Abnormal alterations in the Ca^{2+}/CaV1.2/calmodulin/caMKII signaling pathway in a tremor rat model and in cultured hippocampal neurons exposed to Mg^{2+}-free solution

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Abstract. Voltage-dependent calcium channels (VDCCs) are key elements in epileptogenesis. There are several binding-sites linked to calmodulin (CaM) and several potential CaM-dependent protein kinase II (CaMKII)-mediated phosphorylation sites in CaV1.2. The tremor rat model (TRM) exhibits absence-like seizures from 8 weeks of age. The present study was performed to detect changes in the Ca^{2+}/CaV1.2/CaM/CaMKII pathway in TRMs and in cultured hippocampal neurons exposed to Mg^{2+}-free solution. The expression levels of CaV1.2, CaM and phosphorylated CaMKII (p-CaMKII; Thr-286) in these two models were examined using immunofluorescence and western blotting. Compared with Wistar rats, the expression levels of CaV1.2 and CaM were increased, and the expression of p-CaMKII was decreased in the TRM hippocampus. However, the expression of the targeted proteins was reversed in the TRM temporal cortex. A significant increase in the expression of CaM and decrease in the expression of CaV1.2 were observed in the TRM cerebellum. In the cultured neuron model, p-CaMKII and CaV1.2 were markedly decreased. In addition, neurons exhibiting co-localized expression of CaV1.2 and CaM immunoreactivities were detected. Furthermore, intracellular calcium concentrations were increased in these two models. For the first time, o the best of our knowledge, the data of the present study suggested that abnormal alterations in the Ca^{2+}/CaV1.2/CaM/CaMKII pathway may be involved in epileptogenesis and in the phenotypes of TRMs and cultured hippocampal neurons exposed to Mg^{2+}-free solution.

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Introduction

Epilepsy is one of the most common neurological conditions and is a set of chronic brain diseases caused by abnormal discharge of neurons within a brief time-frame (1). Voltage-dependent calcium channels (VDCCs) have important effects on neurotransmitter release and membrane excitability (2,3). CaV1.2 is the most predominant VDCC located in certain dendrites of hippocampal and cortical neurons (4-6). Previous studies have demonstrated that abnormal expression of CaV1.2 is present in different epilepsy phenotypes (7-9). At early stages during and following pilocarpine-induced status epilepticus, significant changes of CaV1.2 have been found in different groups of hippocampal neurons (7). However, no changes were observed in the protein expression of CaV1.2 in inferior colliculus neurons of genetically epilepsy-prone rats, compared with control Sprague-Dawley rats (3). There are at least four binding-sites linked to calmodulin (CaM) and several potential CaM-dependent protein kinase II (CaMKII)-mediated phosphorylation sites in CaV1.2 (8-11). Binding of Ca^{2+} produces a conformational change in CaM, exposing hydrophobic residues that promote interactions of the Ca^{2+}/CaM complex with CaV1.2 (12). The Ca^{2+}/CaM complex can enhance the affinity of CaM and the activity of CaV1.2 (13-16). Thus, CaM is important as a Ca^{2+} sensor for Ca^{2+}-dependent facilitation and inactivation (17,18). CaMKII is a multifunctional serine/threonine kinase, which can mediate several Ca^{2+}-dependent neuronal processes, and it accounts for 0.5-1.0% of total brain protein and up to 2% of hippocampal protein (19,20). CaMKII is activated by auto-phosphorylation when it is combined with the Ca^{2+}/CaM complex. Additionally, phosphorylated-CaMKII (p-CaMKII) exhibits its biological activity by the phosphorylation of other target proteins (21,22). With the involvement of CaMKII, the activity of CaV1.2 is promoted by CaM (23,24). A previous

study demonstrated immediate inhibition of cortical and hippocampal CaM kinase II activity in homogenate following the development of status epilepticus in a rat pilocarpine model (25). However, the roles of CaV1.2, CaM and CaMKII, and their interactions in epilepsy are controversial and remain to be fully elucidated. The tremor rat model (TRM; tm/tm) is an animal model of epilepsy, which is a genetic mutant first discovered in a Kyoto-Wistar colony (26). TRM is regarded as a useful model of epilepsy due to its similarity in pathogenesis and treatment to the human condition (27). Hippocampal neurons exposed to Mg^{2+}-free solution are well suited to biochemical and electrophysiological investigations to elucidate the cellular mechanisms, which lead to epileptogenesis (28), as the spontaneous epileptiform discharges produced by hippocampal neurons exposed to Mg^{2+}-free solution is similar to the electrical activity of epileptic seizures in humans. Thus, the aim of the present study was to investigate the alterations of the selected Ca^{2+}/CaV1.2/CaM/CaMKII pathway in the TRM and in cultured hippocampal neurons exposed to Mg^{2+}-free solution, *in vivo* and *in vitro*, respectively. These investigations aimed to obtain an insight into the pathways in producing neuronal excitability.

Materials and methods

Animals. The TRM is an animal model of epilepsy and the tm genetic mutant was identified in a Kyoto-Wistar colony (29). In the present study, 15 normal Wistar rats and 15 TRMs aged between 9 and 12 weeks were housed in individual cages under a controlled environment (12:12 h light/dark cycle, 50-70% humidity, 24˚C) with free access to food and water. The present study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (15,16). The animal use protocol was reviewed and approved by the Institutional Animal Care and Use Committee of China Medical University (Shenyang, China).

Electroencephalographic (EEG) determination and evaluation. The experimental animals (five animals per group) were anesthetized with 10% chloral hydrate (intraperitonal, 0.3 ml/100 g) and the animals were implanted with EEG electrodes (BL-420F; Taimeng Co., Ltd., Chengdu, China). Cortical and hippocampal electrodes were chronically implanted onto the cortex (3.0 mM lateral and 3.0 mM rostral to the bregma on the cranium) and in the left hippocampus (2.0 mM lateral and 4.0 mM caudal to the bregma and 3.0 mM from the cortical surface), respectively (30). Over the 7 days following this procedure, the EEG was recorded using a pen-writing polygraph. Following habituation of the rats in a soundproof box (40x40x40 cm) for >20 min, the EEG was recorded for 30 min. The rat was exposed to puff stimulation on the face by a researcher every 5 min to ensure the animal remained aroused during the recording period. Changes in the EEG during absence-like seizures consistently correlated with the abnormal seizure behavior, as described previously (29). When 5-7 Hz spike-wave-like complexes in the cortical and hippocampal EEG lasted for >1 sec, the response was considered as one absence-like seizure. When the time interval between two independent 5-7 Hz spike-wave-like complexes was <1 sec, the two events were considered as a single seizure.

