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ARTICLES

TRPC channels promote cerebellar granule neuron survival

Yichang Jia[1–3], Jian Zhou[1–3], Yilin Tai[1,2] & Yizheng Wang[1]

Channels formed by the transient receptor potential (TRP) family of proteins have a variety of physiological functions. Here we report that two members of the TRP cation channel (TRPC) subfamily, TRPC3 and 6, protected cerebellar granule neurons (CGNs) against serum deprivation–induced cell death in cultures and promoted CGN survival in rat brain. In CGN cultures, blocking TRPC channels or downregulating TRPC3 or 6 suppressed brain-derived neurotrophic factor (BDNF)–mediated protection, BDNF-triggered intracellular Ca^{2+} elevation and BDNF-induced CREB activation. By contrast, overexpressing TRPC3 or 6 increased CREB-dependent reporter gene transcription and prevented apoptosis in the neurons deprived of serum, and this protection was blocked by the dominant negative form of CREB. Furthermore, downregulating TRPC3 or 6 induced CGN apoptosis in neonatal rat cerebellum, and this effect was rescued by overexpressing either TRPC3 or 6. Thus, our findings provide *in vitro* and *in vivo* evidence that TRPC channels are important in promoting neuronal survival.

The transient receptor potential (TRP) channel was first identified in *Drosophila melanogaster*, when photoreceptors carrying *trp* gene mutations exhibited a transient depolarization in response to continuous light[1,2]. The TRP channels are formed by homomeric or heteromeric complexes of TRP proteins that constitute at least three subfamilies[3–7]: TRPC (TRP-canonical), TRPV (TRP-vanilloid) and TRPM (TRP-melastatin). Members of the TRPV subfamily are involved in thermosensation and nociception, whereas TRPM proteins are implicated in a wide spectrum of physiological processes ranging from cold sensation to cell survival[4,6]. TRPM7 is also important in Ca^{2+}-overload and the death of cortical neurons induced by oxygen glucose deprivation[8]. Although the activation mechanisms of TRPC channels have been extensively examined[4,6], it is still largely unknown whether TRPC channels are involved in regulating neuronal survival and what are the downstream effectors for TRPC channel activation.

In the developing central and peripheral nervous systems, neuronal survival may depend on neurotrophic factors[9–12]. For example, BDNF, a member of the neurotrophin family of growth factors, is essential for the survival of a variety of neurons, including CGNs[13–15]. The neurotrophin effect on neuron survival is mediated by the ERK/CREB and PI3K/Akt pathways[9–11,16]. Many receptors for neurotrophins are receptor tyrosine kinases (RTKs), which can stimulate PLC-γ to activate TRPC channels[4,6]. In pontine neurons, BDNF triggers a nonselective cationic conductance that resembles that of TRPC channels[17], which are permeant to Ca^{2+} (refs. 4,6). Studies show that TRPC channels are essential for BDNF-mediated growth cone turning in CGNs and *Xenopus laevis* spinal neurons[18–20]. Furthermore, BDNF triggers intracellular $[Ca^{2+}]_i$ elevation in the CGN growth cone through TRPC3 and 6 (ref. 19). Because Ca^{2+} is known to mediate cell growth and survival, it is possible that Ca^{2+} influx through TRPC channels is required for the neuronal protection effect of neurotrophic factors, such as BDNF.

In the present study, we have examined the role of TRPC channels in neuronal survival in both CGN cultures and neonatal cerebellum. We found that TRPC3 and 6 are required for BDNF-mediated neuronal protection, BDNF-triggered intracellular Ca^{2+} elevation and BDNF-induced CREB activation. It is known that the cAMP/Ca^{2+}-response element binding protein (CREB) is activated in neurons in response to a diverse array of stimuli, including hormones, growth factors and neuronal activity[21]. In addition to cAMP, either influx of extracellular Ca^{2+} through membrane Ca^{2+} channels or release of Ca^{2+} from internal stores activates CREB, leading to neuronal survival[15,21–29]. Here, we found that overexpressing TRPC3 or 6 markedly enhanced CREB-dependent transcription and increased CREB phosphorylation. Furthermore, overexpressing these channels protected cultured CGNs against serum deprivation–induced cell death via CREB activation. Notably, in neonatal rat cerebellum, we also found that TRPC3 and 6 promoted CGN survival. Taken together, our findings provide *in vitro* and *in vivo* evidence that TRPC channels have a critical role in promoting neuronal survival and indicated that the activation of CREB is a key downstream effector for the neuronal protective effect of TRPC channels.

RESULTS

TRPC3 and 6 are required for BDNF's protective effect

To address whether TRPC channels are necessary for the BDNF-mediated neuroprotective effect on CGNs, we employed a well-established *in vitro* model system, in which BDNF protects cultured

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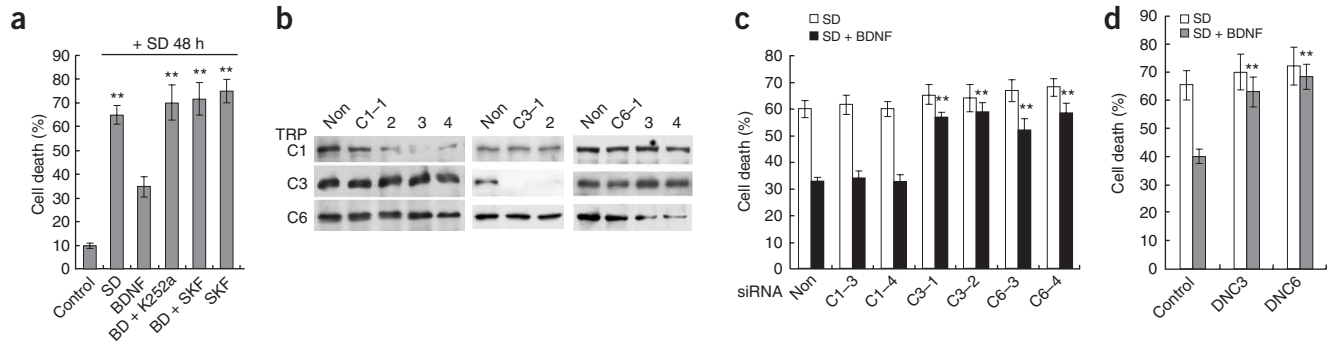

Figure 1 TRPC3 and 6 are required for BDNF's neuroprotective effect. (**a**) Effects of different agents on BDNF-dependent protection against serum deprivation (SD)–induced cell death (control, BME + 10% FBS + 25 mM KCl; K252a, 200 nM; SKF, 20μM SKF96365). Data are mean ± s.e.m. from five experiments in triplicate. ** $P < 0.0001$ versus s.d. + BDNF. (**b**) Western blot of whole cell lysates of CGNs transfected for 4 d with siRNAs against TRPC1 (C1–1 to 4), 3 (C3–1, -2), 6 (C6–1, 3 and 4) or nonsense (Non) siRNA using antibodies against TRPC1, 3 or 6. Data are representative of three independent experiments. (**c**) Effect of downregulation of TRPC1, 3 and 6 by the corresponding siRNAs on BDNF-dependent protection against cell death induced by s.d. Data are mean ± s.e.m. from five independent experiments in triplicate. ** $P < 0.01$ versus s.d. + BDNF with nonsense siRNA. (**d**) Dominant-negative form of TRPC3 (DNC3) and TRPC6 (DNC6) abolished BDNF-dependent protection against SD-induced cell death. Data are mean ± s.e.m. from three independent experiments in triplicate. ** $P < 0.01$ versus s.d. + BDNF with GFP transfection. In **a**, **c** and **d**, s.d. for 48 h, BDNF at 50 ng ml^{-1}, one-way ANOVA test. PI staining was used to detect apoptosis in cultures[30,31].

