Article

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Chrysin Alleviates Maternal Immune Activation-Induced Autistic-Like Behavior by Altering *IL-17A* Levels in Fetal and Offspring Brains of C57BL/6J Mice

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Background: Immune dysregulation is one of the hypotheses brought up to explain autism spectrum disorder (ASD). Interleukine-17A (*IL-17A*), a proinflammatory cytokine, has been demonstrated to be a major mediator of immune-related neurodevelopmental impairment of social behavior, including ASD. Chrysin (CHN) is a naturally occurring hydroxylated flavonoid with antioxidant, anti-inflammatory, anti-asthmatic, anticancer, cardioprotective, and neuroprotective activities. The current study investigated the effects of CHN against Polyinosinic:polycytidylic acid (Poly (I:C))-induced autism-like behavior by modulating the fetal serotonin and *IL-17A* levels in fetal and offspring brains in C57BL/6J mice.

Methods: Pregnant C57BL/6J mice (n = 6) were randomly selected. After the confirmation of pregnancy, female mice were divided into two different experimental groups (n = 3 female/group = 4–8 littermates/group). The pups were randomly divided into 5 experimental groups, namely, control (group I), Poly (I:C) (group II), CHN25 (group III) & CHN50 (group IV), and fluoxetine (group V). Group I and II pregnant mice were pre-treated orally with saline for 12 consecutive days (Estrus Day 0.5 (E0.5) to E12.5) and then challenged with saline (group I) and Poly (I:C) [20 mg/kg Body Weight (BW)] (group II) intraperitoneally on the 12th day (E12.5). Group III, IV & V pregnant mice were administered orally with CHN (25 mg/kg & 50 mg/kg BW) and fluoxetine (10 mg/kg, BW), respectively, for 12 consecutive days (E0.5 to E12.5) and then challenged with Poly (I:C) (20 mg/kg BW) intraperitoneally on 12th day (E12.5). In one set of studies, 1 pregnant mouse from each group was sacrificed after 4 h of Poly (I:C) injection to measure the fetal 5-Hydroxytrptophane (*5-HT*) and *IL-17A* levels in fetal brains using enzyme-linked immunosorbent assay (ELISA) kits. In the second set of experiments, the remaining pregnant mice were allowed to deliver the pups. Offspring were subjected to different behavior tests, including marble burying test (MBT), rotarod test, social interaction test (SIT) and sucrose preference test (SPT) at the age of 6, 7 and 12 weeks to investigate the autistic-like behaviors and associated symptoms. Following behavioral studies, the mice were sacrificed to isolate the prefrontal cortex (PFC), hippocampus (HC) and amygdala (AMG) tissues to measure the *IL-17A* levels through an ELISA kit.

Results: The findings of the present study demonstrated that Poly (I:C) administration to pregnant mice resulted in maternal immune activation (MIA), as evidenced by the significant increase in *IL-17A* ($p < 0.05$) and decrease in *5-HT* levels ($p < 0.001$) in fetal brains. Pre-treatment of CHN and fluoxetine altered the fetal *5-HT* and *IL-17A* levels significantly ($p < 0.001$). Offspring of Poly (I:C) injected pregnant mice showed autistic-like behaviors and associated symptoms as evidenced by an increased number of marbles buried in MBT and decreased fall in time in the Rotarod test, sucrose preference in SPT, and social preference in SIT significantly ($p < 0.001$) which were ameliorated by the chronic pre-treatment of CHN (both the dosages i.e., 25 & 50 mg) and fluoxetine significantly ($p < 0.001$). Further, results showed the significant elevation of *IL-17A* levels in PFC ($p < 0.001$), HC ($p < 0.001$) and AMG ($p < 0.05$) of offspring of Poly (I:C) injected pregnant mice, which were attenuated significantly by the chronic pre-treatment of CHN (both the dosage i.e., 25 & 50 mg) and fluoxetine ($p < 0.001$).

Conclusion: Findings of the study demonstrated that chronic pre-treatment of CHN attenuated autistic-like behavior by altering fetal *5-HT* and *IL-17A* in fetal brain, PFC, HC, and AMG of offspring of MIA pregnant C57BL/6J mice. However, further investigation is required to establish the therapeutic applicability of CHN in ASD.

Keywords: autism spectrum disorder; chrysin; *IL-17A*; maternal immune activation; Poly (I:C)

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Introduction

Autism spectrum disorder (ASD) is a neurodevelopmental disorder characterized by social impairments, communication issues, and restricted and repetitive behaviors [1]. Restricted and repetitive patterns of behavior, interests, or hobbies and chronic impairment in reciprocal social communication and social interaction are necessary diagnostic criteria [2]. It is estimated that the occurrence rate of ASD in the USA and India is 1.5% and ~0.5%, respectively [3]. The occurrence of ASD in Western nations increased by almost 150% between 2000 and 2014 in the average age of 8 years old children, posing a public health concern in North America [4,5]. Thus, a previous study showed that 8-year-old children had the highest prevalence rate of ASD [5]. Further, studies from North America, Asia, and Europe indicate a 1–2% prevalence of ASD [4,5]. Research suggests that prenatal brain inflammation may have a role in the development of neuropsychiatric disorders, including ASD. Further, ASD aetiology includes immunological and autoimmune components [6,7]. The pathophysiology of ASD may be partially explained by interactions between immune cells that cause inflammation and altered cytokine production in individuals with ASD. Additionally, the growing body of research on neuro-immune crosstalk may eventually shed light on some of the mechanisms underlying aberrant brain development and signalling [6]. Scientific literature has long acknowledged a connection between ASD and some autoimmune illnesses. Furthermore, the potential contribution of maternal autoantibodies to the pathophysiology of ASD has been highlighted, as they may impact neurodevelopment by entering the developing fetal brain, thus, maternal immunological activation also appears to be a major risk factor for ASD [7]. Previous research on ASD causes has identified a number of compounds that are important for both genetic and environmental aspects of ASD [8]. Twins having ASD are often associated with genetic components; one such study revealed that monozygotic twins' concordance rates for ASDs were higher than those of dizygotic twins [9]. Recent reports have indicated that the main causative factors include both genetic and environmental factors, provided that environmental factors account for at least 50% of ASD risk [2,6]. The growth in the prevalence of autism spectrum disorder (ASD) coincides with increased accessibility to audiovisual (AV) materials and infants' watching habits, which is an environmental factor [10]. There are numerous modifiable factors at both prenatal and early postnatal ages in different brain areas for ASD. Prenatal exposure to environmental pollution, including air pollution and certain pesticides, has been linked to an increased incidence of ASD. It has also been recognized as a modifiable risk factor affecting mother's health throughout pregnancy [2].

