Acute Nicotine Treatment Attenuates Learning and Memory Impairment in REM Sleep Deprivation through Modulation of CREB and BDNF Protein Expression in Rat's Hippocampus

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A B S T R A C T

Introduction: REM sleep deprivation is associated with learning and memory impairment, and acute nicotine treatment has been shown to attenuate this effect. It involves changes in the DREAM protein expression, which regulates the transcription of others proteins that are important for the learning and memory processes. This study investigates the changes of pCREB and BDNF protein in REM sleep deprived rat's hippocampus upon nicotine treatment.

Methods: Different male Sprague Dawley rats were subjected to normal conditions, REM sleep deprivation and control wide platform conditions for 72 hours. During this procedure, saline or nicotine (1 mg/kg) was given subcutaneously twice a day. The rats were sacrificed, and their brains were harvested for the immunohistochemistry and western blot analysis.

Results: REM sleep deprivation rat (R group) showed a significant decrease of hippocampus pCREB and BDNF protein expression in CA1, CA2, CA3 and DG regions, and the mean relative level of pCREB and BDNF protein, compared to other experimental groups. Treatment with acute nicotine significantly prevented these effects and increased expression of pCREB and BDNF protein in all the hippocampus regions and upregulated the mean relative level of pCREB and BDNF protein.

Conclusion: This study suggests that changes in pCREB and BDNF protein expression in CA1, CA2, CA3 and DG regions of a rat's hippocampus and mean relative level of pCREB and BDNF protein involved in the mechanism of acute nicotine treatment-prevented REM sleep deprivation induced learning and memory impairment in rats.

Key words: BDNF protein; pCREB protein; hippocampus; learning and memory; nicotine; REM sleep deprivation

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INTRODUCTION

Adequate sleep, especially during Rapid eye movement (REM) contributes significantly to the process of memory and neural plasticity[1-3]. Adequate REM sleep is essential for fostering connections among neuronal networks for memory consolidation in the hippocampus[4]. It has been shown that sleep deprivation, especially REM sleep causes impairment in learning and memory[5], reduced level of Brain Derived Neurotrophic Factor (BDNF) in the rat hippocampus[6] and dysregulation of cAMP-response element binding protein (CREB) pathway[7].

Formation of memory in the brain consists of at least three stages: encoding, consolidation and retrieval[8]. Sleep is particularly beneficial to the consolidation stage of memory storage. Manipulation of sleep during this stage will affect the consolidation of memory. The process of learning begins with a transient increase in calcium ions and adenylyl cyclase, an enzyme responsible for the production of the second messenger, cyclic adenosine monophosphate (cAMP)[9]. The second messenger cAMP activates downstream kinases such as calmodulin-dependent protein kinase (CAMKII), mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase (ERK1/2) leads to phosphorylation of transcription factors[10]. Transcription factors such as CREB proteins promote up-regulation of gene expression for a protein that will consolidate labile memories into long-term memories[11]. This consolidation of memory is also dependent on the function of Brain Derived Neurotrophic Factor (BDNF) that plays an important role in synaptic plasticity such as Long Term Potential (LTP) and Long-Term Depression (LTD) during memory consolidation in the hippocampus[4]. For instance, mice lacking BDNF show a reduction in hippocampal LTP[12]. The BDNF gene is considered to be one of the CREB protein targets[13]. Therefore, hippocampus-dependent memory consolidation (formation and working memory) is particularly sensitive to sleep deprivation and changes of CREB and BDNF protein expression.

Acute nicotine treatment has been reported to prevent learning and memory impairment[14-15] and also LTP of the hippocampal CA1 region due to chronic psychosocial stress[16]. In addition, acute nicotine treatment has also been shown to prevent enhancements of LTD in the hippocampal CA1 region due to chronic stress[17]. Previous studies by the author show that acute nicotine treatment prevented impairment of learning and memory and is modulated by the DREAM protein in the hippocampus of REM sleep deprived rats[18]. DREAM protein has been demonstrated to be involved in the mechanism of learning and memory by functioning as a transcriptional repressor for CREB in a Ca2+-dependent manner. Knockout of the DREAM gene can facilitate CREB-dependent transcription and markedly enhance learning and synaptic plasticity with improved cognition[19]. DREAM protein has also been found to act as a negative regulator of the NMDA receptor function such as in the hippocampal synaptic plasticity that is also regulated by BDNF[20]. However, the relationship between DREAM, CREB and BDNF protein in the mechanism of nicotine-prevented learning and memory impairment due to REM sleep deprivation is still unclear and not understood. Therefore, this study was conducted to investigate the changes of hippocampal CREB and BDNF protein expression after acute nicotine treatment-prevented learning and memory impairment in REM sleep deprived rats.

