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Role of DNA Methylation in Hypobaric Hypoxia-Induced Neurodegeneration and Spatial Memory Impairment

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Keywords
Hypobaric hypoxia · DNA methyltransferase · Methyl CpG binding protein 2 · Brain-derived neurotrophic factor · Neurodegeneration

Abstract
Hypobaric hypoxia (HH) is a major stress factor that is associated with physiological, biochemical, molecular and genomic alterations. Brain is the organ that reacts sensitively to oxygen deprivation, which leads to oxidative stress and cognitive function impairment. Our previous studies have reported that downregulation of brain derived neurotrophic factor (BDNF) leads to neurodegeneration and memory impairment. The aim of the present study was to investigate the effect of HH exposure on DNA methylation and its regulation in BDNF expression, neurodegeneration and spatial memory impairment. For this purpose, Sprague Dawley rats were exposed to HH at a simulated altitude of 25,000 feet for 14 days. Real-time polymerase chain reaction was used for transcriptional expression of DNA Methyltransferases (DNMTs) including DNMT1, DNMT3a and DNMT3b, and immunoblotting was used for the translational expression of DNMT1, DNMT3a, DNMT3b, Methyl CpG binding protein 2 (MeCP2), pMeCP2 and BDNF in rat hippocampus. Additionally, neuronal morphology alteration and neurodegeneration in CA1 region of hippocampus were investigated though Cresyl violet (CV) staining and Fluoro-Jade C staining respectively. Results obtained suggested that HH exposure increased the expression of DNMT1 DNMT3b at the mRNA as well as protein level, whereas no significant change was observed in the level of DNMT3a. Furthermore, the level of pMeCP2 and BDNF were significantly decreased; however, the expression level of MeCP2 was significantly increased. The CV and Fluoro-Jade C-positive cells were significantly enhanced in the CA1 region of hippocampus in the HH exposed group as compared to unexposed rats. Thus, the present study concluded that HH decreases neuronal activation by the upregulation of DNA methylation and MeCP2 and decreased the expression of pMeCP2, which result in the downregulation of BDNF. The decreased BDNF expression is associated with neuronal loss and spatial memory impairment. This study highlights that DNMT inhibition could be an important therapeutic target for neurodegenerative diseases.

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Introduction

Hypobaric hypoxia (HH) has been associated with alterations in neurophysiological functions. Exposure to HH leads to sleep disturbance [1], oxidative stress [2] and alteration in acetylcholine neurotransmitter [3]. HH exposure causes cognitive function impairment including learning and memory function, neurodegeneration in CA3 region of hippocampus, glutamate excitotoxicity and high influx of calcium-mediated apoptosis cascade [4]. It also decreases the neurotrophic factor brain derived neurotrophic factor (BDNF), which helps in neuroprotection [21]. It is still unclear how HH induces neurodegeneration and downregulates the BDNF expression that causes memory impairment. Epigenetic changes could be an important aspect in delineating the mechanism behind HH-induced memory impairment.

Recent data suggest that epigenetic modifications in brain play a principle role in the neuropathogenesis of psychiatric diseases [5], neurodevelopmental [6] and neurodegenerative diseases [7]. The mechanism of epigenetic regulation comprise of DNA methylation, histone modification, nucleosome remodelling and RNA-mediated targeting [8]. DNA methylation is the main epigenetic modification, that leads to environment-induced, stable and heritable changes in gene expression. DNA methylation is the covalent addition of methyl group catalysed by DNA Methyltransferase (DNMTs) at cytosine of specially CpG region of DNA that leads to transcriptional inactivation [9, 10]. There are different families of DNMTs that have been identified as DNMT 1, 3a and 3b. The functions of different DNMTs vary with different developmental stages [11]. DNA methylation leads to transcriptional repression by the binding of methyl CpG binding protein 2 (MeCP2) and methyl CpG binding domain proteins on the methylated DNA. Both the methyl CpG binding domain and MeCP2 proteins provide a binding site for histone deacetylase (HDAC) at the CpG dinucleotide and lead to the transcriptional repression of the target gene. However, histone acetylation and deacetylation, which are another type of epigenetic modification, catalysed through histone acetyl transferase and deacetyl transferase (HDAC) respectively [12, 13].

A previous study reported that reduced levels of methyl transferase in the brain protected it from ischemic injury [14]. Furthermore, another research concluded that DNA methylation plays an important role in the mechanism of memory formation and its storage [15, 16]; it has also suggested that DNMT inhibitor altered the methylation label on brain plasticity-related genes such as *BDNF* and *reelin*. Another study has reported that upregulation of DNA methylation led to contextual fear conditioning and inhibition of DNMT led to the blockage of contextual fear conditioning [17, 18]. In a study Kishi et al. [19] reported that MeCP2 is a transcription factor that repressed the expression of targeted genes involved in maturation and function of neurons [20].

Additionally, activity-dependent changes in gene expression occurs due to DNA methylation – for example, changes in BDNF expression due to the phosphorylation of the transcriptional repression complex including MeCP2, CpG and HDAC1 led to the detachment from the BDNF promoter region in addition to the demethylation of CpG of the *BDNF* gene result in transcriptional activation. BDNF is a main moderator of the activity-dependent process in brain and is involved in the regulation of neuronal development and plasticity. Another study has suggested that MeCP2 was found to bind at the promoter region of transcription variant III of BDNF and detached due to MeCP2 phosphorylation that result in transcriptional activation and increased expression [21]. There are several in vivo as well as in vitro studies that reported the increased phosphorylation of MeCP2, which was induced by the neuronal activity in neurons, although, the loss of the MeCP2 phosphorylation associated with the loss of a number of traits of brain functions [22–28]).

