Critical role of TRPC6 channels in the formation of excitatory synapses

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Supplementary Figure 1. Determination of the specificity of the antibody against TRPC6. (a) Western blot analysis of the extracts from HEK293 transfected with TRPC1-HA, TRPC3-Myc or TRPC6-Myc constructs. The TRPC6 antibody can recognize overexpressed TRPC6, but not overexpressed TRPC1 and TRPC3. (b) Western blot analysis of the extracts from P14 rat hippocampus using the antibodies to TRPC1, 3 or 6. No bands were found in the corresponding sites where TRPC1 and 3 were found using TRPC6 antibody. Arrows indicated TRPC1, 3, 6 bands. (c) Western blot analysis of the extracts from P14 rat brain using the TRPC6 antibody or α-Tubulin in the presence or absence of the antigenic peptide (Ag). (d) Representative images of SKOV3 cells transfected with the GFP-TRPC6 and stained with the TRPC6 antibody or antibody against GFP. Antigenic peptide blocked the staining of TRPC6 antibody (red).
Supplementary Figure 2. The effectiveness of the RNAi constructs against TRPC6 and TRPC1. Hippocampal neurons were transfected using the rat neuron Nucleofector Kit (Amaxa). (a) Seventy-two hours after transfection, the neurons were harvested and the cell lysates were western-blotted with specified antibodies. C6i-1 and C6i-2 could specifically knock down TRPC6, but did not affect TRPC1, 3, NR2B or GluR2/3. (b) Quantitative analysis of TRPC6 protein level normalized to α-Tubulin level in a. Data are mean ± S.E.M from five independent experiments. ** P < 0.001 versus scrambled. (c) Verification of RNAi resistant rat TRPC6. HEK293 were transfected with indicated constructs. Mutated rat TRPC6 (RR-TRPC6) (Supplementary methods) could resist C6i-1-induced reduction in TRPC6 protein level. (d) Western blot analysis showed the effectiveness of TRPC1 RNAi constructs (C1i-1 and C1i-2). C1i-1 and C1i-2 specifically knocked down TRPC1, but did not affect TRPC3, 6. (e) Quantitative analysis of TRPC1 protein level normalized to α-Tubulin level in d. Data are mean ± S.E.M from three independent experiments. ** P < 0.001 versus scrambled.
**Supplementary Figure 3.** Knocking-down TRPC6 did not affect peak amplitude, rise time and decay time for mEPSC. Quantification of the mEPSC peak amplitude (a), rise time and decay time (b) from the neurons transfected with scrambled RNAi, C6i-1 and C6i-1 plus RR-TRPC6 in Fig. 2h. n = 12–15.
Supplementary Figure 4. Over-expression of TRPC6 in cultured hippocampal neurons. The neurons were transfected using the rat neuron Nucleofector Kit. (a) Representative images of the neurons transfected at DIV 14 with the GFP construct. (b) Whole cell lysates of the neurons transfected with GFP or wild-type TRPC6 construct (WTC6) were western-blotted with the antibodies to TRPC6 or α-Tubulin.
Supplementary Figure 5. TRPC6 mediated the effect of BDNF on spine formation. Representative images of the neurons transfected with (a) Scrambled RNAi (Scrambled) or (b) C6i-1 and treated with BDNF (25 ng ml⁻¹) for 48 hours. (c) Quantitative analysis of the spine density showed in a,b. n = 40–45, ** P < 0.001. Data represent mean ± s.e.m. Scale bar in (a,b) small panels, 1 μm, in large panels, 10 μm.
Supplementary Figure 6. TRPC6 protein level in TG mice. (a) Quantitative analysis of the TRPC6 and CREB protein levels shown in Fig. 3c, n = 6. (b) Quantitative analysis of the indicated protein levels shown in Fig. 3d. The protein levels of PSD-95 and NR2B in total lysates and in PSD fractions from TG mice were unaltered compared to those from WT mice, n = 5. ** P < 0.001, * P < 0.01 versus WT.
Supplementary Figure 7. Representative images of Golgi-staining on 8-week-old mice. (a) Cortex and hippocampus. (b) The neurons from CA1, CA3 and dentate gyrus.
Supplementary Figure 8. Analysis of the results in Morris water maze. (a) Example traces of the path taken in the probe test of WT or TG mice. (b) Number of crossing the platform in probe test. (c) Quantitative analysis of cumulative distance in probe test of WT or TG mice. Data represent mean ± s.e.m., * P < 0.01 versus WT.
Supplementary Figure 9. Representative images of EM on P14 hippocampus stained with the antibodies against (a) TRPC6 or (b) normal rabbit IgG.
SUPPLEMENTARY METHODS

Production of TRPC6 transgenic mice.
Transgenic mice were generated using the CaMKIIα promoter to drive expression of mouse TRPC6 specifically in forebrain. The linearized DNA containing a genomic fragment including 8.5 kb upstream of CaMKIIα transcription starting site and coding region of mouse TRPC6 was injected into fertilized oocytes of C57BL6 × FBN background. Founders (F0) were crossed onto a C57BL6 background. Three independent founders were used in this study. Mice used in this study were F3 or above. Genotyping was determined by PCR. The 5' and 3' primers for the TRPC6 transgene were GTTCTCCGTTTGCACTCAGG and GCTGGGGTAGTAGCCATAAC, respectively.