Primary neuronal cell cultures. Hippocampal neuronal cells, which were obtained from rats born within 1 day of each other, were dissociated in Hanks' balanced salt solution containing 0.125% trypsin (Sigma-Aldrich, St. Louis, MO, USA) for 10 min at 37˚C. The cells were then plated onto dishes at $2x10^5$ cells/cm^2 in Dulbecco's modified Eagle's medium (Gibco-BRL, Invitrogen Life Technologies, Carlsbad, CA, USA) containing 15% fetal bovine serum and penicillin-streptomycin, (100 U/ml, Gibco-BRL). Following plating for 24 h, the medium was replaced with Neurobasal™ medium supplemented with 2% B27 (Gibco-BRL). The hippocampal neuronal cell media was refreshed every 3-4 days. Hippocampal neurons at the 12th day of *in vitro* culture were used in the following experiments.

Establishment of the hippocampal neuronal culture model. The *in vitro* hippocampal neuronal culture model was established according to Sombati's method (31). For primary neuronal cell cultures, maintenance medium was replaced with physiological recording solution for 3 h (Beyotime Institute of Biotechnology, Haimen, China) with or without $MgCl_2$ (1 mM). The solution contained 145 mM NaCl, 2.5 mM KCl, 10 mM HEPES, 2 mM $CaCl_2$, 10 mM glucose and 0.002 mM glycine (pH 7.3), the osmolarity of which was adjusted to 290±10 mOsm using sucrose. Continuous epileptiform high-frequency bursts were induced by exposing the neuronal cultures to physiological recording solution without $MgCl_2$ (Mg^{2+}-free) for 3 h, following which the culture was exposed to physiological recording solution. For double-labeling immunofluorescence and western blotting, the cultures were exposed to physiological solution for 8 h following treatment with Mg^{2+}-free solution for 3 h prior to use of the hippocampal neurons in these experiments. For measurement of intracellular calcium concentration, the cultures were exposed to Mg^{2+}-free solution of 3 h, following which the hippocampal neurons were used immediately.

Electrophysiological recordings. The electrophysiological recording method used in the present study was that used in our previous study (32). The extracellular bath contained 135 mM NaCl, 5.4 mM KCl, 1.0 mM $MgCl_2$, 0.33 mM NaH_2PO_4, 10 mM HEPES and 5.5 mM glucose (pH 7.4; NaOH). The pipette solution contained 50 mM K-Aspartate, 20 mM KCl, 20 mM HEPES, 1 mM EGTA, 1 mM $MgCl_2$, 0.2 mM $CaCl_2$, 13.6 mM NaCl and 3 mM K2ATP3 (pH 7.4; KOH; Beyotime Institute of Biotechnology). Patch-clamp electrodes were obtained with capillary tubes pulled with a P-97 puller (Gibco-BRL). The electrodes had a resistance of ~2.5-4 MΩ. Patch clamp was performed using an AxoPatch 200B amplifier (Axon Instruments, Foster City, CA, USA). To record action potential, cultures, including the normal cultured neurons and the neurons treated with Mg^{2+}-free solution, were transferred to the stage of an inverted microscope (TMS-1015, Olympus, Tokyo, Japan). Patch-clamp was performed utilizing the whole-cell current-clamp gap-free recording mode.

Western blot analysis. Proteins from the tissues and neurons were extracted by lysis in radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology) with ultrasonication, followed by centrifugation and retrieval of the supernatant. The concentrations of extracted proteins from the

tissues and neurons were determined using a bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology). The protein samples (50 μg per lane) were separated by 10% SDS-PAGE (Beyotime Institute of Biotechnology) and transferred onto polyvinylidene difluoride membranes (Motimo Membrane Technology Co., Ltd., Tianjin, China). The membranes were blocked for 1 h at room temperature with 5% bovine serum albumin (BSA) in tris-buffered saline with Tween 20 (TTBS; Beyotime Institute of Biotechnology) containing 50 mM Tris-HCl; 0.1% Tween-20 and 154 mM NaCl (pH 7.4), followed by 2 h incubation. The membranes were then incubated overnight at 4˚C in TTBS containing the following primary antibodies: Rabbit anti-CaV1.2 (1:500; cat no. ab-81095; Abcam, Cambridge, MA, USA), rabbit anti-CaMKII (1:500; cat no. sc-9035; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), rabbit anti-p-CaMKII (1:500; cat no. sc-3228; Santa Cruz Biotechnology, Inc.; the epitope corresponding to a short amino acid sequence containing phosphorylated Thr-286 of CaMKII α), mouse anti-CaM (1:800; cat no. sc-137079; Santa Cruz Biotechnology, Inc.) and β-actin (1:1,000; cat no. sc-47778; Santa Cruz Biotechnology, Inc.). Following several washes in TTBS, the membranes were incubated for 1 h at room temperature in horseradish peroxidase-conjugated goat anti-mouse IgG or horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5,000; cat nos. sc-2005 and sc-2054, respectively; Santa Cruz Biotechnology, Inc.) for 2 h at room temperature. Immunoreactive bands were visualized using an enhanced chemiluminescence kit. The levels of protein were evaluated by measuring the optical densities of the protein bands using Scion Image (4.03) for Windows image-analysis software. β-actin was used as a control to demonstrate that equal quantities of protein were loaded. Similarly, CaMKII was used as a control to demonstrate that equal quantities of p-CaMKII were loaded.

