CHAPTER ONE

Mechanisms of Gradient Detection: A Comparison of Axon Pathfinding with Eukaryotic Cell Migration

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Contents

Abstract

The detection of gradients of chemotactic cues is a common task for migrating cells and outgrowing axons. Eukaryotic gradient detection employs a spatial mechanism, meaning that the external gradient has to be translated into an intracellular signaling gradient, which affects cell polarization and directional movement. The sensitivity of gradient detection is governed by signal amplification and adaptation mechanisms. Comparison of the major signal transduction pathways underlying gradient detection in three exemplary chemotaxing cell types, *Dictyostelium*, neutrophils, and fibroblasts and in neuronal growth cones, reveals conserved mechanisms such as localized PI3 kinase/PIP3 signaling and a common output, the regulation of the cytoskeleton by Rho GTPases.

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Local protein translation plays a role in directional movement of both fibroblasts and neuronal growth cones. Ca^{2+} signaling is prominently involved in growth cone gradient detection. The diversity of signaling between different cell types and its functional implications make sense in the biological context.

Key Words: Gradient detection, Axon guidance, Cell migration, Cell polarization, *Dictyostelium*, Neutrophil, Fibroblast, Growth cone.

"There is a theory which states that if ever anyone discovers exactly what the Universe is for and why it is here, it will instantly disappear and be replaced by something even more bizarre and inexplicable. There is another theory which states that this has already happened."
—Douglas Adams, *The Restaurant at the End of the Universe*

1. Introduction

Graded distributions of chemotropic factors (referred to as "gradients" herein) are essential guideposts in the development, regeneration, and function of multicellular organisms. They provide directional as well as positional information and steer migrating cells and neuronal growth cones. Detection of gradients allows *Dictyostelium* cells to aggregate and proceed in their life cycle, neutrophils to migrate to sites of infection, fibroblasts to invade and heal wounds, and neuronal growth cones to follow guidance cues and establish the complex connectivity of the nervous system. It should be noted, however, that directed movement can be established by cues other than graded distributions of guidance factors. Likewise, gradients can induce cellular responses different from directed movement. These topics will not be considered in this article.

Gradient detection by eukaryotic cells differs fundamentally from the one in bacteria. Because bacteria are too small to sense a concentration gradient along their cell length, they use a temporal gradient-sensing mechanism. Dependent on temporal changes in the concentration of a chemoattractant or a chemorepellent, they regulate the frequency of random directional reorientation by changing the direction of flagellar rotation. This allows them to bias their overall direction of movement. Although highly efficient in detecting minimal concentration differences over a wide range by means of adaptation, a temporal gradient-sensing mechanism can be only employed by a moving cell and does not guide cells on a straight path toward or away from a chemotactic factor (Baker *et al.*, 2005; Wadhams and Armitage, 2004).

A migrating eukaryotic cell, on the other hand, can sense an external concentration gradient along the cell length and translate it into an internal signaling gradient. The internal gradient leads to a morphological polarization

and, in some cases, an asymmetry of sensitivity. Once polarized, the cell initiates directed movement via cytoskeletal rearrangements. Similarly, a neuronal growth cone responding to an attractive or repulsive gradient activates differing signaling events at the side facing the higher concentration (gradient near side) and the side facing the lower concentration (gradient far side), while performing a turning response. To detect small concentration differences and translate them efficiently into a correlated turnaround, the external gradient has to be amplified by means of signal transduction. Furthermore, a cell or a growth cone migrating in a gradient possibly has to adjust its sensitivity and adapt to detect concentration differences over a broad range of absolute concentrations.

In this chapter, we will exemplarily review these different aspects of gradient detection during chemotaxis in two amoeboid cell types (*Dictyostelium* and mammalian neutrophils) as well as in fibroblasts and compare them with gradient detection of neuronal growth cones. Necessarily, this focus on a few model systems excludes many other important cell types, which perform chemotaxis in response to gradients such as metastatic cancer cells (Condeelis *et al.,* 2005), mesoderm cells during vertebrate gastrulation (Dormann and Weijer, 2006), germ line cells (Kunwar *et al.,* 2006), or mammalian sperm (Eisenbach and Giojalas, 2006).

To give a representative and detailed picture of eukaryotic chemotaxis, *Dictyostelium* cells and mammalian neutrophils are particularly suited, because they have been extensively studied in this regard and can serve as an exemplary model for chemotaxis of other eukaryotic cell types (Dormann and Weijer, 2006; Williams *et al.,* 2006). Although evolutionary distant, *Dictyostelium* cells and neutrophils are morphologically similar and share common pathways for gradient detection (Charest and Firtel, 2006). *Dictyostelium* cells chemotax toward gradients of cAMP, meaning they move up a gradient of a single chemoattractant. Neutrophils are attracted by gradients of different chemotactic factors, which are released in the case of infection or inflammation. Both cell types detect external gradients of a chemoattractant with high sensitivity and perform a strong internal signal amplification by means of feedback loops comprising phosphatidylinositol-3 kinase (PI3K) and its catalytic product, phosphatidylinositide 3,4,5-trisphosphate (PIP3). *Dictyostelium* cells and neutrophils slightly differ, however, regarding the role of the cytoskeleton during gradient detection.

Compared to amoeboid cells, fibroblasts are larger and have a different, more complex cytoskeletal architecture resembling the one in neuronal growth cones. Fibroblasts can sense and migrate up an attractant gradient of platelet-derived growth factor (PDGF) but are much slower than neutrophils. The intracellular signaling underlying gradient detection in fibroblasts shares key components with *Dictyostelium* and neutrophils. However, fibroblasts lack important feedback mechanisms and therefore have a low sensitivity and a strong dependence on the absolute PDGF concentration

when navigating in attractant gradients (Schneider and Haugh, 2005). Interestingly, they employ local protein synthesis at the leading edge to promote directional growth (Shestakova *et al.*, 2001), a principle that is also observed in growth cone guidance (Piper and Holt, 2004).

At first view, neuronal growth cone guidance differs substantially from cell migration. Growth cones advance and navigate relatively independently from the neuronal cell body. Morphologically, growth cones are already polarized and have an intrinsic bias between the axon shaft and the protruding peripheral domain. In the nervous system, growth cones face attractive as well as repulsive gradients and respond to different classes of guidance factors, which can substantially differ in their downstream signaling. Nonetheless, many general features of eukaryotic gradient detection are conserved between migrating cells and growth cones. It is the aim of this review to point out these features and establish common ground between two related fields of research.

2. Mechanisms of Gradient Detection in Selected Eukaryotic Cell Types

2.1. Chemotaxis of *Dictyostelium*

Dictyostelium cells detect and migrate up gradients of cAMP. In a cAMP gradient, the cells adopt a strong internal signaling polarity, which arises from localized activities of phosphatidylinositol-3 kinase (PI3K) and the PI3-phosphatase PTEN as well as a corresponding internal gradient of phosphatidylinositide 3,4,5-tris-phosphate (PIP3) along the cell membrane. As a result, the cytoskeleton is rearranged, morphological polarization becomes apparent, and the cell starts to move in the direction of the gradient source based on actin polymerization and myosin-mediated contraction.

2.1.1. Chemotaxis of *Dictyostelium* relies on spatial gradient sensing

The myxameba *Dictyostelium discoideum* is unicellular in its vegetative cycle. When the food supply is exhausted, the cells start to aggregate to form a multicellular structure (pseudoplasmodium). The aggregation is triggered by cAMP, which is secreted by the cells themselves and functions as a chemoattractant. Thousands of individual cells thus move up a gradient of cAMP and converge at a central point (Strmecki *et al.*, 2005).

The response of *Dictyostelium* cells to cAMP can be easily studied *in vitro* with soluble gradients emanating from micropipettes and has served as an excellent model system for eukaryotic chemotaxis and gradient detection (Van Haastert and Devreotes, 2004). From early experiments, it was deduced that *Dictyostelium* cells employ a spatial gradient-sensing mechanism

because they are able to migrate up a gradient on a more or less straight line. However, because cells experience an overall increase of the surrounding cAMP concentration when they move up a gradient, one could still speculate about a temporal gradient-sensing mechanism. Tani and Naitoh (1999) carefully tested the controversial concepts about spatial and temporal gradient sensing. Strong support for a spatial-sensing mechanism comes from the fact that cells still move up a cAMP gradient when the overall concentration of cAMP decreases over time (Fig. 1.1).

A *Dictyostelium* cell can measure about 1% of concentration difference over its total length (~10–20 μm) in a spatial cAMP gradient (Mato *et al.*, 1975) and move with a speed up to 20 μm/min (Swanson and Taylor, 1982). The efficiency of gradient detection depends on both gradient slope and absolute cAMP concentration. In cAMP gradients with the same slope, chemotaxis is optimal for an intermediate gradient midpoint concentration, that is, there is a biphasic dependence of the efficiency of chemotaxis on the absolute cAMP concentration. Very high or very low cAMP concentrations are suboptimal for chemotaxis even when the gradient slope is optimal. In gradients with the same (optimal) gradient midpoint concentration, cells chemotax more efficiently in steeper gradients. A shallow gradient with

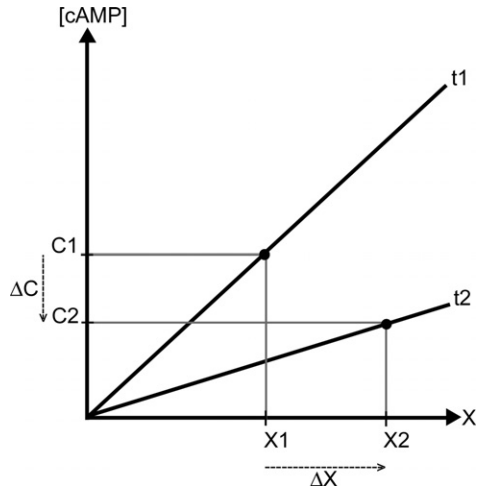

Figure 1.1 Spatial gradient sensing in *Dictyostelium*. At time point t1, the cell is at position x1 on its way up the gradient and is surrounded by the cAMP concentration c1. It faces a steep gradient along its length. Over time, the steepness of the gradient is decreasing while the cell still continuously encounters higher cAMP concentration at its leading edge than at its trailing edge. At time t2, after having crawled a distance Δx up the gradient, the cell is surrounded by the cAMP concentration c2, which is smaller than c1, although x2 is closer to the cAMP source than x1. Successful chemotaxis in the direction of the gradient in the sketched scenario is only possible for cells employing a spatial gradient sensing mechanism.

optimal midpoint concentrations therefore possibly elicits a weaker chemotactic response than a steeper gradient with suboptimal gradient midpoint concentration. This means the cell measures relative and not absolute differences in concentration (Fisher *et al.*, 1989).

2.1.2. Establishment of internal polarity: Feedback loops lead to signal amplification

For spatial gradient sensing, the cell has to translate the external gradient into an internal signaling gradient. Corresponding to the direction of the external gradient, polarity is adopted. In chemotaxing *Dictyostelium* cells, this polarity is morphologically evident. A pseudopod is formed at the leading edge, and a uropod marks the trailing edge. Once polarized, the cell shows an asymmetry in sensitivity toward cAMP: The front is more sensitive than the rear (Swanson and Taylor, 1982). The strong and persistent morphological polarization and the correlated bias in cAMP sensitivity suggest the external concentration gradient is internally amplified.

When does this amplification happen? Extensive studies have revealed the intracellular signaling cascade downstream of cAMP during chemotaxis. In short, cAMP binds to the 7-transmembrane-spanning receptor cAR1, which activates heterotrimeric G proteins. The G$\beta\gamma$ subunit activates the Ras family of small G proteins, which subsequently recruits phosphatidylinositol-3 kinases (PI3K) to the plasma membrane. PI3K converts membrane-residing phosphatidylinositide 4,5-bis-phosphate (PIP2) to phosphatidylinositide 3,4,5-tris-phosphate (PIP3). The produced PIP3 mediates the membrane translocation and activation of a number of proteins containing PIP3-binding plextrin-homology (PH) domains. Among these are protein kinase B (PKB/Akt) and guanine nucleotide exchange factors for Rac (Rac GEFs). The latter are main regulators of the cytoskeletal rearrangements required for cell migration (Affolter and Weijer, 2005). Studies with a functional cAR1-GFP construct revealed that the receptor is uniformly distributed over the cell surface in polarized cells migrating in a cAMP gradient (Xiao *et al.*, 1997). Measurement of G protein activation by FRET further shows that the extent of G protein activation in different regions of the cell reflects the extracellular cAMP concentration of the cAMP gradient faced by the cell (Jin *et al.*, 2000; Xu *et al.*, 2005b). Regarding receptor occupancy and G protein activation, the internal signal output is thus proportional to the input, and there is no strong asymmetric distribution of signaling molecules. The gradient amplification which accounts for a strong and persistent morphological polarization and a bias in sensitivity therefore has to occur downstream of G proteins. Using a GFP fusion of the PH-domain containing protein cytosolic regulator of adenyl cyclase (CRAC) as a sensor for PIP3, Parent *et al.* (1998) showed that PIP3 accumulates in a highly polarized fashion at the cell front and exclusively marks the site of pseudopod formation in cells facing a cAMP gradient. The internal PIP3

gradient is up to sevenfold steeper than the external cAMP gradient (Janetopoulos *et al.,* 2004; Xu *et al.,* 2005b). At the level of PIP3, small concentration differences of the external signal are translated into strong cell internal signaling polarity.

How is this signal amplification achieved? As mentioned earlier, PIP3 is produced by PI3K. *Dictyostelium* has three PI3Ks, of which PI3K1 and PI3K2 are important for chemotaxis. Both PI3K1 and PI3K2 translocate rapidly to the cell membrane after a cAMP stimulus and are localized at the leading edge of chemotaxing cells. PI3K signaling is antagonized by the PI3-phosphatase PTEN, which breaks down PIP3 to PIP2. *Dictyostelium* cells lacking PTEN have defects in chemotaxis toward cAMP due to a rapid, erratic extension of multiple pseudopods and reduced polarity. In resting cells, a small portion of PTEN is membrane associated. Membrane association and function of PTEN are dependent on its PIP2 binding site. In polarizing cells, PTEN dissociates along the leading edge into the cytosol and accumulates at the cell rear: Its distribution becomes reciprocal to the one of PI3K. The inverse PTEN gradient shows little amplification with respect to the external cAMP gradient. However, PI3K and PTEN distributions taken together account for nearly all observed amplification on the level of PIP3 (i.e., PIP3 levels parallel the PI3K/PTEN ratio). PI3K/PTEN/PIP3 participate in a feedback loop system. At the leading edge, recruitment of PI3K enhances local PIP3 production and the simultaneous decrease of PIP2, which is a decrease in PTEN binding sites. This causes PTEN to translocate to the rear. At the rear, PTEN lowers the PIP3 levels while increasing PIP2, creating in this way its own membrane-binding sites. The opposite enzyme activity of PI3K and PTEN sharpen the internal PIP3 gradient and stabilize the signaling polarity of the cell (Fig. 1.2). cAMP molecules binding at the cell rear only lead to a very attenuated signal because high levels of PTEN antagonize PI3K signaling (Funamoto *et al.,* 2002; Iijima and Devreotes, 2002; Janetopoulos *et al.,* 2004).

The signal amplification arising from self-enhanced segregation of PI3K and PTEN potentiates the efficiency of gradient detection. It leads to persistent chemotaxis and makes the system robust for small concentration fluctuations of the attractant cAMP gradient. A bias in sensitivity explains why cells tend to maintain their once-established polarity even when the direction of the gradient is altered. As observed by Swanson and Taylor (1982), chemotaxing cells respond with an L-shaped turn of the pseudopod instead of reorganizing their morphological polarization, when the cAMP-loaded microneedle producing the gradient is shifted to the side of the cell.

