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ORIGINAL ARTICLE

Downregulation of CREB expressions in trimethyltin-induced hippocampus caused memory dysfunction in mice

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ABSTRACT
Downregulation of phospho-cAMP response element-binding (CREB) expression are detected by both western blotting and immunocytochemical analysis in the nucleus hippocampi of trimethyltin (TMT)-exposed mice in time-dependently. A single dose of TMT (2.4 mg/kg, i.p) was applied to mice revealed an increases in phospho-CREB expression at 3h- and 6h-after TMT intoxication, and decreases in p-CREB expression from 12 h to 7 days after TMT. In the forskolin study, we demonstrated that pretreatment with forskolin, an activator of adenylyl cyclases, revealed an increasing in phospho-CREB in the hippocampus of TMT-treated mice as observed by immunocytochemical method using specific antibody of phospho-CREB in accordance with behavioral data showing that pretreated-forskolin mice showed the escape latency faster and the numbers of crossings much more than mice only treatment with TMT.
Keywords: trimethyltin, nuclear extraction, phospho-CREB expression, mice
Abbreviations: *cAMP, 3'-5'-cyclicadenosine monophosphate; CREB, cAMP response element-binding; DG, dentate gyrus; DMSO, dimethylsulfoxide; HPT, hidden platform test; I.P, intraperitoneal; PBS, phosphate-buffered saline; PT, probe test; PVDF, polyvinylidene difluoride; SDS, sodium dodecyl sulfate; SEM, standard error of the mean; TMT, trimethyltin; WMT, working memory test.*

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INTRODUCTION

Trimethyltin (TMT), an organic compound of tin, causes neuronal cell death selectively in the dentate gyrus of hippocampus of mice [6,24] followed by impairment of memory function [13,15]. Damage of cognitive and memory function was demonstrated to relate to changes in cAMP response element-binding (CREB) protein, a constitutively expressed transcriptive agent in the nuclei from hippocampal neurons. Downregulation of immunohistochemical signal of CREB-mediated transcription by oxidative stress and amyloid-beta was demonstrated in the hippocampus and cortex of mice [21]. CREB has been known as a constitutively expressed nuclear transcription factor that play a critical role in the neuronal survival and function including formation and retention of memory in mice [1,17,22]. It was clearly accepted that CREB-mediated gene expression was increased in the hippocampus during long-term potentiation [10] and significant improvement in cognitive function [7]. CREB can form functional homodimers, or heterodimers with other CREB/ATF family proteins. After phosphorylation of CREB at the Ser133 residue and binding to a CRE site, the CREB binding protein, a coactivator of CREB, binds to the phosphorylated CREB. This leads to activation of a transcription factor/RNA polymerase II complex that directly *trans*-actiactivates target gene expression. Ser133 can also be phosphorylated by a number of protein kinases other than protein kinase A, including calcium calmodulin-dependent protein kinase, protein kinase C, and ribosomal S-6 kinase. This suggests that CREB plays an important role in integrating intracellular cAMP and calcium signaling as well as responses to neurotrophic factors [3,18]. Until now, many studies have been done to understand the mechanism by which TMT causes neuronal death especially in the hippocampal dentate gyrus of mice leading to memory dysfuntion. However, changes in phospho-CREB, an activated form of CREB agent in the TMT-exposed hippocampal of mouse's brain were not evaluated in time-dependently. So, this study was performed to extend our earlier findings and to get a better understanding of the changes in phospho-CREB in the nucleus of hippocampi neurons in TMT-exposed mice, western blotting and immunocytochemical analysis with specific antibody for phospho-

CREB was used to detect phospho-CREB changes in time-dependently after TMT intoxication in mice. Simultaneously, the impairments of memory function caused by TMT are assessed using water maze training with or without pretreated of forskolin, demonstrated the increasing in phospho-CREB in the hippocampus of forskolin-treated mice results in enhancing the learning function in TMT model, showing by decreases in the escape latency, and increases in the numbers of crossing in forskolin-treated mice, as compared with TMT-treated mice.

Material and Methods

Animals and drug treatment

All animals were treated in strict accordance with the National Institutes of Health (NIH) Guide for the Humane Care and Use of Laboratory Animals (NIH Publication No. 85-23, 1985; www.dels.nas.edu/ila).

Swiss Albino mice were supplied by the Institute of Vaccines and Medical biologicals at Nha Trang Town, Viet Nam. The male mice, weighing ~22-24g, were maintained on a 12:12 h light: dark cycle and were fed *ad libitum*. They were allowed to adapt to these conditions for 1 week before experiment. Six mice per each group were sacrified at 3 h, 6 h, 12 h, 1 d, 2 d, 3 d, and 7 d after a single dose of 2.4 mg/kg TMT injection for CREB and phospho-CREB detection using immunocytochemical and western blotting analysis. Control mice received an equivalent volume of saline (n=6).

