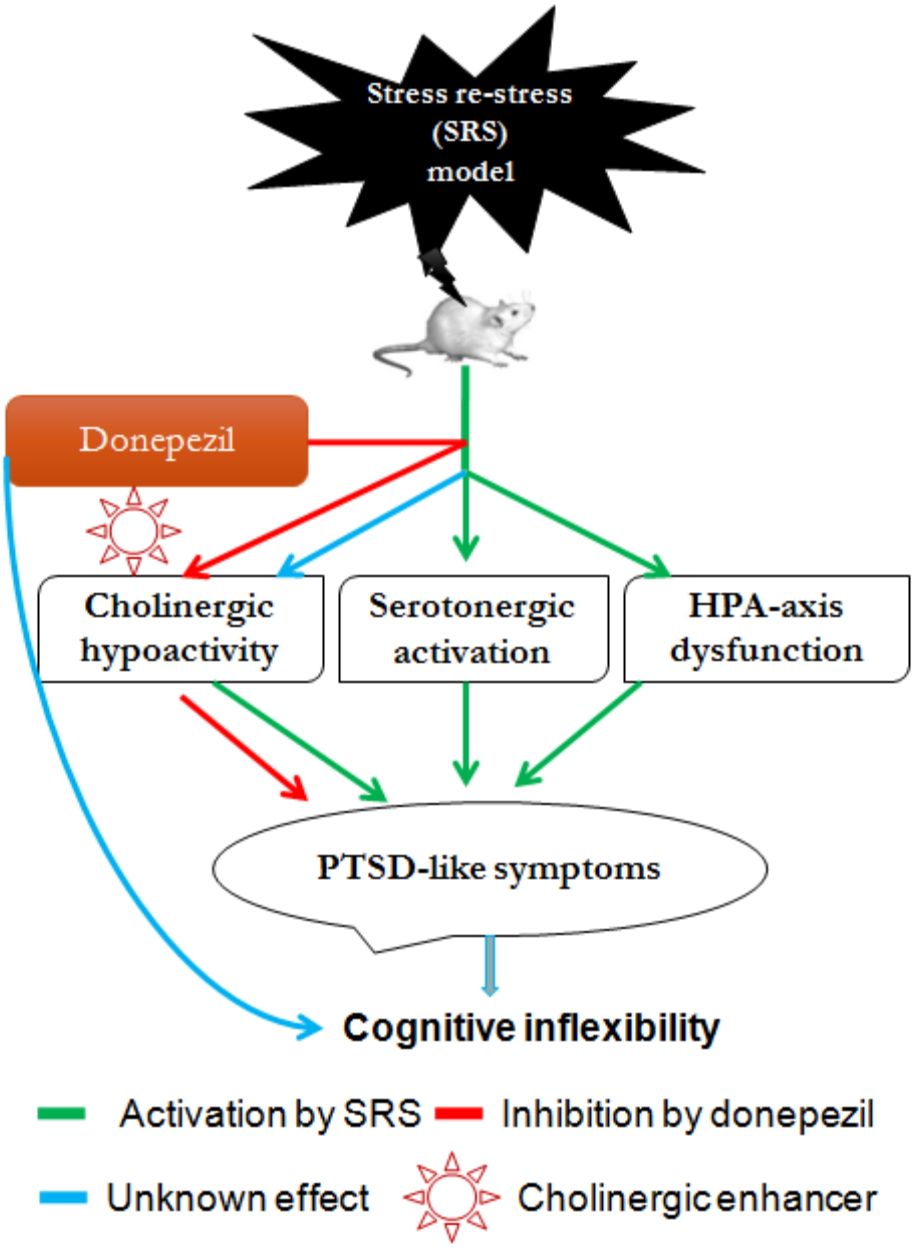


Figure 3.1 Proposed hypothesis of validation of cognitive inflexibility in SRS model of PTSD

The aim of the hypothesis is to evaluate the cognitive inflexibility as a core symptom of PTSD together with fear response and anxiety. The attentional set shifting task was developed as a measure of attention and cognitive flexibility and is modelled after the ID/ED component which is used to identify cognitive dysfunction in humans and non-human primates. Cognitive inflexibility, particularly involving dysfunction of cholinergic circuitry within the PFC. During the PTSD there is a negative alteration on cognitive function due the fear response of traumatic event, so that patients are not able to take a correct decision. Further, the decision made by PTSD patient is based on fear response rather than the execution function as a result of that the discrimination ability or problem solving ability to cope up with traumatic event is lost or suppressed. Deficits in cognitive function also represent the most difficult symptom domain to successfully treat, as serotonin reuptake inhibitors and tricyclic antidepressants have only modest effects. Functional analysis of human brain tissue implicates the hypoactivity of the cholinergic system in PFC in patients with cognitive inflexibility. However, preclinical behavioural assays used to assess these deficits in animal models which can be readily manipulated and could provide the basis for studies of new treatment avenues have been underutilized. Therefore, a well validated SRS model is used to assess effect of variable stresses and re-stress procedure in cholinergic system and associated changes in cognitive flexibility in PTSD.

3.3 Materials and Method

3.3.1 Animals

Male albino Wistar rats (weight between 240±20g) were procured from the Central Animal House, Banaras Hindu University, (Approval No.: Dean/2016/CAEC/324). The principles of laboratory animal care (NIH publication number 85-23, revised 2013) guidelines were strictly followed. In this study, maximum of four animals were accommodated in each polypropylene cages in a controlled temperature of 25±1°C, relative humidity 45–55% and a 12:12 h light/dark cycle. The food (Doodhdharapashuahar, India) and water were available throughout the experiment. The experiments procedures were performed between 08:00 and 18:00 h.

3.3.2 Drugs

Donepezil was procured (Sigma Aldrich) and sertraline was received as a gift sample (Ranbaxy, India). Both the drugs were suspended in 0.5% CMC and were administered orally through gavage. The other chemicals used in the experiment were procured from SRL Pvt. Ltd., India.

3.3.3 Experimental design

The experiment was performed for a period of 32 days. All the animals were randomly allocated to the four experimental groups (n=8, rats/group): Control, SRS, donepezil (3 mg/kg), and sertraline (10 mg/kg). Donepezil (3 mg/kg; *po*) (Pattanashetti et al., 2017) and sertraline (10 mg/kg; *po*) (Kumar and Kumar, 2009, Santiago et al., 2015) were administered orally once in a day, 1 h after re-stress from D-8 to D-32 (figure 3.2) to the respective groups. On D-2, animals were exposed to variable stresses that consisted of 2 h restrain followed by FS and subsequent exposure to 0.8 ml of 4% halothane except the control group. The FS as a re-stress cue was given on D-8, 14, 20, 26 and 32 to all animals at 10:00 h except the control group (Prajapati et al., 2019b). There was a gap of

30 min between variable stresses and the behavioural tests performed on D-2. During behavioural assessment the animals were first subjected to Y-maze test followed by EPM and freezing behaviour on D-2, D-8 and D-32 between 11.00 to 17.00 h. There was a gap of 30 min between each behavioural test (Prajapati et al., 2019a). Test for cognitive inflexibility was performed for five days (D-28 to D-32). All behavioural observations were recorded and analyzed by using ANY-MAZE^{TM} video tracking system (version-3.72; USA). All the behavioural analysis was performed by the blind observer. On the last day of experimental protocol all the animals of each groups (n=8) were decapitated and brain were micro dissected in to PFC, HIP and AMY by following the standard procedure (Paxinos and Ashwell, 2018). The samples were equally divided to perform neurochemical and molecular analysis.