A characteristic of systems containing feedback loops is their self-organizing property. Small stochastic fluctuations in the environment can lead to stable changes of the system. The PI3K/PTEN/PIP3 feedback loop may therefore explain the transient, spontaneous polarization and random migration of *Dictyostelium* cells in the presence of a uniform

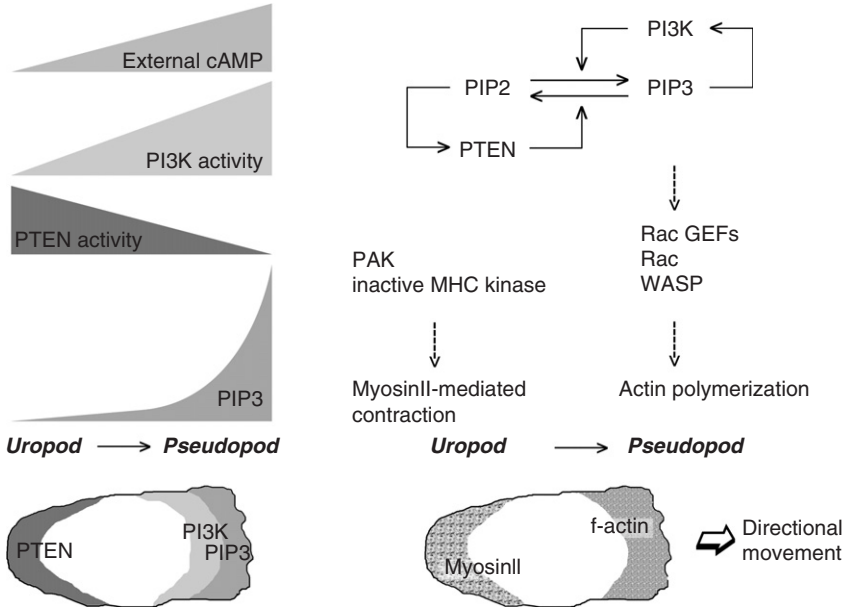

Figure 1.2 Signal amplification in *Dictyostelium* gradient sensing. An external cAMP gradient triggers a slightly amplified gradient of PI3K signaling and a nonamplified, inverse gradient of PTEN signaling inside the cell. A feedback loop comprising PI3K and PTEN builds up a highly amplified gradient of PIP3 at the cell membrane. PIP3 recruits PH domain containing proteins such as Rac GEFs to the membrane at the pseudopod. Activation of Rac and cytoskeletal regulators such as WASP drives actin polymerization and finally leads to directional movement. At the uropod, the inactivation of MHC by PAK enhances myosinII-mediated contraction.

cAMP stimulus. Cells treated with a uniform dose of cAMP show first a rapid (1–10 s) and uniform rise of PIP3 at the cell membrane, which is associated with a peak of unbiased actin polymerization. Subsequently, between 30–60 s after the stimulus, there is a lower second peak of PIP3 accumulation and actin polymerization, which is localized at the sites of randomly emerging pseudopods. For a certain period of time, cells move around in the absence of a cAMP gradient (Chen *et al.*, 2003). Postma *et al.* (2003) further analyzed how the uniform accumulation of PIP3 at the membrane after a uniform cAMP stimulus subsequently organizes in small and distinctly restricted patches. In contrast to the PIP3 accumulation in a gradient, where PIP3 always rises at the gradient near side, PIP3 patches appear randomly at the cell membrane after a uniform cAMP stimulation. At the site of these PIP3 signaling patches, pseudopods are likely to form (Fig. 1.3). After low cAMP doses, the probability of the patches decreases, but their size, intensity, and lifetime is the same as after high cAMP doses. The signal output is partially decoupled from the input. This observation

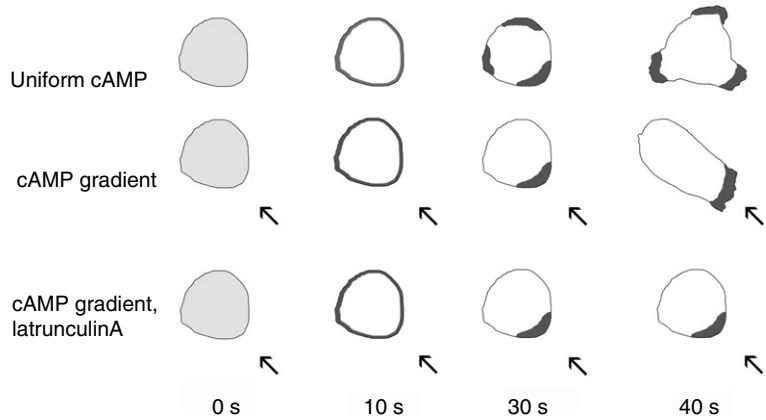

Figure 1.3 Polarization and gradient sensing in *Dictyostelium*. After a uniform dose of cAMP, a GFP-PH domain sensor for PIP3 (shown in gray) is rapidly recruited from the cytoplasm to the cell membrane, indicating a first phase of uniform increase of PIP3 at the cell membrane (10 s). After approximately 30 s, the PIP3 signal has segregated into distinct patches, which precede the formation of pseudopods. In a gradient of cAMP (the source of cAMP is indicated by an arrow), only one PIP3 signaling patch forms after the first uniform rise of PIP3 at the membrane. It points at the uphill direction of the gradient and marks the side of the pseudopod. Cells treated with latrunculinA, which inhibits actin polymerization, are rounded up and cannot form pseudopods, meaning they cannot morphologically polarize. However, the sequence of PIP3 accumulation and polarization at the membrane in response to a cAMP gradient is the same as in untreated cells, indicating gradient sensing precedes morphological polarization.

strongly suggests self-organizing properties of the patches (Postma *et al.*, 2004). In accordance with the notion that this self-organization emerges from the PI3K/PTEN feedback loop, Chen *et al.* (2003) found that abrogation of either PI3K or PTEN affects the localized PIP3 accumulation after a uniform cAMP stimulus.

2.1.3. Cytoskeletal readout: Morphological polarization is not required for gradient detection

Morphological polarization and directed movement during chemotaxis are performed by the cytoskeleton. The pseudopod protrudes through localized actin polymerization, whereas the uropod contracts in a myosinII-dependent fashion, thus enabling the cell to move forward. Actin polymerization at the leading edge is controlled among others by *Dictyostelium* Rac (Chung *et al.*, 2000) and the PIP3 binding adaptor Wiskott-Aldrich syndrome protein (WASP) (Myers *et al.*, 2005). In cells facing a cAMP gradient, myosinII translocates to the cell rear and myosin heavy chain (MHC) kinase to the leading edge. Active MHC kinase at the leading edge is thought to phosphorylate cortical MHC, causing the disassembly of myosinII filaments,

which subsequently reassemble at the cell rear (Rubin and Ravid, 2002). At the cell rear, MHC kinase is inhibited by the ser/thr kinase PAKa. PAKa is activated by Akt after a cAMP stimulus. Although the level of Akt activation is highest at the cell front, active PAKa is predominantly found at the cell rear, where it colocalizes with myosinII. So far, it is not completely understood how activated PAKa translocates to the cell rear (Chung and Firtel, 1999; Chung *et al.,* 2001). Knockout or inhibition of the mentioned cytoskeletal regulators was shown to impair morphological polarization and chemotaxis in *Dictyostelium* cells.

However, one has to be careful to distinguish the difference among gradient detection, morphological polarization, and directed movement (Devreotes and Janetopoulos, 2003). Gradient detection depends on the translation of an external gradient to an amplified internal gradient. This signaling asymmetry leads to an asymmetry in cell shape accomplished by the cytoskeleton. Morphological polarization may be only a passive readout and not a requirement for gradient detection, so that the lack of morphological polarization does not necessarily suggest failing gradient detection. Similarly, the inability to move in a given direction tells nothing about the capability to sense this direction. Signaling events generally required for cell motility are likely to disrupt directional movement, although they may not be required specifically for sensing of direction or maintenance of directionality during movement.

Indeed, experiments with latrunculinA have shown that morphologically unpolarized and immobile *Dictyostelium* cells can still sense a cAMP gradient. LatrunculinA blocks actin polymerization and causes cells to round up. The formation of pseudopods and the general motility of the cells are inhibited. However, in a gradient of cAMP, PIP3 still accumulates in a polarized fashion at the gradient near side of the cell (see Fig. 1.3). The internal PIP3 gradient is an amplified copy of the external cAMP gradient and the extent of amplification is only a little smaller in unpolarized latrunculinA treated cells than in untreated cells (Janetopoulos *et al.,* 2004; Parent *et al.,* 1998). The PI3K/PTEN feedback loop seems to be intact in unpolarized cells because they display the same self-organized formation of PIP3 patches after a uniform cAMP stimulus as it is seen in cells competent to polarize (Postma *et al.,* 2004).

Taken together, gradient sensing and internal signal amplification in *Dictyostelium* is relatively independent of the cytoskeletal rearrangements, which are mainly required for morphological polarization and cell motility.

2.2. Chemotaxis of mammalian neutrophils

Gradient detection in mammalian neutrophils is substantially similar to the one in *Dictyostelium* cells. Neutrophils attracted by gradients of chemotactic factors translate the external gradients into amplified internal gradients of

PI3K activity and PIP3 accumulation. The feedback loops leading to signal amplification are comparable to *Dictyostelium* feedback loops but differ somewhat in their orchestration. Furthermore, neutrophils are specialized to navigate in several superimposed gradients of different chemotactic factors and have developed mechanisms to integrate a complex signal environment.

2.2.1. Gradients guide neutrophils to sites of infection

In the mammalian immune system, an elaborate network of chemotactic factors guides neutrophil granulocytes (polymorphonuclear leukocytes) to sites of infection and inflammation, where they engulf invading bacteria, dead cells, and foreign particulate matter by phagocytosis. Chemotactic factors are either bacterial (e.g., formyl-MetLeuPhe [fMLP]) or host derived (e.g., interleukin 8 [IL-8], leukotriene B4 [LTB4], complement factor C5a).

Chemotaxis of neutrophils toward sources of chemotactic factors has been extensively studied *in vitro* with either primary neutrophils or differentiated human promyelocytic leukemia cells (HL-60 cells), which look and behave like neutrophils. Although evolutionarily distant, mammalian neutrophils and *Dictyostelium* cells share many signaling mechanisms in gradient detection.

When exposed to a uniform dose of chemotactic factor neutrophils morphologically polarize and develop a leading pseudopod and a trailing uropod. They start random migration (chemokinesis) with typical amoeboid movement. Ranking among the fastest moving mammalian cells known so far, neutrophils can reach speeds up to 20 μm/min, which is about the same velocity reached by aggregating *Dictyostelium* cells (Niggli, 2003). Like other chemotaxing eukaryotic cells, neutrophils employ a spatial mechanism of gradient sensing (Zigmond, 1974). In a concentration gradient, they quickly orient their pseudopod toward the source of a chemotactic factor and initiate chemotaxis. Neutrophil orientation in a gradient depends on both steepness of the gradient and the mean concentration of the chemotactic factor. Steeper gradients orient cells more efficiently. For all gradient slopes, orientation is optimal for a medium mean concentration of the chemotactic factor. When the overall concentration of a chemotactic factor gets too high, cellular motility is inhibited. In optimal gradients, neutrophils can detect down to 1% difference in concentration of a chemotactic factor over their length ($\approx$10 μm) (Lin *et al.*, 2004; Zigmond, 1977).

Similar to *Dictyostelium* cells, morphologically polarized neutrophils also show a polarity in sensitivity with a more responsive leading edge. Due to this asymmetry in sensitivity, neutrophils following a gradient frequently make U-turns when the direction of the gradient is reversed instead of reversing their polarity (Zigmond *et al.*, 1981).

Each neutrophil can respond to multiple chemotactic factors and many different chemotactic factors are simultaneously released from a single site

of inflammation. *In vivo,* neutrophils thus have to navigate in a complex environment of superimposed, graded distributions of chemotactic factors. To fulfill their role efficiently, they have to assess a combination of chemotactic factors in a meaningful way. Among chemotactic factors, there is a dominance of so-called "end target chemotactic factors" (fMLP and C5a) over "intermediary endogenous chemotactic factors" (IL-8 and LTB4). Neutrophils can migrate down the concentration gradient of an intermediary chemotactic factor by responding to a distant end target chemotactic factor, but not vice versa. This is true for a wide range of concentration combinations and may be an important mechanism to respond to several sites of inflammation in a sequential way without being trapped in the middle of two opposed gradients. On the other hand, the hierarchy of chemotactic factors can become fatal for septic patients, which have increased levels of fMLP and other dominant chemotactic factors. The concentration of dominant chemotactic factors is far above the optimal midpoint concentration for gradient detection, and neutrophils fail to detect sites of infection because they are inhibited in chemotaxis. In the normal situation, however, neutrophils can very well migrate beyond a saturating concentration of one chemotactic factor (which alone would stop the cell) in response to a second gradient of a different chemotactic factor. Such multistep navigation allows neutrophils to be attracted over a greater distance and arrive at multiple target areas dependent on their expression pattern of receptors for chemotactic factors (Foxman *et al.,* 1997, 1999). The sophisticated integration of several chemotactic signals is based on the resistance of certain, but not all, receptors for chemotactic factors to heterologous desensitization (Richardson *et al.,* 1995) and the employment of different signaling pathways in response to different chemotactic factors (Heit *et al.,* 2002).

2.2.2. PI3 kinase/PIP3 signaling builds up internal polarity

All chemotactic factors involved in neutrophil chemotaxis bind to G protein-coupled receptors (GPCRs). As shown for the receptor for Ca5 (Ca5R), chemotactic factor receptors are homogenously distributed in the plasma membrane of unstimulated as well as gradient stimulated, polarized chemotaxing neutrophils (Servant *et al.,* 1999). Like in *Dictyostelium* cells, the internal amplification of the external concentration gradient takes place on the level of PIP3 accumulation at the cell membrane and can be visualized by the recruitment of PH-domain containing, PIP3 binding sensors such as a construct of the PH-domain of Akt and GFP (PHAkt-GFP). PHAkt-GFP gets locally recruited to the pseudopod of neutrophils stimulated with an external gradient of a chemotactic factor and forms an intracellular gradient. This PHAkt-GFP gradient is approximately sixfold steeper than the external concentration gradient (Servant *et al.,* 2000), which is about the same amount of amplification observed for *Dictyostelium* cells.

Is this signal amplification achieved by the same mechanisms that act in *Dictyostelium?* The relevant isoform of PI3K, which is expressed in neutrophils and signals in chemotaxis is PI3Kγ. Neutrophils from PI3Kγ null mice fail to produce PIP3 after stimulation with various chemotactic factors. The chemotaxis of neutrophils lacking PI3Kγ is impaired because the cells are less motile and cannot stabilize and maintain their leading edge (Hannigan *et al.,* 2002; Hirsch *et al.,* 2000; Li *et al.,* 2000; Sasaki *et al.,* 2000).

Interestingly, PTEN, which is crucial for gradient detection in *Dictyostelium*, does not seem to play a major role in neutrophils. Antibody stainings as well as different PTEN-GFP constructs failed to reveal an asymmetric PTEN localization in polarized and chemotaxing neutrophils (Lacalle *et al.,* 2004; Xu *et al.,* 2003). Recent research strongly suggests that the SH2 domain-containing inositol 5-phosphatase 1 (SHIP1) fulfills the role of breaking down PIP3 and restricting PI3K activity to the pseudopod in neutrophils. Neutrophils lacking SHIP1 have a phenotype reminiscent of *Dictyostelium* cells lacking PTEN, suggesting that SHIP1 and PTEN have similar functions in gradient detection and polarization in the two different cell types (Nishio *et al.,* 2007).

2.2.3. Feedback loops involving f-actin and Rho amplify the signaling asymmetry

Neutrophils respond to chemotactic factors with rapid polymerization of actin. Subsequently, the filamentous actin (f-actin) partially depolymerizes and redistributes focally into the emerging pseudopod while the rounded cells acquire a polarized morphology (Howard and Oresajo, 1985). The microtubule network rearranges in a way that the majority of microtubules gets oriented toward the uropod and is excluded from the actin-rich pseudopod. This rearrangement depends on an intact actin cytoskeleton and activated myosinII. It takes place without local microtubule disassembly (Eddy *et al.,* 2002). The microtubule network was found to stabilize the directionality during neutrophil chemotaxis (Xu *et al.,* 2005a).

Approximately 50% of the neutrophils that get morphologically polarized in response to a uniform dose of a chemotactic factor also display an asymmetric recruitment of the PIP3 sensor PHAkt-GFP to the pseudopod, supporting the notion that they have an intrinsic capacity to build up a signaling polarity in the absence of a polarized external stimulus (Servant *et al.,* 2000). Indeed a uniform dose of membrane permeable PIP3 can mimic a chemotactic factor and induce its own asymmetric accumulation as well as polarized polymerization of actin. PIP3 fails to induce polarization in the presence of PI3K or Rho family kinase inhibitors. This indicates that PI3K signals downstream as well as upstream of PIP3 and thus participates in a positive feedback loop which also includes members of the Rho GTPases (Niggli, 2000; Weiner *et al.,* 2002).