Double-labeling immunofluorescence. The TRMs and control rats were anesthetized with 10% chloral hydrate (i.p., 0.35 ml/100 g). Following intracardial perfusion with 4% paraformaldehyde, the brains of the rats were rapidly removed and placed in 4% paraformaldehyde solution for 24 h at room temperature and 30% sucrose solution for 24 h at 4˚C. The dehydrated-fixed brains were then frozen and 10-μm serial coronal sections were cut using a cryostat (CM1900 UV; Leica Microsystems GmbH, Wetzlar, Germany). These sections, and the Mg^{2+}-free treated and normal hippocampal neurons, were rinsed in phosphate-buffered saline (PBS; 0.01 M; pH 7.4) for 15 min and incubated in PBS (0.01 M; pH 7.4), which was supplemented with 0.25% Triton X-100 (Sigma-Aldrich) and 10% BSA (Gibco-BRL) for 1 h for blocking and penetration. The sections and neurons were then incubated overnight at 4˚C in a mixture of the following primary antibodies: Mouse anti-CaM (1:100; Santa Cruz Biotechnology, Inc.) and rabbit anti-CaV1.2 (1:80; Abcam) or mouse anti-CaM (1:100; Santa Cruz Biotechnology, Inc.) and rabbit anti p-CaMKII (1:100; Santa Cruz Biotechnology, Inc.). PBS without primary antibodies was used as a negative control. Following rinsing with 0.01 M PBS three times, for 5 min each, the sections and neurons were incubated with fluorescein isothiocyanate (FITC)- and Cy3-conjugated goat anti-mouse or anti-rabbit antibodies (1:200; ZSGB-BIO, Beijing, China) for 2 h at room temperature in the dark. Finally, sections from different brain regions, including the hippocampus (CA1, CA3 and DG), temporal cortex and cerebellum, were examined. The sections and neurons were examined using a Confocal laser scanning biological microscope (Fluoview FV500; Olympus Corporation, Tokyo, Japan). CaV1.2 and p-CaMKII were labeled with FITC-emitting green light, CaM was labeled with tetramethylrhodamine-emitting red light, with yellow light representing the co-localization of CaV1.2 and CaM or p-CaMKII and CaM. The co-localized cells that were clearly yellow were selected as positive cells, and the number of positive cells in every 100 cells was determined.

Acute dissociation of neurons. Acute dissociation of neurons was performed using a previous method with certain modifications (33). The experimental animals were anesthetized with 10% chloral hydrate (intraperitoneal, 0.3 ml/100 g) and then quickly sacrificed by decapitation. The brains were rapidly removed and placed in Hank's balanced salt solution (Gibco Life Technologies). The tissues (hippocampus, temporal cortex and cerebellum) were separated and cut into 400 μm-thick slices. These slices were incubated in artificial cerebrospinal fluid (Beyotime Institute of Biotechnology) containing 126 mM NaCl, 5 mM KCL, 2 mM $CaCl_2$, 25 mM $NaHCO_3$, 1.5 mM NaH_2PO_4, 2 mM $MgSO_4$ and 10 mM glucose (32˚C; 5% CO_2; pH adjusted to 7.4 with NaOH) for at least 1 h. The tissue slices were dissociated using 0.125% trypsin (Sigma-Aldrich) for 30 min at 37˚C, and the dissociated tissues were then rinsed three times in extracellular fluid containing 130 mM NaCl; 5.4 mM KCl; 1 mM $MgCl_2$; 1 mM $CaCl_2$; 10 mM HEPES and 25 mM glucose (32˚C; 5% CO_2; pH adjusted to 7.4 with NaOH), and triturated mechanically with a graded series of fire-polished Pasteur pipettes of ~500, 300 and 150 μm (Leiqi Experiment Equipment Co., Ltd., Hangzhou, China, successively. Following standing for 2 min, the upper cells in the cell suspension were plated onto culture dishes ($1x10^6$ cells/ml) in extracellular fluid. The acute isolated neurons were used within 6 h. All experimental procedures were maintained in a humidified atmosphere under 5% CO_2.

Measurement of intracellular calcium concentration ($[Ca^{2+}]i$). The measurement of $[Ca^{2+}]$ I was performed, according to that described in our previous study (34). The acutely dissociated neurons and Mg^{2+}-free treated hippocampal neurons were incubated for 20 min with 5 μm fluo-3 acetoxymethylester (fluo-3/AM; Molecular Probes Life Technologies, Carlsbad, CA, USA; 32˚C; 5% CO_2; pH adjusted to 7.4 with NaOH). The neurons of the control group and model group were plated into two cell culture cover glasses in the same dish. Thus, the control and model neurons were treated using the same procedure. The labeled neurons were then rinsed three times with PBS and cultured for 30 min to exclude non-specific staining of the extracellular fluid (32˚C; 5% CO_2). The preparations were observed and quantitatively analyzed using a confocal laser scanning biological microscope (Fluoview FV500; Olympus, Corporation). The visual field was selected where at least five neurons contained fluo-3/AM, which was excited by the 488 nm line of a 200 mW argon ion laser and captured at wavelengths >505 nm. The data were expressed as relative fluorescence intensities. Confocal imaging was performed on three separate fields of cells for each group. The concentration

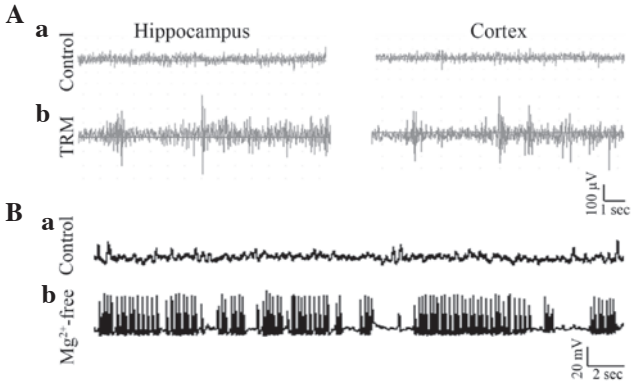

Figure 1. Electroencephalogram recording of the TRM and induction of 'epileptiform discharge' in the cultured hippocampal neurons by Mg^{2+}-free treatment. (A) In the TRM, (a) normal discharge was detected in the control rat hippocampus and cerebral cortex. (b) a 6 Hz spike and wave complex was detected during absence-like seizure in the TRM hippocampus and cerebral cortex. (B) In the neuron model, (a) occasional spontaneous action potentials were detected in the normal neuron; (b) spontaneous recurrent 'epileptiform discharge' was detected in a Mg^{2+}-free treated neuron. TRM, tremor rat model.

