Title: Repetitive transcranial magnetic stimulation (rTMS) modulates hippocampal structural synaptic plasticity in rats.

Author names and affiliations: Yue LI, Lulu LI, Weidong PAN.

Beijing Key Laboratory of Bioelectromagnetics, Institute of Electrical Engineering, Chinese Academy of Sciences. Beijing, 100190, China

Corresponding author:

Name: Weidong PAN

Tel: 86-10-82547168

Fax: 86-10-82547164

Email: panwd@mail.iee.ac.cn

Address: Beijing Key Laboratory of Bioelectromagnetics, Institute of Electrical Engineering, Chinese Academy of Sciences, NO.6 Beiertiao, Zhongguancun, Beijing 100190, China

Short title: TMS modulates synaptic plasticity in rats.

Summary

Repetitive transcranial magnetic stimulation (rTMS) was shown to have therapeutic

potential for some neurological and psychiatric disorders. Previous studies reported that low-frequency rTMS (≤1 Hz) affected synaptic plasticity in rats, however, there were few investigations to examine the possible effects of rTMS on structural synaptic plasticity changes in rats, which included the effects on synaptic morphology in the hippocampus, synaptic protein markers and Ca^{2+}/calmodulin-dependent protein II (CaMKII). Sprague–Dawley rats were subject to 500 pulses of 0.5 Hz rTMS for 15 days, or sham stimulation. After last stimulation, transmission electron microscope (TEM) and real-time PCR were used to determine the effects of rTMS on synaptic plasticity. Results showed that rTMS could cause the change of structural synaptic plasticity, increase the expression of synaptic protein markers: synaptophysin (SYN) and increase the expression of CaMKII, relative to normal rats. suggesting a modulatory effect of chronic rTMS on synaptic plasticity that may be attributed to the increasedexpression of CaMKII in rats.

Key words: Transcranial magnetic stimulation, Synaptic plasticity, CaMKII.

1. Introduction:

Transcranial magnetic stimulation (TMS) was developed by Barker in 1985 as a non-invasive, safe and painless method for the stimulation of the brain. A growing number of studies suggested a therapeutic effect for repetitive transcranial magnetic stimulation (rTMS) on some neurological disorders and other psychiatric disorders,

such as Parkinson's disease, Alzheimer's disease and depression (Avirame et al., 2016; Ba et al., 2017; Cacace et al., 2017; Mi et al., 2017; Reyes-Lopez et al., 2017). The rTMS has been authorized for use in resistant depression patients by US FDA.

Although rTMS has been used broadly, the neurobiology mechanisms underlying these properties still remains to be explored. By inducing electric currents in brain tissue via a time-varying strong magnetic field, which depends on the amplitude, duration, and direction of the currents, rTMS can depolarize cortical neurons and generate action potentials to modulate neuronal circuits. Evidences show that rTMS can activate not only cortical neurons but also cells at some distance from the stimulation via transsynaptic connections (George et al., 1996). Low-frequency rTMS affected electroencephalograms (EEG) and amino acid neurotransmitter (Li et al., 2007; Li et al., 2009). The EEG correlation dimension (*D*2), mean absolute power (MAP) of the gamma band and relative power (RP) of the beta and gamma bands were reduced significantly after low-frequency rTMS, while no changes were observed with sham stimulation. Previous research reported that low-frequency rTMS affected synaptic plasticity in vascular dementia rats (Yang et al., 2015), however, there were few studies to examine the possible effects of low-frequency rTMS on structural synaptic plasticity changes in rats.

Synapse can change its structure and function with external environmental factors, which include synaptic transmission plasticity, synaptic developmental plasticity, and synaptic structures assynaptic loss and formation of new synaptic connections. The synaptic morphology and function changes were observed during physiological

processes as brain development maturation, learning and memory, and recovery of neurological function after brain injury (van Spronsen and Hoogenraad, 2010).

Synaptophysin (SYN) and Calcium/calmodulin-dependent protein kinase II (CaMKII) were important synapse related molecules. SYN, as a general marker protein of pre-synaptic nerve terminals, has been commonly used to quantify the number of terminals during development, aging and following injury (Eastwood, 2004; Masliah and Terry, 1993; Walaas et al., 1988). CaMKII was a ubiquitous serine/threonine protein kinase involved in a vast variety of cellular functions and reduced LTP were observed by application of CaMKII inhibitors (Nayak et al., 1996) or in animals carrying a genetic disruption of the CaMKII gene (Gordon et al., 1996), suggesting the role of CaMKII in LTP induction.

