

# Bacopa monnieri extract (CDRI-08) recovers recognition memory impairment by upregulation of GluR2 AMPA receptor subunit expression and reduced methylation of its cognate promoter in the hippocampus of rotenone-induced Parkinson's disease mouse model

Garima Chandra

Banaras Hindu University

S. Prasad

[s.sprasadbhu@gmail.com](mailto:s.sprasadbhu@gmail.com)

Banaras Hindu University

---

## Research Article

**Keywords:** Parkinson's disease, synaptic plasticity, cognition, glutamate receptors, AMPA receptor, GluR2 subunit, promoter methylation, Bacopa monnieri, CDRI-08

**Posted Date:** December 7th, 2023

**DOI:** <https://doi.org/10.21203/rs.3.rs-3677310/v1>

**License:** © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License. [Read Full License](#)

**Additional Declarations:** No competing interests reported.

---

# Abstract

Parkinson's disease (PD), an age-dependent neurodegenerative movement disorder, is attributed to the loss of dopaminergic neurons in the striatal SNpc, abnormal  $\alpha$ -Synuclein accumulation that leads to complex movement disorders, cognitive dysfunction, and dementia in later stage, however, its precise molecular underpinning is not well known. In the proposed study, we aim to explore the effects of PD on the expression of the synaptic plasticity associated with AMPA receptor subunit GluR2, its epigenetic regulation, and investigate the possible therapeutic role of a well-characterized extract of an ancient Indian medicinal plant *Bacopa monnieri* called CDRI-08 on the PD and memory impairment in rotenone-PD mouse model as presently there is no successful treatment available for curing the PD-induced pathologies. Our immunoblotting and qPCR data showed a significant upregulation of the GluR2 expression in the hippocampus of rotenone-treated mice as compared to the vehicle control group. Our MeDIP-qPCR data revealed that the increased GluR2 expression was significantly correlated with a decline in its promoter CpG methylation in the hippocampus of rotenone-treated mice. *CDRI-08* treatment to the rotenone-treated mice significantly lowered the PD pathologies, and GluR2 expression with a subsequent increase in the GluR2 promoter methylation towards the control values in the hippocampus. The current study suggests one of the precise molecular events underlying PD, its effects on memory impairment, and the neurotherapeutic potential of *Bacopa monnieri* extract, CDRI-08, which might be useful for the treatment of PD-induced movement and cognitive impairments.

## Introduction

Parkinson's disease (PD), a motor associated neurodegenerative disorder, is the second most prevalent chronic neurodegenerative disease after Alzheimer's disease (AD) and is characterized by motor disorders. Its hallmark symptoms are tremors, ataxia, rigidity and bradykinesia. PD is primarily characterized by the loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) of the striatal brain region due to abnormal aggregation of the misfolded  $\alpha$ -Synuclein ( $\alpha$ -Syn) forming intraneuronal Lewy body inclusions and hence the dopamine deficiency in the striatum. However, the genesis of PD involves various mechanisms and disruptions in pathways, including oxidative stress, malfunctioning mitochondria, cellular calcium levels imbalance in cells, neuroinflammation, and alterations in other neurotransmitter systems [1, 2]. These multifaceted elements collectively contribute to the onset and advancement of PD. Oxidative stress, which emerges when there is an imbalance between the production of reactive oxygen species (ROS) and the capability of the body to neutralize them, has been identified as a key player in PD pathogenesis [3]. As a result, the substantia nigra of PD patients has higher quantities of oxidized lipids [4], proteins, and DNA [5], and lower levels of superoxide dismutase (SOD) and glutathione peroxidase (GPx) [6]. Because of this

inherent susceptibility to reactive species, even mild oxidative stress can trigger a number of events that lead to cellular death. In addition to motor abnormalities, PD is linked to a broad spectrum of non-motor symptoms such as sensory dysfunction, autonomic dysfunction, mood disorders, cognitive dysfunction, and hallucination significantly contribute to the overall burden of the disease pathologies [7]. Cognitive decline is reportedly more frequent in individuals with PD, up to six times greater than in the common population making it one of the prominent non-motor symptoms of PD and is intricately linked with the natural progression of the disease [1], however, the precise underlying mechanism of cognitive impairment in PD is still unclear.

Abnormal glutamatergic synaptic transmission and plasticity have been linked with many neurodegenerative diseases including PD. Ionotropic glutamate receptors (iGluRs) such as  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors (AMPA), N-methyl-D-aspartate receptors (NMDARs), and kainic acid receptors (KARs) play a key factor in rapid excitatory neurotransmission in the brain. AMPAR is composed of four subunits (GluR1-4), which are most commonly formed as heterotetramers of GluR1 and GluR2 subunits prevalent in the forebrain [8]. Among them, the GluR2 subunit is vital in synaptic plasticity because it is inserted into the membrane during LTP (Long-term potentiation) production or endocytosed into the cytosol during LTD (Long-term depression) and is involved in the fine-tuning of synaptic transmission and thereby cognition [9]. However, the mechanism of GluR2 alterations in PD and PD-induced cognitive deterioration is not known. Emerging evidence suggests that epigenetic processes play a key role in the transcriptional regulation of genes associated with synaptic plasticity during memory formation. Epigenetic regulation involves processes such as post-translational modifications of histones and DNA methylation resulting in transcriptional alterations in various genes by altering the interactions between the genome and histone cores [10–12]. Moreover, disruption of epigenetic pathways has been widely linked to a variety of disease states ranging from cancer to multiple neurological, psychiatric, and neurodegenerative diseases such as PD [13]. In the proposed study, we have focused on the involvement of alterations in the expression of the role of GluR2 and its correlation with the altered methylation status of its promoter that leads to cognitive impairment in PD and using this as a target for therapeutic intervention as these processes are dynamic in nature.

*Bacopa monnieri* (BM), is an ancient Indian herb known for its nootropic effect on a spectrum of neurological diseases and memory deficits [9]. Based on a number of animal-based pre clinical researches, its ethanolic extract has been proven to have antioxidant activity [14–17] and is employed in neuroprotection [18], cognition [19], depression [20], cancer [21], and inflammation-related disease [22]. The active components of a B Methanolic extract prepared from its stems or leaves have been identified as Bacosides A1-A3, Bacosides I-III, and Bacosaponins A-G [23]. CDRI-08, a specialized extract of *Bacopa monnieri*, rich in Bacosides A and B has been related to decreased oxidative stress

and improved memory through modifying AMPAR and NMDAR expression and hence synaptic plasticity in DM2 (Diabetes mellitus types 2) rodent models [9 ] and many other neurological diseases such as AD [24], HD [25] and epilepsy [24]. However, its effect on epigenetic processes in the light of ameliorating cognitive impairment by modulating AMPA receptors in PD is not well established. Hence, the current study is focused on the investigation of the effects of BM extract (CDRI-08) on the expression of synaptic plasticity-related AMPA receptor subunit GluR2 and its epigenetic regulation in recognition memory of PD mice, which may be associated with the improved motor and related cognitive function.

## Materials and methods

### Chemicals

All Chemicals and reagents used for the experiments were of analytical grades and obtained from Sigma-Aldrich (USA), Antibodies were purchased from ABclonal (USA) or CST (USA). The HRP conjugated secondary antibody (Goat Anti-Rabbit IgG) was obtained from Elab Sciences (USA) and the HRP-labelled anti- $\beta$ -Actin antibody and TRI reagent were procured from Sigma-Aldrich (USA). The RevertAid First Strand cDNA Synthesis Kit and Maxima SYBR Green qPCR Master Mix (2X) were purchased from Thermo Fisher Scientific, (USA). Customized gene-specific primers for RT-PCR were synthesized and obtained from GENE to PROTEIN Pvt.Ltd. (INDIA). Bacopamonnieri extract (CDRI-08) was provided by Lumen Pharmaceuticals, Chennai, India as a gift.

### Animals

The inbred Swiss albino male mice of age  $12 \pm 4$  weeks maintained in the mice colony at the Department of Zoology, Institute of Science, Banaras Hindu University, Varanasi, India at  $24 \pm 2^\circ\text{C}$  with a 12-hour light/dark cycle was maintained for the experiments. Standard mice feed and water was given *ad libitum*. Experiments were performed by sacrificing them following the guidelines approved by the Institutional Animal Ethical Committee of the department for handling and use of experimental animals (IAEC Approval Reference No. BHU/DoZ/IAEC/2019-2020/002) and CPCSEA, Govt. of India.