Which members of the Rho family kinases are involved and what are their individual roles? Trimeric G proteins downstream of the chemotactic factor receptors activate Rac via Gi and RhoA via G12/G13. Activation of Rac has been shown to mediate the so-called "frontness signal," that is, PIP3 accumulation, actin polymerization, and the formation of pseudopods at the leading edge. The stabilization of a single pseudopod depends on the additional activation of cdc42 (Srinivasan *et al.,* 2003). The effect of Rac on the actin cytoskeleton is mediated among others via WAVE (Wiskott-Aldrich syndrome protein family Verprolin-homologous protein) protein complexes and other leading-edge complexes scaffolded by hematopoietic protein-1 (Hem-1) (Weiner *et al.,* 2006). Activated RhoA, on the other hand, translocates to the trailing edge and sets up a "backness signal," which is characterized by activation of Rho kinase (Rock), accumulation of myosinII, and the formation of a uropod (Xu *et al.,* 2003). Frontness (Rac) and backness (RhoA) signals mutually inhibit each other, enhancing their own polarity. The activation of RhoA at the back depends on PIP3-induced activation of cdc42 at the leading edge. The molecular mechanisms, which connect cdc42 to RhoA across the cell's diameter are not yet understood and seem to require intermediate effectors (Van Keymeulen *et al.,* 2006). The exclusion of RhoA signaling from the pseudopod and its segregation to the uropod seem to be partially monitored by the actin cytoskeleton. Actin polymerization suppresses and localizes RhoA activity by a yet-unknown mechanism, corroborating the notion that actin dynamics are not only a passive readout of the system, but also are rather actively integrated into the feedback loop (Wong *et al.,* 2006). Further evidence indicates that the PIP3 feedback loop in neutrophils depends on f-actin. Inhibition of actin polymerization or depolymerization by latrunculinA or jasplakinolide, respectively, leads to a markedly attenuated and transient translocation of PH-sensor constructs to the membrane, indicating that PIP3 accumulation fails to get stabilized at the leading edge in neutrophils with disturbed f-actin organization (Wang *et al.,* 2002).

In summary, signaling pathways during neutrophil chemotaxis (Fig. 1.4) include positive feedback loops between PIP3 accumulation, the activation of Rac, and actin polymerization at the pseudopod. At the uropod, RhoA activity enhances myosinII-mediated contraction.

2.3. Chemotaxis of mammalian fibroblasts

In comparison to amoeboid cells, fibroblasts chemotaxing in attractive gradients of PDGF do not employ a sophisticated and highly specialized signaling network of positive feedback loops. The amplification of the external gradient is mainly based on morphological polarization. In addition to PI3K signaling, localized protein synthesis of β-actin, a mechanism not observed in *Dictyostelium* or neutrophils, affects directional movement in the gradient.

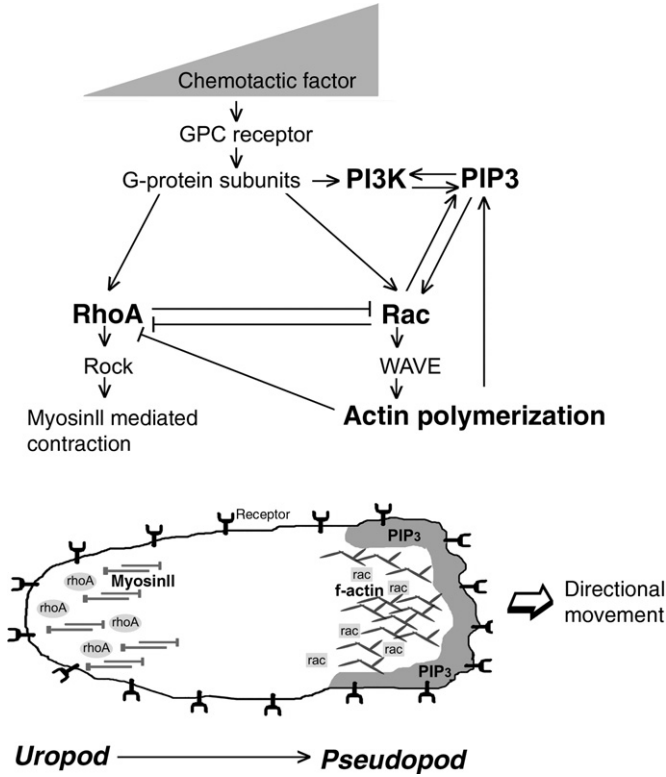

Figure 1.4 Signal transduction during neutrophil gradient sensing. The binding of a chemotactic factor to its G protein-coupled receptor leads to the activation of PI3K and Rho GTPases. At the pseudopod, signaling is dominated by positive feedback loops of PI3K, PIP3, Rac, and f-actin, which mutually enhance their activities. Rac signaling at the pseudopod inhibits RhoA signaling, which is thus restricted to the uropod and there drives myosinII-mediated contraction through activation of Rock.

2.3.1. A gradient of PDGF attracts fibroblasts during wound healing

One step in the complex process of wound healing and tissue repair is the migration of fibroblasts toward the wound. This migration is triggered by PDGF, which is released at the site of injury and forms a concentration gradient in the surrounding tissue (Deuel and Kawahara, 1991).

Like *Dictyostelium* cells or neutrophils, fibroblasts have to detect an external concentration gradient and respond to it with directed locomotion. With respect to morphology and cellular architecture fibroblasts differ markedly from the cell types discussed previously. They are larger (50–250 μm compared to 10–20 μm for amoeboid cells) and migrate at a much lower speed (approximately 2 μm/min compared to 20 μm/min). Fibroblast

polarity and motility depends not only on the asymmetrical distribution of f-actin and myosinII, but also on the microtubule network and local substrate adhesion (Kole *et al.*, 2005; Small *et al.*, 2002; Vasiliev, 1991).

In short, forward migration is driven by localized actin polymerization, which leads to the extension of broad and flattened lamellipodia in the direction of movement. During the protrusion of the leading edge, so-called ''ruffles'' are formed by the bending of the cell membrane (Abercrombie *et al.*, 1970). Addition of PDGF to fibroblasts *in vitro* causes rapid lamellipodial actin polymerization and the appearance of membrane ruffles (Mellström *et al.*, 1988). In the presence of a PDGF gradient, fibroblasts chemotax toward the PDGF source. This chemotactic response is optimal for an intermediate mean PDGF concentration and is inhibited at high concentrations (Seppä *et al.*, 1982).

The PDGF signaling cascade is as follows: PDGF binds to the PDGF receptor (PDGFR), a receptor tyrosine kinase (RTK). Phosphorylated PDGFR binds and activates among others PI3K, phosphatidylinositol-specific phospholipase C-γ (PLC-γ), and Ras-GTPase–activating protein (ras GAP) (Kazlauskas and Cooper, 1990; Kundra *et al.*, 1994). Although PDGF binds to a different receptor type (receptor tyrosine kinase) than the attractants mediating chemotaxis in *Dictyostelium* and neutrophils (GPCRs), it activates the same downstream effector PI3K.

2.3.2. PI3 kinase/PIP3 and Rho GTPase signaling transduces the external gradient

PI3K/PIP3 signaling plays a well-established role in fibroblast gradient sensing. The response of fibroblasts toward PDGF is abolished in the presence of PI3K inhibitors (Derman *et al.*, 1997). A shallow external PDGF gradient triggers a steeper PIP3 gradient in the fibroblast membrane, which can be visualized by a PHAkt-GFP sensor. The region with the highest GFP-AktPH signal exhibits lamellipodia spreading toward the PDGF source (Haugh *et al.*, 2000). The effect of PDGF on fibroblasts can be mimicked by exogenous addition of membrane permeant PIP3. Interestingly, PIP3 does not activate or enhance PI3K signaling like it was shown in *Dictyostelium* cells or neutrophils. In fibroblasts, PIP3 exclusively signals downstream of PI3K and does not seem to be integrated in a positive feedback loop (Derman *et al.*, 1997). In this context, it is noteworthy that different PI3K isoforms are activated by GPCRs (i.e., the receptors for chemotactic factors in neutrophils) and by PDGFR (Vanhaesebroeck *et al.*, 2001). This could explain the differences in the PIP3 signaling cascade in fibroblasts and neutrophils.

The opponent of PI3K, PTEN, is certainly involved in PDGF-induced chemotaxis. However, there is little data about PTEN distribution in chemotaxing fibroblasts and its participation in the development and the maintenance of PIP3 polarity. In unstimulated cells, PTEN is uniformly

distributed (Tamura *et al.,* 1998). Upon PDGF stimulation, PTEN is recruited to the cell membrane by PDGFR via the adaptor protein NHERF (Na^+/H^+ exchanger regulatory factor) into a ternary complex. Fibroblast with disrupted PTEN signaling display prolonged PI3K pathway activation, increased cell motility, and enhanced cytoskeletal rearrangements after PDGF stimulation (Liliental *et al.,* 2000; Takahashi *et al.,* 2006). These findings suggest fibroblast PTEN antagonizes PI3K signaling. It may not exclusively localize to the cell rear like in *Dictyostelium* but rather may have the same distribution as PDGFR.

PIP3 produced by PI3K activates the Rho GTPases Rac and cdc42. Activated Rac stimulates actin polymerization, the characteristic membrane ruffling, and lamellipodia formation in fibroblasts. When microinjected, Rac mimics the effects of PDGF. The action of Rac in fibroblasts is exclusively downstream of PIP3 because the Rac-induced cytoskeletal rearrangements are not prevented by PI3K inhibitors (Nobes *et al.,* 1995). In migrating fibroblasts, a gradient of Rac activation and colocalized actin polymerization peaks near the leading edge, meaning it is correlated with the direction of cell movement (Kraynov *et al.,* 2000). The increase of actin polymerization during ruffle formation is triggered by the Rac-dependent activation of WAVE proteins, which activate, in turn, actin-related protein 2/3 (Arp2/3) complex (Miki *et al.,* 1998; Suetsugu *et al.,* 2003). Rac-null mouse fibroblasts have a dramatically changed morphology. Whereas wild-type cells respond to PDGF with the formation of f-actin rich dorsal and lateral ruffles, Rac-null cells lack this response and only generate small filopodia-like protrusions. However, they show activation of PI3K and PIP3 accumulation to the same extent as wild-type cells. Mutant cells still chemotax in response to PDGF, but they do not translocate via lamellipodia and ruffles, but rather via finger-like protrusions, and their average velocity is reduced. In the absence of Rac, a PDGF gradient can thus still be correctly sensed. Rac is therefore required for the formation of lamellipodia and ruffles mediating efficient cell translocation, but not for gradient detection itself (Vidali *et al.,* 2006). Interestingly, the spatiotemporal dynamics of RhoA activity differ in randomly migrating fibroblasts and fibroblasts chemotaxing toward PDGF. Pertz *et al.* (2006) used a recombinant sensor with intramolecular FRET that responds to RhoA activation. In randomly migrating cells, RhoA activity is concentrated in a sharp band at the edge of protrusions and in peripheral ruffles, but it is very low in the cell body. In cells migrating toward a gradient of PDGF, however, the PDGF-induced protrusions display low RhoA activity. This can be explained by the fact that PDGF activates Rac and activated Rac downregulates RhoA activity.

Taken together, spatial gradient detection in fibroblasts is mediated by PI3K/PIP3, but a positive feedback loop involving PI3K and/or downstream Rho family kinases is apparently missing.

As pointed out by Schneider and Haugh (2005), the absence of positive feedback loops leads to decreased sensitivity and stronger dependence on the midpoint PDGF concentration in a gradient. According to a simple model, fibroblast gradient detection differs depending on the PDGF concentration. At low PDGF concentrations, the gradient sensing is absolute, meaning the level of PI3K activation is directly proportional to the receptor activation because cytosolic PI3K is not depleted. At midpoint PDGF concentrations, the gradient sensing is relative; front and rear of the cell have to share the common PI3K pool according to their receptor activation. At high PDGF concentrations, gradient sensing fails because all receptors are saturated: The PI3K activity at the leading edge is actually lower than in nonsaturating concentrations. Although the classical PIP3 feedback loop is missing, the morphological polarity of the cell, namely, protruding membrane structures at the leading edge may provide an enhancement of PI3K signaling. Once properly aligned with the gradient, the cell displays an intrinsic bias—it is more sensitive at the leading edge.

To overcome the strong dependence on the midpoint concentration of PDGF gradients, fibroblasts may partially decay the encountered PDGF. Haugh (2006) proposes a model in which PDGFR activation is coupled to PDGF endocytosis and intracellular proteolysis. PDGF consumption by the migrating cells might thus allow them to maintain an optimal gradient during the invasion of a dermal wound, where PDGF concentrations are likely to span a large scale.

2.3.3. Local protein translation at the leading edge mediates directional movement

In early studies, inhibition of protein translation was reported to inhibit fibroblasts chemotaxis (Seppä *et al.,* 1982). More recent data show that this inhibition is not a rather unspecific global effect, but point out the importance of localized and controlled protein translation of defined mRNAs at the leading edge of migrating fibroblasts.

Most importantly, β-actin mRNA gets located to the protruding pseudopod via a 3′UTR zip-code sequence. The translocation of β-actin mRNA to the leading edge correlates with the magnitude and direction of cell translocation. When the correct localization of β-mRNA is blocked, cells have collapsed lamellipodia and no leading edge; they lose their morphological polarity (Kislauskis *et al.,* 1994, 1997). More precise analysis shows that directionality and persistence of migration are decreased in fibroblasts with delocalized β-actin mRNA without a decrease in the rate of locomotion. This suggests that mRNA localization is not required in general for cell migration, but rather for directional movement (Shestakova *et al.,* 2001).

The localization of β-actin mRNA requires the zip-code binding protein 1 (ZBP-1). ZBP-1 binds mRNA in the nucleus, prevents premature translation initiation, and regulates its transport. At the destination point, ZBP-1

gets phosphorylated by src, which disrupts RNA binding and activates translation. Local translation can therefore be regulated by spatial restricted activity of src (Hüttelmaier *et al.*, 2005). Notably, src is activated in fibroblasts upon PDGF stimulation. Src family kinases (SFK) can directly bind to activated PDGF receptor and phosphorylate various targets proteins involved in cytoskeletal regulation (Shah and Vincent, 2005). Based on these data, it is conceivable that ZBP-1 is phosphorylated by src upon PDGF stimulation and subsequently triggers the local translation of β-actin at the fibroblast leading edge. Newly synthesized β-actin monomers are a preferential substrate for actin polymerization and therefore establish nucleation sites. Local translation of β-actin may therefore be a more efficient mechanism for driving f-actin accumulation than the usage of the already existing pool of actin monomers in the cell (Shestakova *et al.*, 2001). Local protein translation is required for fibroblast gradient detection, because it sets up the morphological polarization, which plays a crucial role in the establishment and maintenance of the internal signaling gradient.

Taken together, morphological polarity and directional movement of fibroblasts in a PDGF gradient is the result of several signaling pathways converging at the cytoskeleton. Local protein translation as well as the activation of PI3K/PIP3 signaling promotes actin polymerization and membrane ruffling at the leading edge, whereas the trailing edge is dominated by RhoA/myosinII activation (Fig. 1.5).

2.4. Pathfinding of neuronal growth cones

Axon guidance is either attractive or repulsive, and guidance factors fall into distinct classes, such as the netrins, semaphorins, and ephrins, which differ with respect to receptors and downstream signaling (Guan and Rao, 2003). Looking at three major responses of a growth cone to a gradient, attractive turning, repulsive turning, and collapse, it becomes clear that, in part, alternative signaling pathways can execute theses responses. Depending on the guidance factor, signaling during turning or collapse differs and corresponds to different aspects of gradient detection and chemotaxis of migrating cells.