CGNs against serum deprivation–induced cell death[15], and evaluated cell viability by propidium iodide staining[30,31]. When we deprived cultured CGNs of serum for 48 h 66.5 ± 3.1% died, and this cell death was markedly reduced by BDNF application (**Fig. 1a** and **Supplementary Fig. 1** online). Furthermore, the BDNF's protection was abolished by K252a, a tyrosine kinase inhibitor known to block TrkB receptor (**Fig. 1a**), consistent with a specific action of BDNF mediated by TrkB[15]. Notably, SKF96365, an inhibitor known to block TRPC channels[32,33], eliminated BDNF's protection of CGNs (**Fig. 1a** and **Supplementary Fig. 1**) in a dose- and time-dependent manner (**Supplementary Fig. 1**). These results suggest that TRPC channels participate in BDNF neuronal protection.

To avoid potential nonspecific effects of SKF96365, we introduced chemically synthesized siRNAs against TRPC1, 3 and 6 into cultured CGNs, in which these mRNAs were highly expressed[19]. We determined the siRNA delivery efficiency in cultured CGNs by FITC-labeled nonsense siRNA (85 ± 3.3%, $n = 3$, **Supplementary Fig. 2** online). Western blot analysis showed that the amount of TRPC3 protein in cultured CGNs transfected with two different siRNAs against TRPC3, C3–1 and C3–2, was reduced to 10.3 ± 2.1% and 11.4 ± 1.9%, respectively, of that found in cultures transfected with the nonsense siRNA ($n = 3$, **Fig. 1b**). Moreover, siRNAs against TRPC1, C1–3 and C1–4 reduced TRPC1 expression to 8.3 ± 2.7% and 10.8 ± 2.3%, and siRNAs against TRPC6, C6–3 and C6–4 reduced TRPC6 expression to 32.5 ± 3.7% and 22.2 ± 1.8% of the control, respectively ($n = 3$, **Fig. 1b**). These siRNAs against TRPC1, 3 or 6 were specific, as they had no effect on the expression of the other two members of the subfamily (**Fig. 1b**). Application of BDNF protected nonsense siRNA–transfected CGNs deprived of serum (**Fig. 1c**). However, 4 d after transfection with C3–1 or C3–2 siRNA against TRPC3 the protective effect of BDNF was largely abolished ($n = 5$, **Fig. 1c**). Transfection of CGNs with either C6–3 or C6–4 also greatly reduced the BDNF-dependent neuronal protection ($n = 5$, $P < 0.01$; **Fig. 1c**). In contrast, transfection with either C1–3 or C1–4 siRNA against TRPC1 had no effect on protection by BDNF ($n = 5$, $P > 0.1$; **Fig. 1c**). Thus, TRPC3 and 6, but not TRPC1, are necessary for BDNF-dependent protection in cultured CGNs.

We further expressed the dominant-negative form of TRPC3 or 6 (DN-TRPC3 and DN-TRPC6, respectively), which selectively suppresses TRPC3 or 6 currents in a heterologous system[34,35], to study the role of TRPC channels in BDNF protection. The transfection efficiency for cultured CGNs was first determined by transfection of a green fluorescent protein (GFP) construct (75.0 ± 3.7%, $n = 3$, **Supplementary Fig. 2**). Two d after the transfection, application of BDNF for 48 h substantially reduced serum deprivation–induced death in cultured CGNs expressing GFP alone, but not in those expressing GFP together with DN-TRPC3 ($n = 3$, **Fig. 1d**) or DN-TRPC6 ($n = 3$, **Fig. 1d**). Taken together, our results show that TRPC3 and 6 are required for the BDNF neuroprotective effect of CGNs.

PLC and IP_3R are necessary for the BDNF neuroprotection

The TRPC channels can be activated through PLC-IP_3R/diacylglycerol (DAG) pathways[4,6]. If the BDNF neuroprotective effect depends on TRPC3 or 6 activation, inhibition of PLC-IP_3R/DAG pathways should suppress the BDNF-dependent protective effect. We bath incubated BDNF together with U73122, an inhibitor of PLC, which suppressed the BDNF protective effect on cultured CGNs, whereas U73343, an inactive derivative of U73122, had no effect (**Fig. 2a**). To directly address whether PLC-γ1 is necessary for BDNF-mediated protection, we synthesized siRNAs against PLC-γ1. Western blot analysis revealed that PLC-1 or PLC-2 siRNA against PLC-γ1 reduced its expression to 12.3 ± 2.1% or 21.5 ± 3.4% of the nonsense siRNA control ($n = 3$, **Fig. 2b**). Notably, both PLC-1 and PLC-2 siRNAs greatly inhibited the BDNF-dependent protection of CGNs ($n = 3$, **Fig. 2c**).

The neuroprotective effect of BDNF also depended on IP_3R activation, as application of 2-aminoethoxydiphenyl borate (2ApB), an IP_3R antagonist[36], together with BDNF suppressed the BDNF-dependent neuroprotective effect on cultured CGNs ($n = 5$, **Fig. 2a**) in a dose- and time-dependent manner (**Supplementary Fig. 3** online). Similarly, another membrane-permeable IP_3R blocker xestospongin C (Xest C)[37] also abolished BDNF neuroprotective effect ($n = 5$, **Fig. 2a**). However, we found that application of 1-oleoyl-2-acetyl-*sn*-glycerol (OAG), a membrane permeable analog of DAG, did not protect CGNs against serum deprivation–induced cell death and did not facilitate BDNF's protective effect ($n = 5$, $P > 0.1$; **Fig. 2a**), suggesting that the PLC-DAG pathway is not involved in BDNF's protective effect on CGNs. Taken together, these findings support the notion that the PLC-γ1/IP_3R pathway, which activates TRPC channels, is required for BDNF-mediated neuronal protection.

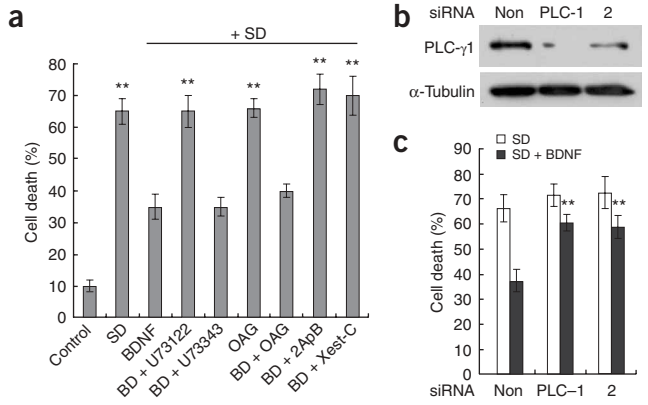

Figure 2 PLC and IP_3R are necessary for the BDNF neuroprotection. (**a**) Effects of the indicated agents on BDNF-dependent protection (100 μM 2ApB; 20 μM xestospongin C (Xest C); 50 μM OAG; 2 μM U73122; 2 μM U73343). Data are mean ± s.e.m. from five independent experiments in triplicate. ** $P < 0.0001$ versus s.d. + BDNF. (**b**) Western blot analysis of PLC-γ1 levels in CGNs transfected with siRNAs against PLC-γ1 (PLC-1, 2) or nonsense (Non) siRNA for 3 d. Data are representative of three independent experiments. (**c**) Effects of downregulation of PLC-γ1 by siRNAs on BDNF-dependent protection. Data are mean ± s.e.m. from three independent experiments in triplicate. ** $P < 0.01$ versus s.d. + BDNF transfected with nonsense siRNA. In **a** and **c**, s.d. for 48 h, BDNF at 50 ng ml^{-1}, one-way ANOVA test.

