MOLECULAR MECHANISMS OF AXON GROWTH AND GUIDANCE

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INTRODUCTION

The mechanisms of axonal growth and guidance have been under investigation for over a century, since Cajal first discovered the growth cone. It was not until the 1980s, however, that much headway was made into the molecular basis of outgrowth and guidance. Since then a multitude of factors appearing on cell surfaces, in the extracellular matrix, and diffusing through the brain have been described. Many of these factors promote axonal outgrowth, and many appear to be involved in growth cone guidance. Here, we attempt to review the status and mechanisms of these factors. We are aware that we have not covered all the putative molecular components that have been implicated in growth promotion and guidance. For example, we do not address, due to our focus on the external factors that promote outgrowth of neurites and guide axons, the possible roles of proteases secreted by the growth cones in neurite advancement and penetration into the extracellular matrix (see Pittman et al 1989). We hope, however, that our effort will provide at least a framework for organizing many of the known growth and guidance factors and for cataloging newly discovered ones.

Early opinions that axonal growth was regulated by gradients of chemo- tropic molecules, by a plethora of labeled pathways, or by expression sequences of one or two cell adhesion molecules (CAMs) have given way to the view that each of these classes of control is exercised, and that the pattern of axonal growth that is found is the result of concerted action by numerous factors. In particular, three major classes of protein have been found to promote and guide neurite growth in vitro and/or axonal growth in vivo: soluble trophic factors and chemotropic agents, various constituents of the extracellular matrix (ECM), and several different categories of cell surface molecules. Recent reviews have discussed these molecules in some detail (Berg 1984; Lander 1987; Jessell 1988; Reichardt et al 1989). In this review we shall use the term “neurite outgrowth-promoting molecules” to refer to molecules encountered by the neurons in their environment, rather than to neuronal cell surface receptors, which are dealt with separately.
PROTEINS THAT PROMOTE NEURITE GROWTH

One of the emerging themes in the study of neurite growth-promoting factors is that regulation of growth involves multiple molecular mechanisms acting in concert. The first suggestion for this idea came from studies using antibodies to block the function of growth-promoting cell adhesion molecules (CAMs) and ECM proteins (Tomaselli et al 1986, 1988; Chang et al 1987; Bixby et al 1987, 1988). These studies demonstrated that several molecular mechanisms, involving both ECM proteins and different types of CAMs, exist on the same cell and can promote growth from the same neuron. For example, ECM proteins, the Ca\(^{2+}\)-independent neural CAM, (NCAM), and the Ca\(^{2+}\)-dependent CAM, N-cadherin, can function to promote neuronal growth on the surfaces of myotubes (Bixby et al 1987). Later similar conclusions from molecular genetic studies in Drosophila showed that mutation of a gene coding for a single adhesion molecule had a small effect on development (Zinn et al 1988; Bieber et al 1989; see also Gertler et al 1989; Elkins et al 1990). One interpretation of these results is that there is redundancy in molecular mechanisms of axon growth, presumably to guard against gross defects caused by a single mutation. Such an interpretation is supported by the results in flies (Bieber et al 1989; Elkins et al 1990). It is also possible, however, that these multiple mechanisms represent not simply redundancy, but a combinatorial process, such that the desired growth is achieved only by the concerted function of several molecule-receptor systems. This idea is consistent with the antibody results mentioned above and is also supported by the observation that the function of the CAM L1 is augmented by binding to NCAM in vitro (Kadmon et al 1990). It has been suggested that one function of NCAM may be to modulate interactions involving many different adhesion molecules (Rutishauser et al 1988). Synergism among growth-promoting molecules need not be limited to those that are substrate-bound. For example, laminin (LN) and nerve growth factor (NGF) may be able to synergize in the induction of sympathetic neurites (Tomaselli et al 1988).

The individual proteins that have been implicated in axon growth are listed in Table 1. In developing a list of neurite growth-promoting proteins, several difficulties arise, some of which are due to our lack of sufficient knowledge, and some of which are due to problems with definitions. For example, the soluble growth-promoting agents listed in Table 1 have generally been demonstrated to have many different actions on the same cell. In many assays, it is difficult to separate the induction of neuronal differentiation or the enhancement of neuronal survival from the induction of neurite growth per se. Similarly, ECM glycoproteins and CAMs often
# Table 1  Neurite growth promoters

