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Research article

# Region specific differential regulation of 5HT-5A and 5B receptor is associated with the difference in stress level between male and female rats

Sarfraj Ahmad Siddiqui [a,*], Sanjay Singh [c], Atul Rawat [c], Md Arshad [b], Sudhir Kumar [a]

[a] Department of Zoology, University of Lucknow, Lucknow, India
[b] Department of Zoology, Aligarh Muslim University, Aligarh, India
[c] Department of Biotechnology, BBAU, Lucknow, India

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ABSTRACT

Stress-related neuropsychiatric disorders affect nearly all people worldwide irrespective of the age and sex of the person. Females are supposed to experience a higher stress and anxiety as compared to the male individuals. The role of serotonin receptor in stress and anxiety condition is supposed to affect this sex-based difference in stress and anxiety condition between male and female animals. Serotonin receptor system is one of the most important molecular mechanism in brain function involved in a number of vital functions such as apetite, sleep, thermoregulation, aggression, learning, mood, cognition as well as in stress and anxiety. The current preclinical study is analyzing the role of serotonin 5HT-5A and 5B receptor in stress and anxiety in male and female rodents. The study suggests here a differential region specific association of both the serotonin receptor under stressful condition between male and female animals.

## 1. Introduction

Stress-related neuropsychiatric disorders affect almost all person irrespective to the sex, age and ethnicity of a person (Salleh, 2008). The impact of stress is multidimensional affecting several physiological functions such as learning, memory, insomnia, anxiety, depression, including basic functions such as digestion and cardiac activity (Godoy et al., 2018). Stress associated alteration of different physiological responses may sometimes progress towards pathological conditions in people. The influence of stress is sometime acute but may also develop into chronic pathological stage, depending upon the type and duration of stressor (Tsigos et al., 2000; Dhama et al., 2019). Stress also has a differential effect which is influenced by the individual difference and the gender or sex to which one belongs (Verma et al., 2011). The effect of stress is not similar to every person as some are severely affected by it while others exhibit lesser or no effect at all (Verma et al., 2011).

Studies have confirmed that the females are comparatively more prone to stress in their life as compared to the male individuals of the same age (Verma et al., 2011; Bale and Epperson, 2015). These studies suggest a sex-based disparity for the stress response between male and females. As compared to the male, females experience a higher chance of stress in their life. Females having work related stress account for almost 50% higher chance of stress as compared to the male person (Chaplin yet al, 2008). Based on these observations on sex-based differences for stress response it is clear that the same type of stress therapy cannot be applied for both the sexes. Unless, we do not understand this difference in detail, it will be an enigma to develop better therapeutics for the treatment of stress and anxiety disorders.

The circuitry of stress involves different brain regions, of which the hippocampus region of the brain expresses receptors for mineralocorticoid and glucocorticoid hormones in higher amount (Koning et al., 2019). Hippocampus in combination with other important brain regions such as amygdala, prefrontal cortex, bed nucleus of stria terminalis etc. functions to drive the stress circuitry (Rajmohan and Mohandas, 2007). The stress circuitry comprises DRN (dorsal raphe nucleus), hippocampus, amygdala and PFC and other brain regions (Shin and Liberzon, 2010). Amygdala (an emotional output region), PFC (a planning and decision-making centre) and hippocampus (processing and storage of information) together forms limbic system, the area most importantly involved in regulation of emotional response under stressful condition, and the circuitry system thus formed is called “stress circuitry” (Rajmohan and Mohandas, 2007; Šimić et al., 2021).

Some of the recent studies have suggested the role of serotonin receptor system with the stress and anxiety including post-traumatic stress disorders (PTSD) and other related disorders. Serotonin receptor system is one of the most important neuronal mechanisms controlling number of

* Corresponding author.
*E-mail address:* sarfrajcbt@gmail.com (S.A. Siddiqui).

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essential brain functions such as learning, memory, stress, anxiety, mood, emotional responses, sleep-wake cycle and other cognitive functions (Frazer et al., 1999). The serotonin system is mainly composed of serotonin receptor which binds with the serotonin neurotransmitter (5-hydroxytryptamine) in our brain (Frazer et al., 1999; Bakshi and Tadi, 2022). There are fourteen different types of serotonin receptors (5HT-1 to 5HT-7) distributed throughout human brain (only 13 in rat brain) and other body parts (Bakshi and Tadi, 2022). These receptors are generally inhibitory in nature, when activated control the release of different neurotransmitters such as glutamate, GABA, acetylcholine etc (Bakshi and Tadi, 2022).

Among these receptors the 5HT-1A, 1B, 2A, 3 and 6 subtypes are known to be highly expressed in brain and are associated with the stress and anxiety condition. Of these, 5HT-2C receptor is known to be associated with the sex chromosomes as its gene is present on X-chromosome (Chaplin et al., 2008). More importantly, other receptors such as 5HT-3 have differential function in female (anxiolytic) and male (anxiogenic) animals (Bhatnagar et al., 2004). The studies on 5HT-1A receptor suggested quantitative variations between male and female brain which again suggested a spatial and functional difference between them. Interestingly, there is also a difference of serotonin neurotransmitter synthesis rate between male and female. Also in human brain, the male exhibit a higher level of serotonin synthesis (52% higher in male) as compared to the female individuals (Nishizawa et al., 1997).

Of these receptors, serotonin 5HT-5A and 5B subtypes are expressed in lower amount in these brain regions and are very less explored in relation to stress and other psychiatric disorders (Nautiyal and Hen, 2017). 5HT-5A receptor is mostly expressed in hippocampus, Cerebral cortex, granular layer of cerebellum and olfactory bulb of the brain, while 5B isoform (not present in humans, Nelson, 2004) in hippocampus, habenula and inferior olive. These receptors have been found to be associated with the different behavioural processes as in spatial memory functions as well as in psychiatric disorders such as depression, anxiety and schizophrenia (Nautiyal and Hen, 2017). Although very limited information is available about the role of 5HT-5A and 5B receptor function in psychiatric disorders. However, some studies through indirect observation have suggested the presence of difference for both the receptor expression in male and female brain. For instance, Karimi et al. (2013) in their study suggested a higher 5HT-5B expression in PFC and hippocampal region in male as compared to the female animals. However, till date no such study worked out to find their sex-based or gender-based association under stress and anxiety condition. Besides this lower expression of these 5HT-5A and 5B receptor, the important regulatory function of these receptors on stress and anxiety cannot be ignored in regulation of stress circuitry under stressful condition.