To study the effect of TMT toxicity on the changes in CREB in the hippocampal mice, forskolin study was performed. Animals received an i.p injection of 5 mg/kg forskolin diluted in a dimethylsulfoxide (DMSO; 3 % of DMSO/PBS; n=6), or vehicle (n=6). Thirty minutes after forskolin or vehicle, mice received a single dose of 2.4 mg/kg TMT. Control mice which were injected forskolin (n=6) or vehicle (n=6) without TMT were used. Animals were performed behavioral test at 3 d after TMT treatment and were sacrified at 20 min after the last session, including CREB and phospho-CREB detection using immunocytochemical and western blotting analysis.

Water maze test

Water maze test was designed as described previously [8]. The swimming pool was a circular water tank 120 cm in diameter and 35 cm deep. It was filled to a depth of 22 cm with water at 25±2°C. A platform 7 cm in diameter and 20 cm in height was placed inside the tank with its top surface being 2 cm below the surface of the water. The pool was surrounded by many cues that were external to the maze. Each animal was received five daily trials for 4 consecutive days of hidden platform test (HPT), the time elapsed until the mouse reached and land on the platform was scored as the escape latency. The first time of the first day of HPT is training time, the mouse was allowed 15 s of rest on the platform after finding it (or after being put on the platform by the experiment if it failed to find it within 60 s). Working memory test (WMT) was performed from 5^{th} to 7^{th} day of water maze test. WMT was performed similarly to HPT (without training time), the location of the platform was randomly varied between the four quadrants but was always placed in the center of the quadrant. Each animal was received five daily trials for 3 consecutive days and escape latency was scored. For the probe test (PT), the mouse swam for 60 s per each of two trials. The numbers of crossings for the location of the platform were scored using an automated tracking system (San Diego Instruments, San Diego, CA).

Immunocytochemistry for phospho-CREB detection

Mice were anesthetized with urethane and perfused transcardially with 50 ml of 50 mM phosphate-buffered saline (PBS), following by 30 ml of a mixture of 4 % paraformaldehyde in PBS. The brains were removed, post-fixed at 4 °C for 6 h in the same fixative and placed in 30 % sucrose in PBS overnight. The brains were cut on a horizontal sliding microtome into 25-μm transverse free-floating sections. Immunocytochemistry was performed as described previously [14]. Briefly, prior to incubation with the primary antibodies, anti-phospho-CREB (pSer133) antibody produced in rabbit C9102 (Sigma) (1:500), sections were preincubated with 0.3 % hydrogen peroxide in PBS for 30 min, then in PBS containing 0.4 % Triton X-100 for 20 min and 1 % normal serum for 20 min. After a 48 h incubation with the primary antibody at 4 °C, sections were incubated with the secondary biotinylated antisera for 2 h, and immersed in avidin-biotin-peroxidase complex for 2 h. 3,3'-diaminobenzidine was used as a chromogen.

The intensity of the immunoreactivity was graded as an absolute value using a computer-based image analysis system. For measurements of the immunoreactive populations, 25-30 cells were examined in each section. The areas examined were marked using a digital tablet and mouse. Within the areas, the immunoreactive cells were labeled and their x/y co-ordinates were recorded. These data were expressed as an absolute value of the immunoreactivity. Each value is the mean ± SEM of six animals.

Western blotting analysis

Western blotting analysis was performed as described previously [24]. Hippocampus were dissected immediately after decapitation and frozen in liquid nitrogen. Nuclear fraction of hippocampal lysates were extracted using the NE-PER nuclear and cytoplasmic extraction kit (Thermo Scientific, Rockford, IL,

USA) according to the manufacturer's instructions. Briefly, hippocampal tissues were homogenized in the provided cytoplasmic extraction reagent using a Dounce homogenizer. The homogenate was centrifuged at 16,000 g for 5 min, and the supernatant (cytosolic) fraction was immediately transferred to a prechilled tube. The pelleted fraction was suspended in the provided nuclear extraction reagent (prechilled), and the resulting suspension was centrifuged at 16,000 g for 10 min. The supernatant (nuclear) fraction was immediately transferred to a prechilled tube. Proteins (20–50 μg/lane) were separated by 8 % sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes. After transfer, the membranes were preincubated with 5 % nonfatmilk for 2 h and incubated overnight at 4 °C with primary antibody against anti-histone H4 antibody produced in rabbit, #2592 (1:1000; Cell Signaling), anti-CREB (48H2) mAb produced in rabbit, #9197 (Cell signaling) (1:1000), and anti-phospho-CREB (pSer133) antibody produced in rabbit #C9102 (Sigma) (1:500). And then, the membranes were incubated with anti-Rabbit IgG - Horseradish Peroxidase (1:1000, Sigma-PM0100) for 2 h. Subsequent visualization was performed using an enhanced chemiluminescence system (ECL Plus; GE Healthcare). Relative intensities of the bands were quantified by PhotoCapt MW (version 10.01 for Windows; Vilber Lourmat, Marne la Vallée, France).

