Review

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Calcium signaling in axon guidance

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Guidance of axons to their targets in the developing nervous system requires a myriad of downstream signaling molecules to coordinate growth cone movement. One of the most important of these is calcium, and over the past few years many new insights have been gained into the role of calcium in axon guidance. In this review we focus on mechanisms of calcium entry into the growth cone and its downstream effects on both growth cone motility and turning. We particularly highlight the role of calcium concentrations in determining attractive versus repulsive responses to graded guidance cues, and their role in guidance by the morphogen Wnt5a.

Introduction

For the brain to be wired correctly during development axons must navigate appropriately to their targets, often over long distances. Axons achieve this feat by detecting and responding appropriately to numerous molecular and physical guidance cues in their local environment, usually via the growth cone [1]. This includes guidance by growth through permissive conduits, by substrate-bound guidance cues, or by gradients of diffusible cues. Several molecular guidance cue families involved in axon guidance have been identified including the netrins, semaphorins, ephrins, slits, neurotrophins, Wnts, and some morphogens [2,3]. These cues are detected by receptors on the axon surface, which then activate a variety of downstream signaling pathways to cause axon turning and/or changes in growth rate [4].

One of the most crucial components of these pathways is calcium [5–8]. Calcium concentrations in growth cones are regulated both by calcium influx through the plasma membrane and by release from intracellular calcium stores [9,10] (see Glossary). A calmodulin-dependent protein kinase II (CaMKII)–calcineurin (CaN) switch often initiates either an attractive (turning towards the highest guidance cue concentration) or repulsive (turning away from the highest guidance cue concentration) response to these intracellular calcium elevations [8]. These responses are effected via the regulation of cytoskeletal components, such as microtubules and actin filaments [11–15], and by membrane dynamics, including vesicle trafficking [16–18]. In the past few years many new insights have emerged regarding the central and multifaceted roles of calcium in mediating axon growth and guidance. We review here some of these developments, focusing particularly on new mechanisms for producing and mediating calcium elevations, the involvement of calcium in the attraction/repulsion switch, how calcium modulates growth cone motility, and the role of calcium in guidance by Wnt5a.

Mechanisms of calcium entry

The spatial distribution and level of intracellular calcium is crucial for axon guidance [19], and regulation of this distribution begins with calcium entry into the growth cone. Methods of entry particularly important for axon guidance include voltage-dependent calcium channels (VDCCs), release of intracellular calcium stores, and store-operated calcium entry (SOCE) from extracellular sources (Figure 1). The most important VDCCs for growth cone turning are the L-type Ca^{2+} channels [20,21]. Inhibition of these channels switches turning in response to netrin-1, an attractive chemotropic factor, to repulsion

Glossary

Axon guidance by growth rate modulation: a type of chemotactic movement whereby axon guidance up the gradient is mediated not by turning, but instead by the axon taking larger steps when it is pointed up the gradient than when it is pointed down the gradient; [67] for more details.
Calcium-induced calcium release (CICR): the release of calcium from intracellular stores in response to rising calcium concentration (i.e., a positive feedback process).
Calcium release-activated channel (CRAC): calcium channels in the cell membrane which are activated in response to depletion of calcium stores in the endoplasmic reticulum (ER) to replenish those stores.
Calcium stores: calcium stored intracellularly in structures such as the endoplasmic reticulum (ER).
Calcium transient: a brief increase in calcium concentration in the cell, which may or may not be spatially localized.
Endocytosis: the absorption of molecules (such as axon guidance ligands) by the cell via engulfment by the cell membrane. The most relevant form of endocytosis for axon guidance is receptor-mediated endocytosis, which takes place via clathrin-coated vesicles.
Exocytosis: the release of molecules from vesicles into the extracellular space (i.e., the opposite process from receptor-mediated endocytosis).
Focal laser-induced photolysis (FLIP): localized release of a caged molecule by light pulses from a narrowly focused laser beam.
IP_3-induced calcium release (IICR): release of calcium from intracellular stores stimulated by IP_3.
L-type calcium channels: a type of voltage-dependent calcium channels which have long-lasting (hence 'L') activation.
ORAI1: calcium release-activated calcium channel protein 1, a calcium-selective ion channel. Activation of ORAI1 channels is caused by depletion of internal calcium stores.
Store-operated calcium entry (SOCE): the influx of calcium across the cell membrane that occurs in response to depletion of calcium in intracellular stores.
Store-operated calcium (SOC) channels: plasma membrane channels activated by depletion of intracellular calcium stores. Some types of SOC channels are selective for calcium itself whereas others are selective for other ions.
Transient receptor potential cation channels (TRPC): a family of ion channels that are generally activated by phospholipase C stimulation.
Voltage-dependent calcium channel (VDCC): calcium channels in the cell membrane whose permeability to calcium is dependent on the voltage across the cell membrane.

