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Directional guidance of nerve growth cones

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The intricate connections of the nervous system are established, in part, by elongating axonal fibers that are directed by complex guidance systems to home in on their specific targets. The growth cone, the major motile apparatus at the tip of axons, explores its surroundings and steers the axon along a defined path to its appropriate target. Significant progress has been made in identifying the guidance molecules and receptors that regulate growth cone pathfinding, the signaling cascades underlying distinct growth cone behaviors, and the cytoskeletal components that give rise to the directional motility of the growth cone. Recent studies have also shed light on the sophisticated mechanisms and new players utilized by the growth cone during pathfinding. It is clear that axon pathfinding requires a growth cone to sample and integrate various signals both in space and in time, and subsequently to coordinate the dynamics of its membrane, cytoskeleton and adhesion to generate specific responses.

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Introduction

Since Cajal's vivid description of growth cones as 'battering rams' that overcome obstacles along their journey to the targets of connectivity, modern microscopy and imaging techniques have enabled us to witness the fascinating behaviors of growth cones in living samples *in vitro* and *in vivo*. Growth cones exhibit a range of behaviors along the path to their targets, including acceleration and diminution of migration, pausing, collapse, retraction and bifurcation. Many of these distinct behaviors reflect the pathfinding responses of the growth cone in searching for its appropriate target. Although axon guidance is essential for the initial wiring of neural circuitry, it is also crucial for the re-wiring of regenerating axons that leads to functional recovery of brain circuitry after injury and disease. It is, thus, of immense importance to understand the

molecular and cellular mechanisms underlying axonal extension and guidance.

In the developing nervous system, axons are guided to their appropriate targets by extracellular molecules, either permissive and attractive or inhibitory and repulsive. Many evolutionarily conserved families of guidance molecules, including the Netrins, Slits, Semaphorins, and Ephrins, have now been identified and characterized [1–3]. However, sophisticated mechanisms appear to be employed by the growth cone to generate distinct responses to various molecules that are often presented in a spatiotemporal, sometimes overlapping, pattern. An excellent example is the guidance of commissural axons during development. In vertebrates and invertebrates, commissural axons are initially lured to the ventral midline floor plate by attractive molecules including netrins and hedgehog [2,4], which are secreted and present at high concentrations in the floor plate. Once they reach the midline, the neurons lose their responsiveness to the attractive cues, and become sensitive to repellents such as Slit proteins, which were first identified in *Drosophila* [1]. Three homologs of *Drosophila* Slit (Slit1–3) and two homologs of its receptor Roundabout (Robo1, 2) have been identified in mammals; the commissural axons fail to cross the midline floor plate in Slit1, 2, 3 triple mutant embryos [5]. Interestingly, Rig-1 (Robo3) functions as a negative regulator to repress premature Slit responsiveness, similar to the function of Commissureless (Comm) in *Drosophila* [6]. Thus, Slit–Robo interactions function to prevent commissural axons from re-crossing the midline, enabling their projections to travel along the anterior–posterior track guided by Wnt signals [7]. Another example involves the guidance molecules ephrins and their Eph receptors. Recent evidence demonstrates that ephrin–Eph interactions elicit bi-directional communications: ephrins function through Eph receptors to exert repulsive activities, whereas Ephs binding to ephrins elicit backward signaling through ephrin ligands for attraction [8]. Because many growth cones co-express both ephrins and Eph receptors on their surface, the puzzling question is how opposite effects are selectively activated. It was recently shown that ephrin molecules and Eph receptors on the same growth cone surface were segregated into distinct membrane microdomains to mediate opposite guidance responses [9**]. These findings illustrate the complexity and sophistication of the responses of growth cones to a variety of guidance cues. It is clear that there is still much to be learnt about how specific guidance signals are transduced from receptor activation to directed growth cone motility. Given that several comprehensive reviews on guidance have been

published recently [3,7,10–13], we only focus here on new findings in three areas of guidance mechanisms: membrane microdomains, Ca²⁺ signaling and focal adhesion.

