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Increased Expression of Phospho-Cofilin in CA1 and Subiculum Areas after Theta-Burst Stimulation of Schaffer Collateral-Commissural Fibers in Rat Hippocampal Slices

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Abstract

Activity-dependent structural plasticity of dendritic spines of pyramidal neurons in the central neuron system has been proposed to be a cellular basis of learning and memory. Long-term potentiation (LTP) is accompanied by changes in synaptic morphology and structural remodeling of dendritic spines. However, there is considerable uncertainty as to the nature of the adjustment. The present study tested whether immunoreactive phospho-cofilin, an index of altered actin filament assembly, could be increased by theta-burst stimulations (TBS), which is an effective stimulation pattern for inducing LTP in the hippocampus. The slope of fEPSPs evoked by TBS to Schaffer collateral-commissural fibers in hippocampal slices was measured, and p-cofilin expression was examined using immunofluorescence techniques. Results indicated that saturated L-LTP was produced by multiple TBS episodes to Schaffer collateral-commissural fibers in the hippocampal CA1 area, and TBSs also increased immunoreactive p-cofilin expression in the stratum radiatum of the hippocampal CA1 area and pyramidal layer of the subiculum. D-2-amino-5-phosphonovalerate (D-APV) prevented LTP and expression of p-cofilin immunoreactive induced by multiple TBS episodes in the stratum radiatum of the hippocampal CA1 area. Two paired-pulse low-frequency stimulation (PP-LFS) episodes to Schaffer collateral-commissural fibers induced long-term depression (LTD), and did not affect p-cofilin expression in the stratum radiatum of the hippocampal CA1 area. These results suggest that LTP induction is associated with altered actin filament assembly. Moreover, the CA1 and subiculum areas of the hippocampal formation possibly cooperate with each other in important physiological functions, such as learning and memory, or in pathological diseases, such as epilepsy.

Key Words: theta-burst stimulation, fEPSP, synaptic plasticity, actin, p-cofilin, hippocampus

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Introduction

Long-term potentiation (LTP), a form of synaptic plasticity implicated in learning and memory formation (4, 12), is commonly proposed to involve changes in synaptic morphology and structural remodeling of dendritic spines (6, 22). However, the nature of these changes remains poorly understood. Several studies have suggested that a simple synaptic change in size and shape is involved (26, 36), or more complicated changes occur, such as formation of perforated or split synapses (25). Other reports proposed that activity-dependent structural plasticity of dendritic spines of pyramidal neurons in the central neuron system (33), which is a cellular basis of learning and memory (34). Matus and Okamoto reported that intense synaptic activity produced modified spine actin networks in cultured cells, which are thought to be related to these morphological observations (24, 27).

Dendritic spines are highly actin-rich sites where the great majority of excitatory synapses are gathered in the mammalian central nervous system (24). Fukazawa and his colleague provided that actin polymerization could be enhanced after LTP in hippocampus (14). Subsequently, other reports also suggest that actin polymerization in dendritic spines is an essential step in the stabilization of LTP in rats hippocampus, which is induced by theta-burst stimulation (TBS) (20, 22). TBS increases the release of brain-derived neurotrophic factor (BDNF) and activates the p21-activated kinase (PAK)/cofilin pathway which regulates extension of actin filaments in dendritic spines (7, 30). A cofilin effect is thought to directly bind to actin filaments and accelerate the removal of monomers from the end of actin filaments, resulting in shortened actin filaments. When LIM kinase, a PAK effector, phosphorylated cofilin at its Ser-3 residue, this effect was blocked. That resulted in inhibited cofilin activity and created conditions favorable for cytoskeleton assembly (6). Moreover, cofilin has been reported to be widely expressed in the mammalian CNS (2) and concentrated in spines within the CA1 area of the adult hippocampus (7).

Fukazawa and colleagues used an *in vivo* dentate gyrus LTP model to show that LTP induction was associated with actin cytoskeletal reorganization within dendritic spines, which was characterized by a long-lasting increase in F-actin content. Increased F-actin content included inactivation of actin depolymerizing factor/cofilin. They also reported that inhibition of actin polymerization inhibited late phase of LTP (14). Chen and colleagues reported that in the hippocampus CA1 area, one episode of theta stimulation markedly increased p-cofilin levels within several minutes, but cofilin phosphorylation faded away 30 min after TBS. They demonstrated that this theta stimulation also increased the probability of an enlarged spine, and that these changes were important for expression and stabilization of late-phase LTP (7).

How structural remodeling supports synaptic plasticity, such as long-term potentiation, at the level of the individual spine has remained poorly understood. The present study investigated whether immunoreactive p-cofilin could be increased by multiple episodes of TBS, which induced saturated LTP, in the Schaffer collateral-commissural pathway of the rat hippocampus.

