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Research Paper

Ginkgolide B as a biopsychosocial treatment salvages repeated restraint stress-induced amygdalar anomalies in mice

Olusegun G. Adebayo [a,b,1,2], Benneth Ben-Azu [c,3,4], Wadioni Aduema [d,5,6], Oyetola T. Oyebanjo [b,e], Emmanuel U. Modo [f], Iheagwam Pauline Ndidiamaka [g], Spiff E. Eleazer [h], Joseph Igbo Enya [i], Abayomi M. Ajayi [j,*,2,7]

[a] Department of Physiology, Faculty of Basic Medical Sciences, College of Health Sciences, Osun State University, Osogbo, Osun State, Nigeria
[b] Neurosciences and Oral Physiology Unit, Department of Physiology, Faculty of Basic Medical Sciences, College of Medicine, University of Ibadan, Ibadan, Oyo State, Nigeria
[c] DELSU Joint Canada-Israel Neuroscience and Biopsychiatry Laboratory, Department of Pharmacology, Faculty of Basic Medical Sciences, College of Health Sciences, Delta State University, Abraka, Delta State, Nigeria
[d] Department of Physiology, Faculty of Basic Medical Sciences, Bayelsa Medical University, Yenagoa, Bayelsa State, Nigeria
[e] Department of Physiology, Faculty of Basic Medical Sciences, Babcock University, Ilishan-Remo, Nigeria
[f] Department of Biochemistry, Faculty of Sciences, Delta State University, Abraka, Delta State, Nigeria
[g] Department of Biochemistry, Faculty of Sciences, University of Port Harcourt, River State, Nigeria
[h] Department of Physiology, Faculty of Basic Medical Sciences, PAMO University of Medical Sciences, Port-Harcourt, River State, Nigeria
[i] Department of Anatomy, Faculty of Basic Medical Sciences, Babcock University, Ilishan-Remo, Nigeria
[j] Department of Pharmacology and Therapeutics, Faculty of Basic Medical Sciences, University of Ibadan, Ibadan, Oyo State, Nigeria

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ABSTRACT

From preclinical and clinical findings, it has been shown that the amygdala is a critical mediator of stress and primary target for stress effects in the brain. We investigated the neuroprotective effect of Ginkgolide B (GB) in repeated restraint stress-induced behavioral deficit and amygdalar inflammation in mice. Mice were orally pre-treated with GB 20 mg/kg 1 h prior to 4 h restraint stress for 21 consecutive days. Behavioural deficit and serum and amygdalar biochemical changes were estimated using spectrophotometric and ELISA techniques. The results showed that GB pre-treatment inhibited spatial memory deficit, renounces neuropsychiatric phenotypes and metabolic redox activity by augmenting the endogenous antioxidant system via Nrf2 levels in the mice. The HPA axis activity impaired by the restraint stress induction was abated with marked reduction of corticosterone, hypertrophy of the adrenal gland and blood glucose level. Meanwhile, our data further reveals that GB pre-treatment inhibited the release of neuroinflammatory mediators (MPO, TNF-α, IL-6, MAPK, COX-2) and elevated CREB production via activation of BDNF protein. Further, the acetylcholinesterase activity was inhibited while the level of glutamate release remains unchanged in the amygdala of the restraint mice. The GB treatment also up-regulate the release of BCL-2 proteins. This study suggests that GB could be considered as a therapeutic agent in the management of memory impairment, neuropsychiatric phenotypes and neuropathological alterations.

* Corresponding author.
E-mail addresses: segsyn07@gmail.com (O.G. Adebayo), pharmben4ever@yahoo.com (B. Ben-Azu), wadioniaduema@gmail.com (W. Aduema), am.ajayi@mail1.ui.edu.ng (A.M. Ajayi).
[1] Orcid: 0000-0001-6654-2564
[2] Postal code- 200005
[3] 0000-0003-3569-3575
[4] Postal code- 330105
[5] 0000-0002-5856-9940
[6] Postal code- 178
[7] Orcid: 0000-0001-6586-0421

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1. Introduction

From preclinical and clinical findings, it has been shown that the amygdala is a critical mediator of stress response and primary target for stress effects in the brain (Liu et al., 2014; Zhang et al., 2021; Kraus et al., 2018). Optogenetics and *in vivo* neuronal imaging has helped in the area of dissecting the input/output signals in the subnuclei structures of the amygdala and also disengaging the neural circuitry or mechanisms used by the amygdala in exerting its effects on stress-related events and behaviours, such as fear and anxiety (Kautz et al., 2017; Zhang et al., 2021). Clinical neuroimaging studies unfolded serious implication of aberrant connectivity found in the amygdala and its sensory/motor regions in line with stress-associated neuropsychiatric diseases, like anxio-depressive disorder, addiction and post-traumatic stress disorder (Henckens et al., 2015). In fact, individual having generalized anxiety disorder, panic-related disorder and social anxiety disorder exhibited hyperactivity of the amygdala in reaction to abnormal amygdala-anterior cingulate cortex coupling during stress (Kraus et al., 2018; Mao et al., 2020).

Biochemical alterations implicated by the generation of reacting oxygen/nitrogen species (ROS/RNS), release of pro-inflammatory cytokines, expression of inflammatory proteins, neurotransmitter interruptions, up-regulation of apoptotic cascade and deactivation of anti-apoptotic signalling molecules, apart from the notable event of the HPA-axis hyperactivation and corticosterone-induced alteration of neuronal proteins in the body have been investigated in preclinical studies when laboratory rats or mice were exposed to extreme stressful cues (Loonen and Ivanova, 2016; Spencer et al., 2018; Ben-Azu et al., 2019, 2020; Ugwu et al., 2022; Li et al., 2022). The activities of inflammatory mediators such as cyclooxygenase-2 (COX-2), neuronal nitric oxide synthase (nNOS) and nuclear factor kappa-B (NF-κB) potentiate the disruption of neurotransmitter secretion such as, glutamate, monoamine, neuropeptides and growth factors in the amygdala as unfolded from recent studies (Liu et al., 2014; Ugwu et al., 2022). These sequalae ultimately mediate the neuropsychiatric disorders associated with stress (Gibney et al., 2013; Jiang et al., 2020; Moradi-Kor et al., 2020). More so, previous investigations reported that anxiety- and depression-related behaviours are linked to decrease cAMP response element binding (CREB) activity, brain-derived neurotrophic factor (BDNF) and tyrosine kinase B receptor (TrkB) (Gibney et al., 2013; Xue et al., 2016; Jiang et al., 2020). Moradi-Kor et al. (2020) reported that adolescent restraint-stress reduces the level of BDNF, length of apical dendrites and branch points in the basolateral amygdala (BLA) neurons in rats (Moradi-Kor et al., 2020). Also, Namgyal et al. (2020) reported that reduced tissue concentration or under-expression of CREB protein elicited poor hippocampal neuronal growth that in turns decreased BDNF protein expression when laboratory rodents were subjected to prolonged stressful cues or toxicant such as cadmium (Namgyal et al., 2020). On the other hand, the deactivation of nuclear factor erythroid 2 related factors 2 (Nrf-2) activated mitogen-activated protein kinase (MAPK) to activate COX-2 inflammatory bodies, deactivates CREB and possibly suppresses neurogenesis and B-cell lymphoma 2 (BCL-2) in the experimental mice amygdala after exposure to the repeated restraint-stress was evident in this study (Choi et al., 2018). In the signal transduction cascade of MAPK, the activation of extracellular signal-regulated kinase (ERK) is involved in cellular proliferation, differentiation, apoptosis and inflammation (Jiao et al., 2019). However, under stressful conditions, the MAPK signalling pathway is usually activated, and the downstream transcription factors such as COX-2 and NF-κB relocate into the nucleus, thereby setting off the expression of similar inflammatory factors (Haftcheshmeh et al., 2022). As reported in this study, alleviating the pathological changes via inhibition of oxido-inflammatory stress and activation of Nrf-2 proteins might serve as a breakthrough for the inhibition of MAPK signalling progression, which in turns switch on CREB system to regulate genes connected with synaptogenesis in the amygdala when individual is exposed to stressful events.

