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SIGNALING AND CELL PHYSIOLOGY

TRPM2 mediates the lysophosphatidic acid-induced neurite retraction in the developing brain

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Abstract Intracellular Ca^{2+} signal is a key regulator of axonal growth during brain development. As transient receptor potential (TRP) channels are permeable to Ca^{2+} and mediate numerous brain functions, it is conceivable that many TRP channels would regulate neuronal differentiation. We therefore screened TRP channels that are involved in the regulation of neurite growth. Among the TRP channels, the *Trpm2* level was inversely associated with neurite growth. TRPM2 was highly expressed in embryonic brain. Pharmacological perturbation or knockdown of TRPM2 markedly increased the axonal growth, whereas its overexpression inhibited the axonal growth. Addition of ADP ribose, an endogenous activator of TRPM2, to PC12 cells significantly repressed the axonal growth. TRPM2 was actively involved in the neuronal retraction induced by cerebrospinal fluid-rich lysophosphatidic acid (LPA). More importantly, neurons isolated from the brain of *Trpm2*-deficient mice have significantly longer neurites with a greater number of spines than those obtained from the brain of wild-type mice. Therefore, we conclude that TRPM2 mediates the LPA-induced suppression of axonal growth, which provides a long-sought mechanism underlying the effect of LPA on neuronal development.

Keywords TRP channels · TRPM2 · Neurite outgrowth · Neuritogenesis · Lysophosphatidic acid

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Introduction

Neuritogenesis is an initial cellular process in neuronal differentiation [5]. During brain development, outgrowing neurites are guided to their targets by extracellular substances, either attractive or repulsive molecules [47]. In addition, the selective elimination of axons or dendrites without death of the parent neurons is also an essential process for pathfinding or axonal connectivity [28]. Thus, balance between axonal outgrowth and retraction appears to be critical for brain development. Cytoskeletal assembly such as dynamic polymerization and depolymerization of actin and tubulin is implicated in the cellular mechanism underlying axonal growth and retraction [23, 28]. In addition, a small GTP-binding protein, RhoA is a key regulator of the cytoskeletal assembly [28]. Activation of RhoA induces neurite retraction in primary cultured neurons and in vivo [2, 27].

Lysophosphatidic acid (LPA) is rich in serum and cerebrospinal fluid in the developing brain [42]. LPA1 receptor is highly expressed in the ventricular zone of the developing cerebral cortex [12]. LPA is secreted from neurons and glia and therefore acts as an autocrine or paracrine mediator that affects neighboring cells during brain development [42, 49].

LPA induces neurite retraction, cell rounding, and growth cone collapse in cortical neurons [8, 45]. This LPA-induced retraction is associated with RhoA activity [48]. Although LPA plays a key role in neuronal differentiation, its signaling pathway has not yet been defined.

In neurons, one of the key regulators of neuronal differentiation is intracellular Ca^{2+} [9, 29]. A source of the intracellular Ca^{2+} signal in neurons is the transient receptor potential (TRP) channels, which are nonselective cation channels permeable to Ca^{2+} [34]. Indeed, a few TRP channels are known to regulate neurite growth. For example, TRPC1 is required for growth cone turning responses to gradients of netrin-1 [43]. TRPC3 mediates the formation of dendritic spines induced by the brain-derived neurotrophic factor [1]. Moreover, TRPV4 promotes nerve growth factor (NGF)-mediated axonal growth in peripheral neurons [18], and their channelopathy is associated with congenital degeneration of peripheral nerve such as scapuloperoneal spinal muscular atrophy or Charcot–Marie–Tooth disease [35]. TRPC5 is also known to suppress axonal elongation and regulate growth cone morphology in neurons [10]. Despite the apparent actions of these TRP channels in brain development, the roles of other TRP channels in neuronal differentiation remain unknown. Hence, we screened TRP channels for determining their roles in regulating axonal growth. Among these TRP channels, we found that TRPM2 was inversely related to neurite outgrowth. Subsequently, we sought to determine the signal pathway upstream of TRPM2. As a result, we found that TRPM2 mediates the LPA-induced neurite retraction.

Results

Screening of TRP channels associated with neurite outgrowth

We first sought to identify TRP channels that were associated with neurite outgrowth. In line with this approach, the mRNA levels of 22 TRP channels were quantified with real-time quantitative polymerase chain reaction (RTQ-PCR) in PC12 cells whose neurites were stimulated by treatment with NGF or inhibited by co-treatment with mitogen-activated protein kinase kinase (MEK) inhibitors [39]. PC12 cells were used because they have been commonly employed as a model for studying the underlying mechanisms of neuronal differentiation [25]. When PC12 cells were treated with NGF for 6 days, outgrown neurites were visible, which were blocked by co-treatment with a MEK inhibitor, UO126 or PD98059 (Fig. 1a). Compared with control values measured in native PC12 cells, NGF treatment notably increased neurite length as well as the number of cells with neurites (Fig. 1a–c). Along