Materials and Methods

Subjects

All animal procedures were conducted in accordance with the guidelines of the Xi'an Jiaotong University Animal Research Advisory Committee. This included efforts to minimize suffering and numbers of rats used in this work.

Hippocampal Slice Preparation and Electrophysiological Recordings

All experiments used young adult (1~2 months old) male Sprague Dawley rats (Animal Experiment Center of Xi'an Jiaotong University, China). Experiments were initiated between 9:00 and 11:00 am. For slice preparation, rats were deeply anesthetized with ether and then decapitated. The brains were quickly removed and placed in ice-chilled oxygenated artificial cerebrospinal fluid (ACSF) which was bubbled with 95% O_2/5% CO_2 (pH 7.4). Transverse hippocampal slices (400 μm thick) through the middle third of the septotemporal axis of the hippocampus were prepared using a vibratome (Campden Instruments, London, UK) and immediately transferred to an interface-recording chamber containing preheated ACSF (95% O_2/5% CO_2, pH 7.4) maintained at a constant temperature (28 ± 1°C). ACSF contained: NaCl 124 mM, KCl 4.4 mM, $NaHCO_3$ 26 mM, $MgSO_4$ 1.3 mM, NaH_2PO_4 1 mM, $CaCl_2$ 2.5 mM, and glucose 10 mM, and had an osmolality of 300~310 mosM/kg H_2O. Slices were allowed to equilibrate in the recording chamber for 2 h before recordings. The upper slice surface was in contact with a humidified, oxygenated (95% O_2/5% CO_2) stream.

Standard extracellular field potential recording techniques were used (35). Field EPSPs (fEPSPs) were recorded from the stratum radiatum of the hippocampal CA1 area using a single glass pipette filled with 2 M NaCl (2~3 MΩ) in response to orthodromic stimulation (bipolar Teflon-coated nickel-chromium

electrode, MicroProbe Inc., Potomac, MD, USA) to the Schaffer collateral-commissural projections in the CA1 stratum radiatum. For baseline recordings, test stimuli of 0.2-ms duration were delivered once per minute (0.017 Hz) for at least 30 min to ensure the stabilization responses. Stimulation intensity was set to yield 40~50% of the maximal response as assessed by a stimulus/response (slopes of fEPSPs) curve. All slices that produced maximal evoked fEPSPs amplitude less than 1 mV were rejected. After obtaining a stable baseline recording, LTP or LTD was induced. Physiologically patterned theta burst stimulation (TBS), which was the same intensity and duration as the test stimulation (TS), was used to induce LTP. A TBS included five trains at 5 Hz with each train including four pulses at 100 Hz. One TBS episode was composed of four TBS separated by 10 s. To induce saturated LTP, six TBS episodes were separated by 10 min (6TBS). Some slices received only one tetanus train (1TBS). Two paired-pulse low-frequency stimulation (PP-LFS) episodes separated by 10 min, which was the same intensity and duration as the test stimulation, were used to induce LTD. One PP-LFS episode included 900 pairs at 1 Hz (paired-pulse interval, 200 ms). The control group slices received only baseline test stimulation (TS), and the experimental time was the same as the corresponding experiment group. Electrical signals were collected and analyzed using an A/D converter (Powerlab 200; AD Instruments, Castle Hill, Australia) with scopes for windows. The electrical signals were filtered at 1 kHz, sampled at 20 kHz, and a personal computer was used to acquire data on-line and analyze off-line.

LTP amplitude (% of baseline) was measured as follows: mean fEPSP slopes were recorded for 3 min (7~10 min after each TBS episode stimulation) and were divided by mean fEPSP slopes recorded for the last 5 min of baseline. The value was then multiplied by 100%.

Immunocytochemical Experiments

After electrophysiological recordings (7 min after the last stimulation), the slices were fixed in 4% paraformaldehyde/0.1 M sodium phosphate buffer (PB), pH 7.2, for 12-16 h, then cryoprotected in 20% sucrose/PB for 2 h at 4°C. Next, the slices were cut into 20-μm thick sections on a freezing microtome, and then washed in 0.01 M PBS (3 × 10 min). Sections were incubated (48 h at 4°C) in rabbit anti-phospho-cofilin (pS3; 1:20; catalog #3133; Cell Signaling Technology, Danvers, MA, USA) in 0.01 M PBS containing 5% (v/v) normal goat serum, 0.3% (v/v) Triton X-100, and 0.05% (w/v) sodium azide (NaN3) (PBT, pH 7.4). The sections were then washed in 0.01 M PBS (3 × 10 min), incubated (2 h at room temperature) with Cy3 anti-rabbit IgG (1:500; catalog #AP187C, Chemicon, Temecula, CA, USA) in PBT and rinsed in 0.01 M PBS (3 × 10 min). The sections were mounted onto clean glass slides, air dried, and cover-slipped with a mixture of 50% (v/v) glycerin and 2.5% (w/v) triethylene diamine (anti-fading agent) in 0.01 M PBS. Control tissue was processed utilizing the same procedures, but the primary antibodies were omitted or replaced with normal rabbit sera. No immunofluorescent histochemical staining was detected for the omitted or replaced antibodies.