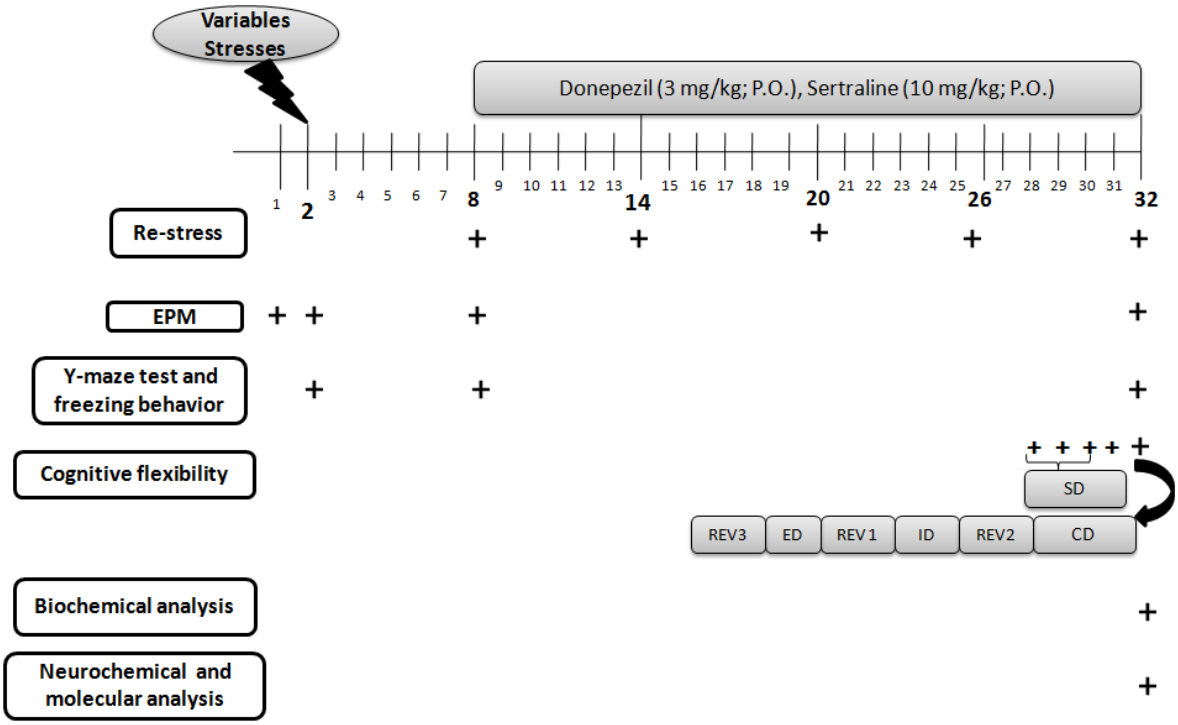


Figure 3.2 represents the experimental design of the study includes variable stresses (2 h restraint, FS, and halothane anaesthesia). On D-2 animals were exposed to variable stress consisted of 2 h restrain stress followed by FS and exposure to 0.8 ml of 4% halothane except for the control group. The foot shock (FS) as a re-stress was given on D-8, 14, 20, 26 and 32 for all animals except the control group. During the behavioural assessment, the animals were first subjected to Y-maze test followed by EPM with the gap of 30 min. Test for cognitive inflexibility was performed for five days (D-28 to D-32).

3.3.3.1 FS as a stress re-stress

The rats of all groups except control groups were individually exposed to the 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 restrain 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 until loss of consciousness. 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 as suggested previously (Prajapati et al., 2019a).

3.3.3.2 Collection of plasma, adrenal gland and brain tissue

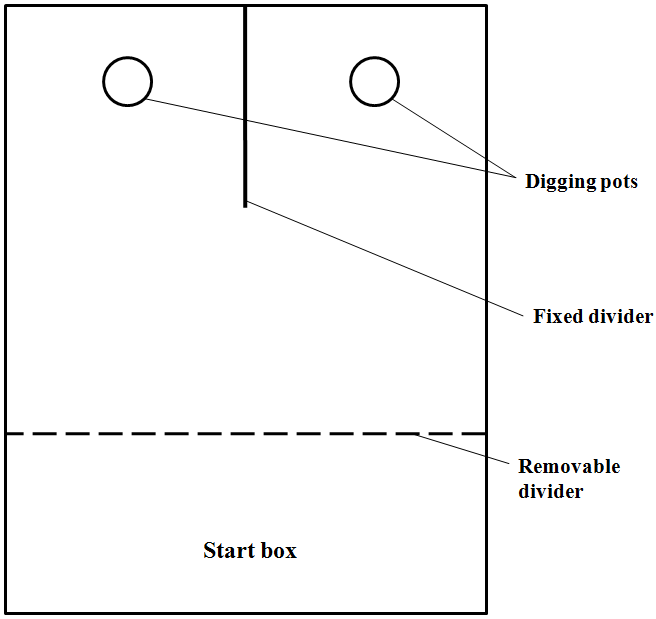
On last day, after the behavioural tests, animals were anaesthetized with 3% v/v isoflurane inhalation (Cat: R620 veterinary anaesthesia machine, RWD life science, San Diego, USA), approximately for 1-2 min to minimize animal suffering and quickly decapitated. The blood was collected just after decapitation and centrifuged at 3000 rpm for 10 min at 4°C. Plasma was collected and frozen until ACTH and corticosterone analysis (Krishnamurthy et al., 2013). PFC, HIP and AMY were microdissected following the standard procedure (Paxinos and Ashwell, 2018) and stored at -80°C until neurochemical and molecular analysis is performed.

3.3.4 Behavioural assessments

3.3.4.1 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 (length, width and height). One-third part of the set-shifting box was separated by removable plexiglass divider and termed as a start box. Another

fixed separator was placed to divide one-third part of the arena into two sections. In each different section, bowl was kept based on tests.



In the trial session, rats were placed in the start box for exploration. During testing session, the bowl with 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 a 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

cue 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, as shown in supplementary fig.1. Testing at each stage continued until the criterion of six consecutive correct trials was reached. The test started with the simple discrimination (SD) stage, involving only one dimension discrimination (for example-odor, and digging medium). For compound discrimination (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).

Discrimination stage	**Dimension**		**Example combination**	
	Relevant	**Irrelevant**	**(+)**	**(-)**
SD	Odor	Medium	Rose/ Sawdust	Clove/Sawdust
CD	Odor	Medium	Rose/ Sawdust	Clove/Wood saving
Rev 1	Odor	Medium	Clove/Wood saving	Rose/ Sawdust
ID	Odor	Medium	Sandalwood/ Thermacolbeads	Mandarin/Plastic beads
Rev 2	Odor	Medium	Mandarin/Thermacolweads	Sandalwood/Plastic weads
ED	Medium	Odor	wood beads/Jasmine	Stone beads/Lavender
Rev 3	Medium	Odor	Stone beads/Lavender	wood beads/Jasmine

Table 3.1 Order of discrimination during cognitive flexibility test. Exemplars of combinations into stimulus pairs are shown for a rat shifting from odour to digging medium. On every trial, the pair of stimuli differed on both the relevant and irrelevant dimensions. The combination of exemplars were presented in pairs and varied such that no two animals within a group received the same discriminations.

3.3.4.2 Cognition measurement in Y-maze test

Y-maze is the validated method to measure general exploratory behaviour and spatial recognition memory (Krishnamurthy et al., 2013). The Y-maze comprises of starting, known arm, and a novel arm having a dimension (50x16x32 cm) with 120^{o} angle to each other. The start arm is the point where rats are introduced to Y-maze for exploration. The known arm is the arm adjacent to starting and novel arm and kept open during both the trials (trial-1 and trial-2). The test was conducted on D-2, D-8, and D-32 after SRS procedure in the light phase. There were two trials, during the first acquisition trial the novel arm was closed, and rats were permitted to explore start and known arms for 10 min. After 4 h, during trial-2, all the three arms of the Y-maze were open and the rats were allowed to explore for 5 min. The number of entries in each arm, total number of entries and percentage entries in known to novel arm were recorded for 5 min. The general exploratory behaviour is measured in terms of total number of entries in all arms (for 5 min period of trial-1 and 2). The spatial recognition memory is measured in terms of mean percentage entries in the known arm to novel arm during trial-2 (Prajapati et al., 2019a).

3.3.4.3 Evaluation of anxiolytic activity on EPM

EPM is used for the measurement of anxiolytic activity (Ojha et al., 2010). The EPM comprises of two closed arms (50x10x40 cm) and two open arms (50x10 cm) at a height 50 cm from the ground. In order to habituate the rats to the laboratory condition, they were shifted to the experimental room, 10 min before the commencement of the experiment. The rats were positioned at the edge of 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, 2, 8 and 32 (Prajapati et al., 2019a).

3.3.4.4 Evaluation of alteration in the extinction of fear behaviour

Enhanced contextual fear response linked to intrusive memory is one of the major symptoms of the pathophysiology of PTSD. This can be directly measured as freezing behaviour. The FS (2 mA for 10 sec) as SRS was given on D-2, 8, 14, 20, 26 and D-32 in (20x10x10 cm) plexiglas chambers with a metal grid floor (Inco Instrument and Chemicals Pvt. Ltd.). In the contextual fear test, rats were exposed to the FS paired context. After FS, rats remained in the chamber for an additional 1 min. The contextual fear response was measured by placing the rats in the identical chamber without applying any shock for 5 min, six-hour post SRS application on D-2, D-8 and D-32 days. The rats were completely motionless (freeze) and even showed the absence of nose twitching. The entire duration of freezing behaviour was counted as an index of contextual fear. The decrease in freezing index was taken as a measure of extinction fear response (Dębiec et al., 2011).