<table>
<thead>
<tr>
<th>Name</th>
<th>(M_r) (kd)</th>
<th>Distribution</th>
<th>Comments</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Neurotrophic factors</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NGF</td>
<td>13 (monomer)</td>
<td>CNS (especially hippocampus), peripheral tissues</td>
<td>Active as dimer</td>
<td>Davies et al (1987)</td>
</tr>
<tr>
<td>BDNF</td>
<td>13</td>
<td>CNS, peripheral tissues</td>
<td></td>
<td>Lindsay (1988)</td>
</tr>
<tr>
<td>NTF-3</td>
<td>13</td>
<td>CNS, peripheral tissues</td>
<td>NGF, BDNF, NT-3 comprise a family</td>
<td>Maisonpierre et al (1990)</td>
</tr>
<tr>
<td>S100β</td>
<td>10</td>
<td>Serum, brain (glia)</td>
<td>Active as dimer</td>
<td>Burgess &amp; Maciag (1989)</td>
</tr>
<tr>
<td>IGF-II</td>
<td>8</td>
<td>Serum, brain</td>
<td></td>
<td>Hatten et al (1988)</td>
</tr>
<tr>
<td><strong>ECM glycoproteins</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LN</td>
<td>850</td>
<td>CNS, peripheral tissues</td>
<td>Family includes s-LN and merosin</td>
<td>Kligman &amp; Marshak (1985)</td>
</tr>
<tr>
<td>TSP</td>
<td>400</td>
<td>Serum, peripheral tissues, embryonic CNS</td>
<td></td>
<td>Rinderknecht &amp; Humbel (1978)</td>
</tr>
<tr>
<td>Collagens</td>
<td>95 (monomer)</td>
<td>Peripheral tissues</td>
<td>Many different forms</td>
<td>Recio-Pinto et al (1986)</td>
</tr>
<tr>
<td>Ig superfamily</td>
<td>NCAM</td>
<td>CAM</td>
<td>CNS, PNS, muscle, glia</td>
<td></td>
</tr>
<tr>
<td>---------------</td>
<td>------</td>
<td>-----</td>
<td>------------------------</td>
<td></td>
</tr>
<tr>
<td>M/L. MAG</td>
<td>180, 140</td>
<td>120</td>
<td>Oligodendrocytes, Schwann cells, Nerve fibers, some glia, subsets of axons</td>
<td></td>
</tr>
<tr>
<td>TAG-1</td>
<td>135</td>
<td></td>
<td>Restricted in CNS</td>
<td></td>
</tr>
<tr>
<td>Others</td>
<td>125</td>
<td></td>
<td>CNS, peripheral neurons, muscle, lens, heart</td>
<td></td>
</tr>
<tr>
<td>Neurofascin</td>
<td>185, 160</td>
<td>80</td>
<td>Subsets of axons, Restricted in CNS</td>
<td></td>
</tr>
<tr>
<td>P54</td>
<td>85</td>
<td>66</td>
<td>Brain (neurons?)</td>
<td></td>
</tr>
<tr>
<td>P30</td>
<td>30</td>
<td></td>
<td>Related to HMG-1 DNA-binding protein</td>
<td></td>
</tr>
</tbody>
</table>

**Notes:**
- Some forms GPI-linked; fasciclin II is homologue
- Neuroginan is homologue of related proteins F3 is homologue, some forms GPI-linked; floor plate expression
have potent adhesion-promoting activity, and many workers have failed to distinguish between this effect and the induction of neurite growth. For the proteins listed in Table 1, evidence suggests a role in neurite growth that is independent of effects on survival or cell adhesion. We now discuss these molecules individually.

**Soluble Neurotrophic Agents**

**NGF, BDNF, NT3** The soluble growth-promoting factors so far identified are called neurotrophic molecules because of their many and various effects on the survival and differentiation of neurons (e.g. Berg 1984). The best characterized of these, nerve growth factor (NGF), was in fact the first molecule described that clearly promoted axon growth (Greene & Shooter 1980). Studies of adult neurons, which do not require NGF for survival but remain responsive, show that NGF can promote growth of differentiated neurons despite being dispensable (Lindsay 1988). NGF, however, may not be involved in the initial axonal growth of responsive cells (Davies et al 1987; Large et al 1986). Recently it has become clear that NGF is a member of a family of growth-promoting molecules, and two other members, brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3), have been identified and characterized (Barde 1988; Leibrock et al 1989; Maisonpierre et al 1990; Rosenthal et al 1990). The three proteins show 50–60% sequence identity in the portion of the precursor molecule believed to be the active factor. NGF, BDNF, and NT-3 have similar but unique temporal and spatial distributions and are apparently active on distinct but overlapping populations of neurons (Barde 1988; Masonpierre et al 1990; Rosenthal et al 1990). There is reason to believe that other members of this family exist (Rosenthal et al 1990).

**FGF** Fibroblast growth factor (FGF) is a member of a family of heparin-binding growth factors with a wide range of specificities and activities, both in the nervous system and elsewhere (Burgess & Maciag 1989). Although it has no signal sequence and its means of secretion is controversial (Abraham et al 1986), it can clearly be found in the extracellular milieu, including the ECM (Rogelj et al 1989), and is a potent effector of neuronal survival and neurite outgrowth in vitro (Unsicker et al 1987; Hatten et al 1988). In some cases the effects of FGF on neurite growth can be separated from those on neuronal survival (Hatten et al 1988).

**S100β** S100β is a glial-derived protein that is a member of a family of small acidic proteins (Van Eldik et al 1988). Its activity was originally defined as neurite extension factor, or NEF (Kligman 1982), and this factor was subsequently shown to consist of homodimers of S100β (Kligman & Marshak 1985; see also Winningham-Major et al 1989). Although this protein
is potent and can initiate a program of neurite growth with a short exposure to the neurons (Winningham-Major et al. 1989), it is not certain whether it is a true neurite induction factor or an inducer of neuronal differentiation. 