To better understand the structural synaptic plasticity changes in response to chronic low-frequency rTMS, we conducted the present study to investigate the synapse structure by TEM as well as measure the expression of synaptic related genes by quantitative real-time PCR.

2. Materials and Methods

2.1. Animals

Sixteen 10-week-old adult male Sprague–Dawley rats (250 g to 300 g) were used in this study. Four rats were housed in each cage. The rats were randomly divided into the following two groups (n = 8 per group): sham group and rTMS-treated group. During the whole experiments, the animals had free access to food and water at constant ambient temperature (23 ± 1°C) with a 12 h:12 h light–dark cycle (08:00—20:00). The

experiment was conducted according to the regulations of the Beijing Laboratory Animal Use and Care Committee. The assessment was made in a double blind way.

2.2. Chronic rTMS treatment

Rats were treated with rTMS daily for chronic condition after a week adaption. The magnetic stimulations were performed on 8 rats with a magnetic stimulator (BEMS-1, IEE, CAS, China) using monophasic pulses. The stimulation was given with a figure-of-eight coil and the frequency of 0.5 Hz. The coil had seven turns in each loop with the inner radius of 3.5cm and the outer radius of 5cm. The stimulation was done once daily on 15 consecutive days. A stimulation train was consisted of 500 pulses. Stimulation intensity was adjusted to 250 V/m at the plane of 0.5cm distance from the coil, which was above the threshold for evoking motor responses in the hindlimb muscles. The rats were placed in the epoxy-made holder with awakeness. The coil was held tangentally to the parietal bones of rats and the coil handle was parallel to the midline of the head. The sham rats were also placed in the holder but with a distance of 20 cm away from the coil as to avoid effective stimulation of the brain.

2.3. Tissue Preparation

Four rats randomly selected from each group were decapitated immediately after the last TMS treatment. The hippocampus was separated from each rat and stored at -70℃ for use in quantitative real-time PCR (qRT-PCR) assay.

2.4. Transmission electronic microscopy (TEM)

After the last rTMS treatment, the specimen preparation was performed for transmission electron microscope (TEM). Under deep anesthesia, four rats randomly

selected from each group were transcardially perfused for 10 min with 0.9% saline and then with 4% cold paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) for 20 min. The brains were removed from the skulls and post-fixed for 2 h in the same fixative condition. The hippocampus were sliced into ultrathin sections and post-fixed with 1% osmium tetroxide for 1 h. After dehydrated with graded ethanol and propylene oxide and embedded in Epon, the sections were stained with uranyl acetate and lead citrate, and observed using a transmission electron microscope (H-7650, Hitachi, Tokyo, Japan). The synapse density was evaluated by using 12 randomized areas in each rat at 20000 times of magnification. The synapse density was recorded as average numbers per μm^2

2.5. Total RNA isolation, reverse transcription, and quantitative real-time PCR

Total RNA was isolated from the hippocampus using TRizol reagent according to the manufacturer's recommendation (Invitrogen/Life Technologies). The RNA (2 μg) was reversely transcribed into cDNA in 20μL of reverse transcription reaction mix using Oligo dT of the FastQuant RT kit (Tiangen Biotech, Beijing, China) according to the manufacturer's instruction. The quantitative real-time PCR was performed using SYBR green (Applied biosystems, CA, USA) as the fluorescence dye according to the following cycle conditions: 30 s at 95 ℃ for 1 cycle, 5 s at 95 ℃, and 30 s at 60 ℃ for 40 cycles (Applied Biosystems StepOneTM Fast Real-time PCR system).

2.6. Data analysis

Statistical analysis was performed with SPSS 20.0 (SPSS Inc., Chicago, IL, USA). The data were expressed as means±SEM and analyzed with two-tail Student's t-test. A

level of $P<0.05$ was considered to be significant in all statistical tests.

3. Results

3.1. rTMS changed the ultrastructure of hippocampus in rats

As shown in Fig.1, synaptic ultrastructure in the CA1 of hippocampus was analyzed by transmission electron microscope (TEM). Compared with the sham group, synaptic density was significantly increased in the rTMS-treated group ($P<0.05$).

