Possible Involvement of Standardized *Bacopa monniera* Extract (CDRI-08) in Epigenetic Regulation of *reelin* and Brain-Derived Neurotrophic Factor to Enhance Memory

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*Bacopa monniera* extract (CDRI-08; BME) has been known to improve learning and memory, and understanding the molecular mechanisms may help to know its specificity. We investigated whether the BME treatment alters the methylation status of *reelin* and brain-derived neurotropic factor (BDNF) to enhance the memory through the interaction of N-methyl-D-aspartate receptor (NMDAR) with synaptic proteins. Rat pups were subjected to novel object recognition test following daily oral administration of BME (80 mg/kg) in 0.5% gum acacia (per-orally, p.o.; PND 15–29)/three doses of 5-azacytidine (5-azaC; 3.2 mg/kg) in 0.9% saline (intraperitoneally, i.p.) on PND-30. After the behavioral test, methylation status of *reelin*, BDNF and activation of NMDAR, and its interactions with synaptic proteins were tested. Rat pups treated with BME/5-azaC showed higher discrimination towards novel objects than with old objects during testing. Further, we observed an elevated level of unmethylated DNA in *reelin* and BDNF promoter region. Up-regulated *reelin* along with the splice variant of apolipoprotein E receptor 2 (ApoER 2, ex 19) form a cluster and activate NMDAR through disabled adopter protein-1 (DAB1) to enhance BDNF. Observed results suggest that BME regulate *reelin* epigenetically, which might enhance NMDAR interactions with synaptic proteins and induction of BDNF. These changes may be linked with improved novel object recognition memory.

**Keywords:** *Bacopa monniera*, Novel object recognition, *reelin*, apolipoprotein E receptor 2 (ApoER 2), N-methyl-D-aspartate receptor (NMDAR), brain-derived neurotrophic factor (BDNF)

**INTRODUCTION**

Long-term memory formation requires fine tuned cellular signaling coordination with transcriptional and translational regulations of gene expression (Hawk and Abel, 2010). Epigenetic mechanisms specifically control transcription through many ways, among which DNA methylation being common (Levenson et al., 2006; Blaze and Roth, 2013; Morris and Monteggia, 2014).
This process involves DNA methyltransferases (DNMTs) that mediates de novo methylation within DNA and alters the chromatin structure (Shilatifard, 2006; Lubin, 2011; Nabel and Kohli, 2011); which directly controls transcription. Several studies have reported that DNA methylation/demethylation in a specific promoter region of reelin and brain-derived neurotrophic factor (BDNF) dictates the transcriptional activity thereby critically regulating synaptic plasticity, learning, and memory (Miller and Sweatt, 2007; Levenson et al., 2008; Lubin et al., 2008; Mizuno et al., 2012; Sui et al., 2012). Reelin is a large secreted glycoprotein richly expressed at hippocampus by a subset of γ-amino butyric acid (GABA)-ergic interneurons (Pesold et al., 1998; Abraham and Meyer, 2003; Ramos-Moreno et al., 2006).

Reelin exerts its effects through two receptors: apolipoprotein E receptor 2 (ApoER 2) and very-low-density lipoprotein receptors (VLDLR) (Weeber et al., 2002; Strasser et al., 2004). ApoER 2 expression in hippocampus promotes neuronal signaling and induction of long-term potentiation (LTP) (Barr et al., 2007). However, only the spliced variant of ApoER 2 with exon 19 (ex 19) (Beffert et al., 2005) interacts with NpX domain of adapter protein disabled-1 (DAB1) to elicit intracellular signaling (Howell et al., 1999; Benhayon et al., 2003; Beffert et al., 2006). Activation/phosphorylation of DAB1 (p-DAB1) by reelin-induced receptor cluster, depends on Src family tyrosine kinases (SFKs). The activated p-DAB1 in turn activates SFKs (Strasser et al., 2004; Kuo et al., 2005). Subsequently, activated SFKs phosphorylate the subunits of N-methyl-D-aspartate (NMDA; NR2A/NR2B) receptors (Yu et al., 1997; Chen et al., 2005; Mota et al., 2014). Further, the activated NMDA receptors target the activation of BDNF (Lubin et al., 2008). On the other hand, subunits of NMDA receptor directly interact with post-synaptic density protein-95 (PSD-95) subunits of NMDA receptor (NR2A/NR2B) receptors (Yu et al., 1997; Chen et al., 2005; Mota et al., 2014). Subsequently, the activated SFKs phosphorylate the subunits of N-methyl-D-aspartate (NMDA; NR2A/NR2B) receptors (Yu et al., 1997; Chen et al., 2005; Mota et al., 2014). Further, the activated NMDA receptors target the activation of BDNF (Lubin et al., 2008). On the other hand, subunits of NMDA receptor directly interact with post-synaptic density protein-95 (PSD-95) subunits of NMDA receptor (NR2A/NR2B) receptors (Yu et al., 1997; Chen et al., 2005; Mota et al., 2014).

Bacopa monniera extract has been traditionally used in Ayurvedic medicine to improve learning and memory. B. monniera extract contains triterpene saponins, which have been named bacosides and bacopasaponins. The major chemical entity shown responsible for neuropharmacological effects of B. monniera are bacoside A (bacogenins A1, A2, A3, and A4) and bacoside B (Pubmed C ID: 53398644); the latter differs only in optical rotation and may probably be an artifact produced during the process of isolating bacoside A (Chatterji et al., 1963, 1965; Basu et al., 1967). CDRI-08 mentioned as BME during the process of isolating bacoside A (Chatterji et al., 1963, 1965; Basu et al., 1967). CDRI-08 is a bacoside-enriched standardized extract of B. monniera (CDRI-08, contains 55 ± 5% bacosides). Earlier studies demonstrate that CDRI-08 significantly improves the cognitive performance in healthy human participants (Downey et al., 2013; Benson et al., 2014); and in elders and patients with neurodegenerative disorder (Barbhiaiya et al., 2008; Calabrese et al., 2008; Stough et al., 2013). Bacosides present in the CDRI-08 are non-polar glycosides, which possibly cross the blood–brain barrier (BBB) by lipid mediated passive diffusion (Pardridge, 1999); and its biodistribution in brain has been confirmed by radiopharmaceuticals (De et al., 2008). Earlier, in rat model, we found that oral treatment of BME elevates the level of serotonin (5-hydroxytryptamine, 5-HT), activates 5-HT3A receptor (Rajan et al., 2011), and regulates cyclic adenosine monophosphate (cAMP) response element binding (CREB) protein through microRNA (miR)-124 (Preethi et al., 2012). Subsequently, we reported that BME regulates extracellular signal-regulated kinase (ERK)/CREB cascade and BDNF through regulation of histone acetylation and protein phosphatases to improve hippocampal memory (Preethi et al., 2014). In the present study, first we tested whether the treatment of BME/DNMT inhibitor (5-azacytidine and 5-azaC) improves novel object recognition. Second, we examined whether BME/5-azaC treatment alters methylation status of reelin and BDNF, and its effect on NMDA (NR2A) receptor interaction with synaptic proteins.