### Induction of PD and tissue preparation

PD was induced in the mice by subcutaneous injection of rotenone at the dose determined by the pilot experiment as described below. For further experiments, the following batches of mice were prepared: Batch 1-Consisted of the mice ( $n = 6$ ) treated with vehicle DMSO and 5% Tween-80 subcutaneously and designated as control group (Cont); Batch 2- Included the vehicle control mice ( $n = 12$ ) were administered subcutaneously with freshly prepared standardized dose of rotenone in DMSO medium (2mg/kg BW) once daily for a

period of 14 days. The drug treatment was given at the same time. The mice of this batch were designated as rotenone-treated mice representing PD mice (Rot); Batch 3 consisted of 6 mice randomly removed from Batch 2 (Rot) and were orally administered with a standardized dose of *Bacopa monnieri* extract (BME)(CDRI-08) dissolved in 5% Tween-80 (200mg/kg BW) once daily for 21 days using a metallic gavage. The said treatment was given at the same time and represented BME-treated mice (R + B) (Fig. 1).

Thereafter, behavioural studies were carried out to analyze the symptoms of PD and memory alterations using suitable methods described in the methodologies section below. After the behavioural studies were completed, the mice were euthanized by cervical dislocation. The brain tissue was removed by careful removal of the skull bone, maintained in ice-cold normal saline, cleaned of the adherent blood, blotted dry within the folds of Whatman 1M filter paper and the hippocampus was carefully dissected out on ice and stored at -80°C Ultra freezer for the further study.

## Rotarod test

The Rotarod test was performed for analyzing the locomotor functions in the mice of all experimental batches. All animals underwent pre-training on the Rotarod equipment separately to ensure steady performance. The training was divided into three sessions over the course of two days, with three different test trials for a duration of 120 seconds each, with a 5-minute break between each trial. The training was given at a speed of 5 RPM on Day 1 followed by 10 and 15 RPM on Day 2, once in the morning followed by once in the afternoon, respectively. On Day 3, the final test was conducted (three sessions, for a duration of 120 seconds each) at 15 RPM. Data were recorded as mean latency to fall [26] in three trials which represented the time duration the animal remained on the rotating rod before losing balance and falling off.

## Novel objects recognition (NOR) test

NOR test was conducted to assess the recognition memory consolidation in mice following the method as described earlier [27, 28] (Fig. 2a). The tests were performed in three phases lasting for three consecutive days. In phase 1 on Day 1, animals were subjected to habituation in an empty wooden box (40×50×50 cm) which was deeper in colour than the animal body colour for 5 min followed by familiarisation for 5 minutes with 2 similar objects of similar shape but different shapes in phase 2 on Day 2 and then the testing phase 3 on Day 3. On the test day, one familiar object was replaced with a novel object, and mice were allowed to explore both objects for a duration of 5 min. Prior to each test, both the objects and box were cleaned with 70% ethanol to get rid of the smell left behind by the previous mouse. An overhead camera was used for the recording of the behaviour of mice during the test, and the video-recorded track was subjected to ANY-maze software Version 4.95 (Stoelting Co., USA) to analyze the data. For calculating the percent time

spent with both the objects,  $TNov / (TNov + TFam) \times 100$  and  $TFam / (TNov + TFam) \times 100$  formula was used where TFam is the time spent with familiar and TNov is the time spent with novel objects and the Discrimination index (DI) was used as the measure of the ability of the mice to discriminate between novel and familiar objects and was calculated as  $TNov - TFam / TNov + TFam$ .

## Antioxidant enzyme assay

The enzymatic activities of antioxidant enzymes SOD and GPx were studied by In-gel assay method as described previously [29]. Briefly, a 10% homogenate of the pooled hippocampus tissue was prepared in a buffer containing 50 mM Tris-HCl pH 7.5, 150mM NaCl, 1mM PMSF and centrifuged at  $12,000 \times g$  for 20 min at 4°C and the supernatant was removed. The protein in the supernatant was estimated using Bradford method [30], and an equal amount of protein (40 µg) was loaded and electrophoresed on a pre-run 10% native polyacrylamide gel at 4°C. Thereafter, the gel was washed with dH<sub>2</sub>O and stained in a staining solution (1.23 mM Nitro blue tetrazolium (NBT), 28 mM Tetramethylethylenediamine (TEMED), and 0.28 mM Riboflavin) in dark for 40 minutes followed by exposure to day light until the appearance of achromatic bands with a dark purple-blue stained background of the gel. In order to assay the GPx activity, the in-gel assay was performed as stated above. The protein sample prepared was resolved on a 10% native PAG as described above. The gel thereafter was incubated in a medium containing 50mM Tris-Cl pH 7.9, 13mM GSH, and 0.004% H<sub>2</sub>O<sub>2</sub> for 30 minutes. Thereafter, the gel was rinsed with dH<sub>2</sub>O and stained in the staining solution (1.2 mM NBT and 1.6 mM PMS) for 10–15 min in the dark. Subsequently, the gel was exposed to light in order to develop the achromatic bands due to GPx activity. The images of the gels were captured separately using the Alpha Innotech Gel Doc system (USA) and subjected to densitometric analysis.

## Determination of total reactive oxygen species (ROS)

The total ROS content in the hippocampus was determined using the method described earlier [31] with slight modification. Briefly, a 10% homogenate of the pooled hippocampus was prepared in Tris buffer (20 mM Tris-Cl pH 7.5). The homogenate was centrifuged at  $12,000 \times g$  at 4°C, the supernatant was carefully removed. In order to measure the ROS content, 40 µg of sample from each group was added to a reaction mix containing 2 mM 2', 7'-dichlorofluoresceindiacetate (DCFDA) per well in a multi well plate and was incubated for 30 min at 37°C in the dark to convert the inert DCFDA into a highly fluorescent 2', 7'-dichlorofluorescein (DCF) by producing the ROS. The level of the total ROS was detected in terms of the DCF fluorescence level at 485 nm (excitation) and 530 nm (emission) using a multimode reader (SpectraMax iD3, USA).

## Malondialdehyde (MDA) assay

The MDA assay was carried to measure the level of lipid peroxidation using the method reported earlier [32, 33]. Briefly, a 10% homogenate of the hippocampus tissue was prepared in Tris-Cl buffer (20 mM Tris-Cl, pH 7.4) medium. 50ul of the extract was incubated with 1 ml of Tris-Maleate buffer (0.2 M, pH 5.9) at 37°C for 30 min. followed by the addition of 0.8% of Thiobarbituric acid (TBA). The resultant mix was heated on boiling water with air-tight caps for 10 min and the mixture was allowed to cool. Thereafter, the pyridine- *n*-butanol mixture (3:1 v/v) and 1 N (w/v) NaOH were added to the above and thoroughly mixed. The mix thereafter was allowed to stand at RT for 10 to 15 min. The level of MDA was measured at A<sub>548</sub> and the result was expressed as nmole MDA/g wet weight of tissue.

## Western blotting

A 10% homogenate was prepared in RIPA buffer as described earlier [29]. Briefly, the homogenate was centrifuged at 12,000Xg for 20 min at 4°C. The supernatant containing the total protein was carefully removed and the protein content was measured by the Bradford method [30]. A uniform amount of protein (50µg) was denatured on boiling water, mixed with 1x sample loading solution (10% SDS, 500Mm DTT, 50% Glycerol, 500mM Tris-HCL, 0.05% bromophenol blue) and was resolved by 15% SDS-PAGE for  $\beta$ -Syn and 8% SDS-PAGE for GluR2. The gel was cleaned by sterile dH<sub>2</sub>O subjected to electro-transfer onto PVDF membrane (Millipore, USA) at 50 volts at 4°C for the overnight period. Thereafter, the blotted membrane was blocked with 5% (w/v) non-fat milk in Tris-buffered saline containing 0.1% Tween 20 (1X TBST) for three hours. The membranes were incubated with primary anti-  $\beta$ -Syn(1:1000) or anti-GluR2(1:1000) antibodies overnight at 4°C. The membranes were then incubated with a secondary antibody (1:2000) for two hours after washing with 1X TBST three times for 5 minutes each. Thereafter, the signal was detected on the X-ray film using ECL reagents. Following the stripping, the above membranes were used for the detection of  $\beta$ -Actin as an internal control using HRP-conjugated anti- $\beta$ -Actin antibody (1:2000).

## RNA Extraction and Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was isolated from the pooled hippocampus obtained from the mice of different groups separately using TRI reagent in accordance with the manufacturer's instructions and its content was spectrophotometrically measured using Nanodrop system (Thermo Scientific NanoDrop One, USA), and its purity/integrity was measured by A<sub>260/280</sub> ratio, and integrity was examined by agarose gel electrophoresis, respectively. 5µg of the total RNA was reverse transcribed to cDNA using the cDNA Synthesis Kit (Thermo Fisher Scientific, USA). For the qPCR, the  $\beta$ -Syn (F- 5'-GAGGGAGTCGTTTCATGGAGT-3'; R- 3'-CATTTGTCACTTGCTCTTTGG-5') and GluR2 (F- 5'-CAGTGCATTTCCGGGTAGG-3'; R- 3'-

TTGGTGACTGCGAACTG-5') primers, respectively were used for synthesis of cDNA as templates. The SYBR Green qPCR Master Mix (2X) (Thermo Fisher Scientific, USA) was employed following the supplier's manual. The cDNA for the  $\beta$ -Actin (F- 5'-CTGTCCCTGTATGCCTCTG-3'; R- 3'-ATGTCACGCACGATTTC-5') was prepared as described above as an internal control to ensure normalization. Change in mRNA level of the above genes was calculated as relative fold change with respect to control which was derived using the  $2^{-\Delta\Delta CT}$  method.