MATERIALS AND METHODS

Animal preparation

Seventy-two adult male Sprague Dawley (230–280 g) rats were obtained from the Animal Research and Service Centre (ARASC), Universiti Sains Malaysia, Kubang Kerian, Kelantan, Malaysia. They were placed in a room with 12 h light/dark (lights on at 7:00 am and turn off at 7.00 pm) cycle at 28°C, with free access to food and water. There were six groups of rats in this study: Control (C, n=12), control treated with nicotine (C+N, n=12), wide platform (W, n=12), wide platform treated with nicotine (W+N, n=12), REM sleep deprivation (R, n=12), REM sleep deprivation treated with nicotine (R+N, n=12). The nicotine groups (C+N, W+N and R+ N) were treated with 1 mg/kg nicotine (Sigma, St. Louis, MO) subcutaneously twice a day, for 72 hours. The non-treatment groups were treated with a subcutaneous saline injection. The nicotine dose in this study was based on a previous study. This dose is known to produce nicotine blood levels similar to those of chronic smokers[21]. All experiments were approved by the Animal Care and Use Committee of Universiti Sains Malaysia [USM/Animal Ethics Approval/2012/ (81) (408)].

Induction of REM sleep deprivation

The REM sleep deprivation induction has followed the author's previous protocol[22]. The modified inverted flowerpot method was used to selectively induce REM sleep deprivation for 72 hours.

Immunohistochemistry analysis

After the Morris Water Maze test (the data was published in the author's previous article), the rats were sacrificed using an overdose intraperitoneal injection of sodium pentobarbitone. This method was used to avoid damage to the spinal cord[23]. Then, thoracotomy, perfusion and fixation of the rat's brains were done using a protocol from the author's previous studies[18]. The brains were dissected from the rats. Following overnight cryoprotection in 20% sucrose in 0.1M PB, the brains were cut using cryostat (30 µm), and the hippocampus regions were collected as free-floating sections in PBS. The sections were then rinsed with Tris buffered saline (TBS) and incubated overnight with rabbit polyclonal primary antibodies (dilution 1:500) for CREB and BDNF (Pierce, USA). Then, the sections were incubated with biotinylated secondary antibody anti-rabbit (dilution 1:200) for 1 hour. After 1 hour, sections were reacted with Avidin-biotin complex (ABC) and stained with diaminobenzidine and hydrogen peroxide until a brown colouration was seen. Then, sections were

mounted on slides, air-dried, dehydrated and covered with cover slips.

Six tissue sections were randomly taken from 1 rat per group, making a total of 36 tissue sections (n =6). The tissue sections were scrutinized at the hippocampal CA1, CA2 and CA3 and dentate gyrus regions. The CREB and BDNF positive neuron in every unit area (360,000 μm^2) were then identified and captured at a 40 x magnification. On average, 4 area units, i.e. 3 in the hippocampus and 1 in the dentate gyrus, could be obtained from each section. The mean number of CREB and BDNF positive neuron per unit area in the hippocampus and dentate gyrus of each animal group were then calculated.