3.3.5 Biochemical and neurochemical analysis

3.3.5.1 Estimation of ACTH and CORT level in plasma

ACTH and CORT concentration was measured through ELISA kit (catalog no. E-EL-R0048 and E-EL-R0269; Elabscience USA). Briefly, plasma samples were incubated with biotinylated primary antibody at 37˚C for 45 min. Plates were washed and incubated with HRP conjugated streptavidin solution for 30 min at 37˚C. Thereafter, the substrate (3, 3, 5, 5-tetramethylbenzidine) was added and incubated for an additional 15 min. Finally, stop solution was added and absorbance was taken at 450 nm using a microplate reader (Bioteck USA). The concentration of ACTH and CORT was determined using a standard.

3.3.5.2 Acetylcholine and acetylcholinesterase assay

The ACh level and AChE activity in PFC, HIP, and AMY were measured through amplex red assay kit (Invitrogen, USA). Briefly, 100 µL of the samples from each group and control (20 mM H_2O_2) was added in to the separate well. Then the reaction is started by adding 100 µL of the Amplex Red reagent containing, 1 U/mL HRP, 0.1 U/mL choline oxidase. Then100 µl of acetylcholinesterase (for ACh measurement) and 100 µl of acetylcholine (for AChE activity) working solution was added to each well. After that reaction mixture was incubated for 30 min at 37˚C. The fluorescence was recorded through spectrofluorometer using excitation wavelength 530 nm and emission wavelength 590 nm.

3.3.5.3 Monoamines and its metabolites measurement

The level of neurotransmitters such as NE, 5-HT and their metabolites 5-hydroxy indole acetic acid (5-HIAA) was estimated in discrete brain regions using HPLC with an ECD and the protein content was determined calorimetrically (Krishnamurthy et al., 2013).

3.3.6 Molecular analysis

3.3.6.1 Western blotting

Brain samples of PFC, HIP and AMY was weighed, washed and lysed in lysis buffer containing 1% protease inhibitor in a ratio of 1:5. Protein content was measured using Bradford reagent as per protocol (López et al., 1993). Lysates were centrifuged at 14,000 rpm for 30 min at 4°C. An aliquot of different brain region protein samples were electrophoresed in 10 % SDS-PAGE gel for choline acetyltransferase (ChAT) and α-7nAChR expression and blotted onto PVDF membrane. The membrane was blocked with 3% bovine serum albumin with shaking for 2 hours and then washed three times with tris-buffered saline containing 0.05% Tween-20 (TBST) and incubated overnight at 4°C with primary anti-ChAT (rabbit) antibody diluted 1:500 (catalogue no. PA5-79038, invitrogen,

USA), anti- α-7 nAChR (rabbit) antibody diluted 1:1000 (catalogue no. ab10096, abcam, USA) and anti-beta actin (catalogue no. E-AB-20094 Elabsciences USA). Then the three washes in TBST, the membranes were allowed subsequently incubated with anti-rabbit IgG antibody-horseradish peroxidase (HRP) diluted 1:200 (catalogue no. E-AB-1003 Elabsciences USA) for 1 h. The whole process was performed in triplicate. The Protein expression of ChAT and α-7 nAChR was detected by chemiluminescence detector (Fusion FX, chemiluminescence-17-200255 vilber lourmat) using enhanced chemiluminescence (ECL) reagents A and B (1:1) (Amersham Bioscience, USA). The densitometric analysis performed using using NIH Image J analysis software (Mousum et al., 2018).

3.4 Data analysis

The sample size for this present study was calculated using G*power analysis. The data of the behavioural, biochemical, neurochemical and molecular studies were assessed by multivariate analysis of variance (MANOVA) and the level of significance was assessed Bonferroni post-hoc test. In this study, the independent variables were the treatment effect, time interval and different brain regions. In the cognitive flexibility test, the dependent variables were attention set-shifting and error criterion during ID, ED and reversal learning in Rev-1, Rev-2 and Rev-3. The dependent variables for the Y-maze test were general exploratory behaviour and spatial recognition in terms of total arms entries in trial-1, trial-2 and percentage known and novel arm entries, respectively. In EPM and contextual fear response tests, the open arm entries, time spent in open arms and percentage freezing response were the dependent variables. In the biochemical analysis, the dependent variables were CORT, ACTH level and ACTH: CORT ratio. For the neurochemical analysis, alteration in 5-HT, 5-HIAA levels, its turnover, NA level, ACh level and AChE activity were the dependent variables. In the molecular analysis, the alteration in ChAT and α-7nAChR expression in different brain regions was considered as the dependent variables. All statistical analyses were performed using the STATISTICA software. All the data are presented as mean±SD and $p<0.05$ was considered significant.

3.5 Results

3.5.1 Donepezil 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 3.3 (a). MANOVA showed a difference in the discrimination trial of criterion among groups ($F_{3,112}$ = 13.5, $p<0.05$), treatment ($F_{3,112}$ = 168.6, $p<0.05$) and their interaction ($F_{9,112}$ = 8.2, $p<0.05$). 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 donepezil significantly decreased the SRS-induced increase in 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 3.3 (b). MANOVA showed that, there was a significant increase in error criterion in ID, Rev-2, ED and Rev-3 following SRS exposure ($F_{3,112}$ = 12.4, $p<0.05$), treatment ($F_{3,112}$ = 141.6, $p<0.05$) and their interaction ($F_{9,112}$ = 4.2, $p<0.05$). Post-hoc analysis showed that donepezil 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.

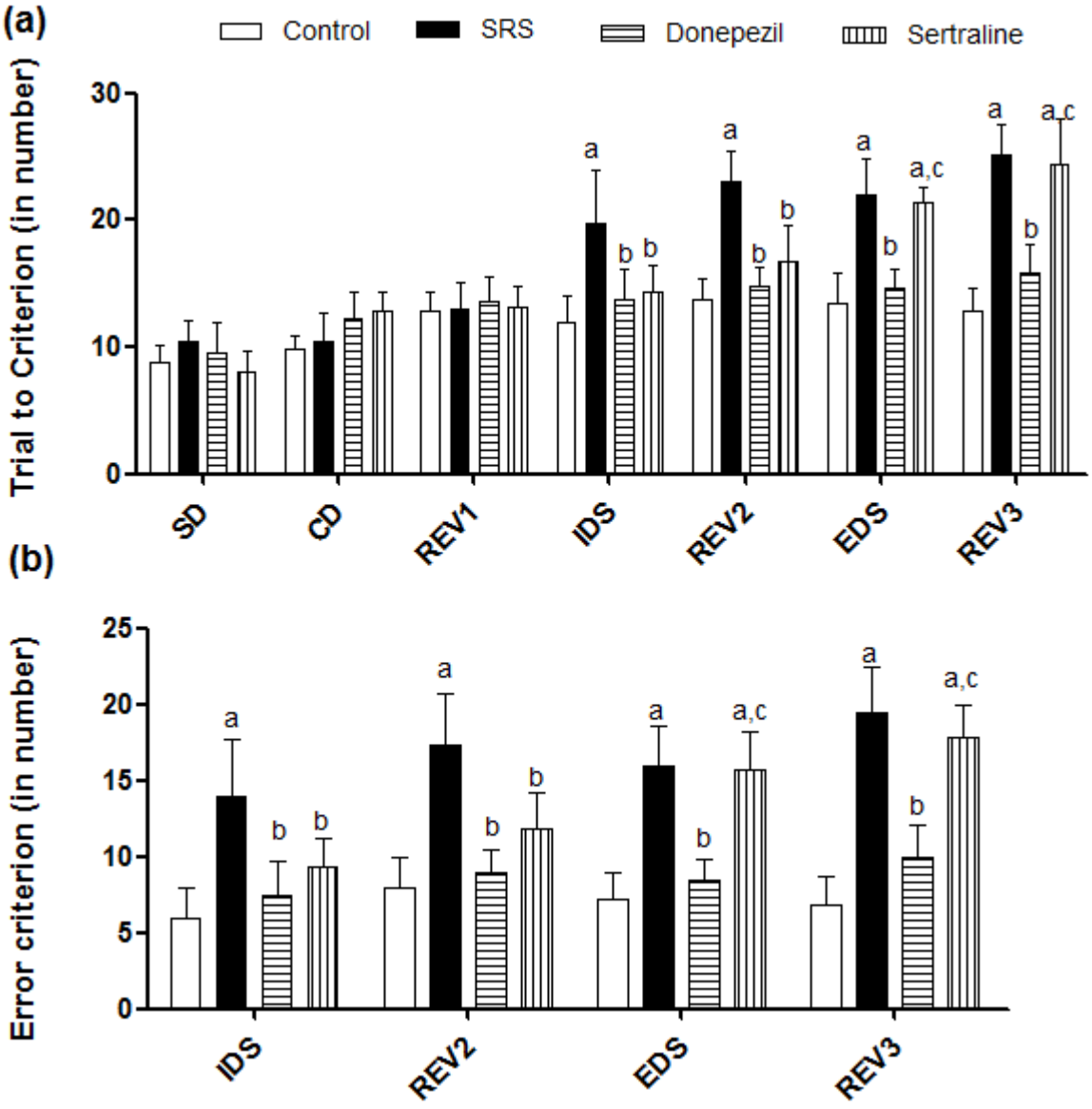


Figure 3.3 represents the effect of donepezil and sertraline on SRS-induced cognitive inflexibility. All values are Mean±SD (n=8). [a]p<0.05 compared to control, [b]p<0.05 compared to SRS and [c]p<0.05 compared to donepezil [MANOVA followed by Bonferroni post-hoc test].