**IGF-II** Insulin-like growth factor II (IGF-II) is a protein closely related to insulin (Rinderknecht & Humbel 1978), found in serum and in the CNS, that has a number of trophic effects on some populations of neurons. IGF-II can promote neuronal growth in a manner that seems to be independent of trophic effects, although this issue is not fully resolved (Recio-Pinto et al. 1986).

**ECM Proteins**

**LN, FN** Several of the most abundant components of the ECM including laminin (LN), fibronectin (FN), and the collagens are capable of promoting neurite growth in vitro (for reviews see Rogers et al. 1989; Lander 1987; Reichardt et al. 1989). These proteins are multifunctional and clearly capable of promoting both cell adhesion and various aspects of cellular differentiation in the nervous system and elsewhere (see Ruoslahti & Pierschbacher 1987, for examples). Nevertheless, the ability of ECM proteins to stimulate neurite growth can be separated from cell adhesion (e.g. Tomasselli et al. 1986). Although the responsiveness of different neuronal types to different ECM proteins varies, all three of these major ECM components can promote growth from several different types of neurons (Rogers et al. 1989; Lander 1987). This, in combination with the broad distribution of the ECM proteins, has led to the view that no specific growth function can be attributed to them. One reason for thinking this conclusion premature is that all three proteins exist in multiple forms and, in the case of LN and FN, a single form can clearly have many different functional domains, possibly with distinct receptors. Examination of the gene for FN reveals that close to a dozen distinct polypeptides can be produced (Kornblihtt et al. 1985), and a single form of FN can have multiple neurite-promoting domains (Humphries et al. 1988; Rogers et al. 1989). Similarly, LN can exist in various combinations of A, B1, and B2 chains (Edgar et al. 1988; Klein et al. 1988), and several different functional domains with different receptors have been described (e.g. Sonnenberg et al. 1990). It is also likely that the association of LN with other proteins and proteoglycans alters its functional state (Chiu et al. 1986). To add to the complexity, it has recently become clear that LN is not an individual molecule, but part of a family of proteins with at least two other members, s-LN (Hunter et al. 1989) and merosin (Ehrig et al. 1990). Both proteins have restricted and unique distributions, and merosin can stimulate neurite growth (Manthorpe et al. 1990). It seems likely that additional members of the LN family will be discovered.
Another component of the ECM, thrombospondin (TSP), has been implicated in the control of neurite growth. TSP, like LN and FN, is a multifunctional glycoprotein that can mediate cell adhesion and spreading of a variety of cell types (Lawler & Hynes 1986; Taraboletti et al 1987). Its distribution during development is consistent with a role in neurite growth (O'Shea & Dixit 1988), and it was recently shown to directly promote axonal growth in vitro (Osterhout & Higgins 1990; Neugebauer et al 1991). Various collagens have limited neurite outgrowth activity in vitro, although some forms may be excellent growth substrates for subpopulations of neurons (e.g. Kleitman et al 1988). It is possible that these proteins act in conjunction with other ECM components.

**CAMs**

**NCAM, MAG**  Many of the proteins now recognized as axonal growth factors are CAMs, and many of these are members of the immunoglobulin (Ig) superfamily. In fact, the regulation of cell adhesion probably represents the original function of Ig superfamily proteins (Williams & Barclay 1988). These molecules can be either integral membrane proteins, or linked to the membrane through glycoprophatidylinositol (GPI). The most extensively characterized of these CAMs, NCAM (Edelman 1986), has a demonstrated ability to promote neurite growth. This has been shown with antibody-blocking studies (Bixby et al 1987) and through transfection of the gene for NCAM into heterologous cells (Doherty et al 1989, 1990), although the purified protein is relatively inactive in most assays (e.g. Lagenauer & Lemmon 1987; Bixby & Jhabvala 1990). Besides its relatedness to Ig superfamily molecules, NCAM has several repeat units homologous to the type III domains of FN (Cunningham et al 1987). The apparent homologue of NCAM in *Drosophila*, fasciclin II, was discovered by a search for axon growth-promoting molecules, and it may serve such a function (Harrelson & Goodman 1988). A CAM with significant homology to NCAM, the myelin-associated glycoprotein (MAG), is traditionally thought of as being involved in myelin formation (see Salzer et al 1987), but this protein has also been implicated in neurite growth through cDNA transfection studies (Johnson et al 1989). It should be noted that Thy-1, a simple Ig superfamily member related to NCAM, has been implicated in neurite outgrowth as well (Leifer et al 1984).

