

Original Article

Activity- and development-dependent down-regulation of TARPy8 and GluA1 in cultured rat hippocampal neurons

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Aim: Transmembrane AMPA receptor regulatory proteins (TARPs) regulate the trafficking and expression of AMPA receptors that are essential for the fast excitatory synaptic transmission and plasticity in the brain. This study aimed to investigate the activity-dependent regulation of TARPy8 in cultured rat hippocampal neurons.

Methods: Rat hippocampal neurons cultured for 7–8 DIV or 17–18 DIV were exposed to the AMPA receptor agonist AMPA at a non-toxic concentration (100 $\mu\text{mol/L}$) for 4 h. The protein levels of TARPy8 and AMPA receptor subunits (GluA1 and GluA2) were measured using Western blotting analysis. AMPA-induced currents were recorded in the neurons using a whole-cell recording method.

Results: Four-hour exposure to AMPA significantly decreased the protein levels of TARPy8 and GluA1 in the neurons at 17–18 DIV, but did not change the protein level of TARPy8 in the neurons cultured at 7–8 DIV. AMPA-induced down-regulation of TARPy8 and GluA1 was largely blocked by the calpain inhibitor calpeptin (50 $\mu\text{mol/L}$), but not affected by the caspase inhibitor zVAD (50 $\mu\text{mol/L}$). Four-hour exposure to AMPA significantly decreased AMPA-induced currents in the neurons at 17–18 DIV, which was blocked by co-exposure to calpeptin (50 $\mu\text{mol/L}$).

Conclusion: The down-regulation of TARPy8 and GluA1 protein levels and AMPA-induced currents in cultured rat hippocampal neurons is activity- and development-dependent, and mediated by endogenous calpain.

Keywords: AMPA receptor; cultured hippocampal neurons; TARPy8; GluA1; calpain; calpeptin; activity-dependent; development-dependent

Acta Pharmacologica Sinica (2016) 37: 303–311; doi: 10.1038/aps.2015.112; published online 4 Jan 2016

Introduction

AMPA-type glutamate receptors (AMPA receptors) play an important role in fast excitatory synaptic transmission in the central nervous system. The functional properties of AMPAR depend on the precise composition of the core subunit (GluA1–4)^[1] and the associated auxiliary subunits, including a family of transmembrane AMPAR regulatory proteins (TARPs)^[2], such as Type I TARPy2 (stargazin), $\gamma 3$, $\gamma 4$ and $\gamma 8$, and Type II $\gamma 5$ and $\gamma 7$ ^[3, 4]. Type I TARPs play pivotal roles in the neuronal trafficking of AMPARs and channel properties^[3, 5, 6], but Type II TARPs mainly modulate AMPAR channel function^[7].

As an AMPAR auxiliary subunit^[8], stargazin delivers AMPARs through the secretory pathway to the plasma membrane, and then shuttles AMPARs to postsynaptic densities (PSDs) by binding to PSD-95 and related PDZ-containing proteins^[5, 9]. Thus, the density of AMPARs at the neuronal membrane and synapse can be controlled by stargazin and PSD-95. Following the discovery of stargazin as an AMPAR auxiliary subunit, three subunits of TARP family ($\gamma 3$, $\gamma 4$, and $\gamma 8$) were identified^[3]. Due to the high level of expression of TARPy8 in the hippocampus, many studies have been focused on the modulation of TARPy8 on AMPAR^[4, 10, 11]. Transgenic mice with a mutation of TARPy8 show a decrease in the expression of the AMPAR protein level^[12]. Knockout of $\gamma 8$ reduces the level of the AMPAR protein and impairs AMPAR-mediated synaptic function^[4, 13]. It also prevents against kainate-induced neuronal death^[12], suggesting that the TARPy8 subunit is criti-

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Received 2015-07-02 Accepted 2015-10-26

cal for the expression and trafficking of AMPAR, which is essential for long-term potentiation (LTP)^[10, 14].

The most efficient way to regulate the level of cellular proteins is through selective proteolysis^[15]. Calpain, a calcium-activated non-lysosomal protease^[16], cleaves many target proteins, such as AMPAR^[17, 18], contributing to glutamate excitotoxicity under disease conditions, including stroke and epilepsy. Caspase, a cysteine-dependent and calcium-regulating protease^[19, 20], was also reported to modulate AMPAR function via activity-dependent AMPAR cleavage^[21-23]. Whether there is a similar regulatory mechanism of the AMPAR-associated protein TARP is unclear.

In this study, we investigated the effects and the cellular mechanism of AMPA exposure on TARP γ 8 expression and AMPA current in different culture stages. We found that pre-exposure of hippocampal neurons to non-toxic concentrations of AMPA^[24, 25] caused a development-dependent decrease in TARP γ 8, which could be prevented by calpain inhibitor but not caspase inhibitor, suggesting that the calpain-mediated cleavage of TARP was dependent on development.

Materials and methods

Animals

Sprague-Dawley rats were obtained from the Experimental Animal Center of Zhengzhou University (Zhengzhou, China). All experimental procedures were approved by the Ethics Committee for Animal Experimentation of Xinxiang Medical University, Xinxiang, China. In addition, all efforts were made to minimize animal suffering and the number of animals used.