## Methylated DNA immunoprecipitation (MeDIP) assay

The level of methylated cytosine in the CpG sequences of the GluR2 promoter was studied using the methylated DNA immunoprecipitation (MeDIP) technique [34–36]. Briefly, the intact genomic DNA was extracted from the hippocampus by the phenol-chloroform method as mentioned above. 10–20  $\mu$ g of the DNA was diluted in TE buffer (10 Tris-Cl, pH 8.0 and 1 mM EDTA) and was subjected to sonication (Sonics Vibra cell, USA) for 5 minutes with a pulse of 30 s at 30% amplitude at 4°C to ensure fragmentation (300–1000 bp fragments). The size of the fragmented DNA was measured by resolving them on 1% agarose gel. Thereafter, the DNA was denatured in boiling water for 10 min, immediately cooled on ice for 5 min, and was divided into input and immunoprecipitation (IP) fractions. In the case of the IP fraction, 100  $\mu$ L DNA was diluted in 10x IP buffer (100 mM sodium phosphate buffer, pH 7.0, 1.4 M NaCl, 0.5% Triton X-100) and 2  $\mu$ g of anti-5-mC antibody and mixed on a rotating platform at low speed at 4°C overnight. Next day, 50  $\mu$ L of pre-washed Protein A-Sepharose affinity resin slurry was added to the mixture and incubated at 4°C under slow rotation for 4 h. Thereafter, the suspension was centrifuged at 6000x g for 10 minutes at 4°C, and the supernatant was carefully discarded. The pellet containing the resin beads was then washed with 1x IP buffer and was suspended in freshly prepared protein digestion buffer [50 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0, and 0.5% SDS] containing Proteinase K [10  $\mu$ g/ml; Qiagen, Netherlands). After the digestion reaction was over, DNA was isolated using the phenol-chloroform extraction method as mentioned above. The DNA thereafter was air-dried and dissolved in 60  $\mu$ L of the TE buffer. The GluR2 promoter-specific primers (F-5'-ATCGAATGCGCAAGACTGGA – 3', R-5'-GAAAGTCACAGAGAGGGGCA – 3') were custom prepared and added to the above in order to amplify the targeted 5-mC containing promoter DNA sequence employing the qRT-PCR method and was normalized using input DNA.

## Statistical analysis

For behavioural studies, each experimental group consisted of 6 mice. The western blot, In-gel assay, and qRT-PCR data were presented as a bar diagram representing mean  $\pm$  SEM of data (RDV, IDV, and fold change respectively) obtained from three independent experiments. For the statistical analysis, Origin Pro Graphing and Analysis Software, USA

was used and data were analyzed by one-way ANOVA followed by post hoc Tukey's test.  $p \leq 0.05$  were considered significant.

## Results

### Effects of rotenone on the motor and cognitive behaviour and alterations by CDRI-08

As rotenone has been reportedly used for developing PD-like symptoms in animal models mimicking the same in human PD patients, it has been used in the current work to generate PD mice models. The mice treated with the drug represent the PD mice. In order to verify the model, the rotenone-treated mice were subjected to a locomotor test as described in the methodology section. Our behavioural data support the symptoms of PD i.e. locomotor disorder as described earlier [26]. Our rota rod test data reveal that the subcutaneous injection of a standardized dose of rotenone caused significantly less prolonged movement on the rotating rod of the equipment (reduced latency) compared to the vehicle-treated control mice ( $p < 0.05$ ). Our data clearly conforms to the results reported earlier [26]. CDRI-08 treated rotenone administered mice exhibited significantly prolonged and sustained movement on the rotating rod and the reduction in latency to fall was mitigated ( $p < 0.05$ ). Our data thereby suggest the development of a successful PD mice model and BM extract CDRI-08 has beneficial effects on balanced motor coordination in PD mice (Fig. 1).

In order to examine whether the PD mice model developed in the current work also possesses cognitive impairment, the control, rotenone treated and CDRI-08 treated PD (rotenone treated) mice were tested for any alterations in the recognition memory using a well-established Novel object recognition (NOR) test as described earlier [27, 28]. The results (Fig. 2) showed that PD mice spent a significantly lower percentage of time with novel objects (35.44%) than the familiar objects (64.55%) as compared to the performance by control mice (88.63% and 11.36% respectively) (Fig. 2b) with a significantly lower discrimination index (DI) (Fig. 2c) due to their diminished ability of discrimination between the novel and familiar objects. However, CDRI-08 treated PD mice significantly improved their DI ( $p < 0.001$ ) (Fig. 2c) with the significantly increased percentage of time spent with the novel objects (76.11%) than the familiar objects (23.8%) (Fig. 2b).

### Effects of rotenone on the activities of SOD and GPx and alteration by CDRI-08

As rotenone is a known inhibitor of the mitochondrial electron transport chain and thereby leads to oxidative stress, we, therefore, examined the activities of two major antioxidant enzymes, SOD and GPx by polyacrylamide gel-based assay. Our in-gel assay data revealed

that the rotenone significantly reduced the activities of SOD and GPx (Fig. 3a and b) ( $p = < 0.001$  and  $0.05$ , respectively) in the hippocampus compared to control mice. The CDRI-08 treatment to the rotenone-treated mice enhanced the activities of both the anti-oxidant enzymes substantially (SOD and GPx in PD mice (56.85% and 77.35%, respectively) towards their levels in control mice (Fig. 3a & 3b).

## Effects of CDRI-08 on the rotenone-induced alterations in the levels of oxidative stress

In order to validate the effect of rotenone-induced decline in the activities of SOD and GPx whether they lead to oxidative stress in the brain, levels of total ROS and lipid peroxidase activities were measured, and the effects of CDRI-08 were studied. We observed that rotenone significantly increased the level of lipid peroxidase activity at the level of malonaldehyde (MDA) ( $p < 0.001$ ) and total ROS ( $p < 0.05$ ) in the hippocampus compared to their values in the control mice. CDRI-08 treatment to rotenone-treated mice significantly mitigated the lipid peroxidase activity and total ROS level which was found significantly high in Rotenone-PD mice with respect to control animals (Fig. 4a and 4b) indicating the antioxidant properties of the CDRI-08.

## Effects of rotenone and CDRI-08 on $\alpha$ -Syn and AMPA receptor GluR2 subunit expression

Our Western blotting data on the expression of  $\alpha$ -Syn revealed that its level is abruptly upregulated by rotenone compared to its level in the vehicle-treated control mice ( $p = < 0.001$ ). The treatment of rotenone-treated mice with CDRI-08 significantly downregulated the expression towards the vehicle-treated control mice ( $p = < 0.05$ ) (Fig. 5a and 5b). The qRT-PCR data on the expression of the  $\alpha$ -Syn gene at transcript level, its expression is similarly increased by three-fold ( $p = < 0.05$ ) which corresponds to the protein level data in the hippocampus after the treatment with rotenone (Fig. 5c). The CDRI-08 treatment to rotenone-treated mice significantly downregulated its expression ( $p = < 0.05$ ) as was evident in case of its effect on the  $\alpha$ -Syn protein level.

Figure 6a shows the immunoblot patterns of the expression level of the AMPA receptor GluR2 subunit. The densitometric scan data obtained from the image clearly suggested that the rotenone treatment resulted in a significant decline in the expression of GluR2 protein compared to the control mice ( $p < 0.05$ ). The administration of CDRI-08 to the rotenone-treated mice significantly upregulated the level of GluR2 ( $p < 0.05$ ) (Fig. 6b). Figure 6c shows the pattern of alterations in the GluR2 transcript as revealed by qRT-PCR. The RT-PCR data are expressed at the level of fold changes in transcript level in respect to endogenous  $\beta$ -actin transcript. The qRT-PCR data revealed that the rotenone treatment significantly downregulated the level of GluR2 transcript as compared to the control mice

( $p < 0.05$ ). However, after the treatment of CDRI-08 to the rotenone-treated mice, the level of GluR2 transcript slightly increased but the effect was found to be not significant (NS).