Statistics

The data were analyzed using a one-way ANOVA followed by post hoc Fischer's PLSD test or two-way ANOVA followed by post-hoc multiple pair-wise comparisons with Bonferroni's correction. *P* values of less than 0.05 were deemed to indicate statistical significance.

RESULTS AND DISCUSSION

Immunocytochemistry for detection of the changes in the phospho-CREB (Ser133), after TMT treatment

Main purpose of this study was to evaluate the changes in cAMP response element-binding (CREB) and its phosphorylated form at serine 133 in TMT mouse model. Additionally, we have evaluated TMT-induced memory impairment in the presence or absence of forskolin, an agent which activated adenylyl cyclases and therefore enhanced cAMP in the hippocampus of mice to prove the role of CREB in the memory impairment induced by TMT. Trimethyltin (TMT) is known as an agent which causes neurodegeneration and memory impairment in murine [2,9,23]. Until now, many TMT studies has been still doing because molecular mechanism by which TMT causes neuronal death and memory dysfunction remained unclear.

Phospho-cAMP response element-binding (CREB) expression in the hippocampal neurons of TMT-induced mice were observed by immunocytochemical analysis using specific anti phospho-CREB (Ser133) antibody. Data was shown in figure 1 revealed that phospho-CREB positive cells were increased in the CA3, CA4 and polymorphic layer of hippocampus at early time-course of TMT treatment (3h and 6h-post TMT), as compared to control group. These expressions showed a significant decrease from 12 h until 7 days after TMT administration, compared to control group. This change was more pronounced at 2 days after TMT injury. At 2 days after TMT, phospho-CREB was almost no detected in CA3, CA4 and polymorphic layer.

Western blot analysis for detection of changes in the phospho-CREB (Ser133) *after TMT treatment*

We examined cAMP response element-binding (CREB), a cellular transcription factor has been shown to be integral in the formation of spatial memory. Western blot analysis revealed no changes in the total cAMP response element-binding (CREB) protein in the nucleus dentate gyrus of mouse hippocampus from early time until 7 days following a single dose of TMT, whereas changes in phospho-CREB were detected from 3 h to 7 days post-TMT administration. A significant increase in phospho-CREB was detected in the nucleus of hippocampus at 3 h after trimethyltin (TMT) administration. (data are shown in figure 2: ratio of phospho-CREB/CREB at 3 h post-TMT: 2.605 ± 0.833, $p = 0.016$, compared with control: 1.296 ± 0.223). From 6 h until 7 days after TMT injury, decrease of phospho-CREB expression was indicated (data are shown in figure 2: ratio of phospho-CREB/CREB of 6h post-TMT: 1.605 ± 0.565, $p = 0.555$, compared with control). Downregulation of phospho-CREB were revealed in figure 2 from 12 h until 7 days after TMT administration (data are shown in figure 2: phospho- CREB/CREB at 12 h post-TMT: 0.222 ± 0.111, $p = 0.045$; 1 d post-TMT: 0.024 ± 0.015, $p = 0.019$; 2 d post-TMT: 0.068 ± 0.039, $p = 0.023$, 3 d post-TMT: 0.050 ± 0.030, $p = 0.021$ and 7 d post-TMT: 0.004 ± 0.003, $p = 0.017$, compared with control).

Accumulating evidences demonstrated that CREB play an important role in different types of learning and memory function, including hippocampal-dependent learning in murine [4,5,11,16,20]. In this study, we found that the phospho-CREB expressions as assessed by western blotting and immunocytochemical analysis were significant decreased in CA3, CA4 subfields and polymorphic layers of TMT-stimulated mouse's hippocampus in time-dependent manner. In the early time after TMT administration, the increases in phospho-CREB were detected (3 h- and 6 h- after injury). After these early time-points,

phospho-CREB expressions were dramatically decreased until 7 days after TMT injury. Although we had not enough evidences to explain the reason why phospho-CREB expressions were increased in the early time-course of TMT insult. Importantly, the decreases in phospho-CREB occurred from 12 h- until 7 days after TMT confirmed that TMT downregulated phospho-CREB lead to memory impairment in mouse model. These results were consistant with results from other study that TMT intoxication downregulated CREB-immunoreactivity neurons of CA1 in rat model [19]. Some evidences showed that TMT decreased CREB-immunoreactivity neurons of the hippocampus in rat. Until now, there has been no paper which revealed CREB-imunocytochemistry in the mouse model with TMT intoxication.