Primary hippocampal neuronal culture

Primary cultures of hippocampal neurons were obtained from Sprague-Dawley rat embryos (E18.5) as previously described^[26]. The hippocampus was isolated under sterile conditions and was enzymatically treated with 0.25% papain in HBSS (5.4 mmol/L KCl, 137 mmol/L NaCl, 0.4 mmol/L KH₂PO₄, 0.34 mmol/L Na₂HPO₄·7H₂O, 10 mmol/L glucose, and 10 mmol/L HEPES) for 10 min. Then, the cells were mechanically dissociated by trituration and seeded at a density of 1×10⁶ cells/cm² onto plates coated with poly-D-lysine (Sigma-Aldrich, St Louis, MO, USA). The neurons were cultured in Neurobasal medium (Gibco, Invitrogen Corp, Grand Island, NY, USA) supplemented with 2% B27 (Gibco), 1% glutamine (Sigma-Aldrich), and 1% penicillin/streptomycin (Sigma-Aldrich Corp) at 37°C in a 95% air/5% CO₂ humidified incubator (Thermo Scientific, Waltham, MA, USA). Half of the medium was changed twice a week. Hippocampal neurons were incubated for at least 7 d *in vitro* (DIV) before being used for experiments.

Western blotting analysis

The proteins extracted from the hippocampal tissues of Sprague-Dawley rats and primary cultured hippocampal neurons were denatured at 95°C for 5 min and were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred onto PVDF

membranes (Millipore, Boston, MA, USA) using a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad, Hercules, CA, USA) following the manufacturer's instructions. Transferred membranes were blocked in 5% skim milk dissolved in Tris-buffered-saline pH 7.5/0.1% Tween-20 (TBST) for 2 h at room temperature and incubated with one of the following primary antibodies: TARP γ 8 (1:1000, 50 kDa, Millipore) at room temperature overnight, or GluA1 (1:1000, 110 kDa, Abcam, Shanghai, China), GluA2 (1:2000, 106 kDa, Abcam), and β -actin (loading control, 1:20 000, 42 kDa) at 4°C overnight. The membranes were washed three times with TBST and then incubated with appropriate conjugated hydrogen peroxidase HRP secondary antibody (1:2000, Abcam) for 1 h at room temperature. Proteins were visualized using the enhanced chemiluminescence (ECL) reagent (Beyotime, Shanghai, China), and band optical density (OD) analysis was performed using Quantity One software (Bio-Rad).

The relative expression of TARP γ 8 and AMPARs was determined using β -actin as a loading control by calculating the ratio of ODs for each protein of interest versus β -actin OD in each individual subject. To avoid interassay variations, the values obtained were normalized with the values measured for the control cultures in each experiment. The data are presented as the mean±SEM from 5 independent experiments.

Whole-cell recordings

AMPA-induced currents were recorded using the whole-cell patch clamp technique under voltage clamp mode from cultured hippocampal neurons 7–8 and 17–18 d after plating. Recordings were performed using borosilicate glass patch pipettes (2–5 M Ω) connected to an Axonpatch 700B amplifier (Axon Instruments, Molecular Devices, Saint Clara, CA, USA). Data acquisition was performed using a digitizer (DigiData 1440, Axon Ins) and off-line analysis was carried out with pClamp10.0 (Axon Ins). The whole-cell currents were filtered at 2 kHz and sampled at 10 kHz. The cells were focused on the stage of a Nikon FN1 (Tokyo, Japan) inverted microscope and was locally superfused with a Tyrode solution containing the following (in mmol/L): 137 NaCl, 2 CaCl₂, 1 MgCl₂, 10 glucose, 5 KCl, and 10 HEPES/NaOH (pH 7.4), 1 μ mol/L tetrodotoxin (TTX), 500 nmol/L MK-801. The whole-cell recording pipette was filled with a solution containing the following (in mmol/L): potassium gluconate 140, KCl 5, HEPES 10, EGTA 0.2, MgCl₂ 2, MgATP 4, Na₂GTP 0.3, and Na₂-phosphocreatine 10 at pH 7.2. Following seal rupture, series resistance (<20 M Ω) or membrane resistance (300–500 M Ω) was monitored throughout the whole-cell recording and data were discarded if the resistance changed by more than 20%. All recordings were obtained at 31±1°C using an automatic temperature controller (Warner Ins).

AMPA-mediated currents were recorded with AMPA (100 μ mol/L) application in cells (held at -60 mV). AMPA was puffed by pressure from a micropipette using a Picospritzer III (World Precision Instruments, Stevenage, Hertfordshire, UK) every 30 s at 10 psi for a duration of 50 ms from patch pipettes of the same dimensions as those used for recording (tip 2–4

$\mu\text{mol/L}$), with the pipette tip located approximately $50 \mu\text{m}$ away from the soma surface, so that the cell recorded could be totally immersed in the drug solution during perfusion.

Antibodies and reagents

We purchased the following primary antibodies: polyclonal rabbit anti-TARP antibody (Millipore), rabbit anti-GluA1 (Abcam), mouse anti-GluA1 (Abcam), and β -actin (Abcam). The following secondary antibodies were used: HRP-conjugated goat anti-rabbit and anti-mouse IgG (Abcam). α -Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX), (2R)-amino-5-phosphonovaleric acid (AP5) and tetrodotoxin (TTX) were purchased from Tocris Bioscience (Bristol, UK). All other reagents were obtained from Sigma-Aldrich.