of intracellular Ca^{2+} was expressed as the relative fluorescent intensity using ImageJ 1.46 software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. Values are expressed as the mean ± standard deviation, and statistical analysis were performed using SPSS 13.0 statistical software (SPSS, Inc., Chicago, IL, USA). Statistical significance was determined using Student's *t*-test and one-way analysis of variance was used for comparisons. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Spontaneous discharge activity in the EEG recording in the TRM and Mg^{2+}-free treatment model. The EEG recording of The control rats and TRMs were detected in the hippocampus and cerebral cortex, respectively. It did not reveal any abnormal discharges in The control rat hippocampus or cerebral cortex (Fig. 1Aa), however, a 6 Hz spike and wave complex was recorded during absence-like seizure in the TRM hippocampus and cerebral cortex (Fig. 1Ab). In addition, the whole-cell current-clamp technique was used to record the electrical physiological activities of normal neurons (n=6) and Mg^{2+}-free treated neurons (n=6). The results demonstrated that representative neurons during 3 h exposure to physiological recording solution containing 1 mM $MgCl_2$ produced a normal baseline activity, presenting with occasional action potentials (Fig. 1Ba). However, exposure of the Mg^{2+}-free treated neurons, which were exposed for 3 h to Mg^{2+}-free recording solution, produced continuous high frequency epileptiform discharges (Fig. 1Bb). These data indicated that the *in vivo* and *in vitro* modelS of epileptic discharge in the present study had been established successfully.

Abnormal protein expression of CaV1.2, CaM, p-CaMKII and CaMKII in the TRM and Mg^{2+}-free treatment model. Western blot analysis was performed to quantify and compare selected the protein expression levels in the TRMs (n=6) and control rats (n=6). The western blot was probed with antibodies to CaV1.2, CaM, p-CaMKII and CaMKII,

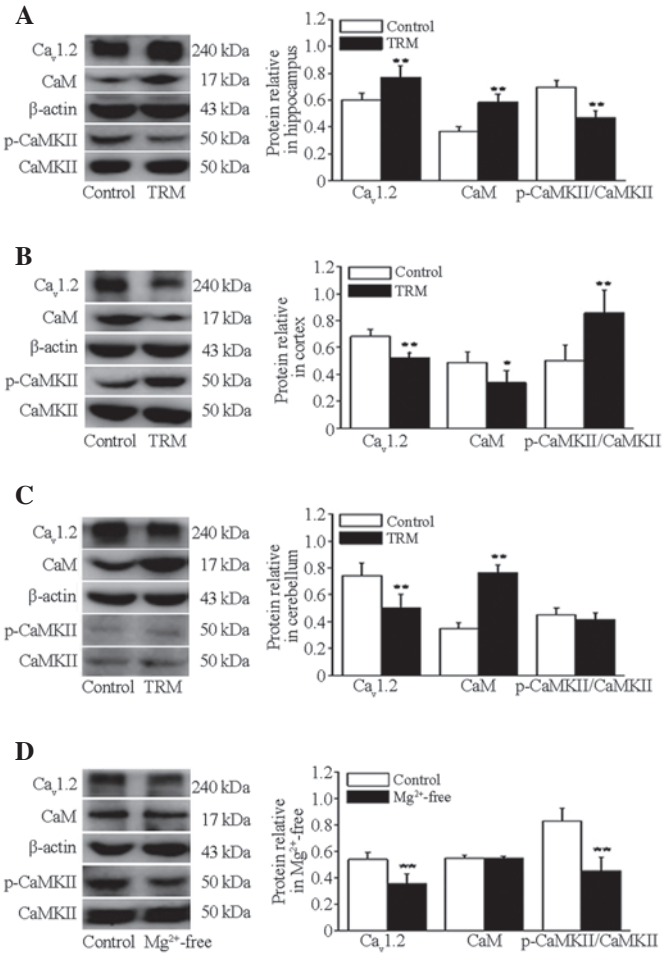

Figure 2. Protein expression levels of CaV1.2, CaM, p-CaMKII and CaMKII in the TRM and hippocampal cultured neurons exposed to Mg^{2+}-free by western blotting. (A) Immunoblots and quantitative analysis of the protein levels of CaV1.2, CaM, p-CaMKII and CaMKII in the TRM hippocampal and control groups (n=6). (B) Immunoblots and quantitative analysis of the protein levels of CaV1.2, CaM, p-CaMKII and CaMKII in the TRM cortex and control groups (n=6). (C) Immunoblots and quantitative analysis of the protein levels of CaV1.2, CaM, p-CaMKII and CaMKII in the TRM cerebellum and control groups (n=6). (D) Immunoblots and quantitative analysis of the protein levels of CaV1.2, CaM, p-CaMKII and CaMKII in the *in vitro* model (n=6) and control groups (n=6). $^{**}P<0.01$, vs. control group; $^{*}P<0.05$, vs. control group (analysis of variance followed by Student's t-test). Data are presented as the mean ± standard deviation. TRM, tremor rat model; CaM, calmodulin; CMKII, CaM-dependent protein kinase II; p-, phosphorylated.