3.2. rTMS regulated the gene expression of SYN

As shown in Fig. 2, the qRT-PCR results analyzed by the $2^{-\Delta\Delta}$Ct method revealed change trends regarding the SYN gene expression. Relative to the sham group, rTMS -treated group displayed a significant increase in the transcription of synaptic markers, SYN ($P<0.05$).

3.3. rTMS modulated the gene expression of CaMKII

As shown in Fig.3, with reference to β-actin for the qRT-PCR assay and compared with sham group, rTMS treated group showed a significant upregulation in the mRNA expression of CaMKII ($P < 0.05$).

4. Discussion

In this study, the effects of rTMS on synaptic plasticity in rats were evaluated. The ultrastructure of hippocampal neurons was examined by transmission electron microscopy and the mRNA expression of SYN and CaMKII were detected via quantitative real time-PCR. Results showed that rTMS could increase the synaptic density and upregulate the gene expression of SYN and CaMKII in rats.

Although rTMS has been used to treat a variety of diseases including depression

and Parkinson's disease, the mechanisms underlying the effects of long-term TMS remain unclear. Most researchers believe that the long-lasting therapeutic effects of rTMS and magnetic stimulation on the above-described processes are related with two phenomena: long-term potentiation (LTP) and long-term depression (LTD) (Chervyakov et al., 2015b). Repetitive TMS could affect dopamine production (Ohnishi et al., 2004) and other neuromediators (Li et al., 2009), which improved expressions of brain derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF) (Dong et al., 2015). High-frequency rTMS could also induce prolonged changes in the gene expression of cyclin dependent kinase 5 (CDK5) and the post synaptic density protein 95 (PSD-95) that caused by a modulation of histone acetylation. Furthermore, these changes were dependent on HDAC and D2 dopamine receptor (D2R) functions. These results showed that rTMS have long-lasting effects on synaptic plasticity and these effects can be mediated by a modulation of dopamine neurotransmission (Etievant et al., 2015). Other studies also provided evidence that the effects of rTMS were associated with neuroplastic changes in synaptic structure that likely occur via the activation of NMDA receptors (Huang et al., 2007).

Synapses played a critical role not only in normal neurophysiology, but also in many neurodegenerative diseases. Synapses loss were found in some neurodegenerative diseases such as Alzheimer's disease (Ingelsson et al., 2004) and Parkinson disease (Finkelstein et al., 2000; Ingham et al., 1997). As synaptic pathology was a common feature of these disorders, it was suggested that synapse might be used as a potential therapeutic target. Avoidance of synaptic loss or strengthening of existing

connections between neurons may delay or even reverse disease-related neurological changes. (Henstridge et al., 2016). In a vascular dementia (VaD) rat model, 1 Hz rTMS could significantly reduce synaptic structure damage (Yang et al., 2015). Vlachos et al reported that a high-frequency (10 Hz) rTMS induced structural remodeling of dendritic spines. In our study, compared with the sham group, synaptic density was significantly increased in the rTMS treated group which was consistent to the previous reports except for the difference in the parameters used, suggesting that rTMS may function with the effectiveness on structural synaptic plasticity.

SYN and CaMKII are key synapse-related molecules. As a presynaptic vesicle protein and a marker of synaptic density, SYN affects the release of neurotransmitters. By using different frequency of rTMS, increased expression of SYN could be observed in cultured hippocampal neurons, normal Wistar rats, aging rats and vascular dementia rats (Ma et al., 2013; Shang et al., 2016; Wang et al., 2015; Zhang et al., 2015). On the other hand, as a calmodulin-dependent protein kinase, CaMKII plays a crucial role in learning and memory by mediating a wide variety of intercellular responses. High-frequency rTMS enhanced the expression of BDNF via activation of Ca^{2+}–CaMKII–CREB pathway in the Neuro-2a cells and *in vitro* neuronal model of ischemia/reperfusion (I/R) injury (Baek et al., 2018a; Baek et al., 2018b). However, this effect was found only in high-frequency rTMS rather than low-frequency rTMS. The investigation using 5 Hz rTMS with 30% maximum output on senescence-accelerated-prone mouse 8 (SAMP8) showed that rTMS significantly upregulated the protein and mRNA expression of SYN and PSD95 in the hippocampus of p8-rTMS

mice (Ma et al., 2017). Our analysis also revealed that the mRNA expression levels of SYN and CaMKII were both significantly higher in the rTMS treated group, suggesting the potential benefit of rTMS for the treatment of some neuropsychiatric disorders.