TRPC3 and 6 contribute to BDNF-induced Ca^{2+} elevation

BDNF triggers $[Ca^{2+}]_i$ elevation in the CGN growth cone through TRPC3 and 6 (ref. 19). However, it is not clear whether TRPC3 and 6 are required for BDNF-induced $[Ca^{2+}]_i$ elevation in CGN soma, where the survival and apoptosis machinery is located[11,12,16].

To investigate this possibility, we directly measured $[Ca^{2+}]_i$ with fura-2AM imaging. We found that application of BDNF led to a substantial $[Ca^{2+}]_i$ elevation in the soma of cultured CGNs (**Fig. 3a**). This elevation was suppressed by Ca^{2+}-free medium in the presence of EGTA (**Fig. 3b**), suggesting that BDNF-induced $[Ca^{2+}]_i$ elevation depends on extracellular Ca^{2+} influx. Treatment with SKF-96365 at the same concentration that abolished BDNF-dependent protection (**Fig. 1a**) markedly reduced BDNF-induced elevation of $[Ca^{2+}]_i$ (**Fig. 3a,b**). In contrast, the L-type voltage-gated calcium channel (VGCC) blocker, nifedipine did not affect BDNF-induced $[Ca^{2+}]_i$ elevation (**Fig. 3b**). Furthermore, PLC and IP_3 R inhibitors U73122, 2ApB or Xest C, all of which abolished BDNF-dependent protection (**Fig. 2a**), eliminated BDNF-induced $[Ca^{2+}]_i$ elevation in the soma of CGNs (**Fig. 3b**).

In addition, because BDNF-induced $[Ca^{2+}]_i$ elevation was reduced in the soma of cultured CGNs that were transfected with TRPC3 or 6 siRNAs ($n = 30$, $P < 0.001$; **Fig. 3c,d**), but was not affected by downregulation of TRPC1 ($n = 30$, $P > 0.1$; **Fig. 3c,d**), TRPC3 and 6 are responsible for the $[Ca^{2+}]_i$ elevation. Furthermore, BDNF-induced $[Ca^{2+}]_i$ elevation in CGNs treated with SKF96365 was similar to that in CGNs treated with U73122, 2ApB or Xest C (**Fig. 3a,b),** and downregulation of TRPC6 reduced BDNF-induced $[Ca^{2+}]_i$ elevation to a level similar to that found in transfectants in which extracellular Ca^{2+} influx was blocked or internal Ca^{2+} stores were depleted by EGTA or thapsigargin (**Supplementary Fig. 4** online). Therefore, it is possible that blockage of TRPC channels may affect internal Ca^{2+} release. Furthermore, BDNF treatment did not affect TRPC3 or 6 membrane expression in CGNs (**Supplementary Fig. 5** online). Taken together, these results support the following model of BDNF action on CGNs: BDNF activates the PLC/IP_3R pathway to induce TRPC channel activation, which causes Ca^{2+} influx and induces Ca^{2+} release from internal store, leading to elevation of $[Ca^{2+}]_i$.

TRPC3 and 6 are required for BDNF-induced CREB activation

BDNF induces phosphorylation of Akt at Ser473, ERK at Thr202 and Tyr204 and CREB at Ser133 to mediate its protective effect[9–11,15,16]. To

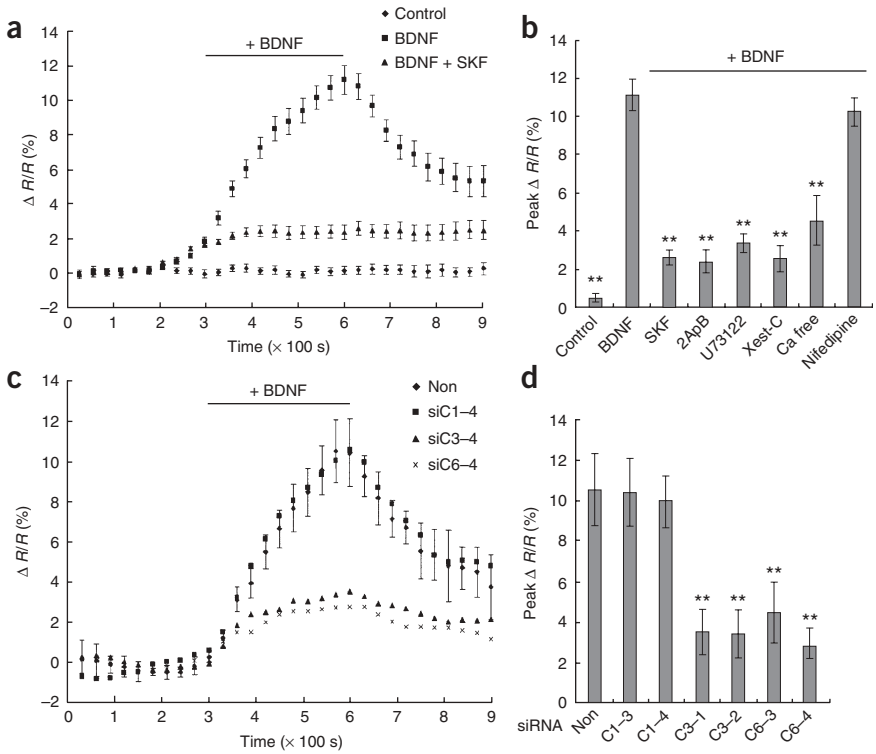

Figure 3 TRPC3 and 6 contribute to BDNF-triggered $[Ca^{2+}]_i$ elevation in CGNs' soma. (**a**) Effect of SKF96365 on the elevation of $[Ca^{2+}]_i$ induced by BDNF. The $[Ca^{2+}]_i$ determined by the ratio F340/F380 was depicted by $\Delta R/R \times 100\%$ and normalized to the ratio of baseline. Each data are mean ± s.e.m. of three independent experiments with 30 cells. (**b**) The effects of indicated agents on peak $\Delta R/R$ triggered by BDNF (100 μM 2-ApB; 2 μM U73122; 5 μM nifedipine). Each data are mean ± s.e.m. from three experiments with 30 cells. (**c**) Effect of downregulation of TRPC channels on BDNF-induced $[Ca^{2+}]_i$ elevation. Intracellular Ca^{2+} levels in CGNs transfected with nonsense siRNA or siRNAs against TRPC1 (C1–3, C1–4), 3 (C3–1, C3–2) or 6 (C6–3, C6–4) were measured as shown in **a**. Each data are mean ± s.e.m. of three independent experiments from 30 cells. (**d**) Quantitative analysis of the peak $\Delta R/R$ stimulated by BDNF in CGNs transfected with siRNAs against TRPC1, 3 or 6. Data are mean ± s.e.m. from three independent experiments from 30 cells. ** $P < 0.001$ versus s.d. + BDNF with nonsense siRNA. In **b** and **d**, ANOVA test.