Changes in the phospho-CREB (Ser133) in forskolin study

Applying with forskolin increases in phospho-CREB (Ser133) protein expressions in the hippocampus (CA3, CA4 and polymorphic layer) of mice as showed in figure 3C. In the presence of forskolin, effect of TMT on the changes of phospho-CREB is ameliorated (figure 3D). Western blotting analysis for CREB and phospho-CREB expression (figure 4) indicated that TMT significant decreased in phospho-CREB in the hippocampus of mice, this change was reversed by forkolin.

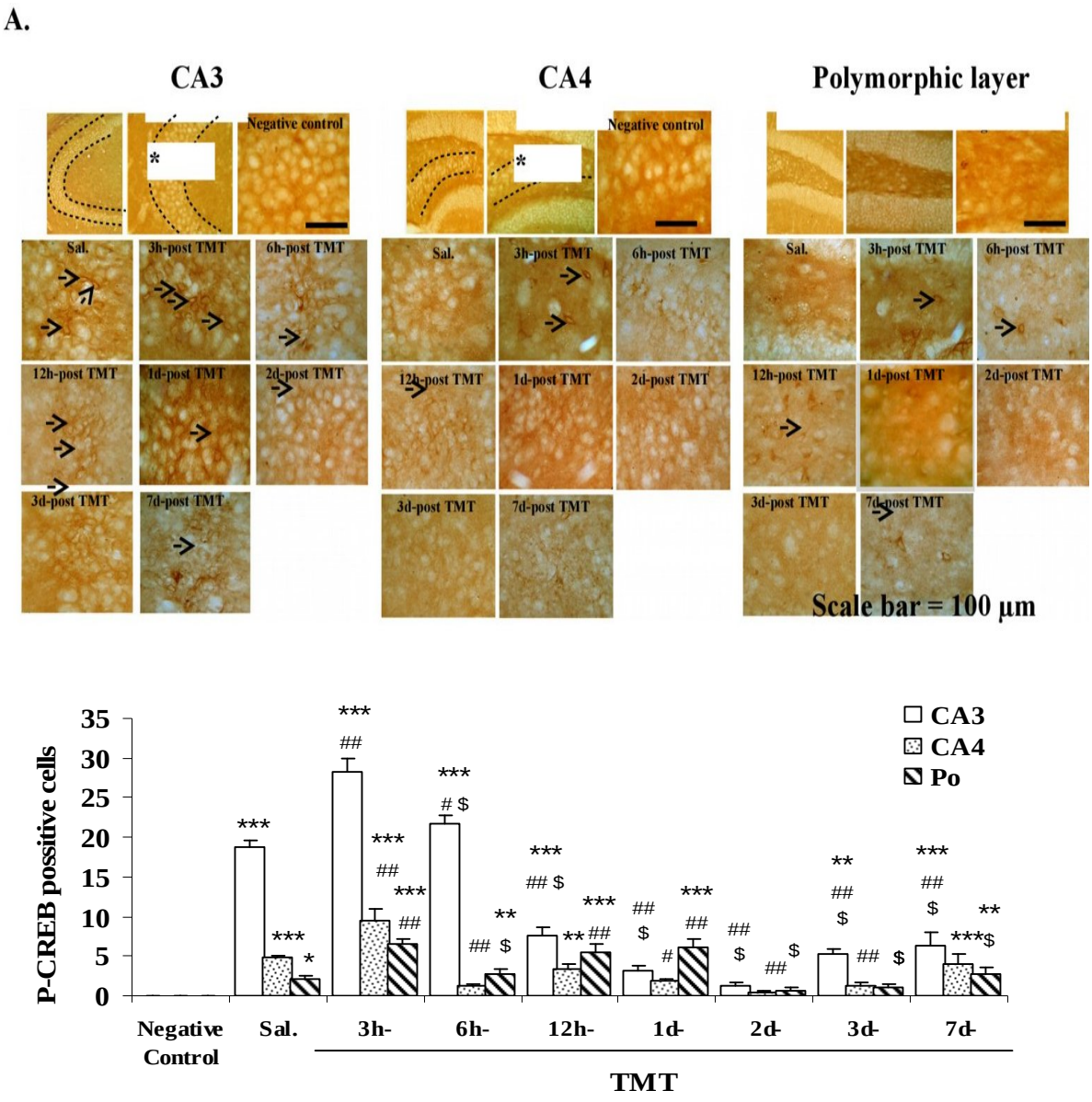


Figure 1. Time-dependent changes in phospho-CREB-immunoinoreactive cells the hippocampus of mice after TMT treatment. Each value is the mean±SEM of six mice. $^{*}P<0.05$, $^{**}P<0.01$, $^{**}P<0.001$ vs corresponding negative-control-treated mice; $^{\#}P<0.01$, $^{\#\#}P<0.001$ vs corresponding saline-treated mice; $^{\$}P<0.05$ vs corresponding 3h after TMT treatment mice (one-way ANOVA followed by Fisher's PLSD test).