**MATERIALS AND METHODS**

**Animals**
Wistar rat pups both male and female (Rattus norvegicus; B.wt: 19.6 ± 0.6 g on PND-15) were housed in a standard laboratory cage (43 × 27 × 15 cm) with paddy husk as a bedding material. Rat pups were maintained at animal house under controlled environmental condition [12-h light/dark cycle (7:00–19:00); temperature: 22 ± 2°C; humidity: 50 ± 5%] with ad libitum access to food and water. Protocols for animal use were performed following the guidelines of Institutional Animal Ethics Committee (BDU/IAEC/2014/OE/08/Dt. 18.03.2014), ensuring that number of animals and pain was kept to a minimum.

**Drugs and Treatment**
Standardized extract of B. monniera (CDRI-08 with 55 ± 5% bacosides) (Gifted by Lumen Marketing Company, Chennai, India), was dissolved in 0.5% gum acacia (Hi-Media Laboratories Pvt. Ltd, Mumbai, India). The DNA methyltransferase (DNMT) inhibitor, 5-azacytidine (5-azaC; ≥ 98%, Sigma–Aldrich, India) was dissolved in 0.9% saline. Drugs were prepared freshly on the day of administration (10:00–11:00 h) and drugs were administered to rat pups during their brain growth spurt period (Dobbing and Sands, 1979). Rat pups from different litters which attained the age of postnatal day (PND)-14 were randomly assigned into three groups: (i) control group (Con, n = 24) received vehicle solution 0.5% gum acacia, per-orally (p.o.) from PND 15–29 and three doses of 0.9% saline intraperitoneally (i.p.) on PND-30 (0, 5, and 23 h), (ii) BME group (BME, n = 24) received 80 mg/kg of CDRI-08 in 0.5% gum acacia (p.o.) from PND 15–29 (Rajan et al., 2011) and three doses of 0.9% saline (i.p.) on PND-30 (0, 5, and 23 h), (iii) 5-azaC group (5-azaC, n = 24) received 0.5% gum acacia (p.o.) from PND 15–29 and three doses of 3.2 mg/kg of 5-azaC (i.p.) on PND-30 (0, 5, and 23 h) (Sales et al., 2011).

**Novel-Object Recognition Test**
The apparatus used for novel-object recognition (NOR) test was conducted in circular open-field board (82 cm diameter) made of wood, coated black surrounded by a wall of 32 cm height (Barbosa et al., 2013). Two sets of objects (Cuboids and Spherical objects) were used. The cuboids (13 × 10 × 10 cm)
were made of plaster of paris and painted with blue color (non-toxic children water color). The plastic balls (radius: 26 cm) were green in color and filled with sand to ensure that animals cannot displace them. The apparatus and objects were cleaned with 75% ethanol after each behavior session. The sessions were recorded by a video camera (Sony megapixel 12.1, Full HD 1080) placed at a distance of 150 cm above the apparatus. In novel object recognition test, rodents’ innate habit of exploring novel object in their environment has been utilized to evaluate their memory (Ennaceur and Delacour, 1988). Exploration time was scored as when the rat nose was touching the object or orienting its head towards the object within a distance of 1 cm. The relative exploration time was recorded and expressed by a discrimination index [D.I. = (t\text{novel} - t\text{old})/(t\text{novel} + t\text{old}) × 100%] as reported earlier (Stefanko et al., 2009). Mean exploration times were calculated and the discrimination indexes between groups were compared.

Rat was allowed to freely explore the experimental area in groups (n = 3) on PND-28, 29 and individually on PND-30 for 10 min. On PND-31, all animals were trained for two sessions (5 min each session) with an interval of 1 h. During training, rats were exposed to four identical objects. On PND-32 during retention session, two new objects were
placed in the position of any two old objects and the exploration behavior of each rat was recorded for 5 min (Barbosa et al., 2013).

**Tissue Collection, RNA and Protein Isolation**

After the NOR test, animals representing each group (n = 6) were euthanized, and the hippocampus tissue was dissected as described by Glowinski and Iversen (1996) for the preparation of RNA and protein. Total RNA was isolated from hippocampus tissue samples using TRIzol (Merck Specialties Pvt. Ltd., India), according to the manufacturer’s instructions and stored with RNase inhibitor (Merck Specialties Pvt. Ltd, India) at −80°C. The hippocampus tissue samples were homogenized in ice-cold lysis buffer (150 mM NaCl, 50 mM Tris–HCl pH 7.5, 5 mM EDTA, 0.1% v/v NP-40, 1 mM DTT, 0.2 mM sodium orthovanadate, 0.23 mM PMSF) and 10 µl/ml protease inhibitor cocktail (Sigma–Aldrich, USA). The homogenates were kept on ice for 30 min and then centrifuged at 10,000 × g for 30 min at 4°C. The clear supernatants were collected in a fresh tube and centrifuged again at 12,000 × g for 15 min at 4°C. The final supernatants were collected and stored as aliquots, in order to avoid repeated freeze–thaw of the samples and were stored at −80°C. The concentration of each protein sample was quantified by measuring the absorbance at 595 nm using Biophotometer plus (Eppendorf Inc., Germany).

