TRPC6 channels promote dendritic growth via the CaMKIV-CREB pathway

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Summary
The canonical transient receptor potential channels (TRPCs) are Ca²⁺-permeable nonselective cation channels with various physiological functions. Here, we report that TRPC6, a member of the TRPC family, promotes hippocampal neuron dendritic growth. The peak expression of TRPC6 in rat hippocampus was between postnatal day 7 and 14, a period known to be important for maximal dendritic growth. Overexpression of TRPC6 increased phosphorylation of Ca²⁺/calmodulin-dependent kinase IV (CaMKIV) and cAMP-response-element binding protein (CREB) and promoted dendritic growth in hippocampal cultures. Downregulation of TRPC6 by short hairpin RNA interference against TRPC6 suppressed phosphorylation of both CaMKIV and CREB and impaired dendritic growth. Expressing a dominant-negative form of CaMKIV or CREB blocked the TRPC6-induced dendritic growth. Furthermore, inhibition of Ca²⁺ influx suppressed the TRPC6 effect on dendritic growth. Finally, in TRPC6 transgenic mice, the phosphorylation of CaMKIV and CREB was enhanced and the dendritic growth was also increased. In conclusion, TRPC6 promoted dendritic growth via the CaMKIV-CREB pathway. Our results thus revealed a novel role of TRPC6 during the development of the central nervous system (CNS).

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Key words: TRPC channels, Dendritic development, Ca²⁺/calmodulin-dependent kinase IV (CaMKIV), CREB

Introduction
The canonical transient receptor potential channels (TRPCs) are nonselective cation channels that are expressed in a variety of multicellular organisms with different functions (Montell et al., 2002). The TRPC family can be divided by homology and function into four subfamilies: TRPC1, TRPC2, TRPC4/5 and TRPC3/6/7 (Clapham, 2003). They can form homomeric or heteromeric channels with distinct properties, such as current rectification and Ca²⁺ permeability (Clapham, 2003). Some TRPC proteins are expressed in the CNS and function as important regulators during development (Jia et al., 2007; Li et al., 1999). TRPC1 is mainly localized to dendritic processes of dopaminergic neurons (Martorana et al., 2006), whereas TRPC6 is localized to proximal dendrites and the axon hillock (Giampa et al., 2007). Interestingly, TRPC5 is preferentially localized to the neuronal nuclei in the substantia nigra (De March et al., 2006), and TRPC3 is mainly found in oligodendrocytes (Fusco et al., 2004). TRPC1 participates in basic fibroblast growth factor (bFGF)-dependent neural stem cell proliferation (Fiorio Pla et al., 2005). TRPC3 and TRPC6 are involved in brain-derived neurotrophic factor (BDNF)-mediated growth cone turning, neuron survival and spine formation (Amaral and Pozzo-Miller, 2007; Jia et al., 2007; Li et al., 2005). TRPC5 inhibits neurite outgrowth in hippocampal cultures (Greka et al., 2003).

The establishment of dendritic morphology is critical for the development of neuronal circuits (Cline, 2001). Dendritic abnormalities are commonly associated with conditions resulting in mental retardation, such as Down syndrome (Becker et al., 1986) and Fragile X syndrome (Dierssen and Ramakers, 2006). A large amount of evidence demonstrates that Ca²⁺ and its signaling control the development of dendrites (Redmond and Ghosh, 2005; Redmond et al., 2002; Wu and Cline, 1998). Elevation in intracellular Ca²⁺ concentration can lead to changes in dendritic morphology. The Ca²⁺ influx through either voltage-sensitive Ca²⁺ channels (VSCCs) or N-methyl-D-aspartate glutamate receptors (NMDARs) is important for activity-dependent dendritic growth (Redmond et al., 2002; Sin et al., 2002). Moreover, transcription factors are involved in the Ca²⁺-mediated changes in dendrite morphology (Aizawa et al., 2004; Gaudilliere et al., 2004; Redmond et al., 2002), among which, cAMP-response-element binding protein (CREB) is regulated by TRPC3 or TRPC6 (Jia et al., 2007). Since TRPCs are Ca²⁺-permeable channels and their activation is less dependent on depolarization (Venkatachalam and Montell, 2007), we asked whether TRPC channels play a role in dendritic development. In the present study, we report that TRPC6 was highly expressed during the period of maximal dendritic growth and promoted dendritic growth through a CaMKIV-CREB-dependent pathway. TRPC6 transgenic mice showed upregulated phosphorylation of CaMKIV and CREB and more complicated dendrite structures. These findings revealed a novel role of TRPC6 during development of the CNS.