Membrane microdomains and growth cone responses

Cells communicate and interact with the extracellular space through the plasma membrane that consists of a mosaic of proteins for receiving stimuli, for signaling in and out of the cell, and for interacting physically with the substrata. Recent studies suggest that the plasma membrane contains microdomains, termed lipid rafts, that are enriched in cholesterol and sphingolipids and, characteristically, are resistant to cold detergent extraction [14,15]. Lipid rafts are thought to be involved in many cellular functions, in particular, signal transduction for extracellular stimuli [14,15]. Many of these functions are also intimately related to the processes involved in neural development, including neurotrophic factor signaling and synaptic plasticity. Recent evidence indicates that membrane lipid rafts play an important part in growth cone migration and pathfinding. Several guidance receptors or receptor complex components associate with the detergent-resistant membrane (DRM) fractions in neurons [9^{••},16[•],17[•],18]. Disruption of lipid rafts by cholesterol and ganglioside manipulation has resulted in attenuation of guidance responses *in vitro* [16[•],19^{••}]. Moreover, recent studies show that ephrin-As and EphA receptors co-existing on the same growth cone surface are probably segregated into microdomains to mediate different signaling pathways [9^{••}], thus reiterating the notion that microdomains on the plasma membrane are functionally important for neuronal signaling. Because ephrin-As are glycosyl phosphatidylinositol (GPI)-anchored proteins, it is likely that lipid rafts are involved in ephrin clusters and/or Eph microdomains on the growth cone surface. It is conceivable that microdomains of lipids and proteins can contribute to spatial control and segregation in distinct signaling cascades underlying growth cone pathfinding.

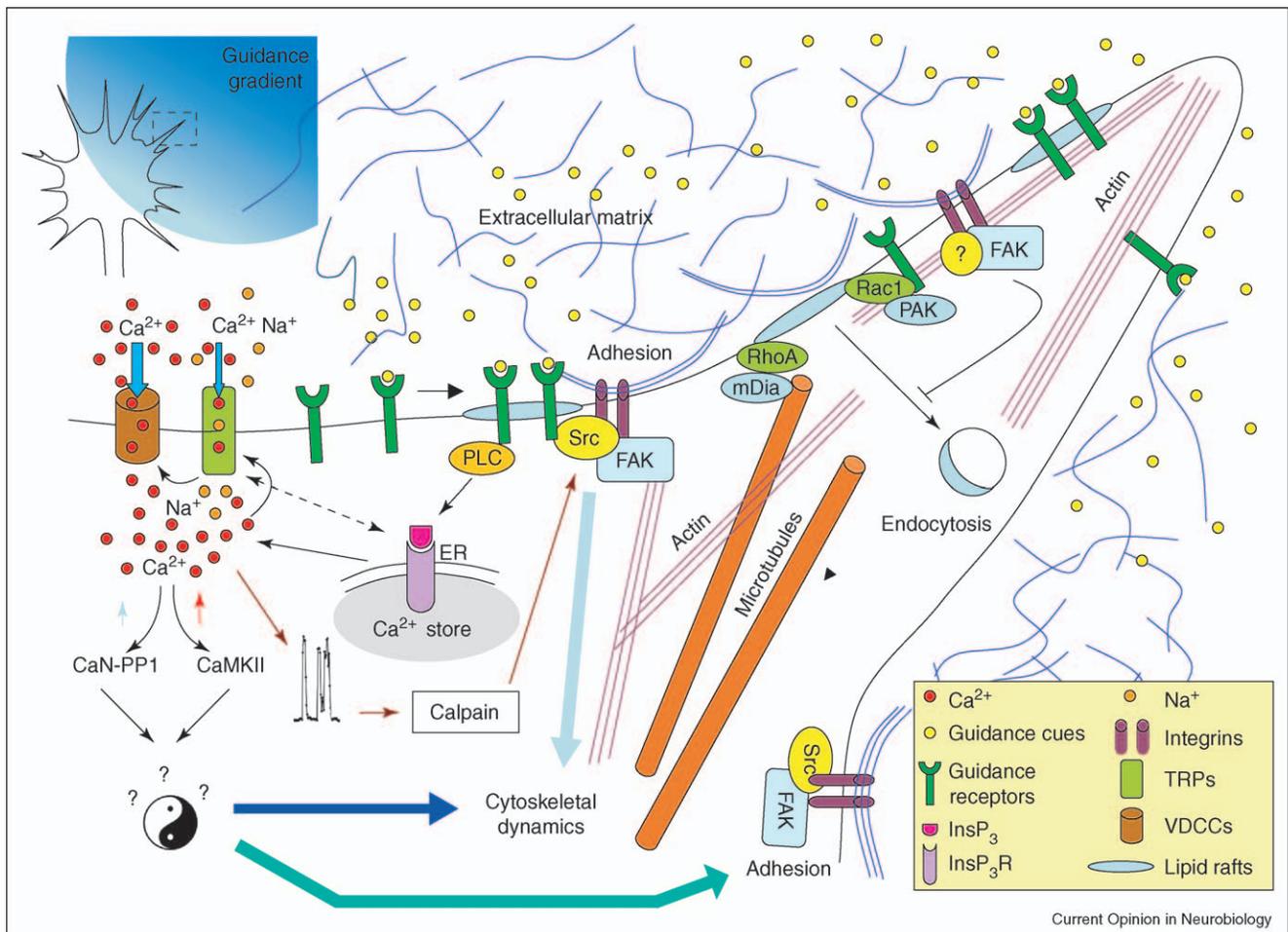
One of the interesting observations on the receptor–raft interactions is that some receptors are only partially associated with lipid rafts at the resting state of the cell, but increase their raft association upon exposure to the ligands [17[•],19^{••},20]. Such ligand-induced translocation might be an important mechanism for selective coupling of extracellular stimuli to distinct signaling pathways. Potentially, receptors in the raft- and non-raft-fractions of the plasma membrane could mediate different effects of the extracellular factors. One piece of evidence came from studies on the raft-dependence of multiple effects elicited by brain derived neurotrophic factor (BDNF) on developing neurons. BDNF enhances cell survival, promotes neurite outgrowth, induces chemoattraction, and potentiates synaptic transmission, all through the high-affinity trkB receptors. Experimental data indicate that