Confocal Microscopy and Quantification of Immunoreactive Puncta

The stained sections were observed with a confocal laser-scanning microscope (LSM, TCS SP2; Leica, Mannheim, Germany) using appropriate excitation laser beams (520 nm for Cy3) and appropriate emission filters (550-600 nm for Cy3).

To quantify the distribution of p-cofilin expression, the confocal images stained with Cy3 were converted to grayscale, and intensity levels were scaled to values determined for each experiment (Photoshop CS, version 8.0; Adobe Systems, San Jose, CA, USA) to visualize low-intensity labeling. Analysis was conducted blindly on batches of slices that were sectioned and stained together. Identified objects measuring $< 0.04\ \mu m^2$ and $>1.2\ \mu m^2$ (7) were excluded from analysis. Image-Pro Plus software (version 6.0; Media Cybernetics Inc., Siler Spring, MD, USA) was used to quantify and determine the area of labeled puncta within the size range.

Neuronal images were acquired using a confocal microscope with 20× and 40× objectives under identical gain. Pixel values for each image were normalized to reduce the impact of background intensity differences across the image, binarized using each intensity threshold, and finally cleaned by erosion and dilation filtering.

Chemicals and Drugs

D-2-amino-5-phosphonovalerate (D-APV), purchased from Sigma (St. Louis, MO, USA), was diluted in ACSF to the desired final concentrations. Rabbit anti-phospho-cofilin and Cy3 anti-rabbit IgG were respectively purchased from Cell Signaling Technology (Danvers, MA, USA) and Chemicon (Temecula, CA, USA).

Statistical Analyses

Analysis of electrophysiological recordings from the final 3-min recordings after each TBS episode

was performed by one-way ANOVA, and *post-hoc* analysis was done using Tukey's HSD test. Statistical significance of puncta counts and fluorescence density mean was determined by one-way ANOVA. The level of significance was assessed at $P < 0.05$. Population counts for these experiments represented numbers of slices. All measures were reported as means ± S.E.M. in the figures.

Results

Saturated L-LTP Induced by Episodes of TBS Was Dependent on NMDARs in the Hippocampal CA1 Area

Characteristics of glutamatergic synaptic plasticity were analyzed in the hippocampal CA1 area by measuring the slope of fEPSPs evoked by test stimulation to Schaffer collateral-commissural fibers in hippocampal slice. Late-phase long term potention (L-LTP) was induced using one episode or multiple episodes of TBS and did not decay over a 3-h period (Fig. 1A). Mean fEPSP slopes were measured at 0.5 h, 1 h, 2 h and 3 h after TBS train in 1TBS (142.87 ± 4.94%, 141.52 ± 6.57%, 140.46 ± 6.15% and 141.99 ± 6.43%, respectively; n = 9, $T_{16} = 9.21$, $P < 0.001$, compared to baseline), and measured at 0.5 h, 1 h, 2 h, and 2.5 h after the last TBS train in 6TBS (307.59 ± 20.36%, 307.22 ± 20.48%, 302.38 ± 22.12% and 303.07 ± 21.15%, respectively; n = 6, $T_{10} = 9.76$, $P < 0.001$, compared to baseline). The mean synaptic response 2 h after the last TBS train in 1TBS was smaller than that of 6TBS. There was a significant difference between the mean synaptic responses 2 h after the last TBS train in 1TBS and 6TBS (Fig. 1A, $T_{13} = 8.54$, $P < 0.001$).

To determine which receptors mediated LTP induction under the present experimental conditions, we used D-APV (50 μM), a competitive N-methyl-d-aspartate receptor (NMDAR) antagonist. When D-APV was present, the same stimulus protocol did not induce LTP (Fig. 1B). Mean fEPSP slopes measured 1 h, 2 h, and 3 h after TBS train in 1 TBS were 104.03 ± 2.77%, 92.74 ± 3.30% and 100.90 ± 3.91% (n = 7, $T_{12} = 0.39$, $P = 0.71$, compared to baseline value), and after the last TBS train in 6TBS were 93.18 ± 3.57%, 94.73 ± 2.8% and 101.78 ± 3.54% (n = 7, $T_{12} = 0.82$, $P = 0.43$, compared to baseline value). Thus, LTP induction under the present experimental paradigm was NMDAR-dependent.

