Title: Altered Serotonin 5HT-1B Receptor Expression in Hippocampus, Amygdala and Prefrontal Cortex May Regulate Sex Dependent Difference for Stress and Anxiety

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Abstract

Stress disorders have multidimentional effect on us. The way we respond to these situations is different, which depends upon our individual difference and our sex or gender. Reports suggests that females are more susceptible to stress disorders as compared to the male person with same age group. Serotonin receptor system is an important mechanism involved in regulation of stress and anxiety in male and female both individuals. Current study incorporates rodent model to study sex-based role of serotonin receptors under chronic restrained stress condition. A chronic restrained stress protocol was used to assign the stress difference between male and female Sprague-Dawley rats. The molecular identification was done using quantitative real-time PCR and immunohistochemistry for serotonin 5HT-1B receptor in rat brain.

Interestingly, 5HT-1B receptor which is one of the most important serotonin receptors, exhibited a sex dependent difference for stress response in male and female rats. Most importantly the trio partners (amygdala, hippocampus and prefrontal cortex), exhibited a region-specific sex dependent difference in 5HT-1B receptor expression which was correlated with the difference in the level of stress response. This biased function of serotonin 5HT-1B receptor might be the reason why females face more stress than male individual.

Overall, the result exhibited a sex dependent difference for stress condition in male and female rats, which was correlated with the spatial expression of serotonin 5HT-1B receptor in brain.

Key words: Serotonin, Serotonin receptors, SD rats, Stress, Amygdala

Introduction

Our daily lifestyle exposes us towards number of environmental factors that may be challenging and problematic in nature. Negative situations we facing in life such as heavy work load, our socioeconomic conditions, accident, trauma, wars, sexual abuse, terrorist attack etc., sometimes have deleterious effect (Stults-Kolehmainen and Sinha, 2014). Stress creates multidimensional adverse effects that causes physiological as well as behavioural and psychological dysfunctions (Schneiderman et al, 2005; Segerstrom and Miller, 2004). If not managed, there is huge possibility for development of various types of psychological disorders such as depression and other related stress and anxiety disorders (Yang et al, 2015).

The consequence of stress is not similar among persons as some people are less affected by it, while other face difficulty. Likewise, the effect of stress and anxiety is also not same between both the sexes as females are more susceptible for stress and anxiety condition (Verma et al, 2011; Bahrami and Yousefi, 2011). The severity of stress and anxiety is also higher in females than the male individual and are comparatively more likely to experience higher stress and anxiety (Verma et al, 2011; McLean et al, 2011). Number of women facing work-related stress is nearly 50% higher when compared with the same age man group (Gino et al, 2015).

Serotonin receptor system is an intricate arrangement of neuronal network involved in regulation of various important brain functions like learning, memory, emotional responses, sleep-wake cycle, anxiety, mood control etc (Jenkins et al, 2016; Bacqué-Cazenave et al, 2020; Charnay and Léger, 2010). This system is formed by the neurotransmitter (5-hydroxytryptamine) serotonin, synthesised by neurons in nervous system, and 14 different serotonin receptors (e.g. 5HT-1 to 5HT-7 receptor) located all over our brain (Yohn et al, 2017; Pithadia and Jain, 2006). It functions to regulate the release of different categories of neurotransmitters (like acetylcholine, GABA, epinephrine/norepinephrine and glutamate) and some hormones, thereby controlling different brain functions such as sleep-wake cycle, mood, learning, anxiety, memory, sleep etc (Ciranna, 2006). Contrary to human, rodent brain contains 13 serotonin receptors (except 5HT-1E) (Berumen et al, 2012; Osredkar and Krzan, 2009). Different animal studies have suggested the involvement of serotonin receptor system in anxiety and stress conditions (Overstreet et al, 2003; Akimova et al, 2009; Bacqué-Cazenave et al, 2020; Karayol et al, 2021). Study involving 5HT-2C receptor overexpression in rodent forebrain region have shown an increased anxiety in these animals (Kimura et al, 2009) while other studies have suggested the role of 5HT-1A, 5HT-2A, 5HT4 receptors in stress and anxiety

condition (Akimova et al, 2009; Xiang et al, 2019, Karayol et al, 2021;). More interestingly, Bhatnagar, 2004 found in their study a difference in role of 5HT-3r between male and female mice (Bhatnagar et al, 2004). 5HT-1B receptor, which is an inhibitory receptor, is highly expressed in the substantia nigra and globus pallidus area of the brain; while in moderate to low level in amygdala, PFC and hippocampus (Tiger et al, 2008; Švob Štrac et al, 2016). Overall, these studies suggested a very specialised function by serotonin receptors regulating stress differently between both the sexes.

The circuitry of stress starts from dorsal raphe nucleus (DRN), from where it innervates hippocampus, amygdala, PFC, and other related brain regions in rodents (Shin et al, 2010). Hippocampus, Amygdala and PFC organized to form an intricate network known as the limbic system; the area mainly associated with the regulation of emotional response in stress condition (Rajmohan et al, 2007; Tiger et al, 2008; Šimić et al, 2021). The circuitry system which is formed by these components is also called "stress circuitry". Hippocampus region expresses comparatively high level of glucocorticoid and mineralocorticoid hormones receptors (Koning et al, 2019). Furthermore, the hippocampus in combination with other brain parts such as amygdala, PFC, brain nuclear stria-terminalis, functions to regulate stress circuitry in brain (Rajmohan et al, 2007).

Although there are known physiological difference among male and female individuals; the difference in serotonin receptor (5HT receptor) is of great importance. In spite of this difference, very little information is available which specify the function of serotonin receptor with stress and anxiety dependent difference in male and female animals. For better understanding such difference, it is requisite to study serotonin receptors in detail with special emphasis on sex-based anxiety difference. Unless we do not understand this biological difference among the brains of male and female individuals, it will be enigma to develop better therapeutic application.