Western Blot analysis

In order to verify the CREB and BDNF protein quantification results, the Western Blot analysis was performed on the hippocampal protein extract. The brain tissue was removed directly from the rats without a fixation process being performed and separated into the hippocampus region. The hippocampus region was immediately deep-frozen in liquid nitrogen and kept at -80ºC until further analysis. Protein was extracted from the hippocampus region using protocols from the author's previous studies[18]. The protein concentration of the extracted samples was measured using the Bicinchoninic Acid (BCA) protein assay kit. Sample protein containing 40-50µg total protein (after optimization) were denatured and subjected to SDS-PAGE using 12% resolving gel. The protein from the polyacrylamide gels was transferred to the nitrocellulose membrane (Bio-Rad, USA). The detailed protocol for the blotting process has been published in a previous article[18] by the author. In brief, the nitrocellulose was then incubated with rabbit polyclonal CREB and BDNF antibody (Pierce, USA) (dilution 1: 500 in TBST) or mouse monoclonal ß actin antibody (Pierce, USA) (dilution 1:2000 in TBST) overnight at 4º C. The nitrocellulose membrane was then incubated with HRP-conjugated goat anti-rabbit antibody (Pierce, USA) (dilution 1:5000 in TBST) or mouse secondary antibody (Pierce, USA) (dilution 1:5000 in TBST) for 1 hour at room temperature. In between the incubations, the nitrocellulose membrane was washed three times in TBS-T20 for 10 minutes each. Finally, the blot was examined using Immobilon western chemiluminescent HRP substrate and an image was taken using an image analyzer. The integrated density values (IDV) of CREB and BDNF protein and ß-actin protein were measured using a software program in the image analyzer. The mean relative intensity or fold change was determined by the following formula:

> Mean Relative Intensity = (IDV DREAM protein/IDV endogenous control) target group/ (IDV DREAM protein/ IDV endogenous control) calibrator group.

Statistical analysis

Statistical analyses were performed using the Statistical Package for Social Sciences (SPSS) software version 21. CREB and BDNF positive neuron expression and mean relative of CREB and BDNF protein level were measured using One-way ANOVA, Bonferroni and Dunnet's post hoc test. All the data are reported as mean ± standard error mean (S.E.M) and the level of significance was set at $p<0.05$.

RESULTS

CREB and BDNF protein expression in hippocampus

CREB and BDNF protein expression was significantly decreased in the R group when compared to the other groups in CA1 ($p<0.001$) (Figure 1 and 3), CA2 ($p<0.001$) (Figure 1 and 3), CA3 ($p<0.001$) (Figure 1 and 3) and dentate gyrus hippocampus ($p<0.001$) regions (Figure 1 and 3). Treatment with nicotine significantly attenuated this effect and increased CREB and BDNF protein expression in CA1, CA2 CA3 and dentate gyrus hippocampus regions as showed in the RN group when compared to the R group ($p<0.001$) (Figure 1 and 3).

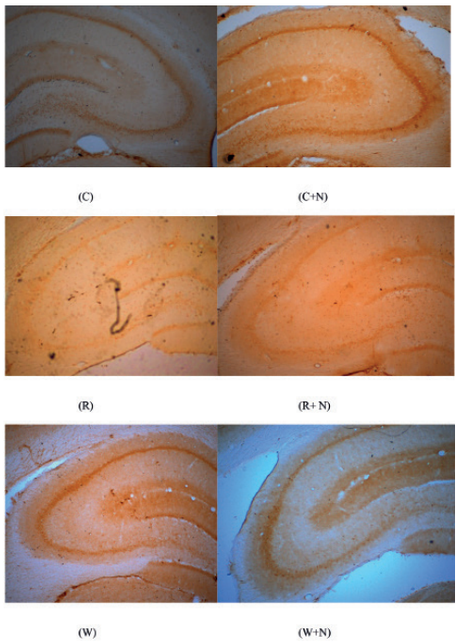


Figure 1 (A)

