Iranian Journal of Basic Medical Sciences

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Allicin attenuates tunicamycin-induced cognitive deficits in rats via its synaptic plasticity regulatory activity

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ARTICLE INFO

Article type:
Original article

Article history:
Received: Jun 23, 2016
Accepted: Jan 12, 2017

Keywords:
Allicin
Dendritic spine density
fEPSP
Hippocampus
Synaptic plasticity

ABSTRACT

Objective(s): To illuminate the functional effects of allicin on rats with cognitive deficits induced by tunicamycin (TM) and the molecular mechanism of this process.

Materials and Methods: 200–250 g male SD rats were divided into three groups at random: control group (n=12), TM group (5 µl, 50 µM, ICV, n=12), and allicin treatment group (180 mg/kg/d with chow diet, n=12). After 16 weeks of allicin treatment, the learning ability and memory were tested using novel object recognition (NOR) testing on rats with 72 hr TM treatment (5 µl, 50 µM, ICV); meanwhile, the variation of field excitatory postsynaptic potential (fEPSP) in the Schaffer Collateral (SC)-CA1 synapse was detected by extracellular electrophysiological recordings and the morphology of dendritic spine was observed by Golgi staining as well as detecting several synaptic plasticity-related proteins by Western blot.

Results: The density of dendritic spine was increased significantly in allicin-treated groups and the correspondence slope of fEPSP in TM-induced cognitive deficits group was enhanced and expression of synaptophysin and glutamate receptor-1(GluR1) in hippocampal neurons was up-regulated.

Conclusion: The results indicate that allicin plays an important role in synaptic plasticity regulation. These finding showed that allicin could be used as a pharmacologic treatment in TM-induced cognitive deficits.

► *Please cite this article as:*
Xiang Q, Li XH, Yang B, Fang XX, Jia J, Ren J, Dong YCh, Ou-Yang Ch, Wang GCh. Allicin attenuates tunicamycin-induced cognitive deficits in rats via its synaptic plasticity regulatory activity. Iran J Basic Med Sci 2017; 20:676-682. doi: 10.22038/IJBMS.2017.8837

Introduction

Several series of evidence indicate that chronic or increased endoplasmic reticulum (ER) stress leads to the pathogenesis of multiple diseases including beta-cell dysfunction and death during the progression of diabetes (1), hepatic steatosis (2), inflammation (3), and homeostatic regulation (4). Our previous paper also reported that endoplasmic reticulum (ER) stress stimulated by tunicamycin (TM) is associated with cognitive deficits (5). Within the hippocampal neurons, synaptic plasticity is thought to be vital in modulating cognitive abilities (6), and both the number of levels of synapses and the levels of synaptic plasticity-related proteins have been shown to decrease in a number of neurodegenerative disease models (7, 8).

Generally, it is known that synaptic plasticity, the ability of synapses, strengthens or weakens over time, in response to increases or decreases in their activation. It includes the morphology and the function of the spit plasticity (6). Dendritic spines act as the storage site which is specific for synaptic strength and transmitting electrical signals to the cell body of neurons, Moreover, they are very plastic, that is to say, spines change significantly in shape, volume, and number in small time courses (9). Long-term potentiation (LTP) is a persistent strengthening of synapses based on recent patterns of activity. There are several phenomena underlying synaptic plasticity, for example, chemical synapses are able to change their strength via synaptic plasticity (10). When it comes to memories, it is thought to be produced by synaptic strength modification, LTP is commonly considered as the cellular mechanism of learning and memory.

Allicin is a natural compound with sulfur, widely distributed in freshly cut or crushed garlic (11), which has been reported for its biological antioxidant, anti-inflammatory (12), anti-proliferative (13), and anti-acrylamide induced toxicity effects (14) in many published papers. In our previous studies, allicin alleviated tunicamycin-induced cognitive deficits and protected neurons against ER stress-related injury by activating the PERK/Nrf2 anti-oxidative signal transduction pathway (5). However, the functional effect and cellular mechanism of allicin in synaptic plasticity in TM-induced cognitive deficits model in rats are still unclear.