Methods

Animals and Groups

The research experiments were executed with healthy adult SD (Sprague–Dawley) rats having 2–3 months age. All the animals were kept under standard laboratory conditions of 12-h light/dark cycle, temperature-23°C, and food and water were available ad libitum. The study

experiments were approved by the Institutional Animal Ethics Committee, KGMU, Lucknow (Ethical no: 169/IAEC/2022). In the study the four groups (Male control-MC, female control-FC, Male stress-CS and Female stress-CS groups) of animals were included. In behaviour experiments there were 10-12 animals in each group (half underwent for EPM test while half OFT), and these animals did not used for molecular study. Another separate group was used for molecular study using IHC and Q-PCR (*n*=10 to 12 animals in each group, half used for IHC and rest for PCR) which there was no OFT or EPM test was performed.

Chronic restrained stress (CRS) in rats

The chronic restrained stress was performed on male and female with the use of 10 days chronic restrained immobilization stress session, 6 hrs per day (8:00 AM to 2:00 PM) (Buynitsky et al, 2009). The process of immobilization in chronic restrained stress was done using a cone made up of polythene (8-10 inches). Followed by stress session the OFT and EPM test was performed in animals to analyse the effect of stress on body physiology. After final behaviour experiment, the animals were perfused transcardially and the brain and blood samples were collected during the perfusion of animals. The samples were stored at -80ºC for further molecular analysis (Fig. 1A).

Elevated Plus Maze (EPM) test

EPM is used to analyse the effect of stress or anxiety on body physiology in rats. The apparatus of EPM consists of four arms of which two are open and two closed (each 40x10*cm* dimension) all joined at the centre thus creating a plus-shaped structure, elevated 50*cm* above the ground and located in a room with low intensity light. During experiment the rats were positioned at the centre area of maze and the face of the animal was towards closed arm. The activity of the animal was observed for up to five minutes and analysed. The entries in open arms were analysed as percent open arm entries with respect to the time spends in each arm during five minutes of exposure in all groups. The analysis for the result obtained by EPM was performed using Any-Maze analysis software (ANY-maze, Stoelting Co., US).

Open field test (OFT)

OFT is used to analyse physiological condition during stress and anxiety in animals (Seibenhener et al, 2015). The apparatus of the OFT was done in a square box (120cm x 120cm x 60 cm dimension) made up of non-transparent material situated in a sound proof room. Whole arena was divided into two region one inner central area and other outer area (Seibenhener et

al, 2015). All the animals were positioned arbitrarily at one of the corners. These Animals explored the arena for up to 10-15 min, and the behaviour of these animals was analysed with the help of monochromic camera situated on the top. The overall time used and the number of entries at centre arena was analysed manually for complete time of the session.

Body weight

For identification and analysis of stress in these animals the animals were weighted after each day of stress session. Previous studies on stress suggested a continuous decreased body weight in experimental animals (Harris, 2015, Yau and Potenza, 2013).

Plasma corticosterone

From both the stress and control groups blood samples (about 1 ml) were collected from these animals through aortic puncture into ice-cooled centrifugal tubes. Blood was kept at room temperature for 1 hour for clotting in a collection tube followed by centrifugation at 1700 ×g, 10 min, 4°C to separate serum from blood. The serum was then stored at -80°C freezer until further experiment.

Brain subregions

Whole amygdala (including BLA and CeA, bregma -2.6 to -3.2), hippocampus (including CA1, CA2, CA3 and DG, bregma -2.6 to -3.2) and prefrontal cortex (including PL and IL, bregma 2.76 to 3.24) brain regions were used for the analysis of serotonin receptor expression through transcription analysis using Q-PCR and translation analysis using IHC. The brain area analysed in this study the key partner for emotional and cognitive responses (Stults-Kolehmainen and Sinha, 2014; Albert et al, 2014; McEwen et al, 2016).

Tissue preparation for Immunohistochemistry and transcription analysis

The rats after stress experiments (2 hrs post experiment) were anesthetized by pentobarbital (60 mg/kg, i.p.), and were transcardially perfused with n-saline solution. This was followed by the introduction of ice-cold 4% paraformaldehyde solution (in 0.1 M phosphate buffer solution, pH 7.4), brains removed after decapitation. Isolated brains were further post-fixed in a 4% paraformaldehyde solution for 24 h, followed by keeping them in sucrose solution (10%, 20% and 30% solutions serially in 0.1M phosphate buffer, pH 7.4). Then the brains were frozen at -30^0 to -35^0C temperature in isopentane solution for 30 min and stored in deep freezer at -80^0C

for IHC. For transcription study, the rats were transcardially perfused with chilled normal saline solution only and the brains were collected and stored in deep freezer at -80^{0}C for Q-PCR.

Isolation of mRNA and cDNA preparation

For transcription analysis Hippocampus, PFC and Amygdala regions were dissected out from all groups. The serotonin receptor mRNA expression was analysed between male and female chronic restrained groups together with their respective control groups. The sample size from each group was 5-6, which was used for the mRNA expression study.

The tissue from each animal (5 mg) was homogenized in 100ul of cell lysis buffer and β-mercaptoethanol (0.7ul).

From these tissue samples total cellular RNA was isolated using GeneJET RNA isolation kit (Thermo, Catalog no. K0731) followed by genomic DNA removal using DNase I treatment. The purity of RNA was analysed using spectrophotometer at 260/280nm which was around O.D. =1.75). cDNA was prepared from these isolated mRNA and oligo-dT primers using RevertAid First Strand cDNA synthesis kit (Thermo, K1622). These cDNA generated were further used as a template in real-time PCR amplification. The reaction mixture for PCR analysis was prepared in a final volume of 15ul as: 5x reaction buffer (3ul), dNTP mixture (1.5ul), oligo dT primer (1ul), reverse transcriptase (0.75ul), RNase inhibitor (0.75ul) and template RNA (3ul). Reaction condition for cDNA preparation was: 60 min at 42^{0}C, 5 min at 70^{0}C and holding at 4^{0}C. The cDNA generated in this reaction was stored at -80^{0}C deep freezer.