In summary, this study revealed that rTMS increased the synapse density in the hippocampus of rats. Moreover, rTMS enhanced synaptic markers-SYN, activated CaMKII, and modulated the fundamental of synaptic structure, revealing neurological basis of the beneficial mechanisms for rTMS. Future studies should be performed to deeply explore the effects of rTMS on structural synaptic plasticity and provide further insights on the molecular mechanism involved in rTMS.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (81200996, 31670855). It is appreciated very much for the technical staff's assistance with the exposure equipment.

References

Avirame K, Stehberg J, Todder D: Benefits of Deep Transcranial Magnetic Stimulation in Alzheimer Disease: Case Series. J ect 32: 127-133, 2016.

Ba M, Ma G, Ren C, Sun X, Kong M: Repetitive transcranial magnetic stimulation for treatment of lactacystin-induced Parkinsonian rat model. Oncotarget 8: 50921-50929, 2017.

Baek A, Kim JH, Pyo S, Jung JH, Park EJ, Kim SH, Cho SR: The Differential Effects of Repetitive Magnetic Stimulation in an In Vitro Neuronal Model of Ischemia/Reperfusion Injury. Front Neurol 9: 50, 2018.

Baek A, Park EJ, Kim SY, Nam BG, Kim JH, Jun SW, Kim SH, Cho SR: High-Frequency Repetitive Magnetic Stimulation Enhances the Expression of Brain-Derived Neurotrophic Factor Through Activation of Ca(2+)-Calmodulin-Dependent Protein Kinase II-cAMP-Response Element-Binding Protein Pathway. Front Neurol 9: 285, 2018.

Cacace F, Mineo D, Viscomi MT, Latagliata EC, Mancini M, Sasso V, Vannelli A, Pascucci T, Pendolino V, Marcello E, Pelucchi S, Puglisi-Allegra S, Molinari M, Picconi B, Calabresi P, Ghiglieri V: Intermittent theta-burst stimulation rescues dopamine-dependent corticostriatal synaptic plasticity and motor behavior in experimental parkinsonism: Possible role of glial activity. Mov Disord 32: 1035-1046, 2017.

Chervyakov AV, Chernyavsky AY, Sinitsyn DO, Piradov MA: Possible Mechanisms Underlying the Therapeutic Effects of Transcranial Magnetic Stimulation. Front Hum Neurosci 9: 303, 2015.

Dong Q, Wang Y, Gu P, Shao R, Zhao L, Liu X, Wang Z, Wang M: The Neuroprotective Mechanism of Low-Frequency rTMS on Nigral Dopaminergic Neurons of Parkinson's Disease Model Mice. Parkinsons Disease 564095, 2015.

Eastwood SL: The synaptic pathology of schizophrenia: is aberrant neurodevelopment and plasticity to blame? Int Rev Neurobiol 59: 47-72, 2004.

Etievant A, Manta S, Latapy C, Magno LAV, Fecteau S, Beaulieu JM: Repetitive transcranial magnetic stimulation induces long-lasting changes in protein expression and histone acetylation. Scientific Reports 5: 16873, 2015.

Finkelstein DI, Stanic D, Parish CL, Tomas D, Dickson K, Horne MK: Axonal sprouting following lesions of the rat substantia nigra. Neuroscience 97: 99-112, 2000.

George MS, Wassermann EM, Post RM: Transcranial magnetic stimulation: a neuropsychiatric tool for the 21st century. J Neuropsychiatry Clin Neurosci 8**:** 373-382, 1996.

Gordon JA, Cioffi D, Silva AJ, Stryker MP: Deficient plasticity in the primary visual cortex of alpha-calcium/calmodulin-dependent protein kinase II mutant mice. Neuron 17: 491-499, 1996.

Henstridge CM, Pickett E, Spires-Jones TL: Synaptic pathology: A shared mechanism in neurological disease. Ageing Res Rev 28: 72-84, 2016.Huang YZ, Chen RS, Rothwell JC, Wen HY: The after-effect of human theta burst stimulation is NMDA receptor dependent. Clin Neurophysiol 118(5):1028-32 2007.

Ingelsson M, Fukumoto H, Newell KL, Growdon JH, Hedley-Whyte ET, Frosch MP, Albert MS, Hyman B T, Irizarry MC: Early Abeta accumulation and progressive synaptic loss, gliosis, and tangle formation in AD brain. Neurology 62: 925-931, 2004.