There are very few studies on epigenetic changes associated with learning and memory under HH. Thus, in the present study, we evaluated the mRNA as well protein levels of different DNMT1, DNMT3a and DNMT3b enzymes in the hippocampus of HH-exposed and -unexposed rats. Our study concluded that HH declines neuronal activation by the upregulation of DNA methylation and MeCP2 and decreased the expression of pMeCP2, which resulted in the downregulation of BDNF. The decreased BDNF expression is associated with neuronal loss and spatial memory impairment.

Material and Methods

Animal

Adult male Sprague Dawley (SD) rats weighing 220 ± 10 g were obtained from the animal house of the Institute. All animals were housed in the control environmental condition with a temperature of 23 ± 2 °C and humidity 50 ± 10%. Rats were supplied food and water available *ad libitum* and the animal room was maintained at a 12 h/12 h light/dark cycle.

All experiments were performed according to the guidelines of the Committee for the purpose of control and supervision of

experiments on animals, Government of India. All experimental protocols were approved by the Institutional Ethical Committee for Animal Care and Use.

HH Exposure

Animals were exposed to chronic HH at 25,000 ft (~7,600 m) in an animal decompression chamber by reducing the ambient barometric pressure. The temperature and humidity of the chamber were maintained at 25 ± 2 °C and 55 ± 5% respectively. Rats were continuously exposed to HH for 14 days (14 DHH) with 10–15 min interval per day for the supply of water, food and changing of cage housing.

Morris Water Maze

Morris water maze (MWM) was used for the estimation of spatial memory impairment. MWM was performed according to the study published by Jain et al. [29], with some modification. MWM was composed of a circular pool with height 0.5 m and diameter 1.6 m filled with water. The temperature of water was maintained at 22–24 °C. The circular pool was divided into four quadrants, designated as Zone 1, 2, 3, and 4. A hidden platform was placed in Zone 4 known as the platform zone and the platform remained 1 cm below water. The behaviour and performance of rats were monitored and recorded by a digital camera. The software of this camera was installed in the monitor with ANY Maze software. During the 7 acquisition training periods, rats were placed in water at midpoint of each quadrant. Rats were freely allowed to swim for 60 s or until they found and climbed on to a hidden platform. If rats were unable to find the hidden platform within 60 s, then it was guided to locate the platform and placed 10 s on platform. When the performance of all rats became equal, they were subjected for probe trial and memory test before HH exposure and after HH exposure for memory retention.

Gene Expression Studies

RNA Isolation

Total RNA were extracted from snap-freezed hippocampus of the unexposed and hypoxia-exposed rats by the TRIzol regent (Sigma) method. RNA quantification was done by Nano drop (Thermo-fisher) and integrity performed by the running of 2 μL RNA on 1% agarose gel. Single stranded cDNA synthesis was performed according to instructions given by the verso cDNA synthesis kit (AB-1453/A). The compatibility of cDNA synthesis was estimated by using the housekeeping gene as GAPDH.

Real-Time Polymerase Chain Reaction

Real time polymerase chain reaction (PCR) was used for the quantification of mRNA levels of DNMT1, DNMT3a and DNMT3b genes. The fold change of these genes was estimated by the use of SYBR green master mix (Thermofisher). The forward and reverse primer of specific genes such as DNMT1 F-3-CTGCGGACCCTGGATGTGTT-5, R-3-GGCTGCTGGTTCCCACATCT-5, DNMT3a F-3-CATCCGCCACCTCTTCGCTC-5, R-3-CTCTCCGTCCTCTCGTTCTTGG-5 and DNMT3b F-3-GTCATCCGCCACCTCTTCGCTC-5, R-3-CTTCTCTCCGTCCTCTCGTTCTTGG-5 was used for the quantification of cDNA expression in hippocampus. GAPDH gene quantification was used as internal control and normalization of the targeted genes expression. The relative quantification (fold change) of mRNA was estimated by the use of the ΔΔCT method.

Immunoblotting

Protein Extraction

Rats were anaesthetized with Xylazine (10 mL/kg body weight) and Ketamine (50 mL/kg body weight) by intraperitoneal (i.p.) injection and decapitated. Immediately whole brains were removed and the hippocampus regions were taken out. They were then snap freezed in liquid nitrogen and stored at –80 °C until use. The hippocampus was homogenized in 1.5 mL micro tube containing the RIPA buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1% Sodium Dodecyl Sulfate (SDS)). A protease inhibitor cocktail (Sigma Aldrich) and a phosphatase inhibitor cocktail (Sigma Aldrich) were added before use. The sample was homogenized by a hand homogenizer followed by centrifugation and collection of supernatant. Protein concentrations of whole tissue homogenates were estimated by the Bradford method.