2.4.1. Growth cones respond to gradients of axon guidance cues

Gradients of axon guidance molecules are essential for wiring up the developing nervous system. For example, an attractive gradient of netrin guides vertebrate commissural axons ventrally to the floorplate in the developing spinal cord (Kennedy *et al.*, 2006). After reaching the floorplate, commissural axons cross the midline and are subsequently directed anteriorly toward the brain by a combination of an attractive Wingless 4 (Wnt4) and a repulsive sonic hedgehog (Shh) gradient (Charron and Tessier-Lavigne, 2005). A repellent gradient of slit regulates midline crossing and defines the distance of longitudinal axon tracts to the midline in *Drosophila*

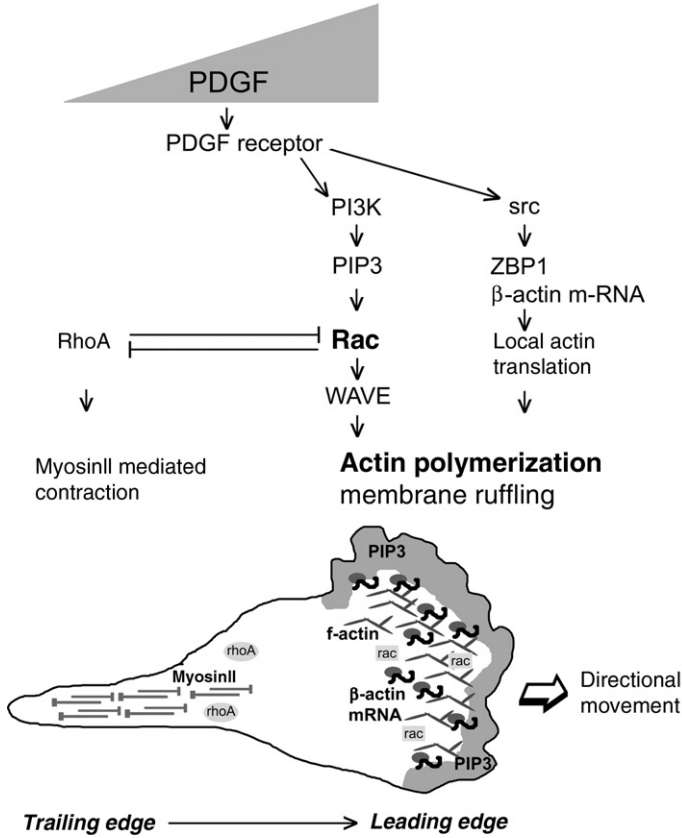

Figure 1.5 Signal transduction in fibroblasts chemotaxing toward PDGF. Downstream of the PDGF receptor, parallel pathways control actin polymerization at the leading edge. Activation of PI3K and subsequent polarized accumulation of PIP3 at the membrane lead to the dominance of Rac versus RhoA signaling. Rac activates among others WAVE and thus enhances the formation of a stable, f-actin–rich leading edge. Mutual inhibition of Rac and RhoA may sharpen the internal signaling polarity between leading and trailing edge. Additional to the action of PI3K, local phosphorylation of ZBP1, which is likely to happen via src downstream of the PDGF receptor, increases the availability of β-actin mRNA for translation. Newly translated β-actin at the leading edge supports actin polymerization.

(Simpson *et al.*, 2000). Besides providing directional information, gradients in the nervous system also confer positional information during topographic mapping. The development of the retinotopic map is controlled by the graded distribution of several different molecules, most prominently ephrinAs, which act as repellents during anterior–posterior mapping (McLaughlin and O'Leary, 2005).

Important insights into how growth cones read and respond to these *in vivo* gradients have mainly come from *in vitro* assays. Chemotactic turning of growth cones was first shown *in vitro* in response to nerve growth factor (NGF). NGF not only has a growth promoting effect, but can also orient growth direction when applied in a soluble gradient emanating from a pipette into the medium. When the pipette is repositioned, growth cones can rapidly follow (in ~10 min) the direction of the NGF source. The chemotactic response is seen over a range of concentrations but gets saturated at high NGF levels (Gundersen and Barrett, 1979; Letourneau, 1978).

A neuronal growth cone responding to a graded distribution of a guidance molecule is at a different starting point compared to the previously discussed cell types. A neuron with a growing axon is already polarized, and the growth cone has the intrinsic property to advance on a more or less straight path. Gradients of guidance factors alter this path by causing attractive or repulsive turning, by stimulating further outgrowth, eliciting sidebranching, stopping the growth cone at a specific position, or causing collapse and subsequent retraction. These manifold and partially interrelated reactions are specialized readouts of gradient detection. For the sake of clarity, we will focus on gradient detection in the context of growth cone turning, which has been studied extensively with the help of simple *in vitro* assays. In addition, we will briefly discuss gradient detection during topographic mapping.

Like in chemotaxing cells, signaling during gradient detection in growth cones has to establish a polarized distribution of intracellular effectors mirroring the external gradient and finally rearranges the cytoskeleton in a localized fashion to trigger directional movement.

For the proper establishment of the neuronal connectivity, different subsets of axons have to react differently to the same guidance factors. Moreover, reactions to attractive and repulsive gradients have to be distinguished. Depending on the context, the same growth cone may react in a different way to the same guidance factor. Signaling cascades elicited by gradients of axon guidance factors therefore have to be versatile and amenable to modulations. Consequently, different intracellular signaling pathways can elicit the same morphological and mechanistic event: a turning reaction of the growth cone. The most important of these pathways will be introduced in the following section.

The growth cone advances by means of actin polymerization, which drives the protrusion of filopodia and lamellipodia. Filopodia play an important role for the turning response: Just before turning, an increasing number of filopodia forms on the side of the growth cone facing the (attractive) gradient. Elimination of filopodia with cytochalasinB inhibits turning without inhibiting axon extension (Zheng *et al.*, 1996). Growth cone turning could thus be interpreted as a localized and biased promotion of filopodia. Alternatively, turning can be conceptualized as a localized

collapse at the side of the growth cone, which points away from the direction of the turn (Fan and Raper, 1995). Local disruption of actin bundles induces a partial growth cone collapse and subsequent repulsive turning. During this process, the local loss of f-actin causes microtubules to disappear and rearrange in the direction of the induced turn. The translocation of f-actin further leads to the appearance of asymmetric lamellipodial protrusions (Zhou *et al.*, 2002).

Both attractive and repulsive external gradients have to elicit intracellular signaling gradients. Theoretically, one can postulate two extreme possibilities: (1) The internal signaling gradients mediating attraction and repulsion differ completely with respect to the components, meaning they activate actin polymerizing and actin depolymerizing factors, respectively. (2) The internal signaling gradients are similar or identical except for their orientation.

As exemplified in the following, these concepts do not mutually exclude each other and can both be supported by experimental data.

2.4.2. Signaling events during growth cone turning

PI3 kinase/PIP3 signaling: Polarity versus gradient detection PI3K/PIP3 signaling is essential for polarization and gradient detection in *Dictyostelium* and neutrophils and is also implicated in fibroblast chemotaxis. It is therefore tempting to investigate the role of PI3K/PIP3 signaling in neuronal polarization and growth cone guidance.

In neuronal cells, there are several levels of polarization during differentiation. First, a rounded cell initiates the outgrowth of neurites. Subsequently, one of the neurites specifies as an axon, which becomes distinct from the dendrites. Once the axon has adopted its identity and developed a growth cone, which turns in response to guidance factors, polarity can be also found inside the growth cone. Interestingly, PI3K/PIP3 signaling in neuronal cells is involved in all of these cell polarity decisions.

NGF stimulation of PC12 cells, a neuronal cell line, leads to neuronal differentiation and neurite outgrowth. Neurite outgrowth and maintenance depends on PI3K activation and a subsequent rise of PIP3, or the injection of activated PI3K into PC12 cells is sufficient for the formation of neurites (Carter and Downes, 1992; Jackson *et al.*, 1996; Kimura *et al.*, 1994; Kita *et al.*, 1998). Interestingly, PI3K/PIP3 signaling during neurite outgrowth closely resembles the formation of a leading edge in *Dictyostelium* cells or neutrophils. NGF stimulation of PC12 cells results in an early phase of global PI3K activation (0–10 min). PI3K activity subsequently gets restricted to the protruding neurites. The local accumulation of PIP3 leads to activation of Rac1/cdc42, thus promoting process outgrowth. Rac1/cdc42 further activates PI3K at the outgrowing protrusions and establishes a positive feedback loop similar to the one observed in *Dictyostelium* cells and neutrophils (Aoki *et al.*, 2005).

The same feedback loop functions again in the later step of axon specification. In hippocampal neurons, PI3K activity and PIP3 are selectively localized at the tip of the axon and act upstream of the polarity protein mPar3. The specification of the axon is strengthened by a positive feedback via Rac1/cdc42. A small rise in PIP3 in one neurite induced by a stochastic fluctuation or an external cue such as laminin will be strongly enhanced and can thus specify the axon (Ménager *et al.,* 2004; Shi *et al.,* 2003). PI3K signaling causes phosphorylation and inactivation of GSK-3. In the absence of active GSK-3, APC can bind to the plus end of microtubules, trigger microtubule polymerization, and thus enhance fast axonal growth. In the dendrite, in contrast, PTEN signaling dominates, increasing the ratio of activated GSK-3 and attenuating the rate of elongation (Jiang *et al.,* 2005; Zhou *et al.,* 2004).

The described development of polarity during neuronal differentiation is not necessarily linked to gradient detection. However, it can be regarded as a step toward the establishment of the axonal growth cone, a cellular structure specialized for the detection of guidance cues during axonal pathfinding. Once the axon is specified, PI3K signaling plays a role in growth cone collapse as well as growth cone turning.

In an advancing axon of chick dorsal root ganglion cells, active PI3K keeps GSK-3 inactive in the f-actin–rich peripheral domain of the growth cone. PTEN is normally sequestrated to the microtubule-rich central domain of the growth cone. Stimulation with the repulsive cue semaphorin3A (Sema3A) causes an accumulation of PTEN at the membrane of the peripheral domain of the growth cone, where it antagonizes PI3K signaling and activates GSK-3. Active GSK-3 leads to decreased microtubule polymerization via interactions with microtubule binding proteins and induces growth cone collapse (Fig. 1.6A). The Sema3A-dependent collapse is prevented by either inhibition of GSK-3, stimulation of PI3K, or knockdown of PTEN. Contrariwise it can be mimicked by inhibitors of PI3K (Chadborn *et al.,* 2006; Eickholt *et al.,* 2002). Because Sema3A-induced collapse is mediated by PTEN/PI3K signaling, repulsive turning away from a Sema3A gradient could possibly be realized by localized recruitment of PTEN to the peripheral membrane at the side of the growth cone facing the repulsive gradient and a subsequent partial growth cone collapse.

Attractive turning toward NGF gradients, on the other hand, was shown to depend on the activation of PI3K downstream of trkA receptor tyrosine kinase (Ming *et al.,* 1999). Furthermore, attractive gradients of brain-derived neurotrophic factor (BDNF) or netrin-1 induce an accumulation of PIP3 on the gradient near side of the growth cone in *Xenopus* spinal neurons. Addition of membrane permeant PIP3 to the medium can induce intracellular PIP3 accumulation and chemoattraction. This PIP3-induced chemoattraction depends on Akt and PI3K activity, indicating a possible positive feedback loop reminiscent of the one in *Dictyostelium* and neutrophils.

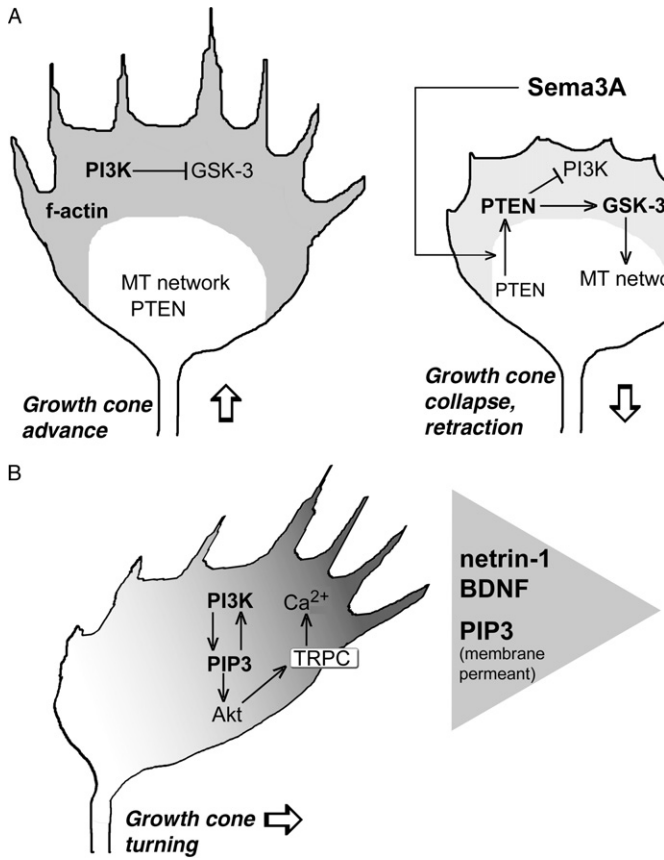

Figure 1.6 PI3K signaling in growth cone collapse and growth cone turning. (A) In an advancing growth cone, PI3K activity is localized to the f-actin–rich peripheral domain of the growth cone, where it inhibits GSK-3 signaling. PTEN resides mainly in the central domain of the growth cone and is thought to be sequestered to the microtubule network. During Sema3A-induced collapse, PTEN moves into the peripheral domain of the growth cone, antagonizes PI3K signaling, and activates GSK-3. Active GSK-3 acts on microtubules and positively affects growth cone collapse and retraction. (B) During attractive turning toward a gradient of netrin-1 or BDNF, PI3K signaling gets elevated at the gradient near side of the growth cone and supports asymmetric accumulation of PIP3. PIP3 signaling may elevate local Ca^{2+} via Akt dependent opening of TRPC channels in the cell membrane and thus trigger attractive turning. Because PI3K and PIP3 engage in a positive feedback loop, an external gradient of membrane permeant PIP3 can mimic netrin-1– or BDNF-induced attraction.

Moreover, it seems that PIP3/Akt signaling can activate transient receptor potential channels (TRPCs), meaning it is located upstream of Ca^{2+} signaling (Fig. 1.6B) (J. Henely, personal communication). As detailed in the next section, high local Ca^{2+} elevations trigger attractive growth cone turning.

Taken together, it seems a modulation of PI3K/PIP3 signaling in the peripheral domain of the growth cone can account for turning or collapse (see Fig. 1.6). In line with this, the peripheral domain of the growth cone might be comparable to the leading edge of a neutrophil or a *Dictyostelium* cell, whereas the central domain might correspond to the uropod. During Sema3A-induced growth cone collapse, the peripheral domain of the growth cone basically abandons its leading edge equivalence, becomes dominated by ''uropod signaling,'' and retracts. In an attractive gradient, PI3K/PIP3 signaling seems to be elevated on the gradient near side of the growth cone. In this way, the orientation of the peripheral domain with respect to the axon shaft is possibly shifted toward the source of the gradient and predates the turning of the whole growth cone.

Although the picture of PI3K/PIP3 signaling in a growth cone responding to guidance cues fits well with models explaining polarity and chemotaxis in *Dictyostelium* and neutrophils, there are still many open questions. Strangely, it was reported that inhibition of PI3K reduces the collapse caused by ephrinA5 or slit2 in chick retinal ganglion cell axons (Wong *et al.,* 2004). Furthermore, PI3K activity seems to be required for repulsive turning in a gradient of slit-2 (Ming *et al.,* 1999). In contrast to the previous mentioned findings that stimulation of PI3K signaling prevents Sema3A-dependent growth cone collapse, rather these data suggest that PI3K signaling positively affects growth cone collapse or repulsive turning. Further experiments are needed to elucidate these seemingly contradictory results.

Ca^{2+} and cyclic nucleotides On the search for intracellular effectors of the observed turning in response to guidance molecules, it was discovered that growth cones also turn toward external gradients of Ca^{2+} plus Ca^{2+} ionophore or membrane permeant analogs of cAMP (Gundersen and Barrett, 1980; Lohof *et al.,* 1992). cAMP and Ca^{2+} play the role of an intracellular messenger in the growth cone during detection and interpretation of gradients of many axonal guidance factors. Focal laser-induced photolysis (FLIP) of caged intracellular Ca^{2+} or caged intracellular cAMP leads to an attractive growth cone turn toward the release site of the caged component (Munck *et al.,* 2004; Zheng, 2000).