Results
Expression of TRPCs in rat hippocampus during development

To explore the possible roles of TRPC channels during dendritic development, we first examined the expression of TRPC proteins in the developing rat hippocampus by western blot. TRPC1 and TRPC3 were detectable at postnatal day 14 and 7, respectively (Fig. 1A), and their expression levels remained high into adulthood. By
contrast, the peak expression of TRPC4, TRPC5 and TRPC6 was between postnatal day 7 and 14. During this period, the maximal dendritic growth is found in the cortex (Miller and Peters, 1981). Since TRPC5 inhibits neurite outgrowth (Greka et al., 2003) and TRPC4, which belongs to the same subfamily of TRPC5 (Vazquez et al., 2004), also inhibited the dendritic growth in our experiment (supplementary material Fig. S1), we further examined the spatial distribution of TRPC6 in rat hippocampus. In situ hybridization with a digoxigenin-labeled probe against the N-terminus of TRPC6 mRNA revealed that TRPC6 was highly expressed in all regions of P14 rat hippocampus (Fig. 1B). Consistent with the mRNA result, the immunohistochemical study also showed that TRPC6 was expressed in all regions of hippocampus (Fig. 1C). Our dual-labeling studies showed that TRPC6 immunoreactivity was colocalized with MAP2 staining in proximal dendrites of CA1 pyramidal neurons (Fig. 1D). These results suggested that TRPC6 might affect dendritic growth during the development of the brain.

**TRPC6 promotes dendritic growth**

We next studied whether TRPC6 affected dendritic growth. The hippocampal cultures were transfected with a TRPC6 construct together with GFP at 5 days in vitro (DIV) and the neuronal morphology was observed after 2 days. The protein level of TRPC6 was similar in neurons transfected with TRPC6 antibody. As shown in Fig. 2A, B, the number of primary dendrites was similar in neurons transfected with TRPC6 (4.333±0.403, n=45) compared with that in neurons transfected with control vector (4.133±0.255, n=45). However, the total number of dendritic tips was increased by 45.1% in neurons transfected with TRPC6 (1122.869±87.631 μm, n=45, P<0.0001) compared with that in neurons transfected with control vector (773.778±55.129 μm, n=45). These results suggest that TRPC6 promoted dendritic growth.

This notion was further tested in loss-of-function experiments. We made two short hairpin RNAi (shRNAi) sequences against rat TRPC6: one was designed to target to both rat and human TRPC6 (shTRPC6i-1) and the other specifically to rat TRPC6 (shTRPC6i-2). Both constructs suppressed the expression of TRPC6 in the primary hippocampal cultures (Fig. 3A). In order to test the specificity of the RNAi constructs, we transfected HEK293 cells with these two constructs plus wild-type rat TRPC5 or wild-type human TRPC6. The constructs did not affect TRPC5 expression, and as expected, shTRPC6i-1 inhibited human TRPC6 expression, whereas shTRPC6i-2 did not. The hippocampal neurons at 3 DIV were then transfected with a control RNAi vector targeting firefly luciferase, shTRPC6i-1 or shTRPC6i-2, and neuronal morphology was examined 4 days after transfection. As shown in Fig. 3C, total dendritic tips in the neurons transfected with shTRPC6i-1 (17.955±1.024, n=44, P<0.0001) or shTRPC6i-2 (17.400±1.210, n=45, P<0.0001) were 37.4% or 39.3% less than those in the neurons transfected with control vector (28.679±1.556, n=45). Moreover, neurons transfected with these RNAi constructs showed a 37.2% or 38.1% drop in the total dendritic length (shTRPC6i-1, 500.471±20.895 μm, n=44, P<0.0001; shTRPC6i-2, 493.688±34.422 μm, n=45, P<0.0001) compared with those transfected with control vector (798.218±25.445 μm, n=45, P<0.0001). We then performed the rescue experiment to make sure that the change in phenotype was not due to the nonspecific effect of RNAi. As shown in Fig. 3B, cotransfection with wild-type human TRPC6 and shTRPC6i-2 eliminated the RNAi inhibitory effect on dendritic growth. However, introduction of human TRPC6 did not prevent shTRPC6i-1, which could also knock down human TRPC6, from inhibiting dendritic growth. Together, these results suggested that TRPC6 promoted dendrite growth.
TRPC6 promotes dendritic growth through CaMKIV