SDS Page for Immunoblotting

Protein levels in hippocampus were analysed through western blotting and analyses were performed according to previously described methods [30, 31]. An equal amount of protein extract was loaded on the SDS page gel that led to the separation of proteins according to their molecular weight. These separated proteins were transferred on to the nitrocellulose membrane followed by 2-h blocking in 3% BSA, which was prepared in 0.1% PBST (tween 20). After completion of blocking, the membranes were washed with 0.1% PBST and incubated with primary antibody anti DNMT1, DNMT3a and DNMT 3b, GAPDH MeCP2, BDNF and pMeCP2for overnight at 4 °C. After the completion of primary antibody incubation, the membranes were washed thrice with 0.1% PBST followed by incubation in a horseradish peroxidase-linked secondary antibody (1:5000, Millipore) for 2 h at room temperature. The immunoblots were developed with chemiluminescence reagents (Enhanced chemiluminescence (ECL); Sigma Aldrich) and image captured with MultiDoc-It™ Imaging System. The band size of appropriated protein was identified by the use of prestained protein ladder (Thermo Scientific). The concentration of the identified protein was quantified through densitometry of immune blots by using Image J analysis software.

Histological Studies

Cresyl Violet Staining

Rats were anaesthsised with Xylazine and Ketamine and then perfused transcardially with chilled 1X PBS (pH = 7.4) and 4% para formaldehyde (pH = 7.4). After perfusion, the whole brain was stored in the same 4% para formaldehyde for 3–5 days. The brain was dehydrated in the series of 10, 20 and 30% sucrose dilution. Serial coronal sectioning of 10 and 30 μm was performed using cryostat. Coronal sections were taken on gelatin-coated slides and washed with distilled water followed by the incubation in Cresyl-Violet (CV) staining for 10 min. After the completion of incubation, slides were rinsed with water followed by dehydration in graded series of alcohol, that is, 50%, 70%, 90% and absolute alcohol. After dehydration, slides were incubated in Xylazine solution for 2 min and mounted on DPX mounting media.

Fluoro-Jade C Staining

Apoptotic neurons were estimated by the use of Fluoro-Jade C staining according to the previously described method [32]

with some modifications. The procedure for cryo-sectioning was used similarly as described in Cresyl violet staining. Sections were immersed in 1% sodium hydroxide solution and then washed with 70% ethanol followed by distilled water washing. After washing, sections were incubated in 0.06% potassium permanganate solution for 20 min and rinsed twice with distilled water. Then sections were incubated with 0.0001% Fluoro-Jade C (Millipore, USA) staining for 20 min followed by 3 times distilled water rinsing. After washing, sections were dried on slides and incubated with Xylene for 2 min and mounted with DPX (Millipore, USA). The Fluoro-Jade C positive cells were manually counted in the CA1 region of HH exposed and control rats.

Statistical Analysis

Data was statistically assessed by the unpaired Student *t* test using graph Pad Prism software version 5. The value were represented as mean ± SEM of 4 animals for real-time PCR and western blotting and 6 animals per group for histological and behavioural study. The level of significance was set at $p < 0.05$.

Results

Effect of HH Exposure on Spatial Memory Impairment

To explore the potential role of HH exposure on spatial memory, impairment was evaluated using MWM. After completion of 7 days training, probe trial and memory test were performed before and post HH exposure. A significant spatial memory impairment was observed in HH-exposed rats as compared to control (unexposed) rats. The probe trial showed that HH exposure resulted in a significant increase in path-length (Fig. 1Aa; $p < 0.05$) and latency (Fig. 1Ab; $p < 0.01$) as compared to unexposed rats. Furthermore, memory test after HH exposure showed that rats spent significantly less time in the targeted zone (Fig. 1Ba; $p < 0.05$) and number of platform entries (Fig. 1Bb; $p < 0.01$) as compared to control group.

Effect of HH Exposure on DNMT1, DNMT3a and DNMT3b mRNA Expression and Protein Level in Rat Hippocampus

The mRNA expression of different DNMTs including DNMT1, DNMT3a and DNMT3b in rat's hippocampus was quantified through RT PCR. The bar diagrams in Figure 2a–c represent the fold change at the mRNA level of DNMT1, DNMT3a and DNMT3b in the hippocampus. HH exposure significantly increased the mRNA level of DNMT1 (Fig. 2a; $p < 0.01$) and DNMT3b (Fig. 2c; $p < 0.01$) as compared to control (unexposed) rats. However, there was no significant change observed in DNMT3a mRNA (Fig. 2b) in HH-exposed rats as compared to control (unexposed) rats.

Furthermore, the protein levels of DNMTs (DNMT1, DNMT3a and DNMT3b) in hippocampus were quantified through western blotting using hippocampus lysates. The band of different DNMTs at appropriate molecular weight on nitrocellulose membrane is shown in Figure 3a. The expressions of DNMT1, DNMT3a and DNMT3b are represented in Figure 3a–c respectively. HH exposure resulted in a significant increase in levels of DNMT1 (Fig. 3b; $p < 0.05$) and DNMT3b (Fig. 3d; $p < 0.001$) as compared to control (unexposed) rats, although no significant changes were found in the level of DNMT3a (Fig. 3c) in HH-exposed rats as compared to control (unexposed) rats.