After 30 min baseline recording, multiple TBS episodes induced a marked increase in synaptic responses measured 10 min after each TBS episode. The mean fEPSP slopes during 7~10 min after each TBS episode were 152.84 ± 9.91% (named LTP_1), 190.91 ± 7.99% (LTP_2), 228.44 ± 10.44% (LTP_3), 265.41 ± 10.88% (LTP_4), 299.55 ± 13.72% (LTP_5) and 307.36 ± 15.95% (LTP_6), respectively ($F_{6, 35} = 43.85$, $P < 0.001$) (Fig. 1C). The mean fEPSPs potentiations of the last 3 min after each TBS train were significantly different compared with values after the previous TBS train except for between the last two episodes (LTP_2 compared with LTP_1, $P < 0.05$; LTP_3 compared with LTP_2, $P < 0.05$; LTP_4 compared with LTP_3, $P < 0.05$; LTP_5 compared with LTP_4, $P < 0.05$; LTP_6 compared with LTP_5, $P = 0.65$) confirming that LTP was saturated after the fifth TBS train (3). These results indicated that multiple TBS episodes to Schaffer collateral-commissural fibers induced saturated L-LTP in the hippocampal CA1 area, which depended on NMDARs.

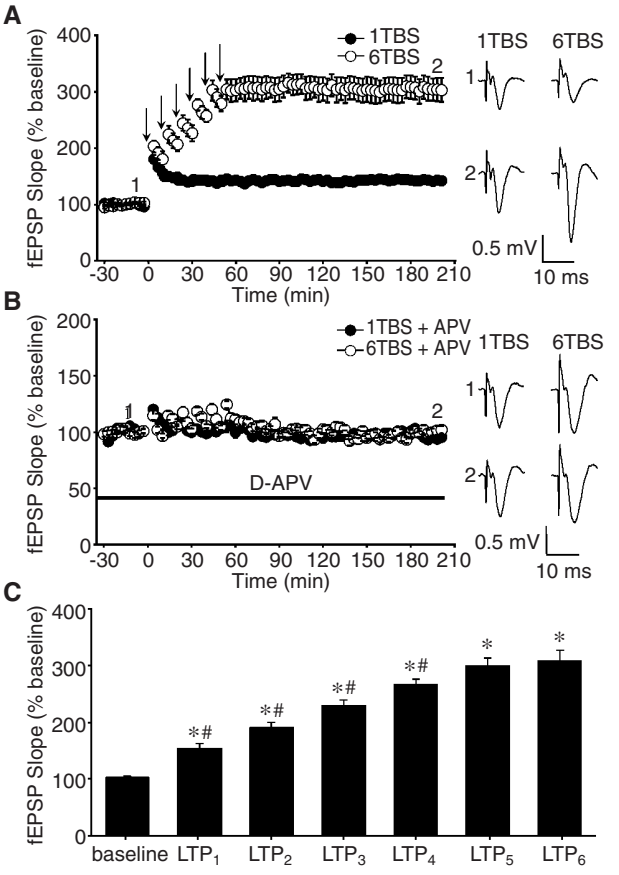

Fig. 1. Saturated L-LTP induced by episodes of TBS was dependent on NMDARs in the hippocampal CA1 area. (A) L-LTP was evoked with one episode (n = 9) or multiple episodes of TBS (downwards arrow, n = 6) in hippocampal slices from adult rats. (B) D-APV (50 μM) prevented L-LTP induction in 1 TBS (n = 7) and 6TBS (n = 7). (C) Histogram representing the baseline and 6 mean potentiations after each TBS. *Statistically different, $P < 0.001$ *vs.* baseline, #Statistically different, $P < 0.001$ *vs.* 6TBS. In this and subsequent figures, the insets are sample fEPSP traces taken at the times indicated by the graph numbers.

Episodes of TBS Induced NMDAR-Time-Dependent p-Cofilin Expression in the Adult Hippocampal CA1 Area

Hippocampal slices stimulated by six episodes of TBS were fixed at 0.5 min, 2 to 7 min, and 30 min after the last TBS train. The slices were then processed for localization of p-cofilin expression using immunofluorescence techniques. The slices exhibited a time-dependent increase in the number of p-cofilin-immunoreactive (p-cofilin-ir) puncta in the stratum radiatum of CA1 innervated by stimulated afferents with no expression in the pyramidal cell layer of CA1 (Figs. 2 B, C and D). There was a significant difference in the number of p-cofilin-ir puncta in 0.5 min, 2 to 7 min, and 30 min after the last TBS train and test stimulation (Fig. 2E, $F_{3, 12} = 181.74$, $P < 0.001$). A more than fourfold increase in p-cofilin-ir puncta was counted in slices collected from 2 to 7 min post the last TBS (n = 4, 20.96 ± 1.05/100 μm^2) compared with the control slices receiving only test stimulation (n = 4, 5.13 ± 0.45/100 μm^2, $P < 0.001$), slices 0.5 min after the last TBS train (n = 4, 5.43 ± 0.28/100 μm^2, $P < 0.001$), or slices 30 min after the last TBS train (n = 4, 5.22 ± 0.35/100 μm^2, $P < 0.001$, followed by Tukey's HSD test).