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In our studies here, we have explored the functional effect of allicin on dendritic spine formation and LTP in TM-induced cognitive deficit rats and cognitive-related behavioral performance, in parallel with the underlying molecular mechanisms. Additionally, we observed the effect of allicin on GluR1, the AMPA receptor subunit, so as to gain a new insight into the role of allicin in the modulation of cognitive deficits.

The aim of this study was to indicate the functional effects and molecular mechanism of allicin on cognitive deficits. *In vivo*, the effects of allicin were examined in primary hippocampus neurons so as to confirm the function of allicin in the modulation of cognitive deficits.

Materials and Methods

Animals and behavioral measurements

200–250 g male Sprague-Dawley rats (the Experimental Animal Center of Xiangya Medical College) were used for this experiment. All interventions and animal care were performed in accordance with the policy of the Society for Neuroscience (USA) on the use of animals in neuroscience research and the Care and Use of Laboratory Animals of Jishou University. The number of animals used and the potential discomfort of the animals during the behavioral experiments were minimized.

Drug treatment

Rats were kept 4 per cage at room temperature (25 ± 2 °C) and relative humidity (55 ± 5%) under a standard 12-hr/12-hr light-dark cycle. The rats were habituated for 2 weeks and randomly divided into three groups: control, TM, and allicin groups (n=12 per group). All rats were fed standard rat chow diet. In addition, the rats in the allicin group were given chow diet supplemented with 180 mg/kg/d of allicin for 16 weeks. Allicin usage at this dose was performed in accordance with some previously published works (15-19). Rats were anesthetized with 10% chloral hydrate (400 mg/kg) and fixed in a stereotaxic instrument (Ruanlong Technology Development Co Shanghai, China) at 17th week. After the scalp was incised (5-8 mm), the skull was cleaned and a hole (diameter 1.0 mm) was made for lateral ventricular infusion. The infusion coordinate was 0.8 mm posterior, 1.5 mm lateral, and 4.0 mm ventral to bregma. A sterilized needle connected to a Hamilton syringe was used to deliver 5 µl of TM (50 µM) into the lateral ventricle of rats in TM and allicin groups. An equal volume of solvent was infused in control rats. The rats in allicin group were still fed allicin after TM administration.

Novel object recognition testing for cognitive ability

After 72 hr TM infusion, the NOR test was performed, which is based on the animals' innate ability to explore novel objects more than familiar ones (20, 21). Behavioral testing was performed in a clear open area. For the sample phase, rats were put in the center of the area and there were also two identical objects in the corners of the sidewall oppositely. The rats were permitted to explore the area and objects for 5 min. 24 hr after the training session, the animals explored the open field for 5 min in the presence of one familiar and one different screened (novel) object. Rats were permitted to explore for another 5 min in the testing phase.

Exploratory behavior was considered as direct touching of the objects (22, 23). Discrimination index (DI) was defined as the preference for the novel object. Recognition index (RI) showed the ability for the novel object recognition.

DI=b-a/a+b×100; RI = b/a+b×100;

a = time with familiar object; b = time with novel object;

a+b=total exploration time of both objects; time=distance/speed;

Electrophysiological recording of long-term potentiation in hippocampal CA1 region

Hippocampal slice preparation

After behavior testing, all animals were decapitated. The entire brain was rapidly removed. Coronal hippocampal brain slices (400 um) were cut using a vibratome (VT1000S, Leica, Germany) in ice-cold artificial cerebrospinal fluid (ACSF) containing (in mmol/l) 125 NaCl, 3.25 KCl, 2 $CaCl_2$, 25 $NaHCO_3$, 1.25 NaH_2PO_4 and 11 glucose (pH 7.4) that was saturated with 95% O_2 and 5% CO_2. After 1 hr of recovery at 34 °C, fresh slices were used for experiments.

Electrophysiological recordings

The constant stimulation in the SC with a bipolar electrode was used to induce the field fEPSPs and was recorded in the stratum radiatum layer of CA1 with a glass micropipette filled with 3 M NaCl (24). The intensity of stimulation was selected to induce an fEPSP with a slope, which was 30%-35% of that obtained with maximal stimuli.