Current study is thus investigating the role of serotonin 5HT-5A and 5B receptor subtypes on sex-based stress and anxiety difference in rodent model and may be useful for development of specialised therapeutics among both the sexes.

## 2. Methods

### 2.1. Animals

All experiments were performed on 2–3 months old healthy adult male and female SD (Sprague–Dawley) rats. The animals were housed under standard conditions of 12-h light/dark cycle, temperature maintained at 23 °C, water, and food available *ad libitum*. All experiments in the study were carried out in accordance with the ethics guidelines and approved by the Animal Care and Use Committee (P/BL/18–19/0581/2019).

### 2.2. Chronic restrained stress (CS/CRS)

Animals (both male and female) underwent 10 days chronic restrained stress (6 h daily) by keeping animals under immobilization condition (Buynitsky and Mostofsky, 2009). The stress session started at 8:00 AM morning up to 2:00 PM afternoon. For immobilization stress in animal, a polythene (appx 10–15 cm long) cone was used. The EPM (elevated plus maze) test and open field test was performed 30 min prior to the commencement of CRS session on day 1 to monitor any stress or anxiety in animals. After commencement of last day experiment EPM and OFT (Open field test) was performed to measure the effect of anxiety, if any, in these animals. Following behavioural experiments animals were perfused to isolate brain for further molecular studies using real-time PCR and Immunohistochemistry in these animals. There was total four groups (male control, female control, male CRS and Female CRS groups) of animals in the study. For behaviour experiments each group contains 10–12 animals (half for CRS + EPM and half for CRS + OFT) which were not used for molecular study. For molecular analysis through IHC ($n$ = 5–6 in each group) and Q-PCR ($n$ = 5–6 in each group), separate group of animals (n = 10–12, half for IHC and half for PCR) were used, which undergone for CRS training without EPM and OFT tests.

### 2.3. Elevated plus maze test

For understanding the effect of chronic stress on anxiety in rats EPM test was performed. EPM apparatus includes two open and two closed arms ($40 \times 10cm$) connected at the centre forming a plus-shaped structure. The apparatus was elevated above 50cm from ground and placed in the dim-light room. Rats were placed at the centre of maze facing closed arms and were observed for up to 5 min. The number of entries rats entered into open arms were recorded as time spends within each arm. The percent open arm entries was recorded during 5 min of exposure with EPM from all groups. The analysis for the EPM and OFT was done using Any-Maze analysis software.

### 2.4. Open field test

OFT is one of the methods to measure stress and anxiety in animals (Seibenhener ML and Wooten, 2015). The OFT was performed in a non-transparent square box ($120 \times 120 \times 60$ cm dimension) present in a sound attenuated room with a house light on ceiling. The field was divided into central and outer region (Seibenhener ML and Wooten, 2015). Animals were placed randomly at one corner of the arena. Animals were allowed to explore the field for up to 15 min, while their behaviour was observed using a monochrome camera located at the top of apparatus. Total time spend and number of entries at centre was analysed manually for overall duration of the session. The field was cleaned with 70% ethanol after each experiment.

### 2.5. Body weight measurement

Measuring body weight is one of the methods to quantify and analyse the effect of anxiety and stress on animal physiology. Generally, stress shows a reduction in body weight gradually from day 1 to the last day (Yau and Potenza, 2013; Harris, 2015).

### 2.6. Plasma corticosterone

Blood sample were collected from these animals in each experiment during perfusion (4:00 pm) from the aortic puncture (about 1 ml) into ice-cooled centrifugal tubes. Blood was allowed to clot at room temperature for 1 h in siliconized serum collection tubes and centrifuged (1700 ×g, 10 min, 4 °C) to separate serum. The collected serum was stored at -80 °C until experiment. The isolated serum sample was used for the identification of corticosterone hormones involved in stress and anxiety. The samples were analysed through an ELISA kit (EIACORT, invitrogen) according to manufacturer's instruction for the identification of corticosterone level between both the sexes under CRS condition in both the objectives.

### 2.7. Details of brain subregions under study

The amygdala, hippocampus and prefrontal cortex (PFC) brain regions were analysed for serotonin receptor expression under stress in both the sexes. These brain regions are well known as a key partner for emotional as well as stress and anxiety responses (Rajmohan and Mohandas, 2007).

### 2.8. Tissue preparation

Two hours following stress session, rats were anesthetized with pentobarbital (60 mg/kg, i. p.), perfused transcardially with n-saline, followed by ice-cold 4% paraformaldehyde (prepared in 0.1 *M phosphate* buffer, pH 7.4), decapitated and brains removed. These brains were then post-fixed in 4% paraformaldehyde for 24 h and then in 10%, 20% and 30% sucrose solution (in 0.1M phosphate buffer, pH 7.4) serially till settle down. Brains were then frozen in isopentane at -30° to -35 °C for 30 min and kept at -80 °C for immunohistochemistry. For mRNA analysis rats were perfused with normal saline only and brains were isolated and frozen at -80 °C for real-time PCR.

### 2.9. mRNA isolation and cDNA preparation

The frozen tissue stored at –80 °C (5 mg) was homogenized with 100ul lysis buffer and 0.7ul $\beta$-mercaptoethanol. Total RNA was isolated from the rat brain tissues using Stratagene absolute RNA isolation kit (Agilent technologies, Catalog no. #400800, CA, USA) and treated with DNase I to remove genomic DNA. Total mRNA was checked for its purity (spectrophotometer 260/280nm, O.D. = 1.69–1.82) and integrity. Genesure first-strand cDNA synthesis kit (Genetix, PGK-162B, New Delhi, India) was used to synthesize cDNA from the total tissue mRNA using oligo-dT primers. Amplified products (6.5 ng/ul) were then used for the real-time PCR amplification. The reaction mixture was prepared using template RNA (3ul), oligo dT primer (1ul), 5x reaction buffer (3ul), dNTP mixture (1.5ul), reverse transcriptase (0.75ul) and RNase inhibitor (0.75ul) with a total rection solution of 15ul. Thermal profile used for the cDNA preparation is 42 °C (60 min), 70 °C (5 min) and 4 °C for holding the reaction. The obtained cDNA was stored at -80 °C for further studies.