Constructs.
The fragment of IRES (pIRES2-EGFP, BD Bioscience Clontech, CA) was inserted into the pCAGGS-GFP vector by EcoRI site to generate pCAGGS-IRES-GFP, which directs target gene expression by the CAGGS promoter and EGFP expression through IRES. The mouse open reading frames (ORFs) of TRPC6 were subcloned into the pCAGGS-IRES-GFP vector. The constructs were confirmed by sequencing and Western blot analysis (Supplementary Fig. 2). Two hairpin siRNA sequences,

TGCTGTTCACATGGACGCAGCTCTCCATATGGAATATGGTAGAAGCCA
CAGATGTAATTCTCATAACATATGGAAGAGGATGCCTACTGCTCCGA (from GeneChem Co., Ltd., Shanghai) against 1158-1178 bp and
TGCTGTTCACATGGACGCACGCTATTAAATCCTGGGATTAGTGAGCCAC
AGATGTAACCCAGGATTATAATGAGATGCCTACTGCTCCGA against 1485-1505 bp in the coding region of rat TRPC6 (NM_053559), were inserted into pPRIME (Potent RNA interference using microRNA expression) construct and named as TRPC6 RNAi-1(C6i-1) and TRPC6 RNAi-2 (C6i-2), respectively.

TGCTGTTCACATGGACGCAGCTCTCCATATGGAATATGGTAGAAGCCA
CAGATGTAATTCTCATAACATATGGAAGAGGATGCCTACTGCTCCGA against 1317-1327 bp and
TGCTGTTCACATGGACGCAGCTCTCCATATGGAATATGGTAGAAGCCA
CAGATGTAATTCTCATAACATATGGAAGAGGATGCCTACTGCTCCGA against 1487-1507 bp in the coding region of rat TRPC1 (NM_053558), were inserted into pPRIME construct and named as TRPC1 RNAi-1(C1i-1) and TRPC1 RNAi-2 (C1i-2), respectively.

In TRPC6 RNAi experiments, scrambled RNAi was designed corresponding to C6i-1.

TGCTGTTCACATGGACGCAGCTCTCCATATGGAATATGGTAGAAGCCA
CAGATGTAATTCTCATAACATATGGAAGAGGATGCCTACTGCTCCGA against 1487-1507 bp in the coding region of rat TRPC1 (NM_053558), were inserted into pPRIME construct and named as TRPC1 RNAi-1(C1i-1) and TRPC1 RNAi-2 (C1i-2), respectively.

In TRPC1 RNAi experiments, scrambled RNAi was designed corresponding to C1i-1.

TGCTGTTCACATGGACGCAGCTCTCCATATGGAATATGGTAGAAGCCA
CAGATGTAATTCTCATAACATATGGAAGAGGATGCCTACTGCTCCGA was inserted into pPRIME construct.

In TRPC1 RNAi experiments, scrambled RNAi was designed corresponding to C1i-1.

TGCTGTTCACATGGACGCAGCTCTCCATATGGAATATGGTAGAAGCCA
CAGATGTAATTCTCATAACATATGGAAGAGGATGCCTACTGCTCCGA was
inserted into pPRIME construct.

The RNAi-resistant rat TRPC6 was made by two point mutations (from ‘c ctc tcc ata tgg tat gag aa’ to ‘c ctc tcg atc tgg tat gag aa’), which did not change the amino-acid sequence.

**Chemical reagents and antibodies**

The Alexa Fluor 488 goat anti-rabbit or mouse IgG (H+L), Alexa Fluor 543 goat anti-rabbit or mouse IgG (H+L) and GFP polyclonal antibodies were purchased from Molecular Probes, antibodies against PSD-95, NR2B, GluR2/3, synaptophysin and synapsin1 from Chemicon, antibodies against α-tubulin, Myc, HA and TRPC3, 6 from Sigma, antibodies against CREB, phospho-CREB Ser133, CaMKIα and CaMKIV from Cell Signaling Technology, antibodies against phospho-CaMKIα Thr286, phospho-CaMKIV Thr196 from Santa Cruz Biotechnology and BDNF from R&D System. All other reagents were from Sangon, Shanghai.