## Effects of rotenone and CDRI-08 on AMPA receptor GluR2 promoter methylation status

In order to study whether alteration in the expression of GluR2 due to rotenone and CDRI-08 is associated with epigenetic changes in its cognate promoter CpG methylation, the MeDIP-qPCR was carried out. Our MeDIP-qPCR data revealed that rotenone treatment resulted in a significant increase in the level of GluR2 promoter CpG methylation in the hippocampus than that of the control mice (5.9 folds;  $p < 0.05$ ). The methylation level of the GluR2 promoter DNA was found to be significantly reduced by 1.17-fold ( $p < 0.05$ ) i.e., it declined towards that in the control mice after the administration of CDRI-08 to the rotenone-treated mice (Fig. 7).

## Correlation between GluR2 promoter DNA methylation and AMPA receptor GluR2 subunit expression

Figure 8 shows the correlation between the level of GluR2 expression and the methylation status of its cognate promoter. Our correlation data suggests that as the expression of the GluR2 is higher, the level of its promoter methylation is low. In the case of rotenone-treated condition, the expression level of the GluR2 subunit is lower which is correlated with higher methylation of its promoter CpG. After CDRI-08 treatment to the rotenone-treated mice, the level of GluR2 expression is upregulated with the significantly declined level of its promoter methylation. Our results clearly suggest that there is a negative correlation between GluR2 expression and its promoter DNA methylation ( $r = -0.904$ ).

## Discussion

PD is a movement disorder and is characterized by neurodegeneration and an oxidative stress-related etiology [37]. Various genetic and neurotoxin-based animal models are employed to study the disease progression of PD [38]. However, these models fail to replicate the persistent progression and substantial pathological involvement observed with Parkinson's disease in humans. Rotenone is a naturally occurring insecticide extracted from the roots of several plant species [39]. The rotenone-based animal models have recently gained particular attention as it replicates most of the histopathological features of PD such as Lewy bodies and motor symptoms [40]. Rotenone exerts its action by inhibiting complex one of the electron transport chains in mitochondria. This inhibition impairs ATP production which in turn affects the functioning of the sodium-potassium ATPase pump causing dysregulation in calcium homeostasis and inducing excitotoxicity

leading to enhanced oxidative stress [41]. All these factors contribute to the aggregation of misfolded  $\alpha$ -Syn protein [42] and its failure to degradation leads to the death of dopaminergic neurons and the onset of motor and cognitive dysfunctions. However, the precise mechanism of their loss is still unknown.

At present, there is no definitive cure for PD, and the current treatment options mainly involve dopamine agonists and monoamine oxidase-B (MAO-B) inhibitors which only offer symptomatic relief from motor symptoms and very little help in improving the non-motor symptoms of the disease [43]. Even then they come with the cost of problematic adverse side effects. Recognizing the promising potential demonstrated by natural products in addressing various health issues, there is an urgent need to explore therapeutic possibilities of herbs, extracts from medicinal plants, and other botanical substances in the context of neurological disorders. In this pursuit, certain herbs have proven to be neuroprotective agents. One such herb is *Bacopa monnieri*, belonging to the Scrophulariaceae family, commonly referred to as Brahmi, which is an ayurvedic medicinal plant with known antioxidant, antidepressant, anti-inflammatory, and antimicrobial abilities [43]. CDRI-08 has proven to be effective in memory recovery following hypoxia-induced impairment [44]. Additionally, studies have shown that the Bacopa extract modifies GABA receptor function in the cortex of rats with epilepsy [45]. Furthermore, it elevates the presence of NMDAR1 on the postsynaptic membrane in different brain regions associated with memory in a rat model of schizophrenia [46]. Previous research also suggests that CDRI-08 could potentially restore spatial memory induced by STZ by modifying either the structure, properties, or quantity of AMPA receptors and consequently, altering the characteristics of the postsynaptic membrane in the hippocampus [9]. Therefore, extracts derived from this plant, CDRI-08, have demonstrated cognitive-enhancing effects in animal studies but the precise mechanism in general and the epigenetic mechanism in particular by which it exerts its effect is not yet known. As a result, we conjectured that it might yield advantageous outcomes in enhancing both the motor and non-motor manifestations of PD, with a specific focus on alleviating cognitive decline. We delved into the epigenetic processes associated with the memory-linked gene AMPA receptor GluR2 subunit to ascertain whether it could counteract the changes, as these processes are dynamic and reversible, and potentially serve as a therapeutic intervention for PD.

The data from our rotarod experiment indicate that CDRI-08 enhanced balance and motor coordination in mice injected with rotenone. This improvement holds potential as a therapeutic approach for alleviating the motor symptoms witnessed in PD. Also, SOD and GPx are antioxidant enzymes that not only play a fundamental but also a vital role and serve as a first line of defense of the antioxidant protective capability of biological systems against free radical assault [47]. Reactive oxygen species (ROS) initiate the oxidation of diverse biological macromolecules including proteins, lipids, and nucleic acids. This

process leads to alterations in the structure and function of these molecules. The oxidation of lipids results in the creation of hydroperoxides, which then break down into various reactive compounds such as malonaldehyde (MDA) which then serves as a measurement of lipid peroxidation and thus the total oxidative stress [48]. In our study, the results obtained from the In-gel assay, used to assess the enzymatic activity of SOD and GPx, indicated a reduction in their activity levels in mice with rotenone-induced PD compared to the control mice group which was restored to normal, following CDRI-08 treatment. Also, our results from the lipid peroxidation assay and total ROS estimation suggest that CDRI-08 was able to alleviate MDA and total ROS levels in the PD mice group further supporting its antioxidant characteristics.

Despite the fact that the discovery of the important function of  $\alpha$ -Syn in the etiology of PD is already twenty-five years old, it remains an important juncture in PD research [49]. Our study demonstrates that CDRI-08 mitigates the increased aggregation of  $\alpha$ -Syn observed in the group of mice with PD compared to the control group. This effect is apparent from the levels of protein and mRNA expression. These findings could potentially unveil a novel neuroprotective action for CDRI-08 suggesting its possible utility as a therapeutic option for synucleinopathies, which encompass PD. Backed by existing literature, it is now clear that progressive PD is linked to cognitive decline. Our findings further confirm this by indicating compromised recognition memory in mice with PD, a conclusion substantiated by the NOR test. CDRI-08 exhibited the potential to improve this condition, as indicated by the discrimination index (DI). This index measures the capability of mice to differentiate between a novel and a familiar object. Mice possessing unimpaired recognition memory tend to allocate more time to explore the novel object compared to the familiar one, guided by their inherent ability, and the same pattern was observed following the administration of CDRI-08 suggesting its memory-boosting ability and thereby improved cognition.

The GluR2 subunit of AMPAR is responsible for key biophysical properties of the receptor at the glutamatergic synapse in the hippocampus. It significantly influences receptor assembly, trafficking, and has a critical role in various types of long-term synaptic changes [50]. These GluR2-containing AMPA receptors have recently gained considerable interest for their implications in modulating synaptic transmission activity. In this context, we investigated epigenetic mechanisms, particularly promoter DNA methylation regulating the expression of the GluR2 subunit of AMPAR, and the implication of CDRI-08 on this mechanism. Our data indicates decreased expression of GluR2 in PD with respect to control which was upregulated after CDRI-08 treatment as evidenced by our Western blotting data and qRT-PCR data. However, the level of increase in GluR2 transcript was not significantly evident from our qRT-PCR data which may indicate the high turnover rate of GluR2 transcripts. The half-life of mRNA molecules can vary greatly. Some mRNAs are very stable and can persist in the cell for an extended period, allowing for sustained

protein production even when transcription rates are low. Conversely, other mRNAs may have a short half-life and degrade quickly after transcription, resulting in low RNA transcript levels. DNA methylation, a widely studied epigenetic process, plays a pivotal role in regulating the transcription of genes associated with memory, often leading to transcriptional suppression and a decline in gene activity [51]. Our MeDIP-qPCR data revealed decreased methylation level at the CpG islands of the promoter of the GluR2 gene after CDRI-08 administration, which was elevated in rotenone-treated PD mice. Thus, there is an inverse correlation between DNA methylation and expression of memory-associated gene GluR2 as indicated by our correlation analysis (Fig. 8) suggesting CDRI-08 reverses DNA methylation which serves as a contributing mechanism in improving memory and hence cognition.

## Conclusion

From our data it can be concluded the possible role of epigenetic events especially promoter DNA methylation in modulating the GluR2 gene associated with memory impairment in rotenone-induced cognitive decline in PD. The *Bacopa monnieri* extract, CDRI-08 administration to rotenone-PD mice effectively counteracts the PD-related pathological hallmarks and loss of recognition memory. Consequently, our comprehensive results propose CDRI-08 as a neuroprotective therapeutic agent that has potential to alleviate cognitive impairment in PD mice through targeted DNA methylation intervention.