BDNF induced synaptic potentiation depends on trkB in lipid rafts but BDNF enhancement of neuronal survival involves trkB outside the rafts [17[•]]. In nerve growth cones, BDNF-induced attraction could be abolished by cholesterol depletion but acceleration of growth cone motility was not affected by cholesterol extraction [19^{••}]. These results suggest that membrane rafts might mediate distinct signaling pathways underlying various neuronal functions.

Directional guidance of nerve growth cones requires spatial and temporal control of intracellular signaling that targets growth cone motility for steering. Membrane microdomains have been implicated in the generation of localized signaling in the growth cone. For example, ligand-induced recruitment of guidance receptors could contribute to an asymmetric association of guidance receptors with membrane rafts, thus resulting in localized signal transduction that is essential for translating the extracellular guidance cues into the turning response of the growth cone (Figure 1). Indeed, imaging studies using *in situ* cold detergent extraction and immunostaining on *Xenopus* growth cones demonstrated that raft-association of trkB receptors became asymmetrically localized to the side exposing to a higher concentration of BDNF in a gradient [19^{••}]. The notion that membrane microdomains contribute and control spatial signaling across the cell surface has been strongly supported by studies on cell chemotaxis [21]. Similar to growth cone guidance, cell chemotaxis involves the sensing of extracellular chemokine gradients and polarization of the cell for directed movement [22]. In cell chemotaxis induced by the chemokine stromal cell derived factor-1 (SDF-1), a persistent redistribution of lipid raft-associated green fluorescent protein (GFP)-GPI to the cell edges was observed [21]. Interestingly, the SDF-1 receptor CCR5 became highly associated with the ganglioside GM₃-rich rafts at the leading edge, followed by recruitment and activation of phosphatidylinositol-3 kinase γ (PI3K γ). Such polarized or asymmetric distribution of different membrane microdomains could be the key for polarization of signaling and adhesion components in migrating cells. Whether microdomains are involved in recruitment and localization of different signaling components across the growth cone during guidance awaits future study.