Statistical analysis

All statistical tests were performed using SigmaStat software (SPSS Inc, Chicago, IL, USA). The results are expressed as the mean \pm SEM. Differences between mean values were evaluated using Student's *t*-test or one-way analysis of variance (ANOVA) followed by Tukey's HSD test. Differences were accepted as significant when $P < 0.05$.

Results

Pretreatment with AMPA decreased TARP γ 8 protein expression level

To study the potential impact of AMPAR activation on TARP γ 8 expression, we measured the TARP γ 8 protein level in neuronal culture at different stages. As shown in Figure 1, Western blotting analysis indicated that TARP γ 8 expression did not differ between neurons cultured for 7–8 DIV (100% as the control) and 17–18 DIV (87.8% \pm 9.4% of control, $n=4$) in the control conditions. AMPA (100 $\mu\text{mol/L}$) pre-treatment induced a significant decrease of TARP γ 8 expression in neurons cultured for 17–18 DIV (47.4% \pm 8.8% of control, $n=4$) compared with the neurons cultured for 7–8 DIV (83.2% \pm 11.1% of control, $P < 0.05$, $n=4$, ANOVA). In the 7–8 DIV cultures, TARP γ 8 expression was not significantly different between the control (100%) and AMPA group (83.2% \pm 11.8% of control, $n=4$). However, in the 17–18 DIV cultures, TARP expression was significantly decreased in the AMPA group (47.8% \pm 8.3%, $P < 0.05$ vs 87.5% \pm 9.6% for non-agonist group, $n=4$, ANOVA). These results indicate that prolonged AMPAR stimulation causes a down-regulation of TARP γ 8 levels in rat hippocampal neurons.

AMPA-induced decrease of TARP γ 8 levels can be blocked by AMPA and NMDA receptor antagonists

To confirm the activity-dependent decrease of TARP γ 8 levels in the prolonged cultures, the antagonist of AMPA and/or NMDA receptors was applied. As shown in Figure 2, the AMPA-mediated decrease of TARP γ 8 expression can be prevented by the AMPA/KA receptor antagonist NBQX (10 $\mu\text{mol/L}$) and NMDA receptor antagonist D-AP5 (20 $\mu\text{mol/L}$). Compared with controls, the TARP γ 8 expression in the pres-

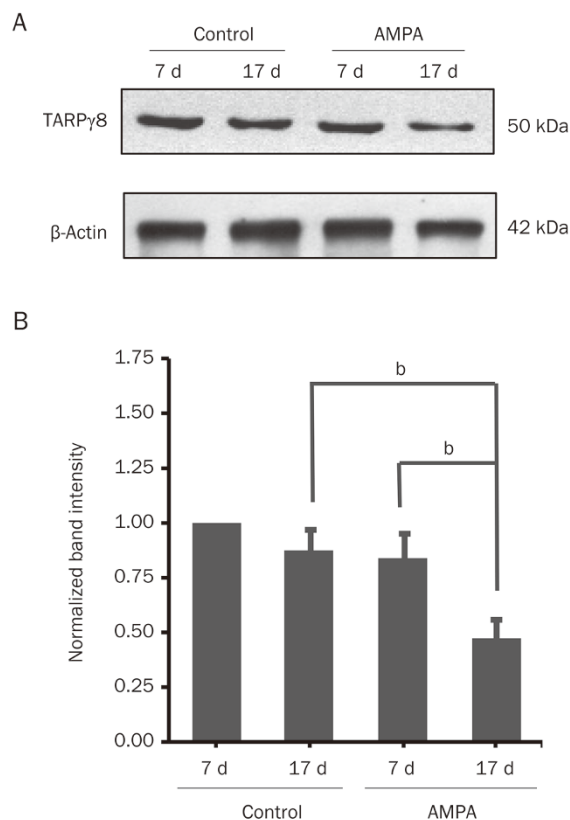


Figure 1. AMPA-induced decrease in TARP γ 8 protein expression in prolonged cultures of rat hippocampal neurons. (A) Western blots show the levels of TARP γ 8 (50 kDa) and β -actin (42 kDa) of hippocampal neurons cultured for 7–8 DIV and 17–18 DIV in the absence and presence of AMPA (100 $\mu\text{mol/L}$, 4 h). (B) Bar graph shows the quantification of TARP γ 8 band densities. The normalization ratio in this case was obtained using β -actin. The data are presented as the mean \pm SEM, ^b $P < 0.05$, $n=4$, one-way ANOVA.

ence of AMPA was significantly decreased (64.4% \pm 5.6% of control, $P < 0.05$, $n=5$, ANOVA), and the TARP γ 8 level was not significantly altered in the presence of AMPA+NBQX (94.3% \pm 5.2% of control, $n=5$). However, compared with control, TARP γ 8 expression was increased in the presence of AMPA+D-AP5 (151.7% \pm 5.5% of control, $P < 0.05$, $n=5$, ANOVA) or in the presence of AMPA+NBQX+AP5 (141.4% \pm 4.7% of control, $P < 0.05$, $n=5$, ANOVA). These results indicated that the AMPA-mediated decrease of TARP γ 8 can be blocked by NBQX treatment and reversed by AP5 treatment, which suggested that both AMPAR and NMDA receptor activation is involved in the AMPA-induced decrease of TARP γ 8 expression.

