Mechanisms by which molecules guide axons

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Current Opinion in Cell Biology 1990, **2**:907–913

Introduction

The ability of growing axons to find their way has captivated biologists throughout this century. Numerous experiments with vertebrate and invertebrate embryos have suggested that axons navigate by recognizing local cues, i.e. signals present on cell surfaces, in extracellular matrices, or diffusing from nearby sources. Such signals are currently thought to consist of molecules to which axons are specialized to respond. Currently, numerous candidates for axon guidance molecules have been proposed and include extracellular matrix proteins, integral membrane proteins, and several classes of secreted proteins, some of which can also associate with cell surfaces or extracellular matrices. Much about the structure and expression of these 'potential axon guidance molecules' has been reviewed elsewhere (e.g. Jessell, *Neuron* 1988, 1:3–13; Sanes, *Annu Rev Neurosci* 1989, 12:491–516; Lander, *Trends Neurosci* 1989, 12:189–195; Schachner, *Ciba Found Symp* 1989, 145:156–169). Such molecules are typically expressed at times and locations *in vivo* where growing axons encounter them, and display biological activities appropriate to the task of guiding axons. Generally, these biological activites have been studied in tissue culture systems, although genetic approaches are also being used now (Hedgecock *et al.*, *Neuron* 1990, 2:61–85; Elkins *et al.*, *Cell* 1990, 60:565–575). *In vitro* observations that suggest that a molecule could guide axons include: growth of neurites (the *in vitro* counterparts of axons and dendrites) towards or away from a source of the molecule; accurate tracking of neurites along a substratum path to which the molecule has been absorbed; acceleration or inhibition of neurite outgrowth in response to the molecule; specific adhesion of neurites to cells or other neurites that bear the molecule. Establishing such *in vitro* effects has, in the case of some molecules, required the development of ingenious assays. As even more sophisticated tests are devised, the number of molecules considered to be potential axon guidance cues will no doubt increase. Yet, the fact that the current list of these molecules is already rather substantial has prompted some investigators to consider what mechanisms underlie the actions of these molecules.

Interest in this question has been growing in recent years. One probable reason for the interest is the increased attention that cell biologists are paying to the structure and function of growth cones, the motile organelles that steer axons (e.g. Smith, *Science* 1988, 242:708–715; Mitchison and Kirschner, *Neuron* 1988, 1:761–772; Bray, *Curr Opin Cell Biol* 1989, 1:87–90; Lankford *et al.*, *Curr Opin Cell Biol* 1990, 80–85). A second reason is the growing evidence that axon guidance molecules are structurally complex (e.g. possessing multiple kinds of binding sites), and variable (e.g. alternatively spliced and/or modified), which suggests that their molecular interactions are not simple (Lander, 1989). A third reason is the growing suspicion that old assumptions about the mechanism of action of some of these molecules are in need of revision. A fourth, and particularly important reason, is that most of the known putative axon guidance molecules are not unique to the nervous system, but are encountered in a wide variety of normal and neoplastic tissues; accordingly, it is reasonable to suspect that the cellular mechanisms by which molecules guide axons are merely examples of the mechanisms by which cells, in general, respond to morphogenetic signals.

The following review presents some of the issues that have been raised by investigations into the mechanisms by which molecules guide axons. Most of these investigations have focused on cell-surface and extracellular matrix molecules, and the discussion below also emphasizes these 'surface-bound' molecules, a partial list of which is given in Table 1. For the sake of brevity, these proteins are sometimes referred to in the following paragraphs as 'axon guidance molecules'. The reader should keep in mind, however, that 'potential axon guidance molecules' would be a more accurate term, because none of these molecules has yet been proved to act as a guidance cue *in vivo*.

The dynamic behavior of growth cones

The responsibility for steering axon growth falls almost entirely on growth cones. Located at the tip of growing axons, these structures receive the signals that guide growth. They are also the primary locus of growth, movement and shape change in elongating axons, insofar as they are the site of motility, secretion, and assembly of organelles and macromolecules into the nascent axon.

Abbreviations

G-protein—GTP-binding protein; **NCAM**—neural cell adhesion molecule.

 ISSN 0955-0674

Table 1. Cell surface and extracellular matrix proteins suspected to control the guidance of axons. The developmental expression, localization, biological activities, and deduced structures of these molecules suggest that they have a role in guiding axons.

