The molecular basis for calcium-dependent axon pathfinding

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Abstract | Ca^{2+} signals have profound and varied effects on growth cone motility and guidance. Modulation of Ca^{2+} influx and release from stores by guidance cues shapes Ca^{2+} signals, which determine the activation of downstream targets. Although the precise molecular mechanisms that underlie distinct Ca^{2+}-mediated effects on growth cone behaviours remain unclear, recent studies have identified important players in both the regulation and targets of Ca^{2+} signals in growth cones.

Intracellular Ca^{2+} signalling influences a broad range of biological events in most, if not all cells, beginning with fertilization and continuing through development, into adulthood and concluding with cell death (for reviews, see REFS 1,2). That this simple ion can mediate such a vast spectrum of physiological events is remarkable. One particularly well-studied, yet highly confounding cellular location where Ca^{2+} has been reported to have many unique effects is in the terminal growth cones of extending axons and developing dendrites. Although Ca^{2+} has been recognized as an important mediator of the morphological differentiation of neurons for more than 25 years, the molecular basis for its diverse effects remains elusive.

Neurons maintain a baseline intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) at the resting state, termed the resting $[Ca^{2+}]_i$. Ca^{2+}-mediated signalling involves fluctuations of $[Ca^{2+}]_i$ above the resting level. At the growth cone, Ca^{2+} fluctuations are often linked with changes in morphology and motility. Correlations between motile behaviour and $[Ca^{2+}]_i$ come from both experimental manipulations of cytosolic Ca^{2+} and from Ca^{2+} imaging studies. However, treatments that elevate or depress $[Ca^{2+}]_i$ often have wide-ranging effects on motility. Effects on growth cone behaviour depend on several factors, including the spatial and temporal characteristics of the Ca^{2+} changes, the Ca^{2+} effectors present in the domain of elevated Ca^{2+}, and possibly the particular Ca^{2+} channel types involved in generating $[Ca^{2+}]_i$ changes. One challenge in elucidating the mechanisms of Ca^{2+}-mediated motility is the difficulty of linking individual versus cumulative Ca^{2+} homeostatic processes with the activation of particular downstream targets. Often, treatments that alter one intracellular process rapidly affect homeostatic processes. For example, a reduction in overall Ca^{2+} influx or influx through specific channels might trigger homeostatic release of Ca^{2+} from stores in an attempt to maintain baseline $[Ca^{2+}]_i$. However, as new Ca^{2+} channels, buffers and transporters are identified together with their specific downstream effectors, we are beginning to understand how diverse behaviours derive from this simple ion.

In this review, we first describe the functional effects that distinct Ca^{2+} signals have on the motility and guidance of axonal and dendritic extensions. Second, we discuss the mechanisms responsible for altering $[Ca^{2+}]_i$ in growth cones and dendrites. Recent findings indicate that several unexpected channels are gated downstream of chemotropic axon guidance cues. After Ca^{2+} enters the cytosol it must act on specific downstream Ca^{2+}-binding partners and subsequent cytoskeletal effectors. We discuss recent findings suggesting that Ca^{2+} signals are decoded by Ca^{2+}-binding proteins with varying affinities and on/off rates. Finally, we present a unifying model that attempts to reconcile disparate data collected from studies using various cell types under distinct conditions over the years. Although good progress has been made recently, several key questions remain unanswered, which we address at the end of the article.

Functional effects of Ca^{2+} on axon outgrowth

Global Ca^{2+} signals regulate neurite outgrowth. A large body of evidence indicates that cytosolic Ca^{2+} has important and diverse roles in the control of axonal and dendritic growth and guidance. Remarkably, different Ca^{2+} signals can have varying behavioural effects, often opposite, on growth cone motility. On the one hand, elevations of growth cone Ca^{2+} have been associated with reduced motility in many neuronal types (for reviews, see REFS 3–5). When growth cones are exposed to stimuli that produce a large, sudden $[Ca^{2+}]_i$ elevation, they typically slow down, stop or retract in a Ca^{2+}-dependent manner[6–11]. Consistent with the idea that Ca^{2+} serves as a negative

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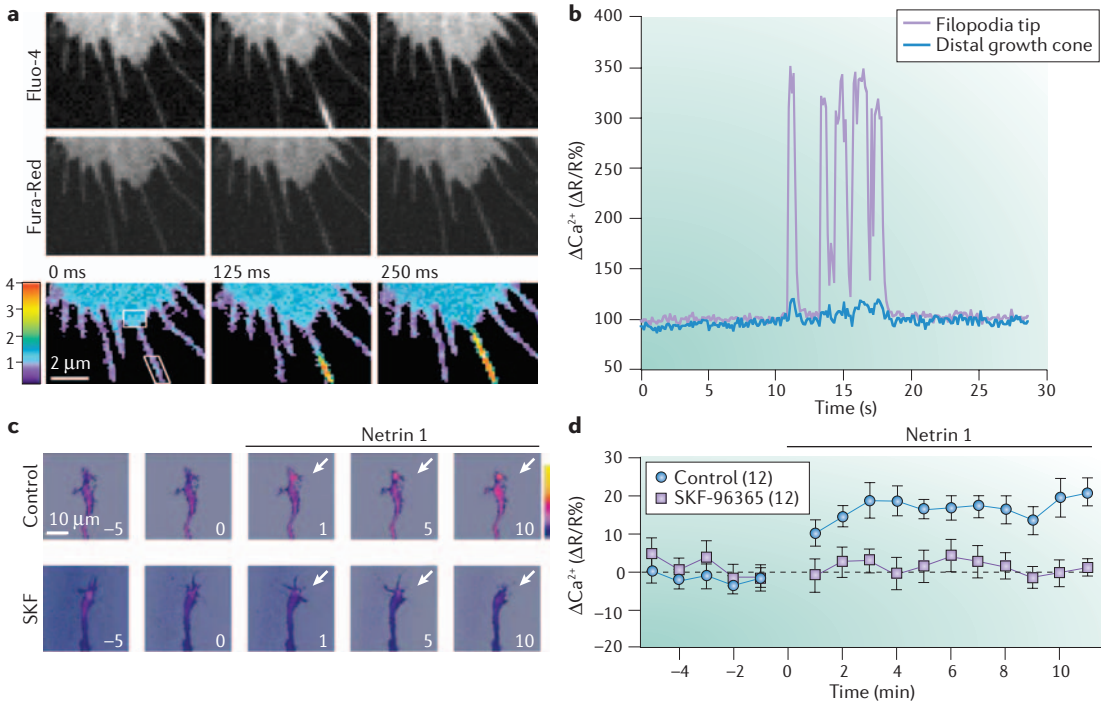