Ginkgo biloba, also known as EGb 761, with a promising chemical compounds which include flavonoids (quercetin, kaempferol, and isorhamnetin), terpenoids (bilobalide and ginkgolides), bioflavonoids (ginkgetin, sciadopitysin, and isoginkgetin), and organic acids (ginkgolic acid) that positively mediates varieties of biological processes in the body (Sherif et al., 2019; Ben-Azu et al., 2022; Adebayo et al., 2022a, 2022b, 2022c, 2022d; Adebayo et al., 2023). The clinical efficacy of the standardized *Ginkgo biloba* extracts has been published and highly recommended for use in a prescribed therapy (Maurer et al., 1997; Singh et al., 2019; Liu et al., 2020; Al-Haddad et al., 2023). Of note, it remains one of the only herbal alternative therapies adopted as a nootropic to synthetic anti-dementia drugs in the management of cognitive deficit and Alzheimer's diseases (Maurer et al., 1997; Singh et al., 2019; Liu et al., 2020). Ginkgolide B (GB), one of the active compounds extracted from the *Ginkgo biloba* plant has shown strong characteristic properties capable of attenuating several neuropathology. Wang et al. reported that in rats, GB is metabolised to its hydroxyl metabolites and that CYP2D6 is the main rat's CYP enzyme usually responsible for the metabolism of GB in rat's liver microsomes (Wang et al., 2008). In the *in situ* closed loop, the absorption rate of GB within the upper intestine was much significantly higher than that within the lower intestine due the varying intestinal pH gradients (Lv et al., 2008). Pharmacologically, GB has been reported to possess significant therapeutic benefit against cognitive impairment in senescence-accelerated P8 mice, microgliosis-mediated neuroinflammation in the hippocampus of mice, amyloid-β_{1-42} induced oxidative damage and altered cellular responses in human neuroblastoma SH-SY5Y cells, and myocardial infarction-induced depression-like behaviors (Gill et al., 2017; Ge et al., 2020; Shao et al., 2021; Luo et al., 2022). Thus, the goal of this study is to investigate the effect of Ginkgolide B on repeated restraint stress-induced amygdala alterations and the mechanism through which this effect is mediated.

2. Materials and methods

2.1. Drugs, reagents and antibodies

GB (purity $\geq$ 98 %) was purchased from Chengdu Dexter Biotechnology Co., Ltd (Sichuan, China). All other reagents, chemicals and antibodies purchased and used for this experiment were at highest purity and analytically graded.

2.2. Animal management

Twenty-four male Swiss mice (26–29 g) were purchased from the Central Animal Housing facility, PAMO University of Medical Sciences, and maintained according to the laboratory standard (12 hr/12 hr light/dark cycle; 23 $\pm$ 1 °C; 55 $\pm$ 5 % humidity) for one week and continued until the termination of experiment. Treatment was done between 7:00 and 14:00 hr according to the Institutional ethical committee (PUMS-AREC/App/03/011/01) approval which is in line with the NIH protocol for the Care and Use of Laboratory.

2.3. Restraint stress induction and experimental protocol

Ginkgolide B (GB) dose selection was carefully done according to Gill et al. (2017) and Wang et al. (2021). 20 mg/kg of GB was administered in this study according to preliminary study with modification and as previously reported by Shao et al. (2021); Lv et al. (2021). The repeated restraint stress (RRS) induction was in accordance with previously reported protocol with some modification (Coballase-Urrutia et al., 2018; Casaril et al., 2019). Conventional method was used to expose the restrain stress induction to the mice for 4 h/day for 21 days between 9:00 am and 1:00 pm by confinement in a 50 mL centrifuge tube sufficient with numerous holes at the other end of the tube to ensure proper ventilation in order to prevent change in body temperature. 1 hr after treatment with oral administration of GB to achieve drug absorption,

restraint stress was induced in the mice. However, in order to ensure psychological stress rather than physical stress, the mice were carefully placed in the tube without squeezing or compression until the restraint period is achieved. After the restraint period, the mice were gently removed and then housed in their cages to allow free movement to avoid motor effect caused by the stress. The non-stressed mice were not subjected to any restraint stress and were left in their cage undisturbed without food and water to ensure the same feeding condition during the experiment. The mice were divided into 3 groups (n = 8) and orally treated for 21 days. Group 1 serve as the non-stressed (normal control) and received vehicle orally (10 mL/kg distilled water), group 2 was considered as the restraint stress control (negative control) and was given vehicle orally (10 mL/kg distilled water), while group 3 was treated with GB 20 mg/kg prior exposure to restraint stress induction. All the mice were treated between 8:00 am and 9:00 am prior restraint stress exposure. The experimental design is shown in Fig. 1.

2.4. Behavioral examination

Behavioral test to evaluate the level of learning and memory, locomotor impairment and neuropsychiatric symptoms was employed in this study. Y-maze test was done as earlier described to measure immediate short-term spatial working memory in the experimental mice, which depends on the total number of alternation by each mice calculated as percentage alternation following treatment and stress exposure (Monte et al., 2013). The long-term spatial learning & working memory deficit was also evaluated in the experimental mice by using Morris water maze (MWM) and its protocol according to the description previously established, dependent on the ability of each mice to located an escape platform hidden in one of the four quadrants in a pool of water after consecutive period of training taken as the total time spent in the platform quadrant in the test phase (Morris, 1984). Locomotor behaviour: a paradigm used to examine motor dysfunction in mice calculated as vertical and horizontal activities or the total number of lines crossed was done using the Open field test maze (OFT) described by (Izidio et al., 2005), while anxiety-like response was also critically monitored in the mice with elevated plus maze (EPM) which test for aversive behaviour (Pellow et al., 1985). Additionally, tail suspension test (TST) which depends on the increase or decrease immobility (freezing) duration depicting whether the mice are depressed or not was also examined (Dereli et al., 2018) and the social behavioural impairment or social withdrawal response was tested using social interaction test (SIT) to further affirm the level of anxio-depressive-like behaviour in the mice as previously described (Kaidanovich-Beilin et al., 2011).

2.5. Estimation of blood glucose

On day 22, after the completion of the behavioural examination, tail vein blood collection for the estimation of glucose level was done using a commercial glucometer (product of VivaChek) between 7:00 am and 8:00 am before anaesthesia.

2.6. Blood and brain sample processing

At termination, five animals per group were anaesthetised with ketamine hydrochloride (75 mg/kg) and diazepam (2.5 mg/kg) before euthanasia by cervical dislocation. The mice thoracic region was cut opened to collect blood through cardiac puncture while the brain and adrenal gland were excised and weighed. The brain was sectioned to isolate the amygdala. The blood sample was centrifuged for 15 min/3000 rpm at 4 °C to obtain serum. The isolated amygdala was homogenized in phosphate buffer (10 % w/v, 0.1 M, pH 7.4) and then centrifuged (10 min/10,000 rpm, 4 °C) to obtain the homogenates/supernatants. The serum and amygdala homogenates/supernatants were immediately stored at −20 °C until biochemical assay.

2.7. Estimation of oxidative and nitrosative stress

The supernatant of the amygdala collected from the whole brain sample was used for the estimation of biochemical parameter in the mice. Protein level was estimated as described (Bradford, 1976) and amygdalar oxidative/nitrosative stress was performed using the direct measurement of malondialdehyde (MDA) to evaluate the activity of lipid peroxidation and nitrite according to previous protocol (Green et al., 1982; Nagababu and Rifkind, 2009). The procedure for estimating reduced glutathione (GSH) concentration was performed according to the reduction of Ellman's reagent directly through the thiol groups of GSH (Jollow et al., 1974), while the Catalase (CAT) concentration was determined with hydrogen peroxide (H_2O_2) obtained as the substrate as earlier described (Adebayo et al., 2021a, 2021b). In addition, the superoxide dismutase (SOD) activity was performed based on superoxide inhibition of adrenaline (Misra and Fridovich, 1972). The UV/Vis Spectrophotometer (INESA 750 N, China) in their respective wavelengths was used to read all the absorbance then recorded with further calculation done by amygdala protein.

