

Published in final edited form as:

Nat Rev Neurosci. 2011 April ; 12(4): 191–203. doi:10.1038/nrn2996.

Second messengers and membrane trafficking direct and organize growth cone steering

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Abstract

Graded distributions of extracellular cues guide developing axons toward their targets. A network of second messengers, Ca²⁺ and cyclic nucleotides, shapes cue-derived information into either attractive or repulsive signals that steer growth cones bidirectionally. Emerging evidence suggests that such guidance signals create a localized imbalance between exocytosis and endocytosis, which in turn redirects membrane, adhesion and cytoskeletal components asymmetrically across the growth cone to bias the direction of axon extension. These recent advances allow us to propose a unifying model of how the growth cone translates shallow gradients of environmental information into polarized activity of the steering machinery for axon guidance.

The formation of functional neuronal networks depends crucially on the spatial accuracy of axon growth and navigation. Each growing axon in the developing nervous system is tipped by a growth cone, a specialized amoeboid structure that is able to interpret secreted and membrane-bound molecular “guidance cues” that direct its migration along the correct path. A series of these events deliver the axon to an approximate target region, which is followed typically by axonal arborization and contacts with appropriate postsynaptic partners at particular subcellular locations¹. Such specificity of synaptic connections within the target region relies on multiple distinct mechanisms including further cue-mediated axon guidance^{2,3}. In this way, guidance cues in the microenvironment play crucial roles in neuronal network formation.

It is widely accepted that graded distribution of guidance cues controls the direction of axon growth (FIG. 1a). Such gradients can be generated by diffusion of a secreted cue away from its source of synthesis⁴ or by differential expression of non-diffusible cues⁵. When a growth cone migrates in a guidance cue gradient, the side of the growth cone facing higher concentrations of the cue will experience higher receptor occupancy. This asymmetric receptor occupancy polarizes the growth cone for turning either toward increasing concentrations of the cue (attraction) or away from the cue (repulsion), via intracellular generation of second messengers such as Ca²⁺ and cyclic nucleotides^{6–11}. An extracellular shallow gradient can be transformed into steeply graded¹² or, in extreme cases, compartmentalized signals^{11,13} inside the growth cone. The asymmetrically produced second messengers orchestrate multiple cellular machineries including membrane

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trafficking, adhesion dynamics and cytoskeletal reorganization to execute bidirectional turning of the growth cone (FIG. 1b).

These basic mechanisms could be sufficient to explain short-distance guidance of axons such as those of local circuit neurons. However, additional complex mechanisms are required for long projecting axons that are guided by intermittently positioned sources of attractants en route, referred to as “intermediate targets”¹⁴. To leave an intermediate target after passing through this once attractive region, the growth cone must change its responsiveness and even reverse the polarity of guidance. Such switching can be accomplished through multiple mechanisms including an induction of different second messenger profiles that direct opposing steering machinery^{15,16}.

Extracellular diffusible molecules showing axon guidance activities *in vitro* have been regarded as “guidance cues” and have been well documented (TABLE 1), even if their functional significance *in vivo* is less clear. This review will seek to synthesize these findings and provide an integrated picture of how axon guidance works *in situ*. We first suggest, based largely on *in vitro* data, the second messenger network models explaining how the growth cone translates shallow gradients of guidance information into either attractive or repulsive turning. Second, we examine recently identified target molecules and mechanisms that link the second messenger system with the steering apparatus. In addition to established mechanisms that act in parallel to remodel the cytoskeleton and substrate adhesions^{17–21}, we here propose the hierarchical organization model of cellular machineries in which asymmetric membrane trafficking redirects cytoskeletal and adhesion components in the growth cone to drive its bidirectional turning. Finally, we provide our viewpoint on how the growth cone makes guidance decisions *in vivo* where it encounters and integrates multiple cues simultaneously to navigate through complex environmental terrain with high fidelity.

Involvement of second messengers in signal transduction

Second messenger networks involving Ca^{2+} influx and secondary Ca^{2+} release controlled by cyclic AMP (cAMP) and cyclic GMP (cGMP) are the primary means by which growth cones interpret and amplify signals encountered in the local environment. Here we explain how second messenger networks are shaped into growth cone guidance signals.

Generation of second messengers

In vitro studies indicate that Ca^{2+} signals are almost universally required for growth cone guidance with a few notable exceptions (TABLE 1). For all of the guidance cues that have been shown to elicit asymmetric Ca^{2+} elevations across the growth cone when applied directionally to induce axon turning, higher Ca^{2+} concentrations are observed on the side of the growth cone facing the source of the cues, regardless of whether the cues are attractive or repulsive (FIG. 1). Thus, localized Ca^{2+} elevations on one side of the growth cone can act as both attractive and repulsive signals^{22,23}.

How do Ca^{2+} elevations of the same polarity elicit bidirectional turning? This most likely relies on the gating of differential sets of Ca^{2+} channels. The generation of cytoplasmic Ca^{2+} signals takes advantage of a steep gradient of Ca^{2+} concentrations across the plasma membrane that drives Ca^{2+} influx through various cation channels such as transient receptor potential type C (TRPC) channels and L-type voltage-dependent Ca^{2+} channels (L-VDCCs) (REF. 24). Ca^{2+} influx can be further amplified and extended in space and duration by secondary Ca^{2+} release from the endoplasmic reticulum (ER) through ryanodine receptors (RyRs) or inositol 1,4,5-trisphosphate (IP_3) receptors (IP_3 Rs). The limited diffusion of cytoplasmic Ca^{2+} (REF. 25) allows spatial confinement of Ca^{2+} elevation to the vicinity of

single open channels or discrete clusters of open channels, referred to as Ca^{2+} nanodomains or microdomains, respectively²⁶.

These diverse spatiotemporal patterns of Ca^{2+} signals contribute to the growth cone's bidirectional responses to various guidance cues (TABLE 1 and FIG. 2). High amplitude Ca^{2+} produced by Ca^{2+} -induced Ca^{2+} release (CICR) through RyRs or IP_3 -induced Ca^{2+} release (IICR) through IP_3 Rs are sufficient to initiate growth-cone turning toward the side with Ca^{2+} signals^{6,11,13} and can mediate cue-induced attraction (FIG. 2a,c,e). Conversely, repulsion is triggered by lower-amplitude Ca^{2+} signals that are mostly generated by Ca^{2+} influx through plasma membrane channels and not accompanied by CICR or IICR (REFS. 7,27) (TABLE 1 and FIG. 2b,f). The amplitude and nanodomain or microdomain localization of this Ca^{2+} signal can vary depending on the gating of differential sets of Ca^{2+} channels, and mediate bidirectional growth cone turning²³. The differences in amplitude can be detected by at least two Ca^{2+} effectors: Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII) and the Ca^{2+} /calmodulin-dependent protein phosphatase, calcineurin (REF. 28). They are able to do so by virtue of their differing affinity for Ca^{2+} -calmodulin: calcineurin has a higher affinity for Ca^{2+} -calmodulin than CaMKII (REFS. 29,30). In this Ca^{2+} affinity model, low-amplitude Ca^{2+} signals activate calcineurin over CaMKII for repulsion whereas high-amplitude Ca^{2+} signals preferentially activate CaMKII for attraction²⁸. However, the source of Ca^{2+} signals can be another determinant of the turning direction, because Ca^{2+} signals of equivalent amplitude with or without CICR elicit growth cone attraction or repulsion, respectively²⁷.