Attractive gradients of neurotransmitters such as acetylcholine (ACh) and glutamate (Zheng *et al.,* 1994, 1996) cause an asymmetric Ca^{2+} influx in the growth cone, which is higher at the gradient near side. This asymmetric influx creates a local Ca^{2+} elevation and an internal gradient of Ca^{2+} in the growth cone with its high side pointing in the direction of the turn. As shown for the guidance factors netrin-1 and BDNF, Ca^{2+} influx during growth cone attraction is mediated by transient receptor potential channels (TRPCs), a class of Ca^{2+} permeable receptor-operated channels (Li *et al.,* 2005; Shim *et al.,* 2005; Wang and Poo, 2005).

Intriguingly, a local Ca^{2+} elevation can mediate not only attractive, but also repulsive turning. Thus, FLIP of caged Ca^{2+} triggers repulsion away from the site of Ca^{2+} release, when the resting intracellular Ca^{2+} concentration is lowered by removal of Ca^{2+} from the extracellular medium and the local Ca^{2+} elevation generated by FLIP is accordingly lower.

The amplitude of the local Ca^{2+} elevation relative to the global Ca^{2+} level determines the directionality of the response: A high local Ca^{2+} elevation causes attractive turning, and a low local Ca^{2+} elevation causes repulsive turning (Zheng *et al.*, 2000). In line with this, the turning response of a growth cone toward Ca^{2+} dependent guidance factors can be switched from attraction to repulsion by manipulation of intracellular Ca^{2+}. Netrin-1 triggered attraction depends on influx of external Ca^{2+} as well as Ca^{2+} release from internal stores. If one of these sources is blocked, that is, if the induced Ca^{2+} elevation is decreased, attraction is switched to repulsion (Hong *et al.*, 2000).

The extend of a Ca^{2+} elevation caused by a guidance factor is crucially influenced by cAMP signaling. cAMP levels act like a switch determining whether the response to a Ca^{2+}-dependent guidance factor is attractive or repulsive. BDNF, Ach, or netrin-1 gradients, which normally induce attractive turning of *Xenopus* growth cones by high local Ca^{2+} elevation, lead at the same time to an intracellular increase of cAMP. If cAMP signaling is blocked by competitive analogs of cAMP or inhibitors of its downstream effector protein kinase A (PKA), attraction is converted to repulsion (Ming *et al.*, 1997; Song *et al.*, 1997). Gradients of myelin-associated glycoprotein (MAG), on the other hand, which normally trigger repulsive turning, become attractive upon pharmacological activation of cAMP signaling pathways (Song *et al.*, 1998). A repulsive gradient of MAG induces an intracellular Ca^{2+} elevation in the shape of a gradient with the highest Ca^{2+} on the gradient near side of the growth cone. However, this graded elevation of Ca^{2+} is about half of that associated with the attractive turning induced by a netrin-1 gradient. Elevation of cAMP signaling enhances the MAG-induced Ca^{2+} signals up to the level induced by netrin-1 and thus switches repulsion to attraction. The different amplitudes of Ca^{2+} signaling may be explained by the fact that netrin-1 triggers an internal Ca^{2+} release as well as Ca^{2+} influx from the outside, whereas MAG causes only internal Ca^{2+} release. The moderate Ca^{2+} elevation induced by MAG is achieved without cAMP signaling (Henley *et al.*, 2004).

In summary, attractive and repulsive turning are mediated by intracellular Ca^{2+} gradients of the same polarity but with differing magnitudes. The magnitude of the intracellular Ca^{2+} gradient is positively affected by cAMP signaling. Ca^{2+} signals of different magnitude are sufficient to trigger bidirectional turning independently of cAMP signaling, but turning induced by a cAMP gradient needs downstream Ca^{2+} signals (Henley *et al.*, 2004). Indeed cAMP regulates Ca^{2+} levels by activating via PKA L-type Ca^{2+} channels

(Nishiyama *et al.*, 2003). These findings suggest cAMP is upstream of Ca^{2+} signaling. However, Ca^{2+} can activate the Ca^{2+}-dependent adenyl cyclase and thus increase cAMP levels (Song *et al.*, 1997). It therefore cannot be excluded that cAMP and Ca^{2+} act in a feedback loop and possibly amplify the external gradient of a guidance factor through their concerted action. This could be the case during attractive turning, where both cAMP and Ca^{2+} levels are high at the gradient near side of the growth cone. In repulsive turning, however, cAMP and Ca^{2+} levels might not reach the threshold required for the proper functioning of the feedback loop.

Notably, the described Ca^{2+}/cAMP switch was proven to be physiologically relevant and regulates different responses to a single guidance factor *in vivo*. In the nervous system, the cAMP level of a neuronal growth cone depends on substrate-specific signals and/or the developmental stage. When growing on poly-D-lysine or fibronectin, *Xenopus* retinal growth cones have high intracellular cAMP levels and show attractive turning toward netrin-1. On a laminin substrate, however, netrin-1 is repulsive because laminin lowers the cAMP levels (Höpker *et al.*, 1999). The bifunctionality of MAG is developmentally regulated in rat dorsal root ganglion (DRG) neurons. Although MAG promotes neurite outgrowth in cells from early postnatal animals, it is repulsive for neurites from DRGs from slightly older animals, which display a correlated developmental decrease in endogenous cAMP levels (Domeniconi and Filbin, 2005).

Downstream of Ca^{2+} signals, there are alternative pathways mediated by two different Ca^{2+}-regulated kinases. High local Ca^{2+} elevations preferentially activate Ca^{2+}-calmodulin–dependent protein kinase II (CaMKII) and induce attraction; lower local Ca^{2+} elevations activate calcineurin-phosphatase-1 (CaN-PP1) and induce repulsion. Elevated cAMP levels may have a dual role in effectively converting repulsion to attraction. First, they amplify the Ca^{2+} signal in the suggested feedback loop. Second, they lead to activation of PKA, which inhibits CaN-PP1 and blocks the repulsion pathway. Upon activation of the repulsion pathway, on the other hand, PP1 acts negatively on CaMKII and thus blocks attraction (Han *et al.*, 2007; Wen *et al.*, 2004). The mutual exclusion of attraction and repulsion signaling pathways may explain why the growth cone is either attracted or repulsed at borderline conditions and does not display an intermediate response (Ming *et al.*, 1997). CaMKII and CaN-PP1 regulate cytoskeleton-associated proteins, tubulin and Rho GTPases, which effect the cytoskeletal rearrangements required for growth cone turning (Jin *et al.*, 2005). Figure 1.7 summarizes the alternative signaling downstream of an intracellular Ca^{2+} gradient of different magnitude leading to either attraction or repulsion.

Data suggests that attractive but not repulsive Ca^{2+} elevations in the growth cone also mediate asymmetric transport and exocytosis of membrane vesicles. The vesicle transport toward the side of the growth cone

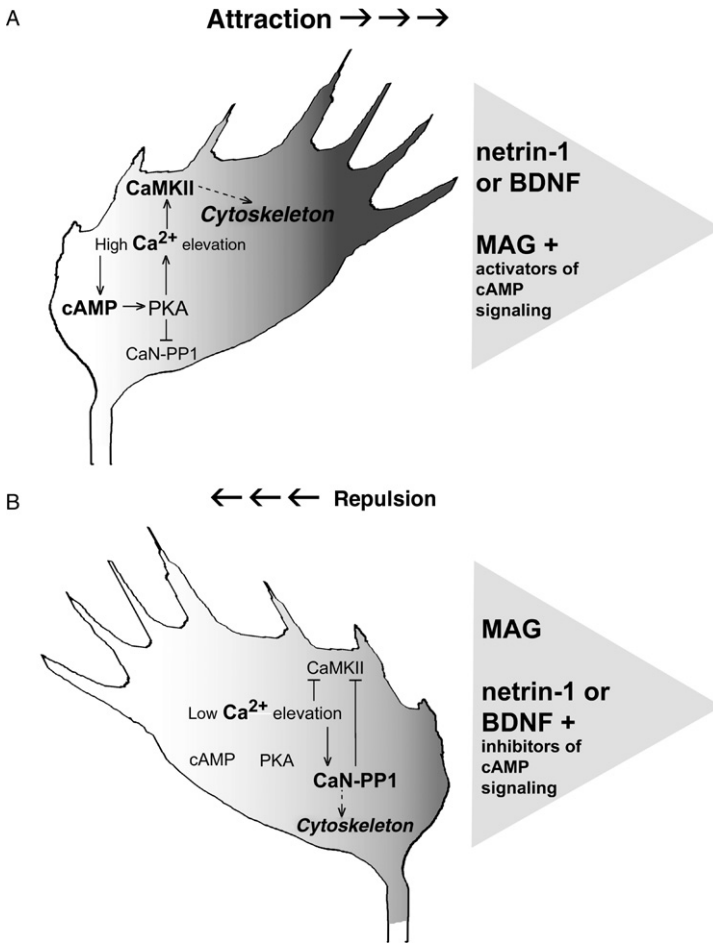

Figure 1.7 Intracellular Ca^{2+} gradients mediate both attractive and repulsive growth cone turning. (A) A high Ca^{2+} elevation leads to growth cone attraction toward the site of the elevation. High Ca^{2+} levels support a positive feedback between Ca^{2+} and cAMP via PKA and activate CaMKII while inhibiting CaN-PP1. CaMKII regulates the cytoskeleton in a way that affects attractive turning. This first scenario is triggered by the attractive guidance cues netrin-1 and BDNF. It can be also induced by the typical repellent MAG, when MAG is applied in combination with activators of cAMP signaling. (B) A low local Ca^{2+} elevation, on the contrary, leads to repulsive turning away from the site of the Ca^{2+} elevation. In this situation, there is no activation of a positive feedback loop between Ca^{2+} and cAMP, and CaN-PP1 wins over CaMKII signaling. As a result, the cytoskeleton is differently regulated and directs the growth cone to gradient far side. This second situation dominates in a gradient of MAG or in a gradient of netrin-1 or BDNF under conditions of suppressed cAMP signaling.

generates an asymmetric expansion of the plasma membrane and a subsequent attractive turn (Tojima *et al.*, 2007).

Taken together, Ca^{2+} signaling plays a prominent role in growth cone turning. In combination with cAMP signaling, it can affect attraction as well as repulsion, thus conferring context-dependent bifunctionality to axon guidance molecules.

It should be noted, however, that not all guidance molecules recruit Ca^{2+} in their downstream signaling. Growth cone turning induced by semaphorin (Li *et al.*, 2005; Song *et al.*, 1998), neurotrophin 3 (NT-3) (Song *et al.*, 1997), or ephrinA (Löschinger *et al.*, 1997), for example, is independent of Ca^{2+} and its modulator cAMP.

Local protein translation and degradation The neuronal growth cone has its own local protein translation and protein degradation machinery. Protein levels can thus independently be regulated from the cell soma and axonal protein transport (Piper and Holt, 2004).

Growth cone turning or collapse in response to many guidance cues requires either local protein translation or degradation in the growth cone or both. For example, turning in response to netrin-1 requires translation and degradation, Sema3A-triggered collapse requires translation, and lysophosphatidic acid (LPA)-triggered collapse requires degradation. The translational activation in response to netrin-1 or Sema3A is mediated by the following signal cascade: activation of MAP kinases leads to the phosphorylation of the eukaryotic initiation factor 4B binding protein (eIF-4EBP), which subsequently releases eIF-4E and allows its binding to mRNAs present in the growth cone. Protein degradation after stimulation with netrin-1 or LPA is partially mediated by activation of caspase-3 downstream of MAP kinases (Campbell and Holt, 2001, 2003).

So far, it is not known which proteins get degraded in response to specific guidance cues (Campbell and Holt, 2003). More progress has been made in the identification of specific mRNAs translated in the growth cone. Like in fibroblasts, local translation of β-actin mRNA is an important mechanism to enhance local actin polymerization. An attractive gradient of netrin-1 or BDNF was shown to trigger asymmetric activation of eIF-4E and a subsequent rise in β-actin on the gradient near side. Local protein translation is linked to Ca^{2+} signaling because a high local and therefore attractive Ca^{2+} elevation induces β-actin synthesis (Leung *et al.*, 2006; Yao *et al.*, 2006). There is some controversy, however, regarding how β-actin translation is regulated in a repulsive gradient of netrin-1 or BDNF. Although Leung *et al.* (2006) reported that repulsive turning in a netrin-1 gradient is independent of β-actin translation, Yao *et al.* (2006) suggested a repulsive gradient of BDNF or a low local (repulsive) Ca^{2+} elevation creates a local decrease of β-actin translation. This local decrease on the gradient

near side of the growth cone can be seen as a relative increase of β-actin at the gradient far side, that is, as a kind of inverted attractive turning.

Turning away from a repulsive gradient, however, can also be induced by an increase of proteins negatively affecting growth cone advance. Sema3A causes local translation of RhoA mRNA and a rise in RhoA, which counteracts actin polymerization. This translation is necessary and sufficient for the Sema3A-induced growth cone collapse (Wu *et al.*, 2005). Besides increasing the protein level of RhoA, semaphorin receptors (plexins) can also regulate Rho GTPases via GEFs (Kruger *et al.*, 2005). Local translation of RhoA and local activation of newly synthesized RhoA by specific GEFs can be thus envisaged to cooperate during the Sema3A signaling.

Sema3A, as well as the repulsive cue slit-2, was furthermore found to induce a protein translation dependent rise of the actin-depolymerizing protein cofilin, which is likely due to translation of cofilin mRNA in the growth cone. An increase of cofilin causes a simultaneous decrease in f-actin, accounting in part for the growth cone collapse (Piper *et al.*, 2006).

Although the local translation of RhoA and cofilin was originally observed during growth cone collapse (Fig. 1.8A), it seems plausible that repulsive turning away from Sema3A or slit-2 is triggered by a more localized activation of protein translation leading to a partial collapse coupled with a reorientation of the growth cone.

It was demonstrated that the homeodomain transcription factor engrailed-2 (En-2), in addition to regulating the transcription of ephrins, can also directly guide retinal axons. When applied in an external gradient, En-2 is internalized by the growth cone and regulates local translation by activation of eIF-4E by mechanisms similar to the ones observed downstream of netrin-1, BDNF, or Sema3A (Brunet *et al.*, 2005). It is left to be investigated whether transcription factors other than En-2 can act as secreted cues and adopt a dual role in controlling transcription in the nucleus as well as translation in the growth cone (Butler and Tear, 2007).