Effect of HH Exposure on MeCP2 and Phosphorylation of MeCP2 at Ser. 421 in Rats Hippocampus

The expression level of total MeCP2 and specific phosphorylation of MeCP2 at Ser. 421 regulates the expression of BDNF, as we found that a band value of about 75 and 51 kDa (Fig. 4a) of pMeCP2 and MeCP2, respectively, was obtained on the nitrocellulose membrane. The densitometry quantification of pMeCP2 and MeCP2 showed significant alterations in the level of MeCP2 and pMeCP2 in HH exposed, and control rats were analysed through Student *t* test (unpaired test) using GraphPad Prism software. The levels of pMeCP2 and MeCP2 are shown in Figure 4b and c respectively. The level of MeCP2 was significantly increased (Fig. 4c; $p < 0.01$) in the HH exposed group as compared to control (unexposed) rats. Moreover, phosphorylation of MeCP2 was significantly decreased (Fig. 4b; $p < 0.05$) in the HH-exposed group when compared with control rats.

Effect of HH Exposure on the Expression of BDNF

The expression of BDNF protein was analysed through western blotting in hippocampus. Fig. 5a showed BDNF protein expression in respective groups. The densitometry analysis showed that HH exposure significantly decreased (Fig. 5b; $p < 0.05$) the level of BDNF as compared to unexposed rats.

HH Induces Neuronal Morphology Alteration and Neurodegeneration in the CA1 Region of Hippocampus

Neuronal morphology alteration is the primary step of apoptotic neurons and loss of neurons associated with memory impairment. CV positive cells and Fluoro-Jade C positive cells in CA1 region had been shown in Figure 6a, as we found that HH exposure was associated with neuronal morphology alteration and neurodegeneration in CA1 region. The bar diagram shown in Figure 6b and c

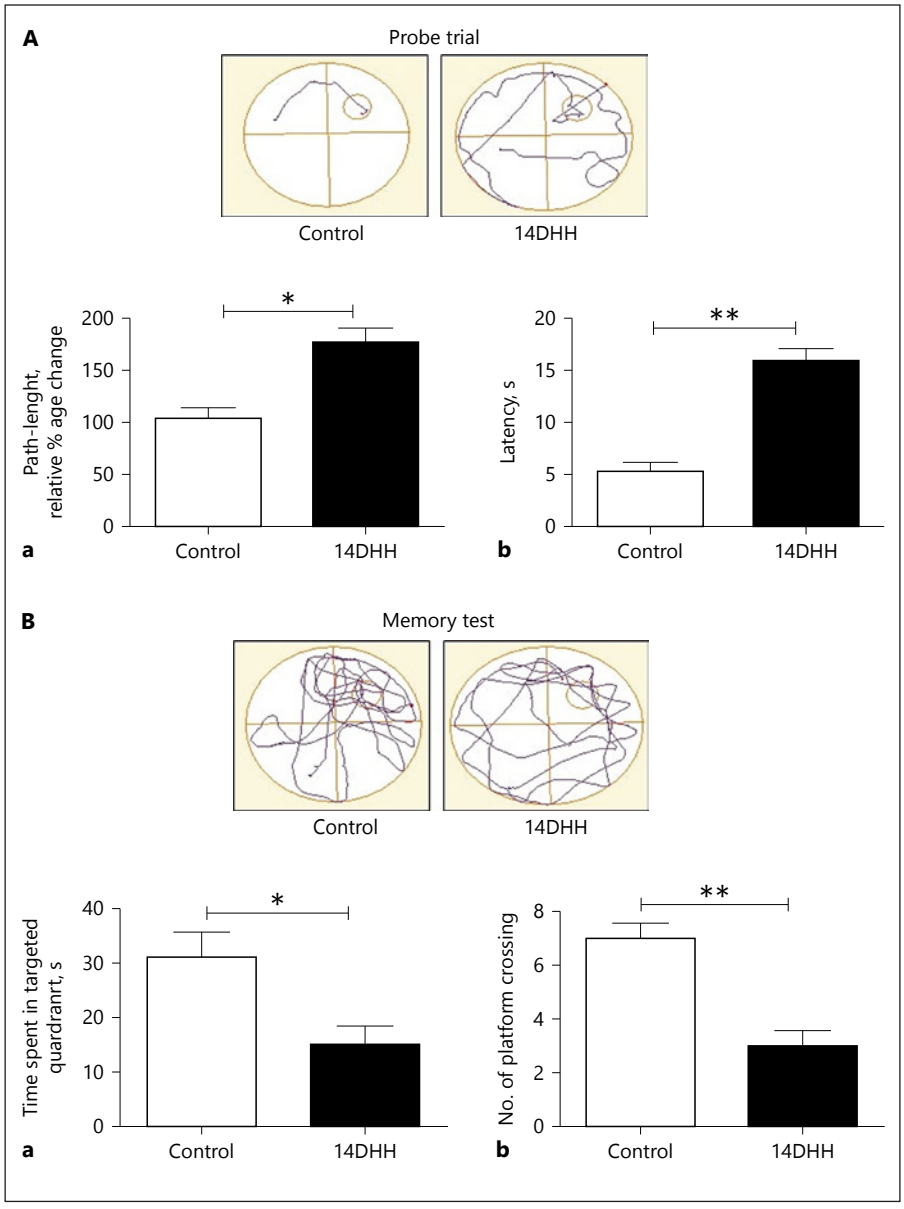

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Fig. 1. Effect of HH exposure on spatial reference memory impairment. HH-exposed spatial memory impairment was estimated through Morris water maze (MWM). **A**, **B** Representative traces plot of MWM for probe trial and memory test respectively. The bar diagram (**Aa**, **Ab**) represent a significant increase in path-length (* $p < 0.05$) and latency (*** $p < 0.01$) in HH-exposed rats as compared to normoxia rats (control) respectively. The bar diagram of (**Ba**, **Bb**) represent a significant decrease in the time spent (* $p < 0.05$) and the number of crossing (** $p < 0.01$) in the targeted zone as compared to Normoxia rats (control) respectively. Data represents mean ± SEM of 4 independent experiments. Student *t* test was used to calculate significance.