To reconcile these seemingly contradictory observations, we propose to refine the Ca^{2+} affinity model²⁸ by assuming that Ca^{2+} effectors for repulsion and attraction are localized to differential subcellular compartments: high-affinity Ca^{2+} effectors such as calcineurin exist diffusely in the growth cone cytosol whereas low-affinity Ca^{2+} effectors such as CaMKII are localized in close proximity to ER Ca^{2+} channels. Both calmodulin and CaMKII have been shown to associate, although not exclusively³¹, with RyRs and IP_3 Rs (REFS. 32,33), and thus are favorably situated for the detection of Ca^{2+} release from the ER. In this refined model, Ca^{2+} influx through plasma membrane channels alone would activate repulsive, but not attractive effectors, leading to growth cone repulsion. Conversely, attractive turning would require activation of attractive effectors by means of Ca^{2+} release from the ER, abundant Ca^{2+} influx across the plasma membrane that can elevate cytoplasmic Ca^{2+} concentrations at the ER, or combinations of the two. Consistent with this model, for example, myelin-associated glycoprotein (MAG)-induced repulsion depends on low-amplitude Ca^{2+} release from the ER (REF. 7) and possibly Ca^{2+} influx through TRPC1 channels (REF. 34) (FIG. 2d), but MAG-induced attraction requires high-amplitude Ca^{2+} signals accompanied by CICR (REF. 7; T. Tojima, R. Itofusa and H. Kamiguchi, unpublished data).

Asymmetric cyclic nucleotide signaling can also mediate axon guidance. For example, activation of the cAMP pathway on one side of the growth cone is sufficient to trigger attractive turning^{35,36}. Such instructive cAMP signaling has been implicated in pituitary adenylate cyclase-activating polypeptide-induced attraction³⁵ and netrin-1-induced attraction³⁶, although there is no direct evidence for asymmetric cAMP distribution across the growth cone in gradients of these cues. The extracellular guidance cue Semaphorin 3A (Sema3A) stimulates the production of cGMP preferentially on the side of the growth cone facing the Sema3A source, leading to a Ca^{2+} influx pattern for repulsive turning¹⁰. Among several guidance cues that have been shown to influence cyclic nucleotide levels, attractive and repulsive cues tend to elevate cAMP and cGMP, respectively (TABLE 1 and FIG. 2).

Second messenger crosstalk for directional switching and signal amplification

Axon pathfinding involves not only attraction or repulsion, but often a switch from one to the other. Cyclic nucleotides can act as such a switch by modulating Ca^{2+} channel activities³⁷. The Ca^{2+} release from the ER that determines growth cone turning polarity is regulated counteractively by cAMP and cGMP pathways^{11,27,38}: cAMP facilitates ligand-dependent activation of RyRs and IP_3 R for an attractive response. Conversely, cGMP inactivates RyRs and blocks CICR although the involvement of cGMP in the regulation of IICR during axon guidance remains to be determined. Biochemical and molecular structural studies have shown that the gating of ER Ca^{2+} channels can be modulated by phosphorylation by the cAMP- and cGMP-dependent kinases protein kinase A (PKA) and protein kinase G (PKG) (REFS. 32,33). The roles of PKA and PKG described in this review, which are based on experimental results using non-specific pharmacological agents, should be confirmed with recently developed cyclic nucleotide analogs and RNA interference technology that can specifically target these kinases^{36,39,40}.

Evidence from other biological systems helps in the development of a hypothetical model of how growth cones can translate and amplify shallow concentration gradients of guidance cues into steeper gradients of intracellular signals. In addition to the pathway by which cAMP facilitates Ca^{2+} mobilization, Ca^{2+} in turn stimulates the production of cAMP via Ca^{2+} -dependent adenylate cyclases (ACs) such as AC1 and AC8 (REF. 41). Indeed, AC8 has been shown to increase cAMP levels in retinal ganglion cells downstream of Ca^{2+} -calmodulin activation⁴². Furthermore, recent work has demonstrated that Ca^{2+} depletion from the ER, an experimental equivalent of the consequence of CICR or IICR, stimulates AC-mediated cAMP production by a mechanism that is independent of the cytoplasmic Ca^{2+} concentration⁴³. If this store-operated cAMP production pathway operates in growth cones, it could mediate CICR- and IICR-elicited cAMP elevations. Such positive-feedback mechanisms would potentially participate in amplifying attractive guidance signals. Both mathematical modeling of cAMP and IICR regulation and imaging of spontaneous cAMP and Ca^{2+} in neuronal cell bodies have shown the existence of positive-feedback regulation, further opening the possibility that this might occur in growth cones⁴⁴.

This positive-feedback augmentation of guidance signals can be terminated by multiple mechanisms. The cytoplasmic Ca^{2+} concentration can regulate the open probability of RyRs and IP_3 R biphasically: open probability increases as Ca^{2+} concentrations elevate, but further Ca^{2+} increase above threshold levels of 0.2 to hundreds of micromolars begins to inhibit opening of these Ca^{2+} channels^{32,33,45}. Also, cyclic nucleotide levels can be self-suppressed via cyclic nucleotide-dependent phosphodiesterases (PDEs) (REF. 46). It is likely that growth cones employ these mechanisms to limit the amplitude of guidance signals.

Importantly, intracellular signals may also be refined via reciprocal inhibition pathways between cAMP and cGMP. Shelly et al⁴⁷ recently identified such pathways in neuronal growth cones: cAMP elevation causes cGMP reduction via cGMP-specific PDE5 whereas cGMP elevation causes cAMP reduction via cAMP-specific PDE4. These reciprocal inhibition pathways allow attractive and repulsive signals to reduce cGMP and cAMP, respectively, and possibly contribute to signal amplification by inhibiting these counteractive regulators of ER Ca^{2+} channels. This may force the guidance machinery toward a clear-cut decision between attractive or repulsive response. It is generally thought that gradient amplification mechanisms in chemotactic cells involve both “local augmentation” and “global inhibition” of the guidance signal, and indeed, such mechanisms appear to be employed by growth cones⁴⁸. This has led to the development of a molecular model that utilizes the positive feedback loops described in this and subsequent sections.

Bidirectional growth cone turning: a molecular model

Although guidance cues signal through structurally diverse receptors, many participating second messengers are shared. This has allowed us to propose a general model for second messenger networks that facilitate attractive and repulsive turning. Signaling networks including positive-feedback loops and reciprocal inhibition help to explain how a growth cone shapes Ca^{2+} signals for precise navigational responses to shallow gradients of attractive cues (FIG. 3). In this hypothetical model, netrin-1, for example, induces primary Ca^{2+} influx through TRPC1 channels (REFS. 9,49) and elevates the cAMP concentration^{15,36,50,51}, preferentially on the side of the growth cone facing the netrin-1 source. The cAMP elevation would cause cGMP reduction via reciprocal inhibition⁴⁷. The resultant increase in the ratio of cAMP to cGMP pushes L-VDCCs and RyRs to the active state and facilitates the generation of secondary Ca^{2+} entry through these channels^{27,37,38}, which in turn stimulates further production of cAMP (REFS. 41–43). Similarly, second messenger crosstalk shapes attractive guidance signals downstream of nerve growth factor (NGF) (REFS. 11,52–54) and brain-derived neurotrophic factor (BDNF) (REFS. 8,52,55) (FIG. 3a). It is tempting to speculate that these positive-feedback loops act as the machinery for “local augmentation” and produce high-amplitude CICR or IICR on one side of the growth cone for attractive guidance.

Conversely, molecular mechanisms underlying “global inhibition” remain more elusive. Recent work⁴⁷ has provided insight into the global inhibition mechanisms: when the cAMP pathway is activated within a single growth cone of a hippocampal neuron, all other neurites of that neuron exhibit cAMP reduction and cGMP elevation presumably involving active transport of unidentified regulators of cyclic nucleotides. If this self-down-regulation of cAMP can operate within an even shorter range, one would imagine that attractant-induced cAMP elevation on one side of the growth cone results in cAMP reduction and cGMP elevation on the other side. This would block the generation of secondary Ca^{2+} signals on the side facing lower attractant concentrations, thereby contributing to the confinement of attractive Ca^{2+} signals to the leading side of the growth cone.

