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REVIEW

# Transient receptor potential canonical channels in angiogenesis and axon guidance

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**Abstract** Wiring of vascular and neural networks requires precise guidance of growing blood vessels and axons, respectively, to reach their targets during development. Both of the processes share common molecular signaling pathways. Transient receptor potential canonical (TRPC) channels are calcium-permeable cation channels and gated via receptor- or store-operated mechanisms. Recent studies have revealed the requirement of TRPC channels in mediating guidance cue-induced calcium influx and their essential roles in regulating axon navigation and angiogenesis. Dissecting TRPC functions in these physiological processes may provide therapeutic implications for suppressing pathological angiogenesis and improving nerve regeneration.

**Keywords** TRPC · Calcium · Endothelial cells · Growth cone · Angiogenesis · Axon guidance

**Abbreviations**

| | |
|---|---|
| BDNF | Brain-derived neurotrophic factor |
| BMP7 | Bone morphogen protein 7 |
| CRAC | Calcium release-activated calcium |
| DAG | Diacylglycerol |
| ERK1/2 | Extracellular signal-regulated kinase 1/2 |
| FKBP | FK506-binding protein |
| GPCR | G-protein-coupled receptor |
| IP3 | Inositol (1,4,5) trisphosphate |
| MAG | Myelin-associated glycoprotein |
| PIP2 | Phosphatidylinositol (4,5) bisphosphate |
| PLC | Phospholipase C |
| ROCE | Receptor-operated calcium entry |
| RTK | Receptor tyrosine kinase |
| SOCE | Store-operated calcium entry |
| STIM | Stromal interaction molecule |
| TRP | Transient receptor potential |
| TRPC | Transient receptor potential canonical |
| VDCC | Voltage-dependent calcium channel |
| VEGF | Vascular endothelial growth factor |

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## Introduction

Blood vasculature and nervous systems are both highly branched and stereotyped networks, which support and regulate physiological functions of most organs and tissues [1–3]. While blood vessels supply the body with oxygen and nutrients, neural networks control and coordinate a variety of behaviors by transmitting electric signals [4]. The vertebrate vasculature, which is composed of arteries, veins, and capillaries, is formed via two successive processes [1, 2]. During early development, mesoderm-derived hemangioblasts first differentiate in situ and coalesce into primitive vascular plexus, a process called vasculogenesis [1, 2]. This early vascular network further undergoes expansion by angiogenesis, during which new vessels sprout from preexisting ones and grow toward their targets to make connection [1, 2]. Similar to angiogenesis, establishment of neural circuits also involves navigation of growing axons through the complex tissue environment to reach their correct targets [5, 6]. Both angiogenesis and

axon guidance share many similarities [2, 4]. Morphologically, specialized endothelial cells called tip cells with numerous dynamic filopodia, which are comparable to axonal growth cones, locate at the angiogenic fronts and actively detect molecular cues in their surroundings [2, 7]. Mechanistically, guidance of vessels and nerves share common underlying signaling pathways [2–4, 7–10]. Some axon guidance cues, such as netrins, ephrins, slits and semaphorins, are also pro- or anti-angiogenic factors [2, 3, 7, 8, 10]. Recent advances have shown that transient receptor potential canonical (TRPC) channels, which are calcium-permeable cation channels, mediate calcium influx in both axon growth cones and endothelial cells, and thereby regulate navigation of neural axons and blood vessels, respectively. In this review, we first provide an overview of transient receptor potential (TRP) superfamily of ion channels, especially TRPC channels, and then discuss the function of TRPC channels in chemotropic guidance of both neural axons and vessels.

## TRPC channels

Calcium ions function as an important second messenger in cells via regulating a broad range of physiological events. Cells express a great variety of calcium-permeable ion channels in the plasma membrane to control intracellular calcium at a highly spatial and temporal resolution. TRP channels are a superfamily of cation channels, which are permeant to calcium ion [11, 12]. They were first identified in *Drosophila*, where photoreceptors with mutated *trp* gene display transient, instead of sustained, response to continuous light stimulation [11–13]. TRP channels were further found to be existing in many other organisms, such as worm, zebrafish, mouse and human [12, 14]. On the basis of the sequence homology of their amino acid, TRP channels are classified into seven families, including TRPC (Canonical), TRPA (Ankyrin), TRPM (Melastatin), TRPML (Mucolipin), TRPN (no mechanoreceptor potential C, NompC), TRPP (Polycystin) and TRPV (Vanilloid) [11, 12, 15]. Numerous studies have revealed the important functions of TRP channels in diverse biological processes [16, 17]. For example, TRPV1 is a mediator of thermal sensation [18, 19]. TRPN is essential for the mechanotransduction of zebrafish hair cells and its deficiency leads to defects in hearing and balance [20].