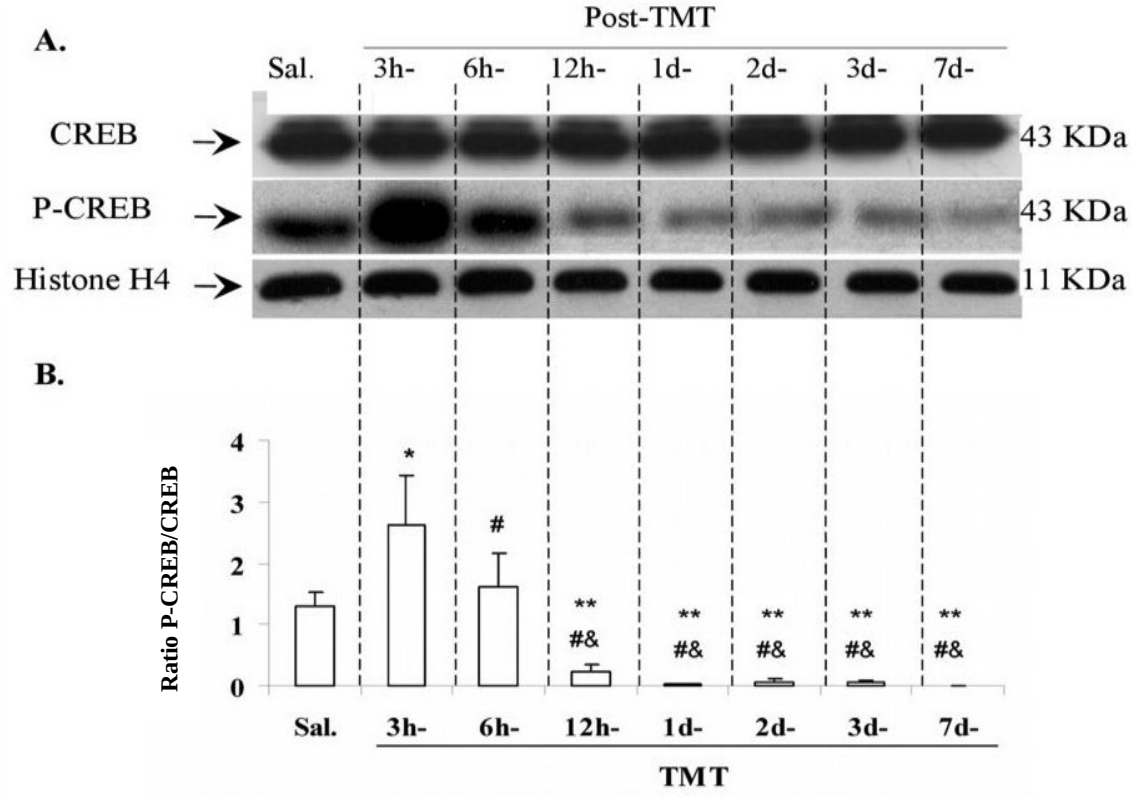
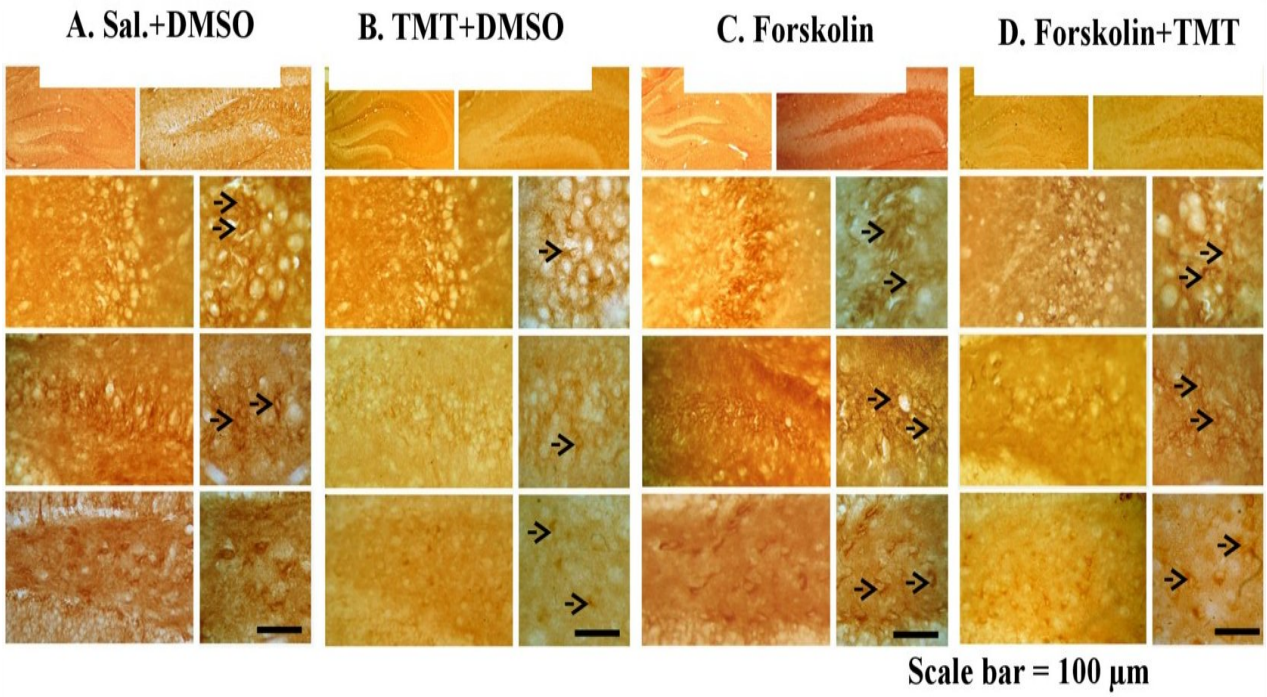