During repulsive guidance, cGMP levels are elevated by Sema3A (REF. 10) and probably by netrin-1 (REF. 37). The cGMP elevation likely causes cAMP reduction via reciprocal inhibition⁴⁷. In this situation, cue-induced primary Ca^{2+} influx would fail to trigger further Ca^{2+} entry through L-VDCCs, RyRs or IP_3 Rs (REFS. 11,27,37,38) (FIG. 3a). Such Ca^{2+} influx without Ca^{2+} release from the ER acts as a repulsive signal. The signaling network for repulsive guidance may lack a positive-feedback loop between Ca^{2+} and cyclic nucleotides (FIG. 3b), which is consistent with the concept that the amplitude of repulsive Ca^{2+} signals is low. Thus, an intriguing model that remains to be thoroughly tested is that amplification of repellent gradients occurs downstream of Ca^{2+} signals.

There are exceptions, however, such as Sema3A, which attracts growth cones if cGMP levels increase to levels that are high enough to activate PKG, which induces membrane depolarization and high-amplitude Ca^{2+} influx through voltage-dependent Ca^{2+} channels (REF. 56). The proposed model (FIG. 3b) can be scrutinized by simultaneous imaging of multiple second messengers and analyses of their spatiotemporal dynamics in growth cones during attraction and repulsion.

Steering machinery for growth cone guidance

In order to accomplish growth cone turning, the external signals that have been interpreted, amplified and transduced need to result in changes in the growth cone morphology. The classic machinery that drives such morphological changes includes the cytoskeleton and adhesion complexes, where the former facilitates plasma membrane protrusion and the latter

mediates membrane anchoring to extracellular matrix. Adhesion receptors like integrins link to the underlying actin cytoskeleton at adhesion complexes by means of adaptor proteins including talin (REF. 57). Together, these machineries cooperate to drive migration. Accumulating evidence highlights the importance of their asymmetric reorganization during bidirectional guidance (FIG. 1b): the growth cone turns preferentially toward the side with cytoskeletal and adhesion complex assembly or away from the side with disassembly. Thus, effector processes that control cytoskeletal and adhesion dynamics must be regulated asymmetrically across the growth cone to initiate chemotactic turning. A growing number of effector proteins have been implicated in growth cone guidance, including Rho-family GTPases (REFS. 13,58–62), actin-depolymerizing factor/cofilin (REFS. 63,64), Enabled/vasodilator-stimulated phosphoprotein (REF. 65), Mical (REFS.66,67), adenomatous polyposis coli (REF. 68), calpain (REF. 69), focal adhesion kinase (FAK) and Src-family kinases (REFS. 70–78). Local translation or degradation of cytoskeletal components and their regulators also participates in growth cone guidance^{79–85}.

Remarkably, several recent studies^{86–89} have demonstrated that regulated membrane trafficking is a critical effector process for chemotactic guidance by gradients of diffusible cues. In these circumstances, membrane trafficking becomes asymmetric across the growth cone immediately after the onset of guidance signals, preceding any detectable changes in cytoskeletal dynamics. These findings are consistent with the hypothesis that regulated membrane trafficking facilitates the subsequent cytoskeletal remodeling that is necessary for axon guidance, although future studies are warranted to determine the causal relationship between these cellular processes. In the following sections, we will examine how asymmetric membrane trafficking can be an instructive step in the initiation of growth cone steering.

Exocytosis and endocytosis in the growth cone

Developing neurons undergo dramatic morphological changes that require alterations in the surface area by means of exocytosis and endocytosis^{90–95}. Axon extension depends on the expansion of growth cone plasma membrane⁹², which can be mediated by vesicle-associated membrane protein 7 (VAMP7)-dependent exocytosis⁹⁶. Regulated exocytosis can also occur locally in the growth cone, whereby synaptic protein-containing vesicles migrate along filopodia and fuse with the filopodial surface⁹⁷. Conversely, growth cone collapse is accompanied by a reduction in the surface area via endocytosis^{87,93,94}. These findings have led to the hypothesis that axon guidance involves asymmetric membrane trafficking across the growth cone^{98,99}.

It is now evident that regulated exocytosis and endocytosis occur asymmetrically across the growth cone in response to guidance cue gradients^{86–89} (FIG. 4). A gradient of chemoattractants promotes vesicle transport and subsequent VAMP2-mediated exocytosis on the gradient side of the growth cone. Conversely, a chemorepellent gradient causes asymmetric clathrin-mediated endocytosis on the side facing the gradient. It is likely that differential regulation of these opposing forces remodels the composition of the surface membrane and associated molecules locally to initiate turning. Regulated membrane trafficking can participate in multiple steps in the axon guidance process such as receptor-mediated signal transduction and downstream mechanical processes.

The participation of exocytic and endocytic trafficking in the control of signaling events in the growth cone has been well documented. For example, the growth cone's responses to guidance cues can be modulated by membrane trafficking that alters the surface availability of relevant receptors such as deleted in colorectal cancer, uncoordinated 5 and neuropilin 1 (REFS. 100–102). The growth cone can adjust its sensitivity to guidance cues by the process of adaptation¹⁰³, which depends on endocytosis-mediated desensitization¹⁰² and protein

synthesis-dependent resensitization^{102,103}: endocytosis of the relevant receptors transiently desensitizes the growth cone to collapse-inducing activities of Sema3A and netrin-1 (REF. 102). Receptor endocytosis may be necessary for the full generation of intracellular guidance signals from early endosomes^{104–106}. Also, Ca²⁺-permeable cation channels packaged into VAMP-positive vesicles are carried to the growth cone, which leads to changes in morphology¹⁰⁷.

In addition to a role in signaling, emerging evidence suggests that membrane trafficking can be a primary and instructive driving machinery for growth cone turning^{86,89}. First, asymmetric exocytosis and endocytosis are necessary for growth cone attraction and repulsion, respectively, induced by direct Ca²⁺ manipulation that can bypass receptor activation and any upstream signaling events. Second, when the turning polarity is switched between attraction and repulsion by modulating second messenger profiles, the balance of exocytosis and endocytosis is also reversed. Importantly, the reversed turning polarity also depends on the newly acquired dominant trafficking mechanism, with attractive Ca²⁺ signals favoring exocytosis and repulsive Ca²⁺ signals triggering endocytosis. Finally, asymmetric perturbation of the balance of exocytosis and endocytosis is sufficient to initiate turning toward the side with more exocytosis or less endocytosis. Taken together, these findings indicate that the balance of exocytosis and endocytosis can dictate bidirectional growth cone turning downstream of second messenger signaling.

How do second messengers control membrane trafficking?

Asymmetric Ca²⁺ signals attract and repel growth cones via CaMKII and calcineurin, respectively²⁸ (FIG. 5a). Both the magnitude and subcellular localization of Ca²⁺ signals might dictate which effector pathways are activated, and consequently favor either exocytosis or endocytosis. During attractive turning, Ca²⁺ signals promote microtubule-based centrifugal transport of membrane vesicles and their subsequent VAMP2-mediated exocytosis on the side with elevated Ca²⁺ (REFS. 86,108). The attractive Ca²⁺ signals might locally activate CaMKII, which could phosphorylate motor proteins like myosin-V and KIF17 and trigger release of these motors from cargo vesicles^{109,110}. After releasing these motors, the vesicles could be incorporated into a readily-releasable pool of vesicles docked beneath the plasma membrane, followed by Ca²⁺-dependent activation of synaptotagmins and other effector proteins on the vesicle membrane that regulate exocytosis¹¹¹. Although the exact mechanisms remain to be elucidated, it is likely that attractive Ca²⁺ signals facilitate at least two distinct but successive processes: vesicle translocation into the growth cone periphery and regulated exocytosis. Repulsive Ca²⁺ signals elicit asymmetric clathrin-mediated endocytosis in a calcineurin-dependent manner⁸⁹. The role of calcineurin in clathrin-mediated endocytosis has been extensively studied in presynaptic terminals¹¹². Calcineurin dephosphorylates, and thereby activates, dephosphins, a group of at least eight endocytic-adaptor proteins^{113,114}. In synaptic transmission, activated dephosphins facilitate synaptic vesicle endocytosis after exocytic neurotransmitter release. In growth cones, the requirement for calcineurin activity in repulsive turning²⁸ and clathrin-mediated endocytosis⁸⁹ suggests that dephosphins play a similar role in axon guidance. Taken collectively, repulsive Ca²⁺ signals facilitate clathrin-mediated endocytosis via calcineurin, whereas attractive Ca²⁺ signals promote exocytic trafficking possibly via CaMKII (FIG. 5a).