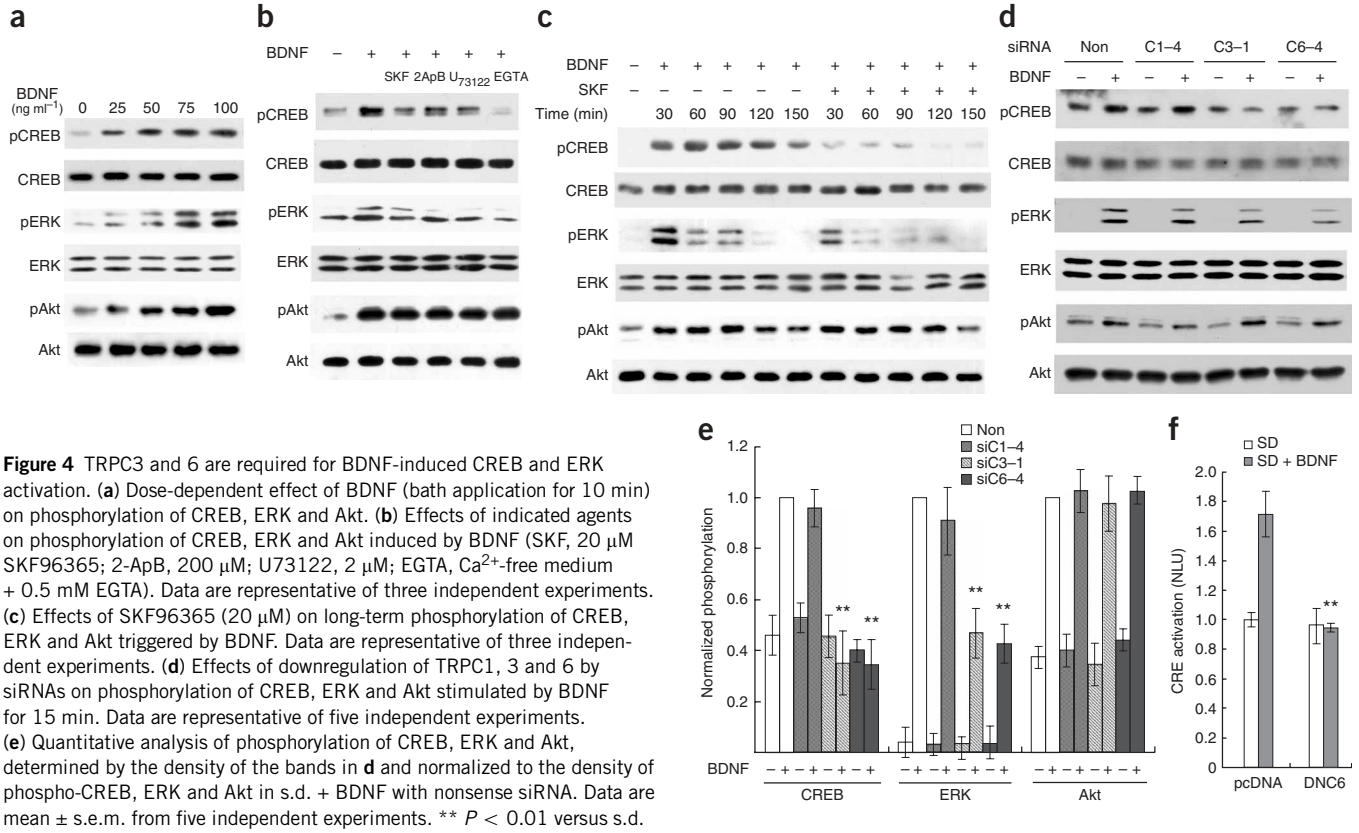

Figure 4 TRPC3 and 6 are required for BDNF-induced CREB and ERK activation. (**a**) Dose-dependent effect of BDNF (bath application for 10 min) on phosphorylation of CREB, ERK and Akt. (**b**) Effects of indicated agents on phosphorylation of CREB, ERK and Akt induced by BDNF (SKF, 20 μM SKF96365; 2-ApB, 200 μM; U73122, 2 μM; EGTA, Ca^{2+}-free medium + 0.5 mM EGTA). Data are representative of three independent experiments. (**c**) Effects of SKF96365 (20 μM) on long-term phosphorylation of CREB, ERK and Akt triggered by BDNF. Data are representative of three independent experiments. (**d**) Effects of downregulation of TRPC1, 3 and 6 by siRNAs on phosphorylation of CREB, ERK and Akt stimulated by BDNF for 15 min. Data are representative of five independent experiments. (**e**) Quantitative analysis of phosphorylation of CREB, ERK and Akt, determined by the density of the bands in **d** and normalized to the density of phospho-CREB, ERK and Akt in s.d. + BDNF with nonsense siRNA. Data are mean ± s.e.m. from five independent experiments. ** $P < 0.01$ versus s.d. + BDNF with nonsense siRNA. (**f**) Effects of DNC6 on CRE-luciferase activity stimulated by BDNF. Data are mean ± s.e.m. from five independent experiments. ** $P < 0.001$ versus s.d. + BDNF. The concentration of BDNF in **b**, **c**, **d** and **f** is 50 ng ml^{-1}. In **e** and **f**, Student's *t*-test and ANOVA were used.

explore the signaling pathways responsible for the TRPC-mediated protective effect of BDNF, we examined whether TRPC channels mediate the BDNF effect on the phosphorylation of these kinases. Application of BDNF in CGN cultures for 10 min induced a dose-dependent phosphorylation of CREB at Ser133, ERK at Thr202 and Tyr204 and Akt at Ser473 (**Fig. 4a**). However, Ca^{2+}-free medium or SKF96365 treatment markedly attenuated BDNF-induced phosphorylation of CREB and ERK, but not that of Akt (**Fig. 4b**). Furthermore, inhibition of PLC and IP_3R, by U73122 and 2ApB also eliminated the phosphorylation of CREB and ERK, but not that of Akt (**Fig. 4b**). A time course experiments showed that Akt phosphorylation triggered by BDNF was entirely insensitive to SKF96365, even after 150 min (**Fig. 4c**). However, in the presence of SKF96365, the phosphorylation of CREB and ERK was suppressed or returned to basal levels after 30 and 60 min, respectively (**Fig. 4c**). These results suggest that phosphorylation of CREB and ERK triggered by BDNF depends on TRPC channel activation, whereas Akt phosphorylation does not.

We further tested this notion by transfection of CGNs with siRNAs against TRPC1, 3 and 6. The siRNA against TRPC3 (C3–1) and TRPC6 (C6–4) abolished BDNF-induced phosphorylation of CREB and greatly suppressed phosphorylation of ERK ($n = 5$, **Fig. 4d,e**), but did not affect Akt phosphorylation ($n = 5$, $P > 0.1$; **Fig. 4d,e**). In contrast, transfection of CGNs with the siRNA against TRPC1 (C1–4) did not affect BDNF-induced phosphorylation of these proteins ($n = 5$, $P > 0.1$; **Fig. 4d,e**). Furthermore, treatment with SKF96365 or transfection with the DN-TRPC6 on cultured CGNs abolished CREB-dependent reporter gene transcription induced by BDNF ($n = 5$, **Fig. 4f** and **Supplementary Fig. 6** online). Therefore, these results indicate that phosphorylation or activation of CREB and ERK, but not Akt phosphorylation, triggered by BDNF depends on TRPC3 or 6 activation.

TRPC3 and 6 protective effect depends on CREB activation

Our loss-of-function experiments showed that downregulation or blockade of TRPC3 or 6 suppressed BDNF-dependent survival (**Fig. 1**), BDNF-triggered $[Ca^{2+}]_i$ elevation (**Fig. 3**) and BDNF-induced CREB and ERK activation (**Fig. 4**).

We then examined whether activation of TRPC channels is sufficient for neuronal survival in gain-of-function experiments. We first transfected cultured CGNs with wild-type TRPC3 or 6 (**Supplementary Fig. 2** and **7** online) and pulse-labeled these transfectants with ^{45}Ca to measure the steady state Ca^{2+} influx. We found that overexpressing TRPC3 or 6 increased ^{45}Ca uptake ($n = 5$, **Fig. 5a**). Cotransfection of TRPC3 or 6 with the CRE-luc reporter plasmid greatly enhanced CREB-dependent reporter-gene expression by about five- or sevenfold relative to control, respectively ($n = 5$–10, **Fig. 5b**). In contrast, transfection of TRPC1 had no effect on CREB-dependent reporter-gene expression ($n = 5$, $P > 0.1$; **Fig. 5b**), indicating that TRPC3- and 6-induced CREB activation is specific. Consistent with the results of the luciferase assay, western blot showed that overexpressing TRPC3 or 6 enhanced phosphorylation of CREB and ERK (**Fig. 5c** and **Supplementary Fig. 8** online).