Figure 1 | **Measurements of Ca^{2+} using fluorescent Ca^{2+} indicators in *Xenopus* spinal neurons show that Ca^{2+} signals can vary widely in amplitude, spatial spread and kinetics. a** | Individual fluorescent channels of a growth cone co-loaded with Fluo-4 and Fura-red at three time intervals (top). The pseudocolour ratio images (bottom) illustrate normalized Ca^{2+} signals. A large Ca^{2+} elevation initiates at the tip of a single filopodium at 125 ms and diffuses towards the body of the growth cone. **b** | Fluorescence ratio (ΔR) measurements (normalized to baseline) determined from regions of interest at the filopodial tip and distal growth cone (regions used indicated on the 0 ms ratio image in **a**). Changes in fluorescence ratio relative to the baseline ratio (R) are indicative of changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). Note that the amplitude of Ca^{2+} transients at the filopodial tip exceed 350% of baseline and lead to small elevations in Ca^{2+} of ~10% above baseline at the distal region of the growth cone. **c** | Ca^{2+} measurements of a growth cone on exposure to a netrin gradient. The assays were conducted using similar techniques to those used in **a**. Pseudocolour ratio images (of Fluo-4/Fura-red signals) illustrate normalized Ca^{2+} signals during exposure to netrin. Inhibition of transient receptor potential TRPC channels with SKF-96365 (an inhibitor of store-operated Ca^{2+} influx) prevents Ca^{2+} elevation in response to netrin. Arrows indicate site of netrin application; numbers represent time in minutes. **d** | Average fluorescence ratio measurements in a growth cone show that netrin modestly elevates $[Ca^{2+}]_i$ (10–20% above baseline) as compared with spontaneous Ca^{2+} transients in filopodia.

regulator of process extension, reductions in $[Ca^{2+}]_i$ were found to promote neurite outgrowth from both invertebrate and vertebrate neurons[12–14]. One interpretation of these results is that there is an optimal range for $[Ca^{2+}]_i$ (REF. 5) that supports maximal outgrowth. Moreover, as reducing Ca^{2+} influx accelerates axonal outgrowth from some types of neuron, it seems that resting $[Ca^{2+}]_i$ is above the optimal range for maximal neurite extension for these neurons. Interestingly, blocking Ca^{2+} influx through certain channel types seems to be more effective than other approaches in accelerating outgrowth[13,14], and partially blocking Ca^{2+} influx provides better results than completely eliminating it. For example, a low concentration of polyvalent cation Ca^{2+}-channel blockers accelerates neurite outgrowth, whereas a high concentration slows outgrowth[13]. These results suggest that different plasma membrane channels contribute to slowing neurite growth rate to different degrees and that some Ca^{2+} influx is necessary for optimal outgrowth. However, other studies have found that elevating $[Ca^{2+}]_i$ in growth cones promotes neurite outgrowth[15–17]. Many factors might explain these discrepancies, such as differences in resting $[Ca^{2+}]_i$ relative to the optimal 'set point' for different types of neuron, or the experimental conditions used. As Ca^{2+} is known to control gene expression, conditions in which chronic manipulations of Ca^{2+} signalling are used can alter cellular differentiation independent of the local effects of Ca^{2+} on the motility machinery of growth cones[18,19]. For example, growth cones can adapt to elevated baseline $[Ca^{2+}]_i$ conditions[19], which suggests that the Ca^{2+}-dependent targets that define the 'optimal range' can adjust their sensitivity or be downregulated.

Growth cone motility is not only regulated by prolonged shifts in Ca^{2+} influx or baseline $[Ca^{2+}]_i$ changes, but also by temporal patterns of $[Ca^{2+}]_i$ fluctuations (FIG. 1a). Neuronal growth cones show spontaneous and agonist-induced transient elevations in $[Ca^{2+}]_i$ that reduce the rate of neurite extension both in culture[14,18,20–23] and *in vivo*[24]. Ca^{2+} transients come in many forms, which have distinct kinetic characteristics and spatial spread. Ca^{2+} transients range from global events involving the entire neuron[16,21] to highly localized signals at the tips of axonal

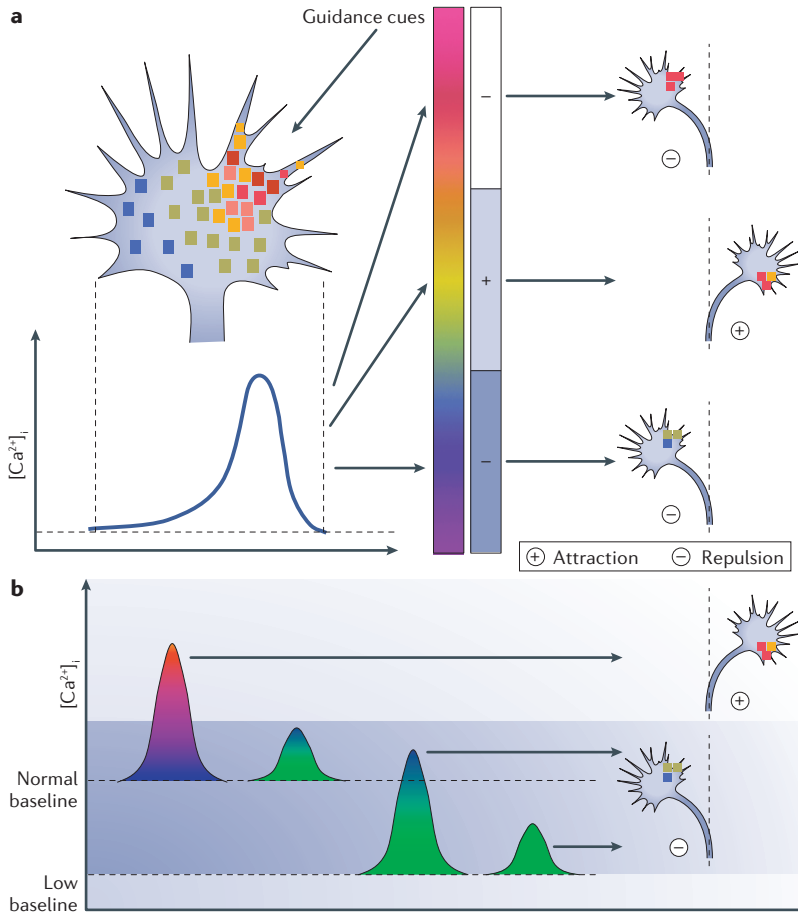

Figure 2 | **Regulation of growth cone turning by local and global Ca^{2+} signals.** **a** | Elevation of the local intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) can occur across three ranges to produce repulsion or attraction. Current data support the idea that small and large elevations in $[Ca^{2+}]_i$ result in repulsion, whereas modest elevations induce attraction. **b** | The baseline $[Ca^{2+}]_i$ can also affect the turning responses induced by local elevations in $[Ca^{2+}]_i$. Experimental data show that lowering the baseline tends to shift the response to local $[Ca^{2+}]_i$ elevation to repulsion.

or dendritic filopodia[25,26] (FIG. 1a). Distinct Ca^{2+} transients are generated by activation of specific Ca^{2+} channels on the plasma membrane[14,20], intracellular stores[6] or both. In some cases, the channels responsible for generating Ca^{2+} transients are unidentified, non-traditional channels that are not gated by transmitters or changes in voltage[25].