2.7.1. Enzyme-linked immunosorbent assay

Serum corticosterone (Oxford Biomedical Research (USA)), TNF-α, and IL-6 (BioLegend (USA)), glutamate (Rocky Mountain Diagnostic, Colorado Springs, (USA)), MAPK (Biosciences, USA), COX-2, BDNF, Nrf-2 and BCL-2 (Elabscience Biotechnology (USA), and CREB (Ray Biotech Pvt. Ltd. India) were all estimated at room temperature as described in

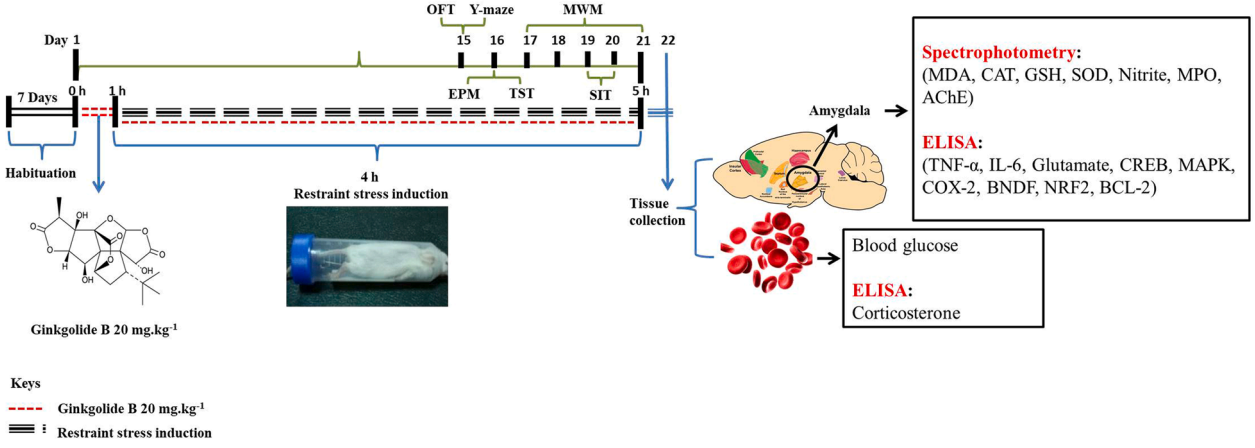

Fig. 1. Experimental design.

the manufacturer's protocol of the ELISA kit and read with a Spectramax M-5 (Molecular Devices, Sunnyvale, CA) plate reader. The level of TNF-α, IL-6, glutamate, MAPK, COX-2, BDNF, Nrf-2, BCL-2, and CREB in the amygdala were collected and calculated from the standard curve of the kits and then expressed as pg/mg protein.

2.7.2. Estimation of acetylcholinesterase activity

The activity of the acetylcholinesterase (AChE) in the amygdala was performed according to Onasanwo et al. (2021) protocol. Aliquot of mygdalar supernatant (0.4 mL) was added to phosphate buffer (2.6 mL, 0.1 M, pH 7.4). The mixture was added to 0.1 mL of 5,5-dithio-bis (2-nitrobenzoic acid) (DTNB) followed by the addition 0.1 mL of acetylthiocholine iodide and read at 412 nm in a UV/Vis Spectrophotometer to measure the level of absorbance at 10 min. Change in bsorbance was obtained at every 2 min. Finally, AChE enzyme activity was recorded at increase colour change obtained from the thiocholine after reacting with DTNB. Changes in absorbance level per minute was recorded and calculated as μmol/min/mg protein (Onasanwo et al., 2021).

2.7.3. Estimation of myeloperoxidase activity

The activity of myeloperoxidase enzyme was measured in the amygdala as described by Bradley et al. (1982). The amygdala supernatants were allowed to be suspended in extraction buffer (0.5 % hexadecyltrimethylammonium bromide in 50 mM potassium phosphate buffer at pH 6.0) frozen at 20 °C. The process to freeze-thawed with sonication procedure for 10 s cycle was repeated three times. The suspension was later centrifuged for 15 min (15,000 rpm; 4 °C) and the supernatant (0.2 mL) taken with the mixed solution (containing 0.167 mg/mL O-dianisidine in 50 mM potassium phosphate buffer and 0.15 mM H_2O_2) (2.8 mL) to calculate the MPO activity. The change in absorbance at 450 nm was thoroughly monitored within 3 min interval in a spectrophotometry (INESA). The unit of MPO was defined at absorbance change of 0.001/min while the activity was taken as the unit of MPO/mg of protein (Bradley et al., 1982).

2.8. Statistical analysis

Data collected was analysed with Graph Pad Prism software, Inc., Lajolla, USA (version 5.0). Analysis of variance (ANOVA) followed by Bonferroni post hoc test was adopted to compare groups and represented as Mean ± SEM. Statistical differences obtained at $p < 0.05$, $p < 0.01$ and $p < 0.001$ were considered significant.

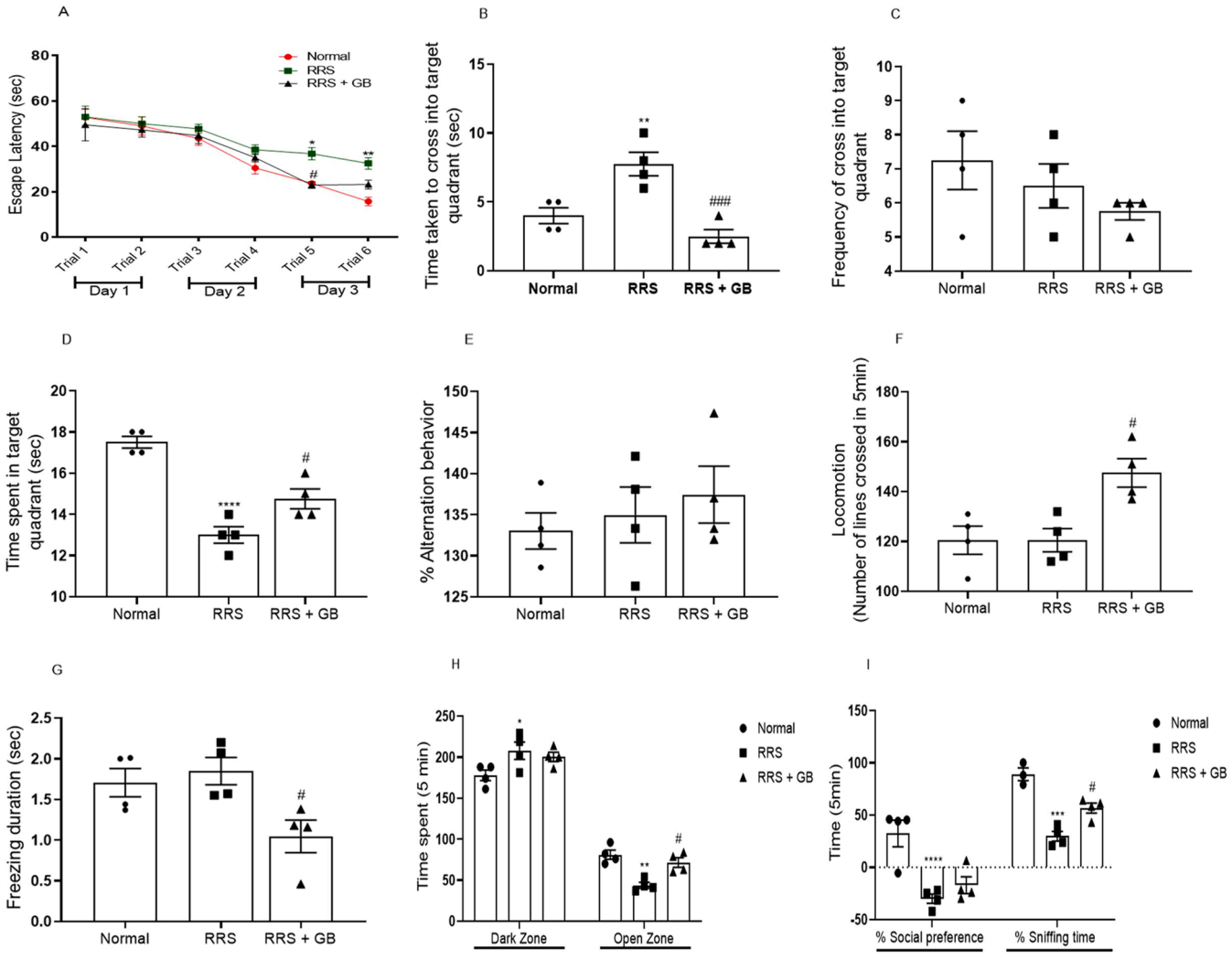

Fig. 2. Ginkgolide B pre-treatment reverses spatial working memory deficit, alteration in locomotor activity and neuropsychiatric phenotypes in RRS-induced mice. Escape latency (a), time taken to cross into target quadrant (b), frequency of cross into target quadrant (c), time taken in target quadrant (d), % alternation behavior (e) locomotion (f), freezing duration (g), time spent in dark and open zone (h), and % interaction time (i). Data (mean ± SEM; n=8) are considered significant at $^{*}p<0.05$, $^{*}\,^{*}p<0.01$, $^{*}\,^{**}p<0.001$ vs normal; $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ vs RRS.