Hippocampal slices stimulated by one episode of TBS (1TBS), six episodes of TBS (6TBS) and 6TBS in the presence of D-APV (50 μM) were fixed at 7 min after the last TBS train at which time cofilin phosphorylation was at maximum (7, 30). As shown in the photomicrographs of Fig. 3A, p-cofilin-ir puncta were more numerous in the stratum radiatum of CA1 in slices that received one TBS and six TBS compared with those that received only test stimulation (TS). When D-APV was present, the effect of 6 TBS episodes on p-cofilin-ir expression was blocked. One-way ANOVA demonstrated a significant TBS effect ($F_{3, 12} = 101.76$, $P < 0.001$) on p-cofilin-ir expression (puncta counts). The differences between p-cofilin-ir puncta numbers were highly significant in TS (n = 4) *versus* TBS1 (n = 4) or TBS6 (n = 4), and in D-APV (n = 4) *versus* TBS1 or TBS6 ($P < 0.001$, followed by Tukey's HSD test). There were no significant differences in the numbers of p-cofilin-ir puncta between slices that received one TBS and six TBSs ($P = 0.35$), or between TS and APV ($P = 0.98$, followed by Tukey's HSD test, Fig. 3). These results indicated that one episode TBS was effective in increasing p-cofilin-ir expression in the hippocampal CA1 area, and this expression depended on NMDARs.

Multiple TBS Episodes Resulted in NMDAR-Dependent p-Cofilin Expression in the Subiculum

As shown in Fig. 4A, slices that received six TBS episodes to Schaffer collateral-commissural

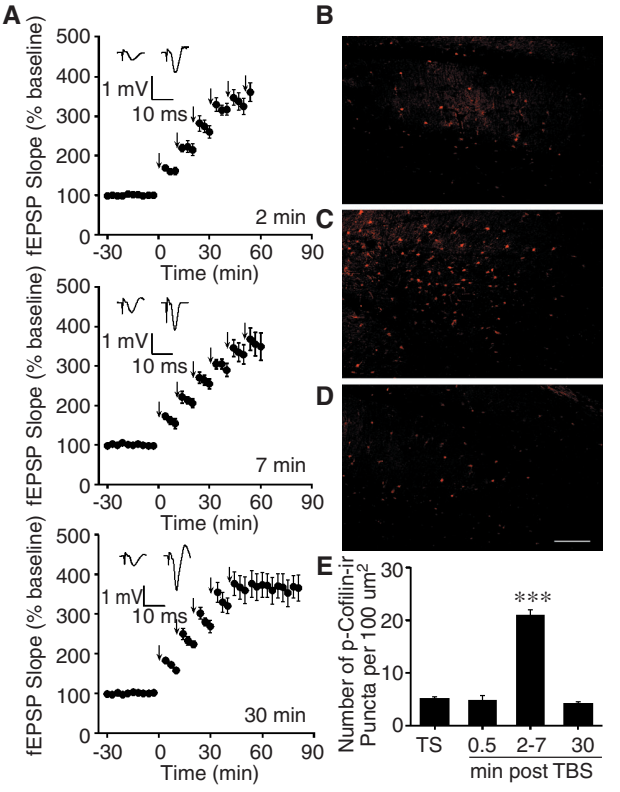

Fig. 2. Episodes of TBS induced time-dependent p-cofilin expression in the adult hippocampal CA1 area. (A) LTP was induced by episodes of TBS (downwards arrow). The insets are sample fEPSP traces taken at the times immediately before delivery of the first TBS (left side) and from the minute before the slices were collected (at right). (B, C, D) Laser-scanning confocal micrographs showing p-cofilin immunostaining in slices that received either test stimulation (TS; B), 2-7 min after six theta-burst stimulation (C), or 30 min after six theta-burst stimulation (D). Scale bar, 20 μm. (E) Histogram representing the number of p-cofilin-ir puncta (means ± SEM) in 100-μm^2 thick slices in the various groups (TS, 0.5 min, 2-7 min, and 30 min post the last TBS. n = 4 in all the groups). ***Statistically different, $P < 0.001$ *vs.* TS, 0.5 min, 30 min group.