In addition to regulating the Ca²⁺ signal within growth cones, cyclic nucleotides can regulate membrane trafficking. For example, cAMP activation of PKA elicits phosphorylation of synapsin I (REF. 115), which then dissociates from the cytoplasmic surface of VAMP2-positive synaptic vesicles¹¹⁶. This potentiates regulated exocytosis in presynaptic terminals¹¹⁶ and facilitates vesicle translocation from the growth cone center toward the periphery¹¹⁷. Furthermore, the importance of synapsin phosphorylation in growth cone motility has been supported by the observation that expression of a synapsin mutant

mimicking constitutive phosphorylation by PKA is sufficient to stimulate axon extension even in the presence of a PKA inhibitor¹¹⁸. These results suggest that cAMP might collaborate with Ca²⁺ to facilitate centrifugal vesicle transport and exocytosis during growth cone attraction. cGMP elevation can accelerate synaptic vesicle endocytosis via elevating presynaptic levels of phosphatidylinositol 4,5-bisphosphate (REF. 119), but the role of cGMP in membrane trafficking in growth cones remains undefined.

Polarized remodeling of adhesion and cytoskeletal machinery

Growth cone turning depends on membrane trafficking, adhesion dynamics and cytoskeletal reorganization. While these mechanical processes can function independently, membrane trafficking might also polarize the activity of adhesion and cytoskeletal machinery by redistributing their components and regulators. For example, clathrin-mediated endocytosis retrieves integrins from the cell surface and sorts them into early endosomes for subsequent degradation or recycling back to the cell surface¹²⁰. In non-neuronal cells, clathrin mediates β 1-integrin endocytosis, a process that is required for focal adhesion disassembly and efficient cell migration¹²¹. Endocytosed β 1-integrin is also present in VAMP2-positive vesicles in these cells, and depletion of VAMP2 reduces the amount of β 1-integrin on the cell surface, which inhibits cell adhesion and chemotactic migration on a fibronectin substrate¹²². These findings indicate that cell adhesion to extracellular matrix can be controlled by endocytic and exocytic trafficking of integrins. An analogous mechanism might operate in axon guidance whereby growth cone repulsion would be driven by clathrin-mediated endocytosis of β 1-integrin and asymmetric adhesion-complex disassembly⁸⁸, whereas growth cone attraction would require exocytic trafficking of VAMP2-positive vesicles⁸⁶ that could carry β 1-integrin (REF. 122) (FIG. 5a). In this way, integrins may be transported to the growth cone periphery in a targeted manner and undergo exocytosis in regions where integrin-containing adhesions assemble, such as the lamellipodia and filopodia^{97,123,124}. Importantly, asymmetric alterations in growth cone-substrate adhesion by blocking β 1-integrin are sufficient to initiate growth cone turning away from the side with reduced β 1-integrin function⁸⁸. Thus, asymmetric trafficking of β 1-integrin and potentially other adhesion molecules would be expected to cause polarized adhesion and turning of growth cones.

Downstream of repulsive guidance signals, endocytic removal of integrins must be coupled spatiotemporally with adhesion complex disassembly that often accompanies cell retraction and detachment from extracellular matrix⁵⁷. As expected, a gradient of the chemorepellent Sema3A causes polarized endocytosis⁸⁹ and asymmetric growth cone detachment from a laminin substrate as assessed by internal reflection microscopy¹²⁵. Moreover, activation of the Ca²⁺-calcineurin pathway leads to disappearance of phosphorylated FAK, a component of integrin-based adhesion machinery, and growth cone detachment from a laminin substrate⁷³. These findings imply that repulsive guidance signals can orchestrate multiple effector processes including dispersal of adhesion structures and selective retrieval of integrins. One possible mechanism underlying this orchestration is that Ca²⁺-dependent effectors modify components of integrin-containing adhesions for subsequent integrin internalization. For example, spontaneous Ca²⁺ transients in growth cone filopodia promote repulsive turning when generated asymmetrically, through local activation of the Ca²⁺-dependent protease calpain (REF. 69) that cleaves several components of the adhesion machinery such as FAK (REF. 126), Src (REF. 127) and talin (REF. 128). Thus, calpain may mediate the linkage between Ca²⁺-induced integrin endocytosis and integrin disengagement from the underlying cytoskeleton.

Cytoskeleton-associated proteins can also be a cargo of intracellular vesicles. Activation of the GTPase Rac can occur on early endosomes in non-neuronal cells¹²⁹. Intracellular vesicles containing the activated Rac then move toward the cell periphery where actin-based

membrane protrusions form¹²⁹. Similarly, the GTPase Cdc42 can localize to intracellular vesicles and be delivered to the leading edge for directed cell migration¹³⁰. Proteomic analyses have identified a large number of proteins including actin, tubulin, Rac and actin-related protein 2/3 complex that associate with VAMP2-positive synaptic vesicle and clathrin-coated vesicle preparations isolated from the brain^{131,132}, supporting the notion of membrane trafficking as a means for the spatial restriction of cytoskeletal remodeling. Furthermore, phosphatidylinositol 3,4,5-trisphosphate (PIP₃) is delivered to neuronal growth cones through microtubule-based anterograde vesicle transport¹³³, where PIP₃ facilitates axon formation via multiple signaling cascades including Cdc42 and Rac1 activation¹³⁴, opening the possibility that asymmetric delivery of lipid mediators across the growth cone might participate in axon guidance. Therefore, it is reasonable to speculate that polarized trafficking of cytoskeletal regulators in the growth cone causes asymmetric cytoskeletal remodeling and growth cone turning (FIG. 5a). Another possibility is that local exocytosis and endocytosis influence the rate of actin polymerization by decreasing and increasing tension in the growth cone plasma membrane, respectively. This possibility is consistent with the Brownian-ratchet model of actin polymerization¹³⁵ and has been supported by experimental results that the plasma membrane tension is inversely correlated with the rate of lamellipodial protrusion¹³⁶.

We would like to propose a mechanistic model for bidirectional axon guidance, in which asymmetric membrane trafficking acts, downstream of second messengers, as a “master organizer” to spatially localize the growth cone driving machinery (FIG. 5b). In response to attractive Ca²⁺ signals, exocytic trafficking of VAMP2-containing vesicles would deliver positive regulators of adhesion complexes, cytoskeletal machinery and bulk membrane preferentially to the side of the growth cone with elevated Ca²⁺. Conversely, repulsive Ca²⁺ signals enhance endocytic removal of adhesion molecules, cytoskeletal components and membrane to reshape the growth cone. In this way, attractive and repulsive Ca²⁺ signals are transformed into exocytosis and endocytosis, respectively, which in turn potentiate asymmetric traction and protrusion forces for growth cone turning through polarized targeting of the driving machinery.

Future prospects: how does a growth cone integrate multiple guidance signals?

A growth cone must integrate signals of multiple guidance cues when navigating through complex environmental terrain *in vivo*. For example, commissural axon pathfinding along the dorsal-ventral axis of the spinal cord requires the release of multiple cues from either the ventral midline floor plate or the dorsal roof plate. The floor-plate-derived attractants netrin-1 (REF. 137) and Sonic hedgehog (REF. 138), and the roof-plate-derived repellents bone morphogenetic proteins (REF. 139) and possibly draxin (REF. 140) cooperate to guide commissural axons toward the ventral midline. After midline crossing, commissural axons turn rostrally, guided by counter gradients of the repellent Sonic hedgehog (REF. 141) and an attractive Wnt activity^{142,143} along the longitudinal axis. Another example comes from studies of the thalamocortical tract where axons are guided by counter gradients of the repellent ephrin-A5 (REF. 144) and the attractant netrin-1 (REF. 145). The growth cone's detection of multiple attractants and repellents with complementary gradient polarities likely serves to ensure correct pathway choices.