**DNA Methylation Assay**

Individuals representing each group (n = 6) were euthanized and DNA was isolated from hippocampus tissue (UltraClean® Tissue & Cells DNA isolation kit, Cat no. 12334-50, MO BIO laboratories, Inc., USA) and processed for bisulfite modification (EpiTect Bisulfite kit, Qiagen, Cat no. 59104).

**Quantitative Real-Time PCR (qRT-PCR)**

The qRT-PCR was performed using real-time reaction mixture (SsoAdvanced™ SYBR® green supermix, Bio-Rad Laboratories, Inc., USA) with specific primers (50 pmol/µl) and DNA (0.4 ng/µl) primers (200 pmol/µl) and cDNA (0.2 µg/µl) in CFX-96 Touch™ Real-time PCR (RT-PCR) Detection System (Bio-Rad Laboratories, Inc., USA).

The detection of unmethylated DNA in the gene promoter was performed using the specific primers for reelin (For 5′-TG TAAATTTTTTGTAGTTTGGGGATGT-3′; Rev 5′-TCCTTTA AATAATCCAAACAACACC-3′) and BDNF (CpG island 1) (For 5′-GGGTGTTATTTGGGGAGGAAGTAT-3′; Rev 5′-TCCTTAAAT CATCCAA CAACACGC-3′) and CpG island 1 of BDNF (For 5′-GTA GCGATTTTGGGGAGGAAGTAC-3′; Rev 5′-CA CACCCTCTATACGGGACTAAATTCCG-3′) (Sui et al., 2012). Reactions were performed with the following cycling conditions: 95°C for 3 min, 40 cycles of 95°C for 15 s, 60°C (reelin)/61°C (BDNF) for 1 min and 72°C (reelin)/74°C (BDNF) for 15 s. To verify specificity, the final product was electrophoresed on 2% agarose gel stained with ethidium bromide (0.5 µg/ml), and amplification and band intensity was examined in ChemiDoc XR + System with image lab 2 (Bio-Rad Laboratories, Inc., USA).

To examine the expression level of mRNA, total RNA (2.0 µg/sample) was reverse transcribed into cDNA using random/oligo-dT primers (iScript cDNA Synthesis Kit, Bio-Rad Laboratories Inc., USA). The expression level of specific genes was estimated using specific primers reelin (For: 5′- AAACCTACAGGTTGGAAGAC-3′ and Rev: 5′-ATTTGAGG CATGA CGGACCTATAT-3′) (Sui et al., 2012)/BDNF (For: 5′-GTAACGTCCACCGGACAAG-3′ and Rev: 5′-TATGTTTTT CTTCGTTGGC-3′), Total ApoER 2 (For: 5′-AGTGTCCCG ATGGC TCGAC-3′ and Rev: 5′-CAGCTAATCTTCTCGGC AGGA-3′), ApoER 2 (ex19) For: 5′GCC CTCAAGGAGCTTTTT GTG-3′ and Rev: 5′-AGGGTTCTTCGGGAGTTGGT-3′, Apo ER 2 (Δ) (For: 5′-CAGGTACAGGAAACAGCAAGAAG-3′ and Rev: 5′-TGACCTCTGCTGC GG-3′ (Beffert et al., 2005) with Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (For: 5′-ACATCATCCTCGATCCAC-3′ and Rev: 5′-AGG AACACGGAAGGCAATG-3′) as internal control. Conditions for the RT-PCR reactions were as follows: initial denaturation at 94°C for 3 min followed by denaturation at 94°C for 5 s, annealing (64.5°C for reelin, 60°C for BDNF, 55°C for total ApoER 2, 60°C for ApoER 2 (ex19), 57°C for ApoER 2 (Δ) and 56°C for GAPDH) for 5 s, extension at 72°C for 5 s. The amplification of the single PCR product was confirmed by monitoring the dissociation curve followed by melting curve analysis. Each reaction was performed in triplicates and normalized with internal control GAPDH. The data were presented as mean fold change relative to the control group (CFX Manager™ version 2 software; CFX-96 Touch™ RT-PCR Detection System Bio-Rad Laboratories, Inc., USA).

**Western Blot Analysis**

Equal concentrations (30 µg) of proteins were resolved on 9% SDS–polyacrylamide gel. The separated proteins were transferred electrophoretically onto polyvinylidene difluoride (PVDF) membrane (Merck Millipore, India) using a semi-dry western apparatus (SD 20; Cleaver Scientific Ltd, UK). The membranes were blocked and incubated at 4°C for 9 h with one of the following specific primary antibodies (Santa-Cruz Biotechnology, Cell signaling solutions, USA): rabbit polyclonal anti-BDNF (N-20) [SC-546, 1:500], anti-PSD-95 [SC-28941, 1:200], anti-Dab1 [Cat #3328, 1:1000], anti-Phospho-Dab1 [Cat #3327, 1:1000], anti-NMDAR2A [Cat #4205, 1:1000], anti-NMDAR2B [Cat #4207, 1:1000] antibodies, mouse monoclonal anti-p-Tyr antibody [SC-7020, 1:300] and rabbit polyclonal anti-β-actin antibody (SC-130656; 1:200) was used as control for each sample. The membrane-bound antibodies were detected
FIGURE 3 | Effect of BME/5-azaC on regulation of DNA methylation in the reelin promoter and reelin expression. BME/5-azaC treatment significantly (A) increased unmethylated DNA, (B) and decreased methylated DNA in reelin promoter region relative to control group. (C) reelin mRNA was significantly higher in BME/5-azaC treated groups compared to control group. Data were shown as mean ± SEM, asterisk indicates significant difference (*P < 0.05; **P < 0.01; ***P < 0.001). Comparisons between groups are represented as a = Con verses BME; b = Con verses 5-azaC; and c = BME verses 5-azaC.