Local Ca^{2+} signals regulate growth cone morphology and steering. Although transient or prolonged global elevations of growth cone $[Ca^{2+}]_i$ often slow or halt axon outgrowth, localized Ca^{2+} signals seem to promote growth cone turning (for reviews, see REFS 4,27). Many studies have shown that when Ca^{2+} is directly elevated locally on one side of the growth cone, these local signals can promote protrusion of filopodia[28–31] and lamellipodial veils[32], and, over time, orient axon outgrowth[28,33,34]. The effects of Ca^{2+} signals on filopodial protrusion and stability are of particular interest, as, again, seemingly discrepant findings have been described. Local and global elevations of Ca^{2+} have been shown to promote filopodial formation and extension in some circumstances[30,31,35–38], but to cause collapse or stabilization of filopodia in other situations[8,25,26,39]. The cause of these opposing responses by filopodia is not clear, but the characteristics of the Ca^{2+} signals, such as amplitude, frequency, number and mechanism of generation, might account in part for the observed differences. For example, a single short duration pulse of Ca^{2+}, produced by photolysis of caged Ca^{2+}, is sufficient to stimulate filopodial protrusion[31,35], but repetitive spontaneous transients stabilize existing filopodia[25]. Interestingly, a recent study investigating Ca^{2+} signalling in developing hippocampal neurons *in situ* found that low-frequency spontaneous Ca^{2+} transients in dendritic filopodia correlated with the formation and extension of nascent filopodia[26]. However, at higher Ca^{2+} transient frequencies, filopodia became stabilized much as observed in axonal growth cones[25]. Although Ca^{2+} transients in growth cone filopodia guide axonal outgrowth and might contribute to early synapse formation, in dendrites these signals probably function to stabilize competing synaptic contacts[26,40].

Chemotropism of axons toward certain guidance cues depends on Ca^{2+} signalling in growth cones. For example, chemoattraction towards nerve growth factor[33,41], brain-derived neurotrophic factor (BDNF)[42] and netrin[43,44] depend on Ca^{2+} signalling. Ca^{2+} imaging studies show that during chemotropism, modest Ca^{2+} gradients form across the growth cone, with the highest $[Ca^{2+}]_i$ on the side toward the source of the guidance cue[28,43,45] (FIG. 1b). Interestingly, chemorepulsion from myelin-associated glycoprotein (MAG) also involves Ca^{2+} gradients in growth cones with the elevated side toward the source of the guidance cue[46]. However, the amplitude of local Ca^{2+} signals at the growth cone seems to be lower during chemorepulsion, which suggests that the slope of the $[Ca^{2+}]_i$ gradient is somehow a determinant for steering direction[43,46] (FIG. 2a). On the other hand, studies using direct manipulation of $[Ca^{2+}]_i$ indicate that the extent of the local Ca^{2+} elevation, which is coupled with baseline $[Ca^{2+}]_i$, controls the direction of growth cone steering[34,47]. Although the combination of a local Ca^{2+} concentration and baseline $[Ca^{2+}]_i$ constitutes the slope of the gradient across the growth cone, it is not clear whether the slope or the individual Ca^{2+} components (local versus baseline) determines the direction of growth cone steering. In the former case, for example, a small local Ca^{2+} elevation produces a larger amplitude difference across the growth cone at low resting $[Ca^{2+}]_i$, which should result in attractive turning. However, lowering the baseline $[Ca^{2+}]_i$ typically promotes repulsive turning in response to both large and small local Ca^{2+} signals[34,46,47]. These results indicate that local and global Ca^{2+} signals in the growth cone might separately determine which downstream signalling cascades are activated to mediate repulsion or attraction (FIG. 2b). Two outstanding questions, which we discuss below, are how different guidance cues generate Ca^{2+} gradients of varying amplitude across growth cones, and which downstream processes transduce these Ca^{2+} signals into attractive or repulsive turning.

Ca^{2+} and axon branching. The proper development of complex neural networks occurs not only through guidance of terminal growth cones, but also by local control of axon branching patterns[48–50]. Axonal and dendritic

Chemotropism
The movement or orientation of an extending axon or cell along a chemical concentration gradient either towards or away from a chemical stimulus.

branching are determined by various Ca^{2+}-dependent processes that seem to control the initiation, elimination and stability of branches (for a review, see REF. 51). Competition between eye-specific retinal ganglion cell axons leads to selective elimination and stabilization of branches in response to Ca^{2+} influx through NMDA (*N*-methyl-D-aspartate) receptors[52]. On the other hand, neuronal activity also seems to be required for branch formation and motility[53]. Although the specific Ca^{2+} signalling characteristics and/or channels involved were not identified in the latter study, it is tempting to speculate that distinct Ca^{2+} signals coordinate the opposing behaviours described in these two studies. These results are similar to the bifunctional effects of Ca^{2+} signals on growth cone turning and filopodial formation described above. Branching factors might control the expansion of new branches into unoccupied territory through activation of specific Ca^{2+} signalling pathways, whereas competition for target-derived factors might stabilize or eliminate branches by activating distinct Ca^{2+} signals. In support of this idea, netrin was recently shown to promote branch formation in cortical neurons by stimulating local Ca^{2+} transients[54]. In this study, local Ca^{2+} transients were produced in response to netrin by Ca^{2+} release from inositol-1,4,5-trisphosphate ($Ins(1,4,5)P_3$) receptors. Local Ca^{2+} signals correlated with rapid initiation of new filopodia and branches, which depended on Ca^{2+} release from stores. It would be interesting to know whether NMDA receptor activity modulates the positive effects of netrin in this *in vitro* paradigm, as this would support the idea that distinct Ca^{2+} signals or pathways promote different phases of branch development.

Cytoplasmic Ca^{2+} concentration in growth cones

Ca^{2+} influx and release mechanisms. The $[Ca^{2+}]_i$ in growth cones is tightly controlled by various channels, pumps and buffers (FIG. 3). Fluctuations in $[Ca^{2+}]_i$ occur when channels on the plasma membrane or on intracellular stores open to allow Ca^{2+} to flow into the cytosol. One of the best-studied plasma membrane channel types on growth cones is gated by voltage changes[55]. Voltage-operated calcium channels (VOCCs) function throughout the stages of neural development that precede synaptogenesis. For example, VOCCs function in neural induction[16,56,57], selection of transmitter phenotype by neurons[58,59], and also in the regulation of growth cone pathfinding behaviours downstream of axon guidance cues[43,60]. Early studies showed that VOCCs were partially, but not completely, responsible for Ca^{2+} elevations induced by netrin 1, as Ca^{2+} signals in response to netrin 1 were reduced, but not eliminated, when L-type VOCCs were blocked[43]. Interestingly, chemoattraction towards netrin is also switched to chemorepulsion when L-type Ca^{2+} channels are blocked. However, some Ca^{2+} influx is still required for chemorepulsion, as all turning (but not outgrowth) is prevented when Ca^{2+} influx is reduced by low extracellular Ca^{2+} conditions[42,61]. These findings suggest that Ca^{2+} influx through other pathways must be responsible for repulsive turning when L-type channels are inhibited.