Ca^{2+} influx from L-type VGCCs and Ca^{2+} release from internal stores can activate Ras, resulting in the phosphorylation of ERK[24–26,28], and the Ras/MEK/ERK pathway leads to the activation of Rsk, which phosphorylates CREB at Ser133 or activates CREB-dependent gene

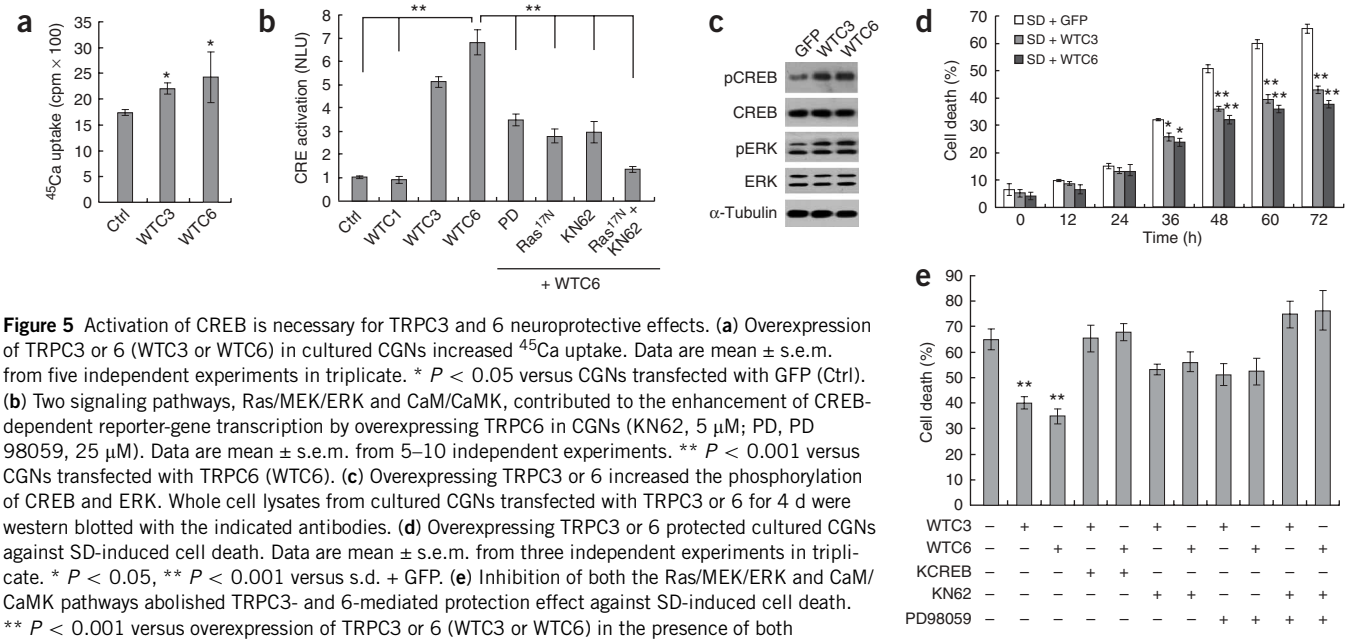

Figure 5 Activation of CREB is necessary for TRPC3 and 6 neuroprotective effects. (**a**) Overexpression of TRPC3 or 6 (WTC3 or WTC6) in cultured CGNs increased ^{45}Ca uptake. Data are mean ± s.e.m. from five independent experiments in triplicate. * $P < 0.05$ versus CGNs transfected with GFP (Ctrl). (**b**) Two signaling pathways, Ras/MEK/ERK and CaM/CaMK, contributed to the enhancement of CREB-dependent reporter-gene transcription by overexpressing TRPC6 in CGNs (KN62, 5 μM; PD, PD 98059, 25 μM). Data are mean ± s.e.m. from 5–10 independent experiments. ** $P < 0.001$ versus CGNs transfected with TRPC6 (WTC6). (**c**) Overexpressing TRPC3 or 6 increased the phosphorylation of CREB and ERK. Whole cell lysates from cultured CGNs transfected with TRPC3 or 6 for 4 d were western blotted with the indicated antibodies. (**d**) Overexpressing TRPC3 or 6 protected cultured CGNs against SD-induced cell death. Data are mean ± s.e.m. from three independent experiments in triplicate. * $P < 0.05$, ** $P < 0.001$ versus s.d. + GFP. (**e**) Inhibition of both the Ras/MEK/ERK and CaM/CaMK pathways abolished TRPC3- and 6-mediated protection effect against SD-induced cell death. ** $P < 0.001$ versus overexpression of TRPC3 or 6 (WTC3 or WTC6) in the presence of both PD98059 and KN62. In **a**, **b**, **d** and **e**, ANOVA test.

expression[15,21,24–26]. Application of PD98059, an inhibitor of MEK, the upstream kinase of ERK, markedly inhibited CREB-dependent transcription induced by overexpressing TRPC6 ($n = 5$, **Fig. 5b**). Similarly, the increased CREB-dependent transcription was also inhibited by cotransfection with Ras^{17N}, a dominant negative form of Ras ($n = 5$, **Fig. 5b**, ref. 24). Because inhibition of the Ras/MEK/ERK pathway, either by PD98059 or by Ras^{17N}, did not abolish CREB-dependent transcription, a pathway independent of Ras/MEK/ERK may exist to activate CREB. $[Ca^{2+}]_i$ elevation also activates the Ca^{2+}/calmodulin-dependent kinase (CaMK), which then phosphorylates CREB at Ser133 in the nucleus and induces CREB-dependent transcription[21,26,27,38,39]. Thus, we applied the CaMK inhibitor KN62 and found that the enhancement of CREB-dependent transcription was markedly suppressed ($n = 5$, **Fig. 5b**). Notably, the CREB-dependent transcription induced by TRPC6 was reduced to control levels in cultured CGNs cotransfected with Ras^{17N} and incubated with KN62 ($n = 5$, $P > 0.1$; **Fig. 5b**).

We further found that overexpressing TRPC3 or 6 prevented apoptosis of CGNs deprived of serum. The protective effect appeared more effective 48–72 h after deprivation ($n = 3$, **Fig. 5d**). Indeed, the TRPC3 and 6 neuroprotective effect depended on CREB activation, as their protective effects were completely blocked by cotransfection with