TRPC channels are the first described mammalian homologs of *Drosophila* TRPs [21–23]. Mammalian TRPC family comprises seven members (TRPC1-7), which can be subdivided into four groups based on similarities in their sequence and function: TRPC1, TRPC2, TRPC3/6/7, and TRPC4/5 [24, 25]. TRPC channels in mammals are putative six-transmembrane proteins, and expected to form homo- or hetero-tetramers with various properties [15, 26, 27]. In the rat brain, TRPC1 interacts with TRPC5 to form heteromeric channels [28]. Heterologous expression of TRPC5 and/or TRPC1 in HEK293 cells showed that co-assembly of TRPC1 and TRPC5 generates a novel non-selective cation channel, which displays different biophysical properties compared with TRPC5 homomeric channels as exemplified by the altered current–voltage relationship of whole-cell currents and smaller single-channel conductance [28].

TRPC channels can be gated by activation of receptor tyrosine kinases (RTKs) or G-protein-coupled receptors (GPCRs), suggesting that TRPC may act as receptor-operated calcium entry (ROCE) channels [29, 30]. While RTKs stimulate the enzymatic activity of phospholipase C (PLC)-$\gamma$ by tyrosine phosphorylation [31], the GPCRs activate $G_{\alpha}$ and $G_{\beta\gamma}$ subunits of heterotrimeric G proteins by catalyzing the guanine nucleotide exchange, subsequently resulting in the activation of PLC-$\beta$ [32]. PLC can modulate TRPC channel activation by hydrolysis of phosphatidylinositol (4,5) bisphosphate (PIP2) with production of the second messengers diacylglycerol (DAG) and inositol (1,4,5) trisphosphate (IP3), however, its underlying mechanism still remains elusive [11, 29, 30].

TRPC channels are also suggested to be activated by store-operated mechanisms [30]. Depletion of intracellular calcium stores (mainly in endoplasmic reticulum), without involvement of PLC activation leads to external calcium entry to replenish the deficits via calcium-permeable channels in the plasma membrane, a process referred to as store-operated calcium entry (SOCE) [33]. Inhibition of sarcoplasmic/endoplasmic reticulum calcium ATPase by application of thapsigargin is often used to examine this phenomenon. Among several reported store-operated calcium currents with distinct biophysical properties, the best characterized one is the calcium release-activated calcium (CRAC) current observed in mast cells and lymphocytes [33–35]. Several studies, either by means of heterologous expression of TRPC channels, or with the help of blocking antibodies or gene knock-down/-out to suppress endogenous TRPC function, have suggested TRPC family members as the candidates mediating SOCE and CRAC currents [30]. RNA-interference screening based on *Drosophila* S2 cells and human HeLa cells has led to the identification of STIM (stromal interaction molecule) and Orai as key components of the pathway responsible for SOCE [36–40]. Further experiments investigating the relationship between TRPC channels, STIM and Orai have collectively led to a model, in which after sensing the depletion of the endoplasmic reticulum calcium store, STIM oligomerizes, traffics to endoplasmic reticulum plasma membrane junction, and organizes Orai and TRPCs to form functional SOC channels [41].

## TRPC channels in axon guidance

### Importance of calcium signaling in axon guidance

Pathfinding of growing axons is guided by a variety of diffusible and membrane-bound molecular cues [5, 42], and calcium has been revealed to play critical roles during this process [43–45]. Calcium imaging studies show that application of netrin-1 gradient induces localized calcium elevation in growth cones of culture *Xenopus* spinal neurons, leading to neurite attraction [46]. A reduction of calcium signaling correlates with the adaptation of nerve growth cones to netrin-1 during chemotactic migration [47]. Myelin-associated glycoprotein (MAG)-caused repulsion also involves the formation of calcium gradients in growth cones [48]. Interestingly, enhancing such calcium signaling by cAMP converts MAG-induced response from repulsion to attraction [48]. These findings suggest that localized calcium signals, depending on their characteristics, can mediate multiple steering responses to extracellular guidance cues. In support of this notion, direct induction of a local large calcium increase by photoactivated photolysis of caged calcium results in growth cone attraction, whereas induction of a modest local calcium elevation leads to repulsion [49, 50].