It is well known that guidance cues can regulate axonal responses to other guidance cues at the level of receptors¹⁴⁶ or second messengers¹⁴⁷. In addition to this hierarchical and regulatory interaction, recent work has demonstrated a “push and pull” mechanism in which both a repellent and an attractant play instructive and mutually supportive roles by confronting the same growth cone from opposite sides¹⁴⁸. This study has also provided *in*

vitro evidence that simultaneous presentation of two opposing cues as counter gradients improves the fidelity of growth cone turning.

Because many guidance cues signal through cytoplasmic Ca^{2+} , counter gradients of two opposing cues could cause Ca^{2+} elevations on both sides of the growth cone (FIG. 6). For the growth cone to turn in this situation, spatial asymmetry in the type of Ca^{2+} and its downstream signaling should form across its axis. One plausible mechanism for this asymmetry is that attractive and repulsive cues differentially regulate cyclic nucleotide signaling such that cAMP and cGMP levels have counter gradients. These counter gradients could be generated as a result of attractant/repellent-induced cAMP/cGMP increases, the reciprocal inhibition between cAMP and cGMP, and the self down-regulation of cAMP as discussed in the previous section. The high cAMP/cGMP ratio on the attractant side would facilitate CICR and IICR while the low cAMP/cGMP ratio on the repellent side would prevent Ca^{2+} release from the ER, which causes the reciprocal distribution of attractive and repulsive Ca^{2+} signals in the growth cone. These reciprocal Ca^{2+} signals would elicit the counter gradients of exocytic and endocytic activities that cause asymmetric alterations in actin assembly and growth cone adhesiveness. In this way, the growth cone could translate multiple guidance signals into turning behavior with high fidelity. Although future studies are necessary to elucidate the growth cone's responses to various combinations of guidance cues, one could speculate that a growth cone would bifurcate in response to attractants on both sides or would stall in response to repellents on both sides. Coexistence of an attractant and a repellent on the same side of the growth cone can be counterbalancing¹⁴⁹ but may induce attraction or repulsion depending on whether the positive-feedback loop between Ca^{2+} and cAMP has been turned on.

Conclusions

Pioneering work in the 1990s led to the discovery of many important axon guidance cues and their receptors. Subsequent studies in the 2000s elucidated signaling mechanisms by which guidance cues polarize the growth cone for turning, e.g., specific ion channels responsible for asymmetric Ca^{2+} signals, a second messenger network that controls switching between attraction and repulsion, and Ca^{2+} -dependent enzymes involved in axon guidance. More recent work has identified target molecules and mechanisms that link the second messenger system with cellular machinery for growth cone motility. Whereas Ca^{2+} and cyclic nucleotides can influence the machinery via multiple pathways, we propose that asymmetric membrane trafficking plays a fundamental role in transforming guidance signals into polarized activity of adhesion and cytoskeletal dynamics for bidirectional turning. These revelations, along with future progress in growth cone research will contribute not only to developmental neurobiology but also to understanding mechanisms of human disorders with aberrant axon connectivity^{150,151} and to technological innovation for guiding regenerating axons to their appropriate targets after injury to the adult central nervous system¹⁵².

Acknowledgments

We apologize to investigators whose work could not be cited owing to space limitations. The authors' work is supported by RIKEN Brain Science Institute (H.K.), Grants-in-Aid from MEXT (H.K. and T.T.), JST PRESTO program (T.T.), funding by the National Institutes of Health (J.R.H.), and a John M. Nasseff, Sr., Career Development Award in Neurologic Surgery Research from the Mayo Clinic (J.R.H.).

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glossary

Local augmentation	A theoretical reaction of chemotactic cells for gradient sensing, in which signals are augmented locally in the cellular area containing higher receptor occupancy.
Global inhibition	Another theoretical reaction for gradient sensing. The locally augmented signals can be further isolated by spreading antagonistic signals over the whole cell.
Adaptation	The ability of a growth cone to readjust its sensitivity over a wide range of guidance cue concentrations during long-distance chemotaxis.
Early endosome	An intracellular membrane compartment where endocytosed molecules are sorted and directed to the plasma membrane for recycling or to lysosomes for degradation. Early endosomes can also serve as a platform where internalized receptors generate signals.
Rac and Cdc42	Rho-family GTPases that link extracellular signals to cytoskeletal rearrangements. Rac and Cdc42 can, for example, promote actin assembly through their major effector actin-related protein 2/3 complex.

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Online Summary

1. During neural development, graded distribution of extracellular cues in the microenvironment causes asymmetric generation of second messengers across the growth cone in order to guide the axon along its correct path. Asymmetrically generated Ca^{2+} signals are sufficient to initiate growth cone turning toward the side with higher Ca^{2+} concentrations (attraction) or with lower Ca^{2+} concentrations (repulsion).
2. Gating of differential sets of Ca^{2+} channels, which are regulated counteractively by cyclic AMP and cyclic GMP, can be responsible for switching between growth cone attraction and repulsion. We propose that high-amplitude Ca^{2+} elevation involving Ca^{2+} release from the endoplasmic reticulum (ER) mediates attractive guidance, whereas low-amplitude Ca^{2+} influx that does not trigger substantial Ca^{2+} release from the ER mediates repulsive guidance.
3. Shallow concentration gradients of guidance cues can shape growth cone Ca^{2+} signals for precise navigational responses. This process may depend on second messenger networks including positive-feedback augmentation between Ca^{2+} and cyclic AMP.
4. Repulsive Ca^{2+} signals cause asymmetric clathrin-mediated endocytosis across the growth cone with more endocytosis on the side with elevated Ca^{2+} . Attractive Ca^{2+} signals promote centrifugal transport of membrane vesicles and their subsequent exocytosis mediated by vesicle-associated membrane protein 2 on the side with elevated Ca^{2+} .
5. We propose that asymmetric membrane trafficking is an early and instructive step in the initiation of growth cone turning. Localized imbalance between endocytosis and exocytosis may trigger redistribution of adhesion molecules, cytoskeletal components and bulk membrane, which would potentiate asymmetric traction and protrusion forces essential for turning.
6. The growth cone in complex environmental terrain *in vivo* must integrate multiple guidance signals simultaneously to navigate with high fidelity. Spatiotemporally regulated interactions among Ca^{2+} and cyclic nucleotides potentially play crucial roles in this integration process, which will need to be scrutinized by quantitative imaging of multiple second messengers in navigating growth cones.

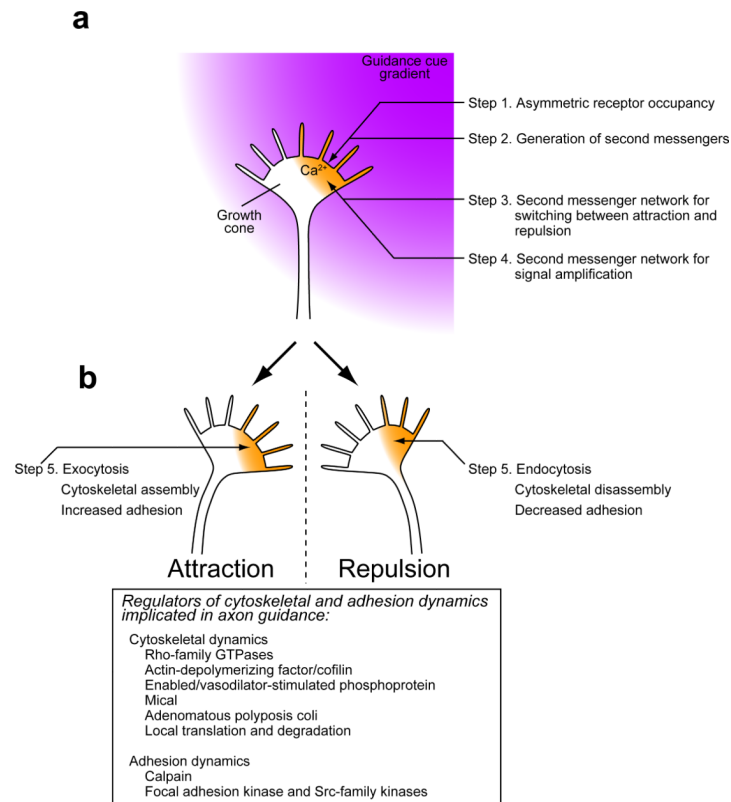


Figure 1. Overview of signaling and mechanical events during bidirectional growth cone guidance