## Declarations

### Acknowledgments

The work was supported by ICMR, CSIR Govt. of India and Institute of Eminence (IoE) program, Banaras Hindu University to SP. The authors gratefully acknowledge Central Instrumentation Facilities of CAS and DST-FIST in Department of Zoology and DBT-ISLS, Faculty of Science, BHU. GC thanks CAS Program in Zoology, Banaras Hindu University for Junior Research Fellowship.

### Authors' Contribution

**Garima Chandra:** Wrote the draft manuscript, Executed experiments, Acquired data, Captured Image Carried out statistical analysis and validation,

**S. Prasad:** Developed concept, Formulated project, Procured Fund, Planned and designed experiments, Interpreted data, Edited and finalized manuscript,

**Both authors:** Planned experiments and contributed preparation and finalization of the manuscript.

**Funding:** Financial support from Indian Council of Medical Research (ICMR), CSIR Govt. of India, Institute of Eminence (IoE) program, Banaras Hindu University Centre of Advanced Study Program (CAS) in Zoology

**Data Availability:** All the data generated during the experiments are incorporated in the manuscript and will be made available on reasonable request from the corresponding author.

### **Ethics Approval**

All the experiments were carried out in accordance with the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India, and approved by the Institutional Animal Ethical Committee of the Department of Zoology, Banaras Hindu University (Registration no. 1802/G0/Re/S/15/CPCSEA).

**Consent to Participate:** Not applicable.

**Consent for Publication:** Authors approve this manuscript and give their consent for publication.

**Competing Interests** The authors declare no competing interests.

## **References**

1. Aarsland D, Batzu L, Halliday GM, Geurtsen GJ, Ballard C, Ray Chaudhuri K, Weintraub D (2021) Parkinson disease-associated cognitive impairment. *Nat Rev Dis Primers* 7:47. <https://doi.org/10.1038/s41572-021-00280-3>
2. Zaman V, Shields DC, Shams R, Drasites KP, Matzelle D, Haque A, Banik NL (2021) Cellular and molecular pathophysiology in the progression of Parkinson's disease. *Metab Brain Dis* 36:815-827. <https://doi.org/10.1007/s11011-021-00689-5>
3. Hwang O (2013) Role of oxidative stress in Parkinson's disease. *Exp Neurobiol* 22:11-17. <https://doi.org/10.5607/en.2013.22.1.11>
4. Bosco DA, Fowler DM, Zhang Q, Nieva J, Powers ET, Wentworth P, Lerner RA, Kelly JW (2006) Elevated levels of oxidized cholesterol metabolites in Lewy body disease brains accelerate  $\alpha$ -synuclein fibrilization. *Nat Chem Biol* 2:249-253. <https://doi.org/10.1038/nchembio782>
5. Nakabeppu Y, Tsuchimoto D, Yamaguchi H, Sakumi K (2007) Oxidative damage in nucleic acids and Parkinson's disease. *J Neurosci Res* 85:919-934. <https://doi.org/10.1002/jnr.21191>
6. Chang KH, Chen CM (2020) The role of oxidative stress in Parkinson's disease. *Antioxidants (Basel)* 9:597. <https://doi.org/10.3390/antiox9070597>

7. Poewe W (2008) Non-motor symptoms in Parkinson's disease. *Eur J Neurol* 15:14-20.  
<https://doi.org/10.1111/j.1468-1331.2008.02056.x>
8. Salpietro V, Dixon CL, Guo H, Bello OD et al (2019) AMPA receptor GluA2 subunit defects are a cause of neurodevelopmental disorders. *Nat Commun* 10:3094  
<https://doi.org/10.1038/s41467-019-10910-w>
9. Pandey SP, Singh HK, Prasad S (2015) Alterations in hippocampal oxidative stress, expression of AMPA receptor GluR2 subunit and associated spatial memory loss by *Bacopa monnieri* extract (CDRI-08) in streptozotocin-induced diabetes mellitus type 2 mice. *PLoS One* 10:e0131862. <https://doi.org/10.1371/journal.pone.0131862>
10. Kim S, Kaang BK (2017) Epigenetic regulation and chromatin remodeling in learning and memory. *Exp Mol Med* 49:e281. <https://doi.org/10.1038/emm.2016.140>
11. Kwapis JL, Wood MA (2014) Epigenetic mechanisms in fear conditioning: Implications for treating post-traumatic stress disorder. *Trends Neurosci* 37:706-720.  
<https://doi.org/10.1016/j.tins.2014.08.005>
12. Maity S, Farrell K, Navabpour S, Narayanan SN, Jarome TJ (2021) Epigenetic mechanisms in memory and cognitive decline associated with aging and Alzheimer's disease. *Int J Mol Sci* 22:12280. <https://doi.org/10.3390/ijms222212280>
13. Hwang JY, Aromolaran KA, Zukin RS (2017) The emerging field of epigenetics in neurodegeneration and neuroprotection. *Nat Rev Neurosci* 18:347-361.  
<https://doi.org/10.1038/nrn.2017.46>
14. Bhattacharya SK, Bhattacharya A, Kumar A, Ghosal S (2000) Antioxidant activity of *Bacopa monniera* in rat frontal cortex, striatum and hippocampus. *Phytother Res* 14:174-179. [https://doi.org/10.1002/\(sici\)1099-1573\(200005\)14:3<174::aid-ptr624>3.0.co;2-o](https://doi.org/10.1002/(sici)1099-1573(200005)14:3<174::aid-ptr624>3.0.co;2-o)
15. Sairam K, Rao CV, Babu MD, Goel RK (2001) Prophylactic and curative effects of *Bacopa monniera* in gastric ulcer models. *Phytomedicine* 8:423-430.  
[https://doi.org/10.1078/S0944-7113\(04\)70060-4](https://doi.org/10.1078/S0944-7113(04)70060-4)
16. Sumathy T, Govindasamy S, Balakrishna K, Veluchamy G (2002) Protective role of *Bacopa monniera* on morphine-induced brain mitochondrial enzyme activity in rats. *Fitoterapia* 73:381-385. [https://doi.org/10.1016/s0367-326x\(02\)00114-4](https://doi.org/10.1016/s0367-326x(02)00114-4)
17. Russo A, Borrelli F (2005) *Bacopa monniera*, a reputed nootropic plant: An overview. *Phytomedicine* 12:305-317. <https://doi.org/10.1016/j.phymed.2003.12.008>
18. Singh HK, Dhawan BN (1997) Neuropsychopharmacological effects of the Ayurvedic nootropic *Bacopa monniera* Linn. (Brahmi). *Indian J Pharmacol* 29:359-365.
19. Prisila Dulcy C, Singh HK, Preethi J, Emmanuvel Rajan K (2012) Standardized extract of *Bacopa monniera* (BESEB CDRI-08) attenuates contextual associative learning deficits in the aging rat's brain induced by D-galactose. *J Neurosci Res* 90:2053-2064.  
<https://doi.org/10.1002/jnr.23080>

20. Liu X, Liu F, Yue R, Li Y, Zhang J, Wang S, Zhang S, Wang R, Shan L, Zhang W (2013) The antidepressant-like effect of bacopaside I: Possible involvement of the oxidative stress system and the noradrenergic system. *PharmacolBiochemBehav* 110:224–230. <https://doi.org/10.1016/j.pbb.2013.07.007>
21. Russo A, Borrelli F, Campisi A, Acquaviva R, Raciti G, Vanella A (2003) Nitric oxide-related toxicity in cultured astrocytes: Effect of *Bacopa monniera*. *Life Sci* 73:1517–1526. [https://doi.org/10.1016/S0024-3205\(03\)00476-4](https://doi.org/10.1016/S0024-3205(03)00476-4)
22. Channa S, Dar A, Anjum S, Yaqoob M, Atta-ur-Rahman (2006) Anti-inflammatory activity of *Bacopa monniera* in rodents. *J Ethnopharmacol* 104:286–289. <https://doi.org/10.1016/j.jep.2005.10.009>
23. Chakravarty AK, Garai S, Masuda K, Nakane T, Kawahara N(2003) Bacopasides III-V: three new triterpenoid glycosides from *Bacopa monniera*. *Chem Pharm Bull* 51:215–217. <https://doi.org/10.1248/cpb.51.215>
24. Abdul Manap AS, Vijayabalan S, Madhavan P, Chia YY, Arya A, Wong EH, Rizwan F, Bindal U, Koshy S (2019) *Bacopa monnieri*, a Neuroprotective Lead in Alzheimer Disease: A Review on Its Properties, Mechanisms of Action, and Preclinical and Clinical Studies. *Drug Target Insights* 13:1177392819866412. <https://doi.org/10.1177/1177392819866412>
25. Vishwas S, Gulati M, Kapoor B, Gupta S et al (2021) Expanding the Arsenal Against Huntington's Disease-Herbal Drugs and Their Nanoformulations. *Curr Neuropharmacol* 19:957-989. <https://doi.org/10.2174/1570159X18666201109090824>
26. Deacon RMJ (2013) Measuring motor coordination in mice. *J Vis Exp*75:e2609. <https://doi.org/10.3791/2609>
27. Baghel MS, Thakur MK (2017) Differential proteome profiling in the hippocampus of amnesic mice. *Hippocampus* 27:845–859. <https://doi.org/10.1002/hipo.22735>
28. Singh P, Thakur MK (2014) Reduced recognition memory is correlated with decrease in DNA methyltransferase1 and increase in histone deacetylase2 protein expression in old male mice. *Biogerontology* 15:339–346. <https://doi.org/10.1007/s10522-014-9504-5>
29. Chandra G, Prasad S (2019) HDAC2 expression upregulation is associated with decreased anti oxidative stress enzymes in the cerebral cortex of drug-induced Parkinson's disease mouse model. *Ageing & society The Indian Journal of Gerontology*Vol. XXIX No. I-IV ISSN-0971-8060:43
30. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*72:248-254. <https://doi.org/10.1006/abio.1976.9999>
31. Das L, Vinayak M (2014) Long term effect of curcumin in regulation of glycolytic pathway and angiogenesis via modulation of stress activated genes in prevention of