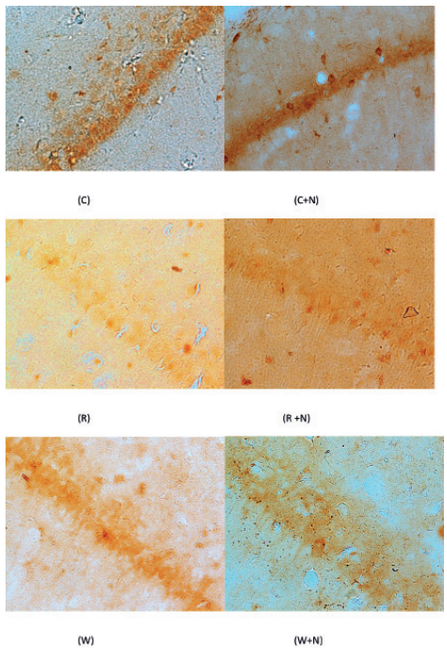
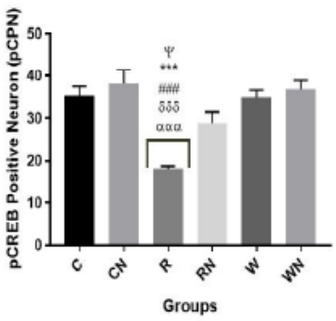
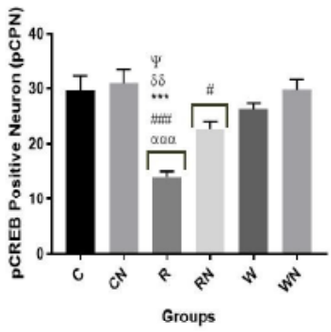
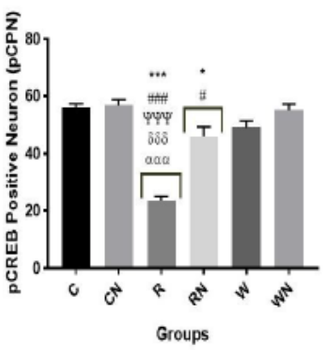
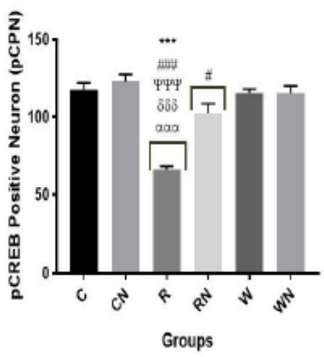


Figure 1 (B)









Figures 1 (C, D, E and F)

* p < 0.05; ** p < 0.01; *** p < 0.001, compared between R and C group

p < 0.05; ## p < 0.01; ### p <0.001, compared between R and CN group

Ψ p < 0.05; ΨΨ p < 0.01; ΨΨΨ p < 0.001, compared between R and RN group

δ p < 0.05; δδ p < 0.01; δδδ p < 0.001, compared between R and W group

α p < 0.05; αα p < 0.01; ααα p < 0.001, compared between R and WN group

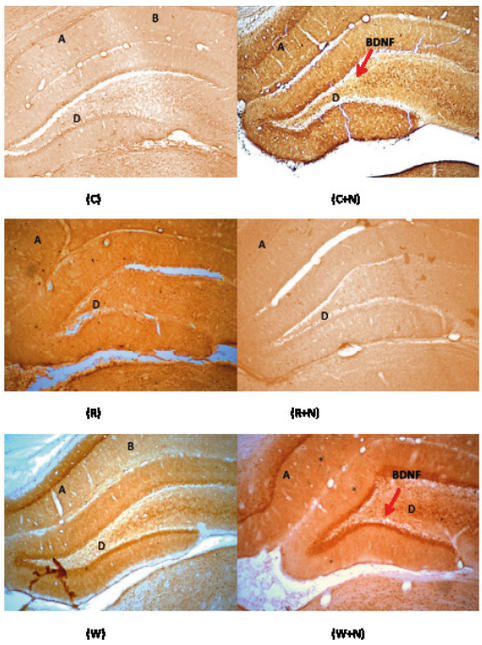


Figure 3 (A)