fibers displayed diffuse p-cofilin-ir in the widened pyramidal cell layer of the subiculum with no expression in the polymorphic and molecular layers. One-way ANOVA demonstrated a significant multiple TBS episodes effect ($F_{3, 12} = 51.24$, $P < 0.001$) on p-cofilin-ir expression (fluorescence density) in pyramidal cell layer of the subiculum. As shown in Fig. 4, p-cofilin-immunoreactivity in the subiculum pyramidal cell layer was significantly greater in slices that received six TBSs (6TBS, n = 4) compared with those that received only TS (n = 4, $P < 0.001$) or one TBS (1TBS, n = 4, $P < 0.001$), or six TBS episodes in the presence of APV (50 μM, lyh, n = 4, $P < 0.001$, ANOVA followed by Tukey's HSD test). The mean

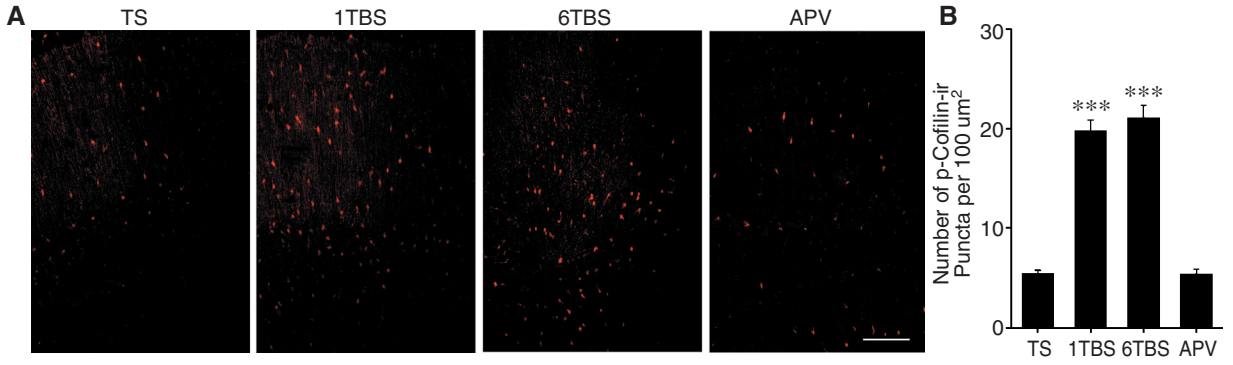

Fig. 3. Episodes of TBS induced NMDAR-dependent p-cofilin expression in the adult hippocampal CA1 area. (A) Laser confocal photomicrographs showing p-cofilin-immunoreactivity in the CA1 stratum radiatum of rat hippocampal slices that received baseline test stimulation (TS), one TBS episode (1TBS), six TBS episodes (6TBS) or 6TBS in the presence of APV (50 μM). Slices were collected 7 min after stimulation. Scale bar, 15 μm. (B) Bar graph showing the number of p-cofilin-ir puncta (means ± SEM) in the stratum radiatum of the hippocampal CA1 area in 100-μm^2 thick slices in TS (n = 4), 1TBS (n = 4), 6TBS (n = 4) and 6TBS in the presence of APV (n = 4), ***Statistically different, $P < 0.001$ *vs.* TS, and APV.

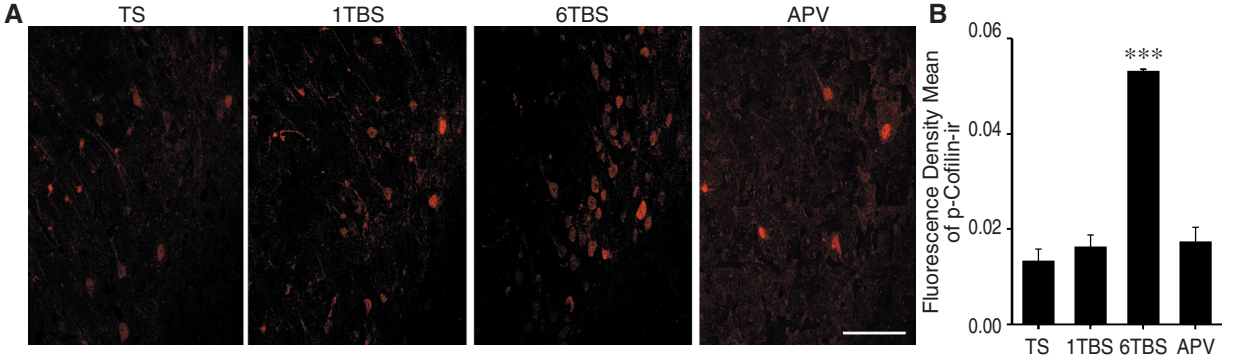

Fig. 4. Multiple TBS episodes-induced immunoreactive p-cofilin increased in the hippocampal subiculum. (A) Laser confocal photomicrographs showing p-cofilin-immunoreactivity in the subiculum pyramidal cell layer of rat hippocampal slices that received either baseline test stimulation (TS), one TBS, six TBS or six TBS in the presence of APV. Slices were collected 7 min after stimulation. Scale bar, 75 μm. (B) Bar graph showing the fluorescent densities of p-cofilin-ir puncta (means ± SEM) in the subiculum pyramidal cell layer (n = 4 in all the groups). ***Statistically different, $P < 0.001$ *vs.* TS, 1TBS, and APV.

fluorescent density values of p-cofilin-ir in the subiculum pyramidal cell layer were not significantly different between slices that received TS and one TBS ($P = 0.41$), and between slices of APV and 6TBS ($P = 0.41$, ANOVA followed by Tukey's HSD test). These results indicated that multiple TBS episodes to the Schaffer collateral-commissural fibers (which induced saturated LTP) increased p-cofilin immunoreactivity in the subiculum pyramidal layer, and this expression depended on NMDARs.