Ingham CA, Hood SH, Mijnster MJ, Baldock RA, Arbuthnott GW: Plasticity of striatopallidal terminals following unilateral lesion of the dopaminergic

nigrostriatal pathway: a morphological study. Exp Brain Res 116: 39-49, 1997.

Li L, Yin Z, Huo X: The influence of low-frequency rTMS on EEG of rats. Neurosci Lett 412: 143-147, 2007.

Li Y, Huo X, Song T: The effects of chronic repetitive transcranial magnetic stimulation on glutamate and gamma-aminobutyric acid in rat brain. Brain Res 1260: 94-99, 2009.

Ma J, Zhang Z, Su Y, Kang L, Geng D, Wang Y, Luan F, Wang M, Cui H: Magnetic stimulation modulates structural synaptic plasticity and regulates BDNF-TrkB signal pathway in cultured hippocampal neurons. Neurochem Int 62 (1): 84-91, 2013.

Ma J, Wang JH, Lv CN, Pang JJ, Han B, Gen Y,Wang MW: The Role of Hippocampal Structural Synaptic Plasticity in Repetitive Transcranial Magnetic Stimulation to Improve Cognitive Function in Male SAMP8 Mice. Cellular Physiology and Biochemistry 41:137-144, 2017

Masliah E, Terry R: The role of synaptic proteins in the pathogenesis of disorders of the central nervous system. Brain Pathol 3: 77-85, 1993.

Mi Z, Biswas K, Fairchild JK, Davis-Karim A, Phibbs CS, Forman SD, Thase M, Georgette G, Beale T, Pittman D, McNerney MW, Rosen A, Huang GD, George M, Noda A, Yesavage JA: Repetitive transcranial magnetic stimulation (rTMS) for treatment-resistant major depression (TRMD) Veteran patients: study protocol for a randomized controlled trial. Trials 18: 409, 2017.

Nayak AS, Moore CI, Browning MD: Ca2+/calmodulin-dependent protein kinase II

phosphorylation of the presynaptic protein synapsin I is persistently increased during long-term potentiation. Proc Natl Acad Sci 93**:** 15451-15456, 1996.

Ohnishi T, Hayashi T, Okabe S, Nonaka I, Matsuda H, Iida H, Imabayashi E, Watabe H, Miyake Y, Ogawa M: Endogenous dopamine release induced by repetitive transcranial magnetic stimulation over the primary motor cortex: an [11C]raclopride positron emission tomography study in anesthetized macaque monkeys. Biol Psychiatry 55: 484-489, 2004.

Reyes-Lopez J, Ricardo-Garcell J, Armas-Castaneda G, Garcia-Anaya M, Arango-De Montis I, Gonzalez-Olvera JJ, Pellicer F: Clinical improvement in patients with borderline personality disorder after treatment with repetitive transcranial magnetic stimulation: preliminary results. Rev Bras Psiquiatr Epub ahead of prin, 2017.

Shang Y, Wang X, Shang X, Zhang H, Liu Z, Yin T, Zhang T: Repetitive transcranial magnetic stimulation effectively facilitates spatial cognition and synaptic plasticity associated with increasing the levels of BDNF and synaptic proteins in Wistar rats. Neurobiol Learn Mem 134 Pt B: 369-378, 2016.

van Spronsen M, Hoogenraad CC: Synapse Pathology in Psychiatric and Neurologic Disease. Curr Neurol Neurosci Rep 10: 207-214, 2010.

Walaas SI, Jahn R, Greengard P: Quantitation of nerve terminal populations: synaptic vesicle-associated proteins as markers for synaptic density in the rat neostriatum. Synapse 2: 516-520, 1988.

Wang F, Chang GM, Yu Q, Geng X: The neuroprotection of repetitive transcranial

magnetic stimulation pre-treatment in vascular dementia rats. J Mol Neurosci 56: 198-204, 2015.

Yang HY, Liu Y, Xie JC, Liu NN, Tian X: Effects of repetitive transcranial magnetic stimulation on synaptic plasticity and apoptosis in vascular dementia rats. Behav Brain Res 281: 149-155, 2015.

Zhang ZC, Luan F, Xie CY, Geng DD, Wang YY, Ma J: Low-frequency transcranial magnetic stimulation is beneficial for enhancing synaptic plasticity in the aging brain. Neural Regen Res 10: 916-924, 2015.