Recent studies on non-neuronal cells demonstrate that lipid rafts mediate integrin-induced membrane targeting of Rho GTPases and their coupling to the downstream effectors [23^{••},24^{••}]. In one of the two complementary studies, integrin-mediated adhesion was found to regulate the coupling of Rac to the downstream effector molecule p21-activated kinase (PAK) by modulating lipid rafts on the plasma membrane [23^{••}]. The working model is that cell adhesion via integrins enables Rac targeting to the membrane rafts and coupling to PAK, whereas cell detachment causes internalization of Rac-associated rafts,

Figure 1



A schematic to illustrate the complex signaling network that controls the response of a growth cone to extracellular guidance cues. A small portion of the growth cone exposed to the guidance gradient is magnified to illustrate different components involved in signal transduction. In this model, guidance molecules induce translocation of their receptors into membrane rafts, generating localized signals to induce growth cone steering. Among several signals, phospholipase C (PLC) activation generates inositol-1,4,5-trisphosphate (InsP₃), which then binds to InsP₃ receptor (InsP₃R) on endoplasmic reticulum (ER) and triggers Ca²⁺ release from intracellular Ca²⁺ stores. Ca²⁺ release from intracellular stores subsequently activates TRP channels on the plasma membrane to enable the influx of both Ca²⁺ and Na⁺, resulting in membrane depolarization and opening of voltage-dependent Ca²⁺ channels (VDCCs) that further enhance the Ca²⁺ signal. Localized elevation of [Ca²⁺]_i can activate different downstream effectors to steer the growth cone. In one model, small and modest local Ca²⁺ signals act on CaMKII or Calcineurin-PP1 to regulate the balance of phosphorylation and dephosphorylation of downstream effectors (indicated by the Yin Yang symbol) to control growth cone attraction and repulsion. The potential targets of this phosphorylation and dephosphorylation could be the cytoskeleton or substrate adhesion of the growth cone. Large Ca²⁺ transients, however, are thought to activate the Ca²⁺-sensitive protease Calpain, which targets Src to induce growth cone repulsion. Guidance receptors (such as DCC) can directly interact with FAK, Src or Fyn to regulate growth cone adhesion or activate downstream effectors such as PAK to control cytoskeletal dynamics during steering. In migrating fibroblasts, cell adhesion involving integrin receptors regulates lipid rafts to enable Rac-PAK or RhoA-mDia coupling, possibly through FAK inhibition of raft internalization. Whether this pathway is involved in growth cone steering remains to be examined. It is apparent that spatiotemporal coordination of these different signals and motility components is crucial for directional movement of growth cones guided by extracellular cues.

leading to de-coupling of Rac and PAK. This model is supported by the second study in which integrin-mediated adhesion induced both localization of G_{M1}-rich lipid rafts at the leading edge of migrating fibroblasts and coupling of Rho to its effector mDia, the mammalian diaphanous-related formins, to stabilize microtubules for cell migration [24•]. Furthermore, focal adhesion kinase (FAK) was found to have a crucial role in this localized

process. Taken together, these findings indicate an exciting role for rafts in controlling polarized signaling and motility in cell migration. Because integrin is involved in many cellular events including axon guidance and, importantly, growth cone turning involves asymmetric adhesion and detachment, it is conceivable that similar mechanisms might also be involved in directional steering of growth cones in response to guidance signals (Figure 1).

Although previous studies showed that growth cone migration and random turning were not affected by raft disruption [19^{••}], different integrin molecules involved in growth cone-substrate adhesion might contribute to the difference in the involvement of rafts in growth cone migration [25]. The possibility that different types of rafts might regulate Rac and Rho coupling to their effectors further indicates the complexity in spatial and temporal control of signaling and motility in migrating cells and growth cones. It will be interesting to see whether what we have learnt from migrating non-neuronal cells can be applied to the growth cone. Finally, it should be pointed out that dynamic lipid microdomains represent an attractive model for spatial control of signal transduction at the plasma membrane, but the concept of lipid rafts is still being debated [26]. The experimental findings discussed above, at least, indicate an important role for membrane lipids and/or the lipid environment in guidance signaling and directional motility. Future studies are clearly required to validate the model and to elucidate the molecular interactions occurring at the plasma membrane that generate intricate networks of intracellular signaling underlying specific neuronal functions.