Because both repulsive and attractive guidance cues regulate translation via eIF-4E and there are a number of different mRNAs present in the growth cone, it is a crucial question how a specific mRNA gets translated in response to a specific cue. Axonal mRNA is incorporated in RNA granules, which contain mRNA, ribosomes, and RNA binding proteins. The latter play a role in mRNA transport as well as in translational regulation and may therefore be involved in the specificity and localization of translation in a turning or collapsing growth cone. In *Xenopus* growth cones, β-actin mRNA colocalizes with ZBP1 in granules. A graded netrin-1 or BDNF stimulus induces the movement of the β-actin mRNA containing ZBP-1 granules into the filopodia of the gradient near side by a yet unknown mechanism. As known from fibroblasts, ZBP-1 releases β-actin mRNA for translation upon phosphorylation by src. Because attractive BDNF gradients trigger a graded activation of src in the growth cone, the release

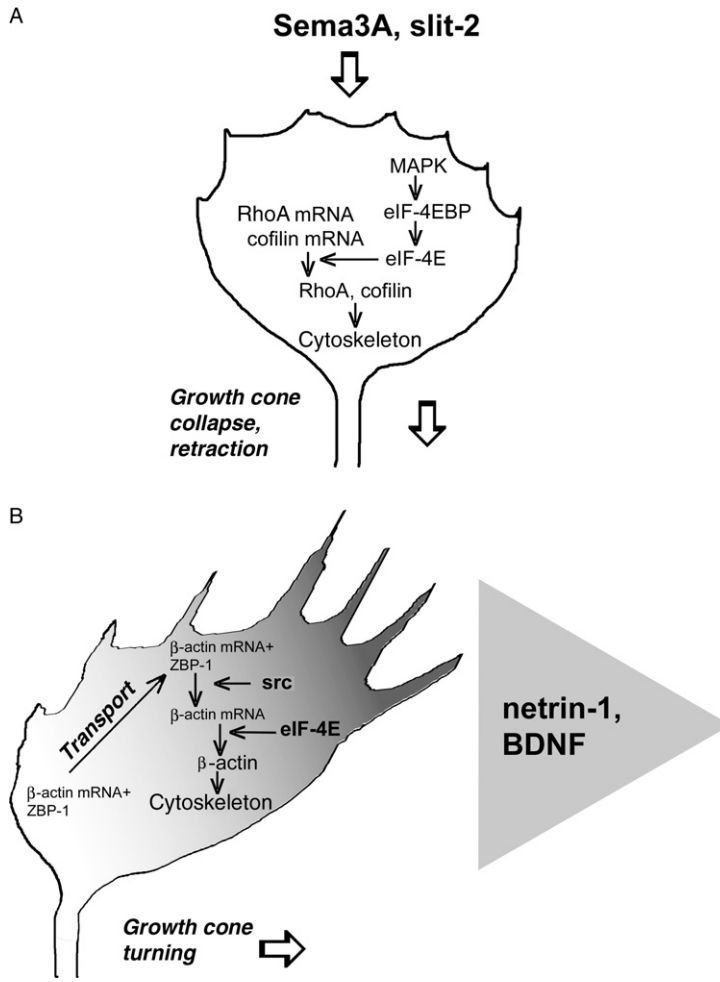

Figure 1.8 Growth cone collapse and turning in response to several guidance cues is mediated by local protein translation. (A) Growth cone collapse triggered by the repulsive cues Sema3A or slit-2 were shown to induce local translation of RhoA and cofilin mRNAs via the activation of eIF-4EBP downstream of MAP kinases. RhoA and cofilin activity lead to cytoskeletal rearrangements causing collapse and retraction. (B) Attractive gradients of netrin-1 or BDNF cause local translation of β-actin. The asymmetry of β-actin translation is achieved by at least three mechanisms. First, RNA granules containing β-actin mRNA bound to ZBP-1 are transported preferentially to the gradient near side of the growth cone. Gradients of src and eIF-4E activation (depicted by gray shading) lead to graded release of β-actin mRNA from its binding partner and graded protein translation, respectively. Newly synthesized β-actin serves as a nucleation site for f-actin and shifts the growth cone toward the source of the gradient.

of β-actin mRNA from ZBP-1 RNA granules is supposed to be graded as well. The asymmetry of β-actin synthesis is thus ensured by directed transport of the RNA granules, graded release of mRNA from translational silencing, and asymmetric activation of eIF-4E (Fig. 1.8B) (Leung *et al.*, 2006; Yao *et al.*, 2006). Both transport of RNA granules and release of mRNA from binding proteins are supposedly differentially regulated dependent on the type of mRNA and the extracellular trigger, which could thus account for different translational readouts in response to different guidance cues.

Rho GTPases and the cytoskeleton: A common signaling output

Interestingly, the signaling events during growth cone turning described in the previous sections are selectively activated in different combinations in response to different guidance cues (Table 1.1) and build up a cascade in some cases.

For example, Ca^{2+} signaling, PI3K/PIP3 pathways, and local protein translation are all implicated in growth cone turning in response to netrin-1 or BDNF. Akt activation at the sites of local PI3K activity and accumulation of PIP3 was suggested to lead to an increase of intracellular Ca^{2+} via regulation of TRPC channels. Ca^{2+} signaling, in turn, is upstream of local protein translation of β-actin. β-actin levels directly affect the rate of actin polymerization and the advance of the growth cone toward the gradient source. Aside from possibly acting upstream of protein translation, both PI3K and Ca^{2+} signaling can directly affect the cytoskeleton. PI3K regulates the microtubule network via GSK-3 and PIP3 may, as in neutrophils, influence the balance of Rho GTPases. Ca^{2+} signaling affects CaMKII and CaN-PP, which regulate Rho GTPases as well as cytoskeleton-associated proteins. Rho GTPases, as elaborated in the following, are additionally activated by Rho GEFs acting immediately downstream of guidance cue receptors.

Taken together, the turning of a growth cone toward netrin-1 or BDNF seems to be achieved by the cumulative action of several signaling pathways on the cytoskeleton, each of which can be essential or even sufficient for turning. The exact interrelation of these signaling pathways, however, is not completely clear. Based on present data, they could be either independently activated by the external cue or rather arranged in a strict hierarchy (Fig. 1.9).

Signaling downstream of Sema3A, on the other hand, is independent of Ca^{2+} but involves the modulation of PI3K/PTEN signaling as well as local protein translation of cofilin and RhoA mRNA. Whereas local protein translation is essential for the response of growth cones to netrin-1, BDNF, or Sema3A, it seems not to be required in ephrinA-triggered collapse (our own unpublished results). However, no matter how the signaling pathways vary dependent on the guidance cue causing growth cone turning or collapse, they commonly lead to rearrangements of the cytoskeleton.

Table 1.1 Signaling events in the growth cone vary dependent on the guidance factor

Guidance cue (receptor)	Response of the growth cone	Intracellular signaling		
		PI3K/PIP3	Ca^{2+}	Local protein translation
Netrin-1 (Dcc)	Attractive turning	Enhanced PI3K activity, PIP3 accumulation	High elevation leading to activation of CaMKII	Increase of β-mRNA translation
	Repulsive turning	Not known	Low elevation leading to activation of CaN-PP1	Not involved?
BDNF (trkB)	Attractive turning	Enhanced PI3K activity, PIP3 accumulation	High elevation leading to activation of CaMKII	Increase of β-mRNA translation
	Repulsive turning	Not known	Low elevation leading to activation of CaN-PP1	Decrease of β-mRNA translation?
MAG (Nogo receptor)	Repulsive turning	PI3K activity required	Low elevation leading to activation of CaN-PP1	Not known
Sema3A (plexinA+ neuropilin1)	Collapse	PTEN translocation and activation	Not involved	Increase of RhoA and cofilin mRNA translation
Slit (Robo)	Collapse	PI3K activity required	Not known	Increase of RhoA and cofilin mRNA translation
EphrinA (EphA)	Collapse	PI3K activity required	Not involved	Not involved

For references, see text.

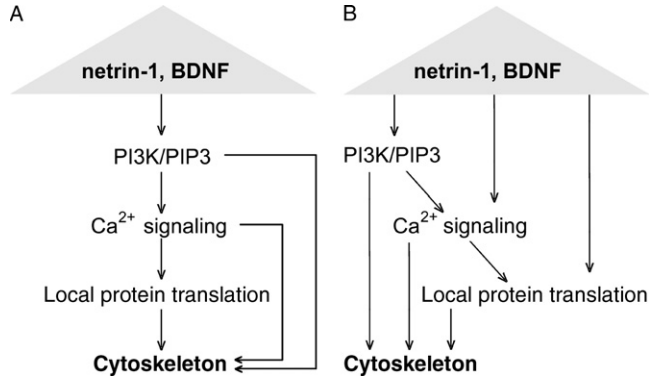

Figure 1.9 During attractive turning toward netrin-1 or BDNF, different interrelated signaling pathways concertedly act on the cytoskeleton. Attractive gradients of netrin-1 or BDNF activate PI3K/PIP3 signaling, Ca^{2+} signaling, and local protein translation of β-actin mRNA in the growth cone. These signaling pathways all act on the cytoskeleton via different downstream effectors. PI3K/PIP3 signals upstream of Ca^{2+}, and Ca^{2+} levels supposedly influence local protein translation. It is not yet known whether the signaling cascade is strictly hierarchical (A) or if the external cues directly activate each pathway (B).

Rho GTPases are probably the best-studied cytoskeletal regulators (Jaffe and Hall, 2005). They play an instructive role in growth cone guidance and are implicated at some point of the signaling cascade in the response to practically every known axon guidance molecule. In general, Rac and cdc42 stimulate f-actin assembly in lamellipodia and filopodia, respectively, whereas RhoA leads to increased contraction of the actin-myosin network. According to a simplified model, Rac/cdc42 signaling dominates in attraction and RhoA signaling in repulsion. Because RhoA and Rac inhibit each other, activation of one Rho GTPase is likely to affect the balance of all three. Asymmetries in the Rho GTPase activities may therefore reinforce and efficiently direct growth cone turning (Dickson, 2001). By doing so, Rho GTPases are not only the output of gradient detection, but also participate in the setup of internal signaling polarity.

Rho GTPases can be regulated via the PI3K pathway, by intracellular Ca^{2+}, or on the translational level as in case of RhoA during stimulation with Sema3A. Moreover, most guidance cue receptors also activate Rho GTPases via Rho GEFs. The netrin-1 receptor DCC is thought to interact with GEFs such as Trio during attractive signaling to activate both cdc42 and Rac1. Cdc42 and Rac1 stimulate an increase of the number of filopodia and the enlargement of the lamellipodial area (Barallobre *et al.*, 2005). TrkB, when stimulated with its ligand BDNF, binds and activates the Rac1 specific GEF Tiam1 (Miyamoto *et al.*, 2006). In the case of ephrinA5, EphA receptors phosphorylate and activate the GEF ephexin, which activates

RhoA and its downstream effector Rock but does not have any effect on Rac/cdc42 (Sahin *et al.*, 2005; Shamah *et al.*, 2001; Wahl *et al.*, 2000). Similarly, RhoA/Rock is activated during Sema3A-induced collapse and retraction (Gallo, 2006) and LPA-induced chemorepulsion (Yuan *et al.*, 2003). Interestingly, there is evidence that growth cone collapse and subsequent retraction is mediated by RhoA activity in the axon shaft rather than in the peripheral domain (Nakamura *et al.*, 2005). This indicates that the effect of RhoA activity depends on its site of action. Repulsive guidance molecules may therefore not globally activate RhoA but lead to a spatially restricted activation pattern. The Rac-specific GEF FARP2 (FERM domain-containing guanine nucleotide exchange factor) is activated immediately downstream of the Sema3A receptor complex. Its activity is required for Sema3A-induced collapse of DRG growth cones (Toyofuku *et al.*, 2005). Because Rac activity has been correlated so far with growth cone extension and attraction, this result is confusing on the first sight. There is evidence that Rac mediates endocytosis of the growth cone plasma membrane rather than promoting actin polymerization during Sema3A-mediated collapse (Jurney *et al.*, 2002). The detailed differences of Rac signaling during collapse versus growth cone extension or attraction await further investigation.

2.4.3. Adaptation in growth cone gradient detection

Adaptation of sensitivity is a common cellular phenomenon in response to a large number of biological stimuli. Adaptation seems especially reasonable during gradient detection of chemotaxing cells or growing axons because they move relative to the external gradient and are thus exposed to changing concentration ranges of the guidance factor. It is therefore not astonishing to observe adaptation under various conditions during axonal guidance.

Adaptation, meaning the readjustment of sensitivity according to the strength of the signal, may be assumed based on different observations. First, a high concentration of a guidance cue may cause a strong response, but a longer exposure to the constantly high concentration may weaken or completely abolish this response. This attenuation of the normal response of a ''naive'' growth cone is referred to as desensitization. Following a period of desensitization, the growth cone may regain its sensitivity (resensitization)—either in the presence of the stimulus or in its absence (Fig. 1.10). Second, a low, subthreshold concentration of a guidance cue, which does not elicit any morphologically detectable response, may be nonetheless sufficient to attenuate the detectable response to a subsequent higher dose of a guidance cue. In short, adaptation can explain why the same growth cone may react differently to the same guidance cue concentration dependent on its ''history.''

Ming (2002) showed a uniform bath concentration of netrin-1 or BDNF leads to a dose-dependent desensitization of the growth cone, which becomes apparent when it is faced immediately afterward to a standard

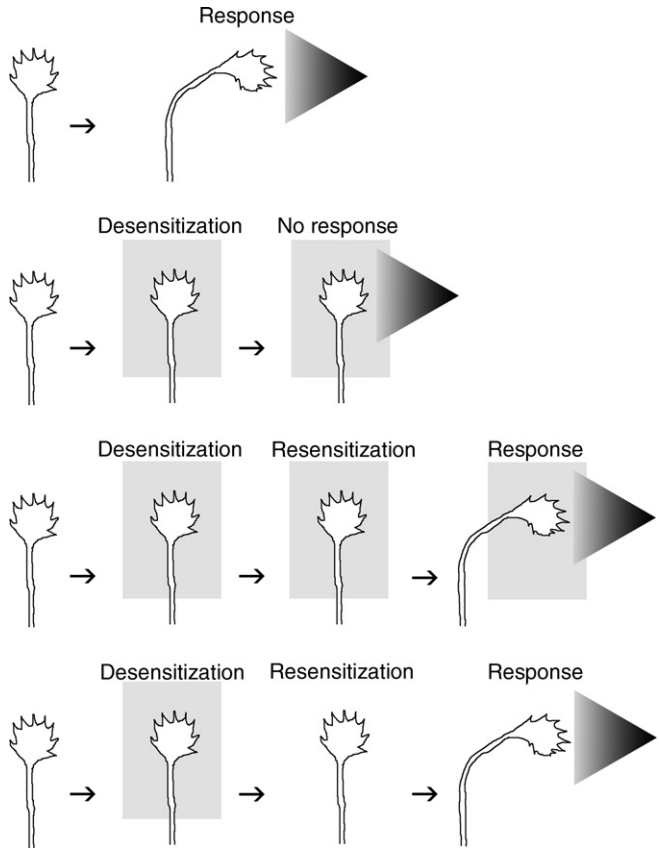

Figure 1.10 Adaptation in gradient detection by growth cones. The turning response toward a gradient of chemoattractant such as netrin-1 (shaded triangle) can be abolished by the application of a uniform low concentration of the attractant (gray rectangle), which leads to desensitization. The growth cone no longer responds to a subsequently applied gradient. If the uniform attractant concentration is maintained during a prolonged time span, the growth cone undergoes a period of resensitization and is again attracted by the gradient. Resensitization can also occur in the absence of the initially desensitizing stimulus.

gradient of chemoattraction, which is no longer able to elicit a turning response under these conditions. The desensitization is reversible, that is, the growth cone regains sensitivity within 90–120 min and seems to readjust its sensitivity to the new basal concentration of chemoattractant. Moreover, when the course of a growth cone in a soluble gradient of netrin-1 is followed over several hours, the extension toward the gradient source shows a "zigzag" pattern. This was interpreted as an indication of alternating periods of attraction and repulsion (Ming *et al.*, 1997). The zigzag course is likely to arise from the higher level of desensitization at the gradient near

side of the growth cone, which results in a higher relative signal at the gradient far side of the growth cone during periods of apparent repulsion. The average interval for the zigzag is approximately 20 min in a netrin-1 or BDNF gradient. In high concentrations of attractant, the desensitization is heterologous, in low concentrations homologous. Desensitization goes along with persistent Ca^{2+} elevation in the growth cone. After resensitization, a gradient can induce a further Ca^{2+} elevation superimposed on top of the elevated basal Ca^{2+} level. Resensitization is dependent on MAPK activity and local protein synthesis (Ming *et al.*, 2002).

In the context of growth cone collapse, desensitization and resensitization can occur very rapidly. The collapse of *Xenopus* retinal axons in response to either netrin-1 or Sema3A is markedly reduced when growth cones are pretreated for 1–2 min with a low dose of the collapse-inducing factor, which itself produces minimal collapse. This desensitization depends on endocytosis. However, if the low dose is maintained for approximately 5 min, the high collapse-inducing dose has the same effect as without pretreatment; namely resensitization occurs in the presence of the low dose after the initial desensitization. This resensitization depends on protein synthesis. During desensitization, the receptors for Sema3A and netrin-1, respectively, disappear from the cell surface in a endocytosis-dependent manner. During the subsequent resensitization, receptors reappear. This reappearance is only partially dependent on protein synthesis (Piper *et al.*, 2005).

It would be interesting to know whether proteins other than the receptors are locally translated during resensitization. Are they the same, which are also translated during the initial response of the growth cone toward the guidance factor? Notably, desensitization and resensitization also occur in growth cones treated with ephrinA5, the action of which seems independent of protein synthesis (von Philipsborn *et al.*, 2006; our own unpublished results). Are there different mechanisms of adaptation depending on the guidance cue?

From a more conceptual point of view, it is also tempting to speculate about the interrelation of signal amplification and adaptation. Signal amplification, which might be crucial for the detection of a shallow external gradient, could have the consequence that a stimulus of intermediate strength already triggers maximal internal signaling in the growth cone and blinds it for further increase of the external stimulus. If desensitization and subsequent resensitization is tightly coupled to signal amplification, such restrictions could be overcome.