The CaMKs play a critical role in dendritic development (Fink et al., 2003; Redmond et al., 2002; Wayman et al., 2006; Wu and Cline, 1998). We therefore examined whether TRPC6 activates CaMKs to promote dendritic growth. Overexpression of TRPC6 increased phosphorylation of CaMKIV at Thr196, a site known to be critical for its kinase activity (Selbert et al., 1995), (1.592±0.323, n=45, P<0.01 versus control). Taken together, these results support the notion that the Ca2+ influx through TRPC6 channels is necessary for TRPC6-mediated dendritic growth.

Fig. 3. Knockdown of TRPC6 impairs dendritic growth. (A) Effectiveness and specificity of TRPC6-shRNAi. The two upper blots are total lysates of hippocampal cultures transfected with the shRNAi constructs targeting rat TRPC6 (shRNA6i-1 or shRNA6i-2) and control shRNAi construct targeting firefly luciferase (pPRIME-FF3) 96 hours after transfection using antibodies against TRPC6 and tubulin. The three lower blots show total lysates of HEK293 cells transfected with rat TRPC6 or human TRPC6 and shTRPC6i-1 (targeting both rat and human TRPC6) or shTRPC6i-2 (targeting only rat TRPC6) 48 hours after transfection detected using the indicated antibodies. (B) Representative images of the neurons transfected at 3 DIV for 4 days with control vector, shTRPC6i-2, shTRPC6i-1 plus human TRPC6 or shTRPC6i-2 plus human TRPC6. Scale bar: 20 μm. (C,D) Quantification of total dendritic tips (C) and total dendritic length (D) of the neurons shown in B. **P<0.01 versus control (ctrl) or shTRPC6i-2 (sh6i-2) plus hTRPC6.

The effect of TRPC6 on dendritic growth is dependent on Ca2+ influx

It is known that Ca2+ plays a critical role in regulating dendritic development (Redmond and Ghosh, 2005; Redmond et al., 2002; Wu and Cline, 1998). We thus investigated whether Ca2+ influx was necessary for TRPC6-mediated dendritic growth. Treatment with 2 mM EGTA, known to chelate extracellular Ca2+ and inhibit dendritic growth (Redmond et al., 2002), caused a 28.5% drop in total dendritic length and a 30.2% drop in total dendritic tips compared with vehicle treatment (Fig. 4A,B). Moreover, overexpression of TRPC6 in the presence of 2 mM EGTA failed to promote dendritic growth (total dendritic length, 612.312±33.049 μm, n=45, P=0.067 versus ETGA treatment), indicating that extracellular Ca2+ was necessary for TRPC6-induced dendritic growth. Then, we used Cd2+, known as a competitive inhibitor of Ca2+ channels (Swandulla and Armstrong, 1989), to block Ca2+ influx. As shown in Fig. 4A,B, treatment with 10 μM Cd2+ reduced total dendritic length by 39.2% and total dendritic tips by 32.9%. Moreover, the TRPC6-induced dendritic growth was abolished by Cd2+ (total dendritic length, 451.952±26.746 μm, n=45, P=0.889; total dendritic tips, 13.669±1.001, n=45, P=0.693 versus Cd2+ treatment). These results showed that Ca2+ influx is critical for TRPC6-mediated dendritic growth.

We used OAG, an agent known to stimulate Ca2+ influx through TRPC3, TRPC6 and TRPC7 (Hofmann et al., 1999; Okada et al., 1999; Venkatachalam et al., 2001), to examine whether Ca2+ influx through TRPC6 was important for TRPC6-induced dendritic growth. In response to OAG, the intracellular Ca2+ level was elevated 1.5-fold. This OAG-induced Ca2+ elevation was attenuated by downregulation of TRPC6 (Fig. 4C), indicating that TRPC6 was partially responsible for the OAG-induced Ca2+ elevation. Consistently, OAG treatment also led to a 26% increase in total dendritic tips and a 36.5% increase in total dendritic length compared with results obtained with DMSO treatment. These OAG-induced increases in dendritic length and branching were abolished by downregulation of TRPC6 (total dendritic tips, 18.272±0.707, n=45, P=0.4; total dendritic length, 689.048±27.473 μm, n=45, P=0.067 versus TRPC6 RNAi). Taken together, these results support the notion that the Ca2+ influx through TRPC6 channels is necessary for TRPC6-induced dendritic growth.