Ca²⁺ signaling in controlling growth cone steering

Ca²⁺ is an important second messenger that relays extracellular information to directional motility [10]. Growth cone turning induced by several guidance molecules, such as netrin-1, BDNF and myelin associated glycoprotein (MAG), depends on localized Ca²⁺ signals in the growth cone [27,28,29[•]]. The intracellular Ca²⁺ concentration ([Ca²⁺]_i) in the growth cone is regulated by several cellular processes such as Ca²⁺ influx through plasma membrane Ca²⁺ channels, Ca²⁺ release from internal stores, and Ca²⁺ extrusion through plasma membrane Ca²⁺-ATPases (PMCA) and a Na⁺-Ca²⁺ exchanger. Previous work indicates that netrin-1-induced growth cone attraction requires Ca²⁺ influx through voltage-dependent Ca²⁺ channels (VDCCs) including L-type Ca²⁺ channels on the plasma membrane [27,30]. Recent studies by three groups demonstrate that transient receptor potential (TRP) channels are involved in growth cone turning responses to netrin, BDNF and MAG [31^{••}-33^{••}]. TRP channels comprise a large group of non-selective cation channels that are involved in regulation of many cellular events such as neurite extension and growth cone morphology [34]. A role of TRP channels in guidance was further supported by the *in vivo* data that knockdown of *Xenopus* TRP channels by morpholino antisense resulted in guidance defects in commissural axons crossing the midline [32^{••}]. These findings thus indicate that members of the TRP channel family function as key mediators for the Ca²⁺ influx that regulates axon guidance during development. It remains to be determined, however, how TRP channels are specifically activated by each guidance molecule. Furthermore, growth cone attraction induced

by BDNF or netrin-1 was completely blocked by TRP channel knockdown in two of these papers [31^{••},33^{••}]. However, knockdown of *Xenopus* TRP1 (XTRP1) only blocked MAG-induced repulsion but, interestingly, converted BDNF- and netrin-1-induced attraction to repulsion [32^{••}]. Such a discrepancy might reflect the difference in the knockdown approaches used and the extent of protein reduction. Because both the baseline [Ca²⁺]_i and the local [Ca²⁺]_i can influence the direction of Ca²⁺-induced growth cone turning [35^{••},36], TRP channels could contribute to either or both of these two Ca²⁺ levels to result in repulsion or blockade of turning responses. More studies are clearly required to understand how exactly TRP channels contribute to Ca²⁺ signaling in growth cone guidance.

Different local Ca²⁺ signals mediate bi-directional responses of the growth cone. Recent studies have begun to illuminate the downstream effectors linking different Ca²⁺ signals to opposite turning responses. On the one hand, large Ca²⁺ transients in filopodia were found to activate Ca²⁺-sensitive protease calpain and resulted in growth cone repulsion [37]. On the other hand, growth cone turning induced by extracellular guidance gradients was found to involve relatively small asymmetric [Ca²⁺]_i elevation that could mediate bi-directional responses [27,28,38]. In particular, a small local Ca²⁺ was found to mediate repulsion, whereas a modest elevation of [Ca²⁺]_i resulted in attraction [29[•]]. Studies using direct focal elevation of [Ca²⁺]_i by photolysis of caged Ca²⁺ showed that Ca²⁺-calmodulin-dependent protein kinase II (CaMKII) and calcineurin (CaN)-phosphatase 1 (PP1) act in concert as a switch that controls growth cone responses to different Ca²⁺ signals [35^{••}]. Although the downstream targets of CaMKII and PP1 are uncertain, these results suggest a model in which CaMKII and PP1 could function as a pair that controls the balance of phosphorylation and dephosphorylation of the downstream components involved in regulating growth motility and directional steering (Figure 1).