Auto-antibodies against embryonic brain proteins have been detected in mothers of children with ASD. Several inflammatory compounds have been shown the elevated levels in brain and cerebrospinal fluid (CSF) of several ASD-suffering patients, including Interleukine-1β (*IL-1*β), IL-6, and Tumor Necrosis Factor (TNF)-α and correlated with regression, as well as impaired communication and aberrant behaviour. Moreover, elevated levels of Interleukine-17A (*IL-17A*) were also seen in the serum of children diagnosed with ASD. Peripheral blood immune cells exhibit heightened *IL-17A* synthesis after mitogen stimulation, and this increase in *IL-17A* was further observed in children with ASD [11]. The deformities observed in children, adolescents, and adults with ASD have similar defects in brain development. Recent research has shown that T-Helper (TH) 17 cells play a crucial role in the progression of ASD by releasing *IL-17A*. The notion is sustained by research findings that indicate a high concentration of *IL-17A*/H17 cells in the bloodstream of individuals with autism as well as in mice exhibiting symptoms like those of autism [12]. In recent years, there has been a growing body of research suggesting a robust inflammatory condition that is linked to ASD. This inflammatory disorder is frequently associated with a malfunction of the immune system. The investigation of pro-inflammatory biomarkers has provided evidence of inflammatory activity in children with ASD. Interleukins are members of the cytokine family that serve as signalling proteins that play a crucial role in immune regulation and responses. The proteins are considered to be key indicators for the investigation of inflammatory conditions. A study revealed that pro-inflammatory cytokines were significantly increased in the plasma of 97 children (22–5 years old) with ASD who were not taking any medication and were in good health [13]. These children were selected from a population-based case management study of genetic and environmental factors contributing to the risk of childhood autism. When peripheral blood monocytes from children with ASD were cultured and stimulated *in vitro*, an excess of pro-inflammatory cytokines was observed [13]. The presence of shared behavior and molecular markers in concomitant diseases such as psychiatric and gastrointestinal problems might complicate the diagnosis process for ASD due to the shared symptoms and neurochemistry observed in these disorders. However, the specific mechanisms that lead to the coexistence of these conditions have not been fully understood. The involvement of 5-Hydroxytrptophane (*5-HT*) in ASD is extensively supported by discoveries that the hallmark behavioral signs of autism, social behavior impairments, and restricted repetitive behaviors are controlled by *5-HT* signalling [6]. Serotonin (*5-HT*) is produced through the enzymatic activity of Tryptophan hydroxylase 1 (TPh1) in the periphery and pineal gland and by TPh2 in the central nervous system (CNS), using L-Tryptophan (TRP) as its precursor. Neuronal activity increases the release of *5-HT* from vesi-

cles within *5-HT* neurons into synapses and the extracellular environment, where *5-HT* can bind to *5-HT* hetero-or-receptors [14].

Maternal immune activation (MIA) during pregnancy can lead to a significant immunological response that causes an increase in inflammatory markers. Numerous factors, such as allergies, autoimmune diseases, oxidative stress, and infections, leads to MIA. Preclinical studies have established connections between unfavourable outcomes for mothers and foetuses and changes in peripheral inflammatory markers caused by MIA [1]. Prenatal immunological exposure and altered fetal brain development may be linked to the maternal immune response, as indicated by the variety of infection agents linked to an increased risk of CNS disease. Animal models have been used to test the MIA hypothesis, using a range of immunogens to stimulate the immune system during pregnancy, which causes abnormalities in the brain and behavior development of the offspring, paralleling aspects of human CNS diseases. Here, we primarily concentrate on MIA models that use Polyinosinic:polycytidylic acid (Poly (I:C)), double-stranded RNA molecules that activate the toll-like receptor-3 (TLR-3) to trigger an immunological response [15]. The Poly (I:C) model has been used by several labs in the last ten years to activate and initiate the mother's immune response in a regulated and time-limited way [16]. Poly (I:C) administration also leads to elevated *IL-17A* Messenger Ribonucleic Acid (mRNA) levels in the tissue of these animals. It was also shown that a prolonged increase in *IL-17A* expression appeared to be detrimental in ASD since *IL-17A* blocking inhibited the development of ASD-like phenotypes. These findings were made through *IL-6* and *IL-17A* signalling inhibitions utilising antibody blocking of the *IL-17A* cytokines. The offspring of mice previously exposed to MIA showed normal behaviors after blocking the *IL-17A*, thus, it might be main diagnostic features of ASD [17].

It has been demonstrated that the toll-like receptor-3 (TLR-3) agonist Poly (I:C) can cause inflammatory reactions akin to those caused by a systemic viral infection [18]. It has been documented that a systemic injection of Poly (I:C) can cause an inflammatory reaction in addition to multiple signs of illness behavior [16]. Poly (I:C) stimulates the immune system by imitating an infection pathogen. More precisely, Poly (I:C) is used to stimulate the acute stages of a viral infection, induce the release of proinflammatory cytokines, and boost the innate immune system [19].

Currently, there are no drugs available to address the fundamental issues linked to ASD. Instead, pharmacological therapies are limited to treating problematic behavior that is not responsive to behavior therapy, as well as co-occurring illnesses like anxiety and sleep disorders [20]. A significant percentage of teenagers with autism are prescribed psychotropic medication, especially when they display disruptive behavior and co-occurring physical and mental health issues [21]. When treating repetitive and stereotypical behavior, doctors have utilized selective serotonin reuptake inhibitors, second-generation antipsychotics, and mood stabilizers like valproate. Naltrexone may help children and adolescents with ASD by reducing impulsivity and hyperactivity; however, this medicine did not seem to help with core symptoms of ASD [22].