Figure 2. Time-dependent changes in CREB and phospho-CREB in the nucleus fraction of hippocampus of mice after TMT treatment. Each value is the mean±SEM of six mice. $^{*}P<0.01$, $^{**}P<0.001$ vs corresponding saline-treated mice; $^{\#}P<0.001$ vs corresponding 3h after TMT treatment group; $^{\&}P<0.001$ vs corresponding 6h after TMT treatment group (one-way ANOVA followed by Fisher's PLSD test).



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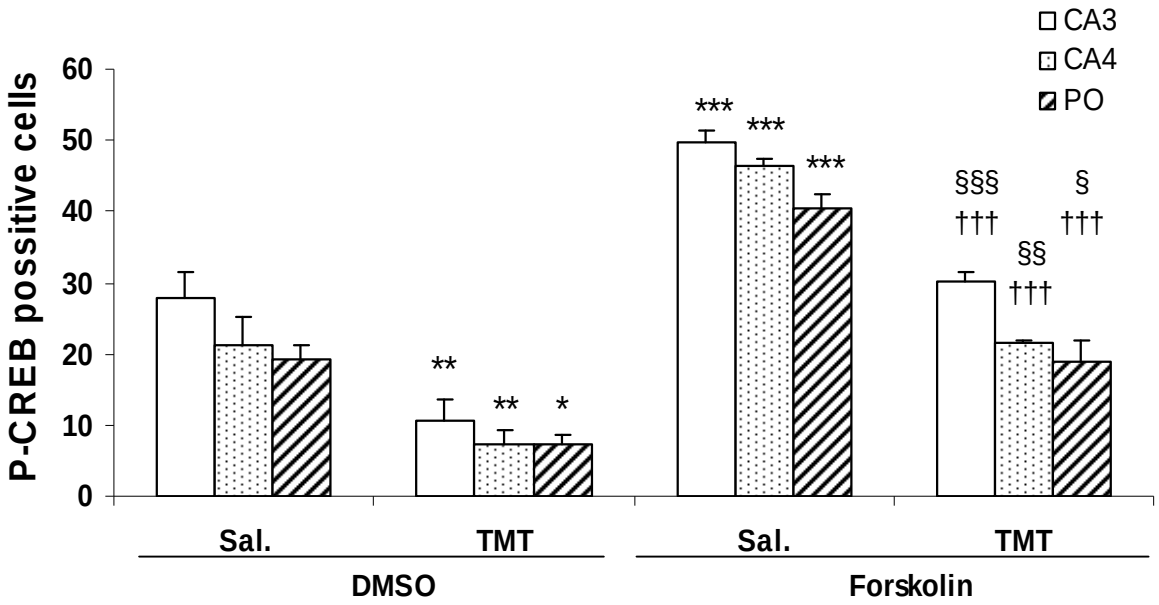


Figure 3. Effects of forskolin on the changes in phospho-CREB-immunoinoreactive cells the hippocampus of mice after TMT treatment. Each value is the mean±SEM of six mice. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs corresponding (saline+DMSO)-treated mice; $^{§}P<0.05$ $^{§§}P<0.01$, $^{§§§}P<0.001$ vs corresponding (TMT+DMSO)-treated mice; $^{†††}P<0.001$ vs corresponding forskolin-treated mice (one-way ANOVA followed by Fisher's PLSD test).