(a) Involvement of second messengers in signal transduction. A guidance cue gradient causes asymmetric occupancy of guidance cue receptors across the growth cone (Step 1) and initial generation of second messengers such as Ca²⁺ (orange) and cyclic nucleotides (Step 2). Second messenger networks determine whether the growth cone turns toward or away from the side with Ca²⁺ signals (Step 3) and may amplify guidance information into steeply graded or even compartmentalized signals in the growth cone (Step 4). Steps 3 and 4 may be functionally coupled and temporally overlapping processes. **(b)** Steering machinery for growth cone guidance. Amplified signals on one side of the growth cone break the symmetry of membrane trafficking, cytoskeletal organization and adhesiveness, which causes attractive or repulsive turning of the growth cone (Step 5). Listed in the box are examples of regulators of the cytoskeleton and adhesion dynamics that are either activated or inactivated by Ca²⁺, cyclic nucleotides, and other signaling components.

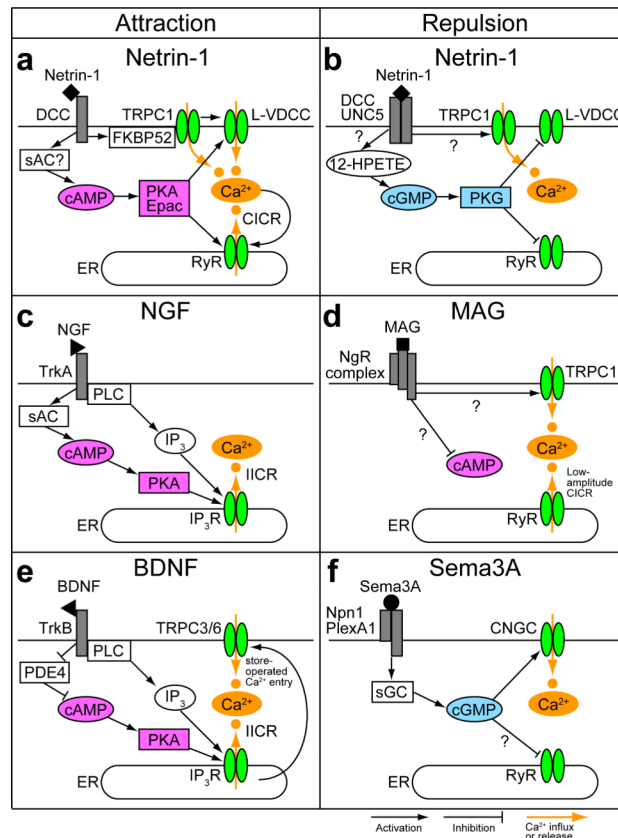


Figure 2. Second messenger systems for growth cone guidance

(a) Netrin-1-induced attraction involves Ca²⁺ influx through transient receptor potential type C1 (TRPC1) and L-type voltage-dependent Ca²⁺ channels (L-VDCC) and Ca²⁺-induced Ca²⁺ release (CICR) from the endoplasmic reticulum (ER) through ryanodine receptors (RyR). Upon netrin-1 binding to the receptor deleted in colorectal cancer (DCC), the peptidyl-prolyl isomerase FK506-binding protein 52 (FKBP52) mediates the isomerization-elicited opening of TRPC1 (REF. 49). The activated TRPC1 triggers membrane depolarization⁹ leading to further Ca²⁺ entry through L-VDCC (REF. 37). This Ca²⁺ entry most likely triggers CICR that causes growth cone attraction⁶. Gating of L-VDCC and RyR requires the activity of the cyclic AMP (cAMP)-protein kinase A (PKA) pathway^{27,37}. A sufficient cAMP level is maintained possibly by the action of soluble adenylate cyclase (sAC) (REF. 50), although conflicting results have been reported⁵¹. The cAMP effector Epac has also been implicated in netrin-1-induced attraction³⁶. (b) Netrin-1-induced repulsion is probably mediated by Ca²⁺ influx through TRPC1 only⁹, because the cyclic GMP (cGMP)-protein kinase G (PKG) pathway inactivates L-VDCC (REF. 37) and RyR (REF. 38). The DCC-uncoordinated 5 (UNC5) receptor complex, which mediates netrin-1-induced repulsion, has been postulated to stimulate the production of cGMP via the lipid mediator 12-hydroperoxyeicosatetraenoic acid (12-HPETE) (REF. 37). (c) Nerve growth factor (NGF)-induced attraction involves the receptor tropomyosin-related kinase A (TrkA) and its downstream effector, phospholipase C (PLC) (REF. 53) that catalyzes the production of inositol 1,4,5-trisphosphate (IP₃) in the cytosol. NGF also increases cAMP levels via sAC (REF. 54), facilitating IP₃-induced Ca²⁺ release (IICR) upon IP₃ binding to the IP₃ receptor (IP₃R) (REF. 11). (d) Myelin-associated glycoprotein (MAG) binds the Nogo-66 receptor complex (NgR complex) and repels growth cones via low-amplitude Ca²⁺ release from the ER (REF. 7) and potentially Ca²⁺ influx through TRPC1 (REF. 34). The TRPC1 may also participate in maintaining ER-stored Ca²⁺. MAG antagonizes neurotrophin-induced cAMP

elevations in postnatal rat neurons under conditions in which MAG inhibits axon growth⁵², suggesting that MAG has a bias toward inhibiting the cAMP pathway during repulsive guidance. **(e)** Brain-derived neurotrophic factor (BDNF)-induced TrkB-mediated attraction requires IICR together with Ca²⁺ influx through TRPC3/6, in which TRPC3/6 may participate in store-operated Ca²⁺ entry for replenishment of the ER with Ca²⁺ (REF. 8). BDNF also increases cAMP levels via inhibition of phosphodiesterase 4 (PDE4) (REF. 55). **(f)** Semaphorin 3A (Sema3A) binds a complex of neuropilin 1 (Npn1) and plexinA1 (PlexA1) and repels growth cones by elevating cGMP levels via soluble guanylate cyclase (sGC), which in turn triggers Ca²⁺ influx through cyclic nucleotide-gated channel (CNGC) (REF. 10). Whether RyR is inactivated downstream of cGMP remains unclear.