3.5.2 Donepezil mitigates SRS-induced cognitive deficits in rats during Y-maze test

Y-maze is a widely used method for measuring cognitive behaviour in rats. In the present study, statistical analysis of MANOVA revealed that significant decrease in exploratory behaviour among the groups during trial-1 and trial-2 [($F_{3,84}$ = 41.5, p<0.05), ($F_{3,84}$ = 37.4, p<0.05) respectively], time [($F_{3,112}$ = 128.2, p<0.05), ($F_{3,112}$ = 300.2, p<0.05) respectively] and their interaction [($F_{3,84}$ = 22.8, p<0.05), ($F_{3,84}$ = 22.7, p<0.05) respectively]. Post-hoc test showed treatment with donepezil and sertraline significantly increased the

exploratory behaviour in SRS-exposed rats (Figure 3.4). Moreover, there were significant differences in arm discrimination behaviour in the percentage of known and novel arm entries among the group [($F_{3,84}$ = 24.6, $p<0.05$), ($F_{3,84}$ = 187.3, $p<0.05$)], time [($F_{3,112}$ = 168.6, $p<0.05$), ($F_{3,84}$ = 76.6, $p<0.05$) respectively] and their interaction [($F_{9,112}$ = 8.2, $p<0.05$) ($F_{6,84}$ = 35.8, $p<0.05$) respectively] in trial-2. Post-hoc analysis showed that exposure of SRS caused a significant decrease in known and novel arm discrimination which was significantly attenuated by both donepezil and sertraline.

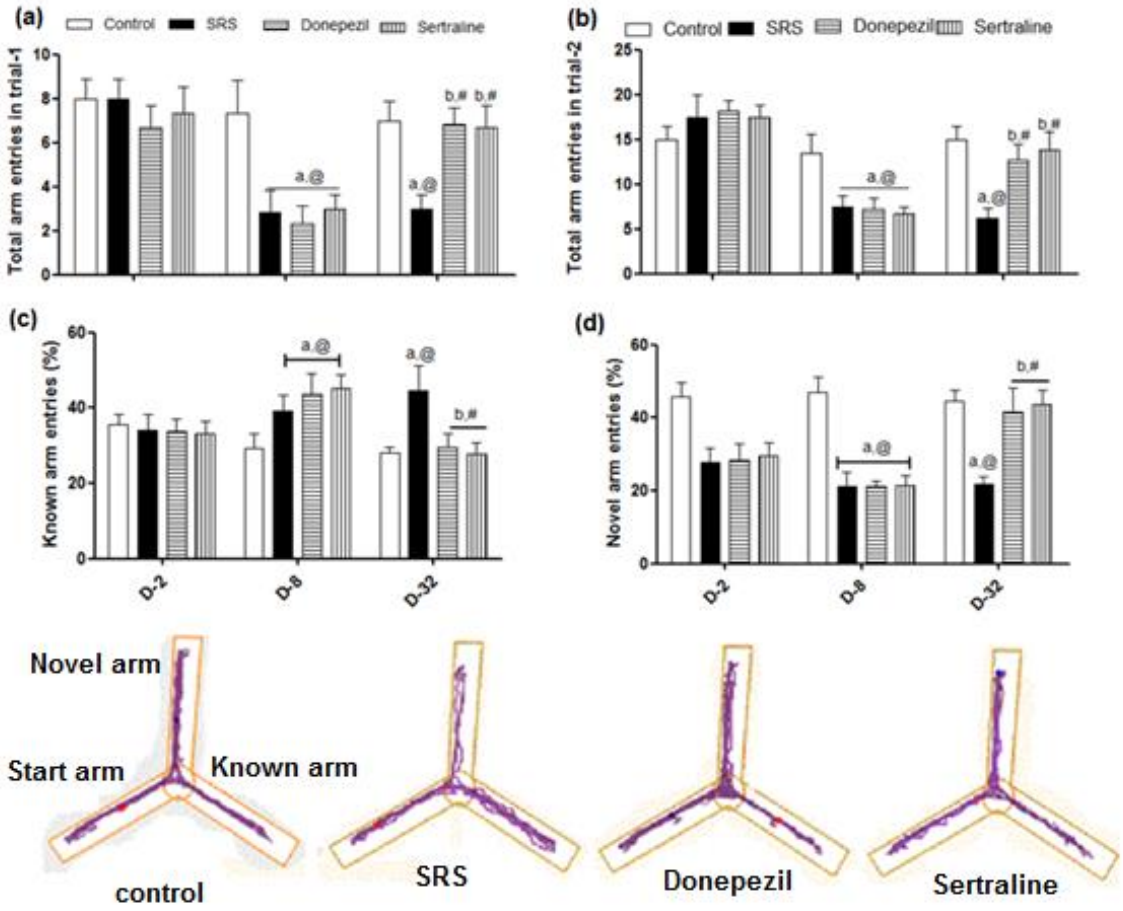


Figure 3.4 represents the effect of donepezil and sertraline on SRS-induced alteration on total arm entries in trial-1 (a), trial-2 (b), known arm entries (c) and novel arm entries (d) in Y-maze tests. All values are Mean±SD (n=8). [a]$p<0.05$ compared to control, [b]$p<0.05$ compared to SRS, [@]$p<0.05$ compared to D-2 and [#]$p<0.05$ compared to D-8 [MANOVA followed by Bonferroni post-hoc test].

3.5.3 Donepezil reduces SRS-induced anxiety-like behaviour in rats

The EPM test was designed to measure anxiety-like behaviour with observed decrease in open arm entries and time spent in open arm (Figure 3.5). On the EPM, three measures were assessed with MANOVA analysis i.e open arm entries, time spent in open arm and total arm entries. The statistical analysis by MANOVA showed there was a significant difference in percentage open arm entries and time spent among the groups [($F_{3,112}$ = 68.1, $p<0.05$), ($F_{3,112}$ = 104.1, $p<0.05$) respectively], time (D-2 to D-32) [($F_{3,112}$ = 80.2, $p<0.05$), ($F_{3,12}$ = 132.5, $p<0.05$) respectively], and their interaction [($F_{9,112}$ = 15.9, $p<0.05$), ($F_{9,112}$ = 37.7, $p<0.05$) respectively]. However, there was no significant effect in the number of total arm entries among the groups ($F_{3,112}$ = 2.1, $p>0.05$), time ($F_{3,112}$ = 0.8, $p>0.05$) and their interaction ($F_{9,112}$ = 2, $p>0.05$). Post-hoc analysis revealed that, there was no significant difference in open arms entry and time spent open arm on D-1 in SRS subjected rats compared to control rodents. However, there were significant decrease in open arms entry and time spent open arm on D-2 to D-32 compared to control rats. Treatment with donepezil and sertraline significantly decreased SRS-induced anxiety-like behaviour in rats.