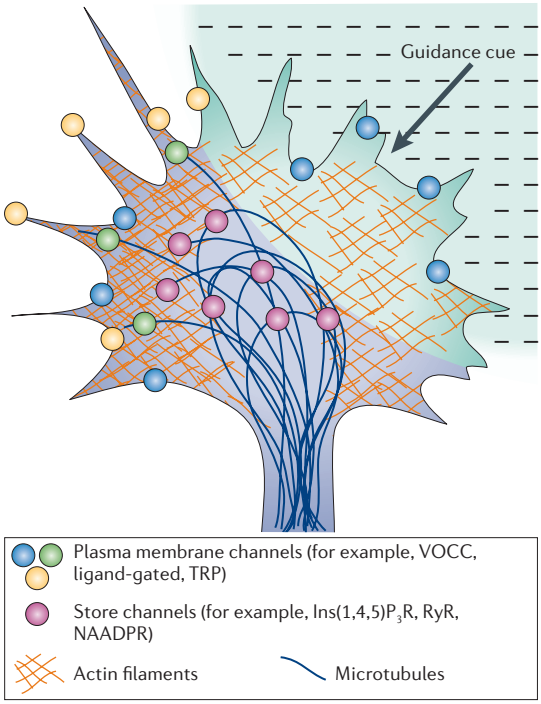

Figure 3 | **Local and global changes in growth cone intracellular Ca^{2+} concentration occur in response to Ca^{2+} influx through plasma membrane channels and release from intracellular stores.** Plasma membrane channels and receptors on Ca^{2+} stores are gated by various stimuli that can be activated locally or globally. For example, neuronal depolarization probably activates voltage-operated calcium channels (VOCCs) across the entire growth cone, leading to an overall cellular Ca^{2+} transient. On the other hand, local activation of channels either directly (for example, receptor channels) or through a receptor-mediated enzymatic process (for example, phospholipase C hydrolysis of phosphatidylinositol-4,5-bisphosphate, $PtdIns(4,5)P_2$, to generate inositol-1,4,5-trisphosphate, $Ins(1,4,5)P_3$) could result in local Ca^{2+} influx or release from $Ins(1,4,5)P_3$ receptor ($Ins(1,4,5)P_3R$) stores. Local signals might be amplified by the recruitment of various channels (for example, Ca^{2+}-induced Ca^{2+} release), resulting in Ca^{2+} gradients of varying slope. As intracellular Ca^{2+} is regulated by various homeostatic processes, various channel types and pumps might contribute to the amplitude, slope and kinetics of the Ca^{2+} signal. Moreover, the functional effectors of Ca^{2+} signals might reside near the site of influx or release in a Ca^{2+} microdomain, leading to variable effects on growth cone motility of seemingly comparable Ca^{2+} signals. The growth cone cytoskeleton — actin and microtubules — might also modulate Ca^{2+} signals by regulating the distribution of plasma membrane Ca^{2+} channels and stores that contain unique receptors involved in Ca^{2+} release. NAADPR, nicotinic acid adenine dinucleotide phosphate receptor; RyR, ryanodine receptor; TRP, transient receptor potential channel.

The presence on growth cones of non-traditional Ca^{2+} channels that are gated by unknown factors has been suspected for years, but the identity of specific channel types has remained elusive. For example, spontaneous Ca^{2+} transients that occur in growth cones[20,21,23]

and locally within individual filopodia[25] depend on Ca^{2+} influx through a non-VOCC. The frequencies of these local Ca^{2+} transients are unaffected by organic VOCC blockers[20,21,25] and ligand-gated channel blockers (T.M.G., unpublished observations), but are inhibited by nonspecific inorganic cations[20,21,23,25]. The triggering mechanism that activates these channels remains unknown, but strong substratum adhesion correlates with higher frequency transients, which suggests that mechanical tension might be involved[25]. Interestingly, in behavioural assays, blocking VOCCs has no effect on the rate of outgrowth, whereas globally inhibiting Ca^{2+} influx accelerates axon outgrowth[21]. This indicates that Ca^{2+} influx through some non-VOCCs might normally suppress axon extension.

Several recent reports have identified new Ca^{2+} influx pathways that are active on growth cones during chemoattraction towards both netrin and BDNF[62–64], and chemorepulsion by MAG[64]. Members of the transient receptor potential (TRP) family of non-selective cationic channels were found to contribute to Ca^{2+} influx and to depolarize neurons sufficiently to activate L-type VOCCs in response to netrin and BDNF[62,63]. Although TRP type C (TRPC) channels were previously shown to regulate neurite outgrowth in hippocampal neurons[65,66], these were the first studies to show that TRP channels are activated downstream of guidance cues. Exactly how these guidance cues activate TRPC channels on growth cones is not clear, but it seems that Ca^{2+} release from $Ins(1,4,5)P_3$ receptor stores triggers TRPC channel opening by store-operated Ca^{2+} influx. The results of earlier studies using turning assays suggested that these guidance cues activate phospholipase C (PLC)[41], which hydrolyses phospholipids into $Ins(1,4,5)P_3$ and diacylglycerol (DAG). $Ins(1,4,5)P_3$ activates Ca^{2+} release from the endoplasmic reticulum and the depletion of these stores can open store-operated Ca^{2+} channels (SOCCs), which include some TRP channels (for a review, see REF. 67). In *Xenopus* spinal neurons, a TRPC1 homologue is necessary for Ca^{2+} changes and chemotropic turning. However, TRPC1 channels do not seem to function as SOCCs in *Xenopus* oocytes[68], which suggests that a different activation mechanism might occur in growth cones[69]. Similarly, chemoattraction of cerebellar granule growth cones toward BDNF seems to require TRPC3 and TRPC6 channels, which act downstream of PLC and $Ins(1,4,5)P_3$ (REF. 62). Together, these findings suggest a signalling cascade that involves guidance receptor activation, PLC activation, $Ins(1,4,5)P_3$ production, Ca^{2+} release from the endoplasmic reticulum and TRPC channel opening.

As previous studies have established that small gradients occurring as a result of locally elevated $[Ca^{2+}]_i$ induce growth cone repulsion and modestly large Ca^{2+} gradients induce attraction, it is puzzling that blocking influx through TRP channels by pharmacological inhibition or antisense nucleotide knockdown completely abolished all the turning responses[62,63]. If Ca^{2+} release occurs when TRP channels are inhibited, then a low amplitude $[Ca^{2+}]_i$ gradient would be generated in response to local guidance cues through Ca^{2+} release alone, which should result in repulsion[46]. Indeed, in a separate study, netrin 1-induced attraction was converted to repulsion after morpholino knockdown of *Xenopus* TRPC channels. Moreover, MAG-induced repulsion was blocked by knockdown of *Xenopus* TRPC protein expression. These results indicate that TRP channels might contribute to the size of local Ca^{2+} elevations or baseline $[Ca^{2+}]_i$. Therefore, TRP channel knockdown could induce repulsion to netrin 1 by reducing either of these two conditions. As MAG-induced repulsion has been shown to involve a small Ca^{2+} elevation[46], *Xenopus* TRPC knockdown would further reduce the Ca^{2+} signals and block all turning responses[64]. It should be noted that protein knockdown by antisense technologies in different cells and by different approaches can result in different degrees of functional loss. Furthermore, chronic inhibition of TRP channel activity could reduce the concentration of Ca^{2+} in intracellular stores, which would, in turn, prevent Ca^{2+} release in response to guidance cues[62–64]. Therefore, differences in the extent of knockdown or homeostatic regulation could account for the discrepant findings.