PP-LFS Induced LTD and Did not Affect p-Cofilin Expression in the Adult Hippocampal CA1 Area

After a 30-min baseline recording, two paired-pulse low-frequency stimulation (PP-LFS) episodes separated by 10 min induced a marked decrease in synaptic responses. The mean fEPSP slopes measured 30 min, 60 min, 90 min and 120 min after the last PP-LFS were 74.18 ± 2.63%, 72.33 ± 3.19%, 68.78 ± 4.41% and 69.11 ± 2.80% (n = 7, $T_{12} = 7.049$, $P < 0.0001$, compared to baseline, Fig. 5A). These results indicated that paired-pulse low-frequency stimulation induced LTD in the hippocampal CA1 area.

As shown in the photomicrographs of Figs. 5B and 5C, the numbers of p-cofilin-ir puncta were not different in the stratum radiatum of CA1 in slices that received two PP-LFS episodes (n = 4) compared with those that received only test stimulation (TS, n = 4). The fluorescence density mean values of p-cofilin-ir in the stratum radiatum of CA1 in slices that received two PP-LFS episodes were no significantly different than slices that received only test stimulation (n = 4, $T_6 = 0.16$, $P = 0.88$, compared to TS, Fig. 5D).

The results from this study suggested that six TBSs to the Schaffer collateral-commissural fibers

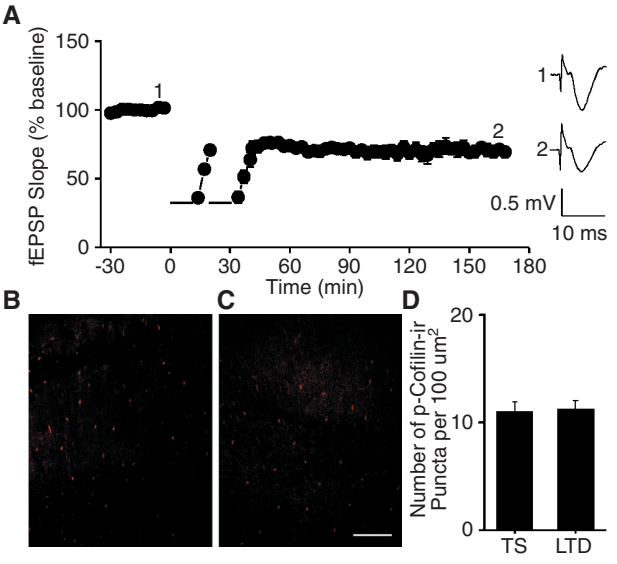

Fig. 5. Two episodes of paired-pulse low-frequency stimulation induced LTD and did not affect p-cofilin expression in the adult hippocampal CA1 area. (A) LTD was evoked with two paired-pulse low-frequency stimulation (PP-LFS) episodes (short line, n = 7 in LTD and TS). (B, C) p-cofilin-immunoreactivity in the proximal CA1 stratum radiatum of rat hippocampal slices that received baseline test stimulation (TS, n = 4), or PP-LFS (n = 4). Slices were collected 7 min after stimulation. Scale bar, 20 μm.

induced saturated LTP in the hippocampal CA1 area, and also produced increased p-cofilin immunoreactivity in the stratum radiatum of the hippocampal CA1 area and the subiculum pyramidal layer, which depended on NMDARs.

Discussion

The present study employed six TBS episodes to the Schaffer collateral-commissural fibers to induce NMDAR-dependent LTP saturation in the CA1 area of rat hippocampal slices. Immunocytochemistry results demonstrated that this mode of stimulation increased p-cofilin-ir expression in the stratum radiatum of the hippocampal CA1 area as well as the subiculum pyramidal layer which depended on NMDARs. Moreover, one TBS episode increased p-cofilin-ir in the stratum radiatum of the hippocampal CA1 area, but not in the subiculum. Besides, two PP-LFS episodes to the Schaffer collateral-commissural fibers induced LTD in the CA1 area of rat hippocampus, and had no effect on the expression of p-cofilin-ir in the stratum radiatum of the hippocampal CA1 area.