The protein levels of TARP γ 8 and GluA1 but not GluA2 are mediated by calpain and lysosomes

Previous studies have demonstrated that calpain mediates the GluA1 expression level^[18]. The AMPA-induced decrease of the TARP γ 8 expression level is involved in NMDA receptor activation and calcium influx, and calpain activity is strongly calcium-dependent; therefore, we further tested whether calpain

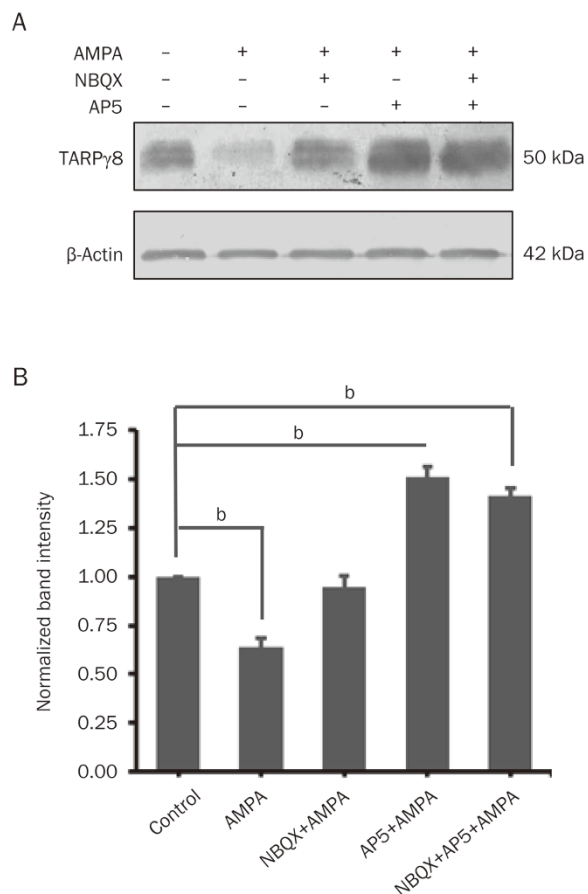


Figure 2. AMPA-induced decrease in TARP γ 8 expression was blocked by AMPAR and NMDAR antagonists. (A) Western blots show levels of TARP γ 8 (50 kDa) and β -actin (42 kDa) of hippocampal neurons cultured for 17–18 DIV following AMPA treatment (100 μ mol/L, 4 h) in the presence of the AMPAR and/or NMDAR antagonists NBQX and/or AP5 (added simultaneously with AMPA treatment). (B) The bar graph shows the quantification of TARP γ 8 band densities. The normalization ratio in this case was obtained using β -actin. The data are presented as the mean \pm SEM, ^b P <0.05, n =5, one-way ANOVA.

contributes to the AMPA-induced decrease of TARP γ 8 expression. As shown in Figure 3, the AMPA-induced decrease of TARP γ 8 expression was blocked by calpain inhibitor, calpeptin (50 μ mol/L). Compared with the control, the TARP γ 8 expression level in the presence of AMPA was significantly decreased (65.4 \pm 3.7% of control, P <0.05, n =5, ANOVA), but TARP γ 8 expression was not significantly different between cultures in the presence of calpeptin (96.1 \pm 6.3% of control, n =5) and in the presence of AMPA+calpeptin treatment (98.3 \pm 5.4% of control, n =5).

We also tested whether lysosomes' enzymatic activity is involved in the AMPA-induced decrease of TARP γ 8 expression. The AMPA-induced decrease of TARP γ 8 expression was blocked by the lysosomal enzyme inhibitor leupeptin (50 μ mol/L). Compared with the control, the TARP γ 8 expression in the cultures in the presence of leupeptin (96.2 \pm 8.5% of control, n =5) and in the presence of AMPA+leupeptin

(96.7 \pm 5.4% of control, n =5) was not significantly different (Figure 3).

Previous studies have demonstrated that caspase mediates the GluA1 protein expression level^[27]. Therefore, we tested whether caspase is involved in activity-dependent decrease in TARP γ 8 expression level. As shown in Figure 3, the TARP γ 8 protein level was not different between control and 50 μ mol/L zVAD, a caspase inhibitor, treatment (94.4 \pm 2.2% of control, n =5). In the activity-dependent condition, the AMPA-induced decrease in the TARP γ 8 expression level was 68.1 \pm 2.3% of control (P <0.05, n =5, ANOVA), and there was no significant difference in TARP γ 8 expression between zVAD+AMPA and AMPA treatment alone. These results indicated that calpain and lysosomes, but not caspase, mediated the activity-dependent decrease of the TARP γ 8 expression level.