**L1, NgCAM, F11/contactin, TAG-1, neurofascin**  A second group of Ig superfamily molecules comprises L1, NgCAM, F11/contactin, and TAG-1, although other members of this group seem certain to be described. A fifth member of this group, called NgCAM-related molecule (Nr), has recently been cloned (M. Grumet et al, submitted). Nr is 40% identical both to
NgCAM and to L1, which themselves are only 40% identical (Burgoon et al. 1991). The members of this group of proteins are all similar in primary sequence (30–50%) as well as overall structure, with each containing six Ig-type repeat units and four or five FN-type III domains (Moos et al. 1988; Brummendorf et al. 1989; Ranscht 1988; Furley et al. 1990). On the basis of sequence identity, it is possible to group L1 (NILE), NgCAM, and Nr in one subfamily, with TAG-1 and F11/contactin in another. A member of this family has also been described in Drosophila, called neuroglian (Bieber et al. 1989), which may be the Drosophila homologue of L1. Another CAM, neurofascin (Rathjen et al. 1987), shows a similar pattern of expression to L1 and F11/contactin (widespread distribution on neuronal fibers) and may be a member of this family, although its primary structure is unknown. In contrast to the broad distribution of these three, TAG-1 shows a greatly restricted distribution during embryogenesis and may be involved in guidance functions of the floor plate (see below). L1, which has been identified a number of times in different species, is likely to be the same molecule as NILE, G4, and 8D9 (see Lemmon et al. 1989, for discussion), although the situation may be more complex (Burgoon et al. 1991). The role of L1 in the induction of neurite growth has been demonstrated through the use of the purified protein as a substrate (e.g. Lagenauer & Lemmon 1987) and by antibody blocking studies (Chang et al. 1987; Bixby et al. 1988; Seilheimer & Schachner 1988). F11/contactin and neurofascin have been implicated in the induction of axon growth by antibody blocking experiments, and purified TAG-1 has been shown directly to promote neurite growth from some (but not all) neurons (Chang et al. 1987; Rathjen et al. 1987; Furley et al. 1990). The mouse homologue of F11/contactin is the protein F3 (Gennarini et al. 1989).

**Cadherins** The members of the Ig superfamily mentioned above belong to the general class of Ca$^{2+}$-independent CAMs. A second major group of CAMs is the Ca$^{2+}$-dependent CAMs, or cadherins (Takeichi 1988), of which five or six members have clearly been identified, and others of which certainly exist (Takeichi 1990; Crittenden et al. 1988; Ranscht & Dours 1989; C. Kintner, personal communication). The major cadherin in the nervous system appears to be N-cadherin, probably identical to molecules originally identified as N-calCAM and A-CAM (see Bixby & Zhang 1990, for references). N-cadherin has been implicated in neurite growth by all three of the criteria currently in use: inhibition of neurite growth by antibodies to the protein (Bixby et al. 1987, 1988; Tomaselli et al. 1988; Neugebauer et al. 1988), enhancement of the neurite-promoting activity of cells transfected with the N-cadherin cDNA (Matsunaga et al. 1988), and
induction of neurite growth using the purified protein (Bixby & Zhang 1990). Other cadherins are likely to be present in the regions through which axons must navigate, and they might also influence growth, either in a positive or a negative fashion (Ranscht & Dours 1989; M. Takeichi, personal communication).

P84, P30 Other proteins listed in Table 1 are apparently unrelated to those mentioned above. P84 is a CAM with a restricted distribution in the embryonic CNS, like that of TAG-1, which later becomes more widely expressed (Chuang & Lagenauer 1990). Like TAG-1, it is expressed in the floor plate region, and promotes neurite growth from some but not all neurons when used as a purified substrate. Preliminary analysis suggests that P84 is not identical to any of the previously characterized CAMs (C. Lagenauer, personal communication), but it remains to be seen whether it may belong to one of the families identified above. P30, a brain protein bound to membranes in a heparin-dependent fashion, promotes neurite growth from brain neurons (Rauvala & Pihlaskari 1987; Rauvala et al 1988). This molecule is widespread in late embryonic rat brain, but is down-regulated shortly after birth. Sequence analysis demonstrates that this protein is identical to the DNA-binding protein HMG-1. Which of the two suggested properties is primary remains unclear (Rauvala et al 1988). Finally the secreted protein, axonin-1, which also has a membrane-associated form (Ruegg 1989), has recently been shown to act as an adhesive and possibly a neurite outgrowth-promoting molecule when purified (Stoekli et al 1991).

Perspectives

How many molecules exist that promote axonal growth? In the past, views on the number varied considerably, from two or three, whose functions are regulated by modulation of their abundance and structure (Edelman 1986), to more than 50 (Bastiani et al 1985). Clearly, this question is unanswerable at present, but several points can be made. First, the number proposed will surely vary depending on one's definition of a growth-promoting molecule. Second, the number of such molecules may not be astronomical because many of those noted in Table 1 have been isolated multiple times, in some cases using quite different strategies. Third, one of the themes that emerges from a perusal of Table 1 is that many of the proteins listed are members of families, including the LNs, the NGF-related molecules, the Ig superfamily molecules, and the cadherins. In several cases, it is clear that different members of these families have subtly different distributions and/or functional properties. On evolutionary grounds, one might predict that different members of a family of related
factors will be used on distinct but evolutionarily related pathways. Whether or not this idea has any merit will surely be uncovered as students of neural evolution turn their attention to these molecules. The actual number of growth-promoting molecules, whatever it turns out to be, will have a much higher functional complexity than indicated by this base number if appropriate consideration is given to (a) temporal and spatial modulation of the expression of these proteins, alone and in combinatorial association, (b) similar regulation of their neuronal receptors, and (c) the potential for functional changes imposed on an individual molecular species by alternative splicing and post-translational modifications.