3. Results

3.1. Repeated restraint stress-induced spatial working memory deficit and alteration in locomotor activity in mice were ameliorated by Ginkgolide B pre-treatment

Morris water maze (MWM) is a neurobehavioural test designed to investigate the neural mechanisms of long-term spatial learning or memory in animals. This test is based on assessing the levels of acquisition of information about the spatial location as well as the ability to recall previous information about a specific location after initial exposure. After the behavioural assessment with MWM, restraint stress exposure significantly ($p < 0.05$) increased the escape latency time, the time taken to cross the platform quadrant and also decreased the time spent in the platform but with no significant effect ($p>0.05$) on the frequency of cross into the platform quadrant (Fig. 2A-D). The treatment with GB 20 mg/kg increased the spatial memory function by significantly ($p<0.05$) decreasing the escape latency time (treatment: F [10,54] = 1.32; p=0.2443, days: F[2,54] = 8.67; p=0.0005 and mean interaction effect of treatment × days: F[5,54] = 37.12; $p< 0.0001$) (Fig. 2A) when compared with the restraint stress control. GB 20 mg/kg significantly ($p< 0.05$) decreased the time taken to cross into the platform quadrant ([F2, 9] = 16.71, p=0.0009) and increased the time spent in the platform quadrant ([F2, 9] = 32.22, $p<0.0001$) during the probe phase when compared with the restraint stress control (Fig. 2B and D). Additionally, the pre-treatment with GB 20 mg/kg did not show any significant ([F2, 9] = 1.397, p=0.2963) effect on the frequency of cross into the target quadrant comparative to the restraint stress control (Fig. 2C).

To substantiate our results on the level of spatial memory function and the effect of GB in the exposed experimental mice, Y-maze test was adopted. The Y-maze test evaluates the short-term spatial learning and memory, and it is based on the natural tendency of the animal to explore and navigate a new environment. As represented in Fig. 2E, exposure to restraint stress showed statistical differences ($p > 0.05$) in alternation behaviour comparative to the normal control. Also, GB 20 mg/kg pre-treatment did not improve the alternation behaviour ([F2, 9] = 0.5183, $p = 0.6123$) when compared with the restraint stress control.

Further, we measured the locomotor activity of the animals in a controlled environment using the open field test. From Fig. 2F, no significant change in locomotor behaviour was observed in the restraint stress control mice comparative to the normal control. However, GB 20 mg/kg pre-treated mice showed a significant ($p<0.05$) increase in locomotor behaviour as indicated by the number of lines crossed ([F2, 9] = 8.501, p= 0.0084) relative to the restraint stress control (Fig. 2F).

3.2. Ginkgolide B pre-treatment reduces repeated restraint stress-induced neuropsychiatric phenotypes in mice

In addition to the characteristic learning and memory impairment and exploratory deficit, the clinical features of stress can be complicated by neuropsychiatric phenotypes such as depression, anxiety and social behaviour. We investigated further on the effect of repeated restraint stress on depression, anxiety and social behaviour. The results showed that GB 20 mg/kg pre-treatment reduces the duration of freezing time, increases the time spent in the light zone and percentage sniffing time (interaction time). Whereas, the RRS-induced mice showed otherwise with significant ($p< 0.05$) neuropsychiatric phenotypes compared to the normal control (Fig. 2H-I). Significant differences were recorded in the experimental mice as shown by TST (Freezing duration for depressive-like behavior) ([F2, 9] = 5.557, p= 0.0268), EPM (Anxiety-like behavior) (Treatment: [F2, 18] = 12.84; $p = 0.0003$, activity: [F2, 18] = 1.19; $p = 0.3257$ and mean interaction effect of treatment × activity: [F1, 18] = 573.94; $p < 0.0001$) and SIT (% social preference and % sniffing time indicating social behavioural response) (Treatment: [F2, 17] = 0.74; $p = 0.4899$, activity: [F2, 17] = 32.31; $p < 0.0001$ and mean interaction effect of treatment × activity: [F1, 17] = 105.19; $p < 0.0001$). Furthermore, our analysis with post hoc test revealed that restraint stress induction altered behaviour and produced neuropsychiatric phenotypes as evident by anxio-depressive-like and social withdrawal behaviour relative to the GB 20 mg/kg pre-treated and normal control mice.

3.3. Repeated restraint stress-induced body and organ weight changes were reversed by Ginkgolide B pre-treatment

Two main front liners that helps to combat stress in the body; the brain and adrenal gland, have been seriously implicated and with their enlargement widely considered as a main indicator following excessive exposure to stressful conditions. The body weight decreases significantly ($p< 0.05$) after two and three weeks, while there was no significant differences in the brain and adrenal weights following exposure to restraint stress comparative the normal control (Fig. 3A-C). However, there were significant differences observed between the experimental mice: Body weight at week 3 (Treatment: [F4, 44] = 9.59; $p < 0.0001$, activity: [F2, 44] = 25.51; $p < 0.0001$, and mean interaction effect of treatment × activity: [F2, 44] = 11.83; $p < 0.0001$) and Adrenal weight ([F2, 6] = 16.80, $p = 0.0035$) when administered GB 20 mg/kg relative to the restraint stress control (Fig. 3A and C). Moreover, no statistical differences was observed in the brain weight ([F2, 6] = 3.750, $p = 0.0878$) when administered GB 20 mg/kg relative to the restraint stress control (Fig. 3B).

3.4. Ginkgolide B pre-treatment reduces blood glucose and serum corticosterone concentrations after repeated restraint stress exposure

Relating the organ hypertrophy with the exposure to restraint stress, we evaluated the concentrations of blood glucose and serum corticosterone levels to further enhance the data of this study (Fig. 3D-E). Exposure to restraint stress increased the blood glucose and serum corticosterone levels compared to the normal control. Meanwhile, the administration of GB 20 mg/kg reduced significantly ($p< 0.05$) the level of blood glucose ([F2, 6] = 26.16, $p = 0.0011$) and serum corticosterone ([F2, 6] = 31.21, $p = 0.0007$) comparative to the restraint stress control.

3.5. Ginkgolide B pre-treatment regulates the antioxidant signalling molecules in repeated restraint stress-induced oxidative disturbances in mice

Oxidative stress has been implicated in the pathogenesis of stress. Herein, restraint stress exposure caused a significant ($p< 0.05$) increase in oxidative damage in the amygdala of the mice brain relative to the normal control (Fig. 4A-E). Pre-treatment with GB 20 mg/kg significantly reduced the rate of lipid peroxidation (MDA) ([F2, 6] = 76.84, $p< 0.0001$; Fig. 4A) and nitrite (NO) ([F2, 6] = 11.62, $p = 0.0086$; Fig. 4B) activities but improved the antioxidant enzyme status. The GB 20 mg/kg increased significant ($p< 0.05$) the levels of catalase (CAT) ([F2, 6] = 14.94, $p = 0.0047$; Fig. 4D) and superoxide dismutase (SOD) ([F2, 6] = 32.44, p=0.0006; Fig. 4E), with no marked effect on glutathione (GSH) level ($p>0.05$) ([F2, 6] = 22.20, p=0.0017; Fig. 4C) comparative to the restraint stress control. Nuclear factor erythroid 2 related factor 2 (Nrf2), a molecular regulator of antioxidant system was estimated in this study. As indicated in Fig. 4F, restraint stress exposure significantly decreased Nrf-2 level ($p< 0.05$) ([F2, 6] = 18.82, $p = 0.0026$) relative to the normal control. Interestingly, administration of GB 20 mg/kg significantly ($p< 0.05$) elevated the Nrf-2 level contrarily to the restraint stress control (Fig. 4F).