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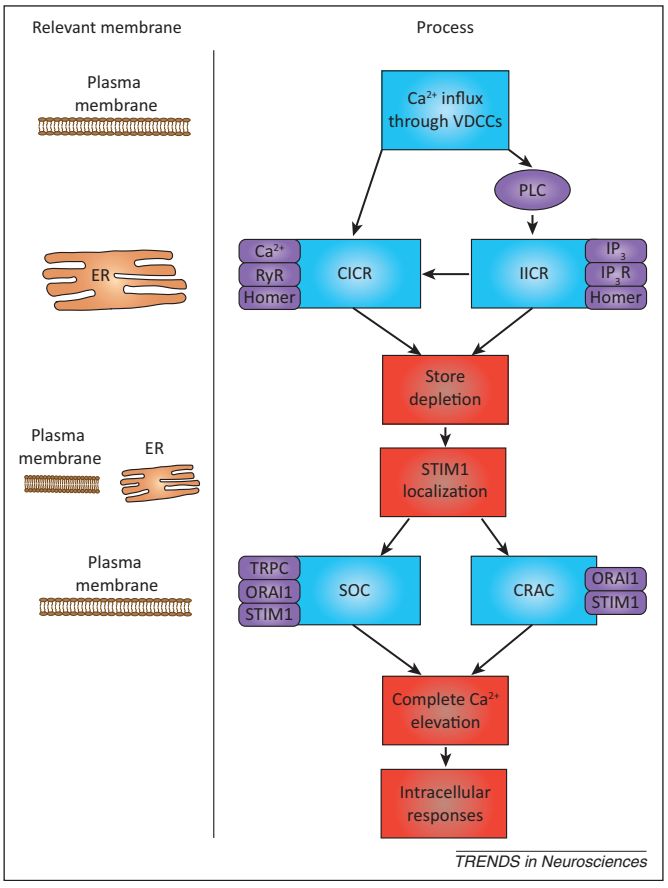

Figure 1. Pathways of calcium entry into growth cones and release from intracellular stores. Calcium entry occurs through VDCCs (in particular L-type calcium channels) in the cell plasma membrane to activate CICR directly, and indirectly initiate IICR. CICR and IICR are both Homer1-mediated and release Ca^{2+} from the ER. CICR is induced by the binding of incoming Ca^{2+} to RyRs on the ER membrane. In the IP_3 pathway, PLC is activated to hydrolyze PIP_2 into IP_3 and diacylglycerol. IP_3 binds to receptors on the surface of the ER, initiating IICR. In response to calcium store depletion, STIM1 becomes localized to the ER and plasma membrane junctions, and leads to SOCE through SOC channels which utilize TRPC and ORAI1, and CRAC channels which depend mainly on ORAI1. SOCE facilitates large influxes of Ca^{2+} from extracellular sources. The resulting asymmetric intracellular calcium elevation leads to asymmetric intracellular responses creating attraction or repulsion. Colors indicate the function of a component: purple for important molecules, blue for calcium-releasing processes and red for other processes. The left column indicates major membranes involved in each step. Abbreviations: ER, endoplasmic reticulum; CICR, calcium-induced calcium release; CRAC, calcium release-activated channel; IICR, IP_3-induced calcium release; IP_3, inositol trisphosphate; IP_3R, IP_3 receptor; ORAI1, calcium release-activated calcium channel protein 1; PLC, phospholipase C; PIP_2, 4,5-bisphosphate; RyR, ryanodine receptor; SOC, store-operated calcium; SOCE, store-operated calcium entry; STIM1, stromal interaction molecule 1; TRPC, transient receptor potential cation channel; VDCC, voltage-dependent calcium channel.

[20]. Activity of L-type Ca^{2+} channels is precisely regulated by cAMP (activation) and cGMP (inactivation): usually high levels of cGMP promote repulsion in response to netrin-1 whereas high levels of cAMP promote attraction [21]. Thus, L-type Ca^{2+} channels are essential for growth cone attraction to a netrin-1 source, with cAMP and cGMP allowing control and modulation of activity.

There are two main mechanisms for triggering release of calcium from intracellular stores: inositol trisphosphate (IP_3)-induced Ca^{2+} release (IICR) [22] and Ca^{2+}-induced Ca^{2+} release (CICR) [23]. In response to a chemotropic factor such as netrin-1, brain-derived neurotrophic factor (BDNF), or nerve growth factor (NGF), phospholipase C (PLC) is activated [24,25], which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) into IP_3 and diacylglycerol. Once created, IP_3 selectively binds to IP_3 receptors (IP_3Rs) located on the surface of the endoplasmic reticulum (ER), initiating IICR [26]. This process is likely mediated by both protein kinase A (PKA) [22] and Homer1 [23,27]. Besides its necessity for growth cone turning, artificially induced IICR is also sufficient for turning [22]. Gradients of NGF produce asymmetric IP_3 signals in a PLC-dependent manner, which then produce asymmetric Ca^{2+} elevations intracellularly [22]. The concentration of both IP_3 and Ca^{2+} are both higher on the side of the growth cone closest to the NGF source [22], consistent with responses to other guidance cues [28]. Inhibition of PLC, or treatment with thapsigargin (which empties intracellular Ca^{2+} stores), inhibits NGF-induced attraction, confirming the PLC-dependence and intracellular source of IICR [22]. To demonstrate sufficiency of asymmetric IP_3 signals to generate turning, Akiyama *et al.* [22] photolytically released IP_3 on one side of the growth cone. This generated matching asymmetric IP_3 and Ca^{2+} distributions and turning towards the side of the growth cone with the larger intracellular Ca^{2+} concentration [22]. IICR is thus a crucial step in attractive axon guidance.

CICR from ER internal stores is caused by an initial release of Ca^{2+}, often small in magnitude, which binds to ryanodine receptors (RyR) to induce release [23,29]. CICR is enhanced by cAMP but inhibited by the cGMP–nitric oxide (NO) pathway: increasing the level of intracellular cGMP directly inhibits CICR [30]. This is sufficient to reverse the effect of attractive guidance cues, indicating the importance of both CICR and the cGMP–NO pathway to growth cone guidance. Both IICR and CICR are dependent on Homer1, a scaffolding protein associated with both RyRs and IP_3Rs. Homer1 knockdown removes intracellular calcium in response to BDNF and switches attraction in response to BDNF and netrin-1 to repulsion [23].