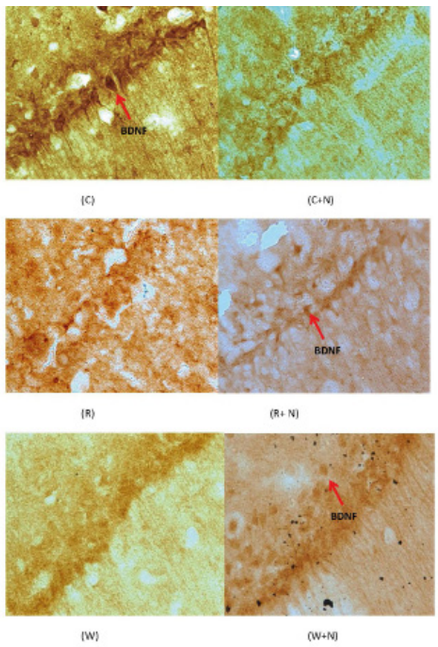
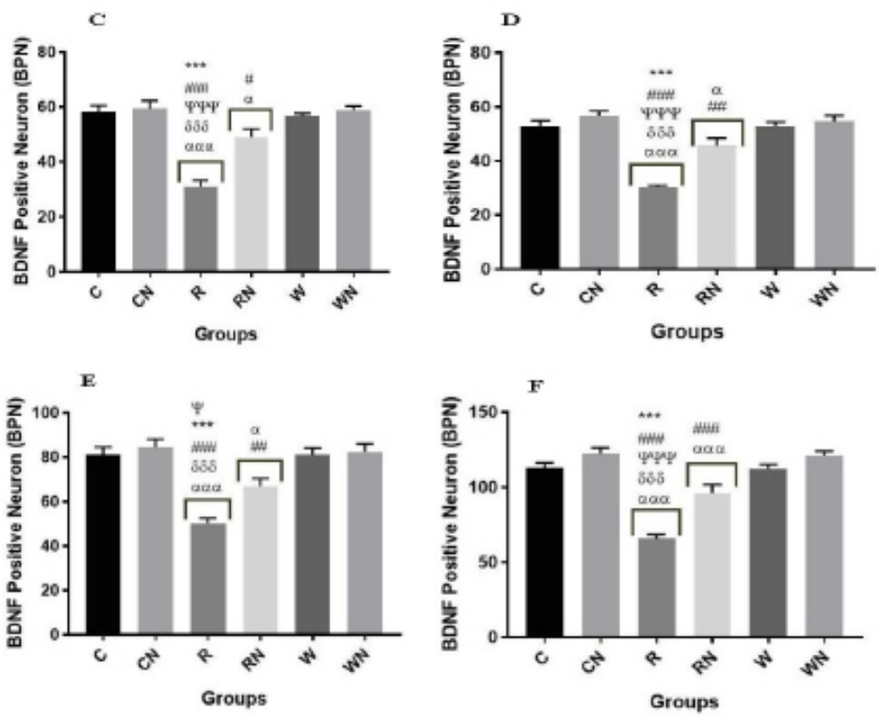


Figure 3 (B)



Figures 3 (C, D, E and F)

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, compared between R and C group

$p < 0.05$; ## $p < 0.01$; ### $p < 0.001$, compared between R and CN group

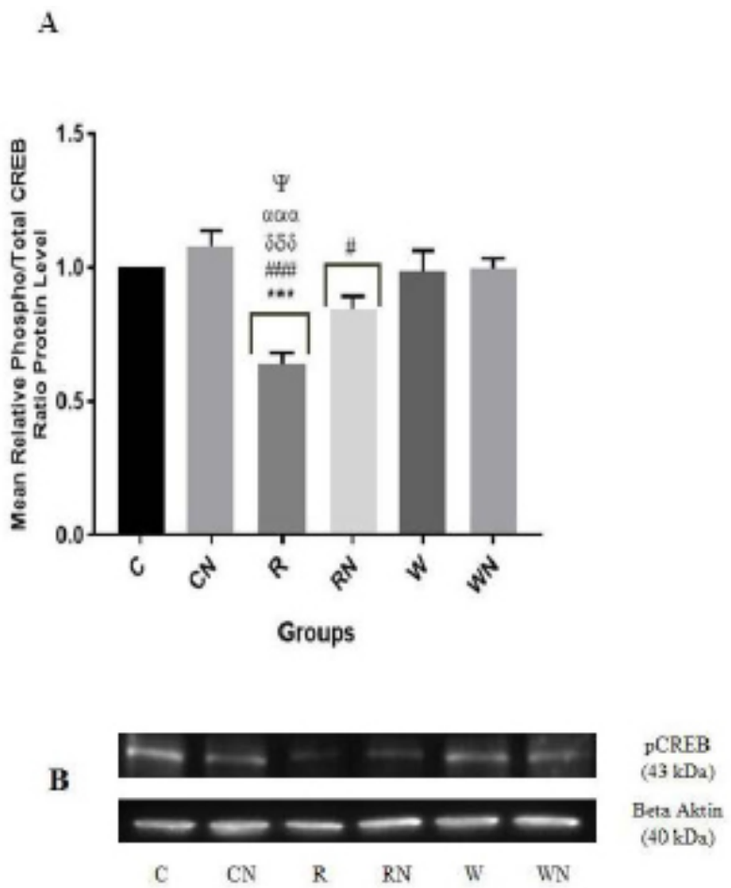
Ψ $p < 0.05$; ΨΨ $p < 0.01$; ΨΨΨ $p < 0.001$, compared between R and RN group