Quantitative Real-time PCR

The level of serotonin receptor mRNA was analysed using Quantitative real-time PCR or Q-PCR. mRNA expression analysis was performed in Stratagene Max-Pro Real-Time PCR detection System using maxima SYBR green/ ROX master mix (Thermo). The quantity of expression was represented as fold change through C_t method. The primers sequences are: c-fos, 5'-CCGACTCCTTCTCCAGCAT-3' (forward), 5' –TCACCGTGGGGATAAAGTTG-3' (reverse); 5HT-1Br, 5`-GGAAAGTCCTGCTGGTTGCT-3` (forward), 5`-CGATCAGGTAGTTAGCCGGG-3` (reverse) and control GAPDH, 5`-AGTGCCAGCCTCGTCTCATA-3` (Forward), 5`-TCCCGTTGATGACCAGCTTC-3` (Reverse). There were 5-6 samples in each study groups and each sample were analysed in triplicate. The reactions mixture consists of 10µl SYBR green master mix (2x), forward and reverse primers (1µl each), cDNA (3µl), final volume 20µl with ddH_2O. The reaction condition

used was: initial denaturation (95^0C, 10 min), denaturation (95^0C, 30sec), annealing temperature (57^0C, 30sec), and extension (72°C, 30sec), total 45 cycles were performed.

Immunohistochemistry (IHC)

The brain was sliced in to 20μm thick brain sections which contain different brain regions such as PFC and Hippocampus, and the serial sections were collected with the help of cryostat (Microm HM 525, Germany). The collected sections were then washed in PBS solution (0.01M) and were blocked in PBST containing 1% normal horse serum solution (Vecta-stain kit). After blockage the sections were incubated in anti 5HT-1Br (1:250 dilution, ASR-022, Thermo), anti-c-fos (1:500, cat. PA5-143600, Invitro) primary antibody, overnight at room temperature. Following this the sections were incubated for 2h at room temperature in biotinylated linked secondary antibody (1:500 dilution, Vector Lab.). After this the sections were washed and incubated with avidin-biotinylated-peroxidase complex (Vectastain Elite ABC Kit) and further stained by the DAB staining solution (Vector lab.). After staining the sections were mounted on glass slides and covered with coverslip using DPX mountant. The images from each section were acquired using compound light microscope (Nikon). The level of serotonin receptor expression was analysed as number of positive neurons in different brain subregions using NIS-Basic Research image analysis system (Nikon).

Statistical analysis

For statistical analysis, the data was presented as means and standard-deviation (± SD) of the means, and analysed by ANOVA (one-way, two-way) or student's t-test using GraphPad Prism-7 statistical software.

Results

Behaviour

EPM test

Both male and female animals underwent for 6 hrs of chronic stress training for 10 days (Fig. 1A). All animals explored the environment but anxiogenic factors caused a decreased exploration for the environment in open arms. Entries in open arm was measured as percent entries in open arm for both the groups for overall duration of time. Tukey's post hoc analysis exhibited decreased entries in open arm by both the male and female chronic stress groups on

day 10, however, control groups exhibited an enhanced open arm entry at day 10 compared to first day test [F (3,16) = 21.15, p<0.001]. (Fig. 1C). The statistical analysis from the result exhibited significant effect of chronic stress on body physiology in both the male and female CS rats when compared with their respective control groups. (Fig. 1B)

OFT for anxiety measurement

When compared, the OFT showed a significant change between CS and respective control groups. The time spent in central area was higher for the control groups than CS group. Tukey's post hoc analysis suggested that the female CS group exhibited comparatively lower entry in the central field as compared to the male CS group (p<0.05). Here, the result suggested a significant effect of behavioural groups on time duration spent in central arena [F (3,16) = 19.42, p<0.001]. When analysed the number of entries in central area significantly lower in male and female CS groups as compared to control groups. (Fig. 1C)

Body weight measurement

Following stress session, the body weight was measured in CRS and control groups (everyday). There was alteration in mean body weight in all groups when compared day1 with the day 10 (p<0.05). In CS groups of male and female both, the body weight declined significantly from first day to day 10 continuously (p<0.0001), however, control groups (Male and Female) exhibited significantly enhanced body weight from day1 to day 10 (all p<0.0001) (Post-hoc analysis- Tukey's). The test compared first and last day trials of all the groups. The result suggested a significant consequence of stress [F (3,15) = 15.95, p<0.0001] and the interaction of stress with number of trials [F (3, 15) = 431.4, p<0.0001] with the body weight in these rats (Fig. 1D).

Plasma corticosterone level

The physiological response caused by stress on animal is measured by the analysing plasma corticosterone level from the blood of these animals. The corticosterone in the male and female animals was higher under CS when compared with the control animals; though, it was much higher in female rats as compared to the male animals under similar stress condition. The result was confirmed by the statistical analysis using Tukey's post-hoc examination. The result revealed a significant outcome of gender [F (1, 4) = 80.09, p=0.0009], stress [F (1, 4) = 445.6, p<0.0001] and interactive effect of gender with the chronic stress [F (1, 4) = 9.9, p=0.05] when

corticosterone level was analysed in both the male and female rats under chronic stress. (Fig. 1E)

Transcriptional analysis

Amygdala

c-fos mRNA expression

The c-fos expression showed a significant fold-change in c-fos mRNA expression in male and female rats when compared with their respective control groups. In amygdala of the male and female rats, the c-fos expression was amplified significantly in chronic stress groups when compared with their respective controls ($p<0.05$, $p<0.01$) (Tukey's post hoc analysis). Interestingly, the change in c-fos mRNA was significantly more in female chronic stress groups than the male chronic stress groups ($p<0.05$). The result exhibited significant outcome of sex of animals [$F(1,4) = 26.25$, $p<0.01$], the stress on rats [$F(1,4) = 58.79$, $p<0.01$] and interaction of sex with the chronic stress [$F(1,4) = 16.78$, $p<0.05$] for c-fos mRNA expression in amygdala compared to control groups. (Fig. 2.B)