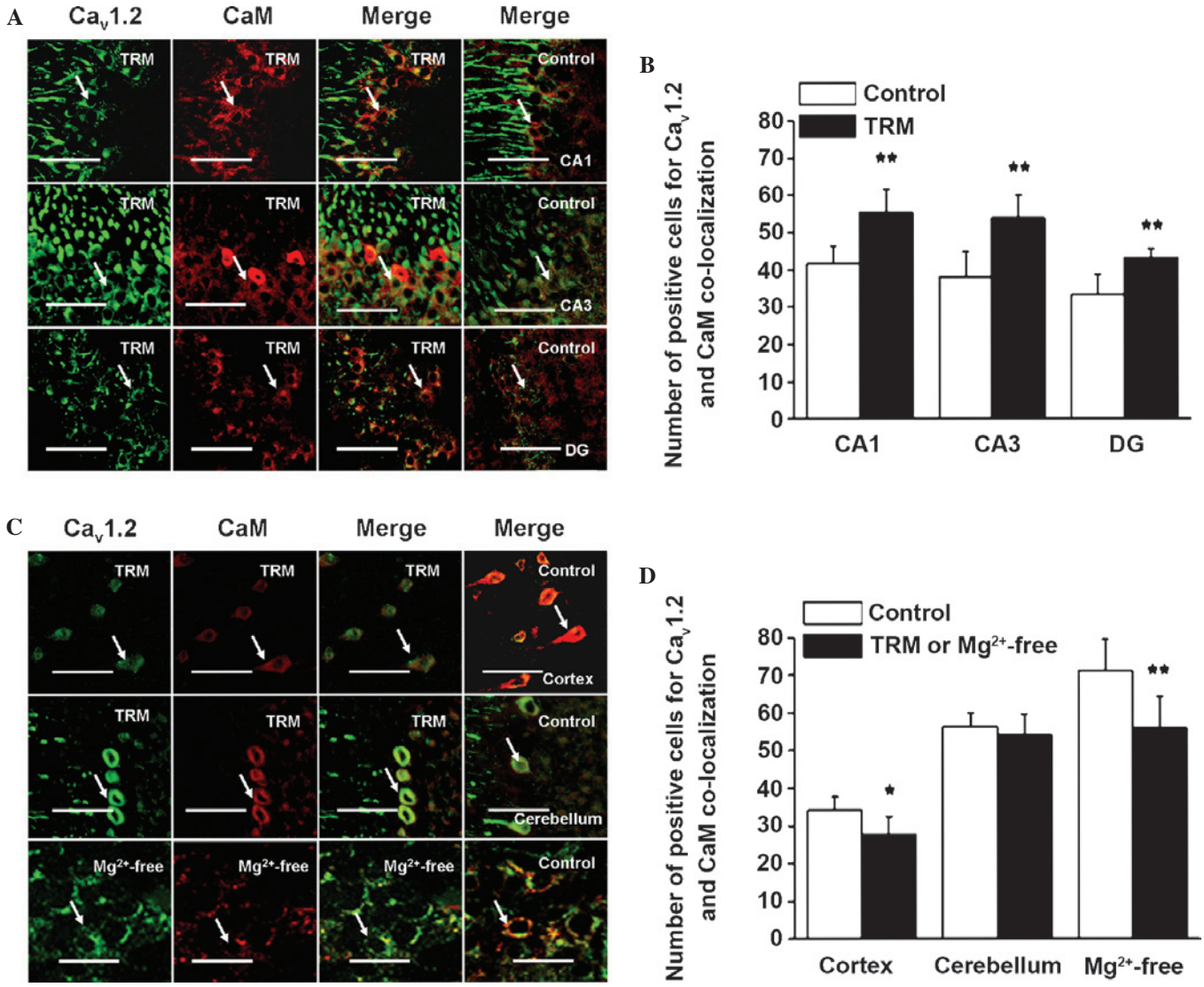

Figure 3. Co-localization of CaV1.2 and CaM in the TRM and *in vitro* model, detected using immunofluorescence. CaV1.2 was labeled with fluorescein isothiocyanate-emitting green light, CaM was labeled with tetramethylrhodamine-emitting red light, with yellow light indicating the co-localization of CaV1.2 and CaM. (A) TRM hippocampus CA1, CA3 and DG region. (B) Number of positive cells for CaV1.2-CaM in the TRM hippocampus. **P<0.01, vs. control group; *P<0.05, vs. control group (ANOVA followed by Student's t-test; n=5). (C) TRM cortex, cerebellum and Mg^{2+}-free hippocampal neurons. (D) Number of positive cells for CaV1.2-CaM in the TRM cortex, cerebellum and in Mg^{2+}-free hippocampal neurons. **P<0.01, vs. control group; *P<0.05, vs. control group (ANOVA followed by Student's t-test, n=5). Scale bar=50 μm. Data are presented as the mean ± standard deviation. TRM, tremor rat model; CaM, calmodulin; CMKII, CaM-dependent protein kinase II; ANOVA, analysis of variance.

and their respective anticipated bands at 240 kDa, 17 kDa and 50 kDa were detected, respectively. In the TRMs, hippocampal quantification analysis revealed that the levels of expression of CaV1.2 (P=0.0017) and CaM (P=0.0001) were increased significantly, while those of p-CaMKII (P=0.0001) were decreased significantly, compared with the control rats. No change was observed in the expression of CaMKII (P=0.08), compared with control groups (Fig. 2A). Notably, the situation was reversed in the protein expression patterns of the TRM temporal cortex. The expression levels of of CaV1.2 (P=0.0001) and CaM (P=0.0130) were significantly lower than those in the control group. Compared with the control rats, the expression of p-CaMKII (P=0.0017) was increased significantly. No change was detected in the expression of CaMKII (P=0.77), compared with the control group (Fig. 2B). In addition, these findings verified upregulation of the expression of CaM (P=0.0001) and downregulation in the expression of CaV1.2 (P=0.0019) at the protein level in the TRM cerebellum. No changes occurred in the protein expression of p-CaMKII (P=0.3700) or CaMKII (P=0.72), compared with the control group (Fig. 2C). In the hippocampal Mg^{2+}-free neuron epileptiform discharge model, no significant differences were observed in the expression of CaM (P=0.9799), compared with the control neurons, while p-CaMKII (P=0.0006) and CaV1.2 (P=0.0001) were decreased, compared with the control neurons. No change was observed in the protein expression of CaMKII (P=0.43), compared with the control group (Fig. 2D).