Figure legends:

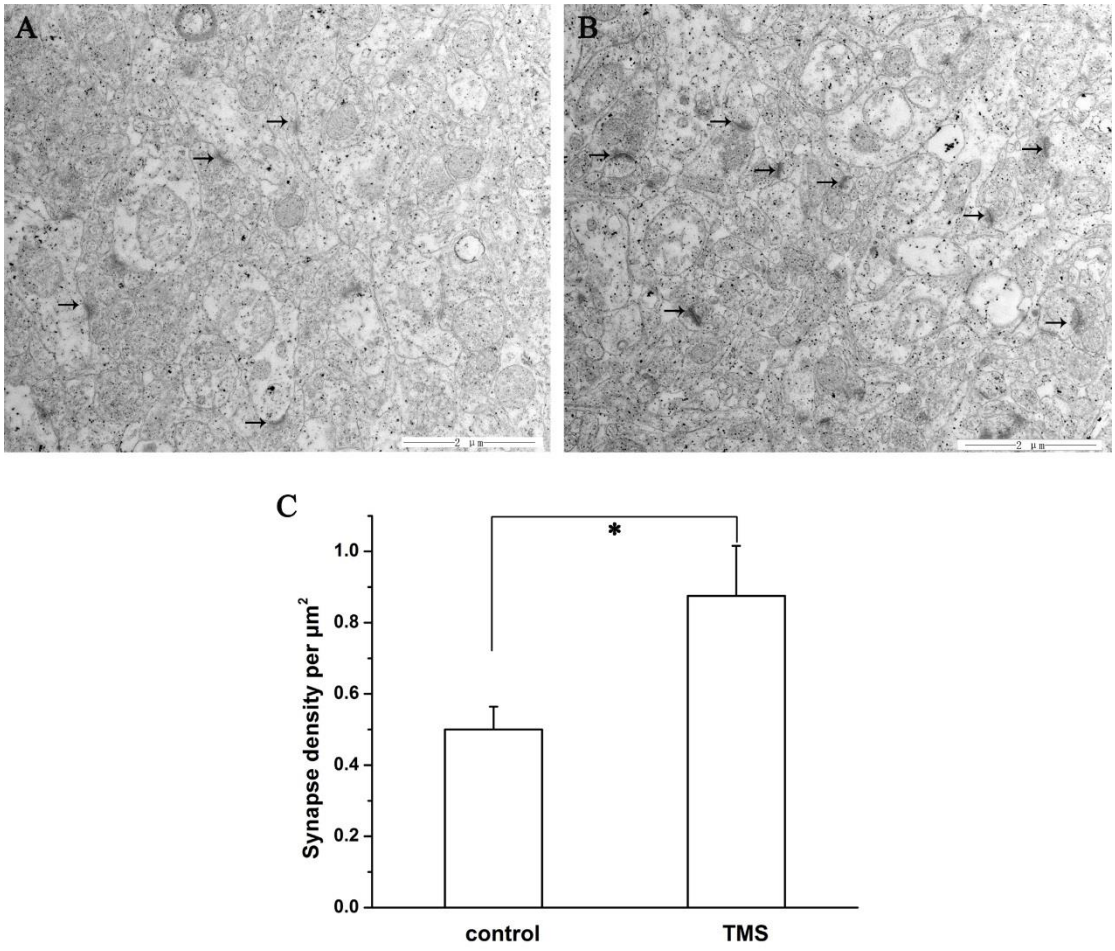

Fig.1. Ultrastructure alterations of the CA1 of hippocampus in rats. A: Synapse structure of sham group rats. Synapses are indicated by arrows. The scale bar represents 2μm. B: Synapse structure of rTMS-treated rats. C: Number synapse per μm^2. Values are presented as mean ± SEM.