5HT-1B mRNA expression

The 5HT-1B expression showed a significant fold-change in 5HT-1B mRNA expression in male and female rats when compared with their respective control groups. In male SD rats the 5HT-1B mRNA was amplified ($p<0.01$) significantly, while in female rats, transcription diminished ($p<0.01$) in CRS groups compared with their respective control animal groups (post hoc analysis by Tukey's). The result presented a significant effect of sex of animals [$F(1,4) = 21.31$, $p<0.01$] and interface of sex and stress [$F(1,4) = 156.3$, $p<0.001$] for serotonin 5HT-1B mRNA expression in amygdala region, compared to the control animals. (Fig. 2.C)

mRNA expression in Prefrontal cortex

c-fos mRNA expression

The c-fos expression showed a significant fold-change in c-fos mRNA expression in male and female rats when compared with their respective control groups. In PFC of the male and female rats, the c-fos expression was amplified significantly in chronic stress groups when compared with their respective control groups ($p<0.01$, $p<0.01$) (Tukey's post hoc analysis). The c-fos expression when compared was higher in female stress group compared to male stress group

(p<0.05). The outcome exhibited a significant consequence of stress [F (1,4) = 85.84, p<0.001] on c-fos mRNA expression in PFC. (Fig. 3.B)

5HT-1B mRNA expression

The 5HT-1B expression showed a significant fold-change in 5HT-1B mRNA expression in male and female rats when compared with their respective control groups. In male and female SD rats the 5HT-1B mRNA was declined (p<0.01; p<0.01) significantly in CRS groups compared with their respective control animal groups (post hoc analysis by Tukey's). The result displayed a significant effect of chronic stress in rats [F (1,4) = 97.04, p<0.001] and sex of animal [F (1,4) = 326, p<0.0001] for expression of 5HT-1B mRNA in PFC when analysed. However, basal serotonin 5HT-1B receptor mRNA level was higher for female group when compared with the male stress group. (Fig. 3-C)

mRNA expression in Hippocampus

c-fos mRNA expression

The c-fos expression showed a significant fold-change in c-fos mRNA expression in male and female rats when compared with their respective control groups. In hippocampus of the male and female rats, the c-fos expression was amplified significantly in chronic stress groups when compared with their respective control groups (p<0.05, p<0.01) (Tukey's post hoc analysis). Though, the change in expression was significantly more in female chronic stress groups than male chronic stress group rats (p<0.05). The ANOVA result revealed a significant consequence of chronic stress [F (1,4) = 13.61, p<0.05] and the sex of animals [F (1,4) = 56.15, p<0.01] and interaction of sex of animals with the chronic stress condition [F (1,4) = 9.62, p<0.05] for the expression of c-fos mRNA in hippocampus region when compared with the control. (Fig. 4.B)

5HT-1B mRNA expression

The 5HT-1B expression showed a significant fold-change in 5HT-1B mRNA expression in male and female rats when compared with their respective control groups. In male and female SD rats the 5HT-1B mRNA was declined (p<0.01; p<0.05) significantly in CRS groups compared with their respective control animal groups (post hoc analysis by Tukey's). The ANOVA result revealed a significant consequence of chronic stress [F (1,4) = 32.61, p<0.01] and the sex of animals [F (1,4) = 133.1, p<0.001] for the expression of 5HT-1B mRNA in

hippocampus region when compared with the control. The 5HT-1B level was significantly lower in female chronic stress group compared with the male chronic stress group, suggesting a sex-based association of this receptor subtype in hippocampus under stress condition. (Fig. 4.C)

Immunohistochemistry

c-fos expression in amygdala

The expression of c-fos protein was analysed in amygdala for both male and female groups to understand amygdala activity under stress condition. The chronic restrained group of male and female rats revealed a significant variance compared to their control group animals. In male and female SD rats the c-fos level was enhanced ($p<0.001$; $p<0.001$) significantly in CRS groups compared with their respective control animal groups (post hoc analysis by Tukey's). Though, a significant difference was observed among male and female animals for c-fos level when animals exposed to stress ($p<0.05$). ANOVA result revealed a significant consequence of chronic stress [$F(1,5) = 24.71$, $p<0.01$] and the sex of animals [$F(1,5) = 54.05$, $p<0.001$] for the expression of c-fos in amygdala region when compared with the control. The c-fos level, suggested a sex-based association of this receptor subtype in amygdala under stress condition. (Fig. 2.D, 2.E)

Serotonin 5HT-1B receptor expression in amygdala

The serotonin receptor 5HT-1B expression was analysed in amygdala between both male and female animals. The chronic restrained group of male and female rats revealed a significant variance compared to their control group animals. In male SD rats the 5HT-1B level was enhanced ($p<0.05$) significantly in CRS groups compared with their respective control animal groups however it declined in female rats ($p<0.05$) when exposed to stress (post hoc analysis by Tukey's). ANOVA result revealed a significant consequence of the sex of animals [$F(1,5) = 40.98$, $p<0.01$] and combined action of chronic stress with sex of animals [$F(1,5) = 35.14$, $p<0.01$] for the expression of 5HT-1B in amygdala region when compared with the control. The 5HT-1B level, suggested a sex-based association of this receptor subtype in amygdala under stress condition. (Fig. 2.F, 2.G)

c-fos expression in PFC

The c-fos expression was analysed in PFC between both male and female animals to understand PFC activity under stress condition. The chronic restrained group of male and female rats revealed a significant variance compared to their control group animals. In male and female SD rats, the c-fos level was enhanced ($p<0.05$; $p<0.01$) significantly in CRS groups compared with their respective control animal groups (post hoc analysis by Tukey's). Though, a significant difference was observed among male and female animals for c-fos level when animals exposed to stress ($p<0.05$). ANOVA result revealed a significant consequence of the chronic stress [$F(1,5) = 21.44$, $p<0.01$], sex of animals [$F(1,5) = 39.89$, $p<0.01$] and combined action of chronic stress with sex of animals [$F(1,5) = 11.26$, $p<0.05$] for the expression of c-fos in PFC region when compared with the control. The c-fos level, suggested a sex-based association of c-fos activity in PFC under stress condition. (Fig. 3.D, 3.E)