2.4.4. Gradient detection during topographic mapping

Gradient detection not only directs growth cone turning, but also is essential in the formation of topographic neural maps. A topographic map consists of a population of projecting neurons, whose axonal connections in the target region reflect their original spatial order. As first proposed by Sperry (1963),

topography can theoretically be established by the graded distribution of a guidance cue in the target region and the graded expression of the corresponding receptor on the ingrowing axons. For example, topography is found in the thalamo-cortical, the hippocampo-septal, and the vomero-nasal projection in the nervous system (McLaughlin and O'Leary, 2005). One of the best-studied neural topographic maps is the connection between the vertebrate eye and the brain, the retino-tectal/collicular projection that is mainly set up by ephrins and Eph receptors. Numerous *in vivo* studies such as ephrinA/EphA knockout or knockin mice have consolidated the notion that temporal retinal ganglion cells (RGCs) axons, which express high levels of EphA receptors, are confined to the anterior tectum/superior colliculus containing low ephrinA levels, whereas nasal RGC axons with less EphA receptor invade the ephrinA gradient in the target region up to the posterior part of the tectum/superior colliculus (Brown *et al.*, 2000; Dütting *et al.*, 1999; Feldheim *et al.*, 2000, 2004; Nakamoto *et al.*, 1996).

Topographic mapping has been extensively reviewed (Flanagan, 2006; McLaughlin and O'Leary, 2005) and explained by different theoretical models (Gierer, 1983; Goodhill and Urbach, 1999; Honda, 2004; Koulakov and Tsignakov, 2004; Löschinger *et al.*, 2000; Reber *et al.*, 2004; Yates *et al.*, 2004). We intend to focus here on a few principal questions with respect to gradient detection during mapping and selected experiments addressing them.

So far, it is still not completely understood how a RGC growth cone reads a repulsive gradient of ephrinA. The process of anterior-posterior mapping in the visual system is far more complicated than growth cone turning. First, growth cones have to follow a repulsive gradient up to a certain point, instead of avoiding it at all and performing a negative turning response as soon as they detect the gradient. Second, growth cones should be able to read not only the directional, but also the positional information of the gradient. Intuitively, one might assume that the sensing of position requires sensing of absolute concentration, which could be in conflict with the concept of signal amplification and adaptation.

Because mapping is accomplished by graded distributions of substrate-bound molecules and implicates the stop of a growth cone rather than its turning, the classical turning assay with soluble gradients can only give limited information. Moreover, ephrinAs require membrane attachment and/or clustering for proper EphA activation (Davis *et al.*, 1994; Egea *et al.*, 2005). Uniform addition of soluble clustered or dimeric ephrinA causes temporal RGC growth cones to collapse. *Xenopus* RGC growth cones were also observed to turn in response to a soluble gradient of dimeric ephrinA5. However, this turning occurs at a high background level of growth cone collapse (Weinl *et al.*, 2003).

The action of substrate bound guidance molecules such as ephrinA has been studied with the so-called stripe-assay, an array of ephrinA-covered

lanes alternating with lanes devoid of ephrinA. EphrinA-sensitive axons avoid the ephrinA-containing lanes and display a striped outgrowth on the permissive lanes (Monschau *et al.*, 1997; Vielmetter *et al.*, 1990; Walter *et al.*, 1987). The stripe assay can impressively demonstrate preference for or avoidance of guidance factors, but provides no information about the behavior of growth cones in gradients of these factors.

EphrinA is a repulsive cue when applied in a nongraded form. However, a growth cone obviously invades ephrinA gradients *in vivo* and switches from advance to stop in the target area. This switch could either result from an inherent, concentration-dependent bifunctionality of ephrinA or from the counteraction of a second, superimposed attractive guidance force. There is experimental data supporting both notions.

Indeed, temporal RGC axons can invade substrate bound gradients *in vitro* and switch at a certain point of the artificial gradient to growth inhibition or avoidance reactions. Early studies using repellent membrane material from chick posterior tectum or ephrinA-overexpressing cells to create gradients of various shapes come to differing conclusions about the question, which parameters of the gradient confer its repulsive properties. Baier and Bonhoeffer (1992) suggest retinal axons stop in gradients depending on the gradient slope. Only sufficiently steep gradients caused a growth inhibition. Rosentreter *et al.* (1998), however, found in a similar assay that temporal retinal growth cones avoid a certain threshold concentration of repellent membrane material when they invade a gradient. This postulated threshold concentration is independent of the gradient slope. When growing out on basal levels of repellent membranes, growth cones travel up the gradient for an additional fixed increment of concentration, indicating a certain adaptation mechanism.

In contrast to diffusible gradients, substrate-bound gradients are far more complicated to fabricate *in vitro*. In gradients of membrane material, it is difficult to quantify the exact amount of a single protein and exclude the influence of other components. Progress has been made by generating concentration gradients in three-dimensional (3D) gels, which are comparatively stable for several hours (Rosoff *et al.*, 2005). To obtain highly reproducible and long-lasting graded distributions of purified ephrinA5, microcontact printing proved to be a useful technique. Gradients fabricated by microcontact printing are discontinuous on a microscale, that is, they consist of a geometric pattern of protein dots or lines, which vary with respect to sizes and spacing. As shown by von Philipsborn *et al.* (2006), temporal retinal growth cones can read discontinuous gradients of ephrinA produced by microcontact printing and stop at a certain point in the gradient. By testing different gradient slopes and concentrations, the influence of gradient steepness versus a threshold concentration on growth cone stop was explored in detail. Because growth cones also stop in nongraded patterns of ephrinA5, they apparently do not solely measure a fixed threshold

concentration but rather employ a kind of summation mechanism to determine their stop position. Interestingly, growth cones encountered higher total amounts of ephrinA5 and stopped at higher local ephrinA5 concentrations in steep gradients than in shallow gradients. Shallow gradients may be more efficient in causing growth cone stop with less repellent material because they cause less desensitization. The authors hypothesize that the growth cone's tendency to stop may increase with the amount of encountered ephrinA5, whereas the local ephrinA5 concentration counteracts this tendency by leading to a constant readjustment of sensitivity. The growth cone stops when both parameters reach a certain ratio. Such an integrative mechanism would be advantageous for the detection of a target zone in a gradient in the *in vivo* situation because it is rather insusceptible to variances in ephrinA expression levels. Moreover, it could explain how growth cones stop in a repellent gradient, although they are able to adapt to the repellent.

Substrate-bound gradients of ephrinA alone can thus be permissive up to a certain point for temporal RGC axons and confer a repulsive stop or avoidance signal beyond this point. Intriguingly, temporal growth cones react in the gradient assays as a uniformly sensitive population, and all nasal axons display the same extend of insensitivity. Along the naso-temporal axis of the retina, there is no graded sensitivity to ephrinA gradients, as one would expect based on the expression pattern of EphA receptors, but rather a sharp binary split in a responsive temporal and an unresponsive nasal half.

A certain graded response to ephrinA across the retina was observed by Hansen *et al.* (2004). When used in a uniform distribution as a substrate, ephrinA2 stimulates neurite outgrowth at low concentrations and inhibits outgrowth at high concentrations. Furthermore, the transition point from outgrowth promotion to inhibition is dependent on the naso-temporal position of the RGCs tested in this assay. Although these results give no direct information about gradient detection, they indicate that ephrinA is not only permissive, but also stimulates axon outgrowth at low concentration. This acts as a bifunctional cue, at least in initial neurite outgrowth, which may be governed by different mechanisms than growth cone guidance.

By no means can it be excluded that the invasion of the ephrinA gradient in the tectum/superior colliculus is also accomplished by other attractive guidance cues. Candidates are EphA receptors, which signal via ephrinAs (''reverse signaling'') and BDNF signaling via trkB (Marotte *et al.*, 2004; McLaughlin and O'Leary, 2005).

Taken together, gradient detection during topographic mapping implicates a higher complexity than gradient detection during a turning response because it not only requires the processing of directional, but also of positional information. Further research is needed to investigate if and how the sensing of direction and position differs with respect to the underlying signaling events.

3. Common Grounds and Diversity

3.1. Signaling pathways

The signal transduction during eukaryotic gradient detection shares a number of conserved pathways (Table 1.2). *Dictyostelium* cells and neutrophils have been frequently compared in terms of signaling, and their mode of gradient detection has been explained based on common models (Charest and Firtel, 2006; Skupsky *et al.*, 2005; Van Haastert and Devreotes, 2004). Fibroblast gradient detection is accomplished by similar signaling pathways, although it is much simpler as in amoeboid cells and restricted with respect to signal amplification, sensitivity, and adaptation (Schneider and Haugh, 2005). *Dictyostelium* cells, neutrophils, and fibroblasts all move toward the source of the gradient.

The picture is more complex in the growth cone because it detects and reacts to both attractive and repulsive graded cues, which moreover fall within several different classes of molecules. The combination of signaling pathways downstream of different guidance cues differs, but the core components are shared with other eukaryotic cell types. Signaling downstream of attractant and repellent gradients are remarkably similar. A switch in the directionality of the response is achieved by modifications of a signaling system rather than by completely novel mechanisms.

In chemotaxing cells, signaling events at the leading edge/pseudopod facing the source of the attractive gradient is generally antagonistic to the ones at the trailing edge/uropod.

Attractive growth cone turning is often effected by signaling events characteristic for the leading edge of chemotaxing cells. Signaling during repulsive turning or collapse in response to some axon guidance cues, on the other hand, resembles trailing edge signaling. However, "front" and "back" signaling are not completely independent pathways but have to interact and balance each other during detection of and response to a gradient. In line with this, the activity of many "front" signaling components is essential for repulsive turning and growth cone collapse and vice versa.

PI3K activity, for example, is required for attractive turning as well as for growth cone collapse in neurons, whereas it is exclusively coupled to the advance of the leading edge in other cell types. Rac is activated predominantly at the leading edge/pseudopod in migrating cells. Despite its front signaling role, it is also activated during growth cone collapse triggered by Sema3A, possibly fulfilling a distinct function.

In summary, growth cones employ the common signaling pathways of eukaryotic gradient detection in a sophisticated and context-dependent manner to respond in various ways to different guidance cues. In specific cases, signaling during growth cone turning or collapse can be directly compared to the scenario in other eukaryotic cells.

Table 1.2 Signaling components in eukaryotic gradient detection and their role in different model systems

	Dictyostelium	Neutrophils	Fibroblasts	Growth cones
PI3 kinase signaling	PIP3 is the first amplified readout of the external gradient. PI3K at the cell front and PTEN at the cell rear participate in a feedback loop to strengthen PIP3 accumulation at the leading edge.	PIP3 is the first amplified readout of the external gradient. PI3K is localized at the cell front; PTEN is distributed uniformly in polarized cells.	Active PI3K and PIP3 are concentrated at the leading edge, but they do not engage in a positive feedback loop.	PIP3 accumulates in attractive gradients of netrin-1 or BDNF at the gradient near side of the growth cone. Active PTEN in the peripheral domain of the growth cone is implicated in Sema3A-induced collapse.
Ca^{2+} signaling	cAMP causes transient intracellular back to rear Ca^{2+} gradients. Ca^{2+} enhances myosinII-mediated contraction.	Chemotactic factors induce an increase in intracellular Ca^{2+}. Ca^{2+} enhances myosinII-mediated contraction.	PDGF induces Ca^{2+} influx. Ca^{2+} enhances myosinII contraction.	Local Ca^{2+} elevations can induce both attractive and repulsive turning dependent on their magnitude. Not all guidance factors signal via Ca^{2+}.

Local protein synthesis	Required for cell motility, but not for gradient sensing.	Not known.	Local translation of β-actin mRNA at the leading edge is required for directionality and persistence of movement during chemotaxis.	Turning toward or away from several guidance factors depends on asymmetric protein synthesis in the growth cone.
Rho family kinase signaling	Rac activity is concentrated at the pseudopod.	Rac signaling at the pseudopod contrasts with RhoA signaling at the uropod.	Rac signaling at the leading edge contrasts with RhoA signaling at the trailing edge.	RhoA activity is implicated in growth cone collapse. Rac/cdc42 can mediate attractive turning.

For references, see text.

3.1.1. PI3 kinase/PIP3 signaling and events at the plasma membrane

PI3K signaling plays a significant role in all cell types discussed here but clearly shows differences with respect to its integration into the whole signaling machinery mediating gradient detection.

After stimulation with an external gradient, PI3K rapidly translocates to the cell membrane of *Dictyostelium* cells and neutrophils. Its product, PIP3, accumulates in a polarized fashion and sets up an amplified internal signaling gradient. PIP3 represents binding sites for a great number of PH domain containing signaling proteins, among those Akt and Rho GEFs. In both cell types, PIP3 enhances its own production by PI3K and thus establishes a positive feedback loop, which has been elucidated in great detail. Polarized PI3K signaling at the leading edge is sharpened by the signaling of its antagonist PTEN at the trailing edge in *Dictyostelium,* whereas in neutrophils the role of PTEN seems to be accomplished by SHIP1. Only found to be important for polarization and chemotaxis, the cellular distribution of SHIP has not yet been investigated. In neutrophils, the accumulation of PIP3 at the leading edge is further sustained by actin polymerization. Inhibition of actin polymerization in *Dictyostelium* cells does not impair PIP3 patterns during signaling polarization.

In fibroblasts chemotaxing toward PDGF, PI3K generates an amplified PIP3 gradient along the membrane as well. In contrast to amoeboid cells, PIP3 cannot enhance its own production by PI3K, indicating the lack of a PI3K/PIP3 feedback loop. PTEN is involved in the chemotactic response of fibroblasts toward PDGF, most likely as a negative regulator. So far, there is no data about its distribution in chemotaxing fibroblasts and its detailed influence on polarized PI3K signaling and local PIP3 accumulation. It is thus not completely understood how the amplified gradient of PIP3 is generated in chemotaxing fibroblasts.

PI3K activity is generally required for axon elongation and growth cone advance. A prevalence of PTEN over PI3K in the peripheral domain of the growth cone plays a role in Sema3A triggered growth cone collapse. In this context, growth cone collapse and retraction is correlated with signaling events typical for the trailing edge/uropod of migrating cells.

PI3K signaling during growth cone turning has been less investigated so far. Asymmetries in PI3K activity and local PIP3 accumulation may lead to attractive turning of the growth cone toward netrin-1 or BDNF. Although PI3K is a positive regulator of axonal elongation, its activity is required for growth cone collapse in response to MAG, slit-2, or ephrinA. The exact role and localization of active PI3K during the collapse response is not known but is likely to differ from the one during attractive turning.

An important feature about PI3K and PIP3 signaling is its localization to the plasma membrane. The establishment and maintenance of locally restricted membrane-linked signals is possibly further enhanced by cholesterol-enriched

microdomains or "lipid rafts." Membrane microdomains have been correlated with the development of cell polarity in neutrophils (Gomez-Mouton *et al.*, 2004; Seveau *et al.*, 2001) and were shown to enhance uropod and restrict pseudopod signaling (Bodin and Welch, 2005). Disruption of membrane microdomains can abolish the response of the growth cone to guidance factors such as BDNF, netrin-1, and Sema3A, most likely because of defective association of the respective receptors with microdomains (Guirland *et al.*, 2004).

3.1.2. Ca^{2+} signaling

Ca^{2+} signaling regulates both attractive and repulsive growth cone turning in response to some, but notably not all axon guidance factors. The magnitude of a local Ca^{2+} elevation determines the direction of the turn and is interrelated with internal cAMP levels. Ca^{2+} levels in the growth cones regulate numerous cytoskeleton interacting proteins (Gomez and Zheng, 2006; Henley and Poo, 2004) and can additionally influence local protein translation required for the response to some guidance factors (Leung *et al.*, 2006; Yao *et al.*, 2006).

Ca^{2+} signaling is far less involved, or at least far less considered during gradient detection of other cell types. Among migrating cells, chemotaxing fibroblasts probably come closest to navigating growth cones with respect to the impact of Ca^{2+} signaling. In fibroblasts migrating toward PDGF, Ca^{2+} influx was shown to signal upstream of calmodulin, an MLC kinase. The phosphorylation of MLC activates myosinII and thus regulates trailing edge contraction. This mechanism is crucial for cell motility and migration (Yang and Huang, 2005). Ca^{2+} elevations after PDGF stimulation activate CaMKII, an activator of the Rac1-specific GEF Tiam1 and cause Rac1-dependent membrane ruffling (Buchanan *et al.*, 2000). As described earlier, CaMKII is an important mediator of attractive growth cone turning toward Ca^{2+} (i.e., guidance cues triggering attractive intracellular Ca^{2+} elevations).