FIGURE 4 | Effect of BME/5-azaC on ApoER 2 splicing of variants. (A) BME/5-azaC treatment significantly increased the level of ApoER 2 (ex 19) mRNA, (B) and decreased levels of ApoER 2 (Δ) exon mRNA compared to control group. Data were shown as mean ± SEM, asterisk indicates significant difference (**P < 0.01; ***P < 0.001). Comparisons between groups are represented as a = Con verses BME; b = Con verses 5-azaC; and c = BME verses 5-azaC.

by incubating for 3 h at 4°C with alkaline phosphatase (ALP) conjugated either with goat anti-rabbit (Cat #621100180011730, MERCK, 1:2000), goat anti-mouse (Cat #621100480011730, MERCK, 1:2000) secondary antibody. Subsequently alkaline phosphatase activity was detected with 5-bromo-4-chloro-3-indolyl phosphate di-sodium salt (BCIP)/nitroblue tetrazolium chloride (NBT) (HIMEDIA, Mumbai, India) according to the manufacturer’s instructions. Images were acquired with a
Chemidoc XR + System (Bio-Rad Laboratories, Inc., USA), and optical density of trace quantity for each band was measured using Image Lab 2 software (Bio-Rad Laboratories, Inc., USA). The trace quantity of each bands were normalized with β-actin bands, following that fold changes were calculated by dividing normalized values of BME/5-azaC groups by control groups.

Co-immunoprecipitation
Spin columns were packed with AminoLink Plus Coupling resin slurry (50 µl) and washed with provided buffer, then 20 µg of anti-rabbit NR2A antibody (Cat #4205, adjusted to 200 µl volume with 1× coupling buffer) was added in the column and incubated on a rotator at room temperature for 2 h to immobilize antibodies. For Co-IP, 200 µg of protein lysates from each groups Con/BME/5-azaC were immunoprecipitated with NR2A antibody in immobilized spin columns overnight at 4°C. Immunoprecipitated protein was eluted using elution buffer. The complete Co-IP experiment performed following the instructions provided with the kit (Catalogue no. 26149, Pierce Co-Immunoprecipitation Kit, Thermofisher, IL, USA). Equal volume of NR2A precipitated protein from each sample elutes was analyzed by immunoblotting using a SFK (SC-7020, 1:300) and PSD-95 (SC-28941, 1:200) antibody. Immunoblotting was performed following the procedure reported in the western blots section.

FIGURE 5 | Effect of BME/5-azaC on induction and phosphorylation of DAB1 in hippocampus. (A) Western blots showing the immunoreactivity to anti-total DAB1, and anti-phosphorylated DAB1. (B) BME/5-azaC treatment increased levels of p-DAB1 compared to control. Data were shown as mean ± SEM, asterisk indicates significant difference (\(P < 0.05\); \(\ast\), \(P < 0.01\); \(\ast\ast\), \(P < 0.001\)). Comparisons between groups are represented as a = Con versus BME; b = Con versus 5-azaC; and c = BME versus 5-azaC.

Statistics
Data were presented as a mean ± standard error of the mean (SEM) and plotted with KyPlot (ver 1.0) for graphical representation. For behavioral analysis (NOR), multivariate ANOVA was performed to test the effect of factors (training sessions, objects) and their interactions during training. One-way analysis of variance (ANOVA) and Two-way analysis of variance (Two-way ANOVA) were used to assess the significance between groups and groups × objects interactions during testing respectively. Post hoc Bonferroni test was used to examine the difference between groups. Association between the recognition memory (DI) and methylation/unmethylation DNA was estimated using Pearson correlations (SPSS, ver.15). For expression data, One-way analysis of variance (ANOVA) was used to observe the significance between groups. Differences were considered significant if \(P < 0.05\).

RESULTS
BME Improves Novel Object Recognition
Novel object recognition test was performed to examine the effect of BME on hippocampus dependent long-term memory. At first, rat pups from experimental groups (Con, BME, 5-azaC) were subjected to training. Multivariate ANOVA analysis revealed that there was no significant difference between training sessions\([\text{Wilks' }\lambda = 0.953; F = 1.016; P = 0.392]\), objects\([\text{Wilks' }\lambda = 0.916; F = 0.614; P = 0.784]\) and interaction of training sessions × objects\([\text{Wilks' }\lambda = 0.9356; F = 0.466; P = 0.895]\). Further, our analysis showed that the time spent by rat pups exploring the objects did not differ significantly between training sessions\([\text{Con: }F(1,71) = 0.493, P = 0.493]; \text{BME: }F(1,71) = 2.48, P = 0.120; \text{5-azaC: }F(1,71) = 0.096, P = 0.758]\), objects\([\text{Con: }F(3,71) = 0.295, P = 0.829]; \text{BME: }F(3,71) = 0.995, P = 0.401; \text{5-azaC: }F(3,71) = 0.691, P = 0.561]\) and interaction of training sessions × objects\([\text{Con: }F(3,71) = 0.672, P = 0.572]; \text{BME: }F(3,71) = 0.446, P = 0.721; \text{5-azaC: }F(3,71) = 0.284, P = 0.837]\)\((\text{Figures 1A,B; Supplementary Table S1})\). During retention test, Con/BME/5-azaC groups spent significantly more time around novel objects than control group. Further
two-way ANOVA revealed that there was a significant difference across the treatment groups \( F_{2,53} = 5.279, P < 0.01 \) and objects \( F_{1,53} = 245.476, P < 0.001 \) in novel object exploration time, and treatment groups × objects interaction \( F_{2,53} = 12.43, P < 0.001 \). In addition, Bonferroni post hoc test confirmed that the novel object exploration time was statistically higher in BME \( P < 0.05 \) and 5-azaC \( P < 0.05 \) compared to control \( (P < 0.01) \). BME/5-azaC treated group spent more time to explore the novel objects compared to old objects. Discrimination indexes were greater than zero for them \( F_{2,53} = 4.985, P = 0.040 \) (Figure 2B). Our results demonstrated that BME treatment effectively establish long-term memory for the familiar object, which was comparable to 5-azaC treatment.