Extracellular matrix glycoproteins	Ca^{2+}-dependent cell adhesion molecules	Ca^{2+}-independent cell adhesion molecules	Cell-surface inhibitors of neurite outgrowth
Laminin	N-cadherin	NCAM	Myelin-associated
S-laminin	Other cadherins	L1	Others
Merosin		F11/contactin/F3	
Fibronectin		G4	
Tenascin		Neurofascin	
J1-160/180		Neuroglian	
Thrombospondin		Fasciclins I, II and III	
Collagen IV		Myelin-associated glycoprotein	
		Axonin I	

Information about the individual properties of many of these molecules has been reviewed elsewhere (e.g. Jessell, *Neuron* 1988, 1:3–13; Sanes, *Annu Rev Neurosci* 1989, 12:491–516; Lander, *Trends Neurosci* 1989, 12:189–195; Schachner, *Ciba Found Symp* 1989, 145: 156–169; Schwab, *Exptl Neurol* 1990, 109:2–5; Sanes *et al.*, *J Cell Biol* 1990, 111:1685–1699). See also [3,5–7,9–13].

Interposed between these functions must lie a sophisticated decision-making mechanism capable of performing under stringent constraints of speed (growth cones respond to many signals in a matter of seconds to minutes) and sensitivity.

The nature of this mechanism can only be guessed at based on what is known about growth cone structure and function. At the very least, the fact that the only access of growth cones to the genetic and protein synthetic apparatus of neurons is via a pathway along which communication (by retrograde and anterograde transport of material along the axon) is relatively slow, suggests that transcriptional and translational events are unlikely to participate in the decision-making functions of growth cones.

Growth cones display a rather modular architecture — a central base from which variable numbers of motile projections, the filopodia and lamellipodia extend. It has been argued (Mitchison and Kirschner, 1988) that the leading, motile regions of growth cones possess a distinctive type of cytoplasm (recently termed 'kinetoplasm'), that is fundamentally different from cytoplasm elsewhere in the axon. The kinetoplasm is highly dynamic, possessing a cytoskeleton rich in bundles and meshworks of actin filaments that appear to be in constant motion. The axonal cytoplasm (axoplasm), in contrast, contains bundled microtubules, and is apparently much more stable.

The relative sharpness of the transition zone between kinetoplasm and axoplasm suggests that the two states of cytoplasm are mutually exclusive (Smith, 1988). Nevertheless, continual conversion of kinetoplasm into stable axoplasm (a process referred to as consolidation or maturation) appears to be a fundamental step in the process of axon elongation (Goldberg and Burmeister, *J Cell Biol* 1986, 103:1921–1931). Interestingly, consolidation has been observed to be a cyclical process. At first, the lamellipodia and filopodia of a growth cone extend, move about, and retract in similar patterns of seemingly random activity. Then, a small region of growth cone, containing perhaps a single lamellipodium with associated filopodia, becomes 'stabilized, i.e. it fails to retract. Next, consolidation of the stabilized region occurs. Finally, other regions of the growth cone resorb, while new kinetoplasm forms at the leading edge of the newly consolidated structure (Goldberg and Burmeister, 1986).

This picture of growth cone behaviour implies that the direction in which an axon grows is continuously determined by the region of the growth cone where stabilization and consolidation take place. Accordingly, for a growth cone to be guided towards an immobilized extracellular guidance cue, the contact of a structure such as a filopodium or lamellipodium with that cue must somehow result in the consolidation of that structure becoming more probable. In addition, some sort of crosstalk must occur among growth-cone structural elements to explain why, usually, only one part of a growth cone consolidates at a time, or why the encounters of one or a few filopodia can sometimes trigger global events such as the collapse of the entire growth cone (Kapfhammer and Raper, *J Neurosci* 1987, 7:201–212).

Thus, two major tasks in elucidating the mechanisms by which molecules guide axons are to understand how events at the plasma membrane act locally to influence the fate of the underlying kinetoplasm, and to identify intracellular interactions that coordinate the activity of growth-cone components. As discussed below, current hypotheses tend to differ primarily in the degree to which these processes are thought to be mediated via mechanical as opposed to chemical means.

The role of adhesion in axon guidance

Long before individual molecules that influence axon guidance were identified, it was appreciated that growth cones recognize and respond to the physical topography of their environment. Bumps, troughs, obstacles, etc.

can all guide neurite growth *in vitro* (cf. Hollerbeck and Bray, *Annu Rev Cell Biol* 1988, 4:43–61). In the mid-1970s, Letourneau (*Dev Biol* 1975, 44:92–100) extended these findings to include a physicochemical feature of the environment: 'adhesiveness'. Using plastic surfaces onto which patterns of molecules such as polyornithine and palladium were deposited, he demonstrated that growth cones, when faced with a choice between regions of the substratum treated with dissimilar molecules, invariably chose the more adhesive (in this context, adhesion is taken to refer to the resistance of growth cones or cells to detachment, by force, from the substratum).