In response to depletion of ER Ca^{2+} stores, a final mechanism of calcium entry, SOCE, is initiated. When ER stores are depleted, the ER-resident stromal interaction molecule 1 (STIM1) localizes to the ER and plasma membrane junctions [31–33]. From here, STIM1 facilitates SOCE [34] through store-operated calcium (SOC) channels which utilize transient receptor potential cation (TRPC) channels and ORAI1, and calcium release-activated calcium (CRAC) channels which depend primarily on ORAI1 [33,35–37]. Importantly, STIM1 localization in response to gradients of guidance cues is biased towards the up-gradient side of the growth cone, facilitating propagation of asymmetric Ca^{2+} elevations [33].

Calcium entry through TRPC channels in response to attractive gradients of netrin and BDNF is PIP_3-dependent [38]. Gradients induce asymmetric PIP_3 levels at the leading edge of the growth cone through activation of phosphoinositide 3-kinase (PI3K), a kinase which catalyzes the production of PIP_3 from PIP_2 [38,39]. PIP_3 production is necessary for attraction and protein kinase B (Akt, a downstream kinase) activation. Perturbation of Akt levels is sufficient to disrupt attraction, indicating that PIP_3 induces guidance via Akt [38]. External gradients of PIP_3 are sufficient to induce calcium entry through TRPC

Box 1. A recently discovered attraction/repulsion switch

Recently a mathematical model has been used to describe the attraction/repulsion switch acting within growth cones [42]. This builds on previous work modeling the switch from long-term potentiation to long-term depression in synaptic plasticity [74], which is controlled by a similar pathway [44,45,47].

Following Ca^{2+} entry in response to a graded guidance cue, the pathway begins with the rapid binding of Ca^{2+} to CaM to form a Ca^{2+}/CaM complex [75]. This activates CaN, PKA, and CaMKII at differing levels on each side of the growth cone, depending on the intracellular Ca^{2+} concentration. At low levels of Ca^{2+}/CaM, CaMKII has low affinity for Ca^{2+}/CaM. However, once a critical threshold level is achieved CaMKII affinity for Ca^{2+}/CaM increases dramatically [76]. Meanwhile, CaN has a constant affinity for Ca^{2+}/CaM at all Ca^{2+} concentrations [45]. At normal levels of cAMP, CaN activity predominates at low cytosolic Ca^{2+} concentrations and CaMKII activity predominates at high concentrations [42]. CaN promotes repulsion whereas CaMKII promotes attraction, and it is the relative activity of these molecules that is important for growth cone turning.

In addition to the bistability of CaMKII, cAMP plays a crucial role in turning [77,78]. Although this role was originally thought to be exclusively PKA-dependent, it has recently been shown to be dependent on Epac, a protein with similar function to PKA [78]. Murray *et al.* [78] have suggested that Epac mediates attraction and PKA mediates repulsion. However, because both PKA and Epac are modulated by cAMP [77,78] the mathematical model [42] does not distinguish between the effects of these molecules. The final two proteins in the model are I1 and PP1. PP1 inhibits CaMKII (thus promoting repulsion) and is inhibited by I1 [47]. I1 activity is inhibited by CaN, and promoted by the cAMP/PKA/Epac group. The protein originally thought to activate I1 was PKA [47]; however, because the inhibitor used was nonspecific, Epac could possibly perform this function [78].

The model splits the growth cone into two compartments (up-gradient and down-gradient). In each compartment a set of differential equations representing the pathway described above is considered independently. The output of the model is the CaMKII:CaN ratio in each compartment, and the model hypothesizes that turning will occur towards the side of the compartment with a greater CaMKII:CaN ratio (Figure 2).

Because the relationship between Ca^{2+} concentration and CaMKII:CaN ratio is nonlinear, the model is able to explain some counter-intuitive aspects of how calcium and cAMP levels control attraction versus repulsion [79,80]. In particular, the model explains why the steepness of the Ca^{2+} gradient across the growth cone is important, and why an abnormally high or low background Ca^{2+} concentration switches the response (Figure 2). These results suggest that it is the resulting Ca^{2+} concentration that is critical rather than the method of calcium entry.

channels and attractive turning (with matching Ca^{2+} elevations), indicating that PIP_3 is involved upstream of Ca^{2+} signaling.

The method of calcium entry has recently been proposed as indicative of turning. Tojima *et al.* [17] found that calcium entry involving CICR and IICR produced an attractive response, whereas Ca^{2+} signals without store entry invoked a repulsive response [17]. Furthermore, the method of calcium entry also affects axon outgrowth and growth cone motility [40,41]. Calcium influx through mechanosensitive stretch-activated channels decreases axon motility [41]. By contrast, calcium influx through other avenues (TRPC channels and intracellular stores) supports and promotes axon outgrowth [41]. It is unclear whether these outcomes are specifically related to the source of Ca^{2+} or simply to the resulting elevation – which is known to have narrow ranges which result in attraction [42] and increased axon outgrowth [43].

Role of calcium in turning

A turning response requires asymmetric deployment of signaling molecules between the two sides of the growth cone. Zheng [44] used focal laser-induced photolysis (FLIP) of caged calcium to generate a spatially restricted elevation of intracellular calcium concentration on one side of the growth cone. This asymmetric calcium elevation is sufficient to cause growth cone turning; however, the direction is dependent on the steepness of the calcium gradient and background calcium concentrations [44]. Large calcium increases (steep gradients) with normal extracellular calcium concentrations cause attraction [44]. Conversely, large intracellular calcium increases in the absence of extracellular calcium, or small intracellular calcium increases (shallow gradients), cause repulsion of the growth cone [45].