Serotonin 5HT-1B receptor expression in PFC

The 5HT-1B expression was analysed in PFC between both male and female animals to understand its activity under stress condition. The chronic restrained group of male and female rats revealed a significant variance compared to their control group animals. In male and female SD rats, the 5HT-1B level was diminished ($p<0.05$; $p<0.05$) significantly in CRS groups compared with their respective control animal groups (post hoc analysis by Tukey's). ANOVA result revealed a significant consequence of the chronic stress [$F(1,5) = 70.25$, $p<0.001$] and sex of animals [$F(1,5) = 7.6$, $p<0.05$] for the expression of 5HT-1B in PFC region when compared with the control. The 5HT-1B level, suggested a sex-based association of 5HT-1B activity in PFC under stress condition. (Fig. 3.F, 3.G)

c-fos expression in Hippocampus

The c-fos expression was analysed in hippocampus between both male and female animals to understand its activity under stress condition. The chronic restrained group of male and female rats revealed a significant variance compared to their control group animals. In male and female SD rats, the c-fos level was enhanced ($p<0.05$; $p<0.01$) significantly in CRS groups compared with their respective control animal groups (post hoc analysis by Tukey's). Though, a significant difference was observed among male and female animals for c-fos level when animals exposed to stress ($p<0.05$). ANOVA result revealed a significant consequence of the chronic stress [$F(1,5) = 14.78$, $p<0.05$], sex of animals [$F(1,5) = 25.85$, $p<0.01$] and combined

action of chronic stress with sex of animals [F (1,5) =43.03, $p<0.01$] for the expression of c-fos in hippocampus region when compared with the control. The c-fos level, suggested a sex-based association of c-fos activity in hippocampus under stress condition. (Fig. 4.D, 4.E)

Serotonin 5HT-1B receptor expression in Hippocampus

The 5HT-1B expression was analysed in hippocampus between both male and female animals to understand its activity under stress condition. The chronic restrained group of male and female rats revealed a significant variance compared to their control group animals. In male and female SD rats, the 5HT-1B level was declined ($p<0.05$; $p<0.05$) significantly in CRS groups compared with their respective control animal groups (post hoc analysis by Tukey's). Though, a significant difference was observed among male and female animals for 5HT-1B level when animals exposed to stress ($p<0.05$). Although both the stress groups exhibit a reduction in serotonin 5HT-1B receptor expression, the basal level for serotonin 5HT-1B receptor was lower in female stress ($p<0.05$) groups. The ANOVA result revealed a significant consequence of the chronic stress [F(1,5) = 29.19, $p<0.05$] and sex of animals [F (1,5) =153.7, $p<0.0001$] for the expression of 5HT-1B in hippocampus region when compared with the control. The 5HT-1B level, suggested a sex-based association of 5HT-1B activity in hippocampus under stress condition. (Fig. 4.F, 4.G)

Discussion

Current study investigated the role of serotonin 5HT-1B receptor subtype on sex-dependent stress and anxiety variation in SD rats. In our rodent model-based study, we found a sex-based spatial function played by serotonin 5HT-1B receptor subtype in chronic restrained stress condition among male and female SD rats. For better understanding the difference of stress circuitry and involvement of 5HT-1B receptor, we first investigated the activity of amygdala, hippocampus and PFC regions of the brain under CRS condition using c-fos expression (an IEG used as a neuronal activity marker gene) (Gallo et al, 2018). We observed a sex-based differences in activation pattern in these brain areas under CRS training.

The c-fos expression analysis confirmed activation of hippocampus, PFC and amygdala brain regions during chronic stress in male and female rats. However, the expression was comparatively at higher level in female CRS animals than male, which was correlated with

comparatively higher stress response in females. The result suggests that comparatively enhanced activity of trio brain areas (i.e., PFC, amygdala and hippocampus) causes hyperactivation of stress circuitry in females stress group in comparison to the male stress group. Although other regions such as hypothalamic–pituitary–adrenal axis (HPA) are also associated with the stress circuitry but our main focus was on trio partners due to their central role in emotional responses (McEwen et al, 2016). The stress circuitry involves DRN (Dorsal raphe nucleus) nucleus, from where it innervates amygdala, PFC and hippocampus through direct and indirect neuronal connections (McEwen et al, 2016; Huang et al, 2019). DRN through its serotonergic innervation regulates the activity of amygdala, PFC and hippocampus regions under stressful condition (Bocchio et al, 2016; Huang et al, 2019).

Current study hypothesised that the difference in serotonergic 5HT-1B receptor between both the sexes may cause differential activation of these brain regions which resulted in differences in stress response (Fig. 5). We started our investigation finding potential role of serotonin receptors in CRS condition which affects internal cellular and molecular difference between both the sexes. Our examination for different serotonin receptors suggested an interesting role of 5HT-1B receptor subtype in both the sexes which was correlated with the difference in stress responses.

It is observed through current study that the expression of serotonin 5HT-1B receptor declined in PFC and hippocampus in male and female both the stress groups, but in amygdala it increased in male CRS group and decreased in females CRS group. Although basal 5HT-1B level in amygdala was similar between both the sexes, however hippocampus exhibited comparatively lower 5HT-1B expression in female control group than the male control group. Compared to male, female CRS group exhibited comparatively lower 5HT-1B receptors expression in hippocampus, which might be one of the reasons why females have a higher stress than male. Some of the recent animal studies have suggested an anxiolytic function for 5HT-1B receptor activation (Yohn et al, 2017; Tiger et al, 2018) which is also in line with our study where decreased serotonin 5HT-1B receptor correlated with the higher stress response. Likewise, hippocampus, a key brain region regulating stress response (Levone et al, 2014; McEwen et al, 2016), exhibited a comparatively reduced 5HT-1B level in female chronic stress group than the male stress group. Anti-anxiety activity of serotonin 5HT-1B receptor might be the main reason why hippocampus exhibited such a reduced 5HT-1B level in females than in male rats.