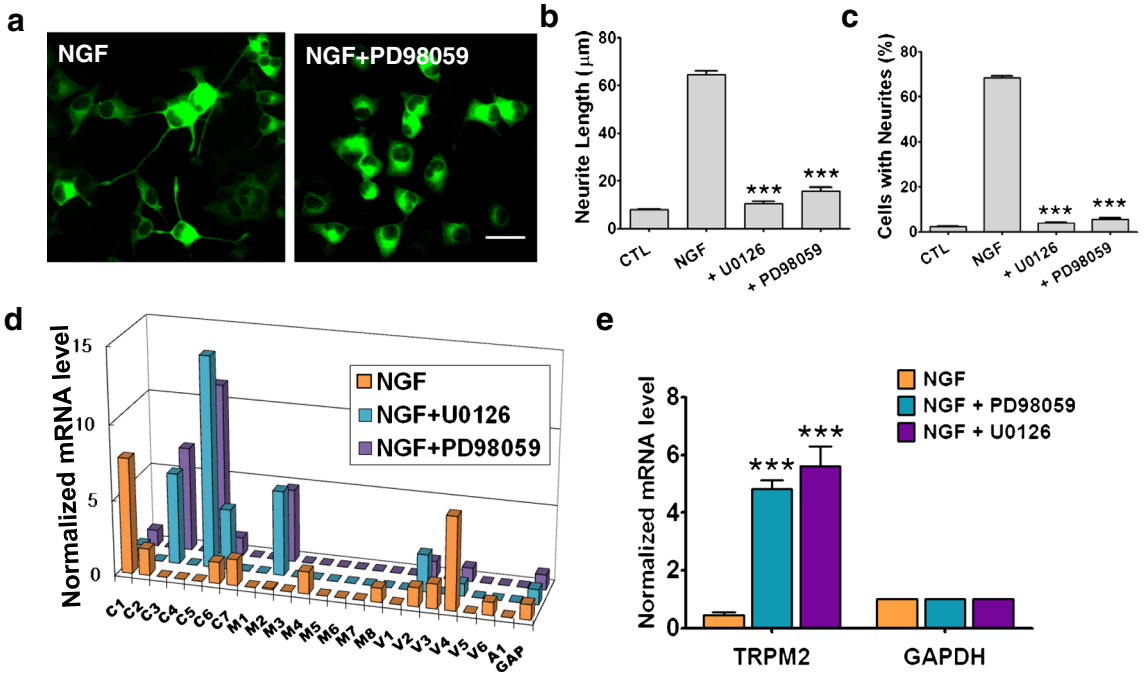


Fig. 1 Changes in mRNA levels of TRP channels along with neurite growth in PC12 cells. **a**–**c** NGF treatment of PC12 cells for 6 days stimulated neurite outgrowth, whereas co-treatment with MEK inhibitors (U0126 or PD98059) prevented neurite outgrowth (**a**, **b**) and reduced the percentage of cells with neurites (**a**, **c**). To visualize neurites, cells were immunostained with antibody raised against neuron-specific class III beta-tubulin, Tuj-1 conjugated with Alexa Fluor® 488 goat anti-mouse IgG. *Scale bar* represents 10 μm. ***$p<0.001$ compared with NGF-treated cells (ANOVA and Tukey's post-hoc test). *Error bars* indicate S.E.M. **d** mRNA levels of 22 TRP channels in NGF-treated or MEK inhibitor-treated PC12 cells were quantified using RTQ-PCR. Transcript levels of TRP channels were normalized by GAPDH amplification. **e** The transcript levels of TRPM2 in PC12 cells treated with NGF or NGF and MEK inhibitors. TRPM2 mRNA levels were significantly increased when PC12 cells were treated with NGF and MEK inhibitors

with the neurite growth, the TRPC1 and TRPV4 transcripts levels were greatly increased, whereas those of TRPC3, TRPC5, and TRPM2 were decreased (Fig. 1d). By contrast, when PC12 cells were co-treated with NGF and MEK inhibitors, the mRNA levels of TRPC3, TRPC5, and TRPM2 were markedly enhanced. These results confirm that TRPC1, TRPC3, TRPC5, and TRPV4 channels are closely associated with modulation of axonal outgrowth as previously reported [1, 10, 18, 43]. However, as the action of TRPM2 on axonal outgrowth is unknown, we sought to determine the function of TRPM2 in neuritogenesis in detail (Fig. 1e).

TRPM2 is expressed in embryonic brain

To be a regulator of neurite outgrowth, TRPM2 is expected to be expressed in the developing brain. Indeed, strong TRPM2 immunoreactivity was observed in the ventricular zone of the lateral ventricle, hippocampus, and neocortex in the brain of 16-day embryos (E16) (Fig. 2a, b). In addition, TRPM2 was highly colocalized with nestin, a marker for neural stem cells in the ventricular zone [24] (Fig. 2a). TRPM2 immunoreactivity was also found in cells stained with neuron-specific class III β-tubulin (Tuj-1), a marker for immature neurons in the intermediate zone, where the cortical and striatal immature interneurons migrated from the piriform cortex (Fig. 2b). TRPM2 was also observed along the dendrites of cultured immature cortical neurons stained by Tuj-1 (Fig. 2c). In addition, the TRPM2 mRNA level in embryonic brain reached a peak at postnatal day 1 when axonal outgrowth was actively generated (Fig. 2d) [6, 26]. These results suggest that TRPM2 is highly expressed in immature neurons in the developing brain.

TRPM2 inversely regulates neurite growth

To investigate further whether TRPM2 influences neurite outgrowth, we then overexpressed mouse TRPM2 in PC12 cells. To visualize neurites, cells were stained with TuJ-1. As TRPC5 is known to inhibit neurite growth [10], it was used as a positive control. When treated with NGF, PC12 cells transfected with an empty vector (pcDNA3.1) developed elongated neurites, whereas PC12 cells transfected with TRPM2 had markedly shorter neurites and a significantly less number of cells with neurites (Fig. 3a, b). To examine whether knockdown of *Trpm2* augments neurite growth, small interfering *Trpm2* RNA (siRNA) or scrambled siRNA was

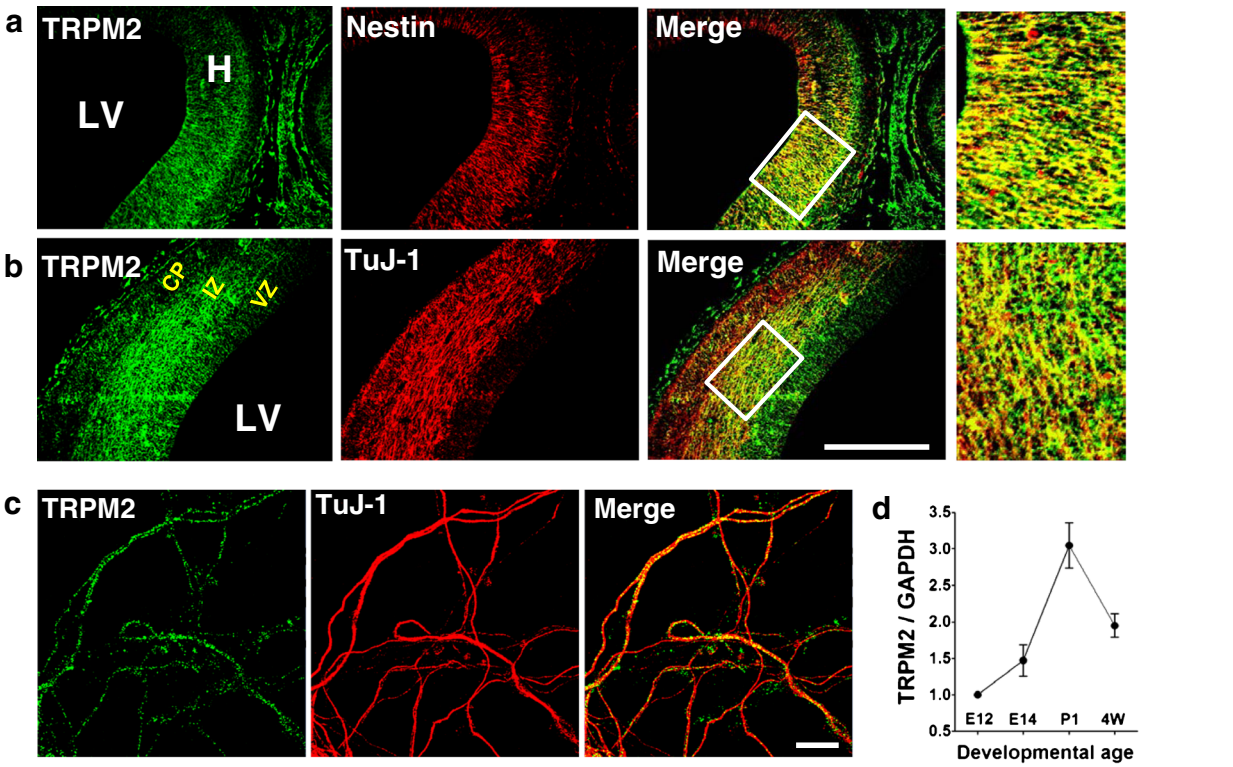


Fig. 2 Expression pattern of TRPM2 in embryonic brain. **a**–**b** Brain sections from E16 mice were stained with antibodies specific for TRPM2 (*green*), nestin (*red*), and Tuj-1 (*red*). TRPM2 was abundant around the lateral ventricle (*LV*) near the hippocampus (*H*). *VZ* ventricular zone, *IZ* intermediate zone, *CP* cortical plate. *Scale bar* represents 100 μm. **c** Primary cultures of early immature neurons isolated from the cortex were stained with antibodies specific for TRPM2 (*green*) and Tuj-1 (*red*). *Scale bar* represents 10 μm. **d** TRPM2 level was steeply increased during brain development period