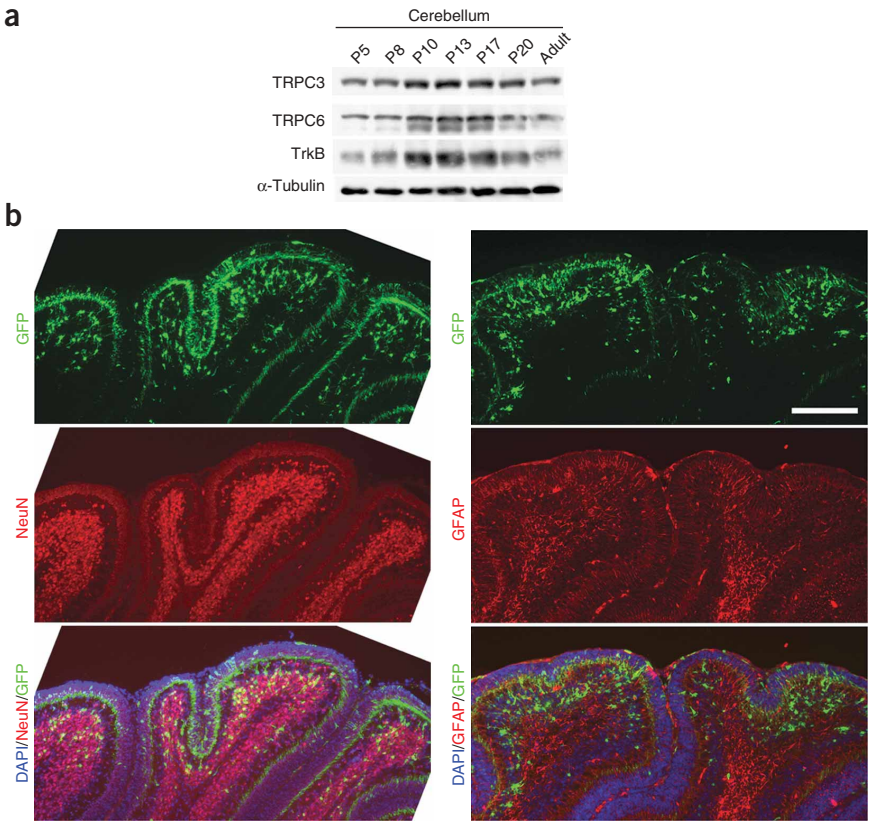

Figure 6 Expression pattern of TRPC channels in developing cerebellum and establishment of *in vivo* electroporation in neonatal rat cerebellum. (**a**) Western blot analysis of postnatal (P) and adult rat cerebellum at the indicated times with the specified antibodies, and the same PVDF membrane was probed again with an antibody specific for α-tubulin. (**b**) The brain sections from P8 cerebella electroporated with pCAGGS-GFP on P4 were stained with antibodies specific for GFP (green), NeuN (red, left) and GFAP, a glial marker (red, right). Scale bar, 200 μm.

K-CREB ($n = 3$, **Fig. 5e**), a dominant negative form of CREB[15,21]. The protection effect of TRPC3 or 6 was partially inhibited when either the Ras/MEK/ERK or CaM/CaMK pathways were inhibited by PD98059 or KN62, but was totally abolished when both pathways were blocked ($n = 5$, **Fig. 5e**). Taken together, these results support the notion that activation of TRPC3 or 6 stimulates two signaling pathways, Ca^{2+}/Ras/MEK/ERK and Ca^{2+}/CaM/CaMK, that converge on CREB activation, resulting in neuronal survival.

TRPC3 and 6 channels promote granule cell survival *in vivo*

Our *in vitro* results show that TRPC3 and 6 is required for BDNF-mediated neuronal protection (**Fig. 1**) and protects CGNs against

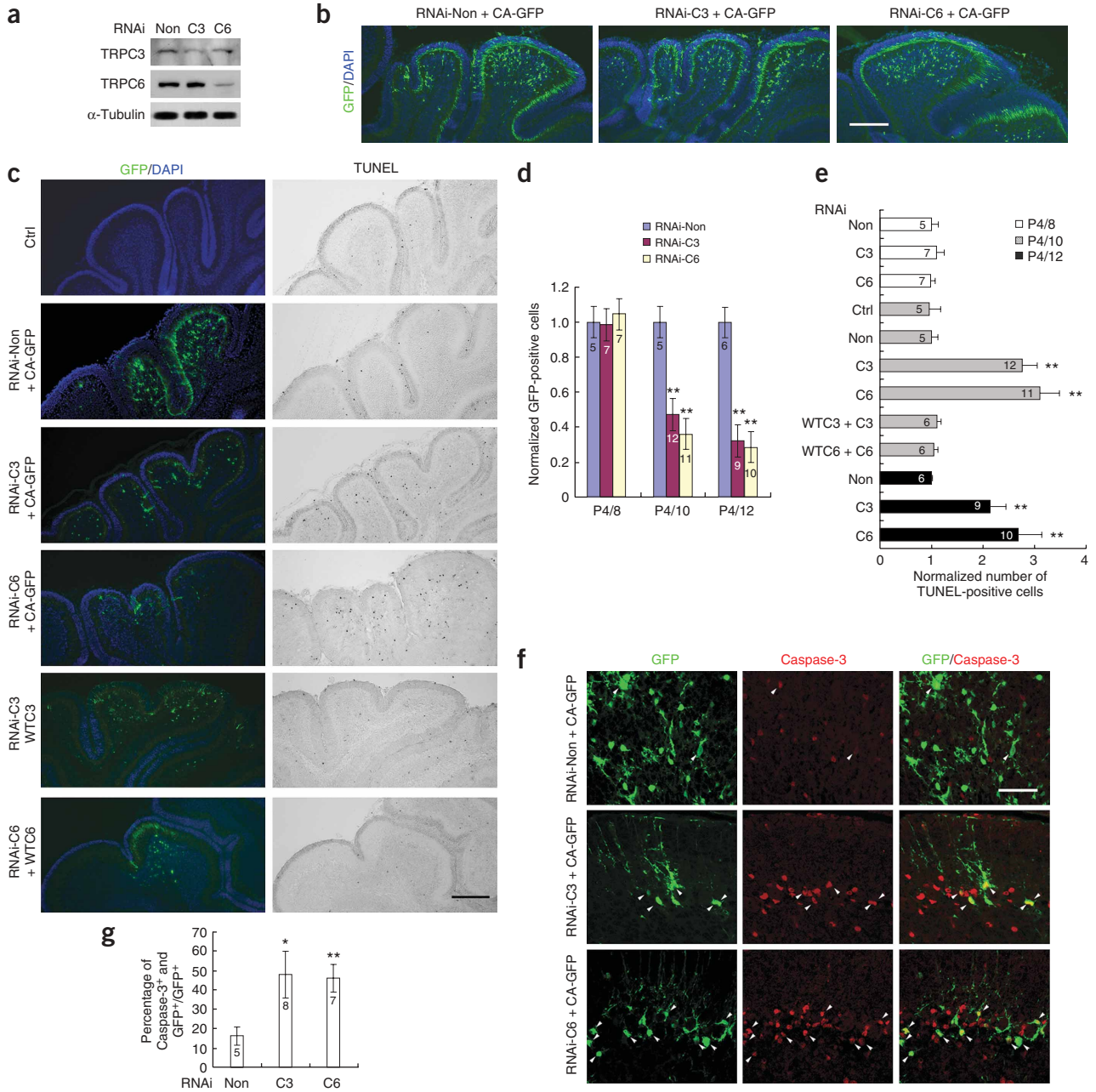

Figure 7 TRPC3 and 6 channels promote CGN survival *in vivo*. (**a**) Western blot of whole cell lysates from cultured CGNs transfected with RNAi constructs against TRPC3, 6 (C3 or C6) or nonsense (Non) RNAi for 4 d using indicated antibodies. (**b**) Representative images of P8 rat cerebella electroporated on P4 with RNAi constructs plus pCAGGS-GFP (CA-GFP). (**c**) Representative images of TUNEL-positive cells in cerebella electroporated with indicated constructs on P4 and observed on P10 (Ctrl, P10 cerebella; WTC3, CA-TRPC3-IRES-GFP; WTC6, CA-TRPC6-IRES-GFP). In **b** and **c**, scale bar is 200 μm. (**d**) Quantification of GFP-positive cells on P8, P10 and P12 cerebella electroporated with indicated RNAi constructs plus GFP on P4. The numbers were normalized to those of nonsense RNAi. Data are mean ± s.e.m. from 5 to 12 animals (the numbers in the bars). ** $P < 0.0001$ versus nonsense RNAi. (**e**) Quantification of TUNEL-positive cells on P8, P10 and P12 after electroporation with indicated constructs on P4. The numbers were normalized to those of nonsense RNAi. Data are mean ± s.e.m. from 5 to 12 animals. ** $P < 0.0001$ versus nonsense RNAi. (**f**) Slides of cerebella electroporated with indicated constructs plus GFP on P4 and harvested on P10 were stained with antibodies against cleaved caspase-3 or GFP. Scale bar, 50 μm. (**g**) Quantification of both caspase-3– and GFP-positive cells (number of both caspase-3 and GFP-positive cells/number of GFP-positive cells) in **f**. Data are mean ± s.e.m. from five to eight animals. In **c**, **e** and **g**, ANOVA test.

serum deprivation–induced cell death (**Fig. 5d**). We then examined whether TRPC3 or 6 has an important role in CGN survival *in vivo*. We first investigated the expression pattern of TRPC3 and 6 in the developing cerebellum by western blot analysis and found that the peak expressions of TRPC3 and 6 were between postnatal day 10 and 17 (P10 and P17; **Fig. 6a**). During this period, there is a proliferation of granule cell progenitors in the external granule cell layer (EGL), followed by maturation of granule cells into the internal granule cell layer (IGL), as well as apoptosis of a subpopulation of these cells[40].