LTP was induced using high-frequency stimu-lation (HFS). The HFS consisted of three 1 sec, 100 Hz clusters, which were separated by a 30 sec interval from each other (10). After obtaining stable baseline responses for 20 min, a single stimulus of 100 Hz (1 sec) was given to the SC-CA1 pathway. The responses were recorded for an hr. Synaptic responses for LTP experiments were normalized by dividing all fEPSP slope values by the average of the five responses recorded during the 5 min immediately before HFS.

Golgi staining and dendritic spines analysis

For Golgi staining, we used the FD Rapid Golgi Stain Kit (FD NeuroTechnologies, Ellicott City, MD, USA). Dissected rat brains were immersed in Solution A and B for 2 weeks at room temperature and transferred into

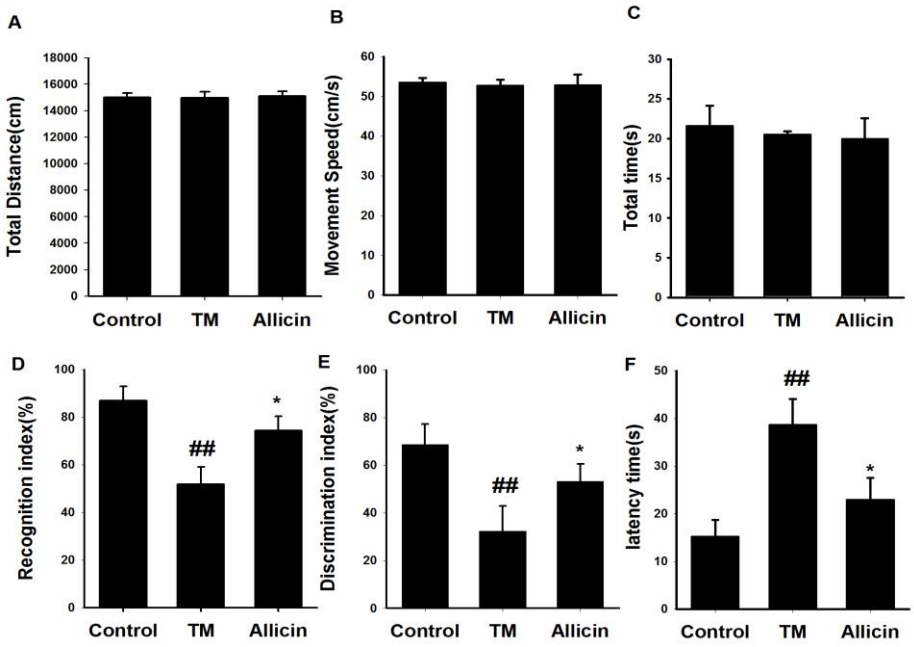

Figure 1. Effect of allicin on TM-related cognitive deficit rats in the novel object recognition test. The NOR test was used to test the function of cognition of rats. (A, B, C) They respectively showed total distance, movement speed and total time object exploration in training period. (D) The recognition index in test period. (E) The discrimination index in test period. (F) Time with familiar object. Data shown as the mean ±SEM. (n=5–7); *P< 0.05; ** P< 0.01 vs TM group; ## P<0.01 vs control group

Solution C for 24 hr at 4 °C. Brains were sectioned at 150 µm thickness using a VT1000S Vibratome (Leica, Bannockburn, IL, USA). Dendritic images were scanned by Axioplan 2 (Zeiss, Oberkochen, Germany) under brightfield microscopy. Spine width, length, and linear density were measured using Scion image software (Scion Corporation, Frederick, MD, USA). Spines from 0.2 to 3 µm in length were included for analysis. All morphological analysis was done blind to genotypes and experimental conditions.