Bray and his colleagues provided a compelling explanation for this phenomenon by pointing out that the structural elements of growth cones (filopodia, lamellipodia) pull against the substratum (Bray, *J Cell Sci* 1979, 37:391–410; Lamoureux *et al.*, *Nature* 1989, 340:159–162), building up a mechanical tension that in itself can be a stimulus for neurite growth (Bray, *Dev Biol* 1984, 102:379–389; Dennerll *et al.*, *J Cell Biol* 1989, 109:3073–3083). The maximum tension that can result when a growth cone pulls against the substratum should be directly related to the degree of adhesion; accordingly, by sensing levels of tension, growth cones indirectly sense differences in substratum adhesiveness. An appealing feature of the tension/adhesion model is that the tensions produced by individual filopodia or lamellipodia are expected to sum, as a result of purely mechanical coupling, in a manner that ensures a single coordinated response by the growth cone as a whole. This effect specifically predicts that, on uniform substrata, growth cones should tend to grow straight ahead (parallel to the axon shaft), branch only rarely, and, when branching split into parts heading in more or less opposite directions (Bray, In *Cell Behaviour* edited by Bellairs R *et al.* Cambridge University Press, 1982, pp 299–317; Bray, *Trends Neurosci* 1987, 10:431–434). These predictions generally fit with observations.

In the late 1970s and early 1980s, several cell culture-derived 'factors' were described that promoted neurite extension when adsorbed to a tissue culture substratum (see Lander, *Mol Neurobiol* 1987, 1:213–245 for discussion). When these substances were applied in distinct patterns, growth cones restricted their growth to those patterns (Collins and Lee, *J Neurosci* 1984, 4:2823–2829), just as they did in Letourneau's experiments when confronted with patterns of adhesive molecules. When the active component of the neurite extension factors was identified as the extracellular matrix protein laminin, a protein capable of mediating cell attachment, it seemed logical to assume that laminin acted upon growth cones by increasing their adhesion to the substratum. Only recently has this assumption been tested, and the results have been surprising.

Both Adler *et al.* (*Dev Biol* 1985, 112:100–114) and Hall *et al.* (*J Cell Biol* 1987, 104:623–634), for example, pointed out that retinal neuron attachment to laminin was relatively weak, compared with other molecules that did not possess the same ability as laminin to stimulate or guide neurite outgrowth. Gundersen (*Dev Biol* 1987, 121:423–431) measured growth cone adhesion directly by comparing the degree to which growth cones grow on substrata containing certain extracellular matrix molecules with the degree of growth cone adhesion shown towards those same molecules. Growth cones were observed to choose substrata containing laminin over other molecules or combinations of molecules that were more, less, or equally as adhesive as laminin. A subsequent study, using interference reflectance microscopy, indicated that the number of close growth cone–substratum appositions formed on laminin substrata was equal to or less than the number formed on other substrata (Gundersen, *J Neurosci Res* 1988, 21:298–306); this result further supported the idea that laminin is not unusually adhesive with regard to growth cones.

These data can be interpreted in two ways. One possibility is that current methods of measuring adhesion, such as cell attachment, growth cone distraction and interference reflectance microscopy, do not accurately reflect the degree of adhesion 'sensed' by the growth cone itself. The other possibility is that, sometimes, mechanisms other than adhesion are involved in the response of growth cones to substratum-associated molecules. Both interpretations are defensible.

Adhesion: a time-dependent process

If growth cones 'sense' adhesion by pulling against the substratum and measuring the tension produced, it is useful to consider which factors influence that measurement. One such factor is the amount of contractile force produced by the growth cone. It is currently suspected that force is produced by the action of a plasmalemmal motor (e.g. myosin I) on the bundles of actin filaments which are so abundant in filopodia and lamellipodia (Mitchison and Kirschner, 1988). A second factor is the position at which the contractile force is applied; moving actin bundles appear to be localized beneath the membranes of filopodia and lamellipodia (Smith, 1988) [1] and some observations suggest that filopodia bear much of the contractile load at their tips (Tsui *et al.*, *Proc Natl Acad Sci USA* 1985, 82:8256–8260). A third factor is the time period over which contractile force is applied.

The potential importance of timing merits special consideration. Because filopodia and lamellipodia undergo a repeating cycle of extension, contact with the substratum and retraction, contraction against the substratum occurs discontinuously. The typical length of this cycle suggests that most of the force exerted by filopodia and lamellipodia is exerted against substratum contacts that are rarely more than a few minutes old (Bray and Chapman, *J Neurosci* 1985, 12:3204–3213). This raises an interesting question: are the measurements of growth cone adhesion that are currently made in the laboratory (most of which measure adhesion mediated by relatively long-lived contacts) good predictors of the level of adhesion experienced by growth cone structures pulling against 'fresh' contacts? The clever work

of McClay and his colleagues [2] strongly suggests not. These investigators have demonstrated that the initial adhesion experienced between a cell and its substratum and the same adhesion experienced many minutes later can differ by more than an order of magnitude [2]. Moreover, the profile of adhesion development over time can be strikingly different for contacts mediated by different substratum-bound molecules. For example, in the case of cells that strengthen their fibronectin-mediated contacts more than 10-fold over 15 min at 37°C, contacts mediated by tenascin actually decline in strength over the same time course [2]. Large time-dependent decreases in adhesive strength have also been observed when cerebellar neurons were allowed to attach to the tenascin-related nervous-system glycoproteins J1-160/180, but not when the same cells were permitted to attach to laminin [3]. Thus, any attempt to order a set of substratum-associated molecules according to their adhesiveness might produce very different results, depending on when and how adhesion measurements are made.