Chrysin (CHN) is obtained from various plant extracts, such as propolis, honey, and blue passion flower (*Passiflora caerulea*), having significant therapeutic and commercial uses. Propolis is another name for bee glue, which is typically given to the material that honey bees (*Apis mellifera* L.) extract from different plants [23]. Furthermore, research on various cellular levels in humans and animals has demonstrated that CHN possesses immunoregulatory, chemoprotective, antioxidant, and anti-inflammatory qualities [24]. The latest evidence proposed that CHN can protect neurons from oxidative stress and pro-inflammatory cytokines. Alleviating effects of CHN in cognitive deficits and brain damage injected by chronic cerebral hypoperfusion in rodents have also been demonstrated [25]. The study showed that CHN alleviated the Poly (I:C)-induced elevation of pro-inflammatory cytokines (TNF-α, Interferon (IFN)-γ, IL-1β, and IL-6) and indolamine-2,3-dioxygenase (IDO), and increased the *5-HT* level in HP patients [26]. Further, previous study demonstrated the neuroprotective effects of CHN in various neurological disorders, especially epilepsy, anxiety, and depression [27]. Earlier studies demonstrated that the pre-treatment of CHN reduces the *IL-17A* level during inflammation and also shows anti-depressant activity by enhancing the *5-HT* level [28–30]. Based on the anti-inflammatory and neuroprotective potential, we investigated the effect of CHN against maternal immune activation (MIA)-induced autistics-like behavior in C57BL/6J mice.

Materials and Methods

Chemicals

Poly (I:C) (CAS no. 42424-50-0, Lot no. 019M4060V, Sigma Aldrich, St. Louis, MO, USA), chrysin (CAS no. 480-40-0, B.no. STBH6761, Sigma Aldrich, St. Louis, MO, USA), fluoxetine (CAS no. 56296-78-7, Lot no. DBY2H-QE, Sigma Aldrich, St. Louis, MO, USA) and Protease inhibitor cocktail (Catalogue no. P8340-1ml, Sigma Aldrich, St. Louis, MO, USA) were purchased. Sodium Hydroxide (CAS No. 1310-73-2, B.no. PF123HF01, QualiChem's Fine Chem Pvt. Ltd., Vadodara, India), Tris-Buffer (CAS no,77-86-1, Lot no. 0000379979, HiMedia Pvt. Ltd., Mumbai, India), and Propylene glycol (CAS no. 57-55-6, B.no. L300051905, Loba Chemie Pvt. Ltd., Mumbai, India) was purchased. 5-HT (Lot no. 5HT1119, KRISHGEN Biosystems, Mumbai, India) and *IL-17A* (Catalog No: E-EL-M0047, Lot no. UX14XZ262346, Elabscience, Houston, TX, USA)

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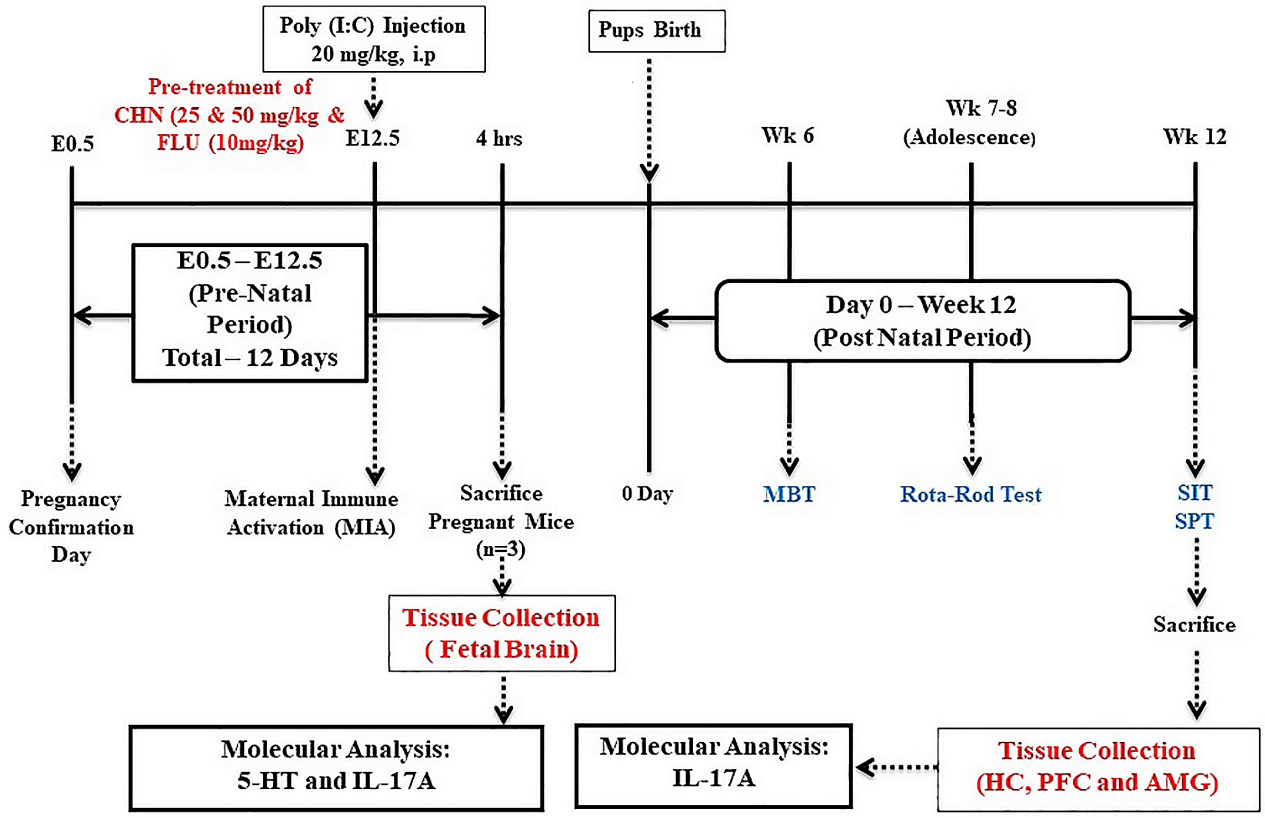

Fig. 1. Diagrammatic representation of the experimental design. E0.5, Estrus Day 0.5; Poly (I:C), Polyinosinic:polycytidylic acid; MIA, maternal immune activation; Wk, Week; MBT, marble burying test; SPT, sucrose preference test; SIT, social interaction test; HC, hippocampus; PFC, prefrontal cortex; AMG, amygdala; IL, Interleukin; *5-HT*, 5-Hydroxytrptophane; CHN, chrysin; FLU, fluoxetine.

enzyme-linked immunosorbent assay (ELISA) kits were also purchased. All other used chemicals were of analytical grade and purchased from reputed companies.