Primary neuron culture and immunostaining

Primary cultures of rat hippocampal neurons were prepared from the hippocampus of E18-E19 Sprague-Dawley rat embryos. In short, the rats were sacrificed and the hippocampus was dissected. We washed twice with D-Hank's buffer and digested the hippocampus with equal volumes of 0.125% trypsin/EDTA for approximately 15 min at 37 °C and then washed the hippocampus with Hank's solution containing 10% fetal bovine serum (Biochrom, Cambridge, UK). Then, neurons were plated in six-well plates (8.75×104 cells/cm^2) or in 96 well plates (0.85×104 cells/cm^2). The neurons were cultured with DMEM for 6 hr, after that DMEM was replaced with Neurobasal/B27. After 7 days in culture, cells were incubated with various concentrations of allicin (1.235, 3.705, 12.35, 37.05, and 123.5 µM) and TM (4 µg/ml) and maintained for 40 hr, allicin was dissolved in DMSO and DMSO and used as the vehicle. The rabbit anti-GluR1 (EMD Calbiochem, Gibbstown, NJ, USA) antibody was used. Immunostaining of GluR1 in hippocampal neurons was performed. In short, live neurons were incubated with N-terminal GluR1 antibodies (10 g/ml in conditioned medium) for 10 min, fixed with 4% PFA. GluR1 was detected with Alexa fluor-555 secondary antibodies.

Neurons were then permeabilized in methanol (20 °C, 90 sec), and incubated with anti-GFP antibody to identify transfected neurons.

Statistical analysis

Data was presented as mean±SEM. Statistical analysis was performed using Student's t-test. P<0.05 and P<0.01 were considered as having statistical significance.

Results

Effect of allicin on NOR in TM-induced cognitive deficits rats

As illustrated in Figure 1, rats in the TM group performed with significantly decreased levels of recognition index and discrimination index (Figure 1D,E; P<0.01), while they exhibited significantly increased latency times as compared to control rats (P<0.01, Figure 1F). There was a considerable increase in the levels of recognition index in the allicin-treated rats compared with the TM groups (Figure 1D; P<0.05). The discrimination index in the allicin-treated rats also increased significantly compared with that of no-treatment-TM groups (Figure 1E; P<0.05). The latency time for the novel object increased sharply in the TM groups compared with that of the allicin-treated rats (Figure 1F; P<0.05).

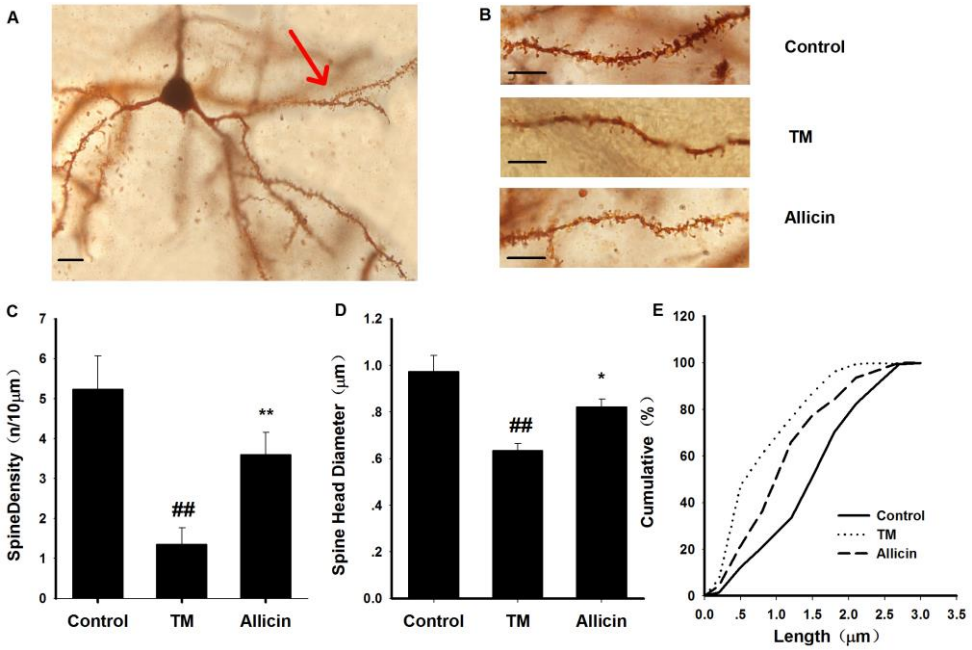

Figure 2. Effect of allicin on spine density, spine head diameter, and length in TM -related cognitive deficits rats. (A) Representative micrograph of a hippocampal CA1 neuron illustrating dendritic (apical) regions analyzed. (B) Representative dendritic segments of CA1 pyramidal neurons from control, TM, and allicin animals (n=6 rats). Scale bar, 5 μm. (C) Mean spine density from control, TM, and allicin animals in CA1 regions (30 neurons/rat, * P<0.05; ** P<0.01 vs TM group; ## P<0.01 vs control group). (D) Spine head diameter from control,TM, and allicin animals in CA1 regions (30 neurons/rat, * P<0.05vs TM group; ## P<0.01 vs control group). (E) Cumulative distribution plot of hippocampal spine length from control, TM, and allicin groups (180–200 spines/rat, Kolmogorov–Smirnov Test, P<0.001)