Does focal adhesion function as a convergence point?

The growth cone advances by cytoskeleton-driven motility that is coupled by selective adhesion to the extracellular matrix. Transmission of growth cone traction force through adhesion-cytoskeletal linkage is essential for growth cone migration [39]. Although it is known that guidance signaling often targets the cytoskeletal dynamics in the growth cone to exert its influences on growth cone motility and behaviors (for a recent review, see Kalil and Dent [11]), recent studies indicate that cell adhesion could also be the primary target of guidance signaling. For example, activation of the repulsive Robo receptors inhibited N-cadherin-mediated cell adhesion to induce repulsion [40]. Moreover, the netrin-1 receptor deleted in colorectal cancer (DCC) directly interacts with

adhesion components FAK, Src and Fyn, and DCC is tyrosine phosphorylated upon netrin-1 stimulation. Disruption of FAK, Src or Fyn signaling was able to block the netrin-induced axon outgrowth and turning response [41^{••}–43^{••},44]. Because cell adhesion and cytoskeletal dynamics are tightly linked, spatial and temporal regulation of growth cone–substrate adhesion might be one important mechanism that regulates guidance responses.

The findings that DCC is tyrosine phosphorylated and that netrin-1 stimulates tyrosine phosphorylation of FAK and Src families of kinases reinforce an important role for tyrosine phosphorylation in growth cone motility and guidance. Tyrosine phosphorylation is involved in the downstream signals of guidance receptors [45[•],46–48]. For example, tyrosine phosphorylation of UNC-40, a *C. elegans* homolog of DCC, has been observed, and UNC-40 signaling is regulated by the receptor protein tyrosine phosphatase (RPTP) CLR-1 [49[•],50]. Previous work indicates that FAK might bind to cytoskeleton-associated proteins such as paxillin to modulate the cytoskeletal dynamics [51]. Recently, Robles *et al.* [45[•]] demonstrated that BDNF can stimulate Src kinase to phosphorylate Cdc42 effector p21-activated kinase (PAK) and regulate the growth cone motility. These results suggest that FAK–Src might act as a crucial mediator between guidance cue receptors and rearrangement of the cytoskeleton to regulate growth cone responses. It should be mentioned that both BDNF and netrin-1 involve Ca²⁺ and CaMKII in inducing growth cone turning [10]; therefore, these findings indicate a potential crosstalk of signaling pathways involving Ca²⁺-serine and threonine phosphorylation and tyrosine phosphorylation. FAK interacts with multiple signaling pathways that affect various cellular processes [52,53]. In particular, FAK contains several tyrosine and serine phosphorylation sites, activated FAK recruits Src, and FAK and Src stimulate each other. Therefore, it is plausible that FAK could function as the converging point of signaling pathways involving serine and threonine phosphorylation (e.g. by Ca²⁺–CaMKII) and tyrosine phosphorylation through Src (Figure 1). Previous studies have shown a role for FAK Ser732 phosphorylation by cyclin-dependent kinase 5 (Cdk5) in neuronal migration [54]. Whether serine phosphorylation of FAK plays a role in growth cone guidance remains to be examined. Nonetheless, a growth cone migrates by integrating distinct signaling pathways and by coordinating its cytoskeletal dynamics and adhesion to complete its journey for specific connections.

Conclusions and future directions

Although several components in the signaling pathways that control the steering of growth cones have been identified and described, none of these pathways are yet complete. It is still not well understood how extracellular guidance cues generate specific signals inside the growth cone, how different signaling pathways are coor-

inated, or what key components downstream of the Rho GTPases regulate growth cone turning. A deeper understanding of the guidance mechanisms will require a detailed understanding of intricate signaling cascades that propagate from the membrane through the intracellular space to target the dynamics of the cytoskeleton and of adhesion. The challenge is that many of these events are spatially and temporally controlled in the growth cone and a complete understanding will require arrays of analyses on the chemical reactions occurring inside the cell in real time.

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