Co-localization of CaV1.2 with CaM, and p-CaMKII with CaM in the TRM and Mg^{2+}-free treatment model. Immunofluorescence analyses were performed in the two model (n=5) and control group (n=5) using anti-CaV1.2, anti-CaM and anti-p-CaMKII to detect the distributions and expression levels. As shown in Fig. 3A, in the TRM

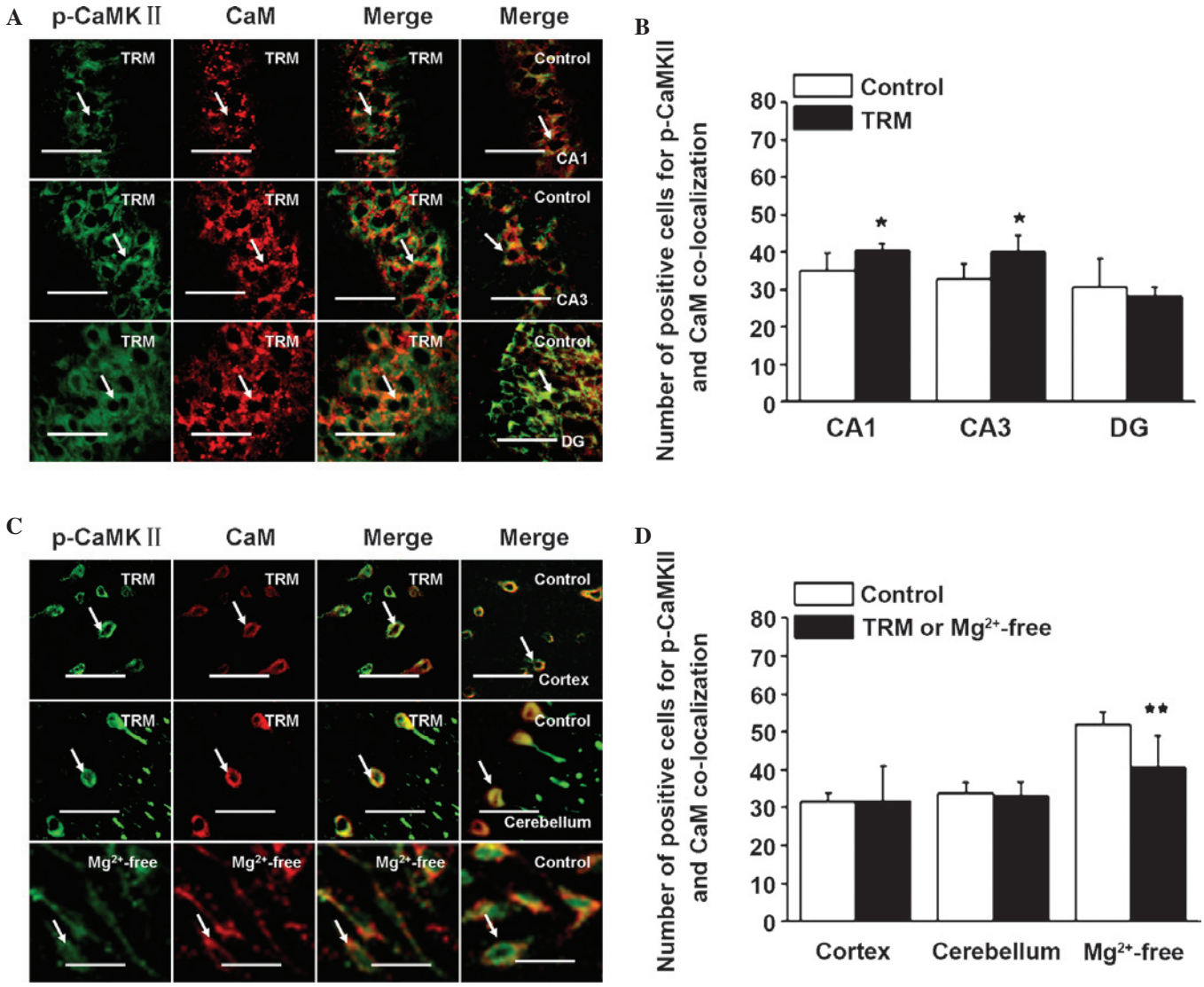

Figure 4. Co-localization of p-CaMKII and CaM in the TRM and *in vitro* model, detected using immunofluorescence. The p-CaMKII was labeled with fluorescein isothiocyanate-emitting green light and CaM was labeled with tetramethylrhodamine-emitting red light, with yellow light indicating the co-localization of p-CaMKII and CaM. (A) TRM hippocampus CA1, CA3 and DG regions. (B) Number of positive cells for p-CaMKII-CaM in the TRM hippocampus. **P<0.01, vs. control group; *P<0.05, vs. control group (ANOVA followed by Student's t-test; n=5). (C) TRM cortex, cerebellum and Mg^{2+}-free hippocampal neurons. (D) Number of positive cells for p-CaMKII-CaM in the TRM cortex, cerebellum and in Mg^{2+}-free hippocampal neurons. **P<0.01, vs. control group; *P<0.05, vs. control group (ANOVA followed by Student's *t*-test; n=5). Scale bar=50 μm. Data are presented as the mean ± standard deviation. TRM, tremor rat model; CaM, calmodulin; CMKII, CaM-dependent protein kinase II; p-, phosphorylated.