RECEPTORS FOR THE GROWTH PROMOTERS

The growth-promoting molecules discussed above must influence axon elongation through binding to specific cell-surface receptors, which in turn must transduce this signal to the neuron's interior. Early hypotheses about the mechanism of this transduction focused on neuron-substrate adhesion, and the specific hypothesis developed that growth promotion (and guidance) by molecules like LN and FN was due to their ability to increase this adhesion (Letourneau 1975; Hammarback et al 1988). The studies cited (and others) clearly demonstrated the necessity for adequate adhesiveness if neurites were to form. Now the strict adhesion hypothesis seems unlikely to be correct on the basis of several lines of evidence. (a) For physiologically relevant substrates, there is little or no correlation between the strength of neuron-substrate or growth cone-substrate adhesion and the ability of the substrate to induce process outgrowth (Gunderson 1987; Hall et al 1987; Lemmon et al 1990). (b) Antibodies to neuronal receptors for these proteins can inhibit neurite growth even in conditions in which strong adhesion to the substrate and lamellipodial formation is allowed (e.g. Tomaselli et al 1986). (c) Antibodies to some, but not all, neuronal cell surface receptors can promote neurite growth when used as culture substrates (Leifer et al 1984; Hall et al 1987). The data therefore suggest that the involved receptors transduce growth signals through intracellular messenger systems and the cytoskeleton, just as is the case for other cell surface receptors. What are the receptors involved in transduction of the signals provided by the proteins in Table 1?

Neurotrophic Receptors

NGF-R In the case of NGF, a 75-kd receptor (NGF-R) has been purified and cloned, and a great deal is known about its synthesis and regulation (for review, see Springer 1988). Although two affinity forms of the NGF-R
have been described, it seems likely that only one receptor protein exists and that its function is modulated by association with other proteins (Hempstead et al 1989). The NGF-R is not related to any other family of cell surface receptors, so the transduction mechanism associated with it cannot be inferred by analogy. Indeed, the key to this transduction may lie in the proteins with which NGF-R associates. The structures of the receptors for BDNF and NTF-3 have not been described, but we may guess that they will prove to have some functions in common with the NGF-R. The recent finding that a receptor-class tyrosine kinase is activated by NGF suggests the possibility that there is more than one high affinity NGF receptor protein (Kaplan et al 1991).

**ECM Receptors**

**INTEGRINS** A number of potential receptors have been described for the ECM proteins, LN, FN, and the collagens (Edgar 1989). Of these, the receptors whose function is best correlated with the induction of neurite growth belong to a superfamilly of cell-surface heterodimers known as the integrins (Hynes 1987; Ruoslahti & Pierschbacher 1987). Integrins are segregated into families based on different α subunits that have a common β subunit. Antibodies to the β1 integrin subunit have been shown to inhibit neurite outgrowth on LN, FN, collagens, and complex ECMs (Bozyczko & Horwitz 1986; Tomaselli et al 1986; Hall et al 1987). In studies of cell adhesion to these proteins, it has been found that several different integrin heterodimers can recognize the same ECM protein, including receptors with distinct β subunits (e.g. Wayner & Carter 1987; Sonnenberg et al 1990; Tomaselli et al 1990). In general, it is not known which particular integrins are involved in neurite growth of a neuronal cell type on a particular ECM protein (but see Tomaselli et al 1990), however, it seems reasonable to expect that at least some of the transduction mechanisms discovered may be shared by different integrins.

**CAM Receptors**

**HOMOPHILIC RECEPTORS** Members of the Ig superfamily often bind to other Ig superfamily molecules (Williams & Barclay 1988), either to a second member protein (heterophilic interaction), or to themselves (homophilic interaction). Intercellular homophilic interactions have been demonstrated for most of the Ig-related CAMs listed in Table 1 (Edelman 1986; Grumet & Edelman 1988; Filbin et al 1988). Homophilic interactions are also implicated in cadherin function (Takeichi 1990; Nose et al 1990). These observations suggest that for Ig-related CAMs and cadherins, the receptors for these molecules are the cognate molecules themselves in the neuronal membrane. The importance of such homophilic interactions in the induc-
tion of neurite outgrowth has been directly demonstrated in the case of L1 and NCAM (Lemmon et al 1989; Doherty et al 1990).

**HETEROPHILIC RECEPTORS** For molecules like TAG-I, which are not transmembrane proteins, the receptors would be expected either to be other (heterophilic) molecules, or to be functionally coupled to a membrane protein, so that the signal they provide could be transduced into the activation of growth cone advancement. Recent studies suggest that while TAG-I can act as a homophilic adhesion molecule when transfected into *Drosophila* cells, its ability to promote neurite outgrowth is heterophilic. DRG cells are not inhibited from extending neurites on a substrate of TAG-1 by treatment with phospholipase C, which cleaves TAG-1 from their membranes (T. Jessell, personal communication). Since TAG-1 has an RGD sequence in its FN-type repeats, and since TAG-1 is secreted (Karagogeos et al 1991), perhaps the receptor for this outgrowth-promoting activity is an integrin (currently under investigation, J. Dodd & T. Jessell, personal communication). There is a precedent for receptor interaction between integrins and Ig superfamily molecules in the interaction of ICAM-1 with LFA-1 (Springer 1990).