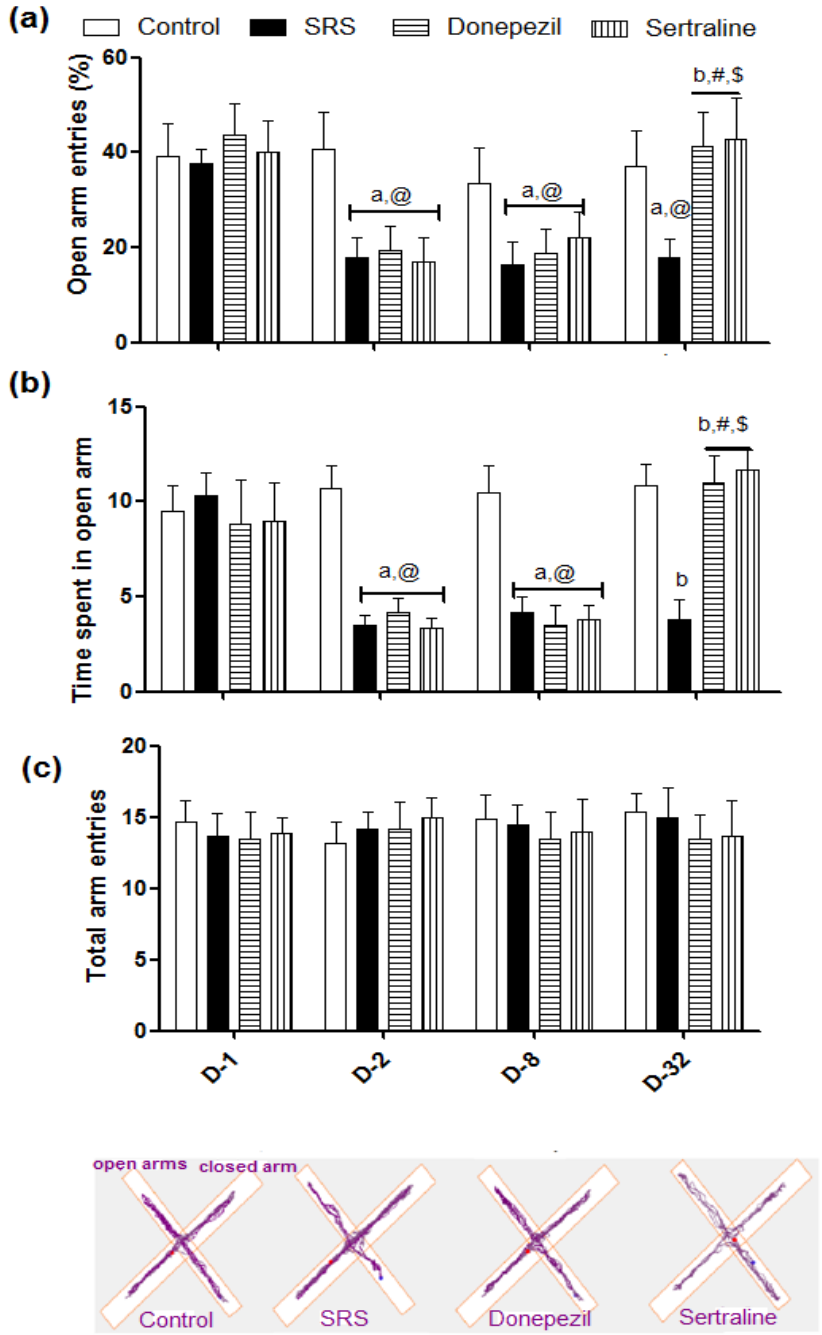


Figure 3.5 represents the effect of donepezil and sertraline on SRS- induced alteration on open arm entries (a), time spent in open arms (b) and total arm entries (c). All values are Mean±SD (n=8). $^{a}p<0.05$ compared to control, $^{b}p<0.05$ compared to SRS, $^{@}p<0.05$ compared to D-1, $^{\#}p<0.05$ compared to D-2 and $^{\$}P<0.05$ compared to D-8 [MANOVA followed by Bonferroni post-hoc test].

3.5.4 Donepezil reduces the contextual fear response in rats

The contextual fear conditioning followed by extinction testing was performed on D-2 (stress day), D-8 (re-stress day) and D-32 (last day) shown in Figure 3.6. Statistical analysis revealed that increase in freezing behaviour following SRS exposure among the

groups ($F_{3,84}$ = 51.5, p<0.05). This effect was observed from D-8 to D-32 ($F_{3,84}$ = 8.5, p<0.05). There was a significant interaction between groups and time ($F_{3,84}$ = 6.5, p<0.05). Post-hoc analysis showed treatment with donepezil (3 mg/kg) and sertraline (10 mg/kg) significantly decreased the in freezing response in SRS exposed rats.

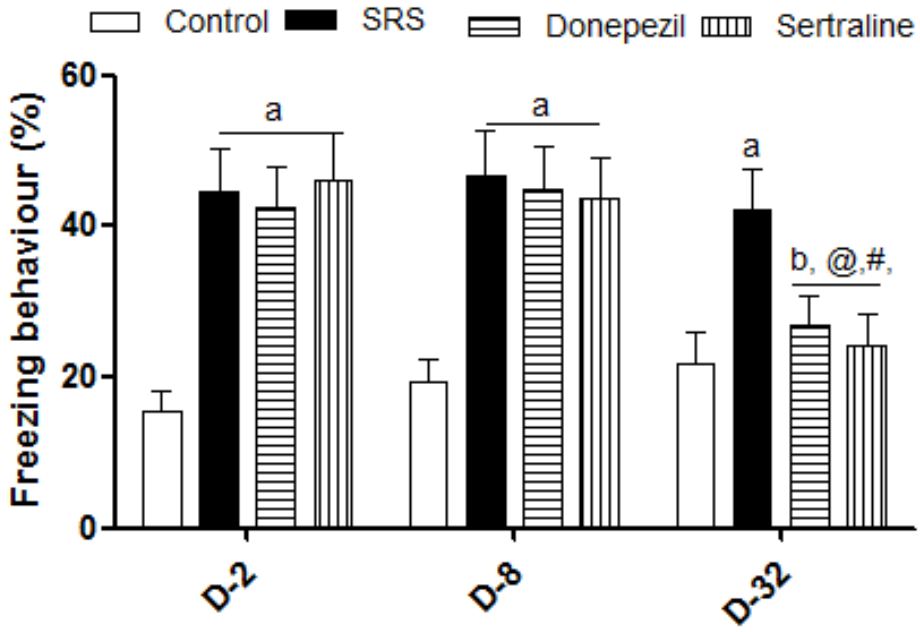


Figure 3.6 represents the effect of donepezil and sertraline on SRS-induced decrease in extinction fear response in rats. All the values are represented as Mean±SD (n=8). [a]p<0.05 compared to control, [b]p<0.05 compared to SRS, [@]p<0.05 compared to D-2 and [#]p<0.05 compared to D-8 [MANOVA followed by Bonferroni post-hoc test].

3.5.5 Donepezil did not modulate SRS-induced HPA-axis dysfunction in rats

Between-group comparisons were performed by MANOVA followed by Bonferroni post-hoc test. The effect of donepezil on SRS-induced alteration in the weight of adrenal gland, plasma CORT, ACTH level and ACTH: CORT ratio is depicted in the Figure 3.7. Data analysis with MANOVA showed a significant difference in adrenal weight among the groups ($F_{3,28}$ = 22.2, p<0.05). Post-hoc analysis represented that, exposure of SRS caused significant decrease in adrenal weight compared to control in rats. Treatment with sertraline and donepezil did not show any effect on SRS-induced increase in adrenal weight in rats.

Similarly, plasma corticosterone was measured to analyze HPA axis sensitivity in response to SRS. MANOVA showed a significant difference in corticosterone level ($F_{3,28}$ = 162.9, $p<0.05$) among the groups. Post-hoc test revealed that, donepezil did not cause any significant effect on plasma corticosterone whereas; sertraline significantly increased the SRS-induced decrease in plasma corticosterone up to the basal level (Figure 3.7). Further, the SRS exposed rats showed an increase in ACTH level ($F_{3,28}$ = 46.44, $p<0.05$) compared to control rats. Only sertraline was able to mitigate the SRS-induced increase in plasma ACTH level.

Assessment of ACTH: CORT ratio gives a direct measurement of the negative feedback mechanism. It also differentiates the central regulation of corticosterone from the peripheral system. In the present study, MANOVA followed by post hoc analysis showed an increase in ACTH: CORT ratio in response to SRS ($F_{3,28}$ = 123, $p<0.05$). Donepezil did not cause any changes in ACTH: CORT ratio. However, sertraline significantly attenuated SRS-induced modulation.

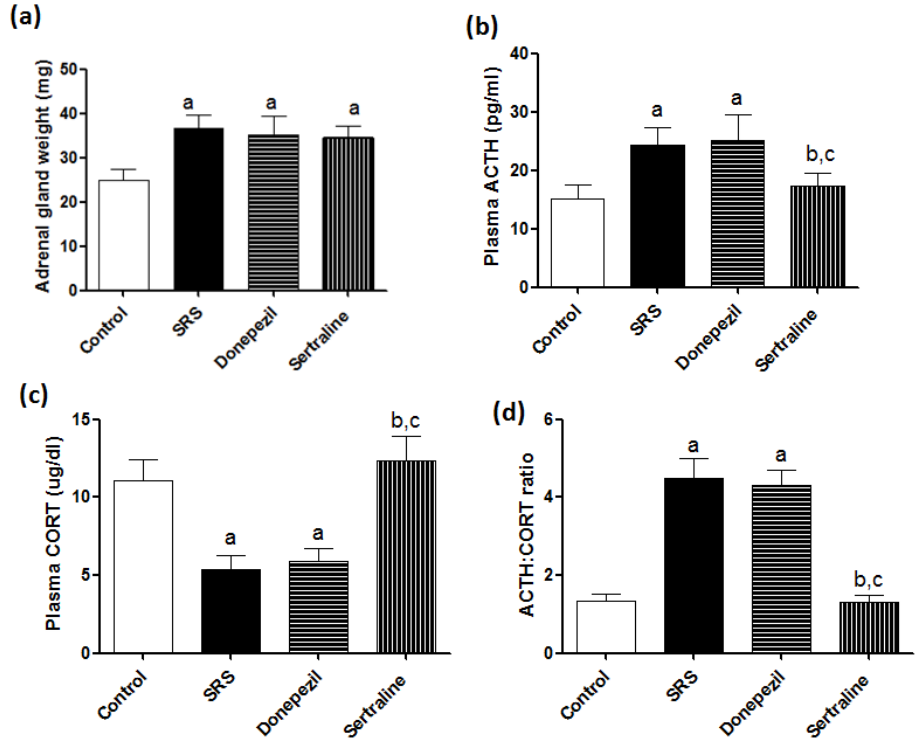


Figure 3.7 represents the effect of donepezil and sertraline on SRS-induced alteration in the weight of adrenal gland, plasma ACTH level and ACTH: CORT ratio. All values are represented as Mean±SD (n=8). [a]p<0.05 compared to control, [b]p<0.05 compared to SRS and [c]P<0.05 compared to donepezil [MANOVA followed by Bonferroni post-hoc test].