An important function of receptors for chemotactic guidance cues, such as BDNF, NGF, netrin-1, or myelin-associated glycoprotein (MAG), is thus to generate asymmetric Ca^{2+} elevations across growth cones in response to chemotropic gradients [46]. It is at this stage that different methods of calcium entry into the cytosol are crucial because control of entry mechanisms allows precise modulation of calcium elevation magnitude and distribution in response to different guidance cues. This begins a cascade of secondary reactions, some of which serve to amplify the initial elevation through processes including CRAC and CICR. Other responses to the calcium elevation propagate the signal along a pathway beginning with Ca^{2+}/calmodulin (CaM), and result in either attraction or repulsion of the growth cone along the gradient. Attraction is primarily mediated by CaMKII, whereas repulsion is mediated by CaN in conjunction with protein phosphatase 1 (PP1) [45]. Other crucial components of this pathway include PKA, exchange protein directly activated by cAMP (Epac), inhibitor 1 (I1), and the second messenger cAMP. A recent mathematical model has proposed a mechanism for this molecular switch [42], based on a pathway uncovered by Wen *et al.* [45] and Han *et al.* [47] (Box 1 and Figure 2).

Similar turning mechanisms have been proposed to underlie the response of various other cues, in particular morphogens [48,49]. Under normal conditions bone morphogenic protein (BMP) 7 activates cytoskeletal components through LIM kinase (LIMK) to induce attractive turning [48]. However, when TRPC channels are opened, Ca^{2+} induces CaN activity which counters the effects of LIMK, converting attraction to repulsion in a Ca^{2+}-dependent manner [48].

Downstream effects of calcium

Although a molecular switch mediated by CaMKII and CaN likely determines the growth cone response to intracellular calcium distributions, it is downstream effectors which induce membrane trafficking and cytoskeletal changes that create this response [16,17,48]. Repulsive guidance cues usually induce asymmetric endocytosis [17], whereas attractive cues induce asymmetric exocytosis