transfected into PC12 cells. To identify siRNA-transfected cells, *Trpm2* siRNAs were labeled with Fluorescein® (Fig. 3c). The transfection of *Trpm2* siRNA to PC12 cells effectively reduced its mRNA levels (Fig. 3d). As shown in Fig. 3, PC12 cells transfected with scrambled siRNA had a neurite length comparable to that in nontransfected cells. By contrast, cells transfected with TRPM2 siRNA showed a marked increase in neurite length and in the percentage of cells with neurites (Fig. 3e). In cells transfected with scrambled siRNA but without NGF treatment, no elongated neurites were observed (data not shown). Furthermore, the presence of functional endogenous TRPM2 in PC12 cells was also confirmed because application of 300 μM ADP ribose (ADPR), an endogenous activator of TRPM2, activated robust currents in PC12 cells (Fig. 3f).

To determine whether pharmacological inhibitors of TRPM2 promote neurite growth, we treated PC12 cells with flufenamic acid (FFA), clotrimazole, or econazole [13, 14]. Application of 100 μM FFA, 20 μM clotrimazole, or 20 μM econazole significantly increased the neurite length and proportion of cells with neurites (Fig. 4a, b). Furthermore, 50 μM FFA, 5 μM clotrimazole, or 5 μM econazole also elicited a significant increase in the neurite length in primary cultures of rat embryonic cortical neurons (Fig. 4c, d). These results further support that TRPM2 suppresses axonal growth in PC12 cells and cortical neurons.

ADPR is known to be an endogenous activator of TRPM2 [36, 41]. Therefore, application of ADPR to cells should suppress neurite growth. However, the cell membrane is impermeable to ADPR, and therefore it cannot be delivered inside the cells when applied extracellularly. Utilizing its nucleotide-like structure, we transfected Cy3-labeled ADPR into PC12 cells with lipofectamine. Cy3-labeled ADPRs were visible throughout the cytosol in neurons after transfection

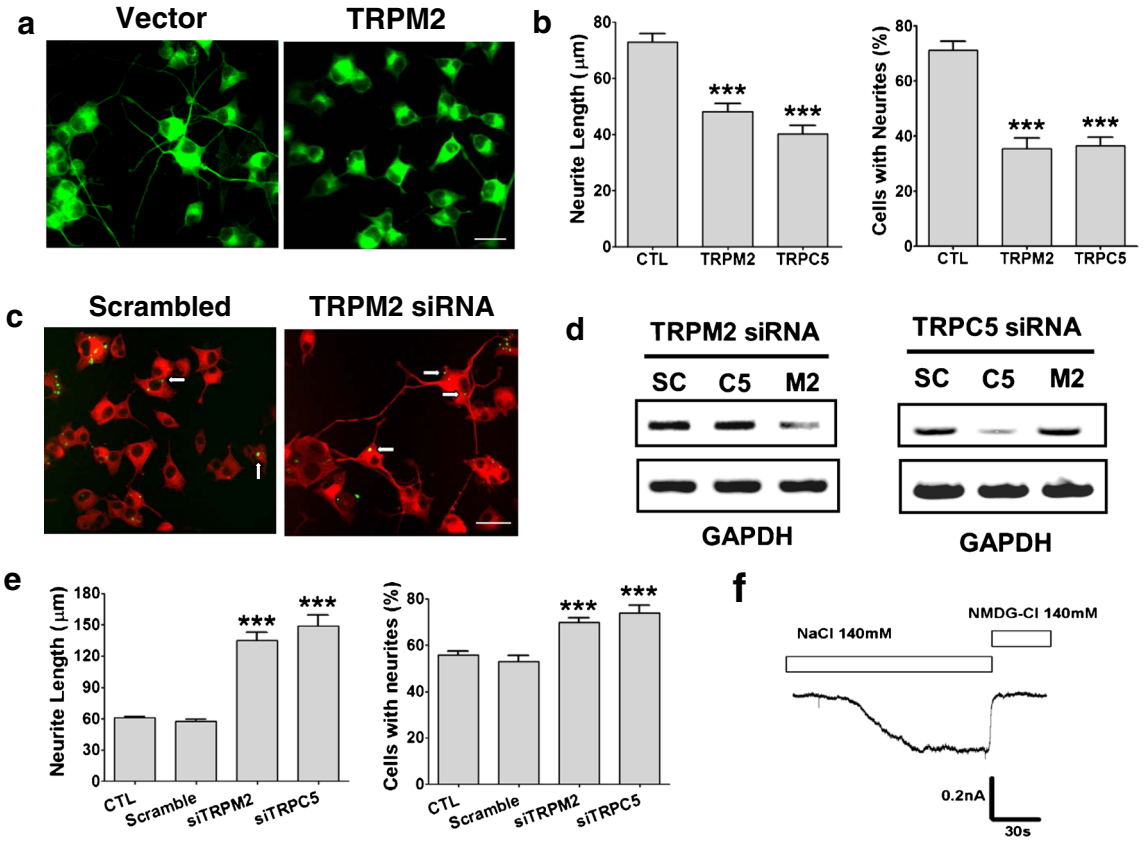


Fig. 3 TRPM2 negatively regulates neurite growth. **a** The expression of mouse TRPM2 suppressed neurite growth. PC12 cells were cultured for 6 days after NGF supplementation. pCDNA3.1-TRPM2 (*right*) or empty vector (*left*) was transfected into PC12 cells 1 day before the NGF supplementation. **b** Effect of TRPM2 transfection on neurite length (*left*) and on percentage of cells with neurites (*right*). ***$p<0.001$ compared with mock-transfected cells (*CTL*). **c** Knockdown of TRPM2 by TRPM2 siRNA augmented neurite growth. Mouse TRPM2 siRNA and scrambled siRNA were labeled with Fluorescein®. Cells were immunostained in red with anti-Tuj1 conjugated with Alexa Fluor 546 goat anti-mouse IgG. Green granules (*arrows*) indicate transfected siRNAs. siRNAs were transfected 1 day before NGF supplementation. **d** The levels of TRPM2 or TRPC5 (for control) transcripts in PC12 cells after siRNA transfection. mRNA levels of TRPM2 (*M2*) or TRPC5 (*C5*) were measured by reverse transcription PCR reaction. *Left lanes* represent the levels of PCR products of scrambled siRNA-transfected cells (*SC*). GAPDH was chosen as a negative control. **e** Effects of TRPM2 and scrambled siRNAs on neurite length (*left*) and on percentage of cells with neurites (*right*). ***$p<0.001$ versus cells transfected with scrambled siRNA. **f** A whole cell current of a PC12 cell was activated by ADPR, suggesting that TRPM2 is functional in PC12 cells. Pipette solution contained 300 μM ADPR and 1 μM Ca^{2+}. NMDG-Cl was perfused to the bath to block the cation current