- cancer. PLoS One 9:e99583. <https://doi.org/10.1371/journal.pone.0099583>
32. Placer ZA, Cushman LL, Connor B (1966) Estimation of product of lipid peroxidation (malonyl dialdehyde) in biochemical systems. *Anal Biochem* 16:359-364. [https://doi.org/10.1016/0003-2697\(66\)90167-9](https://doi.org/10.1016/0003-2697(66)90167-9)
  33. Singh S, Koiri RK, Trigun SK (2008) Acute and chronic hyperammonemia modulate antioxidant enzymes differently in cerebral cortex and cerebellum. *Neurochem Res* 33:103-113. <https://doi.org/10.1007/s11064-007-9422-x>
  34. Tost J (ed) (2009) DNA methylation methods and protocols. In: Springer Protocols Methods in Molecular Biology: 2nd edn. Humana Press, New York, pp 3-20
  35. Karpova NN, Umemori J (2016) Protocol for methylated DNA immunoprecipitation (MeDIP) analysis. In: *Neuromethods*. Humana Press Inc., pp 97-114
  36. Guerrero-Bosagna C, Jensen P (2015) Optimized method for methylated DNA immunoprecipitation. *Methods* 82:432-439. <https://doi.org/10.1016/j.mex.2015.10.006>
  37. Chithra Y, Dey G, Ghose V, Chandramohan V, Gowthami N, Vasudev V, Srinivas Bharath MM (2023) Mitochondrial complex I inhibition in dopaminergic neurons causes altered protein profile and protein oxidation: implications for Parkinson's disease. *Neurochem Res* 48:2360-2389. <https://doi.org/10.1007/s11064-023-03907-x>
  38. Khan E, Hasan I, Haque ME (2023) Parkinson's disease: exploring different animal model systems. *Int J Mol Sci* 24:9088. <https://doi.org/10.3390/ijms24109088>
  39. Miyazaki I, Asanuma M (2020) The rotenone models reproducing central and peripheral features of Parkinson's disease. *NeuroSci* 1:1-14. <https://doi.org/10.3390/neurosci1010001>
  40. Xiong N, Xiong J, Jia M, Liu L, Zhang X, Chen Z, Huang J, Zhang Z, Hou L, Luo Z, Ghoorah D, Lin Z, Wang T (2013) The role of autophagy in Parkinson's disease: Rotenone-based modeling. *Behav Brain Funct* 9:13. <https://doi.org/10.1186/1744-9081-9-13>
  41. Moradi Vastegani S, Nasrolahi A, Ghaderi S, Belali R, Rashno M, Farzaneh M, Khoshnam SE (2023) Mitochondrial Dysfunction and Parkinson's Disease: Pathogenesis and Therapeutic Strategies. *Neurochem Res* 48:2285-2308. <https://doi.org/10.1007/s11064-023-03904-0>
  42. Won SJ, Fong R, Butler N, Sanchez J, Zhang Y, Wong C, Tambou Nzoutchoum O, Huynh A, Pan J, Swanson RA (2022) Neuronal Oxidative Stress Promotes  $\alpha$ -Synuclein Aggregation In Vivo. *Antioxidants* 11:2466. <https://doi.org/10.3390/antiox11122466>
  43. Jadiya P, Khan A, Sammi SR, Kaur S, Mir SS, Nazir A (2011) Anti-Parkinsonian effects of *Bacopa monnieri*: Insights from transgenic and pharmacological *Caenorhabditis elegans* models of Parkinson's disease. *Biochem Biophys Res Commun* 413:605-610. <https://doi.org/10.1016/j.bbrc.2011.09.010>

44. Rani A, Prasad S (2015) A Special Extract of *Bacopa monnieri* (CDRI-08)-Restored Memory in CoCl<sub>2</sub>-Hypoxia Mimetic Mice Is Associated with Upregulation of Fmr-1 Gene Expression in Hippocampus. *Evid Based Complement Alternat Med* 2015:347978. <https://doi.org/10.1155/2015/347978>
45. Mathew J, Balakrishnan S, Antony S, Abraham PM, Paulose CS (2012) Decreased GABA receptor in the cerebral cortex of epileptic rats: effect of *Bacopa monnieri* and Bacoside-A. *J Biomed Sci* 19:25. <https://doi.org/10.1186/1423-0127-19-25>
46. Piyabhan P, Wetchateng T (2014) Neuroprotective effects of *Bacopa monnieri* (Brahmi) on novel object recognition and NMDAR1 immunodensity in the prefrontal cortex, striatum and hippocampus of sub-chronic phencyclidine rat model of schizophrenia. *J Med Assoc Thai* 97 Suppl 8:S50-S55.
47. Ighodaro OM, Akinloye OA (2018) First line defence antioxidants-superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX): Their fundamental role in the entire antioxidant defence grid. *Alexandria J Med* 54:287-293. <https://doi.org/10.1016/j.ajme.2017.09.001>
48. Ayala A, Muñoz MF, Argüelles S (2014) Lipid peroxidation: Production, metabolism, and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal. *Oxid Med Cell Longev* 2014:360438. <https://doi.org/10.1155/2014/360438>
49. Calabresi P, Mechelli A, Natale G, Volpicelli-Daley L, Di Lazzaro G, Ghiglieri V (2023) Alpha-synuclein in Parkinson's disease and other synucleinopathies: from overt neurodegeneration back to early synaptic dysfunction. *Cell Death Dis* 14:176. <https://doi.org/10.1038/s41419-023-05672-9>
50. Isaac JTR, Ashby M, McBain CJ (2007) The Role of the GluR2 Subunit in AMPA Receptor Function and Synaptic Plasticity. *Neuron* 54:859-871. <https://doi.org/10.1016/j.neuron.2007.06.001>
51. Ni C, Qian M, Geng J, Qu Y, Tian Y, Yang N, Li S, Zheng H (2020) DNA Methylation Manipulation of Memory Genes Is Involved in Sevoflurane Induced Cognitive Impairments in Aged Rats. *Front Aging Neurosci* 12:211. <https://doi.org/10.3389/fnagi.2020.00211>

## Figures

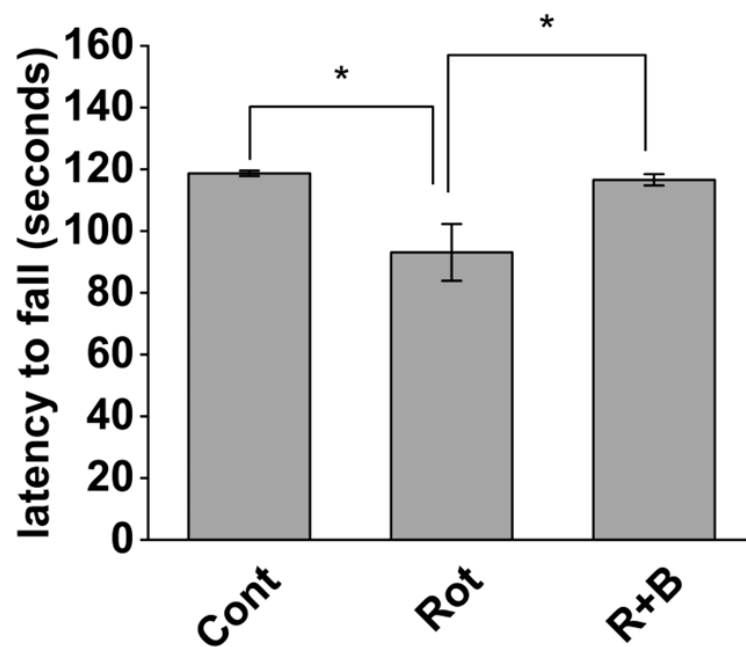


Figure 1

Rotarod test of the locomotor function of the control (Cont), rotenone-treated (Rot), and CDRI-08-treated Rotenone-mice. Histogram representing the result of the rotarod test. n = 6 mice per group. Data are shown as the mean  $\pm$  SEM. \*p < 0.05.