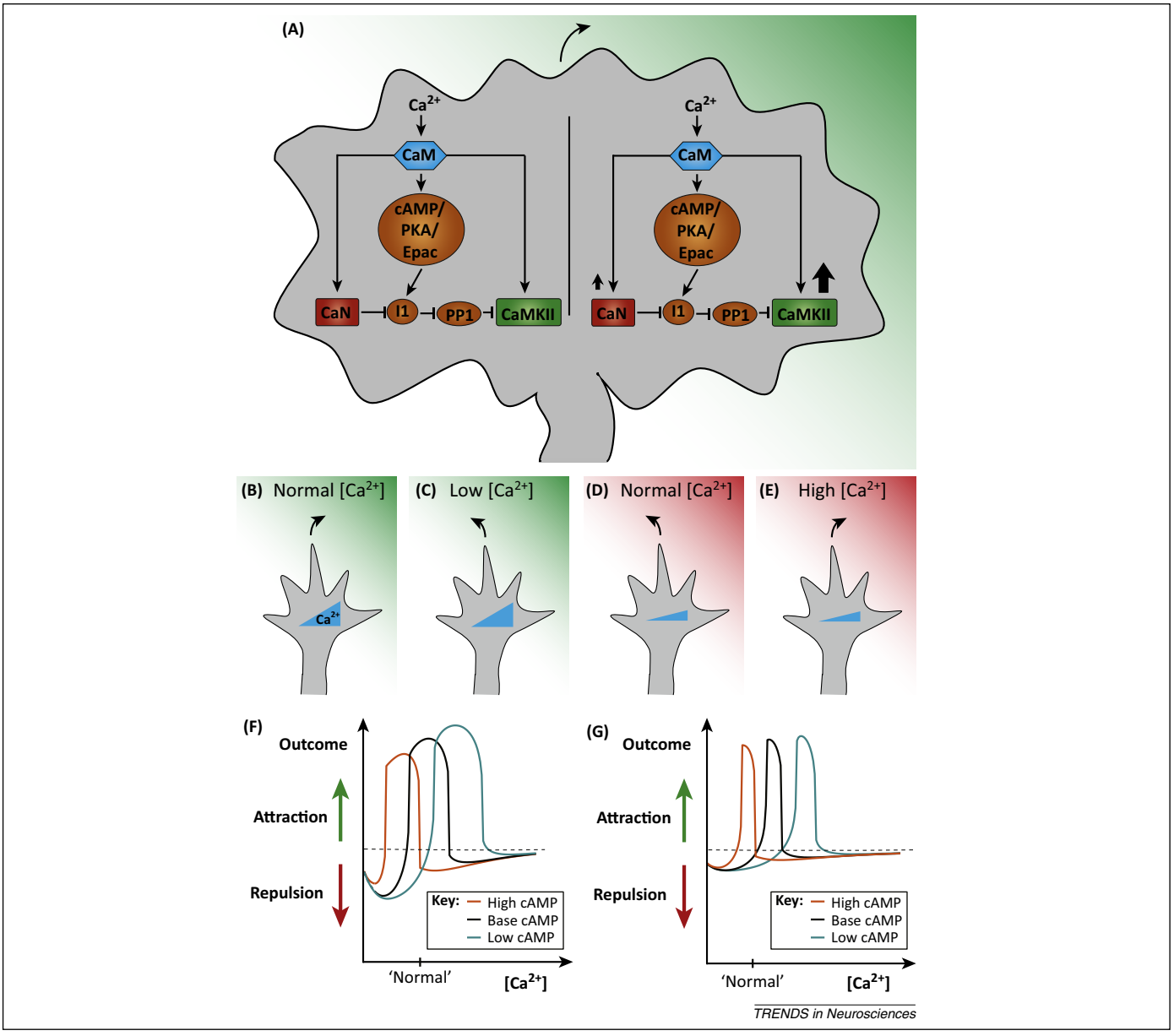

Figure 2. A mathematical model for how calmodulin-dependent protein kinase II (CaMKII) and calcineurin (CaN) activity modulation determines growth cone attraction versus repulsion to a gradient [42]. **(A)** The growth cone is imagined to be split into two compartments (up-gradient to the right, and down-gradient to the left). On each side a Ca^{2+}-dependent switch [47] processes Ca^{2+} elevations. Calcium binds to CaM to form a calcium/CaM complex which activates CaN, protein kinase A (PKA), and CaMKII. At low concentrations of calcium ($[Ca^{2+}]$), CaMKII has low affinity for the calcium/CaM complex, and CaN activity therefore predominates. As the calcium concentration rises above a threshold, the affinity of the calcium/CaM complex for CaMKII increases dramatically, resulting in a higher activity of CaMKII compared to CaN. The model compares the ratios of CaMKII:CaN between the two sides of the growth cone, and hypothesizes that turning occurs to the side with the higher ratio. **(B–E)** Summary of experimental findings regarding how the steepness of the intracellular Ca^{2+} gradient interacts with background concentrations of Ca^{2+} to determine the direction of turning. **(F)** Predictions of the model for a normally attractive gradient. At normal PKA levels (black curve) and normal background Ca^{2+} concentrations the ratio of CaMKII:CaN is higher on the up-gradient side (assumed to have 30% higher Ca^{2+}) compared to the down-gradient side of the growth cone, and thus the response is attraction. However, in low background Ca^{2+}, CaMKII is very low on both sides, whereas CaN is still increasing with Ca^{2+} concentration. This causes the ratio of CaMKII:CaN to be higher on the down-gradient (lower Ca^{2+}) side, leading to repulsion. In high background Ca^{2+} the activity of CaMKII is saturated on both sides, and the steady increase of CaN with Ca^{2+} concentration means that again the CaMKII:CaN ratio is now higher on the down-gradient side, leading to repulsion. The effect of changing PKA activity is primarily to shift the curve along the Ca^{2+} concentration axis. When this shift is large enough it causes both high and low levels of PKA to lead to repulsion. **(G)** Predictions of the model for a normally repulsive gradient. In this case the up-gradient side is assumed to have 10% higher Ca^{2+}. Similar reasoning applies as for the normally attractive gradient discussed above, but the response at normal background Ca^{2+} concentration is now repulsion because the 10% increase is not sufficient to cause a large increase in CaMKII activity between the two sides; [42] for more details. Abbreviations: Epac, exchange protein directly activated by cAMP; I1, inhibitor 1; PKA, protein kinase A; PP1, protein phosphatase 1.

[50]. Notably, these responses are not merely the same response with the sign reversed; attraction to the left of a growth cone invokes a distinctly different response to repulsion from the right.

Growth cones usually respond to chemorepulsion through asymmetric endocytosis on the up-gradient side of the growth cone [16,17]. In response to a repulsive MAG gradient, *Xenopus* spinal neurons modulate the functional distribution of integrin receptors. Asymmetric β_1-integrin function is both necessary and sufficient for repulsive turning in response to MAG [16]. This asymmetry can be created by endocytosis of β1-integrin, which is positively

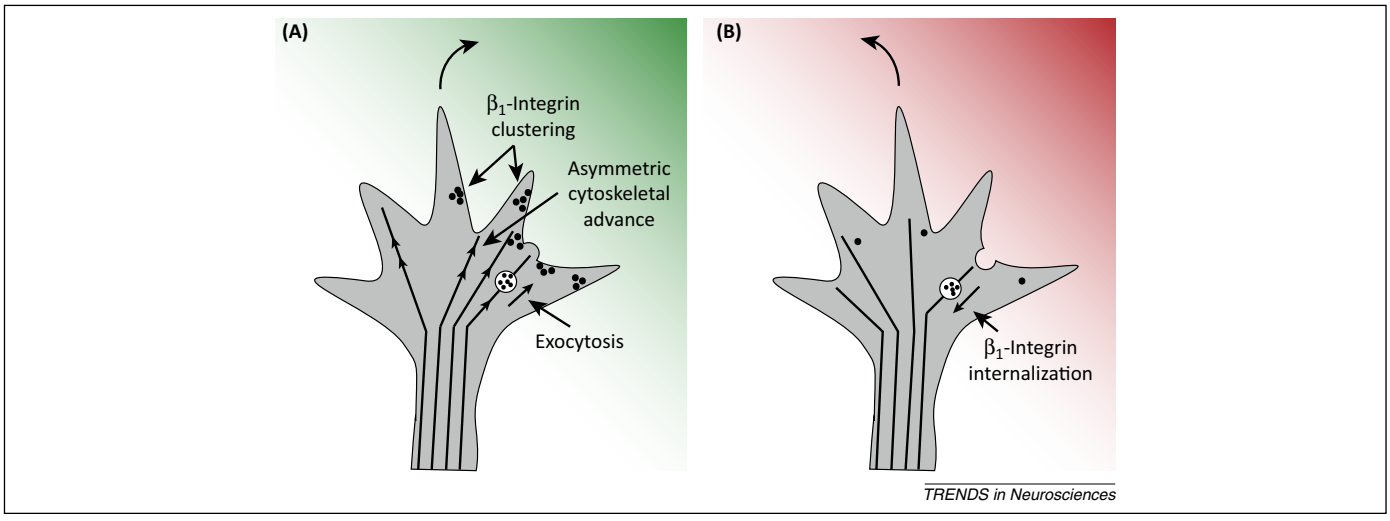

Figure 3. β_1-Integrins and membrane dynamics in response to calcium signaling during growth cone guidance. **(A)** Attractive chemotropic cues induce asymmetric exocytosis in the growth cone mediated by vesicle-associated membrane protein 2 (VAMP2) or syntaxin and neurotoxin-insensitive VAMP (TI-VAMP), as well as by β1-integrin clustering on the cell plasma membrane on the up-side of the gradient. Increased actin polymerization and branching, as well as increased tubulin polymerization, lead to an asymmetric increase in filopodial and lamellipodial activity on the up-side of the gradient. Ca^{2+} functions as a second messenger that directly activates these components. Increased adhesion of the growth cone to the substrate via β1-integrin, and addition of new plasma membrane to the up-side of the gradient, leads to turning and increased axon outgrowth in response to gradients of attractive guidance cues such as brain-derived neurotrophic factor (BDNF; green). **(B)** By contrast, gradients of repulsive chemotropic cues such as myelin-associated glycoprotein (MAG; red) lead to internalization of β_1-integrin via clathrin-mediated endocytosis, as well as the asymmetric endocytosis of cell plasma membrane on the up-side of repulsive guidance cue gradients. Loss of β1-integrin adhesions and removal of the cell membrane leads to turning of the growth cone away from the gradient.