Fig. 4. The Ca2+ influx through TRPC6 is important for dendritic development. (A,B) Quantification of total dendritic tips and total dendritic length of hippocampal neurons transfected with the indicated constructs at 5 DIV for 2 days. Vehicle, 2 mM EGTA or 10 μM Cd2+ was applied for 24 hours before neurons were fixed. Data are the mean ± s.e.m. of 45 cells from three independent experiments. (C) Effect of downregulation of TRPC6 on OAG-induced [Ca2+]i elevation. Intracellular Ca2+ levels in the neurons transfected with pPRIME-FF3 or shTRPC6 RNAi were determined by the F340/F380 ratio. The relative change in [Ca2+]i was depicted by 340/380 ratio normalized to the baseline. Data are mean ± s.e.m. of 45 cells from three independent experiments. (D,E) Quantification of total dendritic tips (D) and total dendritic length (E) of the neurons transfected with the indicated constructs at 3 DIV for 4 days. DMSO or 100 mM OAG was applied for 24 hours before the neurons were fixed. **P<0.01 versus control.
downregulating TRPC6 inhibited phosphorylation of CaMKIV at Thr196 normalized to total CaMKIV (0.693±0.153, n=4, P=0.029). Changing the TRPC6 expression level did not affect the total CaMKIV protein level. Neither overexpression nor downregulation of TRPC6 affected CaMKIIα at Thr286 (supplementary material Fig. S3), a site known to be important for its activation (Colbran et al., 1989). These results indicated that activation of TRPC6 stimulated CaMKIV activity. Since activation of CaMKIV is required for dendritic growth (Redmond et al., 2002), we asked whether TRPC6 affected dendritic growth through activation of CaMKIV. To address this question, we transfected hippocampal cultures with wild-type TRPC6 and CaMKIV-T200A (DN-CaMKIV), which is a dominant-negative mutant of CaMKIV (Lemrow et al., 2004). Expression of DN-CaMKIV reduced total dendritic tips by 28.89% (16.733±1.322, n=45, P<0.0001 versus control) and total dendritic length by 40.9% (496.933±37.642, n=45, P<0.0001 versus control). As shown in Fig. 5D, DN-CaMKIV also abolished the TRPC6-induced increases in dendritic branching and length (total dendritic tips: 16.133±0.895, n=45, P=0.671; total dendritic length: 508.733±41.754 μm, n=45, P=0.813 versus DN-CaMKIV). However, transfection of the constitutive active form of CaMKIV (Ca-CaMKIV) (Yu and Malenka, 2003) rescued the change in phenotype induced by TRPC6 shRNAi. Transfection of TRPC6 shRNAi showed impaired dendritic arbors compared with control vectors (Fig. 5E), whereas cotransfection of TRPC6 shRNAi and CA-CaMKIV (total dendritic tips: 37.750±1.425, n=41, P=0.561; total dendritic length: 1089.833±36.979 μm, n=41, P=0.827 versus CA-CaMKIV) showed no significant difference compared with transfection of CA-CaMKIV alone (total dendritic tips: 39.091±1.749, n=45; total dendritic length: 1101.557±37.892 μm, n=45). Thus, these data provide strong evidence that CaMKIV activity is required for TRPC6-induced dendritic growth.