3.5.6 Donepezil did not modulate SRS-induced alteration on monoamines level in rats

The changes in the amygdalar serotonergic system are directly related to HPA axis hypersensitivity (Krishnamurthy et al., 2013). The effect of donepezil and sertraline on SRS-induced increase in 5-HT, 5-HIAA levels its turnover and NE in PFC, HIP and AMY are analysed by MANOVA and depicted in Figure 3.8. MANOVA revealed that there was a significant difference on 5-HT, 5-HIAA, their turnover and NE levels among the groups [($F_{3,36}$ = 28.9, p<0.05), ($F_{3,36}$ = 17.8, p<0.05), ($F_{3,36}$ = 5.2, p<0.05) and ($F_{3,36}$ = 45.2, p<0.05) respectively], in different brain regions [($F_{2,36}$ = 28.9, p<0.05), ($F_{2,36}$ = 28.9, p<0.05), ($F_{2,36}$ = 28.9, p<0.05) and ($F_{2,36}$ = 64.9, p<0.05) respectively] and their interaction [($F_{6,36}$ = 7.2, p<0.05), ($F_{6,36}$ = 5.4, p<0.05), ($F_{6,36}$ = 5.2, p<0.05) and ($F_{6,36}$ = 8.7, p<0.05] respectively]. Post-hoc analysis revealed that there was significant increase in the 5-HT and 5-HIAA in HIP and AMY but not in PFC in SRS-exposed rats compared to control group. Further, there was an increased in 5-HIAA/5-HT ratio in AMY but not in HIP and PFC. Treatment with donepezil did not cause any change to the SRS-induced increase in the 5-HT, 5-HIAA level and its turnover in any of the brain regions. However, sertraline increased the 5-HT and 5-HIAA level only in HIP. Further, treatment with donepezil and sertraline did not show any changes in 5-HIAA/5-HT level in any of the regions.

Moreover, the effect of donepezil and sertraline on SRS-induced increase in NE level is depicted in Figure 3.8. MANOVA analysis represented significant difference among the group ($F_{3,36}$ = 45.2, p<0.05), different brain regions ($F_{2,36}$ = 65, p<0.05) and the interaction of group and brain regions ($F_{6,36}$ = 8.7, p<0.05). Post-hoc analysis showed SRS exposure caused increase in NE level in PFC and AMY but not in HIP. Treatment

with donepezil and sertraline did not alter SRS-induced increase in the level of NE in any of the brain regions.

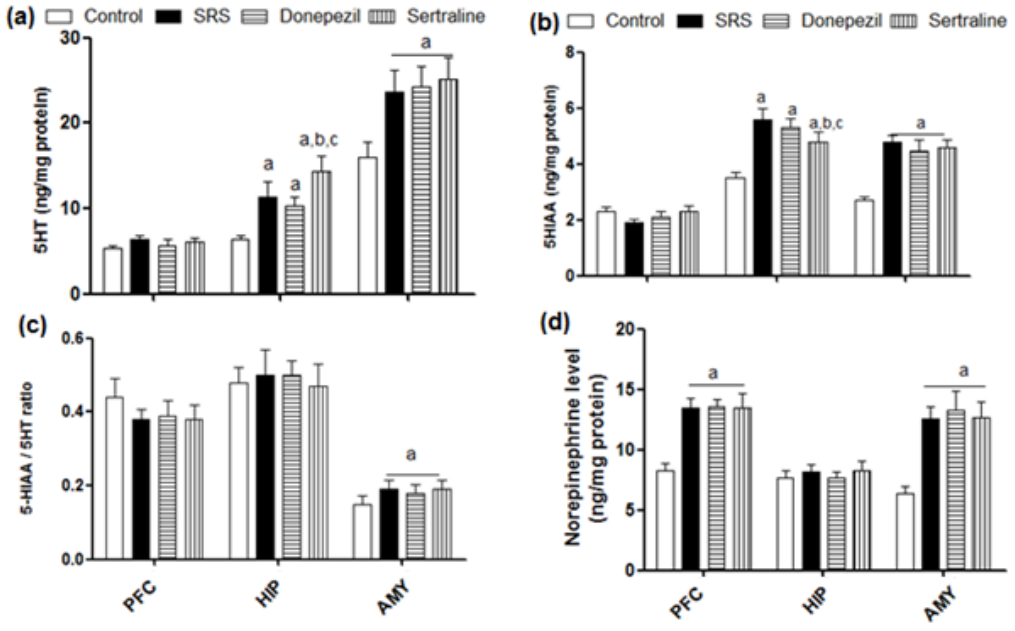


Figure 3.8 represents the effect of donepezil and sertraline on SRS-induced alterations on 5-HT (a), 5-HIAA (b), 5-HIAA/5-HT ratio (c) and NE (d) in different brain regions. All values are Mean±SD (n=4). $^{a}P<0.05$ compared to control, $^{b}P<0.05$ compared to SRS, and $^{c}P<0.05$ compared to donepezil [MANOVA followed by Bonferroni post-hoc test].

3.5.7 Donepezil mitigates the SRS-induced alteration in ACh level and AChE activity in rats

The effect of donepezil on SRS-induced alteration in the ACh level and AChE activity in PFC, HIP and AMY are depicted in Figure 3.9 (a) and (b), respectively. MANOVA revealed that, there was a significant differences on ACh level and AChE activity among the groups [($F_{3,36}$ = 91.2, $p<0.05$), ($F_{3,36}$ = 86.6, $p<0.05$) respectively], in different brain regions [($F_{2,36}$ = 125.6, $p<0.05$) and ($F_{2,36}$ = 198.8, $p<0.05$) respectively] and their interaction ($F_{6,36}$ = 15.4, $p<0.05$] and ($F_{6,36}$ = 31.3, $p<0.05$) respectively]. Post-hoc test represented that the exposure of SRS caused decrease in ACh level and increase in AChE activity in PFC and HIP compared to control rats. Treatment with donepezil significantly attenuated SRS-induced decrease in ACh level and increase in AChE activity in PFC and

HIP. However, sertraline did not show significant effect on ACh level and AChE activity in SRS exposed rats.

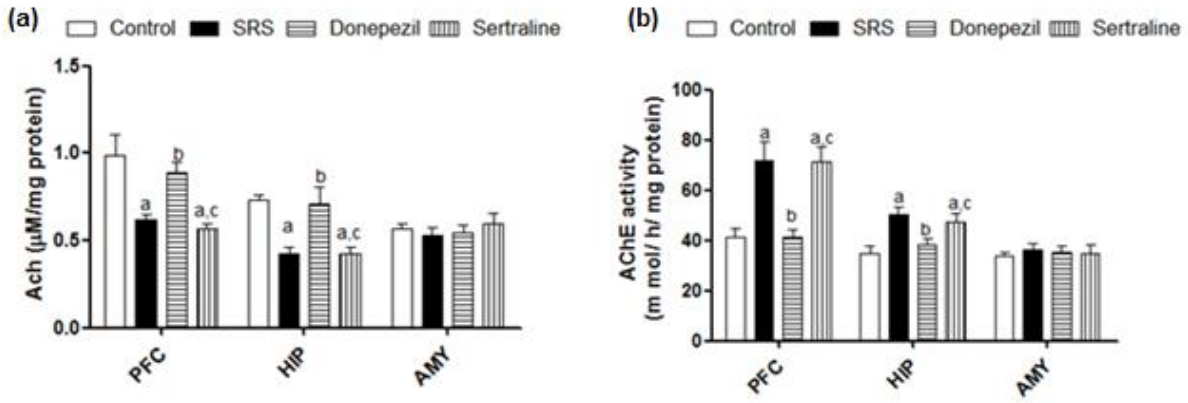


Figure 3.9 represents the effect of donepezil and sertraline on SRS-induced alterations on ACh (a) levels and AChE activity (b) in different brain regions. All values are Mean±SD (n=4). $^{a}P<0.05$ compared to control, $^{b}P<0.05$ compared to SRS, and $^{c}P<0.05$ compared to donepezil [MANOVA followed by Bonferroni post-hoc test].