Various calcium-permeable channels in growth cones provide multiple routes for calcium influx, partially accounting for the origin of the molecular cue-induced calcium signals [43–45]. For example, whole-cell recordings show that growth cones express L-type voltage-dependent calcium channels (VDCCs), whose activity can be enhanced by netrin-1 [51]. Blocking L-type VDCC leads to reduction of the calcium signals and attraction-to-repulsion switch in response to netrin-1 [46].

### Requirement of TRPC channels in chemotropic guidance of growth cones

A TRP-like current is observed in the growth cones of cultured *Xenopus* spinal neurons by whole-cell patch clamp recording [52]. Netrin-1 or brain-derived neurotrophic factor (BDNF) enhances this current, which can be suppressed by further application of the TRP channel blocker SKF96365 or lanthanum [52]. Meanwhile, immunostaining shows that TRPC1 is expressed in growth cones [53, 54]. Consistent with these observations, morpholino-based knock-down of TRPC1 decreases the TRP-like channel activity with or without netrin-1 treatment [52]. These data suggest that functional TRPC channels are present in growth cones and can be modulated by chemotropic guidance cues.

Multiple lines of evidence have shown that TRPC channels are required for axon pathfinding [55]. Down-regulation of TRPC1 or expression of a dominant-negative form of TRPC1 in *Xenopus* spinal neurons abolishes both the attractive growth cone turning elicited by netrin-1 and the repulsive response induced by MAG or bone morphogen protein 7 (BMP7) [52–54]. Importantly, interfering with TRPC1 function results in defective netrin-1-dependent midline axon guidance of commissural interneurons in the *Xenopus* spinal cord in vivo [53]. In agreement with these observations, TRPC3 and TRPC6 are also essential for the BDNF-induced chemo-attractive effect on growth cones of cultured rat cerebellar granule cells [56].

Calcium influx mediated by TRPC channels is an important calcium entry route responsible for molecule cue-induced axon guidance [44], as evidenced by the finding that TRPC activity is required for the calcium elevation elicited by netrin-1 and BDNF [52, 56]. However, it is still unclear how TRPC channels are activated by extracellular molecular cues. In pontine neurons, activation of TrkB by BNDF induces a TRPC3-mediated cation current through PLC [57]. Similarly, based on a series of pharmacological experiments and turning assay, it has also been proposed that following BDNF application, calcium release from endoplasmic reticulum calcium stores, triggered by TrkB—PLC-$\gamma$—inositol-1,4,5-trisphosphate ($IP_3$) signaling, activates TRPC channels, and thus allows calcium entry, which is important for growth cone turning [56]. Further experiments, such as direct examination of the effect of this signaling pathway on the TRPC channel currents, are necessary to support this receptor-operated activation mechanism. A recent finding shows that FK506-binding protein (FKBP) 12 and FKBP 52, which are two types of immunophilins, mediate spontaneous and stimulus-dependent gating of TRPC1, respectively, through their peptidyl-prolyl isomerase activity, thereby contributing differentially to netrin-1-induced axon guidance [58].

TRPC channel opening induced by guidance cues leads to membrane depolarization, which may activate VDCCs and result in calcium influx [52, 53]. TRPC channel and/or VDCC-mediated calcium elevation can activate downstream calcium-dependent signaling pathways to regulate axon guidance [44, 45]. In BMP7 gradient-elicited growth cone repulsion, calcium elevation mediated by TRPC1 stimulates calcineurin phosphatase, which in turn controls actin-depolymerizing factor/cofilin-dependent actin dynamics through Slingshot phosphatase [54]. Interestingly, calcineurin cascade is also required for embryonic axon outgrowth induced by neurotrophins and netrins [59]. Collectively, these findings imply that calcineurin signaling is an important downstream effector, which mediates TRPC function in chemotropic guidance of neuronal growth cones.

### Effects of TRPC channels on neurite outgrowth

In hippocampal neurites, TRPC5 interacts with stathmin 2, which is required for packaging of TRPC5 into vesicles and subsequent transportation into growth cones [60]. Expression of a dominant-negative TRPC5 or a mutant stathmin 2 increases the number of filopodia and the length of neurites [60]. In addition, enhancing vesicular translocation and membrane insertion of TRPC5 inhibit neurite extension [61]. These results reveal a negatively regulatory role of TRPC5 for the formation of hippocampal filopodia and neurites. However, a recent study shows that TRPC5 promotes rather than blocks axon outgrowth of hippocampal neurons by activating calcium/calmodulin kinase I$\gamma$ [62]. This discrepancy may be explained by use of different developmental stages of cultured neurons in those studies, implying that the role of TRPC5 may be stage-dependent. TRPC channels are also involved in axon regeneration. In dorsal root ganglion neurons, nerve injury increases the expression of TRPC4, which is essential for neurite outgrowth of these neurons [63].