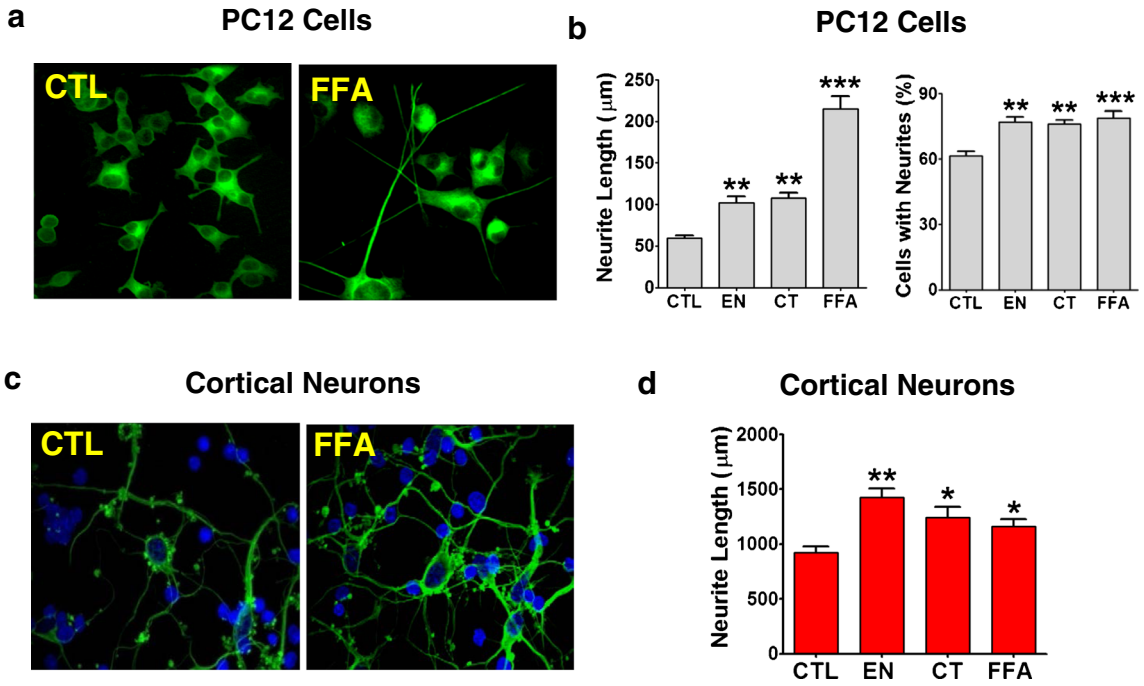


Fig. 4 Pharmacological perturbation of TRPM2 increases axonal growth. **a** The representative picture of treatment with flufenamic acid (*FFA*), a TRPM2 blocker in PC12 cells. **b** Treatment with TRPM2 blockers, econazole (*EN*), clotrimazole (*CT*), or FFA significantly increased neurite lengths (*left*) and percentage of PC12 cells with neurites (*right*). ***$p<0.001$; **$p<0.01$ versus the untreated control (*CTL*; ANOVA and Tukey's post-hoc test). **c** The representative picture of treatment with FFA, a TRPM2 blocker in primary cultured cortical neurons. **d** Treatment with TRPM2 blockers, EN, CT, or FFA significantly increased neurite lengths in primary cultures of cortical neurons

(Fig. 5a). Indeed, PC12 cells transfected with ADPR showed a significantly shorter neurite length ($p<0.001$, $n=207$) and a smaller percentage of these cells with neurites ($p<0.01$, $n=8$) when compared with nontransfected control cells (Fig. 5b).

Neurons from *Trpm2*$^{-/-}$ mice have elongated neurites with a greater number of spines

We now determined axonal growth in cortical neurons isolated from the embryonic brain of *Trpm2*$^{-/-}$ mice. The lengths of

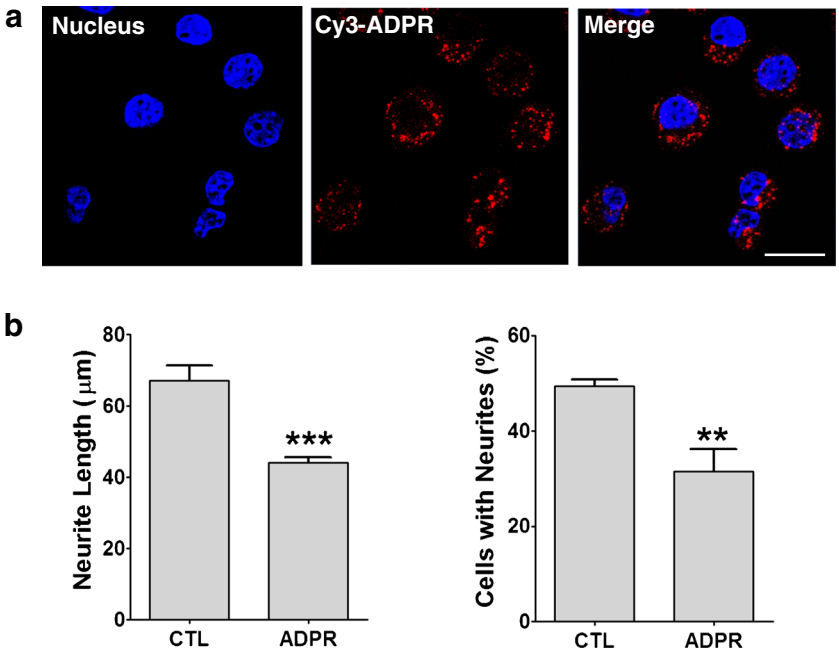


Fig. 5 Cytosolic ADPR regulates neurite growth in PC12 cells. **a** The transfection of Cy3-labeled ADPR (*red*) into PC12 cells was incorporated in the cytoplasm. *Scale bar* represents 10 μm. **b** The ADPR overload in PC12 cells reduced the neurite growth (*left*) and percentage of cells with neurites (*right*). ***$p<0.001$; **$p<0.01$ versus the untreated control (*CTL*' Student's *t*-test)

neurites were automatically calculated by a NeuriteTracer software [38]. As shown in Fig. 6, primary cultured cortical neurons from *Trpm2*$^{-/-}$ mice elicited significantly longer neurites than neurons from wild-type mice ($p<0.05$, $n=15$) (Fig. 6a, b). We also counted the number of spines in the neurites. After neuronal maturation for 16 days, spines were visible in neurites of cortical neurons from WT mice (Fig. 6c). However, the number of spines in neurons from *Trpm2*$^{-/-}$ mice was significantly augmented compared with that in neurons from wild-type mice ($p<0.001$, $n=24$) (Fig. 6d). These results clearly suggest that TRPM2 actively regulates neuritogenesis and synaptogenesis.