indicates that HH exposure significantly increased the CV-positive ($p < 0.05$) and Fluoro-Jade C-positive ($p < 0.01$) neurons, respectively, in the CA1 region of hippocampus as compared to control (unexposed) rats.

Discussions

The effect of HH on the expression of DNMTs and regulation of MeCP2 in BDNF expression is still an enigma. The present study investigated the possible impact of HH exposure on the level of different DNMTs (DNMT1, DNMT3a and DNMT3b) at the level of mRNA and protein and its involvement in alterations in spatial memory. Further, studies have been carried out to find the regulation of MeCP2 phosphorylation through neuronal activation, which regulate the expression of BDNF. BDNF is a neurotrophic factor that works as neuroprotective [33], regulate synaptic plasticity [34] and memory formation. There was an increase in the level of different DNMTs (DNMT1 and DNMT3b) at the mRNA as well as protein level in the hippocampus. HH decreases the neuronal activation that may have decreased the phosphorylation of MeCP2 at Ser. 421

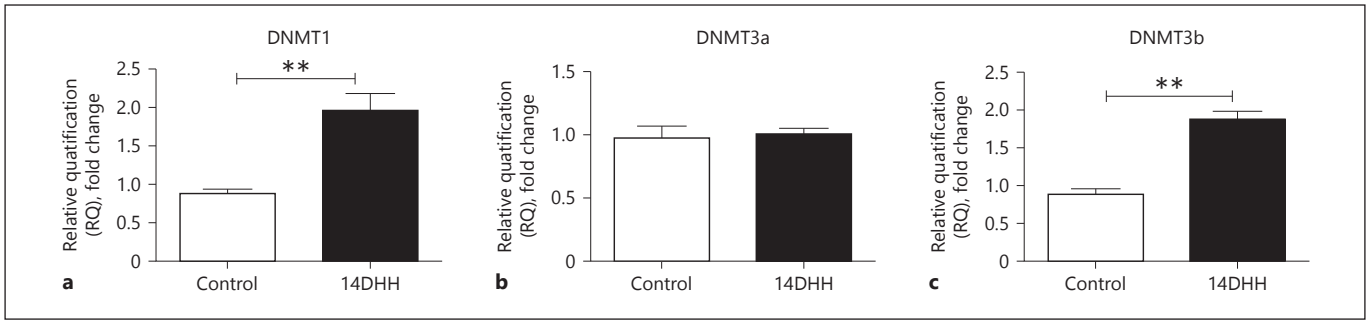

Fig. 2. The effect of HH on the mRNA level of different DNA Methyltransferases (DNMTs) in a rat's hippocampus. The fold change of different DNMTs as DNMT1, DNMT3a and DNMT3b was quantified through real-time PCR in both normoxia and HH-exposed rat's hippocampus. Fold changes of DNMT1, DNMT3a and DNMT3B have been shown through bar diagram as (**a–c**) respectively. Fold change in DNMT1 and DNMT3b was increased significantly ($p < 0.01$) in HH-exposed rats as compared to normoxia rats (Control). Data represents mean ± SEM of 4 independent experiments. Student *t* test was used to calculate significance; ** *p* value < 0.01.