**Perspectives**

As noted above, ECM proteins and CAMs seem to act synergistically in the induction of neurite growth. This suggests that different growth-promoting receptors may transduce signals through common intracellular mechanisms (Bixby et al 1988). Naturally, signals promoting neurite growth must eventually converge on a common pathway to cause neurite elongation. On the other hand, the different classes of receptors discussed in this section have very different structures and seem to have distinct cellular localizations and functions (e.g. Geiger et al 1985). In fact, recent evidence suggests that differences in transduction mechanisms are likely, both in the case of neurotrophic factors (Damon et al 1990) and substrate-acting proteins (Bixby & Jhabvala 1990). In sum, both common and unique mechanisms are likely to be employed, and these can only be sorted out by the examination of receptor interactions with a case-by-case approach.

**SECOND MESSENGERS IMPLICATED IN NEURITE GROWTH**

It is safe to say that for the growth-promoting proteins thus far discussed the transduction mechanisms themselves are poorly defined. Because of the sparsity of information available, our discussion is limited to a brief examination of the intracellular signaling mechanisms that have been
implicated in the control of neurite growth, with reference to pertinent receptor interactions.

**Calcium**

It has been known for many years that manipulations affecting levels of intracellular Ca$^{2+}$ affect neurite growth and growth cone motility, but the precise relationship between intracellular Ca$^{2+}$ levels and neurite growth has proven difficult to define. The data are consistent with the hypothesis that elevations of Ca$^{2+}$ are both stimulatory to neurite growth (Schubert et al 1978; Anglister et al 1982; Suarez-Isla et al 1984) and inhibitory (Bixby & Spitzer 1984; Mattson & Kater 1987). A theoretical resolution of this problem was proposed by Kater and colleagues, who suggested that an optimal Ca$^{2+}$ level exists in growth cones, and that raising or lowering Ca$^{2+}$ from this level will be inhibitory to growth (Mattson & Kater 1987). This hypothesis is attractive, but some data do not fit well (Silver et al 1989). A further complication is that different levels of Ca$^{2+}$ are achieved in different intracellular locations (i.e. neurite vs growth cone, different regions of the growth cone), and there may be unique Ca$^{2+}$ optima in different regions (Silver et al 1990).

Because Ca$^{2+}$ is a regulator of numerous important intracellular functions, it would be surprising if Ca$^{2+}$ levels had no influence on neurite growth. Indeed, signals such as electrical impulses and neurotransmitters can elevate intracellular Ca$^{2+}$ and thereby affect neurite growth (e.g. Mattson & Kater 1987; Lankford et al 1988; Silver et al 1990). What is less clear is whether or not the control of intracellular Ca$^{2+}$ is an element of signal transduction used by growth-promoting proteins. One study using antibodies to CAMs to trigger transduction events found that intracellular Ca$^{2+}$ could be raised by incubating PC12 cells with antibodies to L1 or NCAM (Schuch et al 1989). Further studies will be necessary to allow interpretation of this result and to test its generality.

**cAMP**

Like Ca$^{2+}$, cAMP is a ubiquitous intracellular regulatory molecule that has been implicated in the control of neurite growth. As is also the case for Ca$^{2+}$, studies linking intracellular cAMP levels to regulation of neurite outgrowth have led to differing conclusions. In a number of neuronal types responding to a variety of cues, elevations of intracellular cAMP have been shown to be inhibitory to neurite growth and/or growth cone motility (Forscher et al 1987; Lankford et al 1988; Mattson et al 1988; Bixby 1989). Experiments with neural cell lines, in apparent contrast, suggest that increasing cAMP levels is stimulatory for neurite growth (Nirenberg et al 1983; Greene et al 1986). However, this apparent discrepancy may be
due to a lack of distinction between neuronal axon-like neurites and the processes that are extended from tumor cells (Greene & Tischler 1982; Greene et al 1986).

Whether cAMP is a second messenger for the transduction of the growth-promoting signals under discussion has not been examined in most cases, but it does not seem likely at present. Elevation of cAMP is inhibitory to LN-stimulated neurite growth from parasympathetic neurons (Bixby 1989), and a drug that inhibits cAMP-dependent protein kinase has no effect on this LN-induced growth (Bixby & Jhabvala 1990). Antibodies to CAMs that do trigger other intracellular responses do not affect cAMP levels in PC12 cells (Schuch et al 1989). Damon et al (1990) found that NGF and FGF can each promote neurite growth from PC12 cells that are deficient in cAMP responsiveness.