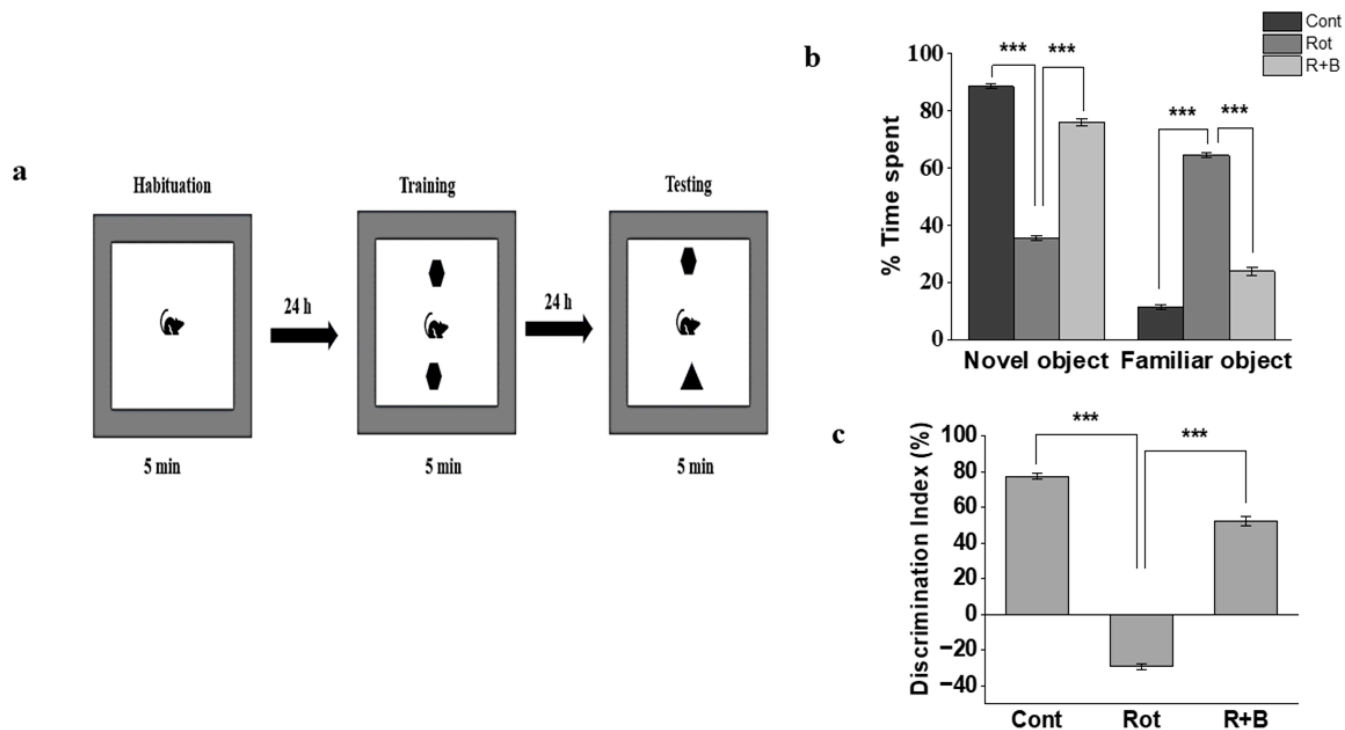


Figure 2

Novel objects recognition (NOR) test. a). Experimental strategy of test, b). % time spent with the novel and familiar objects by control, rotenone-treated and CDRI-08 treated rotenone-administered mice, c) Discrimination index (DI) of the mice of the experimental groups. (n=6/experimental group). Data are shown as the mean  $\pm$  SEM. \*\*\* $p < 0.001$

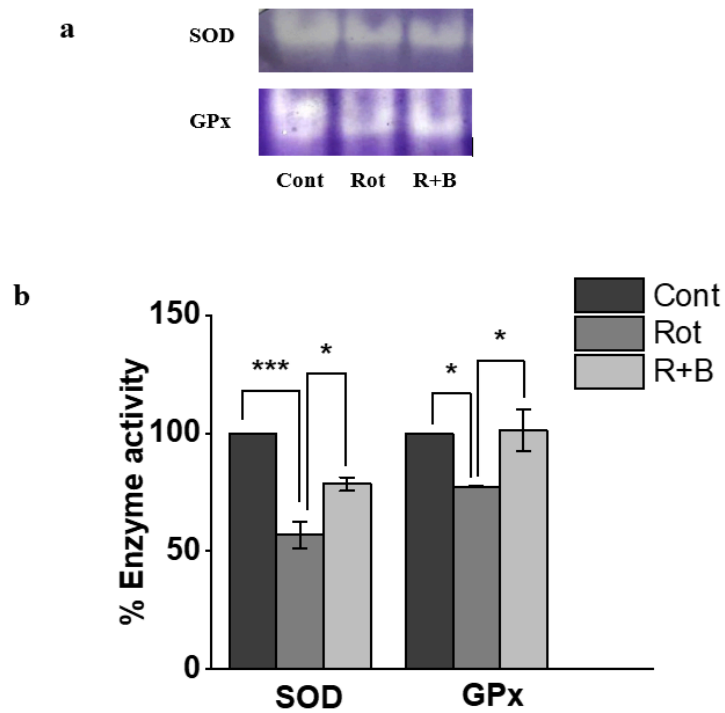


Figure 3

PAGE based in-gel assay of SOD and GPx. activities. a). Histogram showing densitometric scan data of SOD and GPx in-gel assay of hippocampal tissue of the control (Cont), Rotenone treated (Rot) and CDRI-08 treated Rotenone administered mice (n=3 per group.) Data are shown as the mean  $\pm$  SEM. \* $p < 0.05$  and \*\*\* $p < 0.001$

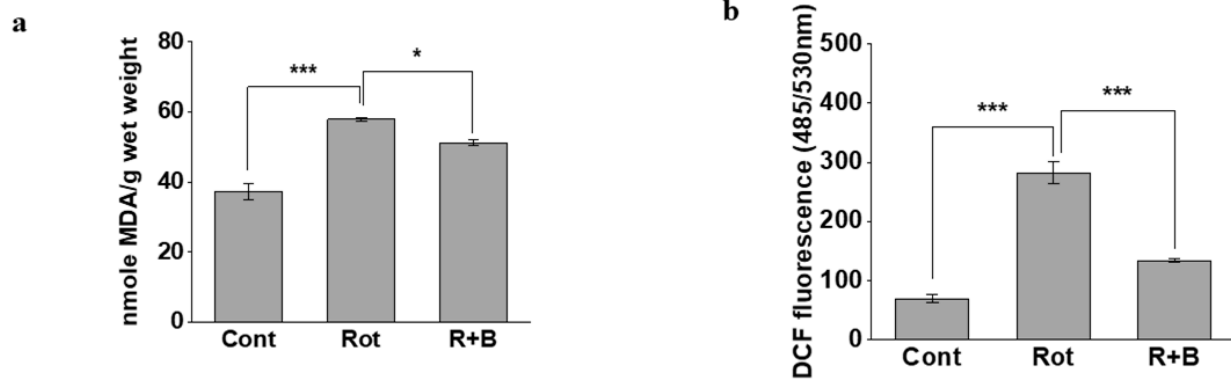


Figure 4

Analysis of the oxidative stress. Histogram showing a). Level of lipid peroxidase (nmole MDA/g wet tissue wt) and total ROS level (DCF fluorescence. Control (Cont), Rotenone-treated (Rot), CDRI-08 treated Rot mice (R+B). (n=3 per group) Data are shown as the mean  $\pm$  SEM. \* $p < 0.05$  and \*\*\* $p < 0.001$