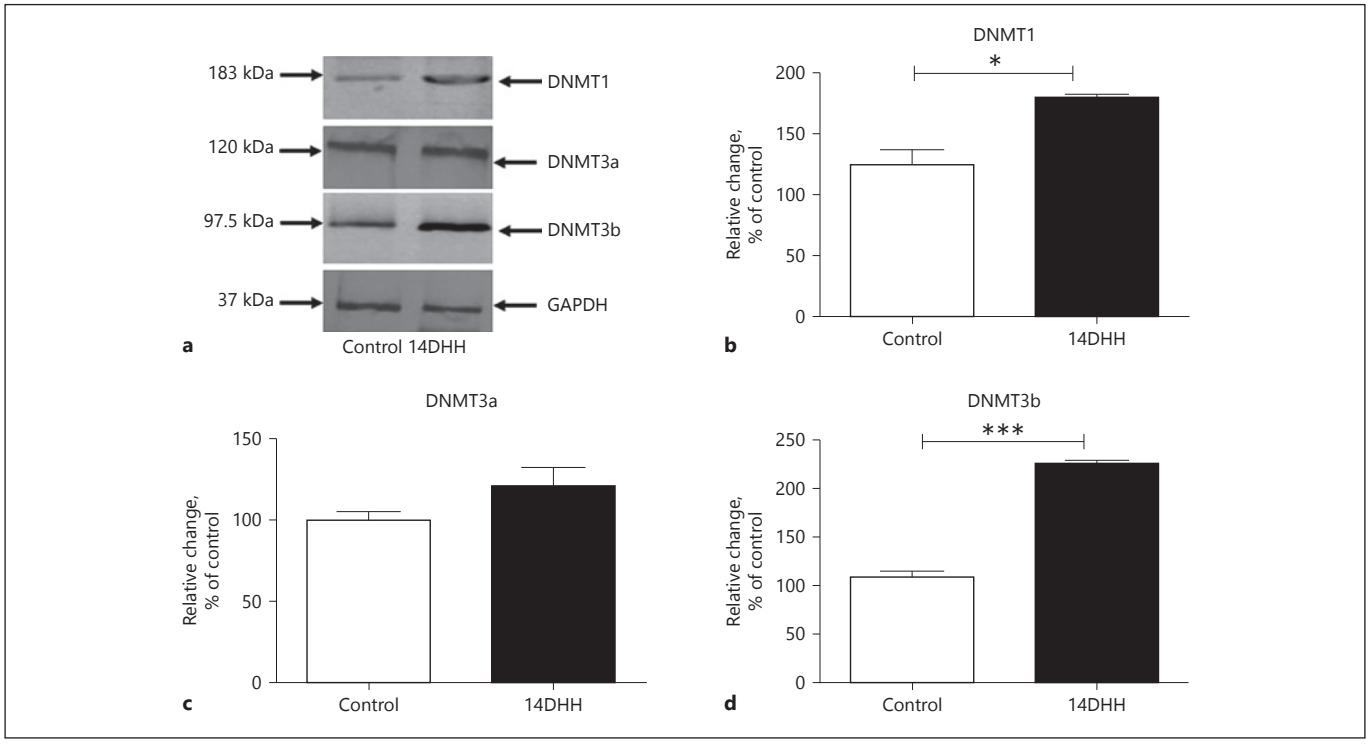

Fig. 3. HH exposure increased the protein level of DNA Methyltransferases (DNMTs) in hippocampus of rats. The levels of DNMTs including DNMT1, DNMT3a and DNMT3b were estimated through western blotting shown in (**a**). The alterations in the level of DNMT1 (**b**) and DNMT3b (**d**) were significantly increased (* $p < 0.05$, *** $p < 0.001$) in HH-exposed rats as compared to normoxia rats (Control), although no significant change was observed in DNMT3a (**c**). Data represents mean ± SEM of 4 independent experiments. Student *t* test was used to calculate significance.

(pMeCP2-S421). Phosphorylation of MeCP2 regulates the activity-dependent expression of BDNF, and decrease in the expression of BDNF could be associated with neurodegeneration and spatial memory impairment under HH exposure.

In our results, we have demonstrated that HH increases the levels of DNMT1 and DNMT3b at both mRNA and protein levels in the hippocampus, whereas there were no significance changes observed in DNMT3a. Our findings are supported by Zhang et al. [35] who reported that hy-

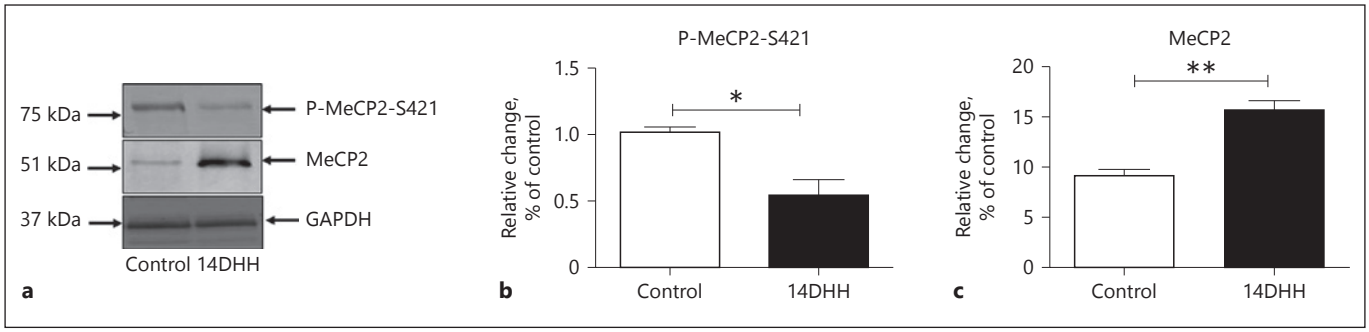

Fig. 4. HH exposure alters the level of MeCP2 and MeCP2 phosphorylation in hippocampus. Alteration in the level of MeCP2 and MeCP2 phosphorylation at position Ser. 421 was analysed through western blotting in both HH-exposed and normal rats. An equal amount of proteins (in both control and HH exposed rats) was loaded for both MeCP2 and pMeCP2 (**a**). A significant decrease in MeCP2 phosphorylation (**b**; * $p < 0.05$) and increase in the level of MeCP2 (**c**; ** $p < 0.01$) were observed in the HH-exposed group as compared to control rats. Data represents mean ± SEM of 4 independent experiments. Student *t* test was used to calculate significance. MeCP2, methyl CpG binding protein 2.