In another set of experiments, we tested whether activity-dependent modulation also occurs in the expression levels of GluA1 and GluA2. As shown in Figure 4A and 4C, AMPA induced a decrease in GluA1 (83.3 \pm 2.2% of control, P <0.05, n =5, ANOVA) in prolonged cultured hippocampal neurons (17–18 DIV), but the GluA1 level did not change significantly following the application of calpeptin (102.1 \pm 3.4% of control, n =5) or calpeptin+AMPA (109.7 \pm 3.6% of control, n =5). Similarly, the GluA1 level did not change significantly following the application of leupeptin (98.5 \pm 4.1% of control, n =5) or leupeptin+AMPA (101.8 \pm 4.3% of control, n =5). zVAD (50 μ mol/L) slightly reduced the GluA1 level without statistical significance (96.9 \pm 1.3% of control, n =5). AMPA induced no change in the GluA2 level (Figure 4B and 4D). On average, the GluA2 level was 98.4 \pm 4.7% for AMPA alone, 104.6 \pm 3.5% for calpeptin alone, 101.4 \pm 2.3% for calpeptin+AMPA, 99.2 \pm 5.1% for zVAD alone, 92.3 \pm 4.8% for zVAD+AMPA, 98.4 \pm 4.4% for leupeptin alone and 98.6 \pm 9.2% for leupeptin+AMPA (n =5 for each group).

Taken together, these data indicate that AMPA reduces the GluA1 expression in cultured hippocampal neurons, which is mediated by calpain and/or lysosome enzyme.

AMPA-induced decrease in AMPA current

To confirm whether there was an activity-dependent functional change in AMPAR under the same conditions of AMPA-induced regulation of TARP γ 8 and GluA1, we examined AMPA-induced whole-cell currents in cultured hippocampal neurons pretreated with AMPA (100 μ mol/L). The application of AMPA induced an inward current, which was completely blocked by the AMPA/kainate receptor antagonist (10 μ mol/L NBQX), indicating that it is mediated primarily by AMPARs. As shown in Figure 5, pretreatment with AMPA did not significantly alter the AMPA-induced current in cultured neurons (7–8 DIV) (14.55 \pm 1.92 pA/pF) compared to those from the sham treatment group (13.81 \pm 1.39 pA/pF, n =12, Figure 5A, B). However, pretreatment with AMPA markedly suppressed the subsequently recorded AMPA-induced current in cultured neurons (17–18 DIV) (8.38 \pm 0.82 pA/pF, n =12, P <0.05, ANOVA) compared to those from the sham treatment group (14.13 \pm 1.34 pA/pF, n =12). The activity-dependent decrease

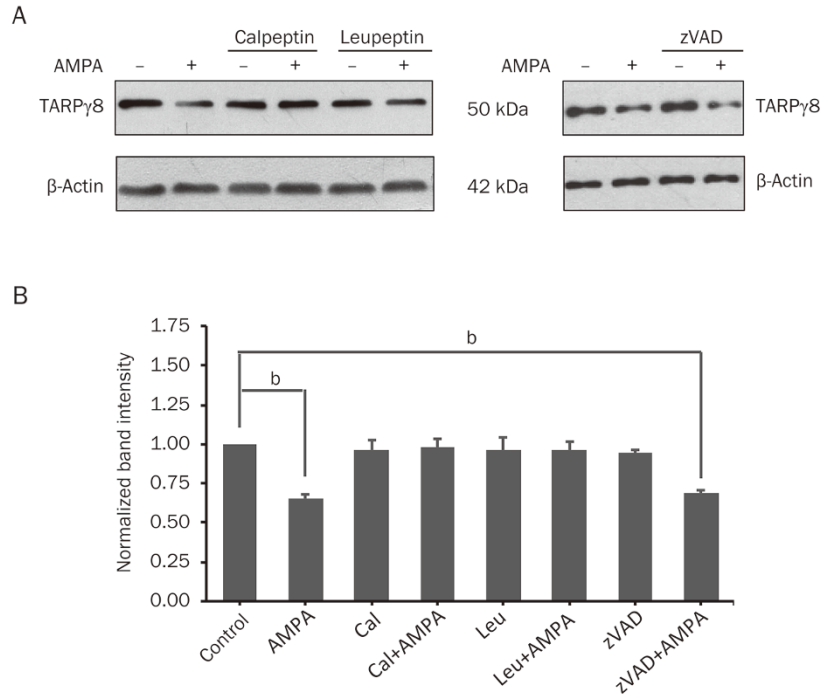


Figure 3. Calpain and lysosome inhibitors blocked the AMPA-induced decrease of TARPy8. (A) Western blots show levels of TARPy8 (50 kDa) and β -actin (42 kDa) of hippocampal neurons cultured for 17–18 DIV in the absence and presence of AMPA (100 μ mol/L, 4 h) following the administration of the calpain inhibitor calpeptin, the lysosome enzyme inhibitor leupeptin, or the caspase inhibitor zVAD (added simultaneously with AMPA treatment, $n=5$). (B) The bar graph shows the quantification of the TARPy8 band densities. The normalization ratio in this case was obtained using β -actin. The data are presented as the mean \pm SEM, ^b $P<0.05$, $n=5$, one-way ANOVA. Cal, calpeptin; Leu, leupeptin.