TRPM2 mediates LPA-induced neurite retraction

LPA is a major determinant of neurite growth inhibition during brain development [12]. This inhibitory action is mediated via a LPA receptor [30]. Despite its long-known role in neuritogenesis, the signaling pathway downstream to the LPA receptor has not been well understood. Because TRPM2 and LPA are negative regulators of neuritogenesis, there could be a possible link between LPA and TRPM2. Indeed, TRPM2 was colocalized with a LPA1 receptor in the developing embryonic brain as well as in the primary cultures of cortical neurons (Fig. 7a, b). As reported previously [30], application of 2 μM LPA to PC12 cells suppressed NGF-induced neurite growth and reduced the percentage of cells with neurites (Fig. 7c). This LPA-induced neurite inhibition was reversed by TRPM2 blockers (Fig. 7c). These TRPM2 blockers also reversed LPA-induced axonal inhibition in primary cultures of embryonic cortical neurons (Fig. 7d). These results suggest that the LPA-induced suppression of neurite growth is mediated by TRPM2.

LPA stimulates poly-ADPR polymerase 1

Therefore, we sought to determine the possible signaling pathway between LPA and TRPM2. We investigated whether LPA directly activates TRPM2. As shown in Fig. 8a, application of LPA to an inside–out patch of HEK 239 T cell transfected with *Trpm2* failed to induce currents whereas application of 30 μM ADPR to the patch readily activated currents. Thus, LPA appears not to activate TRPM2 directly (Fig. 8a).

ADPR is a metabolic degradation product of poly-ADPR that is synthesized by poly-ADPR polymerase 1 (PARP-1)

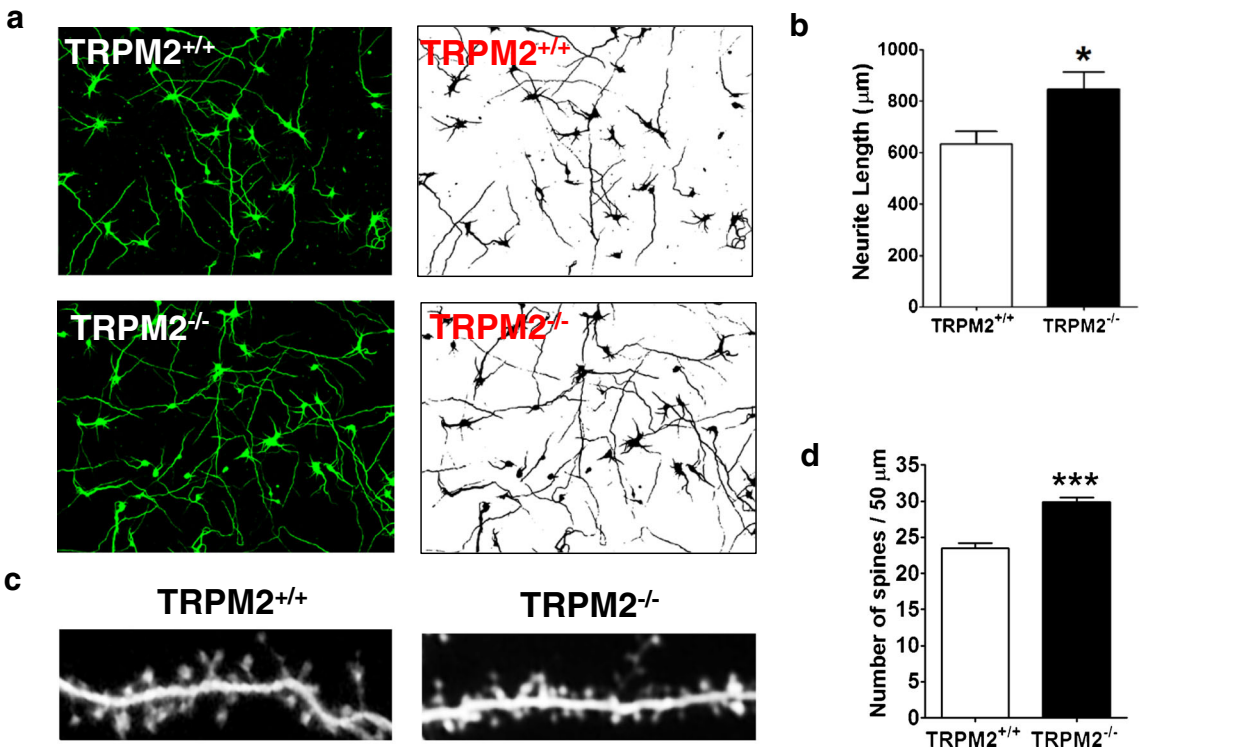


Fig. 6 The genetic deletion of TRPM2 shows longer elongation of neurites and more increased number of spines comparing to wild types as processing neuronal differentiation. **a** The primary cultured cortical neurons from *TRPM2*$^{+/+}$ (*upper*) and *TRPM2*$^{-/-}$ (*lower*) mice were stained with Tuj-1 and reconstructed by the NeuriteTracer software to automatically calculate the axonal length (*upper*and *lower right*). **b** The neurite length of cortical neurons from *TRPM–*$^{-/-}$ mice was longer than that of cortical neurons from *TRPM2*$^{+/+}$ mice. *$p<0.05$ versus *TRPM2*$^{+/+}$ (Student's *t* test). **c** The morphology of spines from *TRPM2*$^{+/+}$ and *TRPM2*$^{-/-}$ after differentiation during 16 days was visible by infused GFP at 10 days after culture. **d** The number of spines was increased in the neurons from *TRPM2*$^{-/-}$ mice compared with that in the neurons from *TRPM2*$^{+/+}$ mice. ***$p<0.001$ versus TRPM2$^{+/+}$ (Student's *t* test)