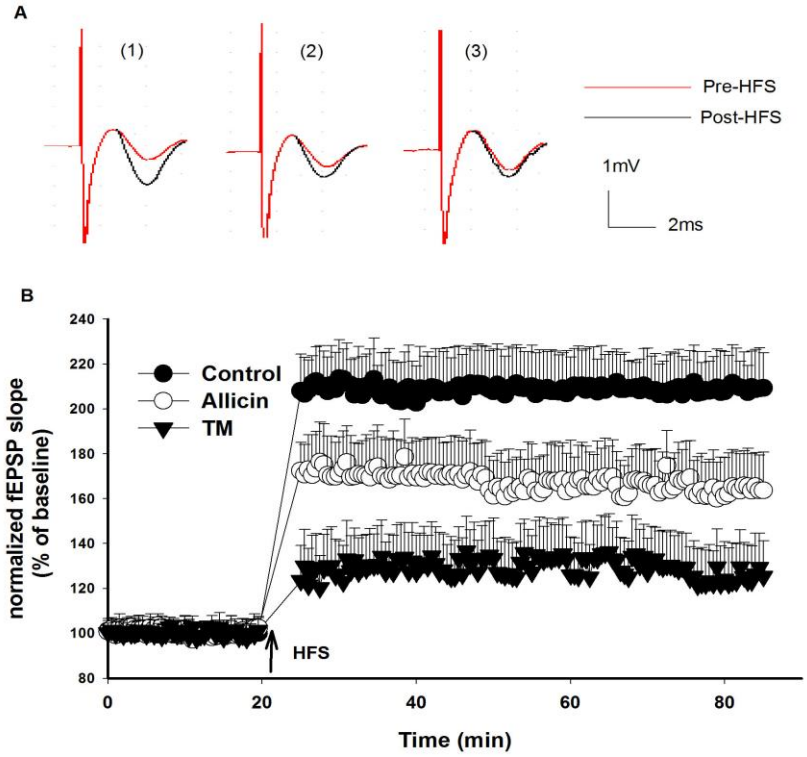

Figure 3. Effect of allicin on LTP at the SC-CA1 synapse in hippocampus slices of TM -related cognitive deficits rats (n=6). (A) A1, A2, A3 respectively show original fEPSPs recorded in control, allicin-treated (180 mg/kg/d with chow diet), and tunicamycin (TM) groups. fEPSPs were recorded before (red line) and after (black line) HFS. Calibration bars: horizontal = 2 ms, vertical = 1 mV. (B) The linear graph illustrates the relative fEPSP slope alteration at different time points within the 85 min pre- and post-HFS. Compared with the control group, the correspondence slope of fEPSP in the Schaffer Collateral-CA1 synapse of TM-related cognitive deficit rats' hippocampus slices was significantly decreased (P<0.01). Meanwhile, the correspondence slope of fEPSP of the allicin-treated group was obviously enhanced compared with that of the TM group (P<0.05). All data is expressed as Mean ± SEM. Multivariate analysis of variance was performed for differences in LTP