In view of these considerations, direct measurements of the changes in the magnitude of adhesion over time at transient filopodial and lamellipodial contacts will need to be made before the role of adhesion and tension in axon guidance can be properly tested. In principle, such measurements are possible, but new methods of measurement may first need to be devised. Until then, one can only speculate about how adhesion develops on the basis of current knowledge. Initial adhesion seems to be an energy-independent process, the extent of which may simply reflect the net energy of the binding of substratum molecules to the receptors that they happen to encounter. In contrast, changes in adhesion that occur over time seem to result from energy-dependent processes which involve the actin cytoskeleton [2]; at least one of these processes is likely to be the active recruitment of receptors from other membrane locations to the site of contact. Thus, the extent to which the magnitude of filopodial and lamellipodial adhesion exceeds the magnitude of initial adhesion may depend on whether or not cell surface molecules are actively translocated in these structures, and if they are, at what rates they are moved. Notably, recent observations indicate that active translocation of cell-surface proteins, including neural cell adhesion molecule (NCAM), occurs in growth cones, both centripetally and centrifugally, at rates of more than one micron per second [1].

The role of intracellular signalling in axon guidance

The idea that the interplay between substratum adhesion and tension controls axon guidance is an attractive hypothesis, but not the only one that is plausible. Growth cones are clearly capable of chemotaxis, i.e. orientated growth towards sources of diffusible, soluble molecules, against which they cannot pull (e.g. Gundersen and Barret, *J Cell Biol* 1980, 87:546–554). Growth cones are also able to adhere strongly without necessarily orientating themselves. For example, when growth cones of retinal neurons contact the neurites of sympathetic neurons, tightly adherent filopodial contacts often form, even though the result of the contact is the inhibition of neurite growth, not the promotion of orientated growth (Kapfhammer and Raper, 1987).

For both of these examples, there are reasons to think that chemical second messengers have a role in mediating growth cone responses. Chemotaxis, for example, is generally thought to result from receptor-mediated signalling events (Devreotes and Zigmond, *Annu Rev Cell Biol* 1988, 4:649–686). Consistent with this view, nerve growth factor, a diffusible molecule towards which axons will grow *in vitro* and *in vivo* (Levi-Montalcini, *Prog Brain Res* 1976, 45:235–258; Gundersen and Barrett, 1980), is thought to act by triggering the production of second messengers, although the identities of these second messengers are not fully known. In the case of cell contact-mediated growth cone arrest, the morphological responses of growth cones are remarkably similar to the responses induced by agents that elevate intracellular calcium levels (Kater *et al.*, *Trends Neurosci* 1988, 11:315–321). Certainly, intracellular calcium appears to play an important role in the morphology and behavior of growth cones (Goldberg, *J Neurosci* 1988, 8:2596–2605) [4]. Significantly, localized increases in calcium concentrations within growth cones have been shown to spread rapidly (Mills and Kater, In *The Assembly of the Nervous System.* Alan R. Liss, 1989, pp 65–80), potentially explaining how contacts made by a few filopodia could influence the motility of an entire growth cone. Whether or not calcium is indeed responsible for the effects of membrane-associated molecules that mediate growth cone arrest should become clearer in the near future, as several groups are now working on purifying and characterizing these molecules (Schwab, *Exp Neurol* 1990, 109:2–5) [5–7].

The possibility that the effects of intracellular second messengers have a role in the navigational machinery of growth cones is also suggested by an examination of the constituents of growth cones. These have been found to be rich in many of the molecules associated with intracellular signalling: GTP-binding proteins (G-proteins) [8], protein kinase C (Girard *et al.*, *Dev Biol* 1988, 126:98–107), *src* kinase (Maness *et al.*, *Proc Natl Acad Sci USA* 1988, 85:5001–5005), ion channels (Cohan *et al.*, *J Neurosci Res* 1985, 13:285–300; Streit and Lux, *J Neurosci* 1989, 9:4190–4199), and enzymes involved in phospholipid and arachidonate metabolism (Van Hooff *et al.*, *J Neurosci* 1988, 8:1789–1795; Frame *et al.*, *J Cell Biol* 1989, 109:212a) are all present, in many cases in considerable abundance.