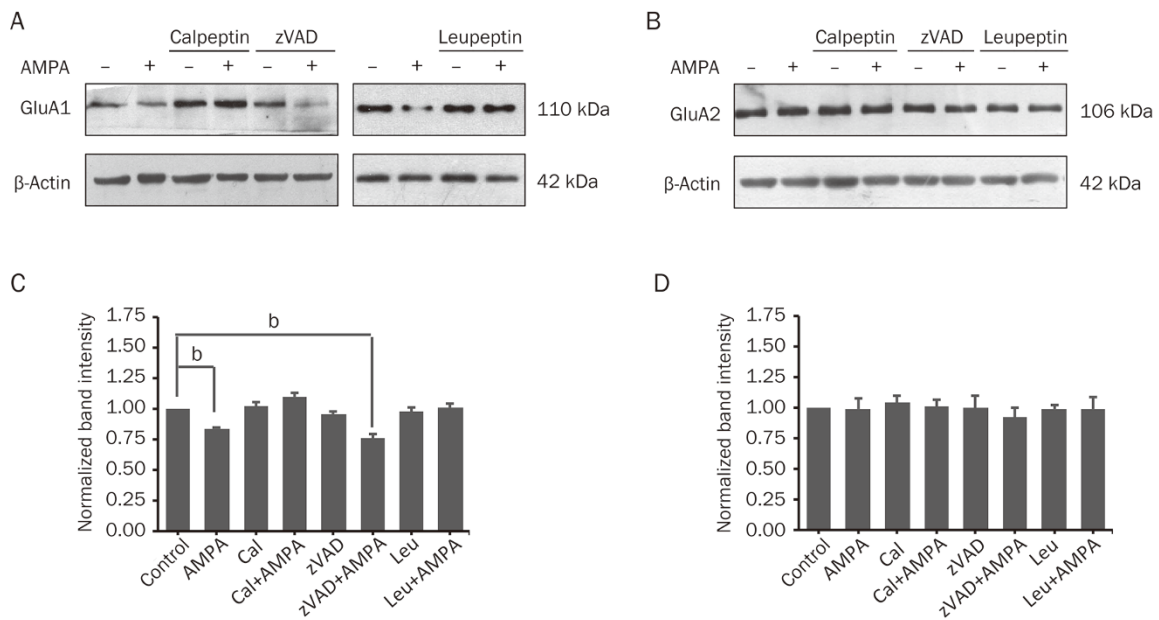


Figure 4. Calpain and lysosome inhibitors blocked the AMPA-induced decrease of GluA1. Western blots show levels of GluA1 (110 kDa) (A) and GluA2 (106 kDa) (B) of hippocampal neurons cultured for 17–18 DIV in the absence and presence of AMPA (100 μ mol/L, 4 h) following the administration of the calpain inhibitor calpeptin, the lysosome enzyme inhibitor leupeptin, or the caspase inhibitor zVAD (added simultaneously with AMPA treatment). Bar graph shows the quantification of GluA1 (C) and GluA2 (D) band densities. The normalization ratio in this case was obtained using β -actin. The data are presented as the mean \pm SEM, ^b $P<0.05$, $n=5$, one-way ANOVA. Cal, calpeptin; Leu, leupeptin.

in AMPA-induced current was blocked by pretreatment with calpeptin (13.88 ± 1.25 pA/pF, $n=12$) or leupeptin (14.13 ± 1.04 pA/pF, $n=12$), but not by zVAD (7.02 ± 0.98 pA/pF, $n=12$, $P < 0.05$, ANOVA), compared to the sham treatment group. The AMPA-induced current in the sham treatment group was not altered by pretreatment with calpeptin (14.38 ± 13.88 pA/pF, $n=12$), leupeptin (13.0 ± 0.76 pA/pF, $n=12$), or zVAD (13.35 ± 0.93 pA/pF, $n=12$, Figure 5C and 5D). These data suggest that the activity-dependent down-regulation of AMPA-induced current is also mediated by calpain.

Discussion

In this study, we found that (1) the pre-treatment of rat hippocampal neurons with the receptor agonist AMPA resulted in the down-regulation of TARP γ 8 and AMPAR subunit GluA1 expression and diminished the AMPA current; (2) the decrease of TARP γ 8, GluA1 expression and AMPA current can be prevented by calpain and lysosome protease inhibitors.

AMPA induced a decrease in TARP γ 8 expression

A major finding in this study was the significant decrease of TARP γ 8 expression in the hippocampus in an activity-dependent manner. To our knowledge, this is the first study

to report the activity-dependent change of the TARP subunit, which suggests that, similar to AMPAR, TARP may also undergo changes in expression level during glutamate-mediated excitation and excitotoxicity and that this change may indirectly affect AMPAR function. The change in the TARP subunit is also development-dependent. The functional consequence of such a change is unknown, but it may be related to the age-related changes in AMPAR function *in vivo*. The amplitudes of spontaneous or miniature EPSCs are reduced in the anterior piriform cortex of aged mice, indicative of an aging-related decrease in AMPAR function^[28]. The decreased AMPAR function in aging cannot be explained by the unaltered expression of GluA1 or by the reduction of GluA2/3 expression in the aged human brain^[29], but it may be associated with the altered trafficking or subcellular location of AMPAR. As TARP contributes to AMPAR trafficking, the decreased expression of the TARP subunit revealed in this study may provide a mechanism for aging-related changes in AMPAR subunits and function.