Animals

6-week-old male or female mice of strain black 6 (C57BL/6j) were used for the experimental activity. Mice were obtained from the National Institution of Nutrition, Indian Council of Medical Research, Hyderabad, India (NIN, ICMR). After arrival, animals have undergone their first 2-week acclimatization in a polypropylene cage with a 12:12 h light and dark cycle. Food and bedding materials for mice were purchased from Kevel Sales Corporation, Ahmedabad, Vadodara, Gujrat, India. Water and food were provided *ad libitum*. All experiments and procedures were performed at Central Animal House Facility (CAHF) of Indira Gandhi National Tribal University, Amarkantak, Madhya Pradesh, India (Reg. No. 2004/GO/ReBi/S/18/CPCSEA) as per the guidelines of the Committee for Control and Supervision of Experiments on Animals (CCSEA), Government of India, New Delhi after duly approved by the Institutional Animal Ethical Committee (IAEC), Indira Gandhi National Tribal University, Amarkantak, India (IGNTU/IAEC/2021/34).

Experimental Design

Based on the results of an earlier experimental study [31,32], the doses of CHN (25 and 50 mg/kg) were chosen. Chrysin was freshly prepared by dissolving in 6.5 mL of fresh Phosphate Buffer Saline (PBS) solution containing 3% (V/V) Sodium Hydroxide (NaOH) (0.01M) as a vehicle [33,34]. Two doses of chrysin, 25 and 50 mg/kg, were administered according to the mice's Body Weight. However, Poly (I:C) was freshly dissolved in saline and administered intravenously (i.p.) at 20 mg/kg based on the weight of the Poly (I:C) itself, excluding the total weight of the potassium salts [35]. The study was designed to investigate the effect of CHN (pre-treatment) at both the dosages against Poly (I:C)-induced autistic-like behaviour and neurochemical changes in the developing fetus, and different brain regions of adult offspring at a different time interval. The females were observed twice a day for the presence of the vaginal plug following mating (three females and one male). After the confirmation of pregnancy, female mice were divided into two different experimental groups (n = 3 female/group = 4–8 littermates/group) [Fig. 1]. Group I: Pre-treatment of normal saline (E0.5 to E12.5 days orally) in pregnant mice and challenged with saline (E12.5, i.p.); termed the control group. Group II: Pre-treatment of nor-

mal saline (E0.5 to E12.5 orally) to pregnant mice and challenged with Poly (I:C) (E12.5, i.p.); termed Poly (I:C) group. Group III & IV: Pre-treatment, 25 & 50 mg/kg of CHN, E0.5 to E12.5 days orally) to pregnant mice and challenged with Poly (I:C) (E12.5, i.p). Group V: Pre-treatment (10 mg/kg of fluoxetine Per Oral (p.o.), E0.5 to E12.5 days orally) to pregnant mice and challenged with Poly (I:C) (E12.5, i.p.). One set of pregnant animals was sacrificed by cervical dislocation after 4 hours of Poly (I:C) injection, and fetal brains were dissected out under the ice with the help of a Stereomicroscope (Quasmo Microscopes & Optics, Mumbai, India) to measure *5-HT* and *IL-17A* levels in fetal brain of pregnant mice. In the second set of animals, pregnant mice were cared for and waited until the delivery of offspring. Different behavioral studies, including the marble burying test (MBT), Rota-Rod test, social interaction test (SIT), and sucrose preference test (SPT), were performed at 6, 7–8, and 12 weeks of age. After behavioral studies, animals were sacrificed by cervical dislocation, and offspring brain tissue [prefrontal cortex (PFC), hippocampus (HC), and amygdala (AMG)] were collected to measure the *IL-17A* levels.

Induction of Maternal Immune Activation (MIA)

Adult female C57BL/6J mice weighing 22 and 30 grams were used. Adult female mice were mated overnight and used to conduct animal testing. Embryonic day 0.5 (E0.5) is the day when pregnancy is verified. MIA was induced in the pregnant mice by administering Poly (I:C). MIA was induced in the pregnant mice by administering Poly (I:C) (20 mg/kg, i.p.) on E12.5. Gestation age E12.5 in mice is thought to be the late first trimester in humans, during which significant brain growth and neurogenesis in the cortical layer occur, leading to an increased prevalence of depression and autism spectrum disorders due to infection. Pups remained with the mother until weaning on a Post Natal Day (PND), 21, i.e., the third week, at which point mice were housed in groups until the day of juvenile period completion and then battery of behavioral tests were started after the completion of fifth week [33,34].

Preparations of Brain Tissue Homogenization

Animals were sacrificed by cervical dislocation after the completion of all behavioral studies, the brain tissues of different parts (PFC, HC and AMG) were quickly isolated on ice and stored at –80 °C until further use. Tissues were homogenized in buffer [50 mM Tris, 10 mM Ethylenediaminetetraacetic acid (EDTA), pH 7.4] containing protease inhibitor cocktail using a small plastic pestle and eppendorf tube through trituration method. Then, tissues were homogenized through the trituration method. The resultant homogenates were incubated for 20 minutes in a cold room on a rotator and centrifuged for 15 minutes at 7000 ×g at 4 °C (NEYA 16R, Remi, Mumbai, India). The supernatant was collected in a fresh and clean centrifuged tube and stored at –80 °C until further use for molecular analysis. Protein estimation was performed using the Bradford Kit (Product code- ML178-1PK, MolBio HiMedia laboratories Pvt. Ltd. Mumbai, Maharashtra, India) with Bovine Serum Albumin (BSA) as a standard [35].

Behavioral Analysis

Marble Burying Test (MBT)

Mice offspring from each group underwent the marble burying test. This test is used to analyse autistic-like behavior in mice. The marble was cleaned using 70% ethanol and placed in an autoclaved (38.5 × 30 cm) mouse polypropylene cage with 3.5 cm of corn cob bedding (Kevel Sales Corporation, Vadodara, Gujrat, India) height. The mouse was given thirty minutes to acclimatize to the lab before the experiment began. Before testing, the bedding was combined, and the marble was put in a 5 × 4 configuration. The mouse spent thirty minutes in the experimental polypropylene cage separately. When the experiment was over, the amount of marble buried was measured. If two to three percent of it was covered in bedding, the marble was also considered for burial. The whole process was video recorded (HDR-PJ675, SONY, Meerut, India), and the number of marbles was counted and compared [36].

Rota Rod Test

The rota-rod test was used to assess motor coordination in seventh-week-old experimental mice. The three trials of the rota-rod (Model no. RR01, Orchid Scientific & Innovative India Pvt. Ltd., Nashik, India) test were conducted daily at intervals of ten minutes. The experimental mice were acclimatized for thirty minutes before the experiment began. The rota-rod testing device's rotor is rough, and the equipment has five compartments. The rotor was cleaned with 70% ethanol before the experiment. Before the main studies, the mice were housed in a rota-rod chamber with continuous speed and time (4 r.p.m. over 180 s on days 1 and 2; after that, on days 3 and 4, 8 r.p.m. over 180 s). The rotation of the rota-rod speed increased steadily over 300 s intervals from the specific r.p.m. 4–40 throughout the first two testing days. Similar to this, the rotation was raised from the specific r.p.m. 8–79 throughout the periods of 300 s in the second phase of the final testing (days 3 & 4). The fall-off time (in seconds) of each mice offspring was recorded and analyzed [35].