**Effect of BME on Methylation of Reelin Promoter and Its Expression**

To test whether the improvement in NOR by methylation status of reelin is due, at least in part by BME treatment, we determine the effect of BME on methylation status of CpG island present in reelin promoter. We found that BME treatment induced a significant effect on methylation status of reelin and its expression. Our analysis showed that significant increase in unmethylated reelin DNA levels associated with BME \( F_{1,5} = 78.132, P < 0.001 \) and 5-azaC treatment \( F_{1,5} = 12.706, P < 0.05 \) than control. There were no changes in the unmethylation level of reelin DNA between BME and 5-azaC groups \( F_{1,5} = 0.667, P = 0.460 \) (Figure 3A). Simultaneously, the level of methylated reelin DNA decreased in BME \( F_{1,5} = 30.867, P < 0.01 \) and 5-azaC \( F_{1,5} = 18.16, P < 0.05 \) compared to control group. Whereas, there was no significant difference between BME and 5-azaC groups \( F_{1,5} = 5.280, P = 0.083 \) (Figure 3B). Interestingly, demethylation of reelin promoter resulted in significant up-regulation of reelin expression in BME \( F_{1,5} = 28.79, P < 0.001 \) and 5-azaC groups \( F_{1,11} = 17.98, P < 0.01 \) compared to control group, but there was no significant difference between BME and 5-azaC groups \( F_{1,5} = 4.299, P = 0.065 \) (Figure 3C). Subsequently, we found a significant positive correlation between recognition memory (discrimination index; DI) and unmethylated reelin DNA (BME: \( r = 0.973, P < 0.001 \); 5-azaC: \( r = 0.878, P < 0.01 \), and a negative correlation between DI and methylated reelin DNA.

**FIGURE 6 | Effect of BME/5-azaC on level of NMDAR subunits.** (A) Western blots showing the immunoreactivity to anti-NR2A, anti-NR2B. (B) BME/5-azaC treated groups showed comparatively increased level of NR2A, (C) and NR2B. (D) NR2A/NR2B ratio was significantly more in BME/5-azaC groups than control group. Data were shown as mean ± SEM, asterisk indicates significant difference \( *(P < 0.05), ***(P < 0.01), ***(P < 0.001)\). Comparisons between groups are represented as a = Con verses BME; b = Con verses 5-azaC; and c = BME verses 5-azaC.
Possible Role of Bacopa monniera in Epigenetic Mechanism

Effect of BME on Splicing of ApoER 2 Receptor

One could expect that, the altered reelin could contribute to the possible changes on the target molecules. Alternative splicing of ApoER 2 was a critical component in the reelin mediated NMDA receptor activity. ApoER 2 (ex 19) mRNA (Figure 4A) significantly increased in BME \( F_{(1,11)} = 23.31, \ P < 0.001 \) and 5-azaC group \( F_{(1,11)} = 31.28, \ P < 0.001 \) relative to control group. Whereas, there was no significant difference between BME and 5-azaC groups \( F_{(1,11)} = 0.832, \ P = 0.383 \). Conversely, the ApoER 2 (Δ) ex 19 mRNA variants were at lower level in BME \( F_{(1,11)} = 19.35, \ P < 0.001 \) and 5-azaC groups \( F_{(1,11)} = 13.2, \ P < 0.01 \) compared to control group, but there was no significant difference between BME and 5-azaC groups \( F_{(1,11)} = 0.0016, \ P = 0.969 \) (Figure 4B). Up-regulated level of ApoER 2 (ex 19) may be linked with recorded improvement in NOR, suggesting that ApoER 2 (ex 19) splicing possibly induced by BME treatment.

Effect of BME on Reelin Mediated NMDA in Phosphorylation of DAB1

Reelin binding to ApoER 2 (ex 19) and VLDLR results in activation of DAB1. To expand our understanding, we tested
whether the BME induced up-regulated ApoER 2 (ex 19) facilitates the phosphorylation of DAB1 (p-DAB1). Total and p-DAB1 level in Con/BME/5-azaC groups were determined. Figures 5A,B showed that BME/5-azaC treatment significantly increased the level of p-DAB1 in both BME \(F(1,5) = 150.662, P < 0.001\) and 5-azaC \(F(1,5) = 14.844, P < 0.05\) compared to control group. The level of p-DAB1 was higher in BME \(F(1,5) = 101.637, P < 0.001\) than 5-azaC group. These results suggested that increased ApoER 2 (ex19) by BME/5-azaC was responsible for elevated levels of total and p-DAB1.

**Effect of BME on Alternation of NMDA Receptors**

We examined whether the BME treatment altered the NMDARs subunits composition (Figure 6A). Western blot analysis showed that the level of NR2A was significantly higher in BME \(F(1,5) = 107.05, P < 0.001\) and 5-azaC \(F(1,5) = 209.707, P < 0.001\) compared to control group. While comparing BME with 5-azaC, the level of NR2A in BME \(F(1,5) = 101.637, P < 0.001\) was higher than 5-azaC group (Figure 6B). Similarly, the level of NR2B was significantly higher in BME \(F(1,5) = 42.446, P < 0.01\) and 5-azaC \(F(1,5) = 12.99, P < 0.05\) compared to control group. Whereas, there was no significant difference between BME and 5-azaC groups \(F(1,5) = 6.931, P = 0.058\) (Figure 6C). Therefore, the NR2A/NR2B ratio was significantly higher in BME \(F(1,5) = 100.439, P < 0.001\) and 5-azaC \(F(1,5) = 103.83, P < 0.001\) compared to control group. Whereas, there was no difference between BME and 5-azaC groups \(F(1,5) = 27.079, P < 0.01\) (Figure 7C). The higher level of SFK/PSD-95 with NR2A in BME/5-azaC groups, further supported NMDAR activation.

**Effect of BME on Interaction of NMDAR Subunit with Synaptic Proteins**

Further, our Co-IP showed that observed alternation in NR2A level concomitantly enhanced interaction with key synaptic proteins (Figure 7A). The analysis revealed that the level of NR2A–SFK proteins was significantly higher in BME \(F(1,5) = 736.071, P < 0.001\) than control group. Surprisingly, it was lower in 5-azaC group compared to control \(F(1,5) = 14.928, P < 0.05\) and BME \(F(1,5) = 202.34, P < 0.001\) (Figure 7B). While considering the level of interacted NR2A–PSD-95 proteins, it was significantly higher in both BME \(F(1,5) = 8.44, P < 0.05\) and 5-azaC treated group \(F(1,5) = 27.079, P < 0.01\) than the control group. Whereas, there was no significant difference between BME and 5-azaC \(F(1,5) = 4.56, P = 0.100\) (Figure 7C). The higher level of SFK/PSD-95 with NR2A in BME/5-azaC groups, further supported NMDAR activation.
The western blot analysis showed that the level of BDNF protein was significantly higher in BME \(F_{(1,5)} = 48.527, P < 0.01\) and 5-azaC \(F_{(1,5)} = 25.766, P < 0.01\) compared to control group, but there was no significant difference between BME and 5-azaC \(F_{(1,5)} = 1.866, P = 0.244\) (Figure 9A,C). These data supported that the NMDAR activation by BME/5-azaC treatment altered the unmethylated BDNF levels and its expression.