### 2.10. Quantitative real-time PCR

Quantitative real time PCR (Q-PCR) was used for comparison of serotonin receptor mRNA between male and female stress groups. Stratagene Max-Pro Real-Time PCR detection System (Mx3000/Mx3005P Real-Time PCR System, CA, USA) and SYBR green (Agilent, Cat: #600883, CA, USA) was used for transcription analysis. Relative quantities or fold change of mRNAs were calculated through comparative $C_t$ method by using $2^{-\Delta Ct}$ equation. Primers used for cDNA amplification were 5HT-5A, 5′-TCAGGTTCTTGGCTCTTGGC -3` (forward), 5′-TAGGCAGATCCATTGCTGGC-3` (reverse); 5HT-5B, 5′-GCAGGGCTGGGGAGATAA -AA-3` (Forward), 5′-GGCCCTACGGTGTGATTTCT-3` (Reverse) and control GAPDH, 5′-AGTGCCAGC -CTCGTCTCATA-3` (Forward), 5′-TCCCGTTGATGACCAGCTTC-3` (Reverse). For each group, 5–6 animals were used and the Q-PCR reaction was performed in a set of triplicates. The reactions were performed in a 20-µl volume containing 10µl SYBR green PCR master mix (2x), 1µl forward and reverse primers each, 3µl cDNA, total volume with dd$H_2O$ 20µl. Samples were made ≤20ml within RNase, DNase-free water. Reactions were carried out in a Stratagene Max Pro PCR machine as follows: an initial denaturation step at 95 °C for 10 min, denaturation at 95 °C for 30sec, annealing at 57 °C for 30sec, and extension at 72 °C for 30sec, repeated 45 cycles.

### 2.11. Immunohistochemistry

20µm coronal brain sections containing Amygdala, Hippocampus and PFC were collected serially using cryostat (Microm HM 525, Germany) from the controls and CRS groups for each antibody. These sections were washed with 0.01M PBS and blocked in PBST (0.01M, Phosphate buffer saline and 0.25% tween-20) containing 1% normal horse serum (NHS, PK-6200, Vecta-stain Elite ABC kit, Burlingame, CA, USA). Sections were then incubated overnight at room temperature with anti-5HT-5A (1:500 dilution, LS-A2119-50, LSBio), anti-5HT-5B (1:300, NBP1-46553, Novus biologicals) and anti-c-fos (1:500, ab7963, Cambridge, UK) primary antibody. Next day these sections were incubated with biotinylated secondary antibody (anti-rabbit IgG, 1:500 dilution, PK-6200, Vecta-stain Elite ABC Kit, Vector Laboratories, USA) for 2h at room temperature. Finally, sections were incubated with avidin-biotinylated-peroxidase complex (PK-6200, Vecta-stain Elite ABC Kit; Vector Laboratories, USA) followed by DAB staining (ab64238, DAB peroxidase substrate, Abcam, UK). Stained sections were mounted on glass slides and images from the sections was acquired using a Nikon Eclipse Ni microscope (Nikon). Expression was analysed as number of positive nuclei in amygdala, hippocampus and PFC subregions of the rat brain using NIS-Basic Research image analysis system (Nikon).

### 2.12. Data analysis

The data was expressed as means and standard error of the means (±SEM), and analysed by one-way ANOVA, two-way ANOVA or student's *t*-test using GraphPad Prism-6 statistical software.

## 3. Results

### 3.1. Behaviour

Animals from chronic stress groups exhibited a higher stress response as compared to their respective control groups. The female stress group exhibited a higher stress level as compared to the male CRS group. The result was confirmed by measuring EPM test, OFT and mean body weight measurement.

### 3.2. EPM test

All CRS animals were trained for 6 h daily stress session for up to 10 days (Figure 1A). Prior to the commencement of behaviour experiment, animals were checked for any anxiety effect using EPM test (Figure 1B). EPM test was also used at last day following behavioural experiment to find out effect of stress on these animals which is the limitation of the study for understanding the effect of stress between and within the groups. Comparable aged control groups were also used for EPM. All animal groups displayed no significant difference at first day. However, result exhibited reduced open arm entries in male and female stress groups following last day training session [$F(3,16) = 13.51$, $p < 0.0001$] as compared to the control groups suggested by Tukey's post hoc analysis (Figure 1B). The result showed significant effect of stress on physiology of both male and female CRS rats as compared to the control groups.

### 3.3. OFT

OFT exhibited a significant difference between CRS and control groups where time spent in central region was significantly lower in CRS group than the control groups. Tukey's post hoc analysis confirmed that in female CRS group the central field entries was comparatively lower as compared to the male CRS group ($p = 0.049$). Results showed a significant effect of groups for the time spent by these groups in centre [$F(3,16) = 21.18$, $p < 0.001$]. The number of entries in central region was significantly lower in CRS groups as compared to the control groups (Figure 1C).

### 3.4. Body weight measurement

All groups exhibited altered body weight when compared day 1 with the last day ($p < 0.001$). Mean body weight decreased