Social Interaction Test (SIT)

The three-chambered apparatus was used for a social interaction test to assess the social preference of 12-week-old offspring adult mice. Mice were placed in the center room and given 10 minutes to explore all three compartments, having two empty boxes in two chambers. The novel (non-familiar) mice were placed in a small box in one chamber, and now again, the experimental mice were placed in a chamber containing the novel mouse for 10 minutes for

Journal of
BIOLOGICAL REGULATORS
and Homeostatic Agents

social interaction. The time spent by mice in each chamber was video recorded (HDR-PJ675, SONY, Meerut, India) and analysed by blind observers. The social preference (in %) was calculated from the time spent with a novel mouse minus time spent with a familiar mouse divided by total exploration time multiplied by 100 [37].

Sucrose Preference Test (SPT)

The sucrose preference test was conducted as per the method of Savalli *et al*. (2015) [38]. In short, mice were fasted for eighteen hours before undergoing a three-hour choice test using two bottles of normal drinking water and a 2% sucrose solution (IK164HD01, QualiChem's, Mumbai, India) The percentage of sucrose preference was computed by evaluating the consumption of sucrose solution by the total amount of liquid consumed [39].

Estimation of 5-HT and IL-17A Level

The *5-HT* level was measured in fetal brain tissues using an ELISA kit (Catalogue no. K12-1316, KRISHGEN Biosystems, Mumbai, India). The *IL-17A* levels were measured in the fetal brain and PFC, HC, and AMG in the offspring brain using an ELISA kit (Catalogue no. E-EL-M0047, Elabscience Biotechnology Inc., Houston, TX, USA) according to the manufacturer's protocol. The 100 µL sample was used to measure the *5-HT* and *IL-17A* levels. All the samples and standards were performed in triplicate, and the readings at 450 nm were obtained using an ELISA Plate Reader (Multiskan SkyHigh Microplate Spectrophotometer, A51119600DPC, Thermo Fisher Scientific India Pvt Ltd, Mumbai, India). The sample values were then obtained by comparing them to the standard curve, and values are expressed in pg/mL.

Statistical Analysis

The data of behavioral and molecular studies were expressed as mean ± standard error mean (SEM) (n = 3–6) and analysed for within-subject effects using repeated measures analysis of variance (ANOVA). Additional statistical approaches were employed, including a one- or two-way ANOVA. A one-way ANOVA was used to illustrate the significance of CHN from the Poly (I:C) group while two-way ANOVA was used to analysis the data of *IL-17A* in different brain areas, and Dunnett's multiple comparison tests ($p < 0.05$) were then performed. The data was examined using GraphPad Prism Statistical Software Version 9.0 (Graph Pad Software, Boston, MA, USA). p-value < 0.05 was considered as a level of significance.

Results

Assessment of Neurobehavioral Functions

Effects of CHN on Poly (I:C) Induced Changes in Offspring Behaviors

When the MIA offspring of Poly (I:C)-treated mice were compared to all other groups in the marble burying assay, the repetitive/compulsive type behavior was observed. Compared to the control group, MIA offspring of Poly (I:C)-treated mice had exceptionally high levels of repetitive behavior in the marble burying test (MBT) which was attenuated significantly ($p < 0.001$) by the pretreatment of both the dosages of CHN (Fig. 2A). Fig. 2B shows that the motor coordination problems induced by Poly (I:C) were corrected by the pre-treatment of CHN at both dosages (25 & 50 mg/kg) when tested in rotarod ($p < 0.001$). Consequently, the administration of Poly (I:C) was associated with decreased time to fall and increased pro-inflammatory cytokine levels in mice compared to control mice. There was a noteworthy reduction in the mice's time to fall in the MIA group ($p < 0.001$). Fig. 2C shows how pretreatment with CHN mitigates the effects of Poly (I:C) on mice's autistic-like behavior during the social interaction test (SIT). Poly (I:C) group showed significant decrease in social preference ($p < 0.001$) compared to Control group. Further, Poly (I:C)-induced decrease in social preference was significantly ($p < 0.001$) improved by the pretreatment of CHN at both the dosages (25 & 50 mg/kg). Fig. 2D, Poly (I:C) treated group exhibited a significant decrease in sucrose preference ($p < 0.001$) that was increased markedly by both the dosages (25 & 50 mg/kg) of CHN pretreatment ($p < 0.001$).

Effects of CHN on Poly (I: C) Induced Changes in *5-HT* Level (Fetal Brain) and *IL-17A* (Fetal and Offspring Brains)

Following the administration of Poly (I:C), there was a significant decrease in the *5-HT* level ($p < 0.001$) in fetal brain compared to control group. CHN pretreatment improved the fetal brain *5-HT* level significantly at both the dosages (25 mg/kg; $p < 0.001$ and 50 mg/kg; $p < 0.05$) when compared to Poly (I:C) challenged group (Fig. 3A). As shown in Fig. 3B, pre-treatment of CHN (25 mg/kg p.o.) significantly reduced *IL-17A* levels in PFC, HC and AMG while the administration of CHN 50 mg/kg did not show significant effect on Poly (I:C)-induced increase in the fetal brain *IL-17A* level. On the other hand, Poly (I:C) enhanced *IL-17A* levels ($p < 0.05$) in the fetal brain mice in comparison to the control group. Poly (I:C) group showed an increase in the level of *IL-17A* in the HC, PFC, and AMG regions ($p < 0.01$, $p < 0.001$ and $p < 0.05$, respectively) than the control group (Fig. 3C). CHN administration at a dose of 25 mg/kg resulted in a considerably decrease in the level of *IL-17A* in HC ($p < 0.001$), PFC ($p < 0.001$) and AMG ($p < 0.01$), as compared to the adult offspring