**Primary cultures and transfection.**

The hippocampal neurons were obtained from embryonic 18–19 days old Sprague-Dawley rats. Briefly, the cells seeded onto 35 mm dishes (50,000 cells per dish) or coverslips (5,000 cells per coverslip) coated with 50 µg ml–1 poly-D-lysine were grown in Neurobasal plus B27 (Gibco, BRL) containing glutamine and penicillin/streptomycin. Twenty-four hours later, the cultures were treated with cytosine-β-D-arabinofuranoside (10 mM) to prevent non-neuronal cell proliferation. The neurons (21 DIV) were fixed for immunostaining in Fig 1d,e. The neurons (14 DIV) were transfected using the calcium phosphate method², fixed and stained in 21 DIV for analysis of spine density, clusters density of PSD-95 and Synapsin1.

**Preparation of synaptosome and post-synaptic density (PSD) fractions.**

Synaptosome and PSD fractions were prepared from rat hippocampus by sucrose gradient method as previously described³⁴. All purification steps were performed at 4°C. The hippocampus of P14 rats or 2-month old transgenic mice were rapidly isolated and homogenized in ice-cold Buffer A (in mM, 5 HEPES, pH 7.4, 1 MgCl₂, 0.5 CaCl₂, 1 NaF, 1 β-glycerophosphate, 0.1 phenylmethylsulfonyl fluoride, 1 benzamidine, 0.1 pepstatin, 1 µg ml⁻¹ aprotinin, 1 µg ml⁻¹ leupeptin, and phosphatase inhibitor mixture I from Sigma). The homogenized extract was spun at 1400 g for 10 min. The supernatant (S1) was saved and pellet (P1) was homogenized using Buffer A again. After centrifugation at 700 g, the supernatant (S1’) was saved and pooled with S1. Pooled S1 and S1’ was centrifuged at 13,800 g for 10 min to collect the pellet (P2). The P2 was resuspended in Buffer B (in mM, 320 sucrose, 6 Tris, pH 8.0, 1 NaF, 1 β-glycerophosphate, 0.1 phenylmethylsulfonyl fluoride, 1 µg ml⁻¹ of aprotinin, 1 µg ml⁻¹ of leupeptin, 1 benzamidine, and 0.1 pepstatin). The P2 suspension was then loaded onto a discontinuous sucrose gradient (0.85 M/1 M/1.2 M sucrose solution in 6 mM Tris, pH8.0), followed by centrifugation for 2 h at 82,500g in a SW-41 rotor. The fraction between 1 M and 1.2 M sucrose was collected and saved as the synaptosome
fraction. The synaptosome fraction was further adjusted to 4 ml with the Buffer B and mixed with equal volumes of Buffer C (6 mM Tris, pH 8.1, and 1% Triton X-100) for 15 min. The suspension was spun at 32,800 g in a Ti70.1 rotor for 20 min. The supernatant was discarded, and the resulting pellet was saved as the PSD fraction. The total lysates of hippocampus, synaptosome and PSD fractions were adjusted to a equal protein concentration (5 µg µl⁻¹) using Bio-Rad Protein Assay (Bio-Rad). Five independent experiments were performed.

**Western blot and immunocytochemistry.**
The cultured hippocampal neurons and P14 hippocampal tissues were extracted at 4°C in a lysis buffer (in mM, 10 Tris-Cl, pH 7.4, 150 NaCl, 5 EDTA, 1% Triton-X100, 1 sodium orthovanadate, 50 NaF, 1 PMSF, 1 aprotinin, 1 leupeptin and 5 DTT). The proteins were separated on SDS-6–12%PAGE, transferred to polyvinylidene difluoride (PVDF) membranes and probed with the indicated primary antibodies and secondary antibodies conjugated with horseradish peroxidase. The proteins were then visualized with the ECL system (Amersham). The bands were scanned and density of the bands determined by ImageQuant (Amersham). For immunostaining experiments, neurons were fixed for 20 min at 25°C with 4% paraformaldehyde and 4% sucrose in PBS. The fixed cells were permeabilized and blocked with 0.1% Triton X-100, 10% goat serum in PBS for 1 hr and then incubated overnight at 4°C in a humidified chamber with primary antibodies (TRPC6, 1:100, PSD-95, 1:100, GFP, 1:1000, Synapsin1, 1:100) followed with the corresponding secondary antibodies with 1:500 dilution.