The AMPA-induced decrease of the TARP γ 8 level can be blocked by AMPA and NMDA receptor antagonists

Although TARP γ 8 expression showed a decreasing tendency

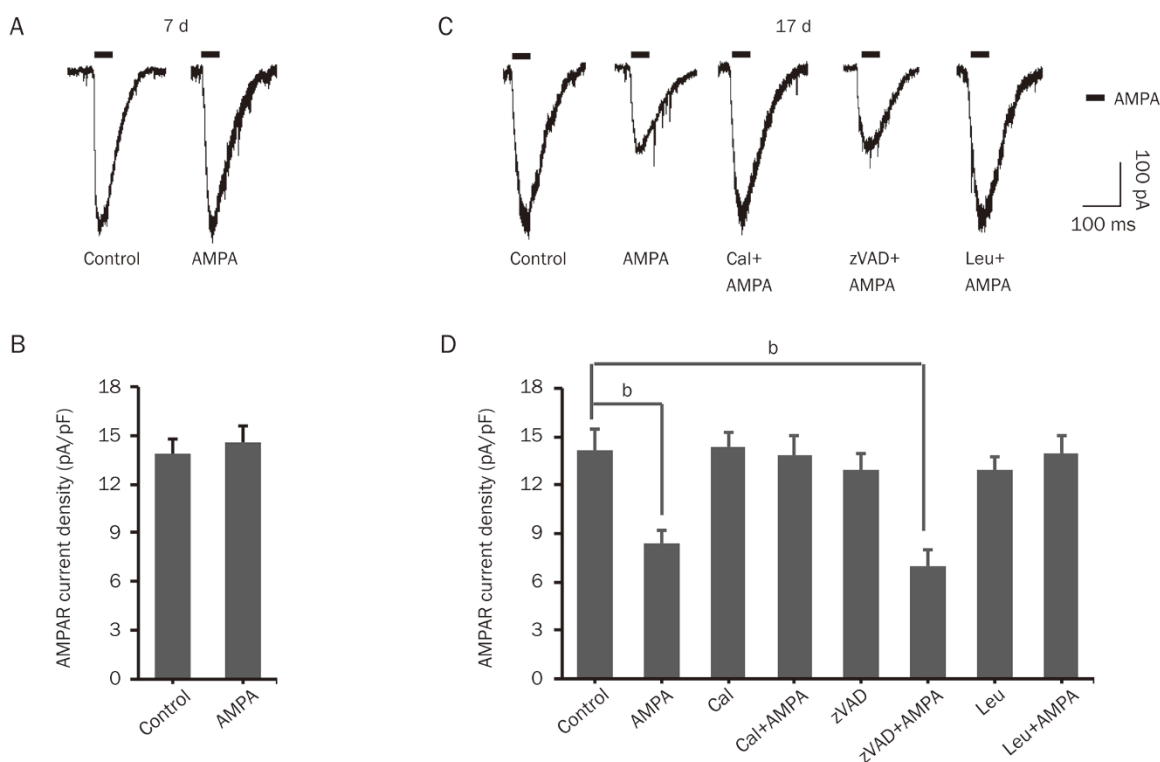


Figure 5. AMPA-induced changes in AMPA-induced inward current in prolonged culture of rat hippocampal neurons. (A) Typical whole-cell currents induced by AMPA ($100 \mu\text{mol/L}$, 50 ms) recorded in hippocampal neurons cultured for 7–8 DIV in the absence and presence of AMPA ($100 \mu\text{mol/L}$, 4 h). (B) Average current density in cultured hippocampal neurons following different treatments. (C) Typical whole cell currents induced by AMPA ($100 \mu\text{mol/L}$, 50 ms) recorded in hippocampal neurons cultured for 17–18 DIV in the absence and presence of AMPA ($100 \mu\text{mol/L}$, 4 h) following the administration of the calpain inhibitor calpeptin, the lysosome enzyme inhibitor leupeptin, or the caspase inhibitor zVAD (added simultaneously with AMPA treatment). The holding potential was -60 mV. (D) Average current density in cultured hippocampal neurons following different treatments ($n=12$ for all groups). The data are presented as the mean \pm SEM, ^b $P < 0.05$, one-way ANOVA.

in prolonged culture, there was no significant difference in TARP γ 8 expression in cultures between 7–8 DIV and 17–18 DIV. AMPA did not change the TARP γ 8 expression level in the cultures for 7–8 DIV, but significantly decreased it in the 17–18 DIV culture. Such a down-regulation of the TARP γ 8 expression level can be prevented by AMPAR or NMDAR antagonist, suggesting that both AMPAR and NMDAR activation were involved. NMDAR's involvement in increased calcium transience in TARP γ 8 over-expressing cells has been reported^[30].

AMPA-induced decreases of TARP γ 8 and GluA1 expression are calpain-dependent.