To examine the role of TRPC3 and 6 in cerebellum development, we used an *in vivo* electroporation method for manipulating gene expression in the developing cerebellum[41]. We electroporated P4 rat cerebella with a GFP construct (pCAGGS-GFP) and found 63.3 ± 2.9% of GFP-positive cells in IGL 4 d after electroporation (P8, mean ± s.e.m., $n = 5$; **Supplementary Fig. 9** online). Furthermore, 85.7 ± 3.8% of GFP-positive cells in IGL on P8 were stained positive with the neuronal marker NeuN, but none of them were positive for the glial cell marker GFAP ($n = 5$; **Fig. 6b**).

To downregulate expression of TRPC3 or 6, we made RNAi constructs against TRPC3 or 6 and tested their effectiveness in cultured CGNs. Western blot analysis showed that TRPC3 or 6 expression was specifically downregulated to 33.5 ± 12.0% or 27.8 ± 5.8% of the control (Non) 4 d after transfection ($n = 3$; **Fig. 7a**). We then electroporated P4 cerebella with these RNAi constructs together with GFP plasmids, and counted the number of GFP-positive cells on P8, P10 and P12 (**Fig. 7b–d**). For cerebella electroporated with nonsense, TRPC3 or 6 RNAi on P4, the number of GFP-positive cells and the proportion of GFP-positive cells found in the EGL, molecular layer and IGL were not altered on P8 ($n = 5$, $P > 0.1$; **Fig. 7b,d** and **Supplementary Fig. 10** online), suggesting that TRPC3 and 6 were not important in granule cell survival, proliferation and migration during P4 to P8. In contrast, the number of GFP-positive cells was markedly reduced on P10 ($n = 5$–12; **Fig. 7c,d**) and P12 ($n = 6$–10; **Fig. 7d**) by electroporation with TRPC3 or 6 RNAi on P4. These results suggest that TRPC3 and 6 are required for granule cell survival from P10 to P12, during which the expression of TRPC3 and 6 is at its peak (**Fig. 6a**).

We further tested this notion by TUNEL (TdT-mediated dUTP nick end labeling) staining. In control experiments, the number of TUNEL-positive cells in P10 cerebella electroporated with nonsense RNAi on P4 was similar to that in P10 cerebella without electroporation, suggesting that electroporation itself did not cause cell death ($n = 5$, $P > 0.1$; **Fig. 7c,e**). However, the number of TUNEL-positive cells was greatly increased in P10 ($n = 5$–12, **Fig. 7c,e**) and P12 ($n = 6$–10, **Fig. 7e**) cerebella electroporated with TRPC3 or 6 RNAi on P4, compared with nonsense RNAi. Consistent with the results that TRPC3 or 6 RNAi did not alter the number of GFP-positive cells on P8 (**Fig. 7b,d**), the number of TUNEL-positive cells in P8 cerebella electroporated with TRPC3 or 6 RNAi on P4 was not increased relative to nonsense RNAi ($n = 5$–7, $P > 0.1$; **Fig. 7e**).

To demonstrate that downregulation of TRPC3 or 6 caused granule cell apoptosis, we generated wild-type human TRPC3 and mouse TRPC6 constructs (pCAGGS-TRPC3 or 6-IRES-GFP) to rescue granule cell apoptosis caused by TRPC3 or TRPC6 RNAi. The wild-type form of TRPC3 or 6 could be expressed in cultured CGNs (**Supplementary Fig. 7**) and the IRES-dependent GFP fluorescence could be observed in cerebellar slices (**Fig. 7c**). Electroporation with these vectors together with TRPC3 or 6 RNAi on P4 cerebella abolished the effect of TRPC3 or 6 RNAi on elevating the number of TUNEL-positive cells at P10 ($n = 6$, $P > 0.1$; **Fig. 7c,e**). These results support the notion that the TRPC3 and 6 RNAi are specific to their corresponding targets. Furthermore, downregulation of TRPC3 or 6 induced activation of caspase-3, one of the main effectors of developmental apoptotic death[42,43], as a marked increase was observed for neurons immunoreactive for both GFP and activated caspase-3 in P10 cerebella electroporated with TRPC3 or 6 RNAi on P4, in comparison with nonsense RNAi ($n = 5$–8, **Fig. 7f,g**). Taken together, these *in vivo* findings show that TRPC3 or 6 promote CGN survival in a specific time window between P10 to P12, during which the expression of TRPC3, TRPC6 and TrkB was at its peak (**Fig. 6a**).

DISCUSSION

The present experiments show that TRPC3 and 6 are required for BDNF-mediated neuronal protection, protect cultured CGNs against serum deprivation–induced cell death via CREB activation and promote CGN survival in developing cerebellum. The current findings led us to propose a model that in addition to the PI3K/Akt pathway, activation of RTKs by neurotrophic factors, such as BDNF, stimulates the PLC/IP_3R pathway to activate TRPC channels and allows elevation of $[Ca^{2+}]_i$ to stimulate the Ras/MEK/ERK and CaM/CaMK pathways that converge on CREB activation, resulting in CGN survival (**Supplementary Fig. 11** online).

Several mechanisms may explain how TRPC3 or 6 activate CREB, resulting in neuronal survival. Influx of Ca^{2+} through TRPC3 or 6 may activate CREB through the Ras/MEK/ERK pathway in a manner similar to that used by L-type VGCCs in promoting neuronal survival in response to high K^+-induced depolarization[25]. This was supported by our findings that activation of both CREB and ERK by BDNF was diminished by inhibition of TRPC channels or by downregulation of TRPC3 or 6 expression (**Fig. 4**), and that the CREB-dependent transcription by overexpression of TRPC6 was suppressed by inhibition of the Ras/MEK/ERK pathway (**Fig. 5b**). Similar to L-type VGCCs, the carboxyl terminus of TRPC channels has CaM binding sites[44], and it is thus possible that TRPC channels use CaM as a local Ca^{2+} sensor to stimulate the Ras/MEK/ERK/CREB pathway. Because downregulating TRPC3 or 6 expression completely abolished BDNF-induced CREB phosphorylation, but only partially reduced ERK phosphorylation (**Fig. 4d,e**), and inhibition of the Ras/MEK/ERK pathway also partially blocked the CREB-dependent transcription by overexpression of TRPC6 (**Fig. 5b**), it is possible that TRPC channel–mediated CREB activation requires both ERK-dependent and independent pathways. We note that inhibition of CaMK by KN62 partially inhibited the enhancement of CREB-dependent transcription (**Fig. 5b**). Furthermore, inhibition of both the Ras/MEK/ERK and CaM/CaMK pathways abolished TRPC-mediated CREB-dependent transcription (**Fig. 5b**) and survival effect (**Fig. 5e**). Thus, both the Ras/MEK/ERK and CaM/CaMK pathways contribute to TRPC3- and 6-mediated CREB activation, resulting in neuronal survival.