In amygdala, the expression of 5HT-1B receptor exhibited a different pattern among male and female rats. In male CRS group, 5HT-1B expression increased, while in female the expression decreased significantly in amygdala region. This lowering of 5HT-1B receptor in female amygdala might be the reason for higher amygdala activity in females under stressful condition as confirmed by c-fos expression. However, this differential expression for 5HT-1B receptor did not correlate with stress response as both the sexes exhibited an enhanced stress response under CRS condition. The result suggests here a difference in intra-amygdala stress circuitry between them for similar type of stress condition. This intra-amygdala difference of 5HT-1B receptor might be a crucial factor which determines the severity of stress between both the sexes. Furthermore, a decreased 5HT-1B expression in amygdala was unable to maintain 5HT-1B level in hippocampus which results in decreased 5HT-1B expression causing a higher stress response in female rats. Contrary to this, an increased 5HT-1B expression in male amygdala instigated a moderate inhibition to the hippocampus that resulted in comparatively lower stress response.

Overall, male CRS group exhibited direct neuronal connectivity between amygdala and hippocampus, where an enhanced 5HT-1B expression in amygdala inhibited 5HT-1B expression in hippocampus. As this serotonergic receptor is inhibitory in nature which function by decreasing the release of other neurotransmitters through postsynaptic neurons once activated (Ciranna, 2006; Tiger et al, 2018). Moreover, in female rats the system exhibited an indirect serotonergic receptor-based stress circuitry where a decreased 5HT-1B expression in amygdala might results in disinhibition of those neurons that inhibit 5HT-1B receptors expression on post synaptic neurons in hippocampus.

The result further suggest that PFC modulated the activity of hippocampus by regulation of amygdala activity. Under CRS condition, PFC was active in both the sexes but female group exhibited relatively higher activation. Although, this PFC activation correlated with decreased 5HT-1B level in PFC of both animals, female chronic stress group revealed significantly lower expression in comparison to its control group. This difference caused comparatively lower 5HT-1B level together with an increased amygdala activity in female animals. This enhanced amygdala activity in females caused a decreased 5HT-1B level in hippocampus and an increased hippocampal activity causing higher stress response in female CRS groups. Contrary to this, comparatively lower amygdala activity in male animal was correlated with an increased 5HT-1B expression, resulting in an enhanced hippocampal activity. The hippocampal activity exhibited a sex-based variation for stress response together with the 5HT-1B expression.

Current study proposed a sex-based variation in stress circuitry, where more interestingly an inhibitory network in female hippocampus might be involved for the regulation of hippocampus activity under stress situation. As a result of decreased 5HT-1B receptors expression, serotonergic neurons might not be able to disinhibit 5HT-1B receptor expression in hippocampus causing higher activation of hippocampus and finally a higher stress in females.

This sex-based variation in anxiety and stress character might be an evolutionary response acquired differently by the females and males based on differences in environmental contingencies. As we know, by the start of human civilization, the condition of women in our society was not so good due to dominancy of males over females (Zhu and Chang, 2019; Martin et al, 2009). Although, the condition of women has improved in our society now a days, but not as good as men are. Both the external environmental factors and internal molecular biology of brain affects the functionality and behavioural aspects of our life equally.

In conclusion, a sex-dependent difference in circuitry of stress among male and female rats incorporating serotonin 5HT-1B receptor system, may plays an important role to regulate this circuitry. The anatomical and neural circuitry-based differences between both the sexes are important aspect for characterization of stress and anxiety. Some of the important outcomes drawn from the study includes: First, there is an internal neuronal difference for stress-circuitry among male and female individuals. Second, the serotonin 5HT-1B receptor is accompanied with anti-anxiety and anti-stress functions irrespective of the sex of the individual. Third, the stress circuitry in females is more complex than the male individual. Fourth, the variation in stress response among male and female animals is associated with the difference in serotonin 5HT-1B receptor-based stress circuitry. Overall, the difference in serotonin receptor system between both the sex is of great importance which promises development of a better therapeutic strategy for stress disorders keeping in mind the importance of sex difference. This is the first study where we studied a spatial association of serotonin receptors in such stressful situation among male and female animals. Future research incorporating other serotonin receptors linked with sex-based stress and anxiety, will be helpful to better characterise this condition and development of potent therapeutic approaches including pharmacological intervention.

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Conflicts of interest

None

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Figures and Legends

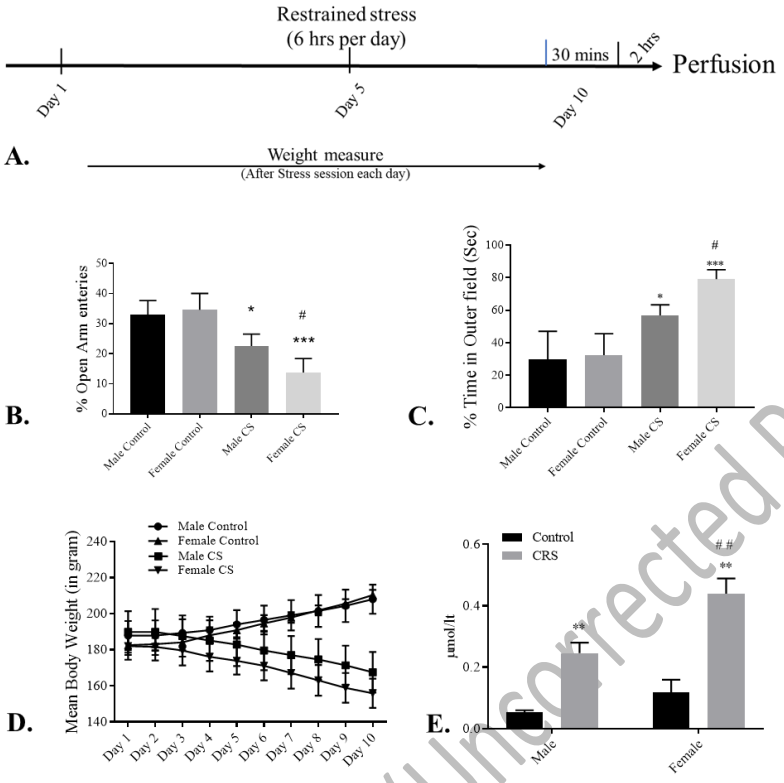

Fig. 1: Animal behaviour experiments. **A.** Behaviour protocol in rats for chronic restrained stress (CRS) experiments. **B.** EPM: % open arm entries at last day of CRS (Following stress). **C.** % time spend in outer field at last day of CRS (OFT, Following stress)**. D.** Mean body weight in CRS session from day1 to last days. **E.** Blood Corticosterone level (µmol/ltr) in male and female animals in CRS training. (* value represent within male and female groups; # represent difference between corresponding male and female groups)