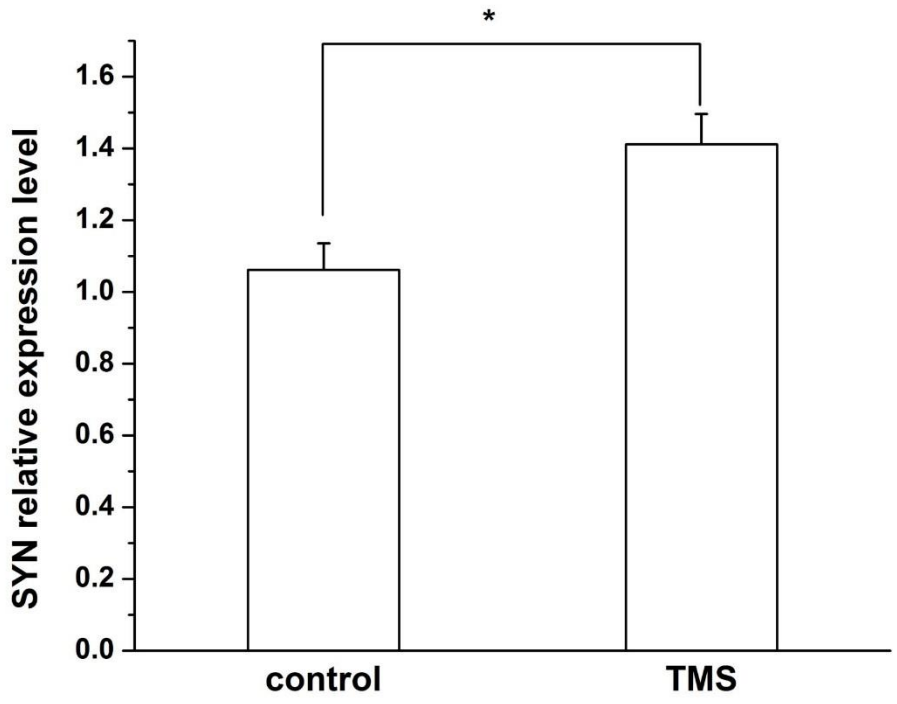

Fig.2. Relative expression levels of SYN gene by quantitative real-time PCR assay. The hippocampus was used in the experiment. Real-time PCR assays were performed to detect the mRNA expression levels of SYN related to β-actin. Data are presented as mean ± SEM. Analysis of relative mRNA level of SYN using $2^{-\Delta\Delta}$Ct method. *$P < 0.05$, compared to sham group. n = 4 for each group.

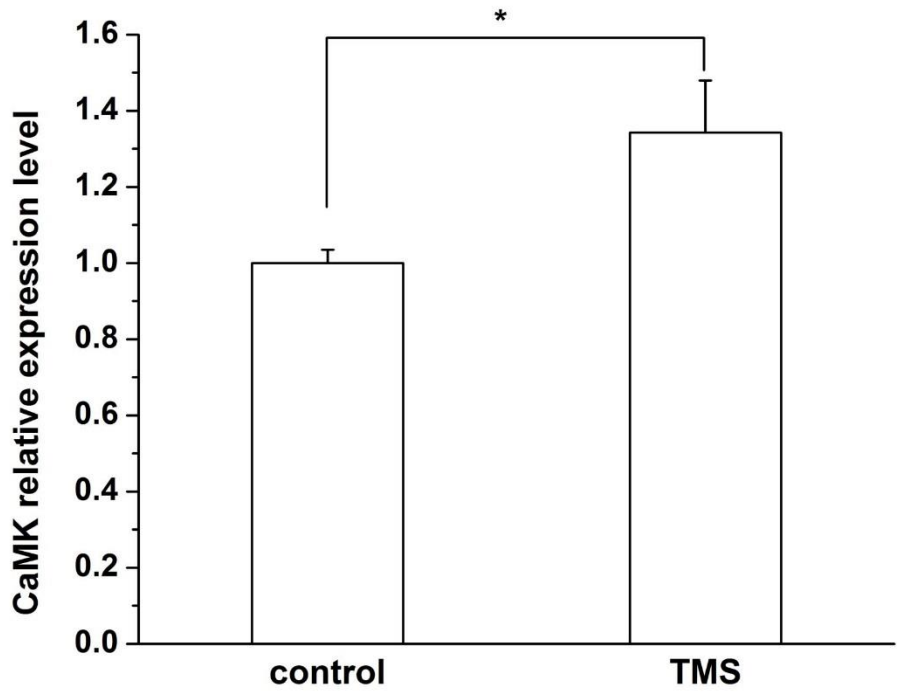

Fig. 3. Relative expression levels of CaMKII gene by quantitative real-time PCR assay. The hippocampus was used in the experiment. Real-time PCR assays were performed to detect the mRNA expression levels of CaMKII related to β-actin. Data are presented as mean ± SEM. Analysis of relative mRNA level of SYN using $2^{-\Delta\Delta}$Ct method. *$P < 0.05$, compared to sham group. n = 4 for each group.