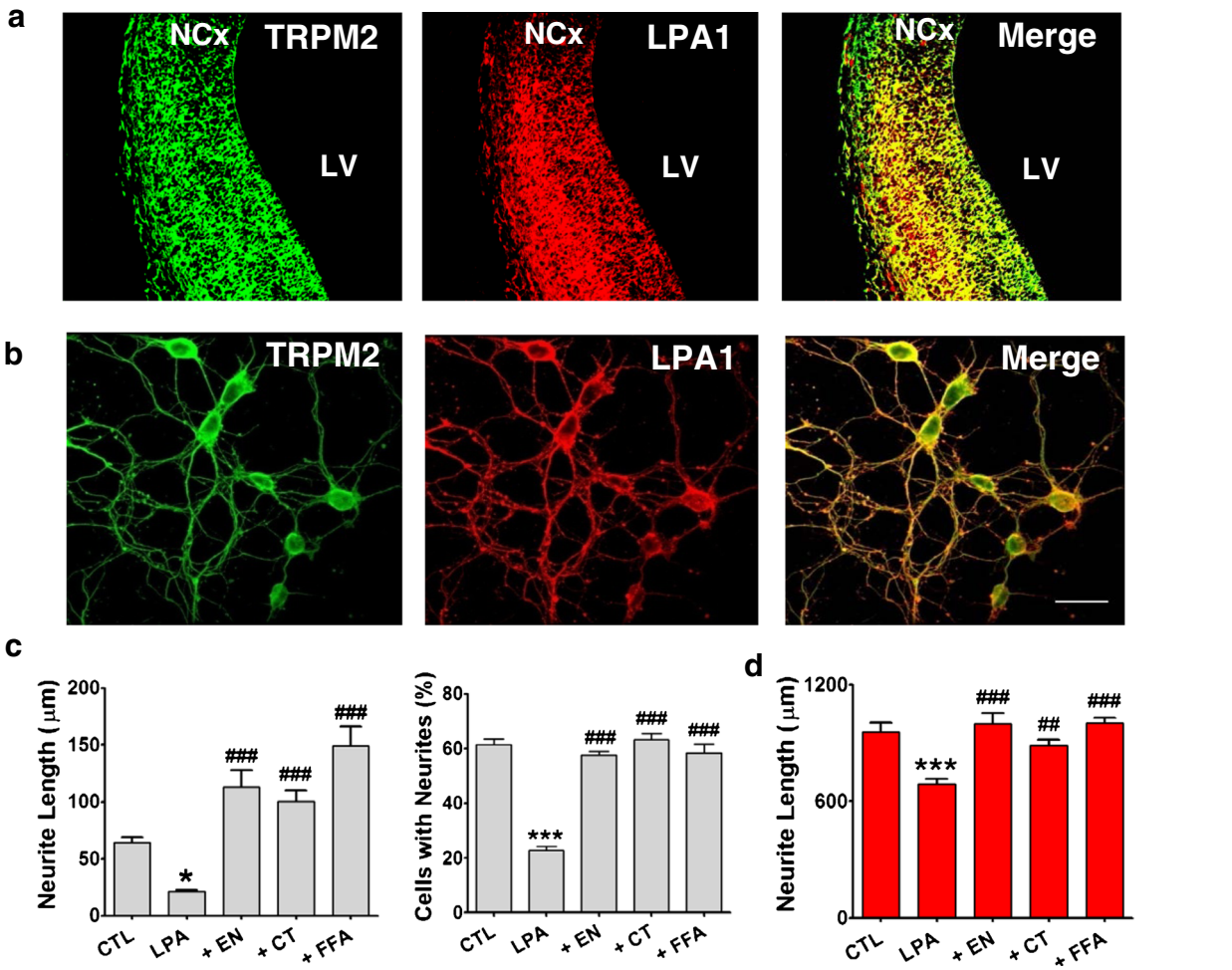
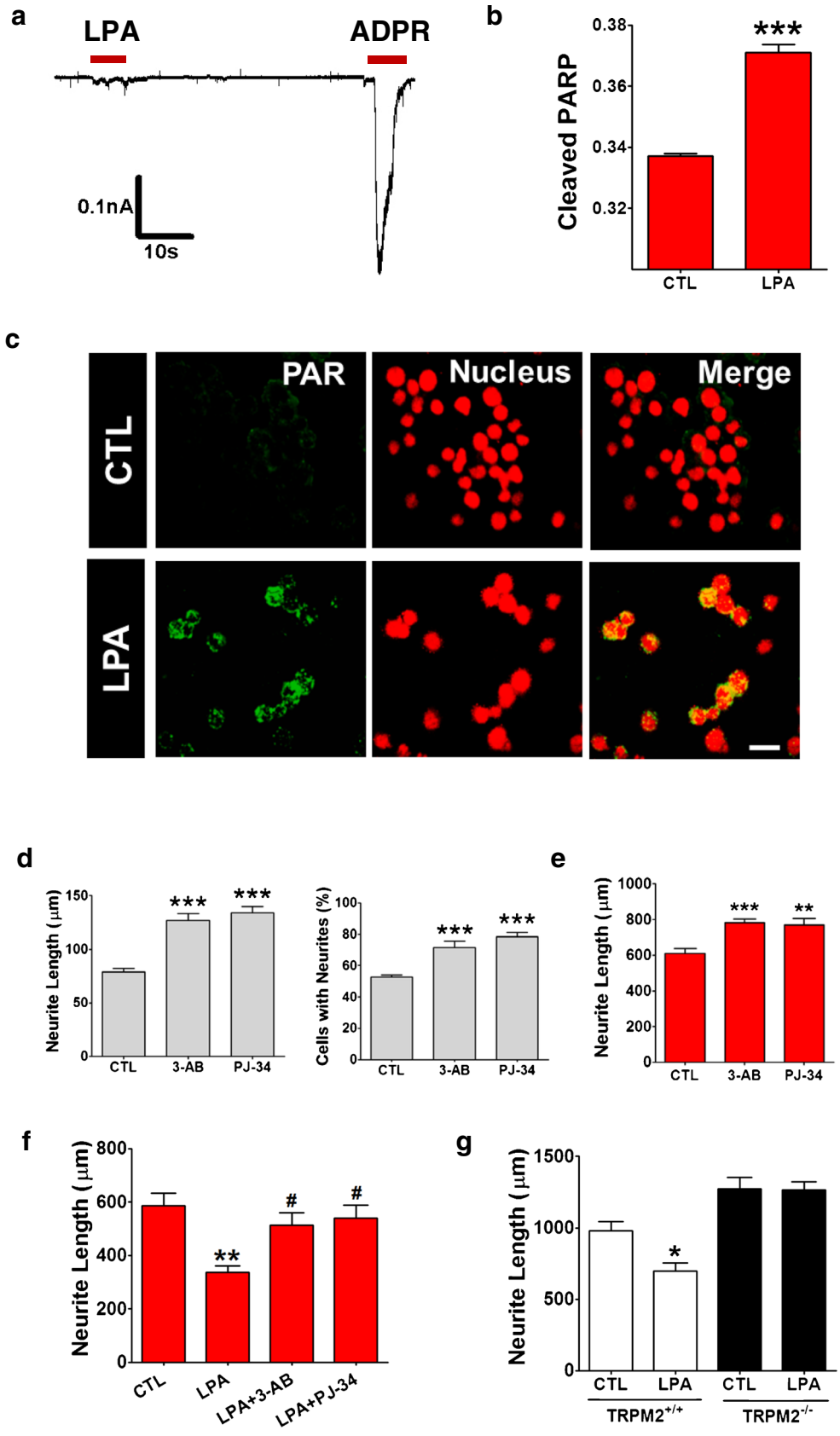


Fig. 7 The blockage of TRPM2 reverses LPA-induced suppression of neurite growth. **a**–**b** TRPM2 and LPA1 receptor were colocalized in the developing embryonic brain (**a**) and cultured cortical neurons (**b**). **c** LPA significantly suppressed neurite growth in PC12 cells, whereas TRPM2 blockers reversed LPA-induced suppression of neurite length (*left*) and the percentage of PC12 cells with neurites (*right*). PC12 cells were cultured with NGF and 2 μM LPA for 6 days before fixation. TRPM2 blockers (20 μM EN, 20 μM CT, and 200 μM FFA) were added to these cells on the second day after culture. *$p<0.05$; ***$p<0.001$ versus the untreated control (*CTL*); $^{\#\#\#}p<0.001$ versus the LPA-treated group (ANOVA and Tukey's post-hoc test). **d** LPA significantly suppressed neurite growth of cortical neurons whereas TRPM2 blockers reversed LPA-induced suppression of neurite growth. Cortical neurons were treated with 5 μM LPA for 2 days. TRPM2 blockers (20 μM EN, 20 μM CT, and 200 μM FFA) were applied to cells 2 days before fixation. ***$p<0.001$ versus the untreated CTL; $^{\#\#}p<0.01$; $^{\#\#\#}p<0.001$ versus the LPA-treated group ($n=15$)