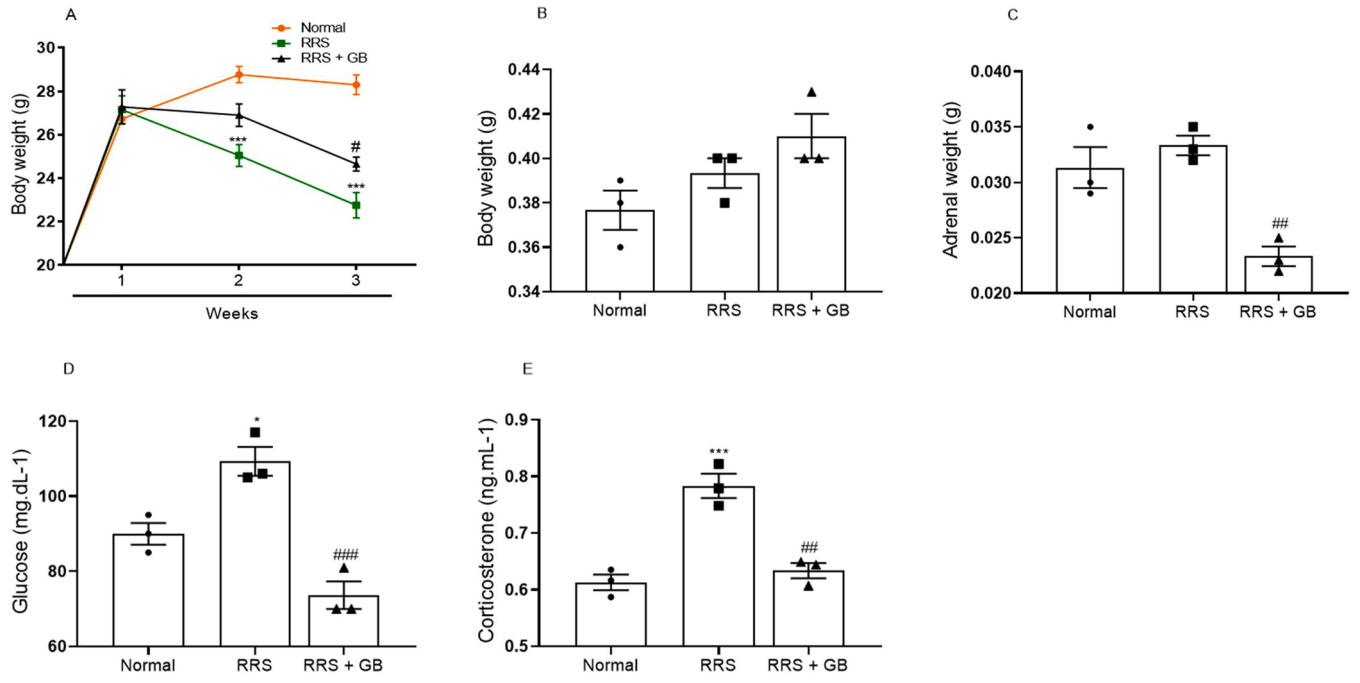

Fig. 3. Ginkgolide B pre-treatment maintains body and organ weight change and reduces blood glucose and serum corticosterone concentration in RRS-induced mice. Change in body weight (a), brain weight (b), adrenal weight (c), blood glucose (d), and corticosterone (e). Data (mean ± SEM; n = 8) are considered significant at $^{*}p < 0.05$, $^{* *}p < 0.01$, $^{* **}p < 0.001$ vs normal; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs RRS.

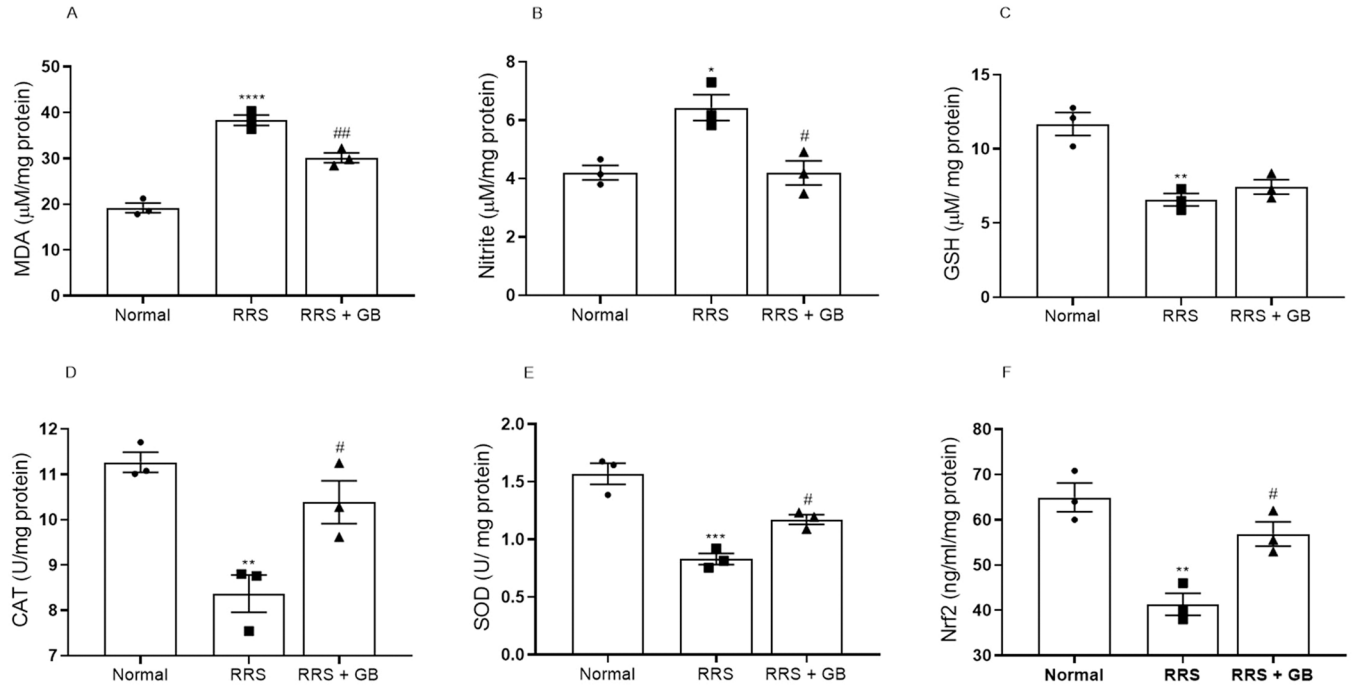

Fig. 4. Ginkgolide B pre-treatment attenuates RRS-induced oxidative disturbances by modulating pro-oxidants and antioxidants enzymes in mice. The amygdaloidal expression of redox imbalances and endogenous antioxidants MDA (a), Nitrite (b), GSH (c), Catalase (d), SOD (e), and Nrf-2 (f) was evaluated by spectrophotometry and ELISA techniques and then normalized by total protein. Data (mean ± SEM; n = 5) are considered significant at $^{*}p < 0.05$, $^{* *}p < 0.01$, $^{* **}p < 0.001$ vs normal; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs RRS.

3.6. Ginkgolide B pre-treatment attenuates pro-inflammatory cytokines, myeloperoxidase, mitogen-activated protein kinase, and cyclooxygenase-2 levels in repeated restraint stress-induced inflammation in mice

Giving some additional evidences to enhance our findings, we extend further our investigations by assessing the inflammatory levels after restraint stress exposure. As represented in Fig. 5A-E, the MPO, TNF-α, IL-6, MAPK, and COX-2 levels in the amygdala of the restraint stress mice were elevated relative to the normal control mice. Pre-treatment with GB 20 mg/kg significantly ($p < 0.05$) inhibited the release of MPO ([F2, 6] = 42.36, p=0.0003), TNF-α ([F2, 6] = 17.84, p=0.0030), and IL-6 ([F2, 6] = 8.251, p=0.0190) in the amygdala compared to the