3.5.8 Donepezil ameliorates the SRS-induced down-regulation of ChAT and α-7nAChR expression in rats

ChAT is a key enzyme for the synthesis ACh and participates in the regulation of cholinergic system. α-7nAChR is also directly involved in the regulation of cholinergic system (Figure 3.10). MANOVA analysis revealed that there was a significant differences on ChAT and α-7nAChR expression among the groups [($F_{3,36}$ = 16.9, $p<0.05$), ($F_{3,36}$ = 39.9, $p<0.05$) respectively], in different brain regions [($F_{2,36}$ = 37.2, $p<0.05$) and ($F_{2,36}$ = 7.2, $p<0.05$) respectively], and the interaction of group and brain region [($F_{6,36}$ = 10, $p<0.05$) and ($F_{6,36}$ = 28.3, $p<0.05$) respectively]. Post-hoc test represented that the exposure of SRS significantly decreased ChAT and α-7nAChR expression in PFC and HIP compared to control rats. Treatment with donepezil significantly increased the ChAT level in both PFC and HIP. However, sertraline did not cause any changes in the ChAT and α-7nAChR expression in SRS-exposed rats (Figure 3.10).

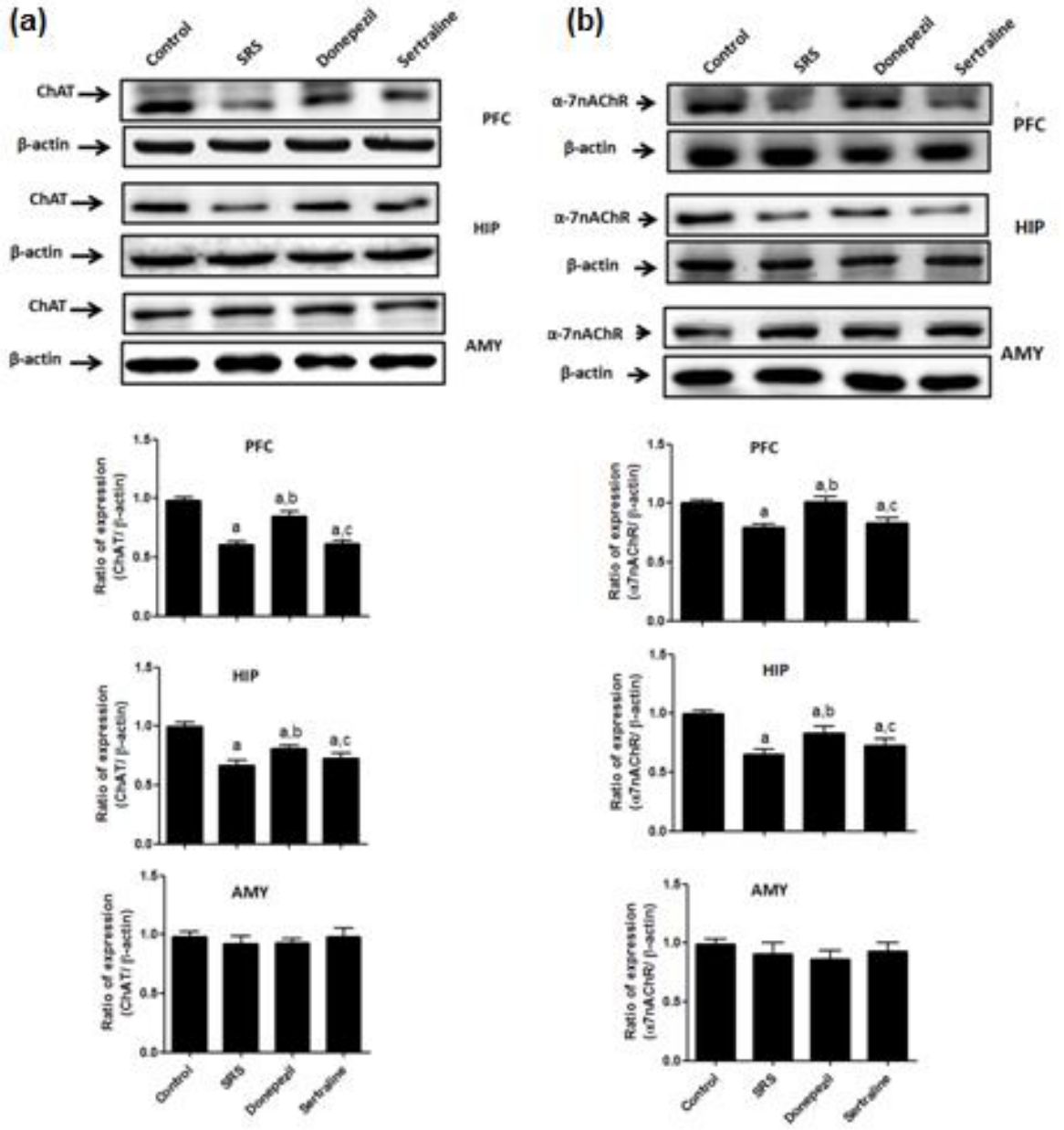


Figure 3.10 represents the effect of donepezil and sertraline on SRS-induced alteration on ChAT (a) and α-7 nAChR (b) expression in different brain regions. All values are Mean ± SD (n=4). $^{a}P<0.05$ compared to control, $^{b}P<0.05$ compared to SRS, and $^{c}P<0.05$ compared to donepezil [MANOVA followed by Bonferroni post-hoc test].

3.6 Discussion

The salient finding of the present study is the development of cognitive inflexibility in the SRS model of PTSD. This was accompanied by hypoactivity of the cholinergic system in the PFC and HIP. Further, donepezil attenuated cognitive inflexibility in rats.

Impairment in cognitive flexibility has been observed in a variety of neuropsychiatric disorders such as attention deficit disorder and PTSD (Fani et al., 2009). Clinically, cognitive inflexibility is characterized difficulty (Herrmann et al., 2011). In the animal model, cognitive inflexibility is the difficulty in the differentiation of ID and ED shift (Birrell and Brown, 2000, Tait et al., 2018). ID shift can be correlated with mild perceptual attention set which requires mild attention for recognition such as colour or shape of 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, Jin et al., 2014). In the present study, SRS-exposed rats showed deficits in ID and ED shifts and its reversal learning, indicating cognitive inflexibility. Reduction in set-shifting behaviour is similar to clinically observed task difficulty discrimination behaviour in PTSD patients (Herrmann et al., 2011). Treatment with donepezil and sertraline attenuated the SRS-induced impairment in ID shifting and its reversal learning. However, the impairment of ED shift and its reversal learning was only attenuated by donepezil indicating the fact that ED shift requires specific executive attention which is regulated by PFC. In support of present finding, previous report suggests that ID shift is regulated by serotonergic and cholinergic circuits in HIP and PFC (Brady et al., 2000). In contrast, ED shift is regulated by the cholinergic circuit in PFC (Mikics et al., 2008). Sertraline-induced attenuation in ID shift may be due to facilitation of serotonergic and cholinergic circuits in HIP and PFC (Steckler and Sahgal, 1995). Therefore, enhancement of cholinergic function by

donepezil ameliorated deficits in several components of cognitive inflexibility in SRS-induced PTSD-like condition.