δ $p < 0.05$; δδ $p < 0.01$; δδδ $p < 0.001$, compared between R and W group

α $p < 0.05$; αα $p < 0.01$; ααα $p < 0.001$, compared between R and WN group

Mean relative protein level of CREB and BDNF in hippocampus

The mean relative protein level of CREB and BDNF in the hippocampus was significantly decreased in the R group when compared to the C+N ($p<0.01$), W ($p<0.01$) and W+N groups ($p<0.01$) (Figure 2 and 4). Treatment with nicotine (R+N) slightly reduced this effect and significantly increased the mean relative protein level of CREB and BDNF in hippocampus when compared to R group ($p<0.05$) (Figure 2 and 4).



Figures 2 (A,B)

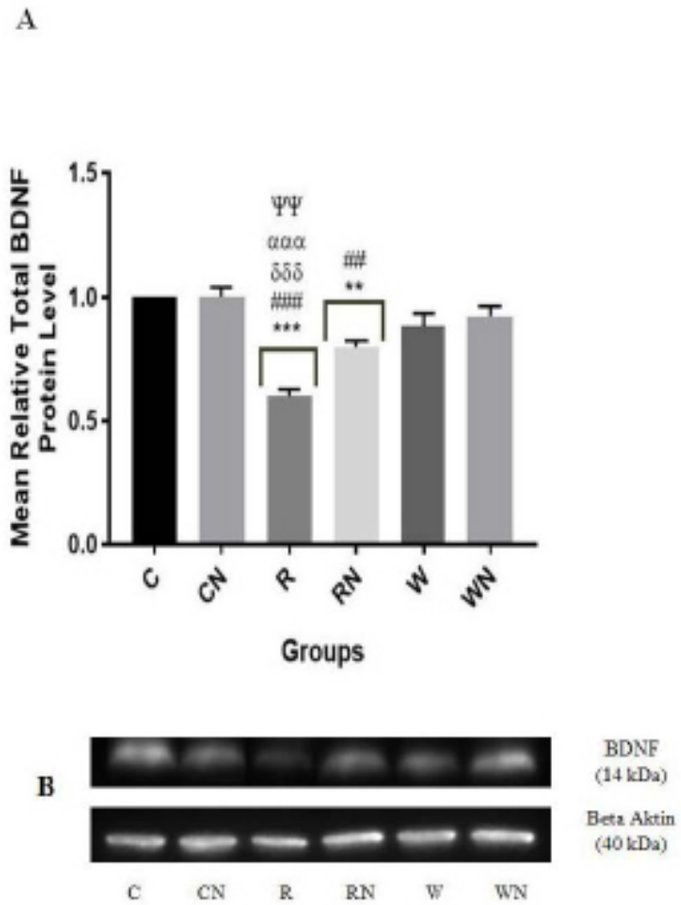
*** $p < 0.001$, compared between R and C group

$p < 0.001$, compared between R and CN group

ΨΨΨ $p < 0.001$, compared between R and RN group

δδδ $p < 0.001$, compared between R and W group

ααα $p < 0.001$, compared between R and WN group



Figures 4 (A,B)

*** p < 0.001, compared between R and C group

p <0.001, compared between R and CN group

ΨΨΨ p < 0.001, compared between R and RN group

δδδ p < 0.001, compared between R and W group

ααα p < 0.001, compared between R and WN group

DISCUSSION

The underlying mechanism of how nicotine treatment can prevent learning and memory impairment due to REM sleep deprivation is still unclear. Studies have suggested that nicotine treatment can activate pre-synaptic nicotine receptors that lead to the increase of glutamate release from the pre-synaptic terminal and as a consequence, increase the activity of excitatory neurons[24]. Nicotine treatment could also facilitate the activity of excitatory neurons through desensitization of α7nAch in GABAergic neuron and reduce the release of GABA. In addition to that, chronic nicotine treatment has been demonstrated to reverse stress-induced reductions in protein levels of the Brain-Derived Neutrophic Factor (BDNF)[16], a key protein in hippocampal synaptic plasticity[26]. Thus, preventing