regulated by Ca^{2+}. Indeed, inhibition of calcium signaling blocks repulsive turning and asymmetry in β1-integrin distributions [16]. Similar studies using semaphorin (Sema) 3A gradients and repulsive Ca^{2+} signals have found similar activation of asymmetric endocytosis [17] and axonal transport [51], indicating that this is likely to be a conserved method for growth cone repulsion that is not exclusive to MAG.

Hines *et al.* [16] found that internalization of β1-integrin occurs through clathrin-mediated endocytosis, and abolishing this process is sufficient to remove turning. Clathrin-mediated endocytosis is initiated by asymmetric formation of clathrin coated pits (the precursor to clathrin-mediated endocytosis) in response to Sema3A gradients or repulsive Ca^{2+} signals generated using FLIP [17]. Surprisingly, inhibition of clathrin-mediated endocytosis switches repulsion to attraction but does not affect growth cone attraction [17].

In contrast to repulsion, attractive signals induce asymmetric exocytosis at the growth cone [17,39]. Tojima *et al.* [18] identified enhanced vesicle-associated membrane protein (VAMP) 2-mediated exocytosis in response to attractive Ca^{2+} signals elicited by FLIP of caged Ca^{2+}. Furthermore, inhibition of VAMP2-mediated exocytosis by tetanus neurotoxin (TeNT) abolished attraction [18]. Interestingly, TeNT did not stop axon growth, suggesting that axon outgrowth mediation is independent of VAMP2 and can be regulated distinctly from attraction [18]. VAMP2-mediated exocytosis is not only necessary but also is sufficient for attraction [17].

In addition to VAMP2-mediated exocytosis, syntaxin-1 (Sytx1) and tetanus neurotoxin-insensitive (TI)-VAMP mediate netrin-1 signals; this complex associates with the netrin-1 receptor in growth cones, deleted in colorectal cancer (DCC) [50]. Noticeably Sytx1 coassociates TI-VAMP, rather than its more common partner VAMP2, in response to netrin-1 signals. This suggests a unique mechanism of exocytosis in netrin-1-induced attraction. Sytx1 and TI-VAMP are required for *in vitro* attraction in response to netrin-1 whereas VAMP2 inhibition (via TeNT) does not affect attraction [50]. Further, TI-VAMP and Sytx1 are both necessary for *in vivo* commissural axon pathfinding (an event known to be mediated by netrin-1) [50,52].

Ca^{2+}-dependent clustering of β1-integrin is essential for growth cone responses to BDNF (normally an attractive cue) (Figure 3) [40]. Bath application of BDNF is sufficient to induce increased β1-integrin clustering at the growth cone, especially within filopodia [40]. Simultaneous immunolabeling of known adhesion components and β1-integrin revealed that β1-integrin clustering in response to BDNF occurs within nascent adhesion complexes [40]. Pretreatment with BDNF was sufficient to abolish β1-integrin internalization in response to MAG. These results are consistent with the hypothesis that growth cone responses are determined by the balance of endocytosis and exocytosis: asymmetric exocytosis adds membrane, receptors, and integrins to the attracted side, whereas endocytosis removes membrane, receptors, and integrins from the repulsed side, and thus turning occurs towards the side with higher exocytosis or lower endocytosis.

Although PI3K is involved upstream of Ca^{2+} activation in response to netrin-1 and BDNF [38], PI3K activation is also required downstream of Ca^{2+} to activate microtubule-dependent membrane transport [22,39]. Inhibition of PI3K abolishes attractive turning (produced by FLIP of Ca^{2+} or IP_3) and inhibits microtubule advance, while leaving IICR unaffected [22,39]. Furthermore exogenous application of PIP_3, the product of PI3K catalyzed reaction, promotes microtubule advance [39] and growth cone turning [38]. Similarly, actin depolymerizing factor (ADF)/cofilin is

essential in regulating the actin cytoskeleton to cause both attraction and repulsion in BMP7-dependent turning of growth cones [48].

Remodeling of the growth cone cytoskeleton is mediated by a host of intermediary molecules. The most prominent of these are members of the small Rho GTPase family, including RhoA, Ras-related C3 botulinum toxin substrate 1 (Rac1), and cell division control protein 42 homolog (Cdc42), which have major roles in regulating actin and microtubule stability [53,54]. Elevation of cytoplasmic Ca^{2+} from intracellular stores can elevate Cdc42 and Rac1, but downregulates RhoA [14] through GTPase-activating proteins and guanine nucleotide exchange factors [55]. Rho GTPases are also activated by protein kinase C and CaMKII [56] which are both activated by Ca^{2+}. Ca^{2+} influx leads to activation of Cdc42 through platelet-activating factor acetyl-hydrolase IB subunit α (formerly known as Lis1) to regulate the cytoskeleton at the leading edge of moving neurons and growth cones by linking microtubule ends to actin [57].