[15]. Therefore, we determined whether LPA stimulates PARP-1. As shown in Fig. 8b, application of LPA to cortical neurons significantly augmented the production of a cleaved PARP-1, an active form of PARP-1. Furthermore, immunofluorescent analysis with anti-poly-ADPR antibody demonstrated a marked increase in the level of poly-ADPRs in the nucleus of cells treated with LPA but not in untreated cells (Fig. 8c). In addition, the length and number of neurites were significantly increased by application of PARP-1 blockers, 3-aminobenzamide (3-AB) and *N*-(6-oxo-5,6-dihydrophenanthridin-2-yl)-*N*,*N*-dimethylacetamide (PJ-34) in PC12 cells (Fig. 8d), as well as in primary cultures of cortical neurons (Fig. 8e). The LPA-induced axonal reduction was reversed by co-treatment of 3-AB or PJ-34 (Fig. 8f). More importantly, this inhibitory action of LPA on wild-type cortical neurons was completely absent in the cortical neurons from *Trpm2*-deficient mice (Fig. 8g). Taken together, these results strongly suggest that LPA regulates neurite retraction via the PARP-1/TRPM2 pathway in embryonic cortical neurons.

Discussion

We identified a new candidate, TRPM2 among the TRP channel family that controls axonal growth. TRPM2 is required for negative control of axonal growth in developing neurons. TRPM2 was expressed in the lateral ventricle of the developing brain. Overexpression of TRPM2 retarded NGF-induced neurite growth, whereas its pharmacological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◀ **Fig. 8** LPA stimulates the activation of PARP-1 and regulates axonal growth. **a** LPA failed to activate TRPM2. LPA and ADPR were applied to an inside–out membrane patch isolated from a HEK-293 T cell transfected with *Trpm2*. Note that application of 30 μM ADPR caused currents while application of 5 μM LPA failed to. **b** The PARP-1 activity was estimated by the level of the active (cleaved) form of PARP-1 measured at 18 h of culture, using specific antibody raised against cleaved PARP-1. LPA (20 μM) was applied to cortical neurons 12 h before the ELISA test. ***$p<0.001$ versus LPA-treated cortical neurons ($n=8$; Student's t test). **c** LPA stimulated the actual level of poly-ADPR (*PAR*) in nuclei. PARs were immunostained in green fluorescence with its antibody. For nuclear localization, nuclei were stained with Hoechst 33258. LPA was applied to PC12 cells 5 min before fixation. *Scale bar* represents 10 μm. **d** PARP-1 inhibitors, 3-AB (10 μM) and PJ-34 (10 μM) caused an increase in the neurite growth (*left*) and percentage of cells with neurites (*right*) in PC12 cells. ***$p<0.001$ versus the untreated control (*CTL*; $n=179$). **e** PARP-1 inhibitors, 3-AB (5 μM) and PJ-34 (5 μM) caused an increase in the axonal growth in primary cultured cortical neurons. **f** PARP-1 inhibitors, 3-AB (10 μM) and PJ-34 (10 μM) reversed the LPA-induced reduction in the axonal growth of cultured cortical neurons. **$p<0.01$ versus the untreated CTL; $^{\#}p<0.05$ versus the LPA NOVA, Tukey's post-hoc test). **g** LPA (5 μM) suppresses axonal growth of cortical neurons from $TRPM2^{+/+}$ but not from $TRPM2^{-/-}$ mice. *$p<0.05$ versus $TRPM2^{+/+}$ cortical neurons

perturbation or genetic disruption enhanced neurite growth. In line with this finding, an elevation in intracellular ADPR caused axonal retraction. Importantly, LPA-induced axonal retraction was completely absent in cortical neurons from *Trpm2*-deficient mice or reversed by TRPM2 blockers, suggesting an active role of TRPM2 in mediating the inhibitory action of LPA on axonal growth. As LPA application upregulated active PARP-1, LPA appears to mediate its modulatory action on neuritogenesis via the PARP-1/TRPM2 pathway.

Consistent with previous reports, the TRPC1 and TRPV4 transcript levels were associated with neurite growth, whereas TRPC5 transcript level was inversely related to neurite growth (Fig. 1). TRPC1 augments axonal guidance by regulating growth cone turning in the developing *Xenopus* spinal cord [43]. *Cis*/*trans* isomerization in a region of TRPC1 is involved in chemotropic guidance of growth cones [44]. TRPV4 also promotes NGF-induced neurite growth in peripheral neurons [18]. It is noteworthy that mutations in TRPV4 are associated with spinomuscular dystrophy such as Charcot–Marie–Tooth disease [35]. TRPC5 is known to suppress axonal elongation in hippocampal neurons [10]. TRPC5 inhibits growth cone via interaction with stathmin 2 and neuronal Ca^{2+} sensor-1 [10, 16]. Furthermore, semaphorin-3A initiates growth cone collapse via the signaling pathway of TRPC5/calpain-1 [20].

TRPM2 was first cloned from brain cDNAs and has been known to be expressed in neurons and immune cells [19, 31]. TRPM2 forms a nonselective, Ca^{2+} permeable channel. TRPM2 is activated by ADPR and oxidative stress such as hydrogen peroxide [3, 37]. In the immune system, TRPM2 regulates chemokine production in monocytes [50]. In addition, TRPM2 mediates ROS-induced activation of inflammasomes in phagocytes [53]. In pancreatic β-cells, TRPM2 is also involved in the release of insulin and thus controls the energy balance and glucose metabolism in obese mice [46, 52]. Given its response to oxidative stress, TRPM2 has been considered as a redox sensor that causes neuronal degeneration in the adult brain [32]. In addition to this, we suggest that TRPM2 seem to be necessary for balancing axonal growth in the developing embryonic brain.

Numerous reports support the role of LPA in controlling neurite growth in the embryonic brain [8, 12, 42, 45]. For example, the application of LPA to intact cerebral cortices rapidly induces abnormal thickening of the cortical wall and causes cortical folds [21]. These effects of LPA are absent in LPA receptor-deficient mice [21]. Moreover, mouse brains exposed to LPA resulted in the development of fetal hydrocephalus, characterized by an enlarged head due to an excessive accumulation of cerebrospinal fluid in the fetal brain [51]. Furthermore, LPA1 receptor-deficient mice elicited changes in the levels of neurochemicals such as glutamate, GABA, and serotonin [11, 40]. Thus, LPA appears to play an active role in balancing axonal growth in the developing brain. Stimulation of LPA receptors induces transient elevation of Ca^{2+} possibly from endoplasmic reticulum via phospholipase C stimulation [7]. LPA is also known to trigger the influx of Ca^{2+} from the extracellular fluid in human neutrophils [17]. Recently, Nieto-Posadas and colleagues suggested that LPA activates the TRPV1 channel in sensory neurons, thereby inducing pain-like behaviors [33]. Thus, it is conceivable that TRPV1 may mediate LPA-induced control of axonal growth in differentiating neurons. However, because the TRPV1 transcript was not detectable in differentiating PC12 cells (Fig. 1), it is less likely to that TRPV1 mediates LPA-evoked axonal retraction.