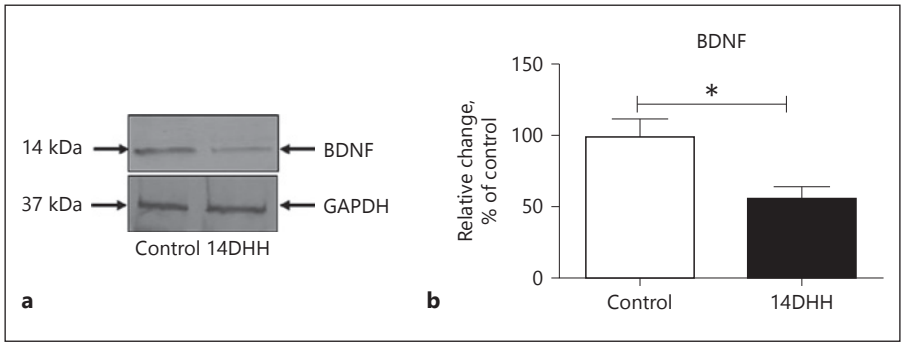

Fig. 5. HH exposure leads to alteration in the BDNF level in hippocampus. Change in the level of BDNF expression was quantified through western blotting in both normoxia (control) and HH-exposed rats. The immunoblotting of BDNF obtained on Nitrocellulose membrane (**a**) at corresponding molecular weight revealed a significant decrease in the level of BDNF expression in the HH-exposed group of rats (**b**; * $p < 0.05$) as compared to normoxia rats (Control). Data represents mean ± SEM of 4 independent experiments. Student *t* test was used to calculate significance. BDNF, brain derived neurotrophic factor.

poxia induces the DNA methylation by increasing the expression of DNMTs in mice hippocampus both at the mRNA and protein levels. DNMT1 predominantly maintained DNA methylation after replication and favourably distinguished hemi methylated DNA [36]. Liu et al. [37] also reported that DNA methylation played an important role in DNA damage and repair under hypoxia/reperfusion-induced brain injuries. So, DNMT1 may play an important role in the methylation of newly incorporated cytosine after repairing [38]. The mechanism behind the upregulation of DNMT1 and DNMT3b under hypoxia is still not clear. A previous report demonstrated that the expression of DNMT1 and DNMT3b was upregulated by hypoxia-induced factor 1α (HIF1α) through the binding of HIF1α at hypoxia responsive element site present at the promoter region of DNMT1 and DNMT3b. The regulation of DNMT1 and DNMT3b through HIF1α provides adaptation to the cell against hypoxia. HH increases the expression of HIF1α [39], which may induce the expression of DNMT1 and DNMT3b. Changes in the expression of DNMT1 and DNMT3b are associated with the alteration of DNA methylation at the CpG region of the targeted genes. Increase in DNMTs activity leads to hypermethylation at the promoter of BDNF exon I and IV that result in the decrease of the BDNF mRNA level.

Additionally, we have found out the consequence of DNA methylation on spatial learning and memory in the rat model. During the probe trial, both the pathlength and latency showed an increase after HH exposure. Furthermore, in memory recognition, the task