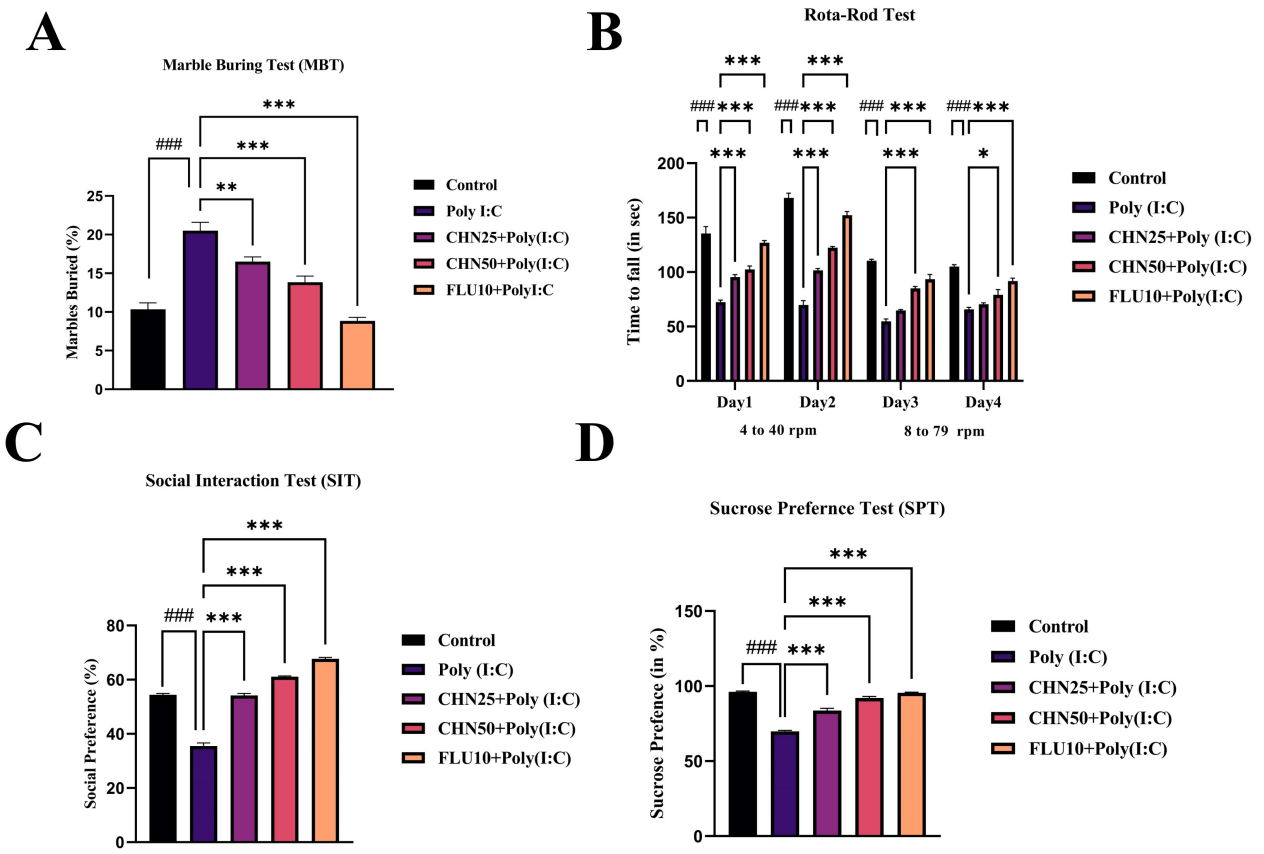

Fig. 2. Effect of chrysin on Poly (I:C)-induced autistic-like behavior in C57BL/6J mice in different behavioral tests. (A) MIA and control offspring were assessed for repetitive behavior in marble buried assay in control and Poly (I:C) treated MIA mice. Results were expressed as Mean ± SEM (n = 6). $^{###}p < 0.001$, vs control $***p < 0.001$, $**p < 0.01$ vs Poly (I:C) group. (B) MIA offspring displayed decreased motor learning, when tested in rota-rod, control and Poly (I:C) treated MIA mice. At 4–40 r.p.m and 8–79 r.p.m. Results were expressed in Mean ± SEM (n = 6). $^{###}p < 0.001$ vs control $***p < 0.001$, $*p < 0.05$, vs Poly (I:C) group. (C) Effect of CHN pre-treatment on Poly (I:C) induced changes in social interaction test. Data were analyzed using one-way ANOVA followed by Dunnett's post-hoc test and results are expressed in the Mean ± SEM (n = 6). $^{###}p < 0.001$ vs control group, $***p < 0.001$ vs Poly (I:C) group. (D) Effect of CHN pre-treatment on Poly (I:C) induced changes in depressive-like behavior in a sucrose preference test. Results are expressed in the Mean ± SEM (n = 6). $^{###}p < 0.001$ vs control group $***p < 0.001$ vs Poly (I:C) group. Poly (I:C), Polyinosinic:polycytidylic acid; CHN, chrysin; FLU, fluoxetine; SEM, standard error mean; ANOVA, analysis of variance.

of the Poly (I:C) treated mice. However, pre-treatment at a dose of 50 mg/kg significantly decreased the *IL-17A* level in HC ($p < 0.001$) and AMG ($p < 0.05$). Significant reductions in HC ($p < 0.001$), PFC ($p < 0.001$), and AMG ($p < 0.5$) were seen with fluoxetine (FLU) (10 mg/kg) administration. Findings demonstrated that pro-inflammatory cytokine levels at PFC, HC, and AMG of the adult offspring of Poly (I:C) treated pregnant female mice have been reduced by CHN pre-treatment.

Discussion

The term "autism spectrum disorder (ASD)" refers to a broad category of disorders marked by challenges with verbal and nonverbal communication, social interaction, and repetitive behaviors. Since rats naturally demonstrate many behaviors that are linked to similar behaviors in people, it is possible to determine whether they exhibit changes in the three defining domains of ASD in animal models, either genetic or due to therapy [40]. One common environmental risk factor for autism spectrum disorder (ASD) during pregnancy is maternal immune activation (MIA). Increased inflammation and oxidative stress in the placenta and fetal brain trigger an immunological response in the pregnant maternal biology [41]. Our findings indicate that Poly (I:C)-induced MIA in this murine model impacts both behavioral and immunological responses in offspring. Pregnant mice treated with Poly (I:C) exhibited acute immunological responses, including early production of fetal brain *5-HT* and *IL-17A*, which interact with fetal brain tissues and influence offspring behavior. The lack of *IL-17A* inhibits behavioral correlates of ASD in this experimental