The Rho GTPases are also themselves regulators of Ca^{2+}. RhoA regulates Ca^{2+} directly and is required for hematopoietic progenitor migration [58] whereas Rac1 modulates Ca^{2+} release in growth cones by enhancing microtubule assembly, which promotes Ca^{2+} release from the ER [59]. These results suggest how asymmetric calcium distributions caused by external guidance cues could lead to asymmetric cytoskeletal remodeling and thus growth cone turning.

Calcium, motility, and growth rate

The role of calcium signaling in overall growth cone motility is less well understood than its role in turning. Calcium signaling has an effect on the growth rate of growth cones, first characterized as a narrow extracellular calcium concentration range favorable for growth [43,60–62]. Surprisingly, calcium transients [63,64] and calcium entry through mechanosensitive channels slow axon growth, whereas calcium release from ER stores enhances growth [41]. The relation between calcium transients and cAMP signaling may be different between the central domain of the growth cone and its filopodia [19]. Further, manipulating growth cone calcium waves is sufficient to affect neurite extension directly [65].

High-frequency filopodial calcium transients can reduce growth cone motility and outgrowth by reducing filopodial motility [5]. Filopodial transients can also be propagated to the rest of the growth cone, initiating global growth cone calcium elevations. Importantly, local transients are not always propagated throughout the axon: local calcium transients can produce differential outgrowth and extension in axons and their branches [66]. Creation of asymmetric calcium transients, by use of multiple different substrates, is sufficient to induce growth cone turning [5]. This suggests that motility changes caused by Ca^{2+} transients and growth cone turning could share common calcium entry and response mechanisms.

It has been suggested that, in shallow gradients, axon guidance occurs primarily by growth rate modulation rather than via growth cone turning [67]. That is, changes in the direction of axon growth are random, but axons move faster when they are directed up the gradient than when they are directed down the gradient. Thompson *et al.* (2011) found that reducing cAMP levels did not convert attraction by growth rate modulation into repulsion, and therefore suggested that this guidance mechanism is controlled by distinct intracellular signaling processes from turning responses [68]. However, the role of calcium in axon guidance by growth rate modulation is unknown.

One way in which calcium signaling is involved in growth cone motility is through BDNF-induced axon outgrowth. Carlstrom *et al.* [40] found that BDNF-induced cytoplasmic calcium signaling leads to remodeling of adhesions containing β1-integrin onto growth cones and results in extension of the axon. Treatment with either a calcium chelator or a non-selective membrane calcium channel blocker is sufficient to block both β1-integrin clustering and the increased axon outgrowth resulting from BDNF application, indicating that Ca^{2+} influx from extracellular sources is necessary for BDNF-induced axon outgrowth [40]. Furthermore, MAG (normally a repulsive guidance cue) exhibited the opposite effect to BDNF, reducing both β1-integrin clustering and axonal extension, in a calcium-dependent manner [16,40].

A second, less direct mechanism which also modulates axonal growth via changes in intracellular calcium concentrations involves eukaryotic elongation factor-2 (eEF2), an essential translation factor modulated by Ca^{2+}/CaM [69]. Iizuka *et al.* [70] and Iketani *et al.* [71] found that particular calcium elevations within growth cones lead to phosphorylation of eEF2 by eEF2 kinase. Dephosphorylated eEF2 promotes mRNA translation whereas phosphorylated eEF2 inhibits translation, and through this mechanism calcium elevations reduce axonal growth [71]. Furthermore, elevation in calcium also leads to a reduction in the total amount of eEF2 within the growth cone, perhaps through a calcium-mediated protein degradation pathway. By contrast, both BDNF and NGF are known to increase the levels of active eEF2 [72,73], and this suggests that phosphorylation of eEF2 may depend on calcium entry mechanisms or the precise magnitude of calcium elevation.

Attractive factors such as BDNF [40], NGF [68], and netrin-1 [45,64], which rely on calcium signaling to produce attractive responses, are also known to promote growth cone motility in a calcium-dependent manner. Similarly, the repulsive guidance cue MAG reduces axon outgrowth in a calcium-dependent manner [40]. Wnt5a, a recently characterized calcium-dependent guidance cue, does not behave in this manner but instead is both a repulsive guidance cue and an axon outgrowth promoter [49] (Box 2).

Concluding remarks

Although Ca^{2+} has long been implicated as a second messenger in growth cone turning, it is only over the past few years that the precise distributions and mechanisms for attraction and repulsion have been elucidated. Many different mechanisms for intracellular Ca^{2+} elevation have been discovered, including release from ER stores (CICR and IICR) and entry from extracellular sources (CRAC, SOCE, and voltage-sensitive Ca^{2+} channels) [22,33,34,38]. A complex pathway which interprets these intracellular

Box 2. A newly discovered chemotaxis molecule – Wnt5a

To demonstrate concepts of Ca^{2+}-mediated axon guidance we consider the Ca^{2+}-mediated mechanisms of axon guidance by Wnt5a. Wnt5a is a member of the Wnt family of morphogens which are involved in a wide range of developmental processes [81]. In addition to its roles during embryogenesis, Wnt5a has recently been implicated in repulsive guidance of post-crossing axons in the corpus callosum in a calcium-dependent manner [49].

Wnt5a creates intracellular responses through the related to receptor tyrosine kinase (Ryk) and Frizzled (Fz) receptors [49]. In particular Li *et al.* [49] demonstrated both Ryk and Fz receptors are necessary for repulsive axon guidance. In contrast to other common repulsive guidance cues (such as MAG and Sema3A), Wnt5a does not decrease axon outgrowth but instead enhances it [49,82]. Increased axon outgrowth is observed both in bath application of Wnt5a and during repulsive turning induced by a Wnt5a gradient. In contrast to repulsive guidance, the Wnt5a-dependent increase in axon outgrowth is mediated exclusively by the Ryk receptor [49].