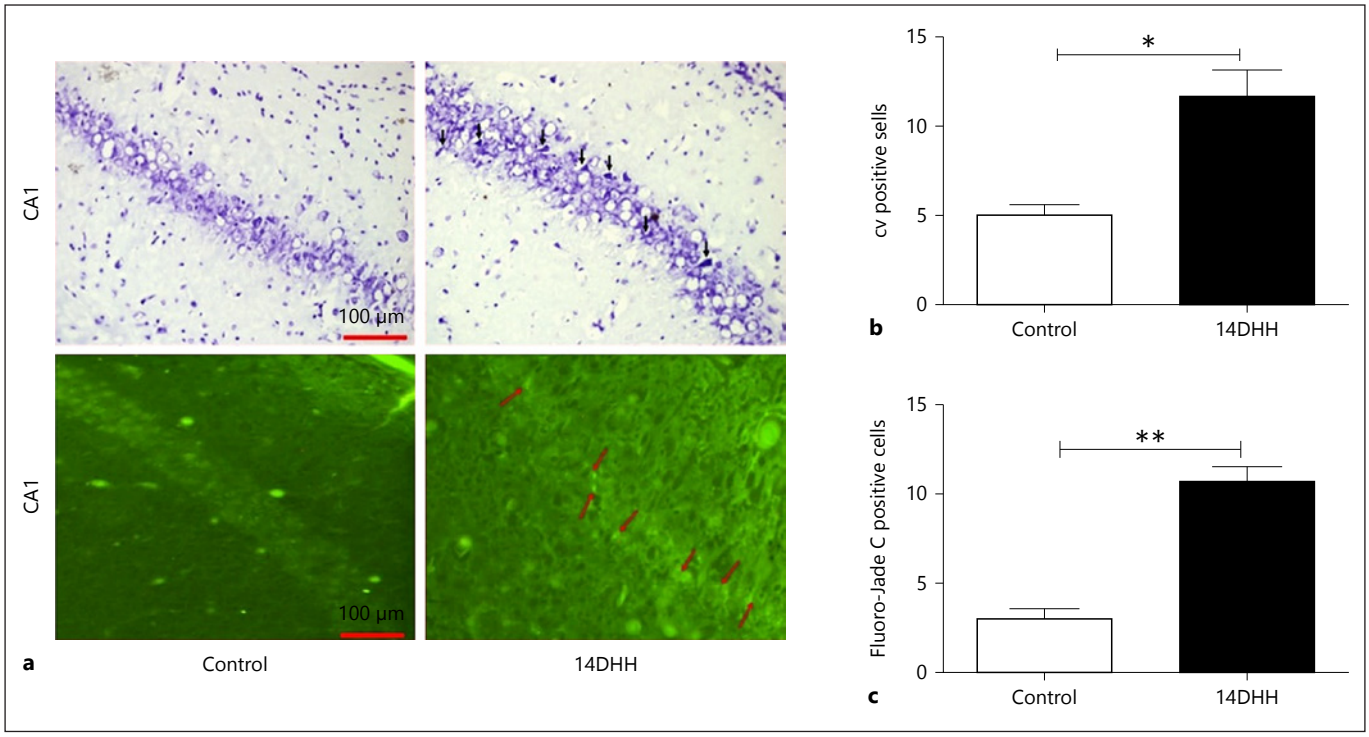

Fig. 6. HH induced neuronal morphology and apoptosis in the CA1 region of hippocampus. Neuronal morphology alteration and apoptotic neurons were examined through CV staining and Fluoro-Jade C staining, respectively, as shown in (**a**). A significant increase in the number of CV positive neurons (**b** ;* $p < 0.05$) and Fluoro-Jade C positive neurons (**c**; ** $p < 0.01$) were observed in the HH-exposed group as compared to Normoxia rats (Control). Data represents mean ± SEM of 6 independent experiments. Student *t* test was used to calculate significance. HH, hypobaric hypoxia; CV, crystal violet.

time spent in the targeted zone and number of crossing in the targeted zone were decreased due to HH exposure. There may be a possible link between DNA hypermethylation and spatial memory impairment under HH. Our results are in agreement with those of Feng et al. [40] where they have also reported that DNMT1 and DNMT3a play an important role in synaptic plasticity and memory formation.

Next, we found out the possible mechanism that regulates the expression of BDNF through MeCP2 and pMeCP2 in hippocampus. MeCP2 is a main transcriptional repressor protein, unfavourably involved in the maturation of nervous system and also involved in the learning and memory formation [41]. MeCP2 controls the expression of genes through the regulation of chromatin structure and alters the interaction of protein at the promoter site of targeted gene i.e. BDNF etc. There was a significant increase in the level of MeCP2 in HH-exposed rats as compared to the unexposed group, which may be due to an increase in DNA methylation. Overexpression of MeCP2 has been associated with transcriptional inactivation and it plays an important role in neurodegeneration [42]. Additionally, our results revealed that HH-induced neuronal morphology alteration and neurodegeneration as quantified through CV and Fluoro-Jade C, respectively, in the CA1 region of hippocampus as compared to control rats. Similar findings are also reported by Dastidar et al. [43]. Furthermore, the study had been extended with these initial outcomes and it explored the possible role of MeCP2 and pMeCP2 in the neurodegeneration as well as regulation of BDNF expression. BDNF genes act as neuroprotective and involve in fear memory [44, 45] and spatial memory [46]. NMDA receptor antagonist MK801 leads to demethylation of BDNF exon III and IV promoter in hippocampus [47]. Next, we found the level of phosphorylation of MeCP2 at Ser. 421 (P-MeCP2-S-421) and BDNF in hippocampus of HH exposed and unexposed rats. Neuronal activation has been associated with the phosphorylation of MeCP2 at Ser. 421 (PMeCP2). The phosphorylation of MeCP2 at the promoter site of *BDNF* gene regulate different processes like neuronal mor-

phology alteration, synapse formation and synaptic plasticity [20, 22, 23, 27]. Demethylation of promoter IV of BDNF resulted in an increased mRNA level of BDNF and phosphorylated MeCP2 [23, 48]. Interestingly, we found the decreased expression of P-MeCP2-S-421, and BDNF in HH-exposed rats as compared to control rats. Neuronal activity dependent increase in MeCP2 phosphorylation at ser 421 leads to removal of MeCP2 from BDNF promoter site which further may attribute to increased BDNF expression [21]. A previous reports suggested that the level of P-MeCP2-S-421 is directly correlated with the expression of BDNF [20, 49]. So, in the summary, HH induces DNA methylation that leads to the downregulation of BDNF expression, which may results in neurodegeneration and spatial memory impairment.

Conclusion

HH increases the expression of DNMT1 and DNMT3b in hippocampus at the mRNA and protein levels. An increase in the expression of DNMTs is associated with hypermethylation that leads to the transcriptional inactivation of the targeted genes by the binding of MePC2 at the hypermethylated site. Increase in DNA methylation is associated with neuronal damage that leads to neuronal morphology alteration and neurodegeneration in CA1 region of hippocampus. HH exposure decreased neuronal activation that is associated with a decrease in the phosphorylation of MeCP2 at Ser. 421 at the promotor of BDNF. This resulted in a decreased expression of BDNF, which worked as a neuroprotective and involved in long-term memory (LTM) formation. Therefore, a decrease in the expression of BDNF may lead to the neurodegeneration and spatial memory impairment under HH exposure. Consequently, MeCP2 and BDNF signaling pathway play an important role in the impairment of hippocampus functions [21].

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Disclosure Statement

The authors have declared that there are no conflicts of interest to disclose.

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