Journal of
BIOLOGICAL REGULATORS
and Homeostatic Agents

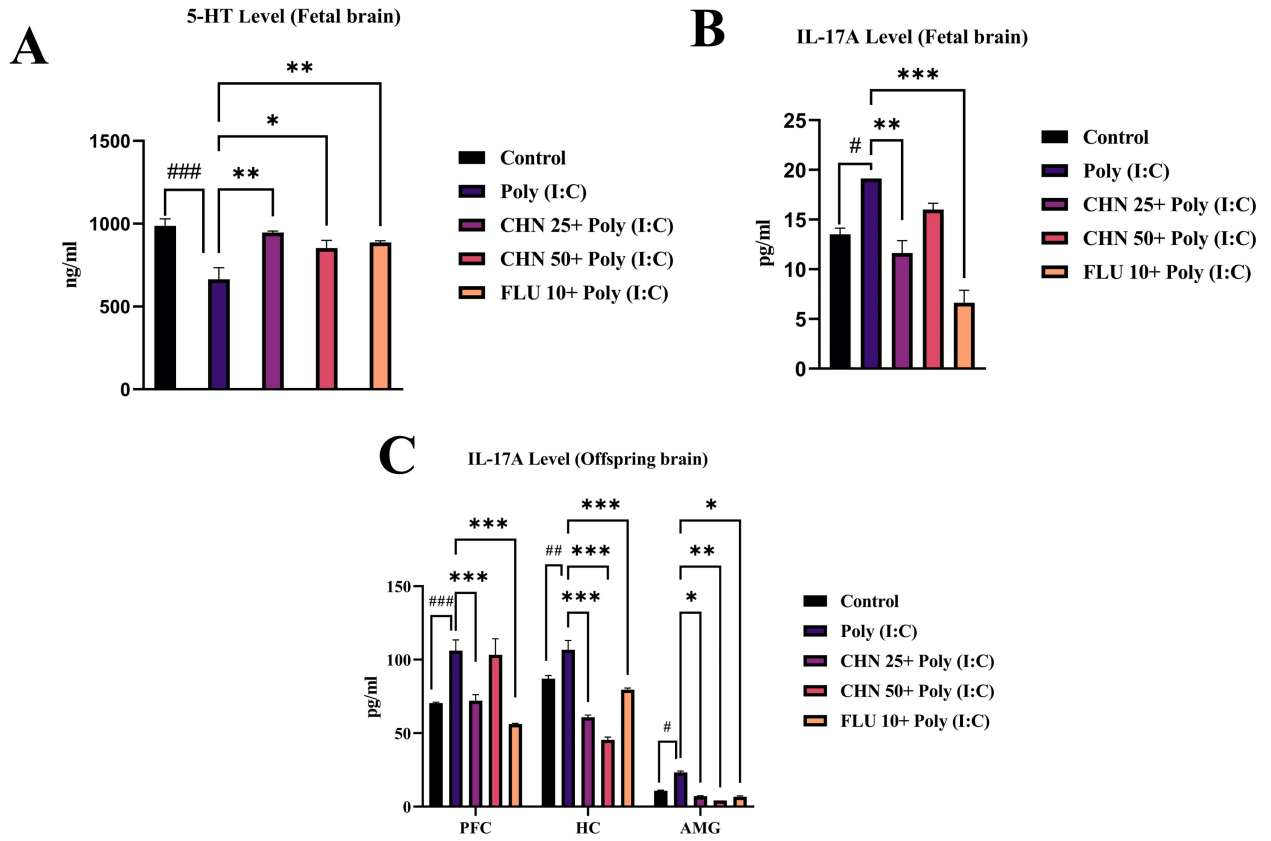

Fig. 3. Effect of chrysin on Poly (I:C)-induced changes in *5-HT* (fetal) and *IL-17A* (fetal and offspring brain regions) in C57BL/6J mice. (A) Effects of CHN on *5-HT* level in fetal brain in mice. Results are expressed in the mean ± SEM (n = 6). $^{###}p < 0.001$ vs control group, $^{*}p < 0.05$, $^{**}p < 0.01$ vs Poly (I:C) group. (B) Effects of CHN on *IL-17A* level in fetal brain in mice. Results are expressed in the mean ± SEM (n = 6) $^{#}p < 0.05$ vs control group, $^{**}p < 0.01$, $^{***}p < 0.001$ vs Poly (I:C) group. (C) Effect of CHN on *IL-17A* level in offspring brain tissue, PFC, HC, and AMG. Data were analyzed using one-way or two-way ANOVA followed by Dunnett's post-hoc test. Results are expressed in mean ± SEM (n = 6) $^{#}p < 0.05$, $^{##}p < 0.01$, and $^{###}p < 0.001$ vs control group, $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ vs Poly (I:C) group.

paradigm. We found a significant rise in *IL-17A* in the embryonic brain 4 hours after treatment with Poly (I:C), a cytokine linked to ASD-like symptoms in similar mice models.

Using the marble burying test, behaviors in the repetitive domain were evaluated. The marble burial test has a low false positive rate, is straightforward to assess, and is trustworthy compared to other tests [42]. Results showed a significant reduction in the marble-burying test, possibly due to CHN's anxiolytic activity. There is evidence to support the theory that by using $GABA_A$ receptors, CHN functioned as an anxiolytic-like drug in a rat model of surgical menopause [43]. Jacqueline Crawley's group devised the three-chambered social preference test to study social preferences like pair bonding, dominance hierarchies, and social memory. This test uses inherent social preference observed in many rodent species [42]. In favour of our findings in the rota-rod and social interaction test, previous studies have shown that sensory impairments and decreased social contact were associated with maternal immune activation-induced deteriorations in an offspring's brain *5-HT* levels from early to mid-gestation [44,45]. On the other hand, *IL-17A*'s effect on social behavior has previously been linked to diminished neuronal activation in the somatosensory cortex, as seen in maternal immune activation-affected mice pups [46]. MIA offspring not only have behavioral deficits but also show alterations in the central nervous system and peripheral immune cells. More specifically, investigations have shown that the cortex and hippocampus of MIA offspring exhibit changes in cytokine and chemokine levels both during and after the period of synaptogenesis [35]. Earlier, it has been reported that Fyn is a useful target for depression intervention and raises the possibility that CHN may be a therapeutic medication for disorders associated with neuroinflammation *via* controlling neuroinflammatory responses. When compared to the Chronic Unpredictable Mild Stress (CUMS) group, the CHN therapy significantly increased the sucrose pref-