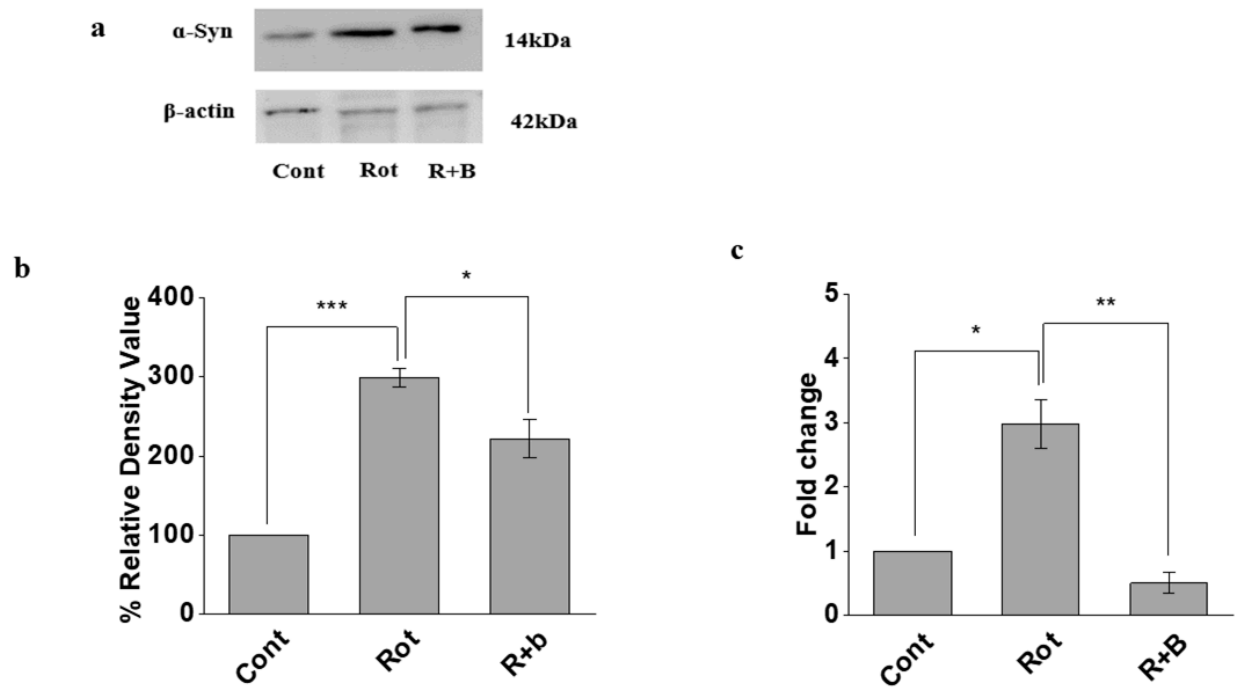


Figure 5

Western blot and qRT-PCR analysis of  $\alpha$ -Syn expression. a) Immunoblot image, and b) histogram showing densitometric scan data as % relative density value of the immunoblot signal; c) Histogram showing the data obtained from qRT-PCR (Fold change in  $\alpha$ -Syn transcript with respect to endogenous control). Data were obtained from control (Cont), rotenone-treated- (Rot) and CDRI-08-treated Rot-mice (R+B) mice. (n=3 per group). Data are shown as the mean  $\pm$  SEM. \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$

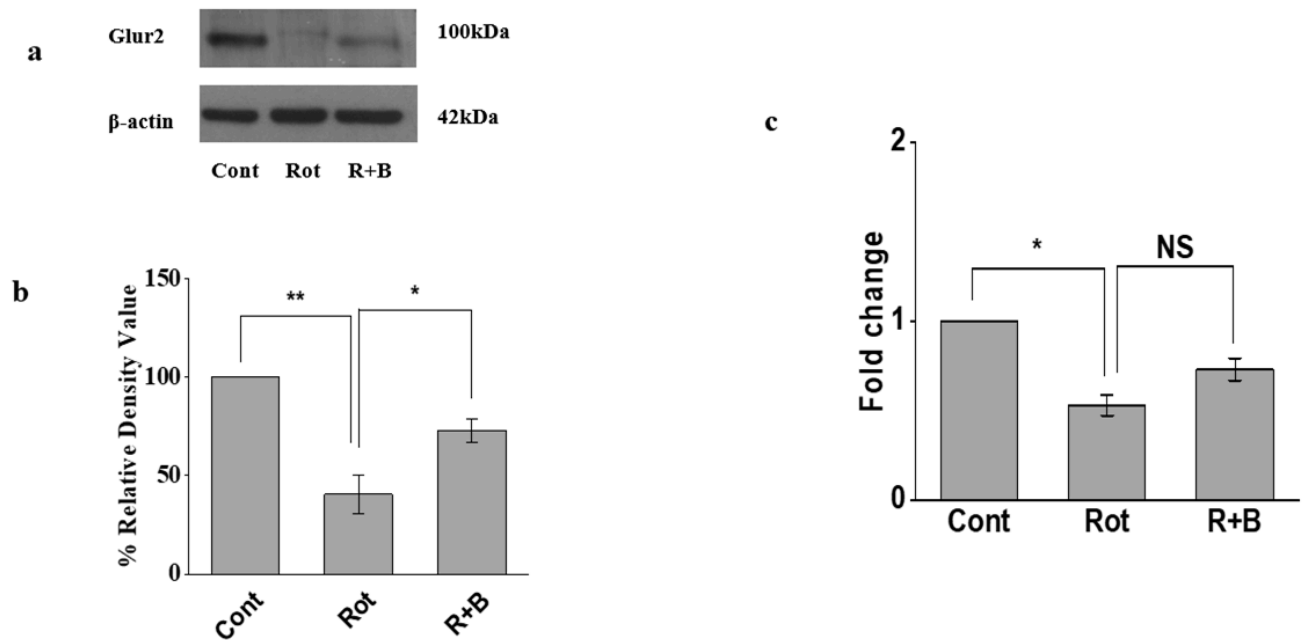


Figure 6

Western blot and qRT-PCR analysis of AMPAR GluR2 protein and transcript, respectively. a) immunoblot image of GluR2, b) Histogram showing the relative density value of the image (a); c) histogram showing the fold change in the level of GluR2 transcript (relative to the endogenous control) in the hippocampus of control (Cont), rotenone-treated (Rot) and CDRI-08 treated Rot-mice (R+B) mice (n=3 per group).Data are shown as the mean  $\pm$  SEM. \*p< 0.05, \*\*p<0.01 and NS not significant

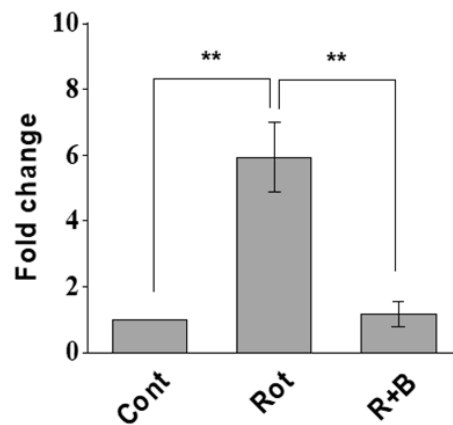


Figure 7

MeDIP-qPCR analysis of GluR2 promoter methylation status. Histogram represents the quantitative data of immunoprecipitated methylated promoter DNA obtained from the hippocampus of Control (Cont), rotenone-treated (Rot) and CDRI-08 treated Rot-mice. alters DNA methylation level at the promoter of GluR2. (n=3 per group). Data are shown as the mean  $\pm$  SEM. \*\*p<0.01

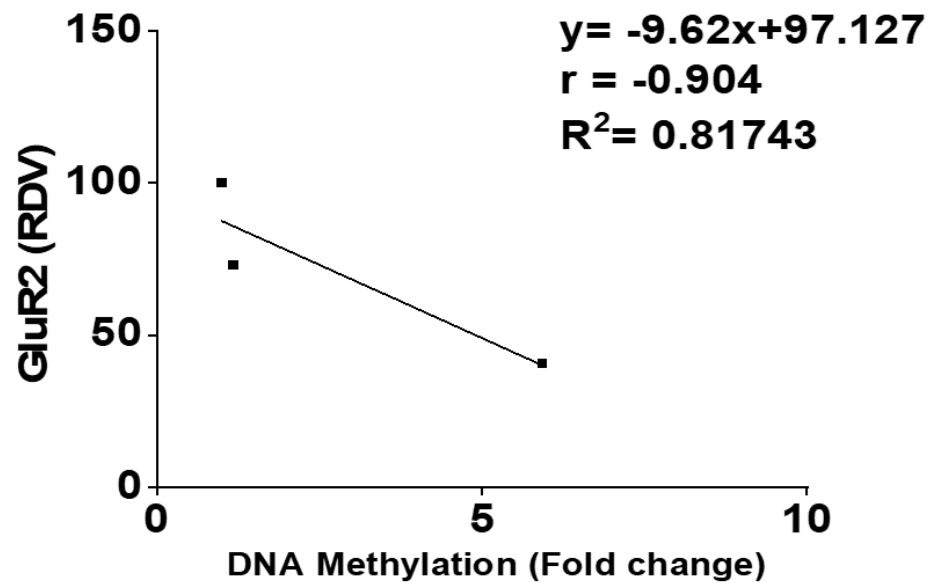


Figure 8

Correlation analysis between GluR2 promoter DNA methylation (fold change) and GluR2 protein expression (relative density value, RDV) obtained from the hippocampus of the control, rotenone-treated and CDRI-08 treated Rotenone administered mice.