**DISCUSSION**

Earlier studies demonstrated that DNA methylation/demethylation involves in the regulation of activity dependent neuronal gene expression (Ming and Song, 2011; Baker-Andresen et al., 2013) and synaptic plasticity (Miller and Sweatt, 2007; Feng et al., 2010). A family of DNMTs (DNMT1, 3a, 3b) can catalyze the process of DNA methylation (Sweatt, 2009). The expression pattern of DNMTs (DNMT1, 3a, 3b) differ in different brain regions following behavioral training and exhibits different behavioral phenotype (Feng et al., 2010; Miller et al., 2010; Morris et al., 2014). In fact, DNMT inhibitors (5-azaC) are known to alter learning and memory/memory consolidation (Miller and Sweatt, 2007). Considering the role of DNA methylation in memory formation, first we showed the effect of BME in novel object recognition by comparing with DNMT inhibitor, 5-azaC. Using NOR test, we found that BME effectively enhanced the NOR memory, this suggested that BME treatment improved the process of recollection and familiarity identification (Squire et al., 2007). It has been shown that methylation status alters rapidly and dynamically within hippocampus (Miller and Sweatt, 2007). The level of DNMT 3a was also significantly elevated following contextual training in NOR test, but its conditional knock-out impaired NOR memory (Morris et al., 2014). Observed behavioral results suggested that BME/5-azaC treatment might optimally regulate the DNMT 3a level at this age of rats and hence improve NOR memory.

Following the improvement in behavioral phenotype; we sought to correlate this with methylation status. As quantified by RT-PCR, a higher level of unmethylated and lower level of methylated reelin DNA was estimated in BME and 5-azaC treated groups when compared to control. Supporting this, earlier studies have shown that 5-azaC treatment reduces the methylated DNA in reelin promoter (Miller and Sweatt, 2007; Sui et al., 2012). Consistent with the demethylation status, the level of reelin mRNA was significantly elevated in both BME and 5-azaC treated rats. The positive correlation between recognition memory (DI) and unmethylated reelin DNA in BME/5-azaC group suggested that BME possibly regulated methylation to enhance memory. These results are in line with earlier reports on methylation status and expression of reelin during neural plasticity (Levenson et al., 2006; Miller and Sweatt, 2007). In addition, observed behavioral data may be linked with the up-regulated reelin expression (Rogers et al., 2011) and increased dendritic spine density in hippocampus (Vollala et al., 2011).

Reelin exerts its effects through ApoER 2 ex 19 receptor (Weeber et al., 2002; Beffert et al., 2005). Interestingly, BME/5-azaC treatment up-regulated the ApoER 2 (exon 19) variant.
and down-regulated ApoER 2(A) transcript. The up-regulated ApoER 2 exon 19 attaches to postsynaptic density protein PSD-95 and forms a signaling complex with NMDARs (Beffert et al., 2005; Hoe et al., 2006). However, to execute the neuronal transmission, NMDAR should be activated by p-DAB1 (Chen et al., 2005; Beffert et al., 2006). Consistently, BME/5-azaC treatment enhanced the level of p-DAB1. In process p-DAB1 activates Src kinase family (SKF) through phosphorylation, which in turn leads to phosphorylation of NMDAR subunit (NR2) (Ballif et al., 2003; Bock and Herz, 2003; Strasser et al., 2004; Chen et al., 2005; Mota et al., 2014). However, synaptic strength is determined by the ratio of NMDAR subunits (NR2A/NR2B) (Quinlan et al., 2004; Lebel et al., 2006; Brigman et al., 2008; Levenson et al., 2008). In this study, the level of NR2A and NR2B subunits and the estimated NR2A/NR2B ratio were higher in BME and 5-azaC groups compared to control. In addition, the co-immunoprecipitation studies further substantiate our hypothesis and showed enhanced interaction of NR2A subunit with SKF and PSD-95 in BME and 5-azaC groups compared to control group. This complex possibly enhances the Ca2+ influx through the release of glutamate, and then induces LTP and LTM (Chen et al., 2005; Qiu et al., 2006). Further, the activated NMDAR is in turn found to mediate events associated with methylation of BDNF and its subsequent regulation of transcripts and translation in the hippocampus during LTM. We found that level of unmethylated BDNF DNA was higher in BME and 5-azaC groups compared to control. Subsequently, the level of Bdnf (exon IV) mRNA and protein were found to be higher in BME and 5-azaC groups compared to control. There could be a possible correlation of unmethylated BDNF with its expression. However, we did not find a significant difference in the level of methylated form (Martinowich et al., 2003; Munoz et al., 2010). Interestingly, we observed a positive correlation between DI with unmethylated BDNF DNA in BME/5-azaC group. BME/5-azaC possibly regulate the exon specific expression by controlling methylation process (Sales et al., 2011), and the expression of BDNF in hippocampus enhances memory (Bekinschtein et al., 2007, 2008a,b; Lubin et al., 2008; Mizuno et al., 2012).

Author Contributions
Conceived and designed the experiments: KER. Performed the experiments: JP. Analyzed the data: KER and JP. Contributed reagents/materials/analysis tools: KER and HS. Wrote the paper: KER and JP.