It is assumed that the AMPA-induced tonic activation of AMPAR and membrane depolarization cause NMDAR activation and Ca²⁺ influx, which may activate a series of calcium-dependent events, such as the activation of calpain and other proteolytic enzymes. Calpain inhibitor III blocks the decrease of stargazin under seizure conditions induced by kainic acid^[31], which demonstrates that calpain mediates TARP expression levels under pathological conditions. In our results, we found that the AMPA-induced TARP γ 8 decrease can be blocked by a pan-calpain inhibitor. One explanation is that the activation of AMPAR and NMDAR may increase cytoplasmic Ca²⁺ concentrations, resulting in the activation of calpain and the subsequent cleavage of TARP γ 8. The calpain-mediated down-regulation of TARP γ 8 in our results was supported by aging-related increases in calpain activity^[32, 33].

Activity-dependent down-regulation of GluA1 observed in this study was in agreement with the results that prolonged glutamate or NMDA treatment reduced the level of GluA1, but not GluA2, subunits in a calpain-dependent manner in cortical cultures^[18, 34]. Previous studies have shown that glutamate or kainate treatment decreased the GluA1 expression level, which can be blocked by calpain inhibitor^[18, 24]. These results indicate that the Ca²⁺-activated protease calpain was involved in GluA1 cleavage. Interestingly, other proteolytic enzymes, such as caspase, cleave GluA1 but not GluA2 in cortical culture^[27, 35], indicating that the activation of these proteolytic enzymes modulates AMPAR function through cleavage of GluA1, but not GluA2.

Previous studies have demonstrated that certain caspases, such as caspase-3, an apoptotic enzyme, cleave GluA1 under apoptotic stimulation^[27, 35]. Our results show that caspase inhibitor did not prevent the AMPA-induced down-regulation of TARP γ 8 and GluA1, suggesting that the activity-dependent conditions do not cause caspase activation, even in prolonged culture (Lu and Mattson, unpublished data). We also found that the lysosome-mediated proteolysis inhibitor leupeptin can block the AMPA-induced down-regulation of TARP γ 8 and GluA1, and this may be related to its non-specific inhibitory role in many other proteolytic enzymes, including calpain^[36]. Thus, the AMPA-induced down-regulation of TARP γ 8 and GluA1 occurs mainly through calpain activation.

AMPA-induced down-regulation of AMPA-induced currents

The whole-cell patch clamp recording in hippocampal neurons

demonstrated that the down-regulation of AMPA-induced current under activity-dependent conditions was blocked by calpain and lysosome enzyme inhibitors, suggesting that there was an activity-dependent functional change in AMPAR under the same conditions as caused the down-regulation of TARP γ 8 and GluA1. Calpain-mediated suppression of AMPAR-mediated currents in cortical pyramidal neurons was also observed in the conditions of prolonged glutamate or NMDA treatment after ischemic insult^[18].

Given the close association between TARPs and AMPAR subunits^[9, 37], the calpain-mediated decrease of TARP γ 8 may contribute to the altered AMPAR function^[10]. Rouach *et al*^[10] found that both GluA1 and GluA2/3 were reduced in TARP γ 8 knockout mice, which was different from the results of this study. We found that the AMPA-induced down-regulation of GluA1 and TARP γ 8, but not GluA2/3, likely occurs through a mechanism of calpain activation in the presence of AMPA. Our results were similar to the reported selective down-regulation of GluA1 but not GluA2/3 under trophic factor withdrawal^[35, 38]. Because the activity-dependent down-regulation of the GluA1 level occurs, it is difficult to define the contribution of the down-regulated TARP γ 8 to the simultaneous down-regulation of GluA1, but not GluA2/3.

Limited studies have adopted prolonged neuronal culture as an aging model *in vitro*^[39, 40]. In this study, we observed the decreased expression of TARP in the prolonged culture of hippocampal neurons, which was consistent with the results found in aged hippocampal tissue *in vivo*. The decreased expression of GluA1 *in vitro* did not agree with the results found in the older human brain. Thus, the *in vitro* model of aging cannot fully mimic the normal aging process. However, the results observed in this study may provide some important clues to better understand the mechanism of normal aging and neurodegeneration and better strategies for preventing and treating aging-related diseases, such as Alzheimer's disease and stroke^[24, 41, 42].

In conclusion, this study demonstrates the activity-dependent down-regulation of TARP γ 8 and GluA1 in prolonged neuronal culture, emphasizing a role of calpain. The down-regulation of TARP γ 8 likely contributed to the impaired AMPAR trafficking and thus impaired fast neuronal transmission, regardless of the changes in AMPAR expression.

Acknowledgements

We sincerely appreciate the constructive discussion from Dr Xiang-ru XU, Department of Anesthesiology, Yale University School of Medicine.

This study was supported by the National Natural Science Foundation of China (NSFC, grant No 31070938 and 81271422), International Collaboration Program of Henan Province Science-Technique Bureau (134300510040), Natural Science Foundation of Hebei Province (H2012203067) and Key Program for Applied Basic Research of Hebei Province (12966119D), Scientific Research Fund of Xinxiang Medical University (2013QN101 and 2013QN114). The authors declare no conflicts of interest.

Author contribution

Jian-gang WANG performed the experiments, analyzed the data and wrote the paper; Ya-li WANG performed the experiments and analyzed the data; Fang XU and Jing-xi ZHAO performed the experiments; Si-yuan ZHOU, Yi YU and Xiao-fang WANG analyzed the data; Paul L CHAZOT designed the research; Cheng-biao LU designed the experiments, performed the experiments and wrote the paper.

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