hippocampus, compared to control group, the CaV1.2 and CaM immunopositive neurons were stained markedly in CA1 and CA3 areas, while in DG areas, they were not expressed as highly as they were in the CA1 or CA3 areas. CaV1.2 was concentrated in the soma and proximal dendrites of the pyramidal neurons (green), and CaM was primarily localized to the cytoplasm (red). It was of interest to note that CaV1.2 co-localized with CaM. Overlapping localization of these proteins (yellow) were evident in the CA1 (P=0.0044), CA3 (P=0.0050) and DG (P=0.0060) areas when the images of their individual staining patterns were merged (Fig. 3B). As indicated in Fig. 3C and D, in the TRM cortex, the number of co-localization cells for CaV1.2-CaM was decreased, compared with that of the normal rats (P=0.0417). In the cerebellum, the number of co-localization cells for CaV1.2-CaM was not changed, compared with the controls (P=0.4771). In the Mg^{2+}-free treated neuron model, CaV1.2 was localized to the cell membrane, while CaM was localized to the cell membrane and the cytoplasm. The number of co-localization cells for CaV1.2-CaM in the Mg^{2+}-free treated model was decreased compared with normal neurons (P=0.0075). In addition, p-CaMKII immunopositive neurons (green) were localized in the cytoplasm of cells in hippocampus. Compared with the controls, the numbers of co-localization cells of CaM-p-CaMKII in the CA1 (P=0.0480) and CA3 (P=0.0289) of the TRM hippocampus were increased, respectively, however, there was no change in the DG (P=0.5675, Fig. 4A and B). It was clear that the distribution pattern of p-CaMKII was widely localized in the cytoplasm of cortical cells, which was similar to that observed in the hippocampus (Fig. 4C). There was no significant change of overlapping localization of p-CaMKII and CaM in the temporal cortex (P=0.9631; Fig. 4C and D). In the cerebellum of the TRM, the number of CaM-p-CaMKII-positive cells was also

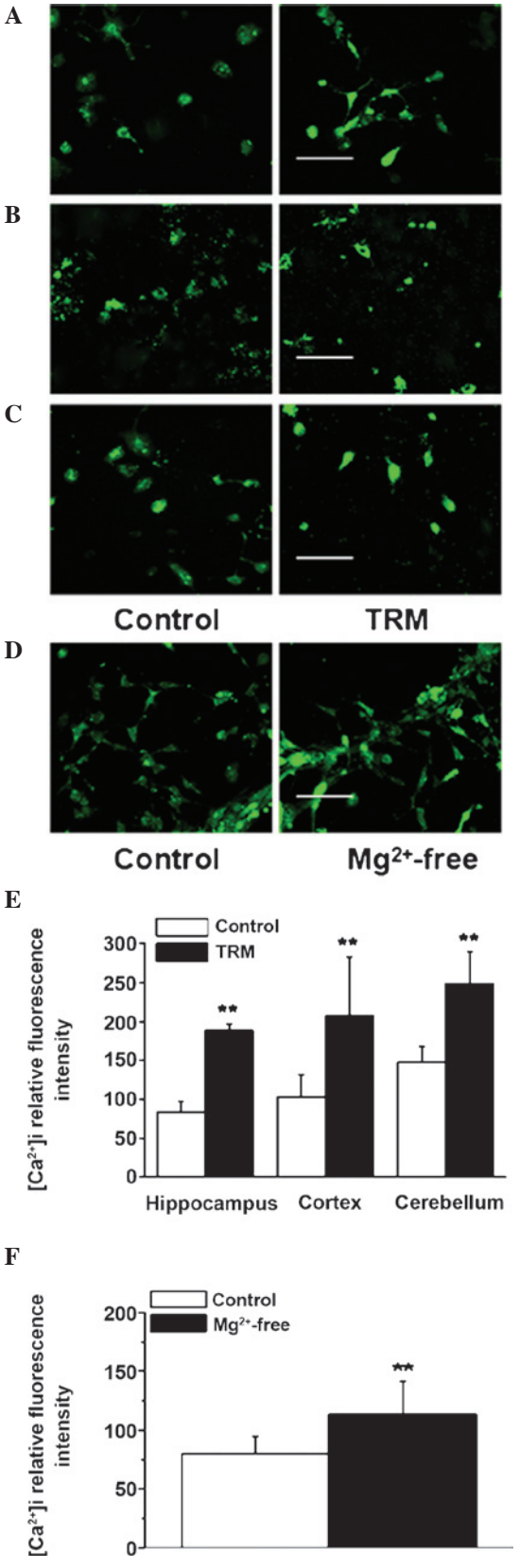

Figure 5. $[Ca^{2+}]_i$ relative fluorescence intensity in the TRM and the cultured hippocampal neurons exposed to Mg^{2+}-free. (A) $[Ca^{2+}]_i$ relative fluorescence intensity in the TRM hippocampus. (B) $[Ca^{2+}]_i$ relative fluorescence intensity in the TRM cortex. (C) $[Ca^{2+}]_i$ relative fluorescence intensity in the TRM cerebellum. (D) $[Ca^{2+}]_i$ relative fluorescence intensity in the Mg^{2+}-free hippocampal neurons. (E) Quantification of the relative fluorescence intensities in the TRM, compared with the control and (F) Mg^{2+}-free hippocampal neurons, compared with the control. $^{**}P<0.01$, vs. control (analysis of variance followed by Student's t-test; n=5). Scale bar=50 μm. Data are presented as the mean ± standard deviation. TRM, tremor rat model.

unchanged (P=0.7792; Fig. 4C and D). However, in the *in vitro* model, there was a decrease in the number of p-CaMKII and CaM co-localization neurons, compared with the controls (P=0.0059; Fig. 4C and D).

Measurement of intracellular calcium concentration in the TRM and Mg^{2+}-free treatment model. Compared with the controls (n=5), the $[Ca^{2+}]$ I relative fluorescence intensity of the model groups (n=5), including the TRMs (hippocampus, cortex and cerebellum; Fig. 5A-C) and Mg^{2+}-free hippocampal neurons (Fig. 5D) were increased, as shown in Fig. 5E and F.

Discussion

The predominant aims of the present study were to examine the changes in Ca^{2+}/CaV1.2/CaM/CaMKII in the TRM and in the hippocampal Mg^{2+}-free neuron epileptiform discharge models; and to demonstrate the possible correlation between CaV1.2 and the CaM/CaMKII pathway in these models. Epileptic models are fundamental to the investigation of the pathogenesis and possible treatments for epilepsy. TRMs were used in the present study as they are similar to the relevant human disease (27). TRM is an ideal model for the investigation of absence-like seizures, and seizures linked with the tm mutant, which was mapped to rat chromosome 10 (35). Furthermore, the paroxysmal occurrence of a 5-7 Hz spike-wave complexes can be recorded in the hippocampal and cortical areas after 8 weeks (29). In addition, the hippocampal Mg^{2+}-free neuron epileptiform discharge model is a common model of epileptiform discharge *in vitro* (36).