Activation of CREB is essential for TRPC6-induced dendritic growth

Having established that activation of CaMKIV was required for TRPC6-induced dendritic growth, we next investigated the downstream signals of CaMKIV. The best-characterized transcription factor target of CaMKIV in dendritic growth is CREB (Matthews et al., 1994; Sun et al., 1994; Sun et al., 1996). It is also reported that TRPC6 induces phosphorylation of CREB at Ser133, a site required for CREB to associate with its coactivator CREB-binding protein (CBP) to promote dendritic growth (Chawla et al., 1998; Hu et al., 1999; Jia et al., 2007). We therefore examined whether CREB was important for TRPC6-induced dendritic growth. Downregulation of TRPC6 inhibited phosphorylation of CREB at Ser133 normalized to total CREB (0.511±0.131, n=4, P=0.004 versus control) (Fig. 6A,B), suggesting that steady activation of TRPC6 regulated phosphorylation of CREB. In parallel with this result, overexpressing TRPC6 enhanced phosphorylation of CREB (1.809±0.378, n=4, P=0.048 versus control), further suggesting that CREB activity was indeed regulated by TRPC6. The protein level of CREB was not changed in neurons overexpressing TRPC6. We then asked whether TRPC6 affected dendritic growth through activation of CREB. As shown in Fig. 6C-E, TRPC6-induced dendritic growth was completely blocked by KCREB, a dominant-negative mutant of CREB (Walton et al., 1992). Neurons transfected with TRPC6 showed more complicated dendritic arborization than those transfected with control vector. However, cotransfection of TRPC6 and KCREB (total dendritic tips: 13.474±0.889, n=45, P=0.086 versus KCREB; total dendritic length: 570.669±34.562 μm, n=45, P=0.288 versus KCREB) showed no significant difference compared with transfection of KCREB alone (total dendritic tips: 12.330±0.616, n=45; total dendritic length: 496.072±26.318 μm, n=45). Taken together, these results indicated the requirement for CREB activity in TRPC6-induced dendritic growth.

TRPC6 promotes dendritic growth in vivo

Our culture results showed that TRPC6 promoted dendritic growth through a CaMKIV-CREB pathway. We then examined whether TRPC6 promoted dendritic growth in vivo. We overexpressed TRPC6 postnatally in the mouse forebrain neurons using the CaMKIIα promoter (Mayford et al., 1995; Tsien et al., 1996). Western blot analysis revealed a 58.8% increase of TRPC6 protein level in transgenic mice (1.588±0.297, n=5, P=0.028) (Fig. 7A,B). Moreover, in the hippocampal lysates of TRPC6 transgenic mice, the phosphorylated forms of CaMKIV and CREB were increased.

Fig. 5. TRPC6-induced dendritic growth depends on CaMKIV activity. (A) Total lysates of cells treated as indicated blotted with antibodies to phosphorylated CaMKIV, total CaMKIV, phosphorylated CaMKIIα or total CaMKIIα. (B) Quantification of CaMKIV phosphorylation in the neurons transfected with the indicated constructs. *P<0.05 versus ctrl. (C) Representative images of the neurons transfected at 3 DIV for 4 days with wild-type TRPC6 (WT6C) and the dominant-negative CaMKIV mutant, or shTRPC6 RNAi construct and the constitutive active CaMKIV mutant. Scale bar: 20 μm. (D,E) Quantification of total dendritic tips (D) and total dendritic length (E) of the neurons shown in C. **P<0.01 versus ctrl. All data are the means ± s.e.m. n.s., not significant.
TRPC6 promotes dendritic growth by 61.6% (1.616±0.289, n = 5, P = 0.027) and 29.4% (1.294±0.161, n = 5, P = 0.044), respectively. To explore the changes in dendritic morphology, Golgi staining was performed at postnatal day 14. Representative images of reconstructed CA1 pyramidal neurons from wild-type and transgenic mice were shown in Fig. 7C,D. Quantitative analysis revealed that there was a 28.9% increase in total dendritic tips (wild-type mice, 42.226±1.293, n = 43; TRPC6 transgenic mice, 54.453±1.836, n = 36, P<0.0001 versus wild-type mice) and a 24.6% increase in total dendritic length (wild-type mice, 2899.617±84.171 μm, n = 43; TRPC6 transgenic mice, 3612.216±92.813 μm, n = 36, P<0.0001 versus wild-type mice). Both apical and basal dendrites in the transgenic mice were longer and more highly branched than those in the wild-type mice (Fig. 7E,F). The dendritic tips of apical dendrites showed a 25.2% increase and dendritic length a 29.3% increase. Similarly, the dendritic tips of basal dendrites had a 28.2% and dendritic length a 27.4% enhancement. Taken together, these in vivo findings were consistent with the in vitro results, suggesting that TRPC6 induces CaMKIV and CREB activation to promote dendritic growth.