erence. Similarly, in our findings, it can be clearly seen that CHN has antidepressant potential against MIA-induced neuroinflammation in the offspring's brain [47]. The current study investigates the molecular processes that underpin the link between brain dysfunction and altered uterine immunity in the emergence of atypical behavioral patterns associated with ASD. In support of our results, the study showed that treatment with chrysin increased *5-HT* levels, as well as raised 5-hydroxyindoleacetic acid/*5-HT* ratio, Kynurenine (KYN)/Tryptophan, and hippocampal levels in a way similar to fluoxetine [27]. Another study showed that the TH17 cell/IL-17 pathway may be pathologically activated during pregnancy in mothers with certain inflammatory diseases. This can change the development of the fetal brain and cause behavioral traits in offspring that resemble autism spectrum disorders [48]. This supports the current study, where we wanted to investigate the potential effect of *IL-17A* on the adult prefrontal cortex, hippocampus and amygdala neurogenesis. In earlier research, researchers have found the decreased neurogenesis in mice prenatally exposed to Poly (I:C) [49,50]. Higher amounts of the pro-inflammatory cytokine *IL-17A* have been connected to an increase in anxiety-like behaviors [51]. This implies that increased marble-burying activity may be correlated with higher *IL-17A* levels, suggesting elevated anxiety or compulsive behaviour [52]. Anxiety and mood are crucially regulated by serotonin. Although the main effects of *IL-17A* are on mood and anxiety, long-term inflammation caused by *IL-17A* may affect general health and physical function, indirectly influencing motor coordination [53]. Direct relationships, however, are less well-established. The social interaction test counts the amount of time mice spend engaging with a conspecific in order to gauge their social behavior and anxiety. Reduced social engagement may be correlated with higher *IL-17A* levels, suggesting heightened anxiety or depressive-like behavior [54]. Anxiety-like behavior has been linked to dysregulation in *5-HT* signalling pathways, specifically changes in *5-HT* gene expression. An increase in marble-burying behavior may be caused by dysregulation or decreased serotonin levels [55]. The rotarod test performance may be impacted by altered serotonin levels brought on by variations in *5-HT* gene expression, which can also impair motor coordination and balance [56]. Social behavior is greatly influenced by serotonin. Changes in the expression of the *5-HT* gene may cause anxiety or depressive-like symptoms, which in turn may cause a decrease in social contact. Therefore, dysregulation of serotonin pathways may reduce rodent social behavior [57]. Anhedonia and other depressive-like behaviors are associated with lower serotonin levels or reduced expression of the *5-HT* gene. Reduced preference for sucrose can be caused by lower *5-HT* gene expression, which indicates a lack of interest in rewarding stimuli [58]. Using a murine MIA model, we found that prenatal MIA leads to aberrant behavioral traits in offspring and immune response dysregulation caused by a single acute viral PAMP exposure during pregnancy [59]. Therefore, some of the ASD-like symptoms can be corrected by pre-treatment of CHN in offspring of MIA mice; however, pre-treatment with CHN may have better therapeutic potential, which can be seen on *5-HT* and *IL-17A* levels in both fetal and offspring brains. Small sample sizes and possible confounding variables like genetic variability or environmental influences are common limitations of current research. Larger, more varied cohorts, controlled settings, and longitudinal studies to confirm and expand findings in order to gain a deeper understanding of the aforementioned mechanisms should all be incorporated into future research to reduce confounding effects.

Conclusion

In conclusion, the investigation showed that CHN considerably reduced the neuroinflammatory reactions and neurobehavioral abnormalities caused by Poly (I:C) in mice. In particular, in Poly (I:C)-treated mice, CHN at both dosages (25 and 50 mg/kg) significantly enhanced motor coordination, social interaction, and sucrose preference in repetitive/compulsive behavior. Significant reductions in repetitive behaviors in the marble burying test, improved social interaction time, corrected motor coordination in the rotarod test, and increased preference for sucrose were all indicative of these findings, which also suggested an overall improvement in depressive and autistic-like behaviors. Moreover, CHN markedly increased the *5-HT* level in fetal brain and decreased *IL-17A* levels in fetal brain and different brain areas, including the amygdala (AMG), prefrontal cortex (PFC), and hippocampus (HC). These findings suggest that CHN has a strong anti-inflammatory effect, adding to its neuroprotective qualities. The study's conclusion, which emphasizes the potential of CHN use in treating ASD associated with prenatal immune activation, shows promising therapeutic potential in reducing neurobehavioral and neuroinflammatory alterations induced by prenatal exposure of Poly (I:C).

Abbreviations

ASD, autism spectrum disorder; MIA, maternal immune activation; CSF, cerebrospinal fluid; IL, Interleukin; *5-HT*, 5-Hydroxytrptophane; MBT, marble burying test; SIT, social interaction test; SPT, sucrose preference test; TNF, Tumor Necrosis Factor; CNS, central nervous system; TRP, Tryptophan; Poly (I:C), Polyinosinic:polycytidylic acid; CHN, chrysin; FLU, fluoxetine; mRNA, Messenger Ribonucleic Acid; TLR, toll-like receptor; IFN, Interferon; KYN, Kynurenine; IDO, indolamine-2,3-dioxygenase; HC, hippocampus; PFC, prefrontal cortex; AMG, amygdala; PBS, Phosphate Buffer Saline; NaOH, Sodium Hydroxide; i.p., intravenously; p.o., Per Oral; Veh, vehicle; PND, Post Natal Day; TPH, Tryptophan hydroxylase; TH, T-Helper; ANOVA, analysis of variance.

Journal of
BIOLOGICAL REGULATORS
and Homeostatic Agents

Availability of Data and Materials

All experimental data included in this study can be obtained by contacting the corresponding author if needed.

Author Contributions

RS and KS conceptualized the idea and designed the whole study. RS did the all experiments and generated data. RS, AK and KS did the statistical analysis and wrote the whole manuscript. RS, AK, and KS contributed to important editorial changes in the manuscript. All authors read and approved the final version of the manuscript. All authors have contributed sufficiently to the work to take public responsibility for appropriate portions of the content and agreed to be accountable for all aspects of the work in ensuring that questions related to its accuracy or integrity.

Ethics Approval and Consent to Participate

All experiments and procedures were performed at Central Animal House Facility (CAHF) of Indira Gandhi National Tribal University, Amarkantak, Madhya Pradesh, India (Reg. No. 2004/GO/ReBi/S/18/CPCSEA) as per the guidelines of the Committee for Control and Supervision of Experiments on Animals (CCSEA), Government of India, New Delhi after duly approved by the Institutional Animal Ethical Committee (IAEC), Indira Gandhi National Tribal University, Amarkantak, India (IGNTU/IAEC/2021/34).

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Conflict of Interest

The authors declare no conflict of interest.

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Journal of
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