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Supplementary Material
The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fphar.2016.00166

Table S1 | Time spent (s) exploring the objects during training by each experimental group (control; BME; 5-azaC) individuals.
Benhayon, D., Magdaleno, S., and Curran, T. (2003). Binding of purified Reelin to Bekinschtein, P., Cammarota, M., Katche, C., Slipczuk, L., Rossato, J. I., Bekinschtein, P., Cammarota, M., Izquierdo, I., and Medina, J. H. (2008a). Frontiers in Pharmacology | www.frontiersin.org 12, June 2016 | Volume 7 | Article 166

Bekinschtein, P., Cammarota, M., Katche, C., Slipczuk, L., Rossato, J. I., Goldin, A., et al. (2008b). BDNF is essential to promote persistence of long-term memory storage. Proc. Natl. Acad. Sci. U.S.A. 105, 2711–2716. doi: 10.1073/pnas.0711863105

Benhayon, D., Magdaleno, S., and Curran, T. (2003). Binding of purified Reelin to the ApoER2 and VLDLR mediates tyrosine phosphorylation of Disabled-1. Brain Res. Mol. Brain Res. 112, 33–45. doi: 10.1016/S0169-328X(03)00032-9

Benson, S., Downey, L. A., Stough, C., Wetherell, M., Zangara, A., and Scholey, A. (2014). An acute, double-blind, placebo-controlled cross-over study of 320 mg and 640 mg doses of Bacopa monnieri (CDRI 08) on multitasking stress reactivity and mood. Phytother. Res. 28, 551–559. doi: 10.1002/ptr.5029

Blaze, J., and Roth, T. L. (2013). Epigenetic mechanisms in learning and memory. Wiley Interdiscip. Rev. Cogn. Sci. 4, 105–115. doi: 10.1002/wcs.1205

Bock, H. H., and Herz, J. (2010). “Role of gene transcription in long-term memory formation.” in Encyclopedia of Behavioral Neuroscience (Oxford: Academic Press), 161–179. doi: 10.1016/B978-0-08-045396-5.00030-0

Brigman, J. L., Feyder, M., Saksida, L. M., Bussey, T. J., Mishina, M., and Holmes, A. (2003). Reelin activates SRC family tyrosine kinases in hippocampal neurons. J. Neurosci. 23, 8209–8216. doi: 10.1523/JNEUROSCI.1951-05.2005

De, K., Chandra, S., and Misra, M. (2008). Evaluation of the biological effect of Bryophyllum pinnatum extract in the prevention and cure of diabetes mellitus. Phytother. Res. 22, 140–144. doi: 10.1002/ptr.2366

Dobbing, J., and Sands, J. (1979). Comparative aspects of the brain growth spurt. Early Hum. Dev. 3, 79–83. doi: 10.1016/0378-7579(79)90022-7

Downey, L. A., Kean, J., Nemeth, F., Lau, A., Poley, A., Gregory, B., et al. (2013). An acute, double-blind, placebo-controlled crossover study of 320 mg and 640 mg doses of a special extract of Bacopa monnieri (CDRI 08) on sustained cognitive performance. Phytother. Res. 27, 1407–1413. doi: 10.1002/ptr.4864

Ennaceur, A., and Delacour, J. (1988). A new one-trial test for neurobiological studies of memory in rats. I: behavioral data. Behav. Brain Res. 19, 31–47. doi: 10.1016/0166-4328(88)90157-X

Feng, J., Zhou, Y., Campbell, S. L., Le, T., Li, E., Sweatt, J. D., et al. (2010). Dnmt3a maintains DNA methylation and regulates synaptic function in adult forebrain neurons. Nat. Neurosci. 13, 423–430. doi: 10.1038/nn.2514

Gloviskins, J., and Iversen, L. L. (1996). Regional studies of catecholamines in the rat brain. J. Neurochem. 63, 655–659. doi: 10.1111/j.1471-4159.1996.tb09873.x

Hawk, J. D., and Abel, T. (2010). “Role of gene transcription in long-term memory storage,” in Encyclopedia of Behavioral Neuroscience, Vol. 3, ed. F. K. George (Oxford: Academic Press), 161–179. doi: 10.1016/B978-0-08-045396-5.00030-0

Hoe, H. S., Pocivavsek, A., Chakraborty, G., Fu, Z., Vicini, S., Ehlers, M. D., et al. (2010). Dnmt1 and Dnmt3a maintain DNA methylation and regulate synaptic function in adult hippocampal neurons. J. Neurosci. 30, 528, 551ñ559. doi: 10.1126/science.11090842

Miller, C. A., Gavin, C. F., White, J. A., Parrish, R. R., Honasoge, A., Yancey, C. R., et al. (2010). Cortical DNA methylation maintains remote memory. Nat. Neurosci. 13, 664–666. doi: 10.1038/nn.2560

Miller, C. A., and Sweatt, J. D. (2007). Covalent modification of DNA regulates multiple roles in memory formation. Neuron 53, 857ñ869. doi: 10.1016/j.neuron.2007.02.022

Ming, G., and Song, H. (2011). Adult neurogenesis in the mammalian brain: significant answers and significant questions. Neuron 70, 687–702. doi: 10.1016/j.neuron.2011.05.001

Mizuno, K., Dempster, E., Mill, J., and Giese, K. P. (2012). Long-lasting regulation of hippocampal Bdnf gene transcription after contextual fear conditioning. Genes Brain Behav. 11, 651–659. doi: 10.1111/j.1601-183X.2012.00805.x

Morris, M. I., Adachi, M., Na, E. S., and Monteggia, L. M. (2014). Selective role for Dnmt3a in learning and memory. Neurobiol. Learn. Mem. 115, 30–37. doi: 10.1016/j.nlm.2014.06.005

Morris, M. I., and Monteggia, L. M. (2014). Role of DNA methylation and the DNA methyltransferases in learning and memory. Dialogues Clin. Neurosci. 16, 359–371.