To evaluate the role of CREB, a transcription nucleus gene, in memory dysfunction caused by TMT, we performed water maze test as described previously [8]. Pretreatment with forskolin ameliorated TMT memory dysfunction as assessed by water maze test. In the hidden platform and working memory test, ANOVA (figure 5A, 5B) revealed significant diffirences between forskolin and non-forskolin groups in the presence of TMT (*P<0.001*). These results were consistant with results from the probe test (figure 5C) These changes were more pronounced in the absence of forskolin, proved that forskolin, a CREB activation enhancer, may play a role in ameliorating TMT-induced memory deficiency.
Forskolin was demonstrated to dramatically activate nuclear CREB phosphorylation in the CA1 and CA3 subfields of the hippocampus of control IκBαAAsingle transgenic mice [12]. Interestingly, in the forskolin study of the present study, our results showed that forskolin increased in phospho-CREB levels in the hippocampal CA1, CA3 subfields and polymorphic layers (as assessed by immunocytochemical method) and in the hippocampal neuronal nuclear (as assessed by western blotting method). Moreover, our data from water maze test demonstrated that TMT-induced memory impairment was ameliorated by forskolin (figure 5). In the hidden platform and working memory test, longer escape time was noted in the TMT-treated mice, as compared to saline-treated mice (figure 5a, 5B). While, escape time was observed in the forskolin+TMT-treated mice was shorter than TMT-treated mice (figure 5). Similarly, data from the probe test showed that no significant changes in numbers of crossings were detected in the forskolin-treated mice, as compared to saline group in the absence of TMT (figure 5C). However, these changes were clearly noted in the presence of TMT (figure 5C). Taken together, our results suggest that TMT-induced memory impairment may closely related to phospho-CREB levels in the mice hippocampus. It means the TMT-induced downregulation of phospho-CREB causes memory loss in mice.

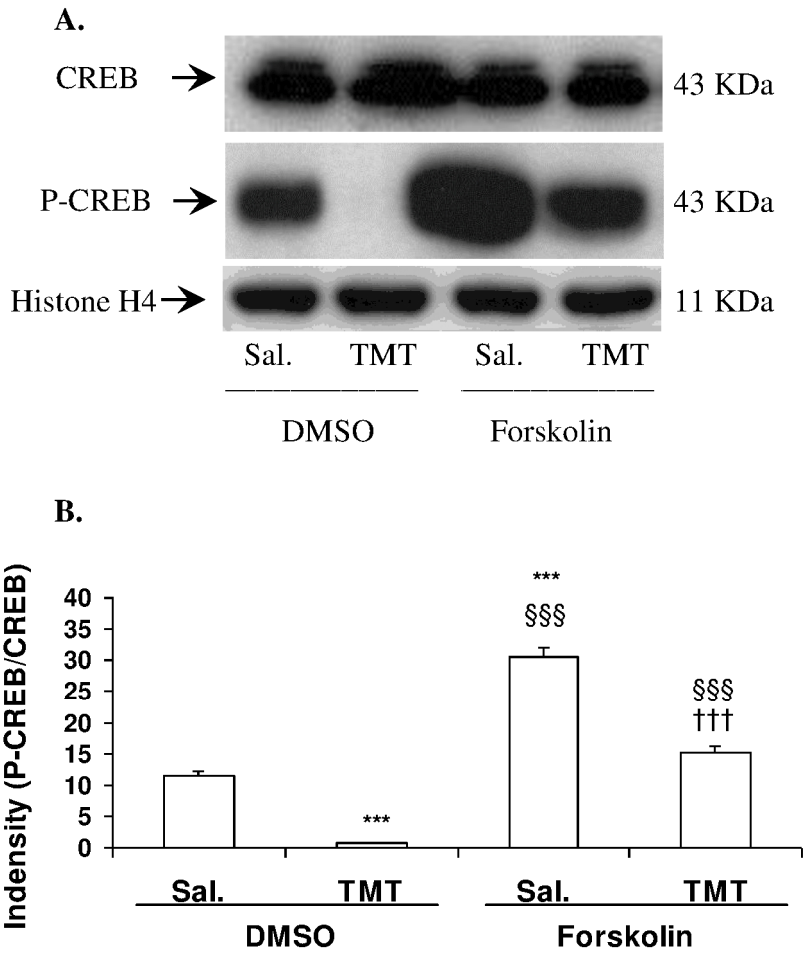
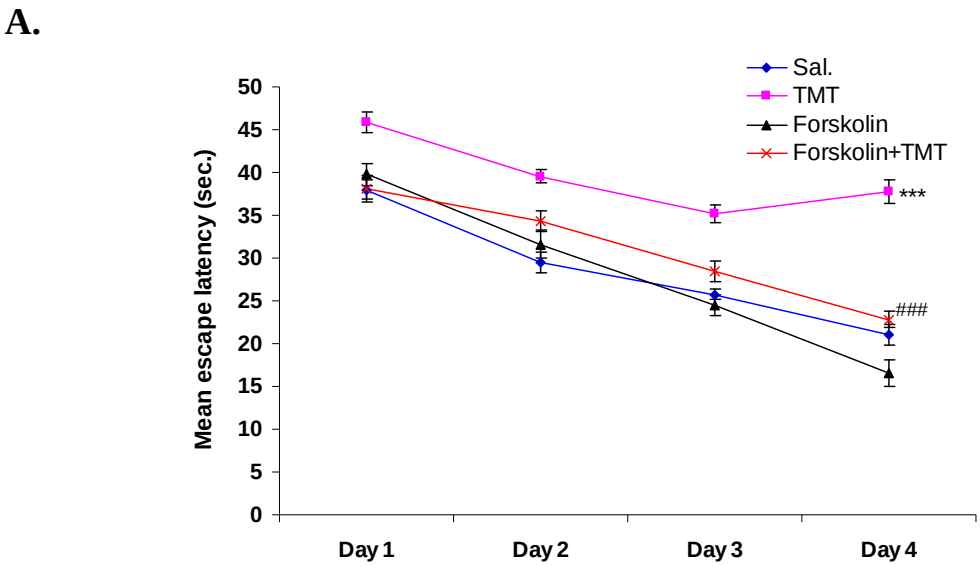