If soluble chemoattractants and growth-inhibitory molecules act on growth cones through second messengers, is it possible that cell-surface and extracellular matrix-associated guidance molecules could work in the same way? There are only a few arguments against this possibility. One is that the interplay between adhesion and tension works so well that there is little need for anything else, i.e. tension itself may be 'the ideal second messen-

ger' as Mitchison and Kirschner (1988) have noted. Another argument is derived from a recent report that the quantitative relationship between the ability of NCAM-expressing fibroblasts to support neurite growth and the amount of surface NCAM they express is a steep one, with an apparent threshold level of NCAM required before any neurite growth effect is seen [9]. Such a relationship would be expected if NCAM acts primarily in an adhesive manner, because receptor-mediated adhesion is inherently a highly cooperative process. In contrast, such cooperativity is not as easy to explain if NCAM functions by producing a chemical signal. A third argument, and probably the most forceful one of the three, is rooted in the structural analysis of receptors for axon guidance molecules. These receptors, which are so far thought to include the integrins, the cadherins, and the cell-adhesion molecules of the immunoglobulin superfamily, have amino acid sequences quite unrelated to those of proteins known to activate second messenger pathways, such as growth factor receptors, oncogenes, ion channels, etc. (Lander, 1989). So far, the biochemical functions that have been directly associated with these receptors have been limited to the binding of extracellular ligands, and the binding of components of the actin cytoskeleton via cytoplasmic domains (e.g. Horwitz *et al.*, *Nature* 1986, 320:531–533; Pollerberg *et al.*, *Cell Tiss Res* 1987, 250:227–236; Hirano *et al.*, *J Cell Biol* 1987, 105:2501–2510).

These arguments notwithstanding, a number of studies suggest a close relationship between the actions of substratum-associated guidance molecules and the generation of intracellular signals. In a timely piece of work, Schuch *et al.* [10] have shown that the treatment of the neuronal cell line PC12 with monovalent or polyvalent antibodies to the neural cell adhesion molecules NCAM and L1 can elicit changes in inositol phosphate levels, an increase in intracellular calcium levels, and a decrease in intracellular pH. Evidence was presented that at least some of these changes were mediated by a pertussis toxin-sensitive G-protein. Other recent reports have suggested that laminin substrata can elicit rapid changes in both protein phosphorylation and inositol phosphate turnover by neurons and neuronal cell lines [Weeks and Kleinman, *J Cell Biol* 1989, 109:322a; Plantefaber and Lander, *J Cell Biol* 1990, 111:(abstract) in press]. Laminin substrata have also been reported to activate tyrosine hydroxylase, an intracellular enzyme that is strongly regulated by phosphorylation (Acheson *et al.*, *J Cell Biol* 1986, 102:151–159). In addition, permitting neurons to attach to laminin or fibronectin substrata appears to alter the way in which they respond to the second messenger-mediated effects of polypeptide growth factors (Edgar *et al.*, *EMBO J* 1984, 3:1463–1468; Millaruelo *et al.*, *Dev Brain Res* 1988, 38:219–228).

If the effects of cell surface-associated and extracellular matrix molecules on cells and growth cones are mediated, at least in part, by second messengers, it will be important to understand the molecular steps that connect recognition events at the cell surface to signalling events inside the cytoplasm. One possibility is that receptors such as integrins and cell-adhesion molecules interact directly with signalling enzymes (kinases, phosphatases, G-proteins, phospholipases, etc.) through interactions that have so far gone undetected. Another possibility is that the interaction with signalling molecules is indirect, perhaps mediated through the cytoskeleton. For example, if some signalling enzymes and their substrates both bind to the cytoskeleton (Scott *et al.*, *Biochem J* 1989, 263:207–214; Papadopoulos and Hall, *J Cell Biol* 1989, 108:553–567; Dingus *et al.*, *J Cell Biol* 1989, 109:190a), then events that organize or cluster cytoskeletal filaments could potentially increase the rates of enzyme–substrate encounters, thereby augmenting signal production. A third possibility is that stretch-sensitive ion channels, which have recently been detected in growth cones (Sigurdson and Morris, *J Neurosci* 1989, 9:2801–2808), directly transduce mechanical tension into membrane depolarization, thereby triggering calcium influx and consequent calcium-mediated signalling.

A final possibility is that the effects of axon guidance molecules may not be entirely mediated by those classes of receptors that are currently believed to do so. There are, in fact, independent reasons to explore this last possibility, as discussed below.