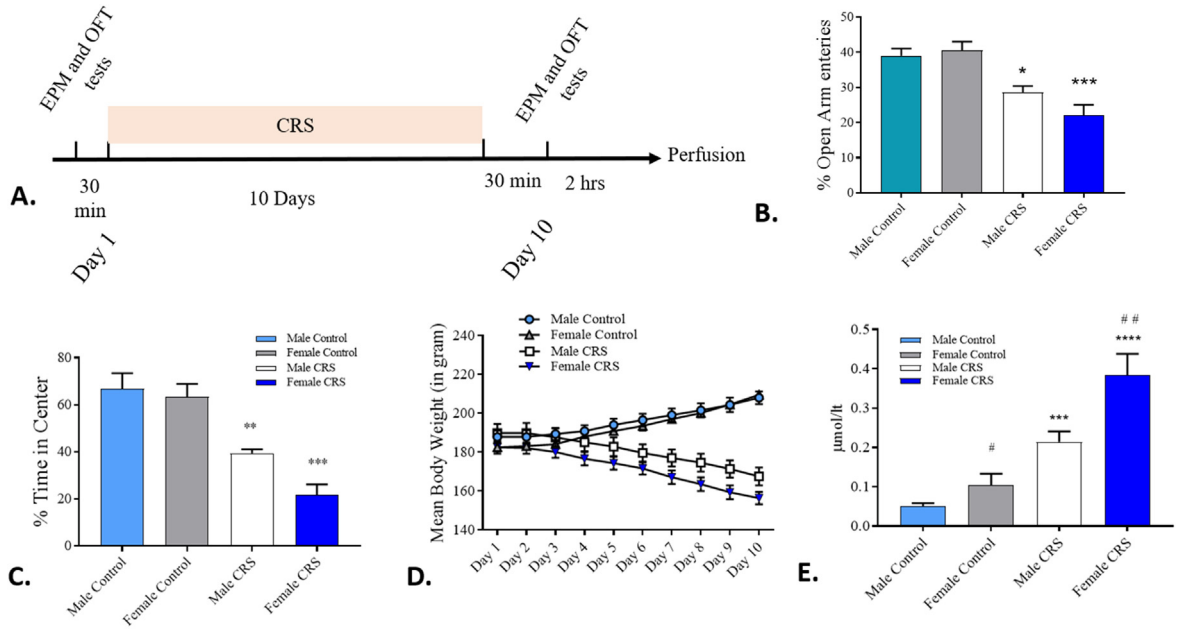


**Figure 1.** Animal behaviour **A.** Behavioural protocol for chronic restrained stress (CRS). **B.** % open arm entries in EPM in male and female groups (before stress) **C.** % time spend in central arena OFT, **D**. Mean body weight change in animals, **E.** Corticosterone level in male and female rats in CRS condition (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$ compared to the control of same sex groups; # = $p < 0.05$, ## = $p < 0.001$ when compared between similar groups of different sexes).

significantly from day 1 to day 10 in both male and female CRS groups ($p < 0.001$), while it increased in control groups from day1 to day 10 (all $p < 0.001$). The result exhibited a significant effect of stress [F (3,15) = 16.05, $p < 0.001$] and interaction of stress and trial [F (3, 15) = 398.01, $p < 0.0001$] on body weight in rats (Figure 1D).

### 3.5. Plasma corticosterone

Stress caused an increased plasma corticosterone level in both the male and female animals; however, the level was significantly higher in females as compared to the male animals as suggested by Tukey's post hoc analysis ($p = 0.0002$). The result confirmed a significant effect of sex of animal [F (1, 4) = 64.4, $p = 0.0013$], stress [F (1, 4) = 333.2, $p < 0.0001$] and interaction of sex with stress [F (1, 4) = 75.19, $p = 0.001$] on corticosterone level in both the male and female animals under stressful condition (Figure 1E).

## 4. Molecular analysis

### 4.1. Amygdala

#### 4.1.1. Altered c-fos expression in amygdala in CRS trained animals

The c-fos expression was analysed to analyse the activity (increased or decreased) of these brain regions under stressful condition. For transcription study the brain regions (i.e., Hippocampus, Amygdala, PFC) were chopped off from the rat brain. A total 5–6 samples were analysed for transcription study from each group. The quantitative real-time PCR exhibited a significant fold change for c-fos mRNA expression between chronic restrained stress group from both the sexes (Figure 2C). In both the male and female amygdala, c-fos mRNA increased significantly in CRS groups as compared to their respective control groups ($p = 0.022$, $p = 0.002$). However, the change was significantly higher in female CRS groups than the male CRS group ($p = 0.028$) as suggested by Tukey's post hoc analysis. Two-way ANOVA analysis exhibited significant effect of stress [F (1,4) = 169.2, $p = 0.0002$] and interaction of sex with stress [F (1,4) = 11.21, $p = 0.029$] for c-fos mRNA expression in amygdala compared to control groups.

The CRS group from both the male and female groups exhibited significant difference when compared with their respective control groups. Tukey's post hoc comparison confirmed a significant increase for c-fos expression among male and female CRS group ($p < 0.01$, $p < 0.001$) as compared to the control group. However, there was a significant difference between both the CRS groups ($p < 0.05$). Two-way ANOVA analysis confirmed a significant main effect of sex of animals [F (1,5) = 7.65, $p < 0.05$], stress condition [F (1,5) = 289.4, $p < 0.0001$] and interaction of sex with stress condition [F (1,5) = 14.69, $p < 0.05$] on c-fos expression in amygdala. In conclusion, c-fos expression as well as activity of amygdala exhibited a sex-based association with the stress in male and female animals (Figure 2B, D).

#### 4.1.2. Serotonin 5HT-5A expression in amygdala in CRS trained animals

In male and female both the 5HT-5A mRNA expression increased in CRS groups as compared to their respective control groups ($p < 0.05$, $p < 0.01$) as suggested by Tukey's post hoc analysis. Two-way ANOVA analysis suggested a significant effect of sex of animal [F (1,4) = 13.16, $p < 0.05$], stress [F (1,4) = 48.49, $p < 0.01$] and interaction of sex with the stress [F (1,4) = 8.78, $p < 0.05$] on 5HT-5A expression in amygdala as compared with the control groups. However, there was no effect of sex of animal on serotonin 5HT-5A expression in amygdala (Figure 2F).

For the identification of the role of serotonin receptor on sex-based stress response, the IHC was performed. The serotonin 5HT-5A receptor expression in amygdala exhibited a significant difference in different groups under chronic immobilisation stress condition. In male and female both the groups, serotonin 5HT-5A receptor expression increased significantly in CRS group ($p < 0.05$, $p < 0.001$) as compared to their respective control groups as suggested by Tukey's post hoc analysis. Two-way ANOVA analysis shows a significant effect of sex of animal [F (1,7) = 20.58, $p < 0.01$], stress [F (1,7) = 163.9, $p < 0.0001$] and interaction of sex with the stress condition [F (1,7) = 12.28, $p < 0.01$] on serotonin 5HT-5A receptor expression in amygdala when compared with the control groups (Figure 2E, G).