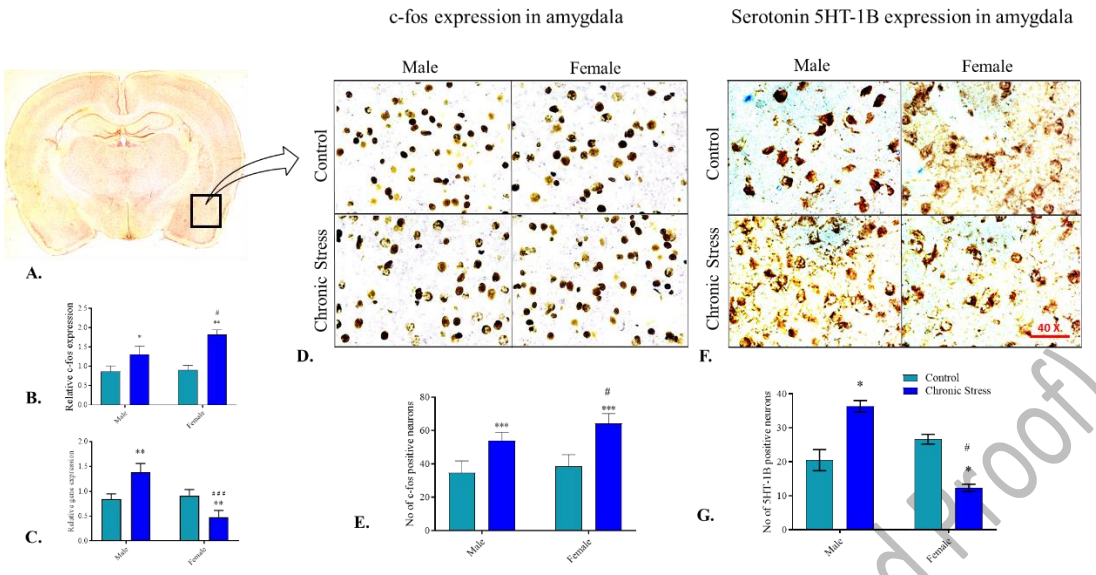

Fig. 2: Effect of CRS training on expression of c-fos and 5HT-1B in amygdala. **A.** Cross-section image of amygdala region. (2x) **B.** Relative c-fos mRNA level in amygdala region. **C.** Relative fold change in 5HT-1B mRNA in amygdala. **D.** and **E.** IHC images for the expression of c-fos in amygdala (40x). **F.** and **G.** IHC images for 5HT-1B expression in amygdala.

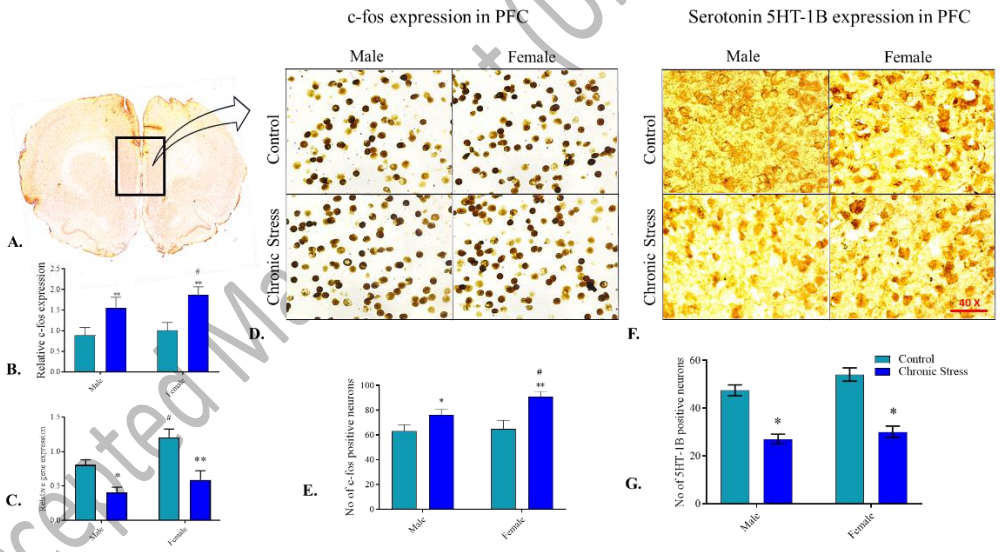

Fig. 3: Effect of CRS training on expression of c-fos and 5HT-1B in PFC (2x). **A.** Cross-section image of PFC region. **B.** Relative c-fos mRNA level in PFC area. **C.** Relative fold change expression in 5HT-1B mRNA in PFC. **D.** and **E.** IHC images for the expression of c-fos in PFC (40x). **F.** and **G.** IHC images for the expression of 5HT-1B in PFC (40x).