In addition to the TRPC/Ca^{2+}/CREB pathway, the PI3K/Akt pathway has a critical role in mediating survival signals in a wide range of neuronal cell types[11,12,16], but blocking TRPC channels or downregulating TRPC3 or 6 expression had no effect on BDNF-induced Akt activation (**Fig. 4**), consistent with the previous reports that Akt activation by BDNF is independent of extracellular Ca^{2+} influx[45,46]. Moreover, PI3K inhibitor, LY294002, known to abolish the BDNF-mediated survival effect, has no effect on BDNF-induced phosphorylation of CREB[15], thus the concurrent requirement of Akt for the survival effect of BDNF may be attributed to an Akt action in parallel with CREB activation[16]. Alternatively, the survival effect of BDNF may require both TRPC/Ca^{2+}/CREB-dependent gene transcription and other Akt-dependent cellular events, such as BAD

phosphorylation[47]. Together, both the TRPC/Ca^{2+}/CREB pathway and PI3K/Akt pathway are required for BDNF-mediated protection (**Supplementary Fig. 11**).

Our *in vivo* findings show that downregulation of TRPC3 or 6 activated caspase-3 and led to CGN apoptosis in a specific time window in IGL (**Fig. 7c–g**). Therefore, activation of TRPC3 and 6 is required for this neuronal survival *in vivo*. Because PLC activation is critical for TRPC channel activation[4,6], it is possible that BDNF binding to its cognate receptor, TrkB, stimulates PLC to activate TRPC3 and 6, leading to neuronal survival *in vivo*. Several lines of evidence support this explanation. First, TRPC3 and 6 are required for the BDNF neuroprotective effect (**Fig. 1**), BDNF-triggered $[Ca^{2+}]_i$ elevation (**Fig. 3**) and BDNF-induced CREB/ERK activation (**Fig. 4**). Second, the peak expression of TRPC3 and 6 in developing cerebellum was similar to that of TrkB (**Fig. 6a**). However, it is also possible that activation of other receptor tyrosine kinases (for example, insulin-like growth factor–1 receptor, **Supplementary Fig. 12** online) or G-protein-coupled receptors may contribute to TRPC3 and 6 activation, resulting in neuronal survival *in vivo*.

In conclusion, our findings point to a previously unknown role of TRPC3 and 6 in promoting CGN survival and the existence of a TRPC3 and 6-dependent CREB signaling pathway that is essential for TRPC-dependent neuronal survival. The demonstration of TRPC-dependent neuronal survival both in cultured neurons and in rat brain further expands our understanding of the diverse functions of TRP channels.

METHODS

DNA constructs and siRNAs. The IRES (pIRES2-EGFP, BD Bioscience Clontech) was inserted into the pCAGGS-GFP vector to generate pCAGGS-IRES-GFP. The TRPC3 and 6 were subcloned into the pCAGGS-IRES-GFP vector. Hairpin siRNA sequence (5′-AACGCCTCACACCGGTTTGAATTCAAGAGATTCAAACCGGTGTGAGGCGTT-3′) against 947–969 bp in the coding region of rat TRPC3 (NM_021771) and siRNA sequence (5′-AACGGCCTCATGATTATTTCTTTCAAGAGAAGAAATAATCATGAGGCCGTT-3′) against 734–754 bp in the coding region of rat TRPC6 (NM_053559) were inserted in pSuper vector[48]. Nonsense hairpin RNAi sequence is 5′-TTCTCCGAACGTGTCACGTTTCAAGAGAACGTGACACGTTCGGAGAA-3′. siRNAs were obtained from GeneChem Co and the sequences of these siRNAs are in **Supplementary Table 1** online. All other reagents were from Sangon.

Chemical reagents and antibodies. Details in **Supplementary Methods** online.

Cerebellum *in vivo* electroporation. Cerebellum *in vivo* electroporation was conducted as described previously[41] and the animal protocol was approved by the Animal Committee of Institute of Neuroscience. Briefly, P4 pups (SLAC Laboratory Animal Co.) were anesthetized, after which the parietal bone was exposed and pierced, and DNA in PBS containing 0.01% fast green was injected on the surface of the cerebellum cortex. Electroporation (550-ms square pulses of 140 V with 1,000-ms intervals) was then carried out using an Electro Square Porator (ECM 830, BTX). We perfused the pups with 4% paraformaldehyde 2 to 8 d after electroporation. We harvested and fixed the cerebellum in 4% paraformaldehyde for 2 h at 4 °C, and cryoprotected in 20% sucrose overnight. Thereafter, the cerebellum was embedded in OCT compound (Sakura) and frozen by dry ice. Sagittal cryosections (14–20 μm) were made and examined by immunohistochemistry and TUNEL staining. We injected 10 μg μl^{-1} and a total 3 μl of target constructs.

Western blot, immunohistochemistry and TUNEL staining. We extracted cultured CGNs and neonatal cerebellum in a lysis buffer (10 mM Tris-Cl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton-X100, 1 mM sodium orthovanadate, 50 mM NaF, 1 mM PMSF, 1 mM aprotinin, 1 mM leupeptin and 5 mM DTT) as described previously[17]. The proteins were Western-blotted with the indicated antibodies and then visualized with the ECL system (Amersham). We scanned the bands and determined the density of the bands by ImageQuant 5.2 software (Amersham). For immunohistochemistry of brain sections, the cerebellum sections were incubated with blocking solution (3% normal goat serum and 0.2% Triton X-100 in PBS) and then with primary antibodies diluted in blocking solution. Sections were then washed with PBS and incubated with the secondary antibody. After three washes with PBS, the cell nuclei were stained with DAPI. The immunofluorescent images were taken. For TUNEL and GFP staining, the slices were incubated with anti-GFP antibody. We used ApopTag *in situ* Apoptosis detection kit (Chemicon) to label the apoptotic cells, following the manufacturer's instructions. Apoptotic cells were visualized as black spots by NovaRED™ Substrate kit (Vector Labs).

Quantification of GFP- and TUNEL-positive cells. We counted the GFP-positive cells in electroporated cerebellum and averaged the numbers from at least three serial slices per animal. To quantify the number of TUNEL-positive cells in GFP-positive slice, we counted the TUNEL-positive cells and averaged from three GFP-positive serial slices per animal. The statistical significance was determined by variance analysis (ANOVA), using the statistical package (SSPS Inc.).

Primary cultures, survival assays and transfection. We obtained CGNs by dissociating the cerebellum of 6-d-old Sprague-Dawley rats as described[49]. The CGNs were grown in basal medium Eagle (BME), supplemented with 10% FBS, 25 mM KCl, 2 mM glutamine, 100 U ml^{-1} penicillin and 0.1 mg ml^{-1} streptomycin. The CGNs cultured for 4 d *in vitro* (P6 + 4 DIV) were washed with BME containing 5 mM potassium without serum (serum deprivation) and then incubated with this medium for 48 h to induce neuronal death in the absence or presence of various agents or vehicles, such as dimethyl sulfoxide. Unless stated, BDNF was used at 50 ng ml^{-1} and SKF96365 at 20 μM. We determined the apoptotic rate by counting the PI-positive cells in all the cells. In biochemical experiments, the BME (10% FBS + 25 mM KCl) was switched to medium without serum for 2 h before BDNF treatment and the inhibitors or dimethyl sulfoxide were added 15 min before BDNF. We transfected CGNs (2×10^6) with 1 μg of the constructs or 2 μg siRNA each by the rat neuron Nucleofector Kit (Amaxa) following the manufacturer's instructions. We performed survival or biochemical analyses 48 h after plasmid transfection or 96 h after siRNA delivery.

Calcium measurement and luciferase assay. Detail in **Supplementary Methods**.

Note: Supplementary information is available on the Nature Neuroscience website.

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AUTHOR CONTRIBUTIONS

Y.J. and J.Z. conducted the experiments and wrote the manuscript. Y.T. prepared some RNAi constructs, and Y.W. supervised the project and wrote the manuscript.

COMPETING INTERESTS STATEMENT

The authors declare no competing financial interests.

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