G Proteins and ras

Two different classes of GTP-binding proteins have been implicated in the regulation of neurite growth, although their relationship to transduction through growth-promoting proteins is not clear. The first of these is the oncogene, p21\textsuperscript{v-ras}. In PC12 cells, introduction of the \textit{v}-ras protein promotes neurite extension (Bar-Sagi & Feramisco 1985; Noda et al 1985), and injection of antibodies to p21

\textit{ras} can block NGF-induced neurite growth (Hagag et al 1986). These results are consistent with a role for p21\textit{ras} in the induction of neurites by NGF, at least in PC12 cells. More recently, Borasio et al (1989) extended these results by showing that intracellular application of the p21\textit{ras}-transforming protein (but not its normal cellular homologue) can potentiate neurite growth in genuine neurons. The fact that transformed cells and/or the transforming version of \textit{ras} are apparently required to implicate \textit{ras} in neurite growth is worth noting, as this makes the physiological relevance less obvious. However, these results may be merely a reflection of the exaggerated activity of an oncogene, which could be revealing an effect too subtle to be seen with the normal proto-oncogene in these relatively crude experiments. Although no evidence exists that CAMs and ECM proteins signal neurite growth through \textit{ras}, at least one member of the Ig superfamily may use \textit{ras} as a messenger. Recently, Downward et al (1990) showed that activation of T cells through the T cell receptor leads to activation of \textit{c-ras} through the inhibition of GTPase-activating protein (GAP).

The so-called large G proteins appear to be involved in the transduction of numerous transmembrane signals (see Casey & Gilman 1988). The predominate G protein in brain is \textit{G}\textsubscript{o} (Sternweis & Robishaw 1984), and \textit{G}\textsubscript{o} is concentrated near cell-cell junctions in vivo and at the tips of growing neurites in vitro (Strittmatter et al 1990). The function of \textit{G}\textsubscript{o} is not clear,
but it has been implicated, among other things, in the control of phospholipase C. Two lines of indirect evidence suggest that Go may be involved in signal transduction leading to axon growth. First, pertussis toxin, which inhibits the function of Go and Gq, blocks some of the responses to anti-CAM antibodies in PC12 cells (Schuch et al 1989). Secondly, the axonal growth-associated protein GAP-43 (Skene 1989) has been implicated in the regulation of axonal growth, as seen in a large body of indirect evidence (for review, see Skene 1989). It was recently shown that GAP-43 can stimulate the binding of GTPγS to Go (Strittmatter et al 1990), which suggests an activating function. However, the relationship of this observation to the control of axon growth is not known.

Tyrosine Kinases and src

A number of growth factor receptors are protein tyrosine kinases, and other receptors have been shown to associate with tyrosine kinases that are themselves not receptors (e.g. Kypala et al 1990). Therefore, although there is no direct evidence that receptors for neuronal growth-promoting proteins are tyrosine kinases, the possibility exists that these receptors associate with the non-receptor tyrosine kinases of the src family (Cooper 1989). The first evidence of this came from the observation that the v-src transforming protein can induce neurites in PC12 cells (Alema et al 1985). Subsequently, Maher (1988) showed that NGF induces tyrosine phosphorylation in PC12 cells. These observations are consistent with the view that NGF may operate to produce neurites through a tyrosine kinase, at least in part. For example, the accessory protein required for the functional NGF-R could be a non-receptor type kinase. A second possibility is that the receptor tyrosine kinase trk comprises another high affinity NGF receptor (Kaplan et al 1991). However, it may be that while tyrosine kinases are involved in the regulation of axon growth, they do not play a direct transducing role. Tyrosine phosphorylation of the β1 integrin receptor subunit (in non-neuronal cells) leads to altered association with cytoskeletal elements (Tappley et al 1989). In Drosophila, the abl tyrosine kinase gene can be deleted without disrupting axonal growth, but if abl and another gene (dab) are both deleted, axonal guidance in the CNS is severely disrupted (Gertler et al 1989; see also Elkins et al 1990). This evidence is in line with a modulatory role for tyrosine kinases in axonal growth.

The actin-binding protein, vinculin, is a major substrate of tyrosine kinase activity in the growth cone (Igarashi et al 1990), which suggests a role in cytoskeletal organization. Recently, Matten et al (1990) showed that tubulin is phosphorylated on tyrosine in neuronal growth cones (probably by c-src) and that this phosphorylation inhibits tubulin polymerization (W. Matten, P. Maness, personal communication). Since tubu-
lin polymerization is obviously critical for axon growth, phosphorylation
by c-src may locally inhibit growth, perhaps by playing a role in axon
guidance. Preliminary evidence using inhibitors of tyrosine phos-
phorylation to affect substrate-induced neurite growth in vitro is consistent
with this possibility (J. Bixby, unpublished observations). In summary,
the scanty evidence available suggests that tyrosine kinases are not the
transducing elements for the growth signal, but probably do play a regu-
larative role.

**Protein Kinase C**

Protein kinase C (PKC) is probably the messenger most often linked
to neurite growth-promoting signal transduction. PKC is in reality a family
of proteins with at least seven isoforms, one or more of which are expressed
predominantly in brain. PKC most likely participates in a variety of
transduction events, usually as a consequence of the receptor-mediated
activation of phospholipase C, which leads to the formation of the PKC
activator diacylglycerol (for reviews of PKC, see Nishizuka 1988; Huang
1989). There are numerous papers documenting a relationship between
PKC and the action of NGF. NGF activates PKC in responsive cells, and
at least some inhibitors of PKC inhibit NGF-induced neurite growth
(Traynor & Schubert 1984; Hama et al 1986; Chan et al 1989; Hashimoto
1988; Koizumi et al 1988). However, experiments using persistent appli-
cation of the phorbol ester TPA (tetradecanoyl phorbol 13-acetate) to
down-regulate PKC in PC12 cells suggest that PKC function is not
required for the neurite-inducing response to NGF or to FGF (Damon et
al 1990). PKC was not directly measured in these experiments, so this
interpretation is not certain. At the moment, the weight of evidence sug-
gests that PKC is involved in transducing the neurite-promoting signal,
but other pathways seem likely to be involved as well.