Ca^{2+} gradients versus Ca^{2+} microdomains. Growth cone steering in response to extracellular gradients requires localized signalling to provide directional instructions. Although most extracellular guidance gradients are quite shallow and spread relative to the width of a growth cone, Ca^{2+} elevation within the growth cone seems to be relatively localized[28,43,46]. By comparison, in chemotactic cells, shallow gradients of extracellular cues are amplified into steep or highly localized gradients of intracellular signals that provide directional instructions for cell migration (for a review, see REF. 70). Although local Ca^{2+} signals have been observed in growth cones by imaging fluorescent Ca^{2+} indicators[25,28,46], the precise nature of the functional Ca^{2+} changes that coordinate directional motility is not clear. For example, the endogenous Ca^{2+} signals in growth cones that activate Ca^{2+}-mediated effectors might occur within Ca^{2+} microdomains, which could be important for localized signal transduction during guidance. Ca^{2+} nanodomains (single channel) or microdomains (clustered channels) are highly localized Ca^{2+} signals generated in cells due to the passive movement of Ca^{2+} through the plasma membrane and store-associated channels coupled with its restricted diffusion by various homeostatic mechanisms[71] (FIG. 3). Cytoplasmic Ca^{2+} buffers, pumps and exchangers help shape the spread, amplitude and duration of local cytosolic Ca^{2+} signals involved in growth cone pathfinding. In fact, many local Ca^{2+} signalling events are undetectable with standard fluorescence imaging techniques, yet have been shown experimentally to mediate important physiological processes[72–75]. It is therefore plausible that past Ca^{2+} imaging data on growth cones have revealed only the overall average of Ca^{2+} signals across the growth cone, and that spatially and temporally-restricted Ca^{2+} signalling domains that are functionally relevant for growth cone motility have remained undetected.

Ca^{2+} microdomains might couple specific Ca^{2+} signals to different downstream effectors. Within local Ca^{2+} signalling domains, spatially restricted Ca^{2+}-binding effector proteins are believed to regulate distinct physiological processes. For example, Ca^{2+} entry through L-type Ca^{2+}

Antisense nucleotide knockdown
The use of an oligonucleotide with a complimentary sequence to a target mRNA to promote hybridization. When antisense DNA or RNA is added to a cell, it binds to a specific mRNA molecule and prevents translation into protein.

Morpholino
A synthetic oligonucleotide with a modified sugar backbone (morphine ring) that is resistant to degradation by nucleases and therefore forms stable translation-blocking hybrids with endogenous mRNA. This form of oligonucleotide is particularly popular for work with zebrafish and *Xenopus* systems.

Ca^{2+} nanodomain
A local Ca^{2+} signal generated by Ca^{2+} influx through a single channel. To encode information, Ca^{2+} sensors must be positioned within 50 nm of the open Ca^{2+} channel.

Ca^{2+} microdomain
A local Ca^{2+} signal generated by integrated Ca^{2+} influx through a discrete cluster of Ca^{2+} channels. To encode information, Ca^{2+} sensors must be positioned < 1 μm from the open Ca^{2+} channels.

channels in cortical neurons activates the Ras–mitogen-activated protein kinase (MAPK) pathway[76]. The physical association between Ca^{2+} channels and signalling intermediaries, such as calmodulin, is believed to account for this selectivity[76]. Conversely, activation of nuclear factor of activated T cells (NFAT) signalling by BDNF specifically requires Ca^{2+} release from Ins(1,4,5)P_3 receptors, but not NMDA receptors or L-type Ca^{2+} channels[77]. Given that Ca^{2+} has been shown to function conclusively within microdomains in the presynaptic terminal, a similar function is anticipated in growth cones.

Downstream Ca^{2+} targets in growth cones

Changes in $[Ca^{2+}]_i$ with different spatial and temporal characteristics can generate diverse cellular responses due to various downstream targets that are modulated by intracellular Ca^{2+} signals. In particular, many Ca^{2+}-mediated target proteins expressed by growth cones regulate motility and axon guidance. For example, Ca^{2+} regulates proteins that are involved in the organization and movement of actin filaments. These include several members of the myosin family that are involved in actomyosin-based retrograde flow, adhesion site formation and vesicle trafficking (for reviews, see REFS 78,79). Other proteins regulated by Ca^{2+} organize actin filament bundles (such as α-actinin and fodrin[80,81]) or promote filament turnover (for example, gelsolin and actin depolymerizing factor (ADF)/cofilin[82,83]), which are two important processes for membrane protrusion and substratum adhesion. In addition to the regulation of the cytoskeleton, which we describe in more detail below, Ca^{2+} might also regulate protein synthesis and degradation[84,85], which have recently been found to occur locally in growth cones in response to axon guidance cues[86,87].

Although it is clear that Ca^{2+} can mediate both attractive and repulsive turning, it is less certain how a single ion instructs opposing motile behaviours of growth cones. Recent studies suggest that different spatiotemporal patterns of cytosolic Ca^{2+} signals are responsible for distinct turning behaviours. In particular, a small local (asymmetric) Ca^{2+} elevation promotes growth cone repulsion, whereas a modest local elevation in $[Ca^{2+}]_i$ induces growth cone attraction[34,43,46,63]. In addition, much larger local Ca^{2+} transients also promote growth cone repulsion[25,88] (FIG. 1a). These findings indicate that at least three types of local Ca^{2+} signal can control attraction versus repulsion: small and large Ca^{2+} signals promote repulsion, whereas modest Ca^{2+} changes promote attraction (FIG. 2). This idea is consistent with previous models of optimal Ca^{2+} range for growth cone motility[89]. A major challenge is to identify the specific downstream effectors that are responsible for translating different Ca^{2+} signals into distinct growth cone responses. Although many proteins could interact with Ca^{2+} to regulate growth cone behaviours (for a review, see REF. 4), we only discuss a few candidates that have been implicated by recent studies.

Ca^{2+}/calmodulin-dependent kinases and phosphatases. The most extensively characterized Ca^{2+}-binding protein in cells is calmodulin (CaM), which functions as an intracellular Ca^{2+} sensor[84,90]. On Ca^{2+} binding, Ca^{2+}/CaM associates with wide-ranging targets, including several kinases and phosphates, to elicit diverse signalling cascades. Ca^{2+}/CaM-dependent protein kinases (CaMKs) represent an important family of Ca^{2+}-activated proteins, and has five identified members[91]. Among the CaMKs, CaMKII is unique for its ability to autophosphorylate and its important roles in neural development and plasticity[92]. Although CaMKI has been shown to be important in Ca^{2+}-dependent regulation of axonal extension[93], CaMKII seems to function in both growth cone turning and branching[28,47,54,94]. Different CaMKs might be involved in regulation of different aspects of axonal and dendritic growth, branching and growth cone steering. Interestingly, isoforms of CaMKII with different affinities for Ca^{2+}/CaM activation are expressed in neurons at different stages of development. Among the CaMKII isoforms, β-CaMKII is of particular interest as it is expressed at early developmental stages, is anchored to the actin cytoskeleton, and is able to regulate neurite extension[94]. Importantly, the concentration of Ca^{2+}/CaM required for β-CaMKII activation is several orders of magnitude less than that required for the α-isoform[95], allowing it to sense smaller Ca^{2+} signals.

CaMKII is crucial for Ca^{2+}-dependent attraction of *Xenopus* growth cones in response to extracellular cues[28,47]. Studies that use direct local elevation of $[Ca^{2+}]_i$ through photolysis of caged Ca^{2+} have shown that CaMKII specifically mediates growth cone attraction that is induced by modest local Ca^{2+} signals. As α-CaMKII is not expressed in *Xenopus*[96], the results of these studies suggest that β-CaMKII might be responsible for Ca^{2+}-dependent growth cone attraction. Whether α-CaMKII has a role in axon guidance of other neuronal types remains to be determined. It was shown recently that α-CaMKII mediates Ca^{2+}-dependent axonal branching, which is activated by netrin 1 in cortical neurons. However, in this case, large Ca^{2+} transients seem to be responsible for α-CaMKII activation in the formation of axon branches in response to netrin 1 exposure[54]. Therefore, it is likely that different CaMKII isoforms serve different functions at distinct stages during neuronal development and in response to Ca^{2+} signals of varying magnitude.