## TRPC channels in angiogenesis

Similar to that in neuronal growth cones, calcium dynamics in endothelial cells is critical for angiogenesis as well [64, 65]. For example, treatment with carboxyamidotriazole, an inhibitor of ligand-evoked calcium influx, impairs endothelial cell adhesion, motility and proliferation induced by fibroblast growth factor 2 in vitro and angiogenesis in the chicken chorioallantoic membrane in vivo [66]. Endothelial cells express a variety of calcium-permeable ion channels in the plasma membrane, including TRPC channels, thus offering diverse calcium entry pathways [67–69].

### Expression and functional characterization of TRPC channels in endothelial cells

Members of TRPC family are expressed in various types of cultured endothelial cells as well as in intact endothelium, revealed by examination of TRPC mRNAs and proteins [68, 70, 71]. Multiple lines of evidence have suggested that TRPC channels may act as SOCE channels in endothelial cells [67, 72, 73]. Both knock-down of TRPC1 by antisense oligonucleotides and inhibition of TRPC function with anti-TRPC1 antibody reduce SOCE induced by thapsigargin and $IP_3$ in cultured endothelial cells [74, 75]. SOCE is regulated by RhoA (a small GTPase protein, which regulates the actin cytoskeleton)-mediated membrane translocation and protein kinase C$\alpha$-dependent phosphorylation of TRPC1 [75, 76]. Tumor necrosis factor-$\alpha$ treatment enhances the expression of TRPC1, and amplifies SOCE [77]. More importantly, endothelial cells from TRPC4 knock-out mice lack store-operated calcium currents and entry [78, 79]. These findings are challenged by a recent paper, which shows that STIM1 and Orai1, rather than TRPC1 and TRPC4, mediate SOCE in endothelial cells [80]. Therefore, further conclusive studies are required to clarify this issue.

### Essential roles of TRPC channels for angiogenesis

Thanks to the availability of transgenic lines with vasculature labeled by fluorescent proteins and establishment of forward and reverse genetics, zebrafish has emerged as an ideal model to study angiogenesis in vivo [81, 82]. We have previously employed zebrafish as an in vivo model to dissect the function of TRPC channels in angiogenesis [83]. Down-regulation of TRPC1 by morpholino oligonucleotides results in defective angiogenic sprouting of intersegmental vessels in the zebrafish trunk. This vascular defect is attributable to impaired filopodia extension, migration and proliferation of tip endothelial cells [83]. Consistent with these observations, knock-down of TRPC1, TRPC4, or TRPC6 also substantially occludes proliferation of cultured human umbilical vein endothelial cells [80].

Vascular endothelial growth factors (VEGFs) are critical mediators for angiogenesis. Disruption of VEGF signaling in mice or zebrafish leads to severe angiogenic defects [84]. Interestingly, TRPC1 and TRPC6 mediate VEGF-induced calcium elevation in endothelial cells, implying TRPC channels are important downstream targets of VEGFs in controlling angiogenesis [85–87]. Further studies have shown that TRPC1 is required for VEGF-enhanced angiogenesis in vivo [83], and TRPC6 is essential for VEGF-elicited migration, proliferation, sprouting and tube formation of endothelial cells in vitro [86, 87], therefore proving a crucial cell-autonomous function of TRPC channels in VEGF pathways.

TRPC channels also regulate angiogenesis in a non-cell-autonomous manner. Secretion of thrombospondin-1, an angiogenic inhibitor, is positively controlled by TRPC4 in renal cells [88]. Normal renal epithelium expresses high level of TRPC4, whereas renal carcinoma cells have low level of TRPC4, which causes impaired thrombospondin-1 secretion, leading to enhanced angiogenesis and facilitated tumor growth [88]. Moreover, inhibiting the hypoxia-induced TRPC6 upregulation and the activation of TRPC6 downstream calcineurin-NFAT pathway in human glioma cells not only decreases tumor growth and invasion, but also markedly impairs angiogenesis, possibly by regulating the expression and/or secretion of pro- and/or anti-angiogenic factors [89].