Multireceptor interactions at the growth cone surface

Although the major cell-surface receptors for molecules of the extracellular matrix are thought to be members of the integrin family, and the major receptors for cell adhesion molecules, such as N-cadherin, NCAM and L1, are thought to be the same or other cell adhesion molecules, the binding of many of these molecules to other types of cell-surface components has also been observed. Most commonly reported has been their binding to glycosaminoglycans, but interactions with glycolipids, cell-surface glycosyltransferases and other cell-surface proteins have also been detected (see Lander, 1989 for discussion).

Laminin is a good example: potential neuronal receptors for laminin include more than four different integrins, heparan sulfate proteoglycans, cell-surface galactosyltransferase, and several other non-integrin proteins (Edgar, *Trends Neurosci* 1989, 12:248–251; Lander, 1989)[11]. Some experiments have suggested that the responses of cells and growth cones to laminin may involve several of these receptors acting in concert. For example, the initiation of neurite extension in response to laminin can be completely blocked by antibodies against the β1 integrin subunit (Bozyczko and Horwitz, *J Neurosci* 1986, 6:1241–1251), but also partially blocked by highly specific inhibitors of cell-surface galactosyltransferase [11], as well as by concentrations of heparin that are sufficient to inhibit laminin–heparan sulfate interactions (Letourneau *et al.*, *Soc Neurosci Abstr* 1986, 12:1334; Chernoff *et al.*, *Tissue Cell* 1988, 20:165–178; O Ariel and AD Lander, unpublished observations). There is also evidence that integrins and heparan sulfate proteoglycans

both have a role in the binding of cells and the extension of neurites in response to fibronectin (Saunders and Bernfield, *J Cell Biol* 1988, 106:423–430; Letourneau *et al.*, 1986), as well as evidence that integrins and a chondroitin sulfate proteoglycan both have a role in the interaction of neural cells with tenascin (Hoffman and Edelman, *Proc Natl Acad Sci USA* 1987, 84:2523–2527; Bourdon and Ruoslahti, *J Cell Biol* 1989, 108:1149–1155).

Among the cell–cell adhesion molecules NCAM-mediated neuronal adhesion is blocked not only by reagents that interfere with homophilic NCAM–NCAM interactions, but also by heparin, antibodies against cell-surface heparan sulfate proteoglycans, antibodies against a heparin-binding domain within NCAM, peptides derived from the heparin-binding domain, and by genetic deletion of the heparin-binding domain (Cole *et al.*, *J Cell Biol* 1985, 100:1192–1199; Cole *et al.*, *Nature* 1986, 320:445–447; Cole *et al.*, *J Cell Biol* 1986, 103:1739–1744; Cole and Akeson, *Neuron* 1989, 2:1157–1165) [12]. Myelin-associated glycoprotein is another example of a heparin-binding neural cell adhesion molecule (Fahrig *et al.*, *EMBO J* 1987, 6:2875–2883). A recent report indicated that N-cadherin, a homophilic calcium-dependent adhesion molecule thought to play a major role in axon outgrowth at cell surfaces, is specifically bound by the cell-surface enzyme N-acetylgalactosaminylphosphotransferase [13]. This finding, along with a study on the role of galactosyltransferase in the recognition of laminin [11], suggests that surface glycosyltransferases in general may have important roles in the recognition of axon guidance molecules.

Why should a growth cone possess a multiplicity of receptors for guidance molecules? Finding an answer to this question depends a great deal on one's choice of model to explain how growth cones respond to guidance cues. For example, if guidance molecules act primarily or in part by generating intracellular signals, then multiple types of cell-surface receptors may exist to extend the range of second messengers that can be produced by a single guidance cue. One difficulty with this explanation is that proteoglycans, glycolipids and glycosyltransferases have not traditionally been thought of as molecules that can mediate intracellular signalling. It has been pointed out recently, however, that binding of molecules to glycolipids on the surface of some cells can trigger calcium influx (Dyer and Benjamins, *J Cell Biol* 1990, 111:625–633), and that some neural proteoglycans are attached to the cell surface via a phospholipid linkage that, when cleaved can generate a second messenger (Carey and Evans, *J Cell Biol* 1989, 108:1891–1897; Herndon and Lander, *Neuron* 1990, 4:949–961).

Alternatively, if growth cones are guided primarily by 'sensing' adhesion, the presence of multiple types of receptors would probably serve to increase adhesion by increasing the avidity of binding. Thus, an NCAM molecule bound to the cell surface via both NCAM–NCAM and NCAM–heparan sulfate interactions would be bound more tightly than an NCAM molecule bound via either interaction alone (Cole *et al.*, 1985). A somewhat more complicated possibility is that cells use multiple receptors not so much to increase the final amount of adhesion they develop, but specifically to regulate the time period over which adhesion develops.