Although CaMKII is involved in Ca^{2+}-dependent attraction, Ca^{2+}-dependent repulsion probably involves different downstream effectors depending on the nature of the repulsive Ca^{2+} signal. Recent studies have shown that both small and large local Ca^{2+} transients induce growth cone repulsion[25,34,43,46,47,88]. These distinct Ca^{2+} signals can be generated in growth cones to promote repulsion by gradients of soluble MAG (which result in a small Ca^{2+} signal) or by filopodial contact with boundaries of the insoluble extracellular matrix protein tenascin (which results in a large Ca^{2+} signal)[25,46]. Recent studies using focal Ca^{2+} photolysis showed that the Ca^{2+}/CaM-dependent phosphatase calcineurin is required for Ca^{2+}-dependent repulsion in response to small local Ca^{2+} signals[47]. Calcineurin is the only known Ca^{2+}/CaM-dependent phosphatase that is highly enriched in the brain[77], and has previously been implicated in Ca^{2+} regulation of axon extension[97]. Although calcineurin has several potential substrates, its activation by Ca^{2+} in growth cones appears

Actomyosin
A motor system composed of actin filaments and myosin, which hydrolyse ATP to produce force in processes such as muscle contraction and retrograde actin flow.

to dephosphorylate and activate another phosphatase, protein phosphatase 1 (PP1), to induce repulsive growth cone turning[47]. Given that calcineurin is activated by smaller Ca^{2+} signals than CaMKII, this kinase–phosphatase pair might function as a bimodal switch to control the direction of growth cone turning in response to different Ca^{2+} signals: small Ca^{2+} elevation preferentially activates the calcineurin–PP1 pathway for repulsion, whereas moderate Ca^{2+} elevation acts predominately through CaMKII for attraction[47]. A bimodal switch could explain the effects of baseline $[Ca^{2+}]_i$ on Ca^{2+}-dependent growth cone turning, as lowering the baseline $[Ca^{2+}]_i$ shifts the local Ca^{2+} signals to favour calcineurin activation[34,46,47]. The integration of local and global Ca^{2+} signals at the growth cone might represent a versatile mechanism for growth cones to respond to diverse extracellular signals that are received simultaneously.

Ca^{2+}-activated proteases. Although small local Ca^{2+} signals can elicit repulsive turning without apparent growth cone collapse, large Ca^{2+} transients restricted to filopodia on one side of the growth cone can also induce repulsion through local inhibition of filopodial motility[25,88]. Recent studies have identified the Ca^{2+}-sensitive protease calpain as one downstream effector of filopodial Ca^{2+} transients. Calpain activity was found to be highest in growth cones on substrata that promote the highest frequency of filopodial Ca^{2+} transients. Moreover, repulsive turning induced by local Ca^{2+} transients in filopodia requires calpain activity. Interestingly, Ca^{2+}-mediated calpain activation seems to regulate integrin-mediated adhesion by inhibiting Src tyrosine kinase activity[88]. Local uncoupling of receptor–cytoskeletal linkages by calpain-dependent cleavage of Src or adhesion proteins such as talin[98] provides a mechanism whereby filopodial contact with inhibitory cues might promote repulsive growth cone turning. These findings point to a potential link between Ca^{2+} signals and the regulation of growth cone–substratum adhesion. Although regulation of the cytoskeleton (microtubules and actin filaments) is recognized as a primary target of biochemical signals during growth cone guidance, there is increasing evidence to indicate that spatiotemporal regulation of growth cone adhesion represents an important mechanism for growth cone steering[99–105]. The finding that local calpain activity is regulated by the frequency of Ca^{2+} transients also indicates the importance of temporal features of Ca^{2+} signals in growth cone guidance.

Crosstalk between Ca^{2+} and small Rho GTPases. Steering of nerve growth cones during axon pathfinding depends on coordination of cytoskeletal dynamics with substratum adhesion. Rapid protrusion and retraction of filopodia and lamellipodia requires dynamic changes in the actin filament network[106]. Regulation of actin filament polymerization and turnover, together with filament crosslinking, membrane coupling and retrograde flow all contribute to the control of growth cone motility. One of the main regulators of actin filament assembly and organization during cell locomotion is the Rho family of small GTPases, which includes RhoA, Rac1 and Cdc42 (cell division cycle 42). The results of several studies suggest that RhoA is involved in growth cone collapse and repulsion whereas Rac1 and Cdc42 participate in growth cone advance — although this is probably an over-simplification considering the many functions of these GTPases in cell motility (for reviews, see REFS 107,108).

Guanine nucleotide exchange factors (GEFs) activate Rho GTPases by facilitating the exchange of GDP for GTP, whereas GTPase-activating proteins (GAPs) inactivate Rho GTPases by increasing their endogenous GTPase activity. Interestingly, GEFs and GAPs often contain other signalling motifs, such as CaMKII phosphorylation sites, which might serve as additional regulatory mechanisms (for a review, see REF. 109). For example, p135 SynGAP localizes to the postsynaptic density and is inhibited by phosphorylation by CaMKII in response to Ca^{2+} influx through NMDA receptors[110]. Ca^{2+}-dependent regulation of Rho GTPase activity *in vivo* is supported by work showing that optic nerve or visual stimulation enhances dendritic growth by increasing Rac1 and decreasing RhoA activity in the optic tectum[111,112].

Evidence is emerging to suggest that Ca^{2+} signals and the Rho GTPases exhibit extensive crosstalk in the control of growth cone motility, which is not surprising given the profound influence of these signals on neurite outgrowth. For example, chemoattraction of growth cones toward BDNF requires Ca^{2+} elevation and activation of protein kinase C (PKC), which, in turn, inhibits RhoA and activates Rac1 and Cdc42 (REF. 113). It is not clear how PKC differentially regulates RhoA, Rac1 and Cdc42, or whether distinct Ca^{2+} signals modulate the activity of the Rho GTPases differentially. Other Ca^{2+}-dependent enzymes have been shown to affect Rho GTPase signalling in non-neuronal cells, which suggests that similar mechanisms might function in growth cones. For example, inactivation of calpain has been shown to promote neutrophil migration by increasing the activity of Cdc42 and Rac1 (REF. 114). Additionally, IQGAPs (IQ motif-containing GTPase-activating proteins) might act as an important signal integrator linking actin filaments with microtubule plus-ends in growth cones in a Ca^{2+}-dependent manner. IQGAPs concentrate at the leading edge of migrating cells, where they crosslink actin filaments and bind active Rac1 and Cdc42, as well as the microtubule plus-tip-associated proteins cytoplasmic linker protein 170 (CLIP170) and adenomatosis polyposis coli (APC) (for a review, see REF. 115). IQGAP1 has been shown to bind and stabilize the active forms of Rac1 and Cdc42, which might aid in the localization of IQGAP to the leading edge of cells and promote actin–microtubule interactions[116,117]. Work with current models suggests that increased Ca^{2+}/CaM binding to IQGAP1 results in decreased Cdc42 binding and disruption of other protein associations[118], which should reduce cell motility. Finally, crosstalk between Ca^{2+} and the Rho GTPase also occurs in the reverse direction, as Rho GTPase signalling can modulate Ca^{2+} influx through VOCCs[119] and membrane insertion of TRP channels[66]. Therefore, Ca^{2+} and Rho GTPase signalling might interact to regulate cell motility at various levels, which suggests that signal integration in this network is extremely complex.