#### 4.1.3. Serotonin 5HT-5B expression in amygdala in CRS trained animals

The quantitative real-time PCR results exhibited no significant change between chronic restrained stress (CRS) group as compared to the control group. In male and female both there was no change in 5HT-5B mRNA

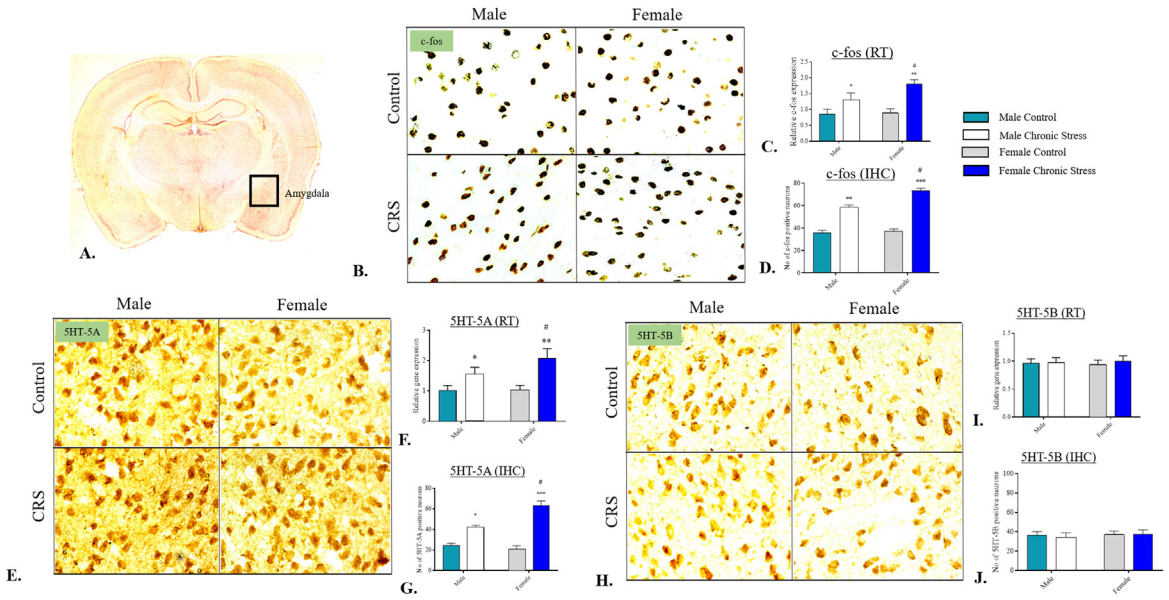


**Figure 2.** Effect of CRS on c-fos 5HT-5A and 5HT-5B expression in Amygdala. **A.** Cross sectional image of amygdala. **B.** IHC image of c-fos expression in amygdala, **C.** Relative c-fos mRNA expression in amygdala. **D.** IHC result of c-fos expression, **E.** IHC image of 5HT-5A receptor in amygdala, **F.** 5HT-5A mRNA expression in amygdala, **G.** IHC result of 5HT-5A expression in amygdala, **H.** IHC image of 5HT-5B expression in amygdala, **I.** Relative 5HT-5B mRNA expression in amygdala. **J.** IHC result of 5HT-5B expression in amygdala (* = p < 0.05, ** = p < 0.01, *** = p < 0.001, **** = p < 0.0001 compared to the control of same sex groups; # = p < 0.05, ## = p < 0.001 when compared between similar groups of different sexes).

expression in amygdala ($p > 0.05$). The result shows no significant effect of stress and sex on serotonin 5HT-5B expression in amygdala (Figure 2I). Moreover, in male and female both, the serotonin 5HT-5B receptor expression did not exhibit any significantly difference between all group ($p > 0.05$) in IHC results (Figure 2H, J).

### 4.2. Prefrontal cortex

#### 4.2.1. Altered c-fos expression in PFC in CRS trained animals

The quantitative real-time PCR results for c-fos mRNA exhibited a significant change between CRS group as compared to control groups (Figure 3A, C). In male and female both the groups, c-fos mRNA increased significantly in CRS groups as compared to the control groups ($p < 0.05$, $p < 0.01$). Female CRS group exhibited comparatively higher c-fos expression in PFC as compared to male CRS group ($p < 0.05$) as suggested by Tukey's post hoc test. Two-way ANOVA analysis exhibited a significant effect of sex of animal [F (1,4) = 17.21, $p = 0.01$], stress [F (1,4) = 88.36, $p = 0.0003$] and interaction of sex with the stress [F (1,4) = 13.85, $p = 0.02$] on c-fos mRNA expression in PFC.

The c-fos expression was further analysed through IHC in PFC of both the male and female animals where Tukey's post-hoc comparison confirmed a significant increase for c-fos expression in both male ($p <$