A still more complicated possibility, but perhaps the most intriguing one, is that, by having more than one type of receptor at their disposal, growth cones can independently modulate the binding of membrane to substratum without coordinately affecting the binding of the cytoskeleton to the cell membrane. As a hypothetical example, if one patch of membrane contains 1000 laminin-binding integrins while another contains 100 integrins and 900 galactosyltransferase molecules, both patches might achieve the same level of adhesion, but the first will be connected to the actin cytoskeleton in ten times as many locations as the second (based on the expectation that integrins associate with actin filaments but glycosyltransferases do not). Why should the number of membrane–cytoskeletal linkages matter to a growth cone? For one thing, Mitchison and Kirschner (1988) have concluded that, in order for the actin filaments in a growth cone to generate tension and still undergo a continual rearward flux, they must constantly slip against their own membrane linkages. Such a necessity for slippage could easily set a limit on the number of membrane connections that are tolerated by an actin filament. If this number is low, growth cones could benefit greatly by achieving a high degree of adhesion through combinations of receptors that do and do not interact with the cytoskeleton.

Conclusions

Clearly, a great deal is known currently about the process of axon guidance: the structure, behavior and cytoskeletal dynamics of growth cones have been subjected to considerable scrutiny, and many molecules with the potential to guide axons have been described. The evidence discussed above suggests that plausible hypotheses do exist for explaining how such molecules guide axons. Testing these hypotheses is an important goal for the future. Achieving this goal, however, will require answers to several difficult questions. What levels of adhesion do constantly moving structures, such as filopodia and lamellipodia, actually experience? What controls the time period over which cell adhesion develops? How do cell–cell and cell–matrix contacts initiate or modify the production of intracellular signals? What happens when cells bear multiple types of receptors for a single cell-surface or extracellular matrix protein? That these are fundamental questions in cell biology should not be surprising, as the molecules that are believed to guide axons are, to a large extent, the same as those that are thought to control the adhesive, motile and morphogenetic behaviors of cells in general.

Annotated references and recommended reading

- • Of interest
- •• Of outstanding interest

1. •• SHEETZ MP, BAUMRIND NL, WAYNE DB, PEARLMAN AL: **Concentrations of membrane antigens by forward transport and trapping in neuronal growth cones.** *Cell* 1990, 61:231–241.

The motions of NCAM and of another cell-surface glycoprotein on the surfaces of living growth cones are analyzed; gold-coupled antibodies are used to bind these proteins, and time-lapse video microscopy is used to follow the movements of the gold particles in real time. In addition to the previously well documented retrograde movement of extracellular particles and intracellular filaments, compelling evidence is presented that proteins can be actively translocated at considerable rates towards leading edges and towards the tips of filopodia. Models of how both forward and backward movements can occur in the same growth cone structures are presented.

2. •• LOTZ MM, BURDSAL CA, ERICKSON MP, MCCLAY DR: **Cell adhesion to fibronectin and tenascin: quantitative measurements of initial binding and subsequent strengthening response.** *J Cell Biol* 1989, 109:1795–1805.

The time-dependence of cell adhesion to fibronectin and tenascin is measured using a centrifugation assay that measures the force required to dislodge cells. Strikingly different responses are seen to these two extracellular matrix molecules; fibronectin contacts strengthen with time, whereas tenascin contacts weaken with time. Remarkably, on mixed fibronectin/tenascin substrata, weakening in cell adhesion still occurs. This work provides an exceptionally clear documentation of what others have called the 'anti-adhesive' effect of tenascin.

3. • MORGANTI MC, TAYLOR J, PESHEVA P, SCHACHNER M: **Oligodendrocyte-derived J1-160/180 extracellular matrix glycoproteins are adhesive or repulsive depending on the partner cell type and time of interaction.** *Exp Neurol* 1990, 109:98–110.

The tenascin-related glycoproteins J1-160 and J1-180 are shown to be adhesive or 'anti-adhesive' depending on the test cell type, suggesting that different cell types may possess different receptors for these glycoproteins. Also documented is the time-dependence of the 'anti-adhesive' effect.

4. • LANKFORD KL, LETOURNEAU PC: **Evidence that calcium may control neurite outgrowth by regulating the stability of actin filaments.** *J Cell Biol* 1989, 109:1229–1243.

Extensive observations are made on the effects of calcium removal and calcium ionophores on the behavior, morphology, and ultrastructure of growth cones. The data support the 'calcium-setpoint' hypothesis: proper growth-cone motility requires an optimum level of calcium, with inhibition of motility resulting from either increases or decreases in free calcium.

5. • DAVIES JA, COOK GMW, STERN CD, KEYNES RJ: **Isolation from chick somites of a glycoprotein fraction that causes collapse of dorsal root ganglion growth cones.** *Neuron* 1990, 2:11–20.

One of several recent reports in which the ability of a molecule to induce growth cone collapse is used as the basis for a partial purification of an active factor. In this case, the molecule derives from somites, the structures through which early dorsal root ganglion axons grow. In development, growth cones appear to avoid the posterior half of each somite. Interestingly, the molecule purified here binds the lectin peanut agglutinin, a specific marker for the posterior half-somite.