**Discussion**

TRPC channels participate in various physiological processes in the developing CNS (Amaral and Pozzo-Miller, 2007; Fiorio Pla et al., 2005; Greka et al., 2003; Jia et al., 2007; Li et al., 2005). The present study revealed a novel role of TRPC6 in CNS development. Our results suggest that TRPC6 may act as a cellular sensor of environmental cues during dendritic development. Consistently, a recent study showed that hyperforin, a key constituent of St John’s Wort, could induce neurite outgrowth in PC12 cells by specifically activating TRPC6 (Leuner et al., 2007). It is know that TRPC6 can be activated by either G-protein-coupled receptors (GPCRs) or receptor tyrosine kinases (RTKs) through phospholipase C (PLC)-dependent mechanisms (Hofmann et al., 1999; Jia et al., 2007; Venkatachalam and Montell, 2007). As a result of PLC activation, phosphatidylinositol(4,5)-bisphosphate (PIP2) is hydrolyzed to produce diacylglycerol (DAG), which can directly activate TRPC6, and inositol trisphosphate (IP3), which can indirectly activate TRPC6 through the Ca2+ release from internal Ca2+ stores (Venkatachalam and Montell, 2007). These characteristics of activation make TRPC6 a candidate for an environmental sensor during dendritic development to translate
extracellular cues into intracellular signals. Since the establishment of the dendritic structure in the CNS is controlled by numerous extrinsic and intrinsic factors (Riddle et al., 1995; Rosso et al., 2005; Whitford et al., 2002), exploring the role of TRPC6 in relaying these signals into intracellular cascades will extend our understanding on dendritic growth.

Previous studies have highlighted the important contribution of Ca\(^{2+}\)-influx, especially through NMDARs and VSCCs, to dendritic growth (Redmond et al., 2002; Sin et al., 2002). In agreement with this notion, we showed here that Ca\(^{2+}\)-influx through TRPC6 (Fig. 4) is also critical for TRPC6-induced dendritic growth. It is known that the activation of both NMDARs and VSCCs are depolarization dependent, whereas that of TRPC6 is less dependent on depolarization. This difference in activation mechanism implies that TRPC6 may have complementary functions in the regulation of dendritic growth. As shown in Fig. 1, TRPC6 is already expressed in utero and in early postnatal days, a period when neuronal activity is relatively low. Given that the activation of TRPC6 is weakly associated with depolarization (Vazquez et al., 2004), it is possible that TRPC6 promotes dendritic development when neurons are relatively quiescent, especially in the early postnatal days. Our results indicate that, in addition to NMDARs and VSCCs, TRPC6 acts as an additional channel to provide Ca\(^{2+}\) in dendritic growth during the development.

One interesting finding of the current study is that among the three TRPC channels including TRPC4, TRPC5 and TRPC6, which are expressed highly during the period of maximal dendritic growth, only TRPC6 promotes dendritic growth (Fig. 2, supplementary material Fig. S1). The developing dendritic arbor is a dynamic structure, thus TRPC channels may participate in both promoting dendritic growth and pruning dendritic arborization, leading to the formation of proper neuronal circuits. Our present study cannot explain why only TRPC6 promotes dendritic growth. One possibility is that different TRPC channels may activate different signal cascades through different local signaling molecules. Alternatively, it is possible that the different Ca\(^{2+}\)-kinetics through these channels activate distinct Ca\(^{2+}\)-sensing molecules, leading to different outcomes. Our results show that overexpression of TRPC6 enhances phosphorylation of CaMKIV, but not that of CaMKII (Fig. 5A). Activation of CaMKII has been reported to inhibit dendritic growth and elevated CaMKII activity can locally stabilize dendritic arbors (Fink et al., 2003; Wu and Cline, 1998). It is possible that CaMKII might be regulated by other TRPC channels. During development, dendrites dynamically change their growth phase, alternating between extension and retraction. It is possible that the dynamics of dendritic growth is regulated through different TRPC channels. In conclusion, we show that TRPC6 proteins are highly expressed in the period of maximal dendritic growth. TRPC6 promoted dendritic growth via the CaMKIV-CREB-dependent pathway. It is possible that TRPC6 plays a critical role in dendritic growth during early development, especially at the stages when neuronal activity is low.