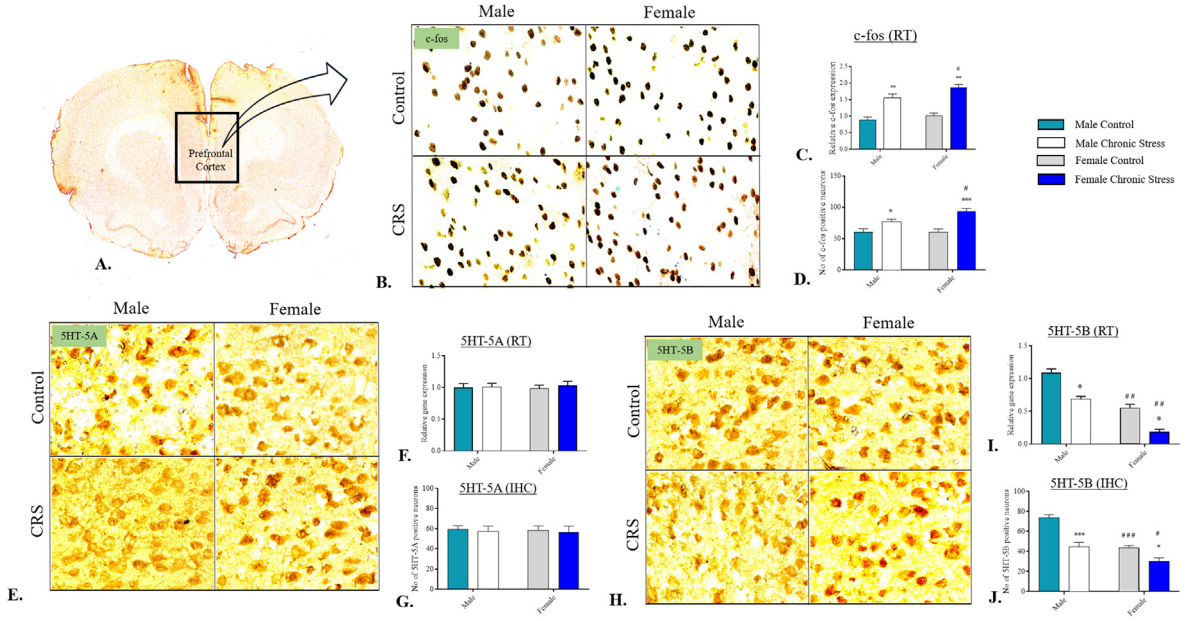


**Figure 3.** Effect of CRS on c-fos 5HT-5A and 5HT-5B expression in PFC. **A.** Cross sectional image of PFC. **B.** IHC image of c-fos expression in PFC, **C.** Relative c-fos mRNA expression in PFC. **D.** IHC result of c-fos expression, **E.** IHC image of 5HT-5A receptor in PFC, **F.** 5HT-5A mRNA expression in PFC, **G.** IHC result of 5HT-5A expression in PFC, **H.** IHC image of 5HT-5B expression in PFC, **I.** Relative 5HT-5B mRNA expression in PFC. **J.** IHC result of 5HT-5B expression in PFC (* = p < 0.05, ** = p < 0.01, *** = p < 0.001, **** = p < 0.0001 compared to the control of same sex groups; # = p < 0.05, ## = p < 0.001 when compared between similar groups of different sexes).

0.05) and female ($p < 0.001$) CRS groups as compared to the control group. However, there was a significant difference between male and female CRS group ($p < 0.05$). Two-way ANOVA analysis confirmed a significant main effect of sex of animal [F (1,5) = 21.14, $p < 0.01$], stress condition [F (1,5) = 121.5, $p < 0.0001$] and interaction of sex with stress condition [F (1,5) = 16.51, $p < 0.05$] on c-fos expression in PFC. In conclusion, c-fos as well as activity of PFC exhibited a sex-based association with the stress in PFC of male and female animals (Figure 3B, D).

#### 4.2.2. Serotonin 5HT-5A expression in PFC in CRS trained animals

The quantitative real time PCR results exhibited no significant change for serotonin 5HT-5A receptor between CRS group as compared to the control group. In male and female both the groups there was no significant effect of stress and the sex of animal on serotonin 5HT-5A mRNA expression as compared to the control groups ($p > 0.05$) in PFC (Figure 3F). Similarly, the IHC result also exhibited no significant difference in different groups under chronic immobilisation stress condition ($p > 0.05$) (Fig, 3. E, G).

#### 4.2.3. Serotonin 5HT-5B expression in PFC in CRS trained animals

The quantitative real time PCR results exhibited a significant change between chronic restrained stress (CRS) group as compared to the control group. In male and female both the groups serotonin 5HT-5B mRNA decreased significantly in CRS groups as compared to the control groups ($p < 0.05$, $p < 0.05$) as suggested by Tukey's post hoc test. Two-way ANOVA analysis showed a significant effect of stress [F (1,4) = 253.6, $p < 0.0001$] and the sex of animal [F (1,4) = 85.09, $p < 0.001$] on serotonin 5HT-5B mRNA expression in hippocampus when compared with the control groups (Figure 3I).

The IHC expression of 5HT-5B receptor in PFC exhibited a significant difference in different groups under chronic immobilisation stress condition. In male and female both, the serotonin 5HT-5B receptor expression decreased significantly in CRS group ($p < 0.001$, $p < 0.05$) as compared to the control groups as suggested by Tukey's post hoc test. Two-way ANOVA analysis showed a significant effect of sex [F (1,7) = 54.35, $p < 0.001$], stress [F (1,7) = 28.41, $p < 0.001$] and interaction of sex with the stress condition [F (1,7) = 8.75, $p < 0.05$] on serotonin 5HT-5B receptor expression in PFC when compared with the control groups (Figure 3H, J).

### 4.3. Hippocampus

#### 4.3.1. C-fos expression in hippocampus in CRS trained animals

In hippocampus the quantitative real-time PCR result exhibited a significant change in c-fos mRNA expression between CRS group as compared to the control group (Figure 4A). In male and female both, the c-fos mRNA increased significantly in CRS groups as compared to the control groups ($p < 0.01$, $p < 0.001$). However, the change was significantly higher in female CRS groups as compared to the male CRS group ($p < 0.01$) as suggested by Tukey's post hoc test. Two-way ANOVA analysis suggested a significant effect of stress [F (1,4) = 146.8, $p = 0.0003$] and interaction of sex with the stress [F (1,4) = 48.03, $p = 0.0023$] for c-fos mRNA expression in hippocampus when compared with the control groups (Figure 4C).

The c-fos expression was further analysed through IHC in hippocampus. Tukey's post-hoc comparison confirmed a significant increase for c-fos expression in both male ($p < 0.05$) and female CRS groups ($p < 0.001$) as compared to the control group. However, there was a significant difference between male and female CRS group ($p < 0.01$). Two-way ANOVA analysis confirmed a significant main effect of sex [F (1,5) = 8.01, $p < 0.05$], stress condition [F (1,5) = 54.92, $p < 0.001$] and interaction of sex with stress condition [F (1,5) = 22.22, $p < 0.01$] on c-fos expression in hippocampus. In conclusion, the c-fos exhibited a sex-based association with the stress in male and female hippocampus (Figure 4B, 4D).

#### 4.3.2. Serotonin 5HT-5A expression in hippocampus in CRS trained animals

The quantitative real time PCR results exhibited a significant change between CRS group as compared to the control group. In male group the serotonin 5HT-5A mRNA decreased significantly in CRS groups as compared to the control groups ($p < 0.05$) as suggested by Tukey's post hoc test. However, there was no significant effect of stress in female groups. Two-way ANOVA analysis showed a significant effect of stress [F (1,4) = 47.65, $p < 0.01$] and the sex of animal [F (1,4) = 27.05, $p < 0.01$] on serotonin 5HT-5A mRNA expression in hippocampus when compared with the control groups (Figure 4F).