sleep deprivation-induced impairment of memory using nicotine is an exciting finding.In this study, it was found that 72 hours of REM sleep deprivation caused a decrease in pCREB and BDNF protein expression and the mean relative protein levels of pCREB and BDNF in all area of the hippocampus. The decrease in pCREB and BDNF protein expression and its mean relative protein levels were accompanied by an increase in DREAM protein expression and its mean relative protein level that could cause a deficit in learning and memory in REM sleep deprived rats as reported in previous studies by our group[18]. Acute nicotine treatment attenuates these effects by increasing the expression of pCREB and BDNF protein expression and its mean relative protein levels in all areas of the hippocampus and reversing the deficit in learning and memory. These results suggest that the expression of pCREB and BDNF protein are associated with changes in the DREAM protein expression in modulating the effects of nicotine treatment in preventing learning and memory impairment due to REM sleep deprivation.

However, the association between DREAM, CREB and BDNF protein and REM sleep deprivation in the mechanism of learning and memory is still uncertain. CREB protein is localized in the nucleus. The phosphorylation at Ser133 residue of CREB protein permitted CREB protein to bind to a cAMP Responsive Element (CRE) sequence on the gene sequences and then activate various downstream kinase cascade and proteins that are dependent on cAMP and calcium signaling and one of them is BDNF protein[27]. The CREB protein is also associated with other co-factors such as CREB binding protein (CBP) and its homologue p300[27]. Due to external stimuli, these co-factors bind to p-Ser133 of CREB protein and trigger the RNA polymerase-II to initiate the process of gene transcription.

DREAM protein or known as KChIP3/calsenilin, is a calcium-regulated transcription modulator that represses transcription when bound to Downstream Regulator element (DRE) in the DNA sequences. DREAM protein is directly regulated by calcium through its four EF hands. Upon the calcium binding to a DREAM protein, DREAM protein unbinds from DRE sites, removing the inactivation of the transcription process[28]. DREAM protein was found higher in the hippocampal dentate gyrus (DG). Studies have shown that during the contextual fear conditioning tests, the DREAM protein translocates from the plasma membrane Kv channels into the nucleus in 6 hours to suppress the target gene expression. The dissociation of DREAM/KChIP3 from the Kv channels will contribute to the enhanced A-currents, which in turn alter general excitability and LTP induction property. This effect negatively regulates target proteins including Prodynorphin, c-fos and BDNF protein[29]. Previous studies have shown that DREAM protein could interact with CREB protein and abolish CREB-CBP interaction by repressing the transcription of genes[30]. However, when the DREAM gene is knocked out or abolished, phosphorylation of CREB will occur and establish the interaction of CREB-CBP and activate the transcription process[31]. Therefore, the DREAM protein can act as a negative regulator to the CREB, by which knocks out the DREAM gene. It facilitates CREB-dependent transcription and markedly increases learning and synaptic plasticity with improved cognition[32].

Studies have reported that chronic caffeine treatment may protect the sleep-deprived rats from impairment in learning and memory by preserving the level of pCREB and BDNF protein expression in the hippocampal region. Caffeine treatment also prevents REM sleep deprivation induced impairment of late phase-LTP in the dentate gyrus which is responsible for the synaptic transmission in the hippocampal brain neuron[32-33]. Therefore, the mechanism of nicotine treatment prevented learning and memory impairment in REM sleep deprived rat seen in this study is similar to the effects of caffeine in previous studies.

CONCLUSION

The present study suggests that nicotine treatment-prevented learning and memory impairment due to REM sleep deprivation by modulating the expression of pCREB and BDNF protein is related to the changes of the DREAM protein, which was previously reported in this study[18]. However, the exact mechanism of how these proteins interact with upstream regulation that is involved in learning and memory during nicotine treatment in REM sleep deprivation is still unclear. Therefore, further investigation is needed, especially on the role of glutamic acid (GLU) and γ-amino-butyric acid (GABA) systems in the nicotine-treated REM sleep deprived rat hippocampus in order to elucidate the mechanism.

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DISCLOSURE OF CONFLICT OF INTEREST

The authors have no disclosures to declare.

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