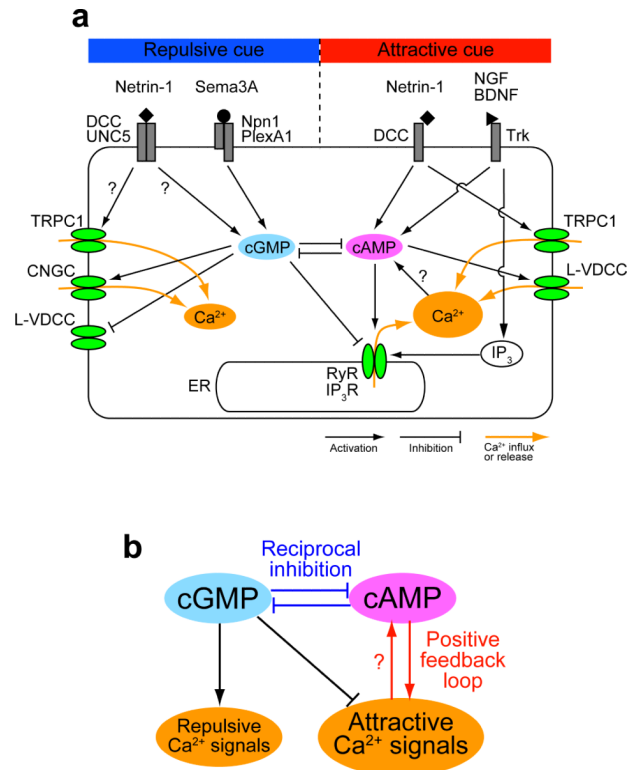


Figure 3. Second messenger network shapes attractive and repulsive Ca²⁺ signals

(a) Signaling network and Ca²⁺ mobilization downstream of guidance cue receptors. A growth cone expresses transient receptor potential type C1 (TRPC1) channels, cyclic nucleotide-gated channels (CNGC) and L-type voltage-dependent Ca²⁺ channels (L-VDCC) in the plasma membrane and ryanodine receptors (RyR) and inositol 1,4,5-trisphosphate (IP₃) receptors (IP₃R) in the endoplasmic reticulum (ER) membrane. Cyclic AMP (cAMP) and cyclic GMP (cGMP) counteractively regulate Ca²⁺ mobilization: e.g., Ca²⁺ release through RyR is facilitated by cAMP and inhibited by cGMP. Ca²⁺ might in turn increase the cAMP level, forming a positive-feedback loop between Ca²⁺ and cAMP. Reciprocal inhibition pathways exist between cAMP and cGMP. BDNF, brain-derived neurotrophic factor; DCC, deleted in colorectal cancer; NGF, nerve growth factor; Npn1, neuropilin 1; PlexA1, plexinA1; Sema3A, Semaphorin 3A; Trk, tropomyosin-related kinase; UNC5, uncoordinated 5. (b) The core signaling network for growth cone guidance. The interactions among Ca²⁺ and cyclic nucleotides, including positive-feedback loops and the reciprocal inhibition, would shape either of the two types of Ca²⁺ signals: high-amplitude Ca²⁺ signals accompanied by Ca²⁺ release from the ER (attractive Ca²⁺ signals) or low-amplitude Ca²⁺ influx that does not trigger Ca²⁺ release from the ER (repulsive Ca²⁺ signals).

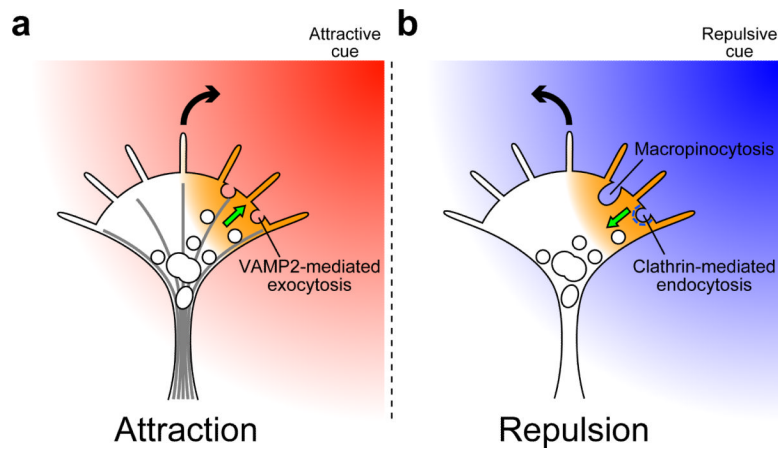


Figure 4. Asymmetric membrane trafficking drives bidirectional growth cone turning
 Extracellular gradients of guidance cues trigger the generation of Ca^{2+} signals on one side of growth cones (orange areas). **(a)** Attractive Ca^{2+} signals promote centrifugal transport of vesicle-associated membrane protein 2 (VAMP2)-containing vesicles (white circles) along microtubules (radial lines), with exocytosis ensuing in the growth cone periphery. **(b)** Repulsive Ca^{2+} signals facilitate the formation of clathrin (white dashes)-coated pits that migrate toward the growth cone center followed by internalization. The migration of clathrin-coated pits depends on retrograde flow of actin filaments⁸⁹. Asymmetric macropinocytosis has been implicated in repulsive guidance⁸⁷, although it remains unclear whether repulsive Ca^{2+} signals enhance this type of endocytosis in growth cones. The curved arrows indicate the direction of growth cone turning. The straight arrows indicate the transport direction of VAMP2-containing vesicles and clathrin-coated pits.

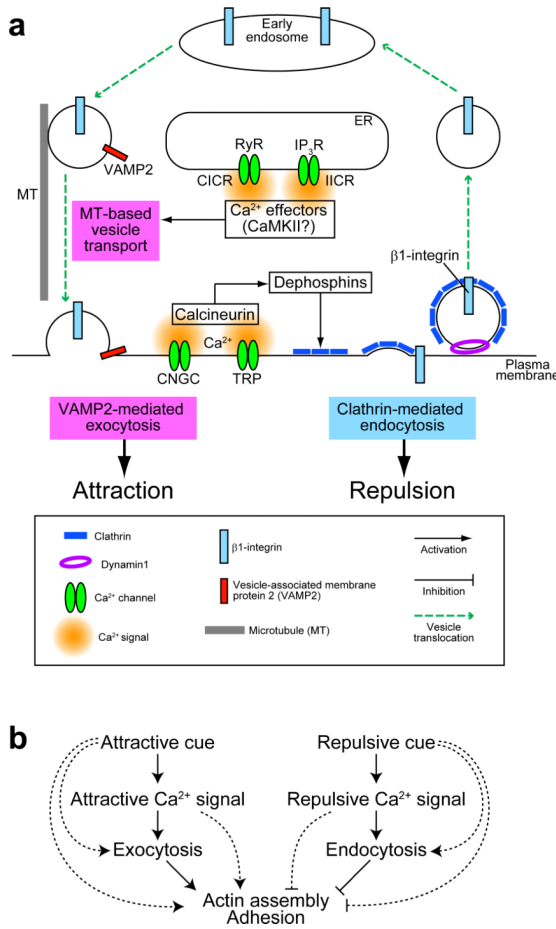


Figure 5. Working model of the growth cone steering machinery: membrane trafficking as a master organizer

(a) Trafficking of membrane vesicles and their cargo proteins in growth cones. Repulsive Ca²⁺ signals, e.g., Ca²⁺ influx through cyclic nucleotide-gated channels (CNGC) or transient receptor potential (TRP) channels, facilitate the formation of clathrin-coated pits perhaps via calcineurin-mediated dephosphorylation of dephosphins. $\beta 1$ -integrin can be captured by coated pits for internalization and most likely sorted into early endosomes. Attractive Ca²⁺ signals, e.g., Ca²⁺ release from the endoplasmic reticulum (ER) through ryanodine receptors (RyR) or inositol 1,4,5-trisphosphate receptors (IP₃R), facilitate microtubule-based centrifugal transport of vesicle-associated membrane protein 2 (VAMP2)-containing vesicles and subsequent exocytosis in the growth cone periphery. Ca²⁺/calmodulin-dependent protein kinase II (CaMKII) might link attractive Ca²⁺ signals to exocytic trafficking. In non-neuronal cells, $\beta 1$ -integrin is recycled to the plasma membrane via VAMP2-dependent exocytosis. Endocytic and exocytic vesicles could also carry cytoskeletal regulators (not shown). CICR, Ca²⁺-induced Ca²⁺ release; IICR, IP₃-induced Ca²⁺ release. **(b)** Mechanistic model for growth cone guidance. Attractive and repulsive Ca²⁺ signals promote exocytosis and endocytosis, respectively, on the side with elevated Ca²⁺. Such asymmetric membrane trafficking is likely to control the surface addition and removal of membrane and cargo proteins including adhesion molecules and cytoskeletal regulators. The polarized targeting of driving machinery would generate asymmetric traction and protrusion forces that are essential for growth cone turning. The parallel pathways also operate that bypass Ca²⁺ signals or membrane trafficking (broken lines).