Cognitive dysfunction such as impaired discrimination ability and reduced exploration is also observed during the Y-maze test in SRS exposed rats (Krishnamurthy et al., 2013). The reduction in arms discrimination ability can be correlated with deficits in mild perceptual attention as it belongs to visual cue. Treatment with donepezil and sertraline significantly attenuated the SRS-induced decrease in general exploratory behaviour and spatial recognition memory. Donepezil facilitates the release of acetylcholine in the brain and thereby improves working memory deficits (Zhao et al., 2017). However, sertraline induced attenuation in cognitive functions may be due to facilitation of serotonin in the HIP which causes activation of cholinergic functions as suggested previously (Steckler and Sahgal, 1995). The cholinergic system regulates the acquisition and retrieval of cognition through cholinergic projections from PFC to HIP (Su et al., 2011). In the current study, weekly exposure of SRS induced a reduction in ACh level and enhancement in AChE activity in PFC and HIP but not in AMY. The reduction in ACh level in the PFC may have caused the difficulty in task discrimination due to dysfunction of executive behaviour (Hasselmo et al., 1996). Another interesting finding is that SRS exposure caused down-regulation of ChAT in PFC and HIP. ChAT is a presynaptic enzyme for ACh synthesis and a marker of cholinergic function and structure (Kakinuma et al., 2009). Therefore, exposure of SRS not only increases the AChE activity but also inhibits the biosynthetic pathway of ACh, indicating that the cholinergic system is critically affected by the exposure of SRS. Donepezil significantly attenuated in SRS-induced abnormalities in ACh level, AChE activity and ChAT in PFC and HIP. The probable mechanism is due to increase in ChAT promoter activity as suggested previously (Kakinuma et al., 2009). The depletion of cholinergic neurons in cognitive

deficits can also be due to the down-regulation of the neuronal α-7nAChR (Shimohama and Kihara, 2001). This receptor is highly expressed in the nervous system and α-7nAChR agonist enhances recognition memory and cognitive flexibility in rats (Corradi and Bouzat, 2016). However, the role of α-7nAChR in PTSD is yet to be studied. In our study, SRS exposure caused down-regulation of α-7nAChR in PFC and HIP, implicating the role of α-7nAChR in the pathophysiology of PTSD. Donepezil but not sertraline attenuated the SRS-induced alteration in α-7 nAChR in PFC and HIP. It may be due to the fact that donepezil facilitated the extracellular ACh level in PFC and HIP in SRS-exposed rats. Further, donepezil up-regulated ChAT and α-7nAChR both of which are responsible for increasing synaptic activity of acetylcholine for the participation of memory formation and execution (Corradi and Bouzat, 2016). Therefore, a cholinergic enhancer can be a potential approach in the treatment of cognitive inflexibility and deficits in decision-making ability in PTSD. The cognitive flexibility regulates fear-induced intrusive memory, and therefore, its reduction is responsible for the development of intrusive memory (Gonzalez and Martinez, 2014). The exaggerated fear response is another cardinal symptom observed in PTSD patients (Gonzalez and Martinez, 2014).

The exaggerated fear response was also observed in SRS subjected rats in terms of increase in freezing behaviour from D-8 to D-32. Further, treatment with donepezil and sertraline significantly reduced fear response in SRS exposed rats which suggests that both cholinergic and serotonergic systems regulate fear response. Moreover, anxiety is another core symptom observed in PTSD patients (Kok et al., 2002). In the present study, exposure of SRS caused anxiety-like behaviour which was significantly attenuated by sub-chronic treatment with donepezil and sertraline. Both the drugs also attenuated the mild perceptual attentional set during ID shift. Further, traumatic event induced cognitive inflexibility and anxiety may prolong HPA axis dysfunction (Hannibal and Bishop,

2014). HPA axis dysfunctions and adrenal dystrophy are some key manifestations observed in PTSD patients (Kasckow et al., 2001). In the present study, SRS subjected rats showed a reduction in adrenal gland weight. Further, SRS exposed rats showed an increase in ACTH to corticosterone ratio. This may be due to adrenal dystrophy where the gland is unable to produce sufficient corticosterone to regulate negative feedback regulation of pituitary ACTH release (Barden et al., 1995). Treatment with sertraline but not donepezil significantly attenuated HPA axis dysfunction and adrenal dystrophy in SRS-exposed rats indicating that donepezil-induced anti-PTSD effect was independent of the HPA axis. Additionally, exposure of SRS increased 5-HT, 5-HIAA and the 5-HIAA/5-HT turnover in HIP and AMY but not in PFC similarly as observed previously (Krishnamurthy et al., 2013). Treatment with sertraline augmented the 5-HT levels in the HIP but not in AMY, suggesting a region-specific effect of sertraline as suggested earlier (Karanges et al., 2011). This could be the reason that sertraline modulated working memory and mild perceptual attentional shift which are mainly regulated by HIP-dependent pathways. On the contrary, donepezil did not modulate serotonergic system in SRS-exposed rats but attenuated working memory in Y-maze and mild as well as higher perceptual, attentional shift during cognitive inflexibility tests. The results indicate that targeting of the cholinergic system is more reliable approach for the treatment of cognitive abnormalities in PTSD. Moreover, exposure of SRS elevated the level of NE both in PFC and AMY. In support of our finding, previous clinical study suggested that stimulation of NE in PFC diminishes its control over amygdala and strengthens amygdalar function (Shin et al., 2006). None of the drugs was able to modulate the SRS-induced activation of the nor-adrenergic system indicating that the effect of donepezil and sertraline were independent of nor-adrenergic system.

3.7 Summary

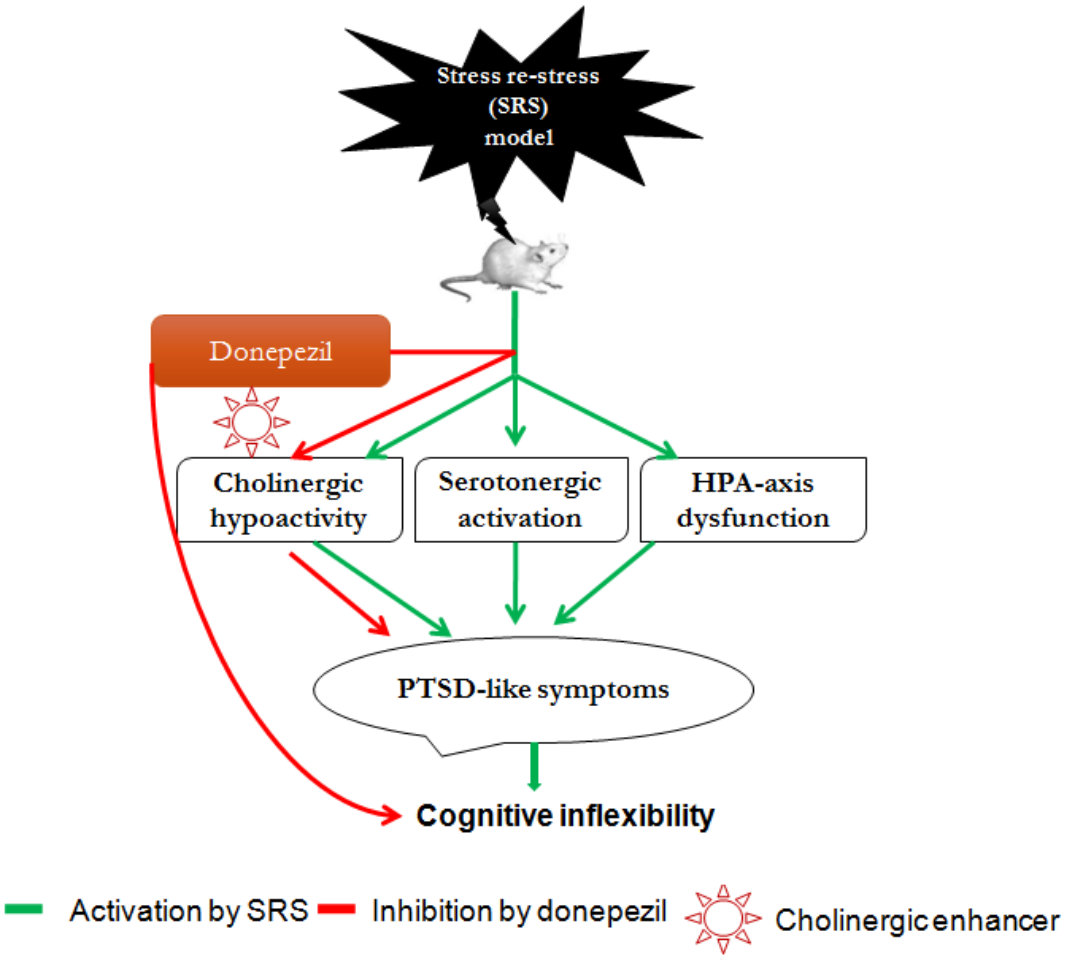


Figure 3.11 Summary of hypothesis

The exposure of SRS caused cognitive inflexibility and hypoactivity of the cholinergic system. Interestingly, exposure of SRS also reduced biosynthesis of ACh and postsynaptically the downregulation of ChAT and α-7nAChR expressions. Unlike sertraline, donepezil reduced AChE activity and enhanced ACh, and thereby mitigated cognitive inflexibility. However, sertraline showed efficacy in reducing mild but not higher perceptual attentional set shift during cognitive inflexibility tests. Hence, cognitive flexibility is important for the development of adaptive behaviour in traumatic stress condition. Therefore, the inclusion of cholinergic drugs such as donepezil in the treatment protocol may be beneficial to PTSD patients where cognitive inflexibility is the major symptom.