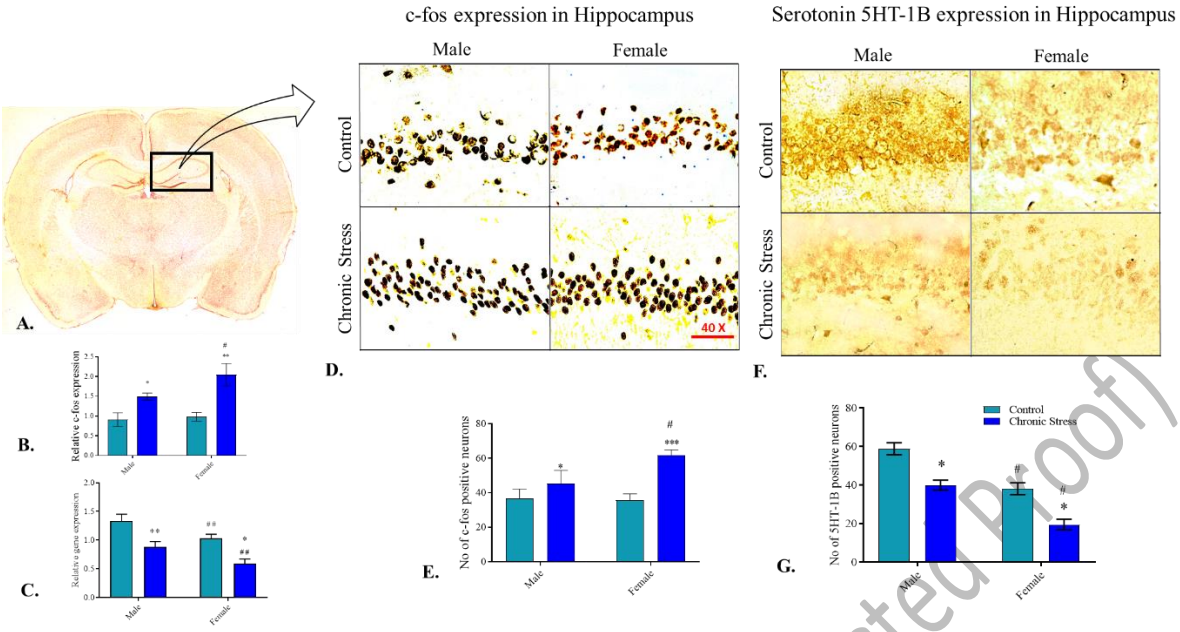

Fig. 4: Effect of CRS training on expression of c-fos and 5HT-1B in Hippocampus (2x). **A.** Cross-section image of Hippocampus region. **B.** Relative c-fos mRNA level in Hippocampus region. **C.** Relative fold change expression in 5HT-1B mRNA in Hippocampus area. **D.** and **E.** IHC images for the expression of c-fos in Hippocampus (40x). **F.** and **G.** IHC images for the expression of 5HT-1B in Hippocampus (40x).

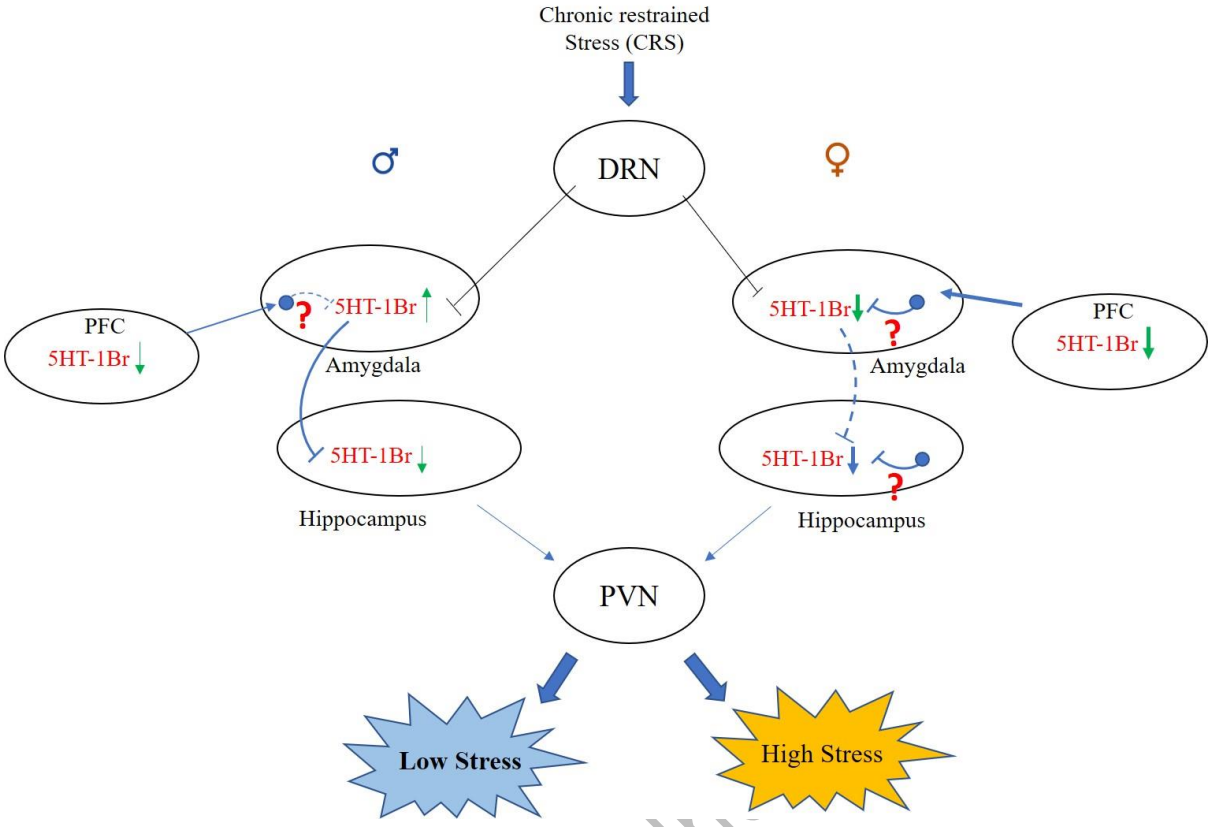

Fig. 5: An illustrative diagram showing the brain circuit for stress behaviour in male and female SD rats. The intensity of arrow signifies the strength of neuronal connections in different brain centres. The lines with solid character (——) describes the stimulated neuronal connection in brain, however, the lines with dash (- - -) represent a lowered neural network. The inhibitory connections are shown as lines with bar-head (T).