Materials and methods

Cell culture

PC12 cells were grown on collagen-coated plates in DMEM supplemented with 10 % horse serum and 5 % fetal bovine serum. DMEM supplemented with 2 % horse serum, 1 % fetal bovine serum, and NGF (100 ng/ml, Invitrogen), was changed every 2 or 3 days until differentiation was complete (5–7 days). Cortical neurons were obtained from brains of rat embryos (E16), passed through a cell strainer (40 μm), plated on poly-L-ornithine-coated coverslips, and incubated at 37 °C in 95 % air/5 % CO_2.

RTQ-PCR

Selected sets of cDNAs and target primers were used for quantitative real-time PCR experiments using iQTM SYBR Green Supermix (BIO-RAD). cDNAs of PC12 cells (1 μl), 5 pmol of forward and reverse primers, and 12.5 μl of 2*SYBR Green Supermix in a total volume of 25 μl were mixed prior to the RTQ-PCR reaction. Using standard curve generated with each PCR product, the initial quantity of cDNA template per well was calculated from the threshold cycle value. To normalize the intersample variation inherently related with each RNA preparation, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) amount per sample was quantified.

Treatment with siRNA

Nucleotide oligomers for siRNAs targeting rat TRPM2: $^{5'}$GAC GAA GCA AUA GCA GUC U$^{3'}$, $^{5'}$UGA GAA GCA AGA AAA CCU U$^{3'}$, and $^{5'}$CUA CAU UGA CGG CGU GAA U$^{3'}$. TRPC5: $^{5'}$TAA TAC GAC TCA CTA TAG GGA GAT CAT TCT CAA CCA$^{3'}$ and $^{5'}$TAA TAC GAC TCA CTA TAG GGG TTT CTT GAT GAA AAG$^{3'}$. Scrambled siRNA: $^{5'}$CCU ACG CCA CCA AUU UCG U$^{3'}$. siRNAs (40 μM) labeled with fluorescein were transfected into PC12 cells using Lipofectamine 2000™ (Invitrogen) a day after plating. To confirm the effect of siRNAs on the transcript level, we performed reverse transcriptase polymerase chain reaction (RT-PCR). Total RNAs were isolated from siRNA transfected cells at 48 h after transfection. RT-PCR was performed with each total RNA using sets of specific primers: TRPM2: $^{5'}$AGC ACT GGG GCC TGG ATG T$^{3'}$ and $^{5'}$TTA GGC TGA AGT CCC GTA CCG$^{3'}$ and TRPC5: $^{5'}$GGG CTG AGA CTG AGC TGT C$^{3'}$ and $^{5'}$TTG CGG ATG GCA TAG AGT AAT$^{3'}$.

Measurement of neurite length

The lengths of neurites of PC12 cells or cortical neurons were measured using *ImageJ* software (National Institute of Health) after images of cells were acquired using a fluorescent microscope. Percentage of cells bearing neurites was determined by counting more than 100 cells in randomly chosen visual fields. Cells with processes longer than a cell diameter were counted as positive. Cells with multiple processes were counted only once. Cell aggregates were disregarded. Neurite lengths of cortical neurons were analyzed by using NeuriteTracer software [38].

Immunocytochemistry

PC12 cells or cortical neurons cultured on round coverslips were fixed with 4 % paraformaldehyde. The mouse monoclonal antibody raised against rat Tuj-1 (neuron-specific class III beta-tubulin; 1:1,000 dilution; Abcam) was incubated overnight at 4 °C. The two primary antibodies were bathed for 1 h at 37 °C with Alexa Fluor 488-conjugated goat anti-mouse IgG (diluted 1:1,000; Molecular Probes).

Immunofluorochemistry

Embryonic brain was extracted from embryos (16-day embryo) and then placed in phosphate-buffered saline containing 4 % paraformaldehyde for 24 h. After fixing, brain tissues were dehydrated in serial concentrations of sucrose from 5 to 30 % and then frozen in the frozen section compound (FSC 22® Clear, Leica). Frozen tissues were sectioned at 8 μm and placed on slides. After thawing the frozen sections at room temperature, sections were blocked for 1 h in a solution containing 4 % bovine serum albumin and 0.05 % Tween 20, and then incubated overnight at 4 °C in a solution containing primary antibodies such as TRPM2 (Bethyl Laboratories), Nestin (Millipore), Tuj-1 (Abcam), LPA1 (Santa Cruz Biotechnology), or poly-ADPR (ALEXIS). After overnight incubation, they were incubated for 1 h at 37 °C with Alexa Fluor 488- or 546-conjugated secondary antibodies.

Current recording

Currents of whole cells or single channels were recorded from PC12 cells or HEK 239 T cells transfected with *Trpm2* using a standard current recording technique [4, 22]. Briefly, for whole cell recordings, pipette solution contained (in mM) 140 K-gluconate, 10 HEPES, 2 $MgCl_2$, 3.13 $CaCl_2$, 5 EGTA, 1 ATP, and 0.33 GTP. To activate TRPM2, 300 μM ADPR was added to the pipette solution. The control bath solution contained (in mM) 140 NaCl, 10 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES), 2 $MgCl_2$, and 2 Ca^{2+}. For single-channel recordings, the pipette solution contained (in mM) 140 Na gluconate, 1 $CaCl_2$, 10 HEPES, and 2 $MgCl_2$. The bath solution contained (in mM) 140 Na gluconate, 10 HEPES, and 2 $MgCl_2$. Currents were recorded with a patch-clamp amplifier (Axopatch 200B, Molecular Device). The output of the amplifier was filtered at 5 KHz, digitized with Digidata 1440A (Molecular Probes), and stored on a computer.

Measurement of PARP-1

Twelve hours after LPA treatment, cultured cortical neurons were lysed. PARP-1 activity in lysates was determined using PathScan® Cleaved PARP Sandwich ELISA Kits (Cell Signaling Technology, Danvers, MA), which detect the cleaved form of PARP protein. This cleavage occurs between Asp214 and Gly215, which separates the amino-terminal DNA-binding domain of PARP (24 kDa) from its carboxy-

terminal catalytic domain (89 kDa). Absorbances were measured at 450 nm spectrophotometrically (Sunrise™, TECAN, Mannedorf, Switzerland).

TRPM2-deficient mice

TRPM2-deficient mice were generated as described previously [50]. TRPM2 heterozygous ($TRPM2^{+-}$) mice were backcrossed to C57BL/6J inbred background over tenth generations. All experiments were performed on neonate from $TRPM2^{+/+}$ and $TRPM2^{-/-}$ mice. The mice were maintained with free access to food and water under a 12:12-h light/dark cycle. Animal care and handling were carried out according to the guidelines of the Institutional Animal Care and Use Committee of the Seoul National University.

Statistical analysis

All results are shown as means ± SEMs. Significances of differences were determined by Student's *t* test or one-way analysis of variance. Statistical significance was accepted at *p* values of *<0.05, **<0.01, and ***<0.001, as indicated.

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