Figure 4. Effects of forskolin on the changes in CREB and phospho-CREB in the hippocampus of mice after TMT treatment. Each value is the mean±SEM of six mice. ***P<0.001 vs corresponding saline-treated mice; §§§P<0.001 vs corresponding (TMT+DMSO)-treated mice; †††P<0.001 vs corresponding forskolin-treated mice (one-way ANOVA followed by Fisher's PLSD test).



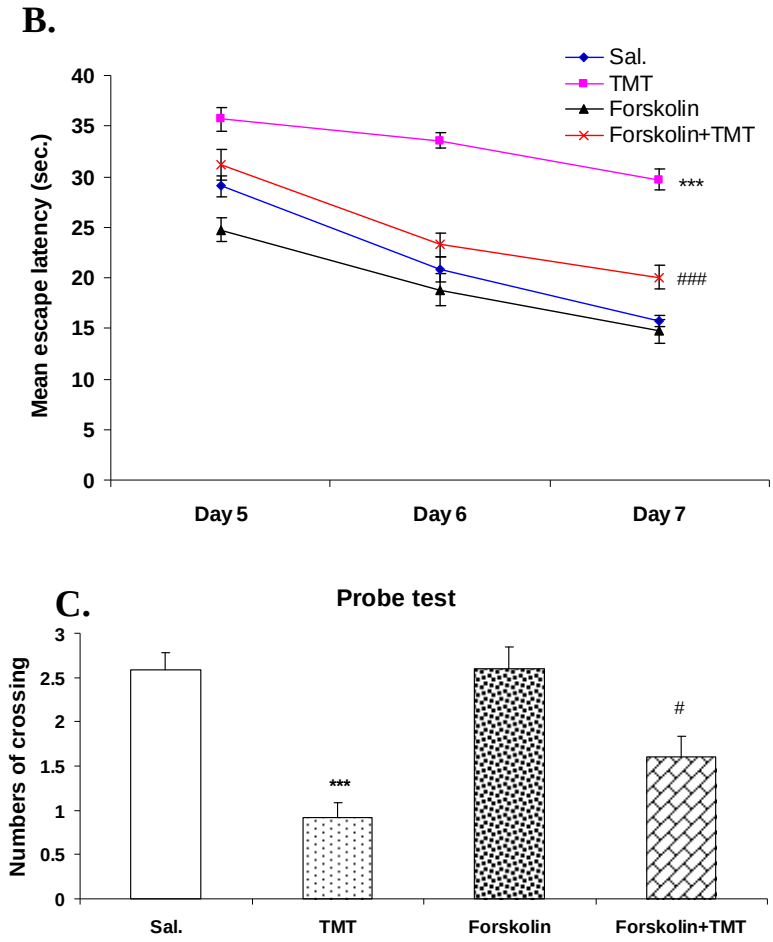


Figure 5. Effects of forskolin on the changes in escape latency (s) (figure 5A, and 5B) and numbers of crossing (figure 5C) of mice after TMT treatment. Each value is the mean±SEM of six mice. $^{***}P<0.001$ vs corresponding saline-treated mice; $^{\#}P<0.05$, $^{\#\#\#}P<0.001$ vs corresponding forskolin-treated mice (one-way ANOVA followed by Fisher's PLSD test).

In conclusion, the results presented here indicate that TMT causes memory impairment via down-regulation of phospho-CREB expressions in the hippocampus of mice. This finding may be part in previous study to account for memory impairment in TMT mouse model.

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