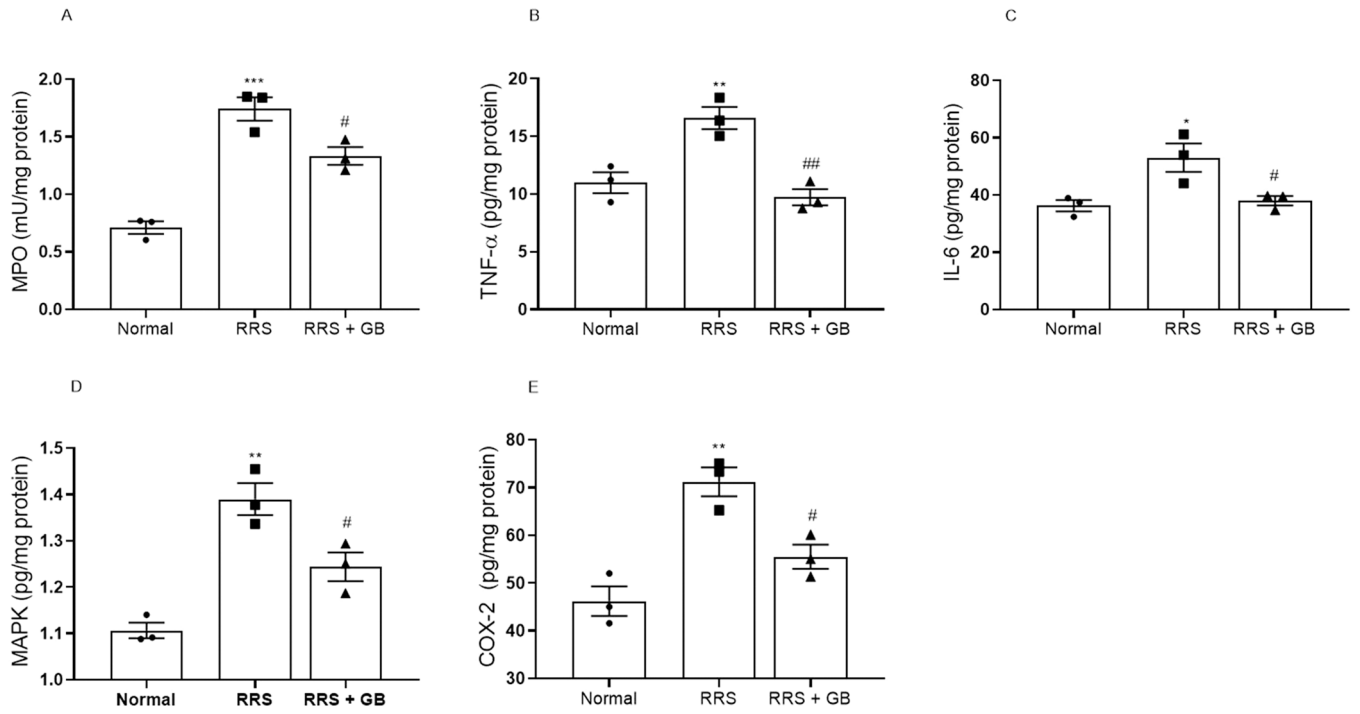

Fig. 5. Ginkgolide B pre-treatment attenuates RRS-induced pro-inflammatory cytokines, mitogen-activated protein kinase, myeloperoxidase and cyclooxygenase-2 activity in mice. The amygdaloidal expression of inflammatory mediators and neutrophil infiltration MPO (a), TNF-α (b), IL-6 (c), MAPK (d), and COX-2 (e) was evaluated by ELISA and spectrophotometry techniques and then normalized by total protein. Data (mean ± SEM; n = 5) are considered significant at $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs normal; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs RRS.

restraint stress control (Fig. 5A-C). The levels of MAPK ([F2, 6] = 24.61, p=0.0013) and COX-2 ([F2, 6] = 19.11, p=0.0025) were further significantly abated by the GB 20 mg/kg pre-treatment when compared with the restraint stress control (Fig. 5D-E).

3.7. Ginkgolide B pre-treatment modulates cholinergic and glutamatergic neurotransmission after restraint stress exposure in mice

The changes in cholinergic and glutamatergic neurotransmission affect learning and memory and other related behaviour in both human and experimental animals. The results shown from this study indicated that acetyl-cholinesterase but not glutamate release were significantly ($p<$ 0.05) elevated by the restraint stress exposure relative to the normal control (Fig. 6A-B). However, GB 20 mg/kg pre-treatment reverses this anomaly significantly ($p<$ 0.05) by reducing acetyl-cholinesterase release ([F2, 6] = 26.04, $p = 0.0011$) suggesting increased cholinergic function, but no change ($p>$ 0.05) in the glutamatergic system ([F2, 6] = 1.790, $p = 0.2457$) was found when compared with the restraint stress control (Fig. 6A-B).

3.8. Ginkgolide B pre-treatment elevates cAMP response element-binding protein and brain derived neurotrophic factor after restraint stress exposure in mice

CREB and BDNF regulate neuronal functions by modulating neurogenesis, although, the activation of CREB regulates the expression of BDNF as aforementioned. From Fig. 7A-B, restraint stress exposure down-regulated the activity of CREB, but not in BDNF expression contrary to the normal control. However, GB 20 mg/kg pre-treatment significantly up-regulated ($p<$ 0.05) the activity of CREB ([F2, 6] = 8.858, p=0.0162) and BDNF ([F2, 6] = 7.824, p=0.0213) when compared with the restraint stress control (Fig. 7A-B).

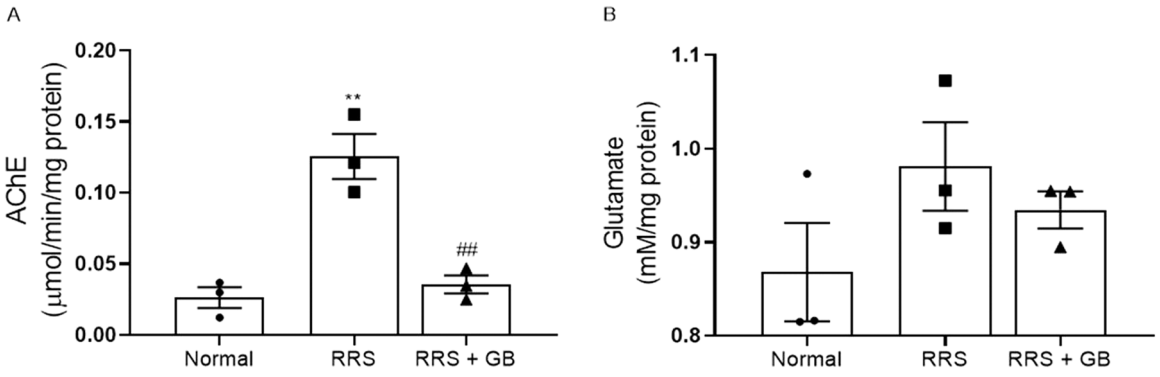

Fig. 6. Ginkgolide B pre-treatment modulates cholinergic and glutamatergic neurotransmission in RRS-induced mice. AChE (a) and Glutamate (b) was evaluated by ELISA and then normalized by protein. Data (mean ± SEM; n = 5) are considered significant at $^{**}p < 0.01$ vs normal; $^{\#\#}p < 0.01$ vs RRS.

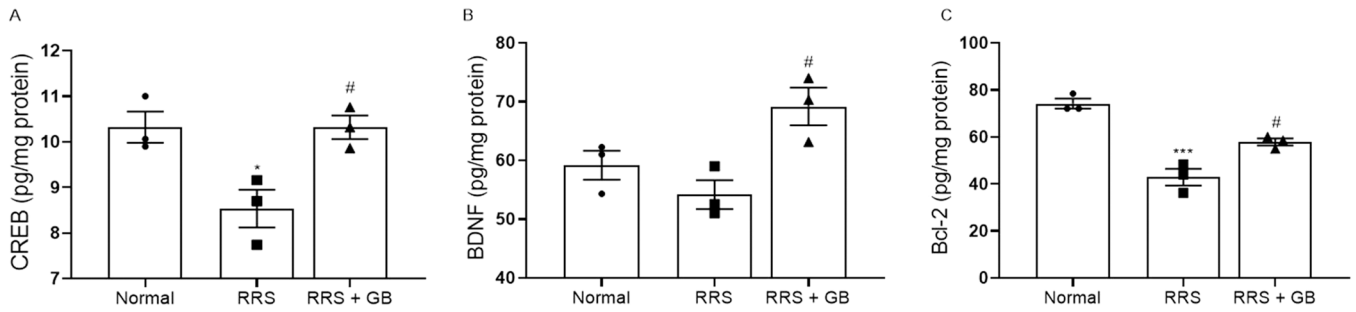

Fig. 7. Ginkgolide B pre-treatment regulates neurogenesis and apoptotic proteins in RRS-induced mice. CREB (a), BDNF (b), and BCL-2 were evaluated by ELISA and then normalized by protein. Data (mean ± SEM; n = 5) are considered significant at $^{*}p < 0.05$, $^{* **}p < 0.001$ vs normal; $^{\#}p < 0.05$ vs RRS.

3.9. Ginkgolide B pre-treatment increases B-cell lymphoma 2 expressions in the amygdala after restraint stress exposure

B-cell lymphoma 2 (Bcl-2) is a regulator protein that controls apoptosis through the inhibition (anti-apoptotic) or inducing (pro-apoptotic) of apoptosis. As represented in Fig. 7C, restraint stress exposure significantly ($p <$ 0.05) down-regulated the Bcl-2 protein levels, thus evoking apoptosis in the amygdala tissue of the mice relative to the normal control. However, the administration of GB 20 mg/kg significantly ([F2, 6] = 38.20, p=0.0004) up-regulated the protein levels when compared with the restraint stress control.