**Materials and Methods**

**Reagents and antibodies**

OAG and EGTA were purchased from Sigma-Aldrich, Fura-2-AM, Alexa Fluor 488-conjugated goat anti-rabbit and Texas-Red-conjugated goat anti-mouse secondary antibodies from Molecular Probes, HRP-conjugated goat anti-rabbit and goat anti-mouse secondary antibodies from Amersham. The following primary antibodies were used: rabbit anti-EGFP (Molecular Probes), mouse anti-MAP2 (Chemicon), rabbit anti-TRPC6 (Alomone Labs), rabbit anti-TRPC5 (Sigma), rabbit anti-TRPC1 (Alomone Labs), rabbit anti-TRPC3 (from Craig Montell, Departments of Biological Chemistry and Neuroscience, Johns Hopkins School of Medicine, Baltimore, MD), rabbit anti-TRPC4 (Alomone Labs), mouse anti-α-tubulin (Sigma), rabbit anti-CalM1 and mouse anti-CalM1(ε) (Cell Signaling), rabbit anti-p-CaMKIV, rabbit anti-p-CaMKIV (Santa Cruz).

**RNAi constructs**

The short hairpin RNA sequence together with a miRNA-30 loop was inserted into the pPRIME vector (Stegmeyer et al., 2005) via Xhol and EcoRI sites. TRPC6-shRNA sequences were designed to target rat TRPC6 mRNA (NM_055359.1) corresponding to nucleotides 1485-1505 (shRNA-A6-1) or 1158-1177 (shRNA-C6-2). The shRNA-A6-2 sequence also targeted against human TRPC6 mRNA (NM_004621.4) corresponding to nucleotides 1915-1935. The negative control was a hairpin sequence targeting the firefly luciferase inserted to the same plasmid by the same sites (a gift from Stephen J. Elledge, Department of Genetics, Center for Genetics and Genomics, Harvard University Medical School, Boston, MD).

**Neuron cultures and transfection**

Primary hippocampal cultures were prepared from embryonic day 18 (E18) rat brains (Shi et al., 2004). Cells were plated on coverslips coated with poly-D-lysine (50 µg/ml) at a density of 100,000/cm\(^2\) and cultured in Neurobasal A supplemented with B-27 (Gibco) and 0.5 mM glutamine. Actin 3 or 5 DIV, neurons were transfected using the Ca\(^{2+}\)-phosphate method (Ramos et al., 2007) for 2-4 days. Briefly, DNA (up to 5 µg/ml) was mixed with 250 mM CaCl\(_2\) and added to the same volume of HEPES (274 mM NaCl, 10 mM HEPES, 1.4 mM Na\(_2\)HPO\(_4\)), 15 mM D-glucose, 42 mM HEPES-free acid, pH 7.4). The DNA mixture was incubated for 25 minutes in the dark and then added to the neurons in MEM without glutamine at 37°C in 5% CO\(_2\) for 15 minutes. For the RNAi verification experiments, transfection was done using the Nucleofector Device II and the Rat Neuron Nucleofector Kit (Amaxa). Briefly, dissociated hippocampal neurons from E18 rat brain was mixed with 100 µl Neuron Nucleofector Solution containing 2 µg DNA. The mixture was transferred to the cuvette and program O-003 was used.

**Cytosolic Ca\(^{2+}\) measurements**

The intracellular Ca\(^{2+}\) change was measured using the Ca\(^{2+}\)-sensitive fluorescent dye Fura-2-AM as described (Jia et al., 2007; Li et al., 2005). Briefly, cells on 12 mm coverslips were incubated with 2 µM Fura-2-AM at 37°C for 20 minutes, washed 3-4 times with the extracellular solution (147 mM NaCl, 2.8 mM KCl, 2 mM CaCl\(_2\), 2 mM MgCl\(_2\), 10 mM glucose and 10 mM HEPES-free acid, pH 7.4) and imaged using a Nikon eclipse TE2000-e microscope with dual excitations of Fura-2-AM at 340/380 nm and detection of fluorescent emissions at 500 nm. The 340/380 nm excitation ratio, which increases as a function of intracellular Ca\(^{2+}\), was captured at 6 second intervals.