**Image analysis and quantification.**
The images were obtained using a Zeiss LSM 510 confocal microscope using a 100 × objective with sequential acquisition settings at 1024 × 1024 pixel resolution. Images for the co-localization analysis were taken with the same exposure parameters. For dendritic spine assays, a z series projection of each neuron was made using approximately 8 to 12 sections (0.4 µm per section), each averaged four times. The resultant stack was merged into a single image using a maximum projection. Synapse density was quantified using Openlab software (Improvision, Lexington, MA). PSD-95 or Synapsin1 clusters greater than 4 pixels in size and localized to the transfected neuron were counted. We observed at least three dendritic segments totaling at least 150 µm of dendritic length per neuron, and the number of spines or clusters was counted and the density was calculated. 10 to 15 transfected neurons were chosen randomly for quantification per experiment, and three independent experiments were done for each construct or treatment. For Figure 1e, the yellow spots showing the co-localization for TRPC6 and PSD-95 were generated using the 'Multiply' function of Metamorph software (Universal Imaging). Percentage of TRPC6 co-localization with PSD-95 was measured as a yellow fluorescence spots number / red fluorescence spots number.
Immunogold electron microscopy.
Immunogold electron microscopy was performed by post-fixation immunogold labeling as described. Briefly, sections (20 µm) were embedded in Epon-Spurr resin. Thin sections (30-40 nm) were collected on nickel mesh grids. The antibodies against TRPC6 (1:100), GluR2/3 (1:200) or rabbit IgG (1:100) were first used. The secondary antibodies conjugated to 10 nm gold particles (Amersham) were then applied. Thirty to 35 immuno-positive excitatory synapses from 2 animals were examined and the numbers of gold particles were counted for each antibody.

Electrophysiological recording of mEPSCs
Whole-cell voltage clamp recordings from cultured hippocampal neurons were obtained using an Axopatch 200B amplifier at 25°C. Extracellular perfusion medium contained (in mM) 125 NaCl, 26 NaHCO3, 1.25 NaH2PO4, 2.5 KCl, 25 glucose, 1 MgCl2, 2 CaCl2, pH 7.4, with 0.001 tetrodotoxin and 0.02 bicuculline. The electrodes were backfilled with (in mM) 115 Cs methanesulfonate, 20 CsCl, 2.5 MgCl2, 4 MgATP, 0.4 Na3GTP, 10 Na phosphocreatine, 0.6 EGTA and 10 HEPES, pH 7.25. Recordings where Rs and Rin were changed by more than 25% were discarded. Miniature excitatory postsynaptic currents (mEPSCs) were recorded from hippocampal pyramidal cells clamped at −70 mV, events were filtered at 1 kHz, series resistance was left uncompensated, and data were analyzed offline using IGOR PRO software (Wavemetrics, OR). Membrane capacitance was measured by applying a 5 mV hyperpolarization step to the cell for 10 ms and calculating the area under the curve. Cumulative averages for each transfection condition were compared using ANOVA test. Twelve to 15 neurons from three independent experiments were recorded for each construct.

Luciferase assay.
Luciferase assay was performed as described previously. Cultured neurons were transfected with the indicated constructs together with the CRE-luciferase reporter gene in 14 DIV. Twenty-four hours after transfection, luciferase activities were determined using the Luciferase assay system (Promega). β-gal genes were co-transfected to normalize the transfection efficiency. Six independent experiments were done for each construct.

Golgi staining.
Golgi staining was performed using Rapid GolgiStain Kit (FD Neurotechnologies) following the manufacturer’s instruction. Briefly, brain from 10 to 12 weeks mice were immersed in the impregnation solution for 2 weeks and then cut into 100 µm sections using a vibratome. Sections were stained and dehydrated in graded ethanol, cleared in xylene and examined with Zeiss LSM 510 confocal microscope.

Morris water maze.
Hidden-platform water maze was performed as described previously and the animal protocol was approved by the institutional animal committee. Eight to 9 weeks old
wild-type mice or TRPC6 transgenic mice were used. A circle pool of 1.2 m in diameter was used and the platform was placed in the centre of any quadrant of the pool and submerged 1 cm underneath the water. The training session consisted of 9 days, four trials per day. The starting quadrant was randomly arranged for each animal, but each day had to contain a starting position from all four quadrants. The time the mice used to find the platform was recorded. During the probe test, the platform was removed and the mice were allowed to swim in the pool for 60 s. The time the mice spent in each quadrant and the number of platform crossing were recorded. Fourteen to 15 mice were tested per genotype. Both wild-type and TRPC6 transgenic mice showed equally swimming distance in probe test and normal acquisition of visible platform, which indicate both of them have normal swimming ability, visual acuity and motivation to escape the water.

Reference