4. Discussion

This present study was undertaken based on ethnopharmacological findings and the natural use of one of the active compounds - Ginkgolide B, found in *Ginkgo biloba* for the treatment and management of neurobehavioral anomalies or neuropsychiatric symptoms. Recent studies have reported that the inability of an animal to respond effectively to extreme physical or psychological stress implicates various neuropsychiatric disorders including anxiety, depression, addiction, loss of cognitive functions and abnormal motor behaviour (Henckens et al., 2015; McEwen et al., 2015a, 2015b; Ben-Azu et al., 2020; Ugwu et al., 2022; Adebayo et al., 2023). From the findings of this study, we recorded that repeated restraint stress (RRS) exposure altered the hypothalamus-pituitary-adrenal axis (HPA), exacerbated markers of oxidative stress and inflammation, agitated the expression of inflammatory and apoptotic proteins, disrupted neurotransmitter homeostasis and machineries of neurogenesis in the amygdala region of the mice brain. However, the administration of GB inhibited all the alterations

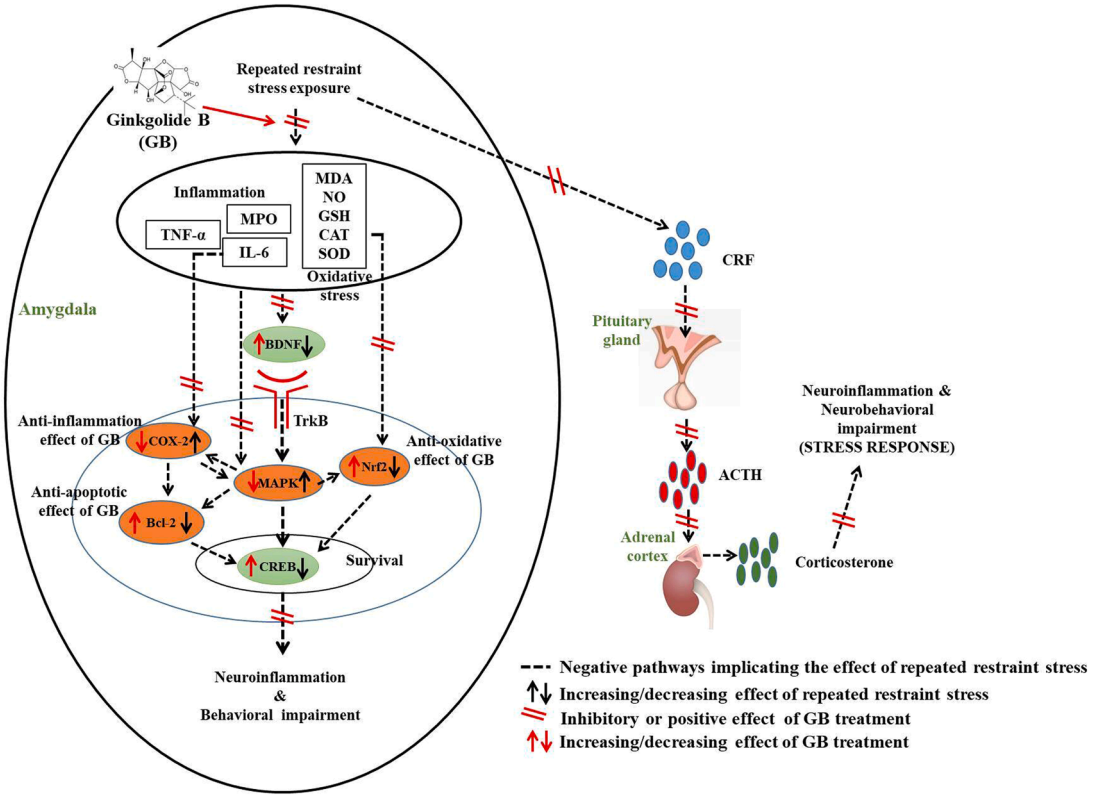

Fig. 8. Diagram to illustrate the mechanism of action of Ginkgolide B pre-treatment in repeated restraint stress-induced amygdala anomalies. Markers of oxidative stress and classic inflammatory cytokines and enzyme released after exposure to restraint stress alter the functions of amygdala such that they implicate BDNF/CREB pathway downregulation. MAPK and COX-2 released from the amygdala suppress the function and survival of the amygdala neurons marked by increased apoptosis and inflammation. The corticosterone released from adrenal cortex interacts with the amygdala to supress the release of Nrf2 protein and promote the production of oxidative and inflammatory markers, which increase apoptosis, inflammation and behavioural impairment. All these effects were abated by the Ginkgolide B pre-treatment.

elicited by the RRS exposure in the animal. The GB pre-treatment mitigated HPA axis alterations, prevented redox reactions via increasing endogenous antioxidant enzymes, inhibited inflammatory cytokines, inflammatory proteins expression and apoptotic events, and up-regulated neurotransmitter secretion and neurogenesis in the stressed mice amygdala.

In this study, we observed that RRS exposure for 21 days impaired locomotor behaviour (decrease in ambulatory activity), decreased spatial learning and memory function, increased anxiety- and depressive-like activity as well as decreased social interaction time in the mice. Recent investigations reported behavioural deficits with signs of depletion of neuronal structures after exposure to unfavourable stimulus which affects neurotransmitter release and endogenous anti-oxidant system in laboratory rodents (Adebayo et al., 2023; Omeiza et al., 2023). However, pre-treatment with GB improved the spatial memory deficit, locomotor impairment and neuropsychiatric symptoms in the mice. This is in line with findings that GB improves neuro-behavioural changes that are linked to decrease locomotor activity, decreased spatial learning and memory function, increased anxiety- and depressive-like activity (Ge et al., 2020; Liu et al., 2021).

The generation of reactive oxygen/nitrogen species (ROS/RNS) and productions of pro-inflammatory mediators have been found to be associated with several health challenges in humans after serious exposure to life threatening or stressful events (Loonen and Ivanova, 2016; Li et al., 2022). The restraint stress model is an acceptable animal model for the simultaneous induction of behavioural impairment or neuropsychiatric phenotypes in rodents. Several studies have reported that RRS-induced oxidative imbalances are associated with redox disruption (Coballase-Urrutia et al., 2018; Casaril et al., 2019). However, the data generated from this study showed that following RRS exposure, the production of ROS/RNS was increased, therefore, suggesting that exposure to RRS may be eliciting the production of ROS/RNS by interrupting the mitochondrial activity in the mice amygdala. Consequently, exposure to RRS inhibited the endogenous antioxidants' activity in the amygdala as evident with marked decrease in glutathione, catalase and superoxide dismutase activities. Of note, the increase in lipid peroxidation and nitrergic activity as well as the decrease levels of endogenous antioxidant enzymes predispose the neurocellular structures of the amygdala to inflammation and degeneration. To substantiate these findings, Nrf2 protein was measured. Nrf2 regulates the expressions of numerous genes that work by repairing homeostasis after oxidative or inflammatory damage in tissues through their promoters "antioxidant response elements" (AREs) (Adebayo et al., 2022a). Several reports have indicated the role of Nrf2 in maintaining neurocellular functions which then serves as a target protein for restoring or treating neuronal dysfunction (Adebayo et al., 2022a, 2022c). From our findings, we discovered that exposure to RRS also supressed Nrf2 protein expression in the amygdala. Conversely, pre-treatment with GB decreased the release of pro-oxidant molecules and increased the production of antioxidant enzymes (enzymic and non-enzymic) in the RRS exposed animal through up-regulation of Nrf2 protein. Therefore, GB pharmacotherapy might be supporting the powerful adaptive response/mechanism of amygdala to oppose the persisting oxidative assaults. In addition, *in-vivo* studies have also made evident the neuroprotective capacity of GB which was attributed to its anti-inflammatory potentials in the brain tissues (Gill et al., 2017; Shao et al., 2021; Lv et al., 2021; Wang et al., 2021; Luo et al., 2022).

Targeting the antioxidant and anti-inflammatory cascade may prevent RRS-induced inflammation and neurodegeneration in experimental animal model. MPO, which mediates neutrophils assaults associated with others markers of inflammation such as TNF-α, and IL-6, were all found to be a key factor in the progression of stress related disorders or neurodegenerative diseases (Zhang et al., 2021). These events trigger the activation of microglia cell and thus exacerbate blood-brain-barrier deformation and increased permeability in the brain tissues such as amygdala (Gu et al., 2021). Other than these, the increase in key inflammatory protein such as COX-2 expression has been reported in patients with neuropsychiatric symptoms (Minghetti, 2004; Sethi et al., 2019). COX-2 is activated by IL-6, IL-1, IL-8 and trauma (such as restraint stress), and it is involved in the control of neurotransmitters and amygdalar activity-dependent synaptic plasticity through marked release of prostaglandin-E2, a molecule also involved in pro-inflammatory mechanism, that triggers other pro-inflammatory cytokines production (Minghetti, 2004). In addition to the release of pro-inflammatory cytokines, the HPA axis known as the first-line neuroimmune system initiates corticosterone secretion which then consequentially activates or deactivates oxido-inflammatory signalling molecules involved in orchestrating or inhibiting stress-induced tissue and organ pathologies (Picard et al., 2021; Marchi and van Eeden, 2021). Adrenal gland activation causes blood sugar (glucose) elevation via gluconeogenesis, lipogenesis, or decrease in cellular glucose uptake or re-uptake (Wilson et al., 2013; Loonen and Ivanova, 2016). Hence, enlargement of the adrenal gland, elevated blood sugar (glucose) and increased corticosterone level are widely accepted as the main indicators of chronic stress states. Herein, we reported that GB inhibited MPO enzyme, TNF-α, IL-6, and corticosterone activity and also down-regulated COX-2 protein that together implicate amygdala neuronal dysfunction in the RRS-induced mice. Thus, we deduce that the anti-oxidation, anti-inflammation, and reduced blood sugar and the adrenal gland weight demonstrated by the GB in the RRS-induced mice are mediated by the inhibition of COX-2 expression and corticosterone release as well as through Nrf2-antioxidant increase in the experimental animal.