Expression of p-cofilin was determined at 7 min after the last TBS (following electrophysiological recording and slice fixation) according to a study showing that one train of theta burst stimulation increased p-cofilin expression within 2 min after stimulation, which peaked at 7 min and dissipated within 30 min (7). Our results also indicated that six trains of TBS had similar results. Previous experiments have shown a marked, region-specific increase in the number of spines with denser concentrations of p-cofilin as well as the formation of larger synapses in the CA1 area in slices that received one train of theta burst stimulation compared with slices with no stimulation (7). The present study showed that altered p-cofilin expression in the CA1 area, which was induced by one train of TBS to the Schaffer collateral-commissural fibers, was the same as expression induced by six trains of TBS. This result was consistent with the previous report (8). The increasing of expression of p-cofilin-ir by six trains of TBS, which was blocked by D-APV, depended on NMDARs. Studies have shown that NMDARs play a critical role in induction of LTP and LTD in the hippocampal CA1 region (23). Our previous study also demonstrated that NMDARs and NR2A subunit are required for induction and partial reversal of L-LTP *in vitro* (35).

In addition, results demonstrated significant increased p-cofilin expression in the widened pyramidal cell layer of the subiculum after six TBS episodes rather than one TBS train or baseline test stimulation. The hippocampal formation consists of the dentate gyrus, hippocampus (areas CA1 and CA3), entorhinal cortex and subiculum (1). The hippocampal CA1 area sends its primary projection to all regions of the subiculum which, in turn, projects to many cortical and subcortical targets, returning a smaller amount of oligosynaptic projections to the hippocampal CA1 area (11). The subiculum is, therefore, the major output structure of the hippocampus (28). Several lines of evidence support the contention that the subiculum is an important mediator of hippocampal-cortical interactions, and that the subiculum is the principal target of CA1 pyramidal cell axons (13, 31) and, thus, represents the final relay in a synaptic loop between the entorhinal cortex and hippocampus. Subiculum activity has been shown to correlate with theta and gamma oscillations as well as with sharp waves of the hippocampal CA1 area (9). Previous studies reported that Schaffer collaterals originating from CA3 pyramidal cells had extensive projections to the stratum radiatum of the hippocampal CA1 area, and several axon collaterals penetrated the subiculum (18, 21). These results suggested that multiple TBS (or saturated LTP in the hippocampal CA1 area) could induce certain pattern activities in the subiculum directly or indirectly. It is probable that a greater amount of activity in the few CA3 pyramidal axons, which was due to more TBS trains to the Schaffer collateral-commissural fibers, increased the phosphorylation level of cofilin in the subiculum.

Previous studies have shown that naturalistic theta stimulation caused both increased BDNF release (26) and actin polymerization in a subpopulation of spine heads in fields of potentiated synapses in adult hippocampal slices (20, 22). BDNF regulates actin assembly in developing filopodia through Rho family GTPases, which promote phosphorylation of actin depolymerizing factor (ADF)/cofilin (16). Reports have shown that both actin polymerization and LTP induction have the same threshold, are stabilized over the same time period, and are disrupted by the same manipulations (20). The present study demonstrated that one TBS episode induced both L-LTP (145.50 ± 4.77%) and cofilin expression which were similar six TBS. This suggested that the intensity of cofilin expression in the CA1 area was likely associated with LTP induction, but not LTP saturation. Some evidence exists that theta burst stimulation plays an important role in stabilizing LTP (5, 7). Our results indicate that NMDARs play a critical role in induction of LTP and p-cofilin expression. The present study shows a relationship between p-cofilin expression and TBS-induced LTP. The actin filament assembly is known to be regulated by the phosphorylation state of cofilin (36). The present results suggested that one TBS episode or LTP induction was likely associated with altered actin filament assembly. Several reports have confirmed that inhibition of actin polymerization affects LTP induction. In 2009, Kramar and his colleagues reported that the effects of estrogen, which caused a pronounced facilitation of LTP in adult hippocampal slices, were completely eliminated by latrunculin, a toxin that prevents actin filament assembly (19). In 2009, Ramachandran and Frey reported that the inhibition of actin polymerization prevented late-LTP, and interfered with synaptic tagging in apical dendrites of hippocampal CA1 (29).

The subiculum provides the major hippocampal output and plays a role in memory formation (15), spatial encoding (32) and the generation of physiological rhythms (9). Furthermore, the hippocampal-subiculum-entorhinal loop has been implicated in temporal lobe epilepsy in animals (17) and in humans (10). The present results demonstrated that six TBS episodes applied to the Schaffer collateral-commissural fibers produced increased expression of p-cofilin in the CA1 area and the subiculum, which suggested that the CA1 and subiculum areas of the hippocampal formation cooperate with each other in certain physiological functions, such as learning and memory, or in pathological diseases, such as epilepsy.

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