Postsynaptic density
An electron-dense complex of proteins located immediately behind the postsynaptic membrane. Proteins in the postsynaptic density have many roles, which include the anchoring and trafficking of neurotransmitter receptors in the plasma membrane, and the clustering of various proteins that modulate receptor function.

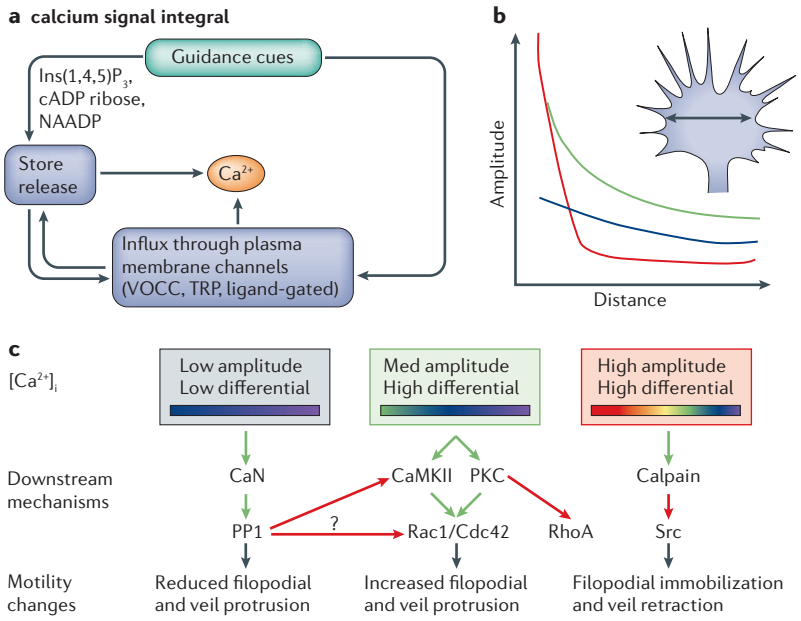

Figure 4 | **Ca^{2+} gradients of various slopes across a growth cone regulate motility by activating different downstream targets. a** | Activation of distinct combinations of Ca^{2+} influx and release pathways by guidance cues can produce a wide range of Ca^{2+} signals in growth cones. Guidance cues can activate Ca^{2+} influx and release directly, through various channel types, or indirectly, through store-depletion-activated influx and Ca^{2+}-induced Ca^{2+} release. Both the particular source of Ca^{2+} and integrated cytosolic Ca^{2+} signal might determine the downstream targets activated. cADP, cyclic ADP; $Ins(1,4,5)P_3$, inositol-1,4,5-trisphosphate; NAADP, nicotinic acid adenine dinucleotide phosphate; TRP, transient receptor potential; VOCC, voltage-operated calcium channel. **b** | Local activation of Ca^{2+} pathways can produce Ca^{2+} gradients that spread varying distances across a growth cone (indicated by double-headed arrow in growth cone) with variable peak amplitude. Hypothetical Ca^{2+} gradients measured across a growth cone are shown on the graph in blue (low amplitude and low differential), green (medium amplitude and high differential) and red (high amplitude and high differential). **c** | Horizontal colour scales represent hypothetical Ca^{2+} gradients across a growth cone (red/orange colours represent high Ca^{2+} concentrations), which vary in peak amplitude and slope (as in **b**). Ca^{2+} signals with these characteristics activate distinct downstream mechanisms. Activation of specific Ca^{2+}-mediated targets can have opposing effects on growth cone motility, which could be locally generated. Cdc42, cell division cycle 42; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; CaN, calcineurin; med, medium; PKC, protein kinase C; PP1, protein phosphatase 1; Rac1, RhoA, small GTPases; Src, non-receptor tyrosine kinase.

Crosstalk between cyclic AMP and Ca^{2+}. It has been shown that Ca^{2+}-dependent growth cone turning is modulated by the cyclic AMP (cAMP) pathway: attraction can be switched to repulsion by inhibition of cAMP-dependent protein kinase (PKA) and vice versa[120]. The switch of guidance cues' repulsive effects to attractive ones by cAMP is of particular interest, as this approach could be used to enhance neural regeneration after brain injury (for example, spinal cord injury)[121]. However, the molecular and cellular mechanisms that underlie cAMP-mediated switching of Ca^{2+}-dependent chemotropism require further investigation. There is little doubt that cAMP and Ca^{2+} signals, including the modulation of Ca^{2+} channel and $Ins(1,4,5)P_3$ receptor function by cAMP and activation of adenylyl cyclase by Ca^{2+}, are intricately interlaced in cells at various sites (for a review, see REF. 122). These cross-regulatory mechanisms might explain correlated cAMP and Ca^{2+} elevations observed in neurons[123]. However, cAMP might also act on downstream effectors to regulate Ca^{2+}-dependent guidance. The existence of extensive crosstalk between the Ca^{2+} and cAMP pathways presents a challenge to establishing the sites where modulation of responses to guidance cues occurs. For example, elevation of cytosolic cAMP was found to increase membrane insertion of DCC (deleted in colorectal cancer) receptors for netrin 1 responses[124]. Moreover, manipulation of the cAMP pathways was found to alter the Ca^{2+} signals induced by extracellular gradients of MAG[46]. Recent evidence also suggests that PKA regulates Ca^{2+}-induced Ca^{2+} release from ryanodine receptors to affect turning responses[125]. On the other hand, PKA has been shown to inhibit the calcineurin–PP1 pathway involved in repulsion to allow the conversion to attraction, which indicates that there is a downstream role for cAMP–PKA in Ca^{2+}-dependent axon guidance. It is proposed that PKA and calcineurin have different effects on PP1 activity through phosphorylation and dephosphorylation of inhibitor 1 to mediate repulsion[47]. Interestingly, a similar mechanism has been observed during synaptic plasticity, as DARPP32 (PP1 regulatory subunit) and inhibitor 1, which are functional homologues, regulate PP1 activity to control long-term potentiation and long-term depression (for a review, see REF. 77).