It has emerged that BDNF, CREB and MAPK are important regulators of synaptogenesis. These proteins promote synaptic plasticity that in turns improves neurobehavioral functions and emotional flexibilities (Rossi et al., 2006; Moradi-Kor et al., 2020). However, the activation of CREB switches on/off the expressions of numerous genes including the BDNF (Xue et al., 2016). Although, MAPK is thought to be involved in oxidative and inflammatory process, it is also known to regulate neurogenesis by activating/deactivating the activity of CREB protein that confers neuronal survival (Kim et al., 2004; Dong et al., 2019). In this study, we observed that aside BDNF protein reduction, the concentration of CREB decreases while MAPK level was found to increase significantly in the amygdala of the RRS-induced mice. Therefore, it is noteworthy to infer that restraint stress induction could exert detrimental changes on the proteins involved in neurogenesis and synaptogenesis and consequentially induce neurobehavioural deficit and neuropsychiatry phenotype in the animals as observed in this study. Interestingly, pre-treatment with GB averted the alterations observed in the experimental animals. The levels of BDNF and CREB protein were up-regulated with a significant down-regulation of MAPK protein in the amygdala. These effects enhanced neurogenesis and synaptogenesis and also improved neurobehavioural deficit and neuropsychiatry phenotypes in the mice. Thus, our findings corroborated previous studies where restraint stress exposure was found to alter neuronal functions in mice different brain regions (Torres et al., 2002; Moradi-Kor et al., 2020).

To strengthen our findings, we measured neurotransmitters implicated in the pathogenesis of neuropsychiatry and neurodegenerative diseases. Glutamate is involved in neurodevelopment, and in mild or chronic neurodegenerative and neuropsychiatric diseases (Ben-Azu et al., 2019; Adebayo et al., 2023). Recent findings have indicated the underlying functions of the glutamate footpath in response to stress (Tiwari et al., 2021). However, GABA and glutamate homeostatic imbalances has been hypothesized to be involved in several disease conditions such as anxio-depression, social behaviour disorders, schizophrenia and learning and memory deficit (Ben-Azu et al., 2019; Adebayo et al., 2023). In addition, AChE release in the cholinergic neurons breaks down acetylcholine (ACh) that as well displays some role in mild and chronic neurodegenerative processes (Ben-Azu et al., 2019; Adebayo et al., 2023). Overproduction of AChE has been previously

reported in rodents (Ben-Azu et al., 2020, 2022; Ugwu et al., 2022) and human (Bosak et al., 2019) exposed to stress. Meanwhile, other reports claimed that the overproduction of AChE may serve as a treatment therapy in the management of neurodegenerative disorder like Parkinson's disease (Farombi et al., 2019), schizophrenia (Ben-Azu et al., 2019), and Alzheimer's disease (Ferreira et al., 2021). In line with the above, amygdala AChE level was found to be increased in the mice exposed to restraint stress model. These effects could be as a result of the immoderate secretion of glucocorticoids (corticosterone) and the BDNF/CREB dissemination under the prolonged stress condition that led to amygdala neurocellular dysfunction and inability to remove/degrade AChE or possibly through the destructive effect of glutamate excitotoxicity in the synapse. Although, the release of glutamate from the amygdala was not significant enough as expected to compensate for the increase in AChE released. Interestingly, the GB treatment maintains glutamate production and further decreased the AChE concentration in the mice amygdala after exposure to stress. This effect further confirms the restorative effect of GB as evident in the neurobehavioral results.

It is worth mentioning that several reports have also revealed anti-apoptotic markers as an important anti-stress molecular marker (Deng et al., 2020). B-cell lymphoma 2 (Bcl-2) is a regulator protein that facilitates anti-apoptosis in animal and human experimental stress-related conditions (Deng et al., 2020). From our findings, we observed that RRS induction reduces Bcl-2 levels, while it was abated by pre-treatment with GB. Since Bcl-2 is confirmed to exhibit anti-apoptotic response when expressed moderately, we therefore report that Bcl-2 confers a neuroprotective function as part of an anti-apoptotic mechanism of GB in this study. Further, Bcl-2 moderates neuronal survival in the amygdala via up-regulation of BDNF/CREB activities. We also predict that Nrf2 proteins in synergy with the Bcl-2 proteins might also be involved in protecting the neurocellular structures of the amygdala. In sum, these effects improve the mice behaviour as observed in this study.

To the best of our knowledge, the applications of Ginkgolide B alone in clinical studies are yet to be reported while several investigations on the use of *Ginkgo biloba* for clinical pharmacotherapy have been published. Currently, *Ginkgo biloba* is adapted as a nootropic drug in Alzheimer's disease, Parkinson's disease, amnesia and dementia treatment (Maurer et al., 1997; Singh et al., 2019; Liu et al., 2020). Due to the results reported in this study, we suggest that future finding on the clinical application of GB potential and how to translate our findings and previously established findings into human treatment strategies should become a critical focus. More so, we proposed that since GB exhibited the potentials to enhance Nrf2-antioxidant and anti-inflammatory signaling axis, and mediators of neurogenesis, isolated GB from *Ginkgo biloba* should be adopted for clinical trial to tackle few neurodegenerative diseases like Alzheimer's disease and Parkinson's disease.

However, we have some limitations which include unavailability to estimate other markers of neurogenesis and TrkB receptor pathway to support our data. This is due to lack of funds and facilities to run the Western blot and RT-PCR assay in our laboratory/department. Having highlighted these weaknesses, the study also has some strength which involves data reproducibility, reliability and accuracy of method, clarity of data analysis, and no study has previously elucidated the repeated restraint stress-induced amygdalar anomalies with GB as pharmacotherapeutic intervention.

In conclusion, the results of this current study demonstrated that GB administration attenuated the homeostatic dissembling and pathobiological sequela associated with stress as evidenced by reduced memory impairment and neuropsychiatric phenotype, regulated HPA-axis, elevated endogenous antioxidant enzyme and Nrf2 expression, and decrement in inflammatory mediators in the amygdala. In addition to this, GB administration improved the survival of amygdala neurons by modulating the neurochemical transmission, increasing CREB and BDNF activity as well as improving anti-apoptotic signalling proteins after exposure to stress. Thus, owing to the efficacy and absorption of GB, this study suggests that GB could be used as a potential pharmacotherapeutic agent for disease intervention in human as well as for the treatment and management of stress.

Ethical consideration

This study was approved by the institutional ethical committee (PUMS-AREC/App/03/011/01) in accordance with the NIH protocol for the Care and Use of Laboratory.

Author contributions

Olusegun G. Adebayo and Joseph Igbo Enya – managed laboratory experiments and the analyses of the study and wrote the first draft of the manuscript. Abayomi M. Ajayi – designed the study, performed the statistical analysis, and wrote the protocol. Benneth Ben-Azu– managed the literature searches and methodology. Emmanuel U. Modo and Wadioni Aduema – provide the material and also managed the literature searches and methodology. Oyetola T. Oyebanjo, Spiff E. Eleazer and Iheangwam Pauline Ndidiamaka – provided the material and also managed laboratory experiments and the analyses of the study. All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.

Financial interest

Authors report no financial or non-financial interest whatsoever.

Conflicts of Interest

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CRediT authorship contribution statement

Iheagwam Pauline Ndidiamaka: Software, Methodology, Investigation, Formal analysis. **Spiff E. Eleazer:** Software, Resources, Investigation, Funding acquisition, Formal analysis. **Benneth Ben-Azu:** Methodology, Data curation. **Wadioni Aduema:** Resources, Methodology. **Oyetola T. Oyebanjo:** Methodology, Funding acquisition, Formal analysis, Data curation. **Modo U. Emmanuel:** Methodology, Funding acquisition, Formal analysis, Data curation. **Abayomi M. Ajayi:** Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Olusegun G. Adebayo:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Formal analysis, Data curation. **Joseph Igbo Enya:** Visualization, Validation, Supervision, Methodology, Data curation.

Declaration of Competing Interest

All the authors involved in this experiment hereby disclose no conflict of interest whatsoever and have hereby given their consent towards the submission of this manuscript to this journal.

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