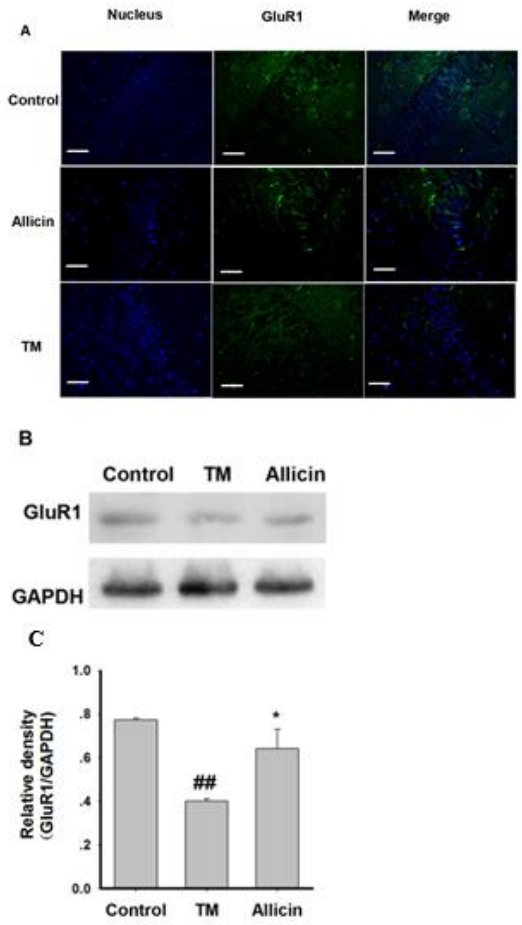

Figure 4. Effects of treatment with allicin on the levels of GluR1 in CA1 pyramidal neurons of TM-related cognitive deficit rats (n=3). The photomicrographs show GluR1 expression (A) in CA1 pyramidal neurons of rats (scale bar = 50 μm). (B) Representative immunoblot for GluR1 in all groups. (C) The relative density is shown as the ratio. Data is shown as mean±SEM of 3 observations, **P*< 0.05 vs TM group; ## *P*< 0.01 vs control group

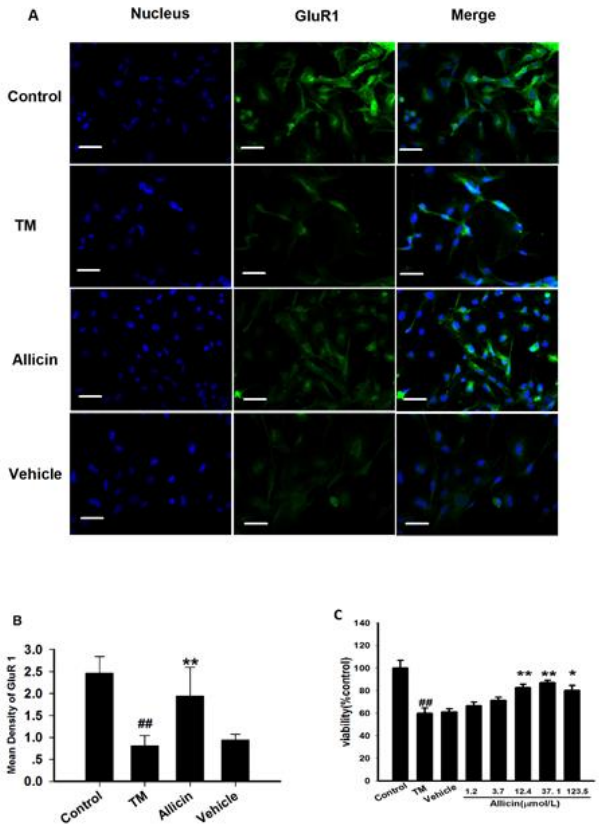

Figure 5. Neuroprotective effect of allicin against TM-induced toxicity. (C) Cells were treated with tunicamycin (4 μg/ml) for a further 40 hr, and then hippocampal neurons were incubated with various concentrations of allicin (1.235, 3.705, 12.35, 37.05, and 123.5 μM). The cell viability was increased compared with the TM glucose group (n=3). (A) The photomicrographs show GluR1 expression in hippocampal neurons (scale bar=50 μm). Histogram B shows the change of mean density labeled GluR1 (n = 3). Each data is shown as mean±SEM, **P*< 0.05 ** *P*<0.01vs TM group; ## *P*< 0.01 vs control group

Effect of allicin on spine density, spine head diameter, and length in TM -related cognitive deficits rats

As shown in Figure 2, in the CA1 region of all groups, the mean dendritic spine density and the spine head diameter were sharply decreased in the CA1 hippocampus of the TM group rats compared with normal control rats, respectively (Figures 2C, D; *P*<0.01). Allicin pretreatment significantly increased the dendritic spine density (*P*<0.01; Figure 2C) and the spine head diameter compared with the TM group (*P* <0.05; Figure 2D). Cumulative distribution plots showed that TM group rats had shorter spines than normal control rats (Figure 2E; *P*<0.01), and TM group rats had longer spines than TM group rats (Figure 2E; *P*<0.01).