6. • RAPER JA, KAPFHAMMER JP: **The enrichment of a neuronal-growth-cone-collapsing activity from embryonic chick brain.** *Neuron* 1990, 2:21-29.

This report details the development of an assay in which the visual assessment of growth-cone collapse is used to purify growth-inhibitory molecules. The partial purification of a factor from the brain is also described.

7. • COX EC, MULLER B, BONHOEFFER F: **Axonal guidance in the chick visual system: posterior tectal membranes induce collapse of growth cones from the temporal retina.** *Neuron* 1990, 2:31–37.

An activity that collapses the growth cones of retinal neurons is detected in membranes derived from the posterior part of the optic tectum (the superior colliculus of the brain). Growth cones from the temporal side of the retina are very sensitive to this activity, whereas nasal retinal growth cones are resistant. In normal development, axons from the retina grow to the tectum, and terminate there in exactly the correct positions to form a map of retinal space on the surface of the tectum. The results in this paper and elsewhere suggest that avoidance responses, between temporal retinal growth cones and the posterior tectum, and between temporal retinal growth cones and nasal retinal axons, may be major forces in building the map.

8. • STRITTMATTER SM, VALENZUEAL D, KENNEDY TE, NEER EJ, FISHMAN MC: **G_0 is a major growth-cone protein subject to regulation by GAP-43.** *Nature* 1990, 344:836–841.

The GTP-binding protein G_0 is shown to be a major component of purified brain growth cones. Evidence is presented that GAP-43, a major growth-cone protein with intriguing properties, but an as yet unknown function, may be an activator of G_0. This report adds to the growing list of signalling molecules that are abundant components of growth cones.

9. • DOHERTY P, FRUNS M, SEATON P, DICKSON G, BARTON CH, SEARS TA, WALSH FS: **A threshold effect of the major isoforms of NCAM on neurite outgrowth.** *Nature* 1990, 343:464–466.

A careful study of the quantitative relationship between the expression levels and the biological activity of NCAM at the cell surface. 3T3 fibroblasts are transformed with NCAM expression constructs, and many different clones are isolated. The levels of NCAM expression by clones are compared with the clones' ability to serve as a substrate for neurite outgrowth, and a steep, highly cooperative relationship is found. The significance of this finding is discussed.

10. •• SCHUCH U, LOHSE MJ, SCHACHNER M: **Neural cell-adhesion molecules influence second messenger systems.** *Neuron* 1989, 3:13–20.

PC12 cells are exposed to antibodies directed against NCAM or L1, and changes in inositol phosphate levels, intracellular calcium and cytoplasmic pH result. Monovalent antibody fragments have a similar effect. This paper is a straightforward and timely attempt to study the relationship between cell contact and intracellular signalling in neurons.

11. •• BEGOVAC PC, SHUR BD: **Cell-surface galactosyltransferase mediates the initiation of neurite outgrowth from PC12 cells on laminin.** *J Cell Biol* 1990, 110:461–470.

A variety of highly specific inhibitors of the cell-surface galactosyltransferase system are shown to partially block the neurite outgrowth-promoting effects of laminin. The effect is primarily due to a decrease in the initiation of neurites in response to laminin. This is one of several recent papers on this intriguing cell-surface enzyme, and a good reminder that more than one type of cell-surface component is likely to participate in the neuronal response to laminin.

12. • REYES AA, AKESON R, BREZINA L, COLE GJ: **Structural requirements for neural cell-adhesion molecule–heparin interaction.** *Cell Regulation* 1990, 1:567–576.

Difficulties in obtaining and structurally defining glycosaminoglycans make it difficult to study their biological activities. Here, the role of a previously identified NCAM–heparan sulfate interaction is studied by manipulating NCAM structure. Substitutions or a deletion in the heparin binding domain of NCAM do not block surface expression, but do eliminate binding to heparin. Intriguingly, these mutant NCAMs were ineffective at promoting retinal cell attachment, suggesting that the NCAM–heparan sulfate interaction may play more of a part in NCAM-mediated cell adhesion than previously predicted.

13. • BALSAMO J, LILIEN J: **N-cadherin is stably associated with an acceptor for a cell-surface N-acetylgalactosaminylphosphotransferase.** *J Biol Chem* 1990, 265:2923–2928.

A cell-surface glycosyltransferase is shown to associate with and act upon (glycosylate) N-cadherin. Although the functional consequences of this interaction are still not known, the apparent parallel with galactosyltransferase and laminin [11] raises the possibility that cell-surface glycosyltransferases are involved in a wide variety of interactions with cell-surface and extracellular matrix proteins.