The 5HT-5A expression in hippocampus exhibited a significant difference in IHC where in male animals, the serotonin 5HT-5A receptor expression decreased significantly in CRS group ($p < 0.01$) as compared

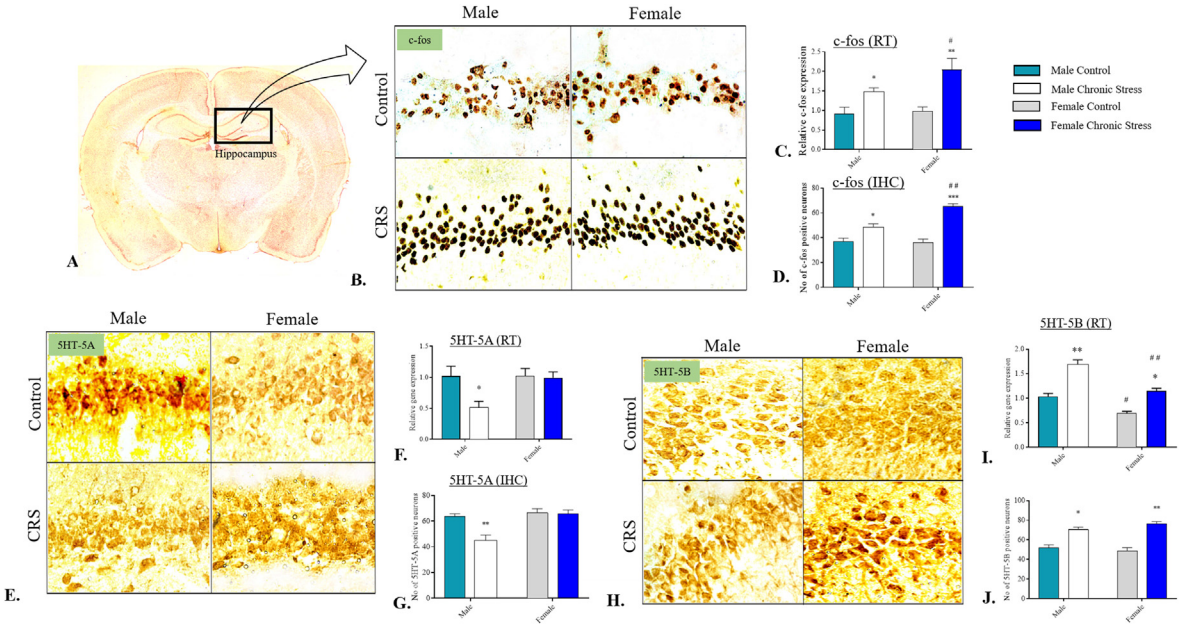


**Figure 4.** Effect of CRS on c-fos 5HT-5A and 5HT-5B expression in Hippocampus. **A.** Cross sectional image of Hippocampus. **B.** IHC image of c-fos expression in Hippocampus, **C.** Relative c-fos mRNA expression in Hippocampus. **D.** IHC result of c-fos expression, **E.** IHC image of 5HT-5A receptor in Hippocampus, **F.** 5HT-5A mRNA expression in Hippocampus, **G.** IHC result of 5HT-5A expression in Hippocampus, **H.** IHC image of 5HT-5B expression in Hippocampus, **I.** Relative 5HT-5B mRNA expression in Hippocampus. **J.** IHC result of 5HT-5B expression in Hippocampus (* = p < 0.05, ** = p < 0.01, *** = p < 0.001, **** = p < 0.0001 compared to the control of same sex groups; # = p < 0.05, ## = p < 0.001 when compared between similar groups of different sexes).

to the control groups as suggested by Tukey's post hoc test. However, female CRS group exhibited no change as compared to the control group. The Two-way ANOVA analysis showed a significant effect of sex of animal [F (1,7) = 7.28, $p < 0.05$], stress [F (1,7) = 10.7, $p < 0.05$] and interaction of sex with the stress [F (1,7) = 32.48, $p < 0.001$] on serotonin 5HT-5A receptor expression in hippocampus when compared with the control groups (Figure 4E, G).

#### 4.3.3. Serotonin 5HT-5B expression in hippocampus in CRS trained animals

In male and female both the group serotonin 5HT-5B mRNA increased significantly in CRS groups as compared to the control groups ($p < 0.01, p < 0.05$) as suggested by Tukey's post hoc test. However, the change was more significant in male groups than the female CRS groups. The Two-way ANOVA analysis showed a significant effect of stress [F (1,4) = 427.8, $p < 0.0001$] and sex [F (1,4) = 39.36, $p < 0.01$] on serotonin 5HT-5A mRNA expression in hippocampus when compared with the control groups (Figure 4I).

The 5HT-5B expression in hippocampus exhibited a significant difference in different groups under chronic immobilisation stress condition in IHC results. In male and female both, the serotonin 5HT-5B receptor expression increased significantly in CRS group ($p < 0.05, p < 0.01$) as compared to the control groups as suggested by Tukey's post hoc test. Two-way ANOVA analysis showed a significant effect of stress [F (1,7) = 45.55, $p < 0.001$] on serotonin 5HT-5B receptor expression in hippocampus when compared with the control groups (Figure 4H, J).

## 5. Discussion

Current work is analysing the role of 5HT-5 receptors subtypes (5A and 5B) in sex-dependent regulation of stress in male and female rats. As suggested by the present study, female rats exhibited a higher stress response and a significantly reduced mean body weight as compared to the male rats, which was also correlated with the change in serotonin 5HT-5A and 5B receptor expression. Behaviour result obtained from the study suggested a sex-based difference in stress response between male and female animals, which is colinear with the previous studies where a higher stress and anxiety was observed in female animals (Verma et al., 2011). The stress caused an alteration in physiological function as exhibited by high corticosterone level in these stressed animals. However, a significantly higher corticosterone level in female stress groups suggest a difference in physiological response between both the sexes, causing a higher stress in females.

Based on our hypothesis we further questioned, what brain parts is/are actually associated with this sex dependent stress and anxiety difference under CRS condition. We analysed amygdala, PFC and hippocampus brain regions reported to be associated with such stressful condition (Rajmohan and Mohandas, 2007). The activity of these brain regions was confirmed by the analysis of immediate early gene c-fos expression. Our study suggested a significantly high activity for these brain regions under stressful condition in both the male and female animals. This is further supported by the studies suggesting a higher activity of these brain regions under different chronic stress related paradigms (McEwen et al., 2016; Arnsten, 2009; Zhang et al., 2018). However, this activation was significantly higher in female group as compared to the male CRS group. Besides this, only few of these studies analysed association of such enhanced brain activity with the higher stress in female animals under stressful condition (Westenbroek et al., 2003). In our result, a higher activity of hippocampus, amygdala and PFC brain regions in female rats suggested a difference in activity of fear circuitry between both the sexes under same stressful event. This variance might be due to the sex-based difference between male and female brains, creating different type of stress response. We further analysed the role of serotonin 5HT-5A and 5B receptors function in these brain regions under stressful condition.