Effect of allicin on LTP in the hippocampal CA1 region

Since hippocampus plasticity is considered the mechanism for learning and memory, we then evaluated the effect of allicin on the CA1 region of the hippocampus *in vitro*. After setting the baseline for 20 min, the fEPSP was recorded for 1 hr under perfusion with ACSF. The results revealed that hippocampal slices of TM group rats weakened the HFS induced LTP in a bell-shaped manner compared with normal control rats (Figures 3A, B; *P*<0.01). The HFS-induced LTP was enhanced by allicin (Figure 3A, B; *P*<0.05 allicin group vs. TM-induced group). These results suggested that allicin affected basal synaptic transmission.

GluR1 expression was significantly increased in allicin-treated cognitive deficits rats. To evaluate the mechanism of the efficacy of synaptic transmission, the expression of GluR1 was analyzed by immunohistochemistry and Western blot (Figure 4). GluR1 is one of the ionotropic receptors of glutamate.

The up-regulation of GluR1 expression in hippocampals was shown in allicin-treated groups (Figure 4A). The total GluR1 protein was also up-regulated (Figure 4B; *P*<0.05, allicin vs. TM groups). Meanwhile, single fluorescent immunofluorescence enabled the

GluR1 expression (green, Figure 5) to be determined in the primary hippocampal neurons. The GluR1-positive neurons were then compared in TM and allicin groups. The number of GluR1-positive neurons was increased significantly in allicin treatment (Figure 5A; $P<0.01$, Allicin vs. TM groups).

Discussion

Based on our previous study, allicin is indicated to be a major factor in potently improving ER stress-related cognitive deficits via the PERK/Nrf2 antioxidative signaling pathway (5). LTP is a manifestation of activity-dependent synaptic plasticity, triggered by HFS in the hippocampal CA1 area via the AMPA receptors activity. It has widely been a target in learning and memory studies. Molecular studies may be useful in elucidating mechanisms (25). We profiled the protein related to the synaptic plasticity in the hippocampus neurons to investigate possible mechanisms by which TM-induced cognitive deficits alters the response to allicin application. Glutamate, one of the major excitatory neurotransmitters (26), which is distributed in the brain is responsible for basal excitatory synaptic transmission and many forms of synaptic plasticity associated with cognitive processes (27, 28). The various biological effects of glutamate are mediated by its receptors. GluR-1 is one of the AMPA receptor subunits involved in the synaptic plasticity regulation (29, 30).

Results of this study showed that the application of allicin to the rats with cognitive deficits induced by TM aggravated the correspondence slope of fEPSP and increased dendritic spine density. Examination of GluR-1 protein levels indicates that up-regulated expression in hippocampal neurons in response to TM-induced neuronal damage may be important in protecting against neurodegeneration and even cognitive deficits (31). At the same time, our *in vivo* study results found that the up-regulated GluR-1 immuno-labeling following allicin treatment are in agreement with the improved cognitive dysfunction behavior observed. Interestingly, some published papers showed that excessive glutamate levels could block the activation of Akt and link to glutamate excitotoxicity and then result in impaired cell survival signaling (32). However, there is not enough evidence in our study to show that survival signaling pathways are involved.

Conclusion

In this regard, we concluded that allicin may be used as a pharmacologic treatment for cognitive deficits induced by TM. Further studies should be needed to fully elucidate the mechanisms involved and should investigate the response to other types of glutamate receptor subtype, which are capable of interaction with each other and cross-talking with other signaling pathways in hippocampal neurons resulting in neurodegenerative diseases including cognitive deficits(32).

Acknowledgment

We thank Professor Zhi-Gang Xiang (School of Medicine, Jishou University) for his great kindness.The work was also supported by the National natural Science Foundation of China (No.81260489; No.81460547; No.81560609); Key Foundation of Education Bureau of Hunan Province (No.14A117); Jishou University Research Award Fund (No.88/8819010) and Innovative Research Training Foundation for medical students of Jisou University (No. JDYXCX201502).

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