**Immunoblotting**

Rabbit anti-TRPC1 antibody (Alomone Labs, 1:200), rabbit anti-TRPC3 antibody (Montell, 1:500), rabbit anti-TRPC4 antibody (Alomone Labs, 1:500), rabbit anti-TRPC5 antibody (Sigma, 1:200), rabbit anti-TRPC6 antibody (Alomone Labs, 1:500), mouse anti-α-tubulin antibody (Sigma, 1:1000), rabbit anti-phosphoCaMKIV (Thr286) antibody (Santa Cruz, 1:500), mouse anti-CaMKIIX antibody (Cell Signaling, 1:1000), rabbit anti-phosphoCaMKIV (Thr196) antibody (Santa Cruz, 1:100) and rabbit anti-CaMKIV antibody (Cell Signaling, 1:500) were used as primary antibodies. HRP-conjugated goat anti-rabbit and goat anti-mouse antibodies (Amersham, 1:3000) were used as secondary antibodies. For the signal detection, ECL Plus Detection System (Amersham) was used.

**Immunohistochemistry**

The brains of neonatal rats were embedded in OCT compound (Tissue-Tek, Miles), and 30 µm frozen sections were transferred to PBS. The sections were washed once with PBS and blocked with 3% goat serum in PBS (PBS plus 0.3% Triton X-100) for 1 hour. The sections were then incubated overnight with anti-TRPC6 antibody (1:100, Alomone Labs) and anti-MAP2 antibody (1:200, Chemicon) at 4°C. The sections were washed in PBST and incubated for 1 hour with Alexa Fluor 488-conjugated goat anti-rabbit antibody (Molecular Probes, 1:5000) and Texas-Red-conjugated goat anti-mouse antibody (1:5000, Molecular Probes) diluted in PBST. The fluorescent signals were examined using an LSM 5 PASCAL laser-scanning confocal microscope.

**In situ hybridization**

Nucleotides 1-1020 of rat TRPC6 were amplified by PCR and subcloned into the T-easy vector. The vector was then digested with BamHI or EcoRI to generate a template for the in vitro transcription of an antisense or sense cRNA probe, respectively. Digoxigenin-labeled cRNA was produced by T7 or SP6 RNA polymerase. In situ hybridization was then performed according to standard procedures (Giger et al., 1996).

**Golggi staining**

Golggi staining was performed using Rapid GolggiStain Kit (FD Neurotechnologies) following the manufacturer’s instructions. Briefly, the brain from postnatal day 14 mice was immersed in the impregnation solution for 2 weeks, then cut into 100 µm sections using a vibratome. The sections were stained and dehydrated in graded
ethanol, cleared in xylene and examined with the Neuro lucida system (MicroBrightField, Williston, VT) with a 50× objective.

Image analysis
Confocal images of the neurons were obtained using a LSM 5 PASCAL laser-scanning confocal microscope with sequential acquisition settings at the resolution of 1024×1024 pixels. Each image was a z-series of 4-6 images at 2 μm depth interval when 40× objective was used. The resultant stack was “flattened” into a single image using the maximal projection. Using the NeuroLucida software (MicroBrightField), the images were traced and reconstructed. The number of primary dendrites, total dendritic tips and total dendritic length was determined by the same software. Transfection experiments were carried out in duplicate wells and all experiments were repeated at least three times. More than 15 neurons per group were obtained each time. Data were mean ± s.e.m. The comparison of results between different sets of experiments was evaluated by Student’s t-test.

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Fig. S1. Overexpression of TRPC4 or 5 inhibited dendritic growth. Quantification of the number of primary dendrites (A), total dendritic tips (B) and total dendritic length (C) of the neurons transfected with the indicated constructs. Data were mean ± s.e.m of at least 45 cells in three independent experiments. ** P < 0.01 versus control (ctrl).
Fig. S2. Protein levels of TRPC6 in overexpression experiments. (A) The total lysates of cells transfected with indicated constructs were blotted with antibodies to TRPC6 or tubulin. (B) Quantification of TRPC6 protein level in neurons transfected with indicated constructs. Data were mean \pm s.e.m of five independent experiments. ** P<0.01 versus control (ctrl).
Supplementary Fig. 3

Fig. S3. Quantification of CaMKIIα phosphorylation in neurons transfected with indicated constructs. Data were mean ± s.e.m of at least four independent experiments. (WTC6: 0.968 ± 0.101, n=5, p=0.729; shC6i: 1.081 ± 0.174, n=4, p=0.882)