Model: Ca^{2+}-dependent growth cone guidance

Guidance of axons to their targets probably involves at least three Ca^{2+}-dependent effects on motility: growth promotion, growth inhibition or collapse, and directional steering (turning). Stimulation and inhibition of outgrowth can be due to overall changes in Ca^{2+} channel activity that may or may not involve chronic shifts in resting $[Ca^{2+}]_i$ or Ca^{2+} transients that spread throughout the entire growth cone. However, axon turning seems to depend on local discontinuities in Ca^{2+} signalling across the growth cone. When presented with a localized stimulus, such as a step or sloping gradient guidance cue, local differences in Ca^{2+} could occur as brief focal transients[25,46] or as a semi-stable variation in $[Ca^{2+}]_i$ across the growth cone (FIG. 1). The threshold of stimulus-activated Ca^{2+} currents across the plasma membrane and from intracellular stores is key in determining the effects on motility (FIG. 4a). Moreover, $[Ca^{2+}]_i$ gradients could spread across a large fraction of the growth cone or could be highly localized to one side, near open channels and possibly within a Ca^{2+} microdomain (FIG. 4b). Differences in the spatial spread of Ca^{2+} signals, which depend on the number and types of channels involved (for example, influx ± release), as well as Ca^{2+} homeostatic mechanisms, determine the shape and magnitude of the local Ca^{2+} elevation. Importantly, the shape and magnitude of the cytosolic Ca^{2+} gradient, rather than its orientation, might determine the polarity of the response (attraction versus repulsion). For example, a small Ca^{2+} gradient produced by modest Ca^{2+} influx or release induces repulsion, whereas a larger Ca^{2+} gradient produced by greater Ca^{2+} influx in combination with release induces attractive turning. In this model, the source of Ca^{2+} is not considered relevant; rather, the overall local

Synaptic plasticity
A process in which the efficacy of signal transmission through a synapse is persistently modified. The modification persists beyond the duration of the stimulus and results from post-translational and/or translational changes in the pre- or postsynaptic cell.

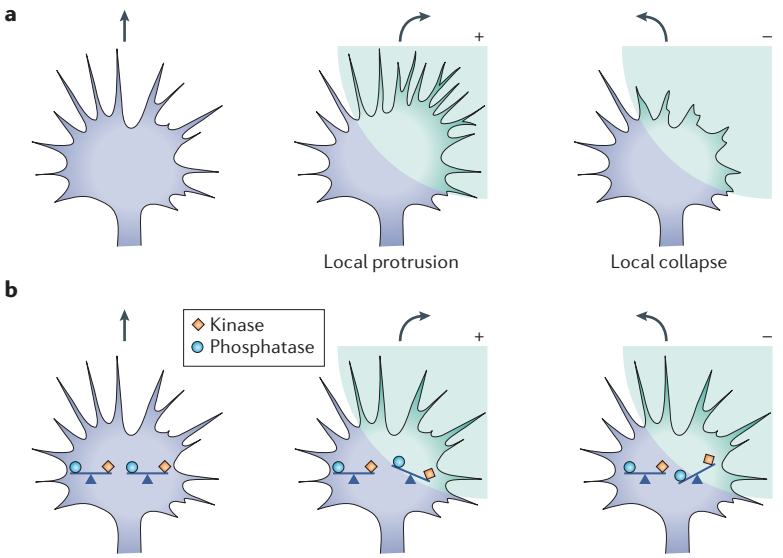

Figure 5 | **A local imbalance of kinase and phosphatase activities downstream of Ca^{2+} signals results in disproportionate effects on growth cone motility, which leads to neurite turning. a** | Intracellular signals activated on one side of a growth cone in response to disproportionate extracellular cues might lead to local effects on motility (increased protrusion or collapse). **b** | Changes in the balance between local kinase and phosphatase activities downstream of Ca^{2+} signals might stimulate local protrusion or collapse. Under the control of local Ca^{2+} signals, Src family tyrosine kinases and serine/threonine kinases such as Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) appear to promote motility, whereas the serine/threonine phosphatase calcineurin inhibits motility. So far, no Ca^{2+}-regulated tyrosine phosphatase has been identified in growth cones.

elevation and percentage difference across the growth cone have a crucial role. However, it is possible that different Ca^{2+} channel types, which reside in distinct Ca^{2+} microdomains, have differential affects on growth cone motility. Linking specific downstream Ca^{2+}-activated targets to highly localized Ca^{2+} sources near channel pores could provide the tight functional connections between Ca^{2+} and its targets that are necessary for different behavioural outcomes. Moreover, as Ca^{2+} stores are mobile in growth cones, behavioural outcomes could be further modulated by the localization of distinct Ca^{2+} stores within growth cones (FIG. 3).

In Ca^{2+}-dependent growth cone guidance, CaMKII and PP1 might serve as a kinase and phosphatase pair that determines the balance of phosphorylation and dephosphorylation of cytoskeletal effectors that control attraction and repulsion (FIGS 4c,5). Clearly, this model is not restricted to Ca^{2+}-mediated pathways, and might be applicable to guidance involving different signalling pathways, as spatial regulation of phosphorylation and dephosphorylation of specific proteins or complexes involved in motility could lead to directional steering. One such example is Src tyrosine kinase, which has recently been shown to promote filopodial extension by phosphorylating targets at the tips of filopodia[105]. Although none has yet been identified, a tyrosine phosphatase might inhibit filopodial extension by dephosphorylating filopodial tip targets. Such a shift in the balance between tyrosine phosphorylation and dephosphorylation was shown to control local filopodial dynamics and the direction of growth cone steering[105]. The complexity of these many biochemical pathways, which certainly interact at several levels within growth cones, is matched only by the complexity of the neural network that these signalling pathways operate to form.

Biosensor
A molecule that reports some aspect of cell physiology or molecular function in living cells. Biosensors are often fluorescent molecules, such as fluorescent fusion proteins with green fluorescent protein or its spectral variants. Fluorescent reporters allow investigators to correlate cellular behaviours with spatial and temporal changes in protein localization and function.

Concluding remarks and future directions

After more than 20 years of study, we have learned much about how Ca^{2+} signals control growth cone motility. However, there are still many unanswered questions. We are beginning to appreciate the complexities of the spatiotemporal patterns of Ca^{2+} signals that occur in growth cones, but the current technology only allows the most extreme changes to be detected. Most current imaging approaches cannot detect rapid Ca^{2+} signals that act on submillisecond timescales and are localized in spatial domains of <200 nm. Faster, brighter and more targeted Ca^{2+} indicators that report more wide-ranging Ca^{2+} concentrations, together with more sensitive and higher resolution imaging instrumentation[126,127], might reveal new dimensions of Ca^{2+} signalling complexities in motile growth cones (and other cells). However, as a complete picture of all Ca^{2+} movements in cells is unrealistic, more refined molecular, cellular and biophysical manipulations must be used to uncover what cannot be visualized. Acute local activation or inhibition of specific Ca^{2+} channels and downstream effectors coupled with behavioural assays can be used to deduce function. Understanding Ca^{2+} signalling pathways in multi-ligand situations is another major challenge, as the combinatorial effects of soluble guidance molecules and substratum-bound adhesion molecules may generate novel Ca^{2+} signalling functions in growth cones. Ultimately, we need to understand how Ca^{2+} signals and downstream effectors function in the context of the developing nervous system, which is a frontier that is now being approached[24,64]. The complex and profound signalling crosstalk between Ca^{2+} and other systems must also be addressed. Here, the development of more sensitive and specific live-cell optical biosensors[128] of other signalling intermediates will allow investigators to directly assess the effects of Ca^{2+}-induced changes on other aspects of cell physiology. With so many challenges ahead, our understanding and appreciation of the remarkable ability of this 'simple' ion to regulate so many aspects of a cell's life should deepen even further during the next decade.

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Competing interests statement

The authors declare no competing financial interests.

DATABASES

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BDNF | β-CaMKII | netrin 1 | TRPC1

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