What is the downstream signaling pathway of TRPC channels in regulating angiogenesis? The MEK-ERK1/2

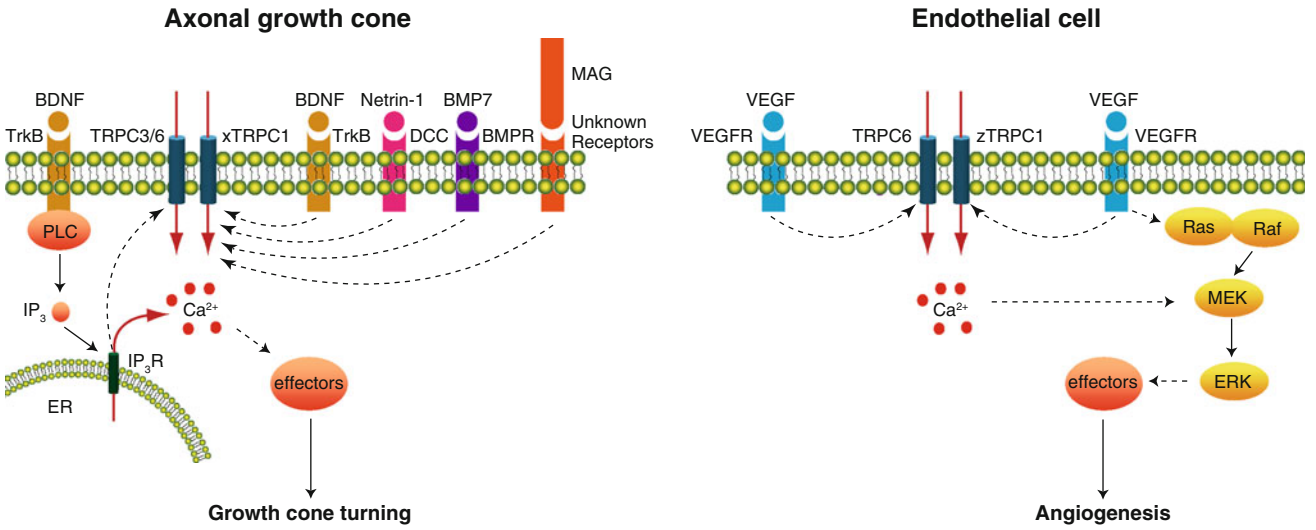


**Fig. 1** A proposed model for TRPC channel-mediated signaling pathways in axonal growth cones and endothelial cells. In axonal growth cone, stimulation of TrkB by BDNF promotes PLC-catalyzed production of $IP_3$, which triggers calcium release from endoplasmic reticulum (ER) via binding to $IP_3$ receptors ($IP_3R$). TRPC3 and TRPC6 are activated downstream to $IP_3R$ activation, leading to growth cone attraction in rat cerebellar granule cells [56, 57]. Netrin-1 through its receptor DCC (deleted in colorectal cancer), and BDNF through TrkB, trigger attractive response by activation of *Xenopus* TRPC1 (xTRPC1) in spinal neurons [53]. BMP7—BMP receptor (BMPR) and MAG—unknown receptor induce repulsive growth cone turning mediated by xTRPC1 in *Xenopus* spinal neurons [53, 54]. In endothelial cells, zebrafish TRPC1 (zTRPC1) regulates angiogenesis by modulating MEK-ERK1/2 cascade downstream of VEGF receptors (VEGFR) [83], whereas TRPC6 mediates VEGF-elicited calcium entry and angiogenic response [86, 87]. *Dashed arrows*, unclear signaling mechanisms; effectors, unknown molecules which mediate upstream signals and lead to growth cone turning or angiogenesis

(extracellular signal-regulated kinase 1/2) cascade has been shown to be one of the underlying mechanisms. In zebrafish, TRPC1 is required for both constitutive and VEGF-induced ERK phosphorylation of intersegmental vessels, and inhibition of MEK-ERK pathway mimics the angiogenic defects caused by TRPC1 knock-down [83]. However, it still remains unknown how TRPC1 activates this cascade.

## Conclusions and future directions

TRPC channels, as the common calcium entry routes in axonal growth cones and endothelial cells, play crucial roles in both axon guidance and angiogenesis (Fig. 1). However, many fundamental questions still remain to be answered: (1) What are the spatiotemporal expression patterns of TRPC channels in growth cones and endothelial tip cells? (2) How are TRPC channels activated by axon and vessel guidance cues? (3) What do TRPC-mediated calcium patterns like in axonal growth cones and endothelial tip cells, particularly in vivo? (4) What are the downstream signaling pathways of TRPC channel activation in regulating axon guidance and angiogenesis? Further efforts to address these questions will deepen our understanding of the TRPC functions in axon guidance and angiogenesis, and may reveal new drug targets for treating nerve injury and tumor.

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