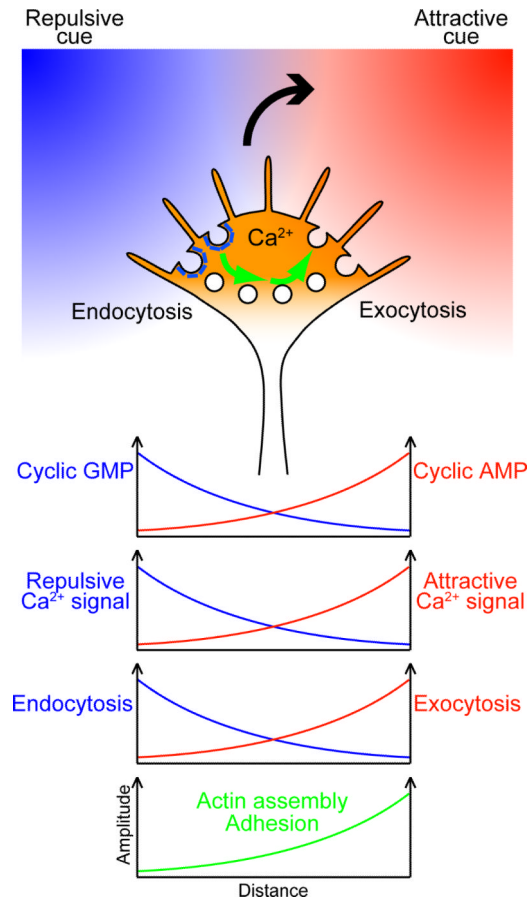


Figure 6. Hypothetical model for growth cone guidance by multiple cues

When a growth cone encounters both an attractant on the right side and a repellent on the left side, counter gradients of cyclic AMP and cyclic GMP could be created via several mechanisms described in the text. These counter gradients control the open probability of ryanodine receptors and inositol 1,4,5-trisphosphate receptors, leading to asymmetric release of Ca^{2+} from the endoplasmic reticulum across the growth cone. Even if the two guidance cues together were to cause symmetric elevations of cytoplasmic Ca^{2+} concentrations, the nature of Ca^{2+} signals would differ between both sides of the growth cone: i.e., attractive and repulsive Ca^{2+} signals would be reciprocally distributed. These reciprocal Ca^{2+} signals elicit the counter gradients of exocytic and endocytic activities that cause relatively increased filamentous actin assembly and growth cone adhesiveness on the attractant side. The larger curved arrow indicates the direction of growth cone turning. The smaller arrows indicate intracellular vesicle trafficking, although it remains unclear whether endocytosed membrane components are recycled directly to the other side of the growth cone. The x-axis of each graph corresponds to the width of the growth cone.

Table 1

Ca²⁺ and cyclic nucleotide signaling elicited by guidance cues

Guidance cues	Cell types and culture substrates	Turning direction	Receptors	Ca ²⁺ channels involved in turning	Cue-induced Ca ²⁺ increase in growth cones	Cue-induced cyclic nucleotide activation
Netrin-1	Stage 22–25 Xenopus spinal neurons 12–26 h on uncoated glass (REFS. 6,9,37,98)	Attraction	DCC	TRPC1 + L-VGCC + RyR (REFS. 6,9)	Asymmetric Ca ²⁺ elevation (REFS. 6,98)	cAMP elevation in Xenopus retinal ganglion cell growth cones (REF. 15)
		Repulsion	DCC/UNC5	TRPC1 with or without L-VGCC (REFS. 6,9) (Involvement of these channels downstream of DCC/UNC5 is unclear)	Not determined	cGMP elevation suggested but not observed (REF. 37)
NGF	E9–10 chicken DRG neurons on L1 or laminin (REF. 11)	Attraction	TrkA	IP ₃ R (REF. 11)	Asymmetric IP ₃ production and IICR (REF. 11)	cAMP elevation determined in rat DRG neurons and PC12 cells by enzyme immunoassay (REFS. 52,54)
BDNF	P0 rat cerebellar granule neurons on Matrigel (REF. 8)	Attraction	TrkB	TRPC3/6 + IP ₃ R (REF. 8)	Ca ²⁺ elevation, asymmetry not determined (REF. 8)	cAMP elevation determined by enzyme immunoassay (REF. 52)
NT-4	E9–10 chicken DRG neurons on laminin (REF. 38)	Repulsion	TrkB	Not determined	Asymmetric Ca ²⁺ elevation (REF. 38)	Not determined
MAG	Stage 22–25 Xenopus spinal neurons 14–20 h on uncoated glass (REFS. 7,34)	Repulsion	NgR complex	TRPC1 (REF. 34) RyR (REF. 7)	Asymmetric Ca ²⁺ elevation (REF. 7)	MAG antagonizes neurotrophin-induced cAMP elevation in rat neurons (REF. 52)
	E9–10 chicken DRG neurons on laminin (REF. 89)	Attraction	Not determined	RyR (T. Tojima, R. Itofusa and H. Kamiguchi, unpublished data)	Ca ²⁺ elevation, asymmetry not determined (REF. 89)	Not determined
Sema3A	Stage 26 Xenopus spinal neurons 16–20 h on uncoated glass (REFS. 10,56)	Repulsion	Npn1/PlexA1	CNGC (REF. 10)	Asymmetric Ca ²⁺ elevation (REF. 56)	Asymmetric cGMP production in growth cones (REF. 10)
Wnt5a	P0–3 hamster cortical neurons on laminin (REF. 153)	Repulsion	Ryk/Frizzled	SKF-96365-sensitive TRP channels (REF. 153)	Ca ²⁺ elevation, asymmetry not determined (REF. 153)	Not determined
BMP7	Stage 20–22 Xenopus spinal neurons 4–8 h on laminin (REF. 63)	Attraction	BMPRII	Ca ²⁺ signals are not required for turning (REF. 63)	No elevation (REF. 63)	Not determined
	Stage 20–22 Xenopus spinal neurons 20–24 h on laminin (REF. 63)	Repulsion	BMPRII	TRPC1 (REF. 63)	Ca ²⁺ elevation, asymmetry not determined (REF. 63)	Not determined

Guidance cues	Cell types and culture substrates	Turning direction	Receptors	Ca ²⁺ channels involved in turning	Cue-induced Ca ²⁺ increase in growth cones	Cue-induced cyclic nucleotide activation
PACAP	One-day-old Xenopus spinal neurons on laminin (REF. 35)	Attraction	PAC1	Ca ²⁺ signals are not required for turning (REF. 35)	No elevation (REF. 35)	cAMP elevation in many cell types (REF. 154)
ACh	One-day-old Xenopus spinal neurons 6–10 h on uncoated glass (REF. 155)	Attraction	nAChR	Not determined	Asymmetric Ca ²⁺ elevation (REF. 155)	Not determined
GABA	E14 rat spinal neurons on serum-coated glass (REF. 156)	Attraction	GABA _A R	Not determined	Asymmetric Ca ²⁺ elevation (REF. 156)	Not determined

ACh, acetylcholine; BDNF, brain-derived neurotrophic factor; BMP7, bone morphogenetic protein; BMPRII, BMP receptor II; cAMP, cyclic AMP; cGMP, cyclic GMP; CNGC, cyclic nucleotide-gated channel; DCC, deleted in colorectal cancer; DRG, dorsal root ganglion; E, embryonic day; GABA, γ -aminobutyric acid; GABA_AR, GABA_A receptor; IP₃, inositol 1,4,5-trisphosphate; IP₃R, IP₃ receptor; IICR, IP₃-induced Ca²⁺ release; L-VDCC, L-type voltage-dependent Ca²⁺ channel; MAG, myelin-associated glycoprotein; nAChR, nicotinic ACh receptor; NGF, nerve growth factor; Ngr, Nogo-66 receptor; Npn1, neuropilin 1; NT-4, neurotrophin-4; P, postnatal day; PACAP, pituitary adenylylate cyclase-activating polypeptide; PAC1, PACAP type 1 receptor; PlexA1, plexin A1; Ryk, receptor related to tyrosine kinase; RyR, ryanodine receptor; Sema3A, Semaphorin 3A; Trk, tropomyosin-related kinase; TRPC, transient receptor potential cation channel, subfamily C; UNC5, uncoordinated 5.