The 5HT-5A and 5B receptors when analysed, exhibited a significant alteration of different brain regions under CRS condition in both the sexes. The result suggested an associated and related function for both the receptors under CRS condition which also exhibited a sex-dependent spatial difference in brain regions. The hippocampus, which is one of the most important brain regions in management of stress, exhibited a higher 5HT-5B expression in females as compared to the male animals. This suggests a difference in activity of stress circuitry between both the sexes which is regulated by a 5HT-5B serotonin receptor subtype. An increased activity (i.e., c-fos expression) in hippocampus under CRS condition in both the sexes suggest an inhibitory regulatory network within hippocampus (Figure 5). Thus, the function of 5HT-5B receptor in

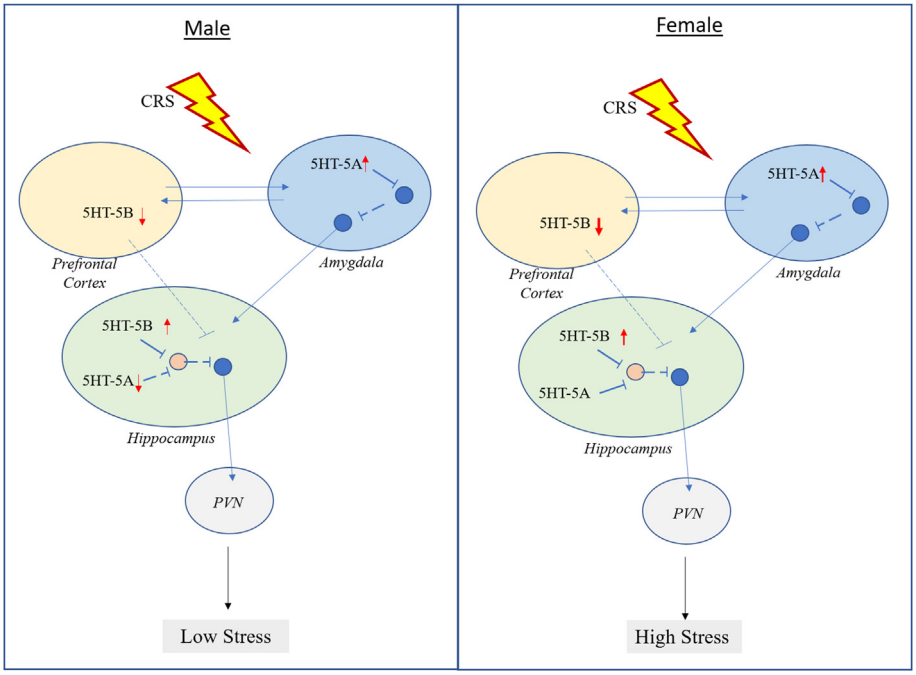


**Figure 5.** Diagrammatic representation of proposed stress circuitry between male and female rat brain involving 5HT-5A and 5B serotonin receptor system. The strength of arrow represents the strength of connection between brain regions. Solid line with arrow represents activated neuronal connection while dashed line with arrow represent inhibited neuronal connection. Inhibitory neuronal innervations are represented by bar headed lines (T).

hippocampus is supposed to disinhibit inhibitory network within hippocampus to promote stress condition. And because of comparatively higher 5HT-5B expression in female hippocampus this disinhibition might be higher as compared to male rats. Besides this, another important regulatory mechanism within hippocampus might be through 5HT-5A expression, which also has an inhibitory function. Although, the 5HT-5A expression decreased significantly in male hippocampus, it was unaltered in females. This suggests comparatively less disinhibition activity on hippocampal inhibitory neurons, thus allowing lower activation of stress pathway (i.e., PVN nucleus) under CRS condition in male rats. This may result in a lower PVN activity as well as stress in males CRS group animals. Contrary to this, no change in female 5HT-5A expression in hippocampus suggests a moderate disinhibitory function, thus a comparatively higher activity of hippocampus-PVN pathway resulted in higher stress response in females.

In PFC, male and female both the groups exhibited a reduced 5HT-5B expression, while female CRS group showed more significant change. When compared the activity of PFC using c-fos expression under CRS condition, female group showed a higher activity than the male group. This PFC activity was negatively correlated with the 5HT-5B expression in both the sexes. Thus, creating a higher stress state in females through lower 5HT-5B expression and thus higher activation of PFC under CRS condition. In contrast, the 5HT-5A expression in PFC did not exhibit any significant change in both the sexes suggesting no spatial function of 5HT-5A.

In amygdala, 5HT-5A receptor exhibited as main receptor for the regulation of stress in both the male and female groups. The 5HT-5A receptor expression increased significantly in both the sexes but was comparatively higher in female CRS groups. The activity of amygdala was also higher in female groups, suggesting a sex-dependent activity of amygdala in the stress circuitry of brain. However, the 5HT-5B receptor expression in amygdala exhibited no significant change between both the sexes, suggesting the role of only 5HT-5A subtype in management of stress in both the sexes.

Overall, the study suggests here a sex-based association of serotonin 5HT-5A and 5B receptor with the difference in stress response between male and female animals. The result also proposes a region-specific spatial association of brain activity as well as serotonin receptor subtype function under CRS condition in both the male and female animals. This activity for both the receptor subtype is supposed to complement each other in governing stress response and suggests a sex-based difference in stress circuitry. Future studies involving spatial and conditional knockout animal models for these serotonin receptor subtypes may be helpful for detail information about the role of these serotonin receptors in management of stress disorders.

## Declarations

### *Author contribution statement*

Sarfraj Ahmad Siddiqui: Conceived and designed the experiments; Performed the Experiment; Analysed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Sanjay Singh, Atul Rawat: Performed the Experiment.

Md Arshad: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Sudhir Kumar: Contributed reagents, materials, analysis tools or data.

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### *Data availability statement*

Data included in article/supp. material/referenced in article.

### *Declaration of interest's statement*

The authors declare no conflict of interest.

### *Additional information*

No additional information is available for this paper.

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