In the present study, Mg^{2+}-free treated hippocampal neurons produced continuous high frequency epileptiform discharges, which corresponded to observations in our previous study using whole-cell current-clamp (32). In the present study, the $[Ca^{2+}]$ I fluorescence intensity was increased in the TRMs (hippocampus, temporal cortex and cerebellum) and in the *in vitro* model, indicating that Ca^{2+} mayt be a key element in the epileptogenesis of these two models. $[Ca^{2+}]$ I led to the depolarization of cells, activating the inflow of Na+ and further enhancing epilepsy (37,38). Previous studies demonstrated that status epilepticus causes sustained elevation of intracellular calcium levels in the hippocampal neuronal culture model (39,40). In addition, calcium influx is enhanced in hippocampal CA3 neurons of spontaneously epileptic rats (41), which is in agreement with the results of the present study. A prominent finding in the present study was that the protein expression of CaV1.2 was increased significantly in the TRM hippocampus. However, the protein expression levels of CaV1.2 were decreased in the cortex, cerebellum and in *in vitro* culture. Previous studies have demonstrated that VDCC is essential in neuronal excitability (42). Transient and selective upregulation of CaV3.2 subunits on the mRNA and protein levels following status epilepticus causes an increase in cellular T-type Ca^{2+} currents and a transitional increase in intrinsic burst firing (43). Enhancements of Ca^{2+} influx into hippocampal CA3 neurons, due to the easier activation properties of VDCCs, are involved in SER epileptic seizures (44). In addition, VDCC currents are enhanced in the hippocampus

of patients with temporal lobe epilepsy (45). Another study reported that the expression of CaV1.2 was significantly reduced in Stargazer mouse cerebellar synapses, compared with their non-ataxic littermates, however, no differences were detected in hippocampal synapses (5). Accordingly, these findings have indicated the importance of CaV1.2 in the epileptic brain. The increased expression of CaV1.2 in TRM may result in an increase in the number of Ca^{2+} channels, following which Ca^{2+} current may be elevated, and a long-lasting depolarization shift accompanied by repetitive firing may be induced, which may contribute to the enhanced neuronal excitability in epilepsy (41). Thus, the present study revealed that the upregulated expression of CaV1.2 in the hippocampus of TRM may be involved in the generation of epileptiform activity and underlie, at least in part, the observed seizure phenotype in TRM.

The present data also demonstrated the upregulation of co-localization of CaM and CaV1.2 in the TRM hippocampus, and the combination of CaM and CaV1.2 may activate the Ca^{2+} channel and, ultimately, elevate the level of excitability in neurons. Notably, the distribution and expression of CaM and CaV1.2 in the cortex was opposite to that observed in the hippocampus in the TRM, indicating a possible compensatory response aimed at counteracting hyperexcitability in the cortex of the TRM. Several previous studies have reported controversy in the change of CaMKII in different models of epilepsy. Selective suppression of CaMKII activity results in alterations in Ca^{2+} homeostasis and the development of SREDs in hippocampal neurons (46). Additionally, the expression of CaMKIIα is decreased in pentylenetetrazol-kindled rats (47). Another study demonstrated that the expression of p-CaMKII is upregulated in dendritic spines during epileptiform activity *in vitro* (48). This may be due to factors including different epilepsy models and brain regions. The present study demonstrated that the expression of p-CaMKII in the hippocampus was downregulated. This may be due to Ca^{2+} being overloaded in epilepsy, which upregulated the expression of CaM, following which Ca^{2+}/CaM-dependent enzymes were adjusted and the activity of CaMKII was restrained. However, the expression of p-CaMKII in the cortex was upregulated, which disagreed with the expression in the hippocampus and may be a compensatory response in the cortex. Notably, the expression levels of p-CaMKII were particularly low in the cerebellum. Thus, sustained Ca^{2+} overload may have caused a sharp increase in CaMKII auto-phosphorylation, which resulted in the reduction in CaMKII and p-CaMKII. In the *in vitro* model, the expression levels of p-CaMKII and CaV1.2 were downregulated, while that of CaM remained unchanged. Similar to previous studies, the reason for this may be that SREDs inhibited the activity of CaMKII and restrained its substrate (23,24).

The limitation of the present study lies in the *in vivo* and *in vitro* models, which may represent different types of models. To a certain extent, the hippocampal neuronal culture model can be considered an acute seizures model, and the neurons treated with Mg^{2+}-free solution for 3 h may be not sufficient to fully undergo the molecular and cellular changes associated with the development of epileptogenesis. By contrast, TRM rats are genetic epileptic animals exhibiting spontaneous seizures, which can be considered a chronic model of epilepsy. Therefore, it is not surprising that the discrepancy was observed in the results of these two models. A noteworthy findings of the present study was that the CaV1.2-CaM and CaMKII-CaM complexes were widely co-localized in the TRM hippocampus, indicating that the association between these proteins may be involved in TRM seizures. The present study hypothesized that when [Ca^{2+}] I increases in TRM, Ca^{2+} and CaM are combined to form the Ca^{2+}/CaM complex, facilitating the affinity of CaM and CaV1.2. Additionally, CaMKII was activated by auto-phosphorylation when it combined with the Ca^{2+}/CaM complex. Furthermore, increased CaM can be co-localized to the membrane with CaV1.2, leading to the upregulation and increased activity of CaV1.2, which contribute to enhanced neuronal excitability and results in TRM seizures. Collectively, the present study demonstrated abnormal changes in the Ca^{2+}/CaV1.2/CaM/CaMKII signaling pathway in TRMs and in the hippocampal neuronal culture model. Altering the expression of CaV1.2, CaMKII and CaM may lead these to become potential targets for therapy in epilepsy or seizures. TRMs and the hippocampal neuronal culture model can be screened for effective specific VDCC subtypes for the treatment of epilepsy or seizures.

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