Wnt5a increases the frequency of calcium transients through both Ryk and Fz receptors; inhibition of either receptor significantly decreases the frequency of calcium transients *in vitro* [49]. In addition, gradients of Wnt5a are sufficient to induce repulsion, accompanied by underlying asymmetric calcium transients across the growth cone such that transients are higher on the opposite side to growth direction [49], confirming the necessary involvement of calcium in the growth cone response to Wnt5a.

The Wnt5a/Ca^{2+} pathway uses both SOC channels (in particular TRPC channels) and IICR to induce calcium signaling. However, axon outgrowth and axon guidance in response to Wnt5a are differentially controlled through these two calcium signaling mechanisms [49]. In particular, axon outgrowth is decreased by inhibition of either TRPC channels or IICR (through inhibition of either IP_3 receptors or PLC), whereas repulsive axon guidance is only affected by TRPC channels. Inhibition of CaMKII, a well-known component of other Ca^{2+} pathways which increase outgrowth, reduces Wnt5a-induced outgrowth [49] but does not affect repulsive axon guidance. These results suggest that two distinct Wnt5a/Ca^{2+} pathways are present. The first, which increases axon outgrowth, involves SOCE (TRPC channels), IICR (IP_3R and PLC), and CaMKII. The pathway controlling Wnt5a-mediated repulsive axon guidance involves TRPC channels, a common component of SOCE, and CaMKII.

Although the Wnt5a/Ca^{2+} pathways were first described *in vitro*, similar results have since been observed *in vivo* [82]. Wnt5a has been implicated in repulsive guidance post corpus callosum crossing [66]. Postcrossing axons have a higher rate of outgrowth than precrossing axons [82], which these authors attributed to the presence of Wnt5a after corpus callosum crossing. Both of the Wnt5a effects in growth cones are influenced by microtubule reorganization [83]. This is induced by CaMKII phosphorylation of tau at Ser262. Tau phosphorylation is necessary for both Wnt5a-mediated turning and axon outgrowth, confirming conserved microtubule action in both responses.

Ca^{2+} signals to determine attraction or repulsion has been described [45,47], together with a recent model which represents this pathway mathematically [42]. Finally, recent work has developed a new understanding of the intracellular mechanisms which produce attraction and repulsion, principally exocytosis and endocytosis (respectively).

Although the findings in recent years have contributed greatly to our understanding of axon guidance and its role in development and regeneration, there are still many outstanding questions (Box 3). One of the most fundamental of these is whether the effects of Ca^{2+} are mediated purely by its spatial concentration distribution, or whether its method of entry is also important (for instance, particular Ca^{2+} release mechanisms could also trigger other signaling pathways which are independent of Ca^{2+}, potentially modulating the effects of the resulting Ca^{2+} increase). We propose here the former hypothesis: that the same responses will be observed in response to a particular Ca^{2+} distribution, irrespective of which of the many ways that distribution could have been produced (e.g., CICR, IICR, and SOCE, via different types of membrane channels). This is consistent with current experimental results, including those utilizing direct elevation of Ca^{2+} concentrations, which are difficult to explain from the alternative standpoint. It is also the assumption at the heart of the mathematical model of [42],

Box 3. Outstanding questions

Although our understanding of calcium involvement in axon guidance has been significantly increased over the past few years, some of the questions that remain unanswered are detailed below.

- Axon guidance via growth cone turning is heavily dependent on calcium concentrations [42,44,84,85], and calcium has been implicated in controlling axon outgrowth [40,71,83,86]. Is calcium signaling involved in axon guidance by growth rate modulation [67,68]? Are mechanisms from steep gradient axon guidance conserved?
- Studies have indicated that many of the inhibitors and activators of PKA are nonspecific and also affect Epac; similarly, the agonist SP-cAMPS also activates Epac [87,88], and Epac knockdown has been demonstrated to abolish attraction in response to calcium-mediated guidance cues [78]. Can the functional effects of PKA and Epac be grouped without losing information? Is PKA or Epac the cAMP effector in processes including initializing calcium entry via IICR [22] and VDCCs [21], and controlling the proposed attraction/repulsion switch [42,47]?
- Although the pathway and model for an attraction/repulsion switch [42,47] does not consider specific methods of calcium entry separately, it has been suggested that the method of entry affects the turning response [30,89]. Can this be understood only in terms of resulting calcium distributions?
- How does cGMP affect parts of the network other than VDCCs and CICR (if at all)?
- Does the pathway described by the model of Forbes *et al.* [42] explain responses to gradients of Sema3A, Wnt5a, and sonic hedgehog [17,90,91]?
- Can the calcium-dependent CaMKII/CaN pathway [45] also explain turning responses to guidance factors such as Sema3A and morphogens (as has been suggested for Wnt5a [83]), or are there distinct non-conserved components in these pathways?
- CaN has been strongly implicated in repulsion [42,45,47] and is sufficient to induce endocytosis in other settings within growth cones [92]; does it directly induce endocytosis in response to repulsive cues [17]?
- What role does calcium signaling play in the response to physical cues?
- Calcium elevations are crucial in the repulsive response to MAG [16,45], and MAG is known to inhibit axonal regeneration *in vivo* [93]. Can controlling calcium concentrations influence axonal regeneration after injury?
- Calcium regulates Rho GTPase function; however, the exact relationship between calcium elevations, Rho GTPase function, and cytoskeletal changes is not entirely clear. Does calcium only induce Rho GTPase activity through molecules such as protein kinase C and CaMKII, or does it also have further downstream roles?

which explains growth cone turning responses as the spatial distribution of Ca^{2+} is varied. However, this is only one of the exciting areas awaiting further exploration in the future study of calcium dynamics in axon guidance.

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