In *Dictyostelium* cells and neutrophils, Ca^{2+} signaling mainly regulates myosin-dependent contraction at the cell rear. When *Dictyostelium* cells respond to a cAMP gradient, internal Ca^{2+} levels transiently increase at the uropod. An internal rear to front Ca^{2+} gradient, which is apparent in some but not all cells, may support the accumulation of contractile myosinII and/or actin depolymerization at the uropod (Nebl and Fisher, 1997; Yumura *et al.*, 1996).

The stimulation of neutrophils with chemotactic factors also causes an increase in intracellular Ca^{2+}, whose polarity has not been investigated. This increase modulates integrin cell adhesion molecules and seems to be crucial for neutrophil migration on adhesive substrates. Like in *Dictyostelium*, Ca^{2+} in neutrophils might furthermore regulate myosinII-dependent contractile forces (Niggli, 2003).

3.1.3. Local protein translation

Localized protein translation is implicated in fibroblast chemotaxis and axon guidance. Synthesis of β-actin and subsequent increase in actin polymerization governs the advance of the fibroblast leading edge and growth cones turning toward attractive gradients of netrin-1 and BDNF. In both systems, src phosphorylates ZBP-1 and leads to the release of β-actin mRNA, which is required for its translation. Local translation of RhoA and cofilin mRNA, which is associated with Sema3A- and slit-2–dependent growth cone collapse, was so far only observed in growth cones. In the future, it will be interesting to investigate whether these mRNAs are also translated during repulsive turning.

Neither *Dictyostelium* (Clotworthy and Traynor, 2006) nor neutrophil gradient detection is substantially dependent on local translation of specific mRNAs. The role of local protein translation during gradient detection may have exclusively emerged in fibroblasts and neurons and is probably correlated with the more complex cytoskeletal organization in these cells. In neuronal growth cones, local translation is the most important way to control protein levels rapidly and independently from axonal transport (Piper and Holt, 2004). This might be the reason why local protein translation is particularly relevant for gradient detection in growth cones.

Although local protein translation is required for the response of the growth cone to guidance cues such as BDNF, netrin-1, and Sema3A, the very same responses (i.e., turning or collapse) can be performed by the growth cone without local protein translation after stimulation with ephrinA or LPA.

Local protein translation is also important for adaptation mechanisms in growth cones. However, local protein translation is not linked to adaptation in chemotaxing fibroblasts, whose ability to adapt to increasing PDGF concentrations seems to be far less prominent (Haugh, 2006).

3.1.4. Rho GTPases

Rho GTPases are common cytoskeletal regulators in migrating cells and steering axons. In neutrophils and fibroblasts, Rac activity at the pseudopod contrasts with RhoA activity at the uropod, leading to f-actin–based protrusion and myosinII-based contraction, respectively. In *Dictyostelium,* RhoA homologs have so far not been identified. The pattern of active Rac, however, resembles the one in neutrophils and fibroblasts. In all three cell types, Rac was shown to promote actin polymerization (Charest and Firtel, 2007).

Rac and cdc42 have been associated with the advance of growth cone filopodia and lamellipodia. Most attractive axonal guidance cues indeed lead to the activation of Rac and cdc42. Two GEFs specific for Rac1, Trio and Tiam1, which are essential for the membrane ruffling of fibroblasts

stimulated with PDGF (Buchanan *et al.*, 2000; Debreceni *et al.*, 2004; Sander *et al.*, 1999), signal also downstream of netrin-1 and BDNF, respectively (Barallobre *et al.*, 2005; Miyamoto *et al.*, 2006). During attractive growth cone turning, Rac and cdc42 seem to play a role in actin polymerization comparable to the one in migrating cells (Dickson, 2001; Yuan *et al.*, 2003).

However, there are also reports that repulsive cues such as Slit (Wong *et al.*, 2001; Yang and Bashaw, 2006), Sema3A (Toyofuku *et al.*, 2005), and ephrinA2 (Jurney *et al.*, 2002) activate Rac and/or cdc42. Whether Rac generally mediates membrane endocytosis during growth cone collapse instead of actin polymerization, as it was proposed for the collapse triggered by Sema3A and ephrinA2 (Jurney *et al.*, 2002), has not been fully investigated. Distinct effects of Rac on growth cone advance versus collapse could also arise by distinct localization of Rac activation in the growth cone or a different intracellular context.

RhoA, which signals predominantly at the back of chemotaxing cells, plays a well-established role in growth cone collapse and repulsive turning. RhoA and Rock signal downstream of ephrinA5 (Wahl *et al.*, 2000), Sema3A (Gallo, 2006), and LPA (Yuan *et al.*, 2003). They enhance myosinII-mediated contraction and thus establish a signaling pathway which is also found in the trailing edge/uropod of migrating cells.

3.2. Signal amplification

Signal amplification is a prevalent property of signaling cascades. During eukaryotic gradient detection, not only the given signal, but also rather small signal differences have to be amplified. There has to be biased signal amplification across the cell to amplify the absolute as well as the relative concentration differences of an external gradient (Fig. 1.11). In the cell types discussed here, this is accomplished to different extents and by slightly differing mechanisms.

Efficient signal amplification arises from feedback loops, which are integrated at different levels into the major signal transduction pathways mediating gradient detection.

In *Dictyostelium* and neutrophils, a small local initial rise in PIP3 at the gradient near side of the cell leads to further accumulation of PIP3 via the activation of PI3K. A similar feedback loop is likely to be present in turning growth cones. In fibroblasts, however, PIP3 does not stimulate the activity of PI3K but signals exclusively downstream of PI3K.

Positive feedback loops can lead to the amplification of absolute concentration differences, but they are not sufficient to explain the amplification of relative concentration differences. For this, signaling downstream of the chemotactic cue has to be selectively enhanced at the gradient near side of the cell and/or selectively inhibited at the gradient far side. As modeled by

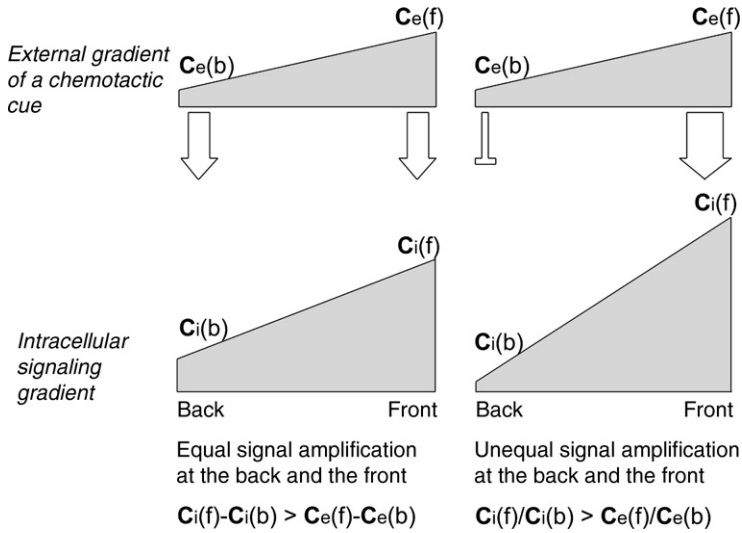

Figure 1.11 Signal amplification during gradient detection. An external gradient of a chemotactic cue is defined by the concentration Ce(b) at the back of the cell and the concentration Ce(f) at the front of the cell. If the extracellular signal gets equally amplified at the back and the front of the cell, the proportion of the concentrations of intracellular effectors at the front Ci(f) and the back Ci(b) stays the same as the proportion of the external concentrations Ce(f) and Ce(b), although the absolute difference of the concentrations increases. To amount to an amplification of the relative difference of the concentrations, there has to be an asymmetry in the signal amplification at the back and the front. In the most extreme case, the signal is highly amplified at the front and not transduced at all at the back.

Meinhardt (1999), a self-enhanced reaction coupled to competing antagonistic reactions is sufficient to establish polarized patterns within a cell. This process, which has been referred to as local activation/global inhibition mechanism (Devreotes and Zigmond, 1988; Parent and Devreotes, 1999; Skupsky *et al.*, 2005), can be achieved by the segregation of signaling domains mutually excluding each other.

In *Dictyostelium* cells, the signaling antagonism between front and rear arises mainly from the reciprocal distribution of PI3K and PTEN activity. Taking the internal gradient of PIP3 as a reference for the internal signaling, it was determined that the relative external cAMP gradient gets approximately sevenfold amplified (Janetopoulos *et al.*, 2004; Xu *et al.*, 2005b). Notably, this amplification is accomplished independently from cytoskeletal rearrangements and morphological polarization.

In neutrophils and fibroblasts, sharpening of the internal signaling gradient seems to also occur at the level of Rho GTPases. Because Rac and RhoA mutually inhibit each other, an initially small bias in their spatial activity pattern can finally establish a strong intracellular signaling asymmetry.

This signaling asymmetry causes asymmetries in cytoskeletal architecture, meaning morphological polarization, which again enhances the signaling polarization. Whereas neutrophils are equal with *Dictyostelium* in their efficiency to detect and amplify a gradient, fibroblasts are less specialized in responding to gradients independently of the absolute PDGF concentration.

Compared with amoeboid cells, which were reported to detect relative concentrations gradients down to 1% across the cell length, a growth cone can only detect a minimal external cAMP concentration gradient of 10% across its length (Lohof *et al.*, 1992). This value may vary for different guidance molecules and crucially depends on the involved signaling pathways. Although it was not directly measured like in *Dictyostelium* cells, an amplification of the relative external gradient is likely to happen in growth cones as well through a number of feedback loops in the different signaling pathways. Preliminary data suggests PIP3 can stimulate its own accumulation in growth cones as it does in chemotaxing cells (J. Henley, personal communication). During attractive growth cone turning caused by high local Ca^{2+} elevations, Ca^{2+} stimulates its own rise via a positive feedback loop between Ca^{2+} and cAMP. A robust asymmetry in local protein translation of β-actin mRNA in gradients of netrin-1 or BDNF is established by the synergistic action of mRNA transport and cooperating gradients of src and eIF-4E activation.

The antagonistic signaling events at the gradient near versus the gradient far side of a turning gradient have been less well described than the front-rear signaling asymmetry in migrating cells.

3.3. Adjustment of sensitivity/adaptation

Signal amplification allows cells to detect minimal concentration gradients. However, as soon as the signal input partially uncouples from the internal signal output, absolute sensing is no longer possible. If the external signal is not amplified proportionally to its strength but rather in a switchlike fashion, the cells respond within certain range to low concentration gradients the same way as to high concentration gradients.

A similar behavior results from adaptation. Adaptation may partially depend on the modulation of signal amplification but can also result from a number of other mechanisms such as the downregulation of signaling at various levels during a strong stimulus. Because a number of phenomena result from or are linked to adaptation, it is helpful to define adaptation as the adjustment of the sensitivity according to the signal strength to prevent conceptual confusion. This adjustment comprises desensitization during strong stimulation as well as sensitization during weak or absent stimulation. Furthermore, resensitization may also occur after a period of desensitization, that is, after the shutdown or absence of an internal signal in the persisting presence of a strong external signal.

Notably, adaptation introduces a temporal sensing element in eukaryotic gradient detection, which is principally based on a spatial-sensing mechanism.

Adaptation has been observed in migrating eukaryotic cells as well as in growth cones, but it is certainly best studied and probably also most prominent in growth cones (Section 2.4.3).

After prolonged stimulation with cAMP, *Dictyostelium* cells display a decreased sensitivity for the chemoattractant, which is coupled to a reduction of cAMP affinity and a loss of ligand binding of the cAMP receptor cAR1 (Caterina *et al.*, 1995).

Similarly, neutrophils can adapt to different chemoattractant concentrations and display a transient loss of responsiveness after rapid concentration changes (Zigmond and Sullivan, 1979). The sensitivity of a neutrophil toward a chemotactic factor and the cell's ability to adapt can also be influenced by the presence of other factors (Foxman *et al.*, 1997; Lin *et al.*, 2005). As mentioned in Section 2.2.1, the balancing of adaptation to a whole set of chemotactic factors is crucial for proper guidance of neutrophils to different sites of infection as well as for the guidance over long distances.

From this point of view, neutrophils face a similar complexity as growth cones, which often have to navigate in superimposed distributions of different guidance cues. Highly suggestive by the *in vivo* expression patterns in the nervous system, the integration of different guidance cues by growth cones has been demonstrated *in vitro*. For example, combined gradients of NGF and NT-3 are synergistic and can guide axons over a longer distance than one gradient alone (Cao and Shoichet, 2003). Furthermore, NGF can counteract repulsive signaling. When applied to axons before treatment with Sema3A, NGF reduces Sema3A triggered growth cone collapse (Dontchev and Letourneau, 2003).

Adaptation mechanisms thus have to be considered not only in response to a single chemotactic factor, but also during cross talk of several factors.

3.4. Biological and functional context

The varieties in eukaryotic gradient detection make sense in the biological context of the different cell types.

The gradient of cAMP attracting solitary *Dictyostelium* cells is relatively simple in regard to its function: It serves to guide the cells to a central point of aggregation. Because the cAMP gradient emitted by the cells serving as an aggregation center oscillates in its concentration (Dormann *et al.*, 2000), the chemotaxing cells have to detect the external gradient over a wide range of concentrations. *Dictyostelium* has developed strong signal amplification and responds to gradients with a substantial internal polarization, which allows maintaining persistently the directionality of migration during changes in the external gradient. There is no requirement for sensing absolute cAMP concentrations or to charge the cAMP signal against other chemoattractants.

Mammalian neutrophils are equally sensitive to gradients as *Dictyostelium* cells and detect the latter by very similar mechanisms. Additionally, they are able to integrate several guidance cues to coordinate the invasion of rivaling infection sites in the organism.

Fibroblasts attracted by PDGF, however, detect gradients in a simpler way than amoeboid cells. Migrating through the tissue and a 3D array of extracellular matrix, their speed of chemotaxis is much slower than the one of neutrophils. The invasion of a dermal wound by fibroblasts takes several days during which the PDGF gradient may adopt an optimal shape for chemotaxis with the help of successive PDGF degradation by the progressing fibroblasts. In addition to the attracted fibroblasts, wound healing is supported by PDGF triggered proliferation of already present cells (Haugh, 2006).

In terms of function, gradients guiding growth cones differ in two major aspects from the discussed gradients relevant for cell migration. First, they are attractive as well as repulsive. Repulsive gradients can either direct the growth cone away from the gradient or allow the axon to proceed up to a certain point, like in the case of the ephrinA gradient in the developing tectum/superior colliculus. Second, gradients in the nervous system provide not only directional, but also positional information because they are implicated in topographic mapping. The requirement to detect positional information entails restrictions in signal amplification and adaptation, which in turn may limit the growth cones sensitivity.

4. Concluding Remarks

Chemotaxis is an important task for eukaryotic cells in different biological and functional contexts. Migrating cells as well as neuronal growth cones are specialized to detect gradients of chemotactic factors by a spatial gradient sensing mechanism. During spatial gradient sensing, the external gradient has to be translated into an internal signaling gradient across the length of the cell or the growth cone, respectively. This signaling asymmetry or internal polarization normally results in a morphological polarization and the establishment of a cytoskeletal architecture capable of directional movement. Signal amplification, and more specifically, biased processing of the external signal at the gradient near side and the gradient far side of the cell provide for a high sensitivity in the detection of shallow and/or low concentrated gradients. Furthermore, adaptation during gradient sensing allows for maintaining this sensitivity over a broad range of concentrations.

Gradient detection during eukaryotic cell migration and axon pathfinding meets similar demands. It is therefore plausible that the underlying signaling is conserved in many aspects and shares common mechanistic features, for example, the employment of feedback loops during signal

amplification and internal signal polarization. Signaling pathways such as the PI3K/PIP3 pathway function in all systems discussed here. Localized protein translation of specific mRNAs plays a role in gradient detection and chemotaxis of fibroblasts as well as neuronal growth cones.

The focus on common ground and diversities during eukaryotic gradient detection tells more about the singularity of gradient sensing in a certain cell type and, at the same time, can fill the gaps in the knowledge about one system with the help of experimental findings from a related system. In the future, it is promising to further explore the similarities and differences in cell migration and axon guidance for new impulses in both fields of research.

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