Mota, S. I., Ferreira, I. L., Valero, J., Ferreiro, E., Carvalho, A. L., Oliveira, C. R., et al. (2014). Impaired Src signaling and post-synaptic actin polymerization in Alzheimer’s disease mice hippocampus—linking NMDA receptors and the reelin pathway. Exp. Neurol. 261, 698–709. doi: 10.1016/j.expneurol.2014.07.023

Munoz, P. C., Aspe, M. A., Contreras, L. S., and Palacios, A. G. (2010). Correlations of spatial learning with the expression of NMDA receptor NR2A subunit. Neurobiol. Learn. Mem. 94, 251ñ258. doi: 10.1016/j.nlm.2014.06.005

Pesold, C., Impagnatiello, F., Pisu, M. G., Uzunov, D. P., Costa, E., Guidotti, A., et al. (1998). Reelin is preferentially expressed in neurons synthesizing gamaaminobutyric acid in cortex and hippocampus of adult rats. Proc. Natl. Acad. Sci. U.S.A. 95, 3221–3226. doi: 10.1073/pnas.95.6.3221

Preethi, J., Singh, H. K., Charles, P. D., and Rajan, K. E. (2012). Participation of microRNA 124-CREB pathway: a parallel memory enhancing mechanism of standardised extract of Bacopa monnieri (BESEB CDRI-08). Neurochem. Res. 37, 2167–2177. doi: 10.1007/s11064-012-0840-z

Preethi, J., Singh, H. K., Venkataraman, J. S., and Rajan, K. E. (2014). Standardised extract of Bacopa monnieri (CDRI-08) improves contextual fear memory by differentially regulating the activity of histone acetylation and protein phosphatases (PP1a, PP2A) in hippocampus. Cell. Mol. Neurobiol. 34, 577–589. doi: 10.1007/s10571-014-0042-0

Qiu, S., Zhao, L. F., Korwek, K. M., and Weeber, E. J. (2006). Differential reelin-induced enhancement of NMDA and AMPA receptor activity in the adult
Possible Role of *Bacopa monniera* in Epigenetic Mechanism

Preethi et al.

*J. Neurosci.* 26, 12943–12955. doi: 10.1523/JNEUROSCI.2561-06.2006

Quinlan, E. M., Lebel, D., Brosh, I., and Barkai, E. (2004). A molecular mechanism for stabilization of learning-induced synaptic modifications. *Neuron* 41, 185–192. doi: 10.1016/S0896-6273(03)00874-2

Rajan, K. E., Singh, H. K., Parkavi, A., and Charles, P. D. (2011). Attenuation of 1-(m-chlorophenyl)-biguanide induced hippocampus-dependent memory impairment by a standardized extract of *Bacopa monnieri* (BESEB CDRI-08). *Neurochem. Res.* 36, 2136–2144. doi: 10.1007/s11064-011-0538-7

Ramos-Moreno, T., Galazo, M. J., Porro, C., Martinez-Cerdeño, V., and Clascá, F. (2006). Extracellular matrix molecules and synaptic plasticity: immunomapping of intracellular and secreted Reelin in the adult rat brain. *Eur. J. Neurosci.* 23, 401–422. doi: 10.1111/j.1460-9568.2005.04567.x

Rogers, J. T., Russiana, I., Trotter, J., Zhao, L., Donaldson, E., Pak, D. T., et al. (2011). Reelin supplementation enhances cognitive ability, synaptic plasticity, and dendritic spine density. *Learn. Mem.* 18, 558–564. doi: 10.1101/lm.2153511

Sales, A. J., Biojone, C., Terceti, M. S., Guimarães, F. S., Gomes, M. V., and Joca, S. R. (2011). Antidepressant-like effect induced by systemic and intrahippocampal administration of DNA methylation inhibitors. *Br. J. Pharmacol.* 164, 1711–1721. doi: 10.1111/j.1476-5381.2011.01489.x

Shilatifard, A. (2006). Chromatin modifications by methylation and ubiquitination: implications in the regulation of gene expression. *Annu. Rev. Biochem.* 75, 243–269. doi: 10.1146/annurev.biochem.75.103004.142422

Squire, L. R., Wixted, J. T., and Clark, R. E. (2007). Recognition memory and the medial temporal lobe: a new perspective. *Nat. Rev. Neurosci.* 8, 872–883. doi: 10.1038/nrn2154

Stefanko, D. P., Barrett, R. M., Ly, A. R., Reolon, G. K., and Wood, M. A. (2009). Modulation of long-term memory for object recognition via HDAC inhibition. *Proc. Natl. Acad. Sci. U.S.A.* 106, 9447–9452. doi: 10.1073/pnas.0903964106

Stough, C., Scholey, A., Crockley, V., Wesnes, K., Zangara, A., Pase, M., et al. (2013). Examining the cognitive effects of a special extract of *Bacopa monnieri* (CDRI08: Keenmind): a review of ten years of research at Swinburne University. *J. Pharm. Pharm. Sci.* 16, 254–258.

Strasser, V., Fasching, D., Hauser, C., Mayer, H., Bock, H. H., Hiesberger, T., et al. (2004). Receptor clustering is involved in reelin signaling. *Mol. Cell. Biol.* 24, 1378–1386. doi: 10.1128/MCB.24.3.1378-1386.2004

Sui, L., Wang, Y., Ju, L. H., and Chen, M. (2012). Epigenetic regulation of reelin and brain-derived neurotrophic factor genes in long-term potentiation in rat medial prefrontal cortex. *Neurobiol. Learn. Mem.* 97, 425–440. doi: 10.1016/j.nlm.2012.03.007

Sweatt, J. D. (2009). Experience-dependent epigenetic modifications in the central nervous system. *Biol. Psychiatry* 65, 191–197. doi: 10.1016/j.biopsych.2008.09.002

Vollala, V. R., Upadhyya, S., and Nayak, S. (2011). Enhanced dendritic arborization of hippocampal CA3 neurons by *Bacopa monnieri* extract treatment in adult rats. *Rom. J. Morphol. Embryol.* 52, 879–886.

Weeber, E. J., Beffert, U., Jones, C., Christian, J. M., Forster, E., Sweatt, J. D., et al. (2002). Reelin and ApoE receptors cooperate to enhance hippocampal synaptic plasticity and learning. *J. Biol. Chem.* 277, 39944–39952. doi: 10.1074/jbc.M205147200

Yu, X. M., Askalan, R., Keil, G. J. I. I., and Salter, M. W. (1997). NMDA channel regulation by channel-associated protein tyrosine kinase Src. *Science* 275, 674–678. doi: 10.1126/science.275.5300.674

**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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