It is natural to wonder whether PKC may also be involved in the
response to ECM proteins and CAMs. This question becomes more rele-
vant because other members of the families involved (i.e. integrins and Ig
superfamily molecules) may well employ PKC as a signal (Banga et al
1986; Burn et al 1988; Cambier & Ransom 1987; Desai et al 1990). Experi-
ments using PKC inhibitors to test its involvement in substrate-induced
neurite growth suggest the following conclusions. ECM proteins like LN,
FN, and collagens induce neurites through a process dependent on PKC
function, while CAMs (N-cadherin and L1) may use both PKC-inde-
pendent (early) and PKC-dependent (late) pathways (Bixby 1989; Bixby
& Jhabvala 1990). Activation of PKC by the substrate was not measured
in these experiments, therefore, whether PKC is actually transducing the
signals remains an open question. Interpretation is further complicated by
the suggestion that integrin receptor function can itself be regulated by PKC (e.g. Shaw et al 1990; but see Danilov & Juliano 1989). If PKC does transduce neurite growth-promoting signals, what are the relevant substrates for phosphorylation? No direct evidence exists on this point. Two proteins associated with the polymerization of microtubules, MAP-2 and tau, are PKC substrates, however, as is the growth-associated protein GAP-43 (Huang 1989). The relationship of this observation to the finding that GAP-43 may activate Go is unclear.

**Nuclear Events**

Substrate-acting neurite-promoting factors like LN and CAMs can induce neurites very rapidly. Nevertheless, these proteins can affect gene expression (e.g. Werb et al 1989), and the possibility of long-term effects on neurite growth remains to be addressed. For the neurotrophic factors, long-term effects on gene regulation have been known for years, and there is evidence that both transcription-independent and transcription-dependent NGF pathways can induce neurites in PC12 cells (Greene & Shooter 1980). The initial messengers involved in the control of gene expression by growth factors are not known. However, NGF and other growth factors can clearly induce the so-called immediate early genes such as c-fos, c-jun and jun-B (Wu et al 1989; Bartel et al 1989). Of these, the c-jun induction shows at least some specificity (Bartel et al 1989). The challenge for the next few years will be to discover the mechanism of this induction and the targets of these early response gene products.

**Perspectives**

Our knowledge of the transduction elements associated with neurite growth is clearly meager. Even for the neurotrophic agents, the signaling of which has been studied for some time, it is not possible to construct a pathway leading from receptor binding to control of the cytoskeletal proteins required for process extension. Nevertheless, there is reasonable evidence that the proteins listed in Table 1 transduce signals through mechanisms common to other, better understood receptor interactions. From what we know now, it seems profitable to focus on GTP-binding proteins, tyrosine kinases, and PKC for the present.

**MOLECULES THAT GUIDE AXONS**

**Guidance vs Growth Promotion**

A factor that guides as opposed to one that simply promotes neurite outgrowth can be defined as an activity that is actually responsible in vivo for steering growth cones along their proper pathways. Steering may take
the form of selective fasciculation of axons or adhesion between growth cones and labeled pathways of guiding cells or ECM factors. It may also take the form of directional orientation up gradients of diffusible chemotropic agents or substrate-bound positional cues. Growth promoters or inhibitors, diffusible or bound, in the right spatio-temporal pattern and context can be guidance molecules. Guidance factors may also be molecules that have no neurite-promoting or inhibiting activity, but that cause axons on a growth-promoting substrate to turn. Guidance factors cannot properly be defined in standard dissociated cell culture conditions, but the possibility of guidance activity can be strongly suggested in complex culture situations where axons make a predictable or physiologically relevant choice while growing on an otherwise permissive substrate.

The selective contacts made by individual filopodia and perhaps the tension generated by them seem to be important for growth cone guidance (Wessels & Nuttall 1978; Bray & Chapman 1985; Bray 1989; Heidemann et al 1990). Growth cones in vitro may extend without filopodia, for instance in the presence of cytochalasin (Letourneau et al 1987), but in situ growth cones become disoriented, although they continue to extend, when filopodia are collapsed (Bentley & Toroian-Raymond 1986). Another mechanism of growth cone turning may involve the selective retraction or micropruning of growth cone branches formed from lamellipodia (Burmeister & Goldberg 1988). Receptor-mediated transduction as well as adhesion in the growth cone can be driven by many different molecular pathways, which raises the possibility that the growth cone may use separate pathways for guidance and growth. For instance, one might imagine that one pathway serves to promote growth by encouraging microtubule insertion into the base of the growth cone (Bamburg et al 1986), while another plays a more important role in guidance by causing asymmetrical cytoskeletal rearrangements at the leading edge of the growth cone. Similar suggestions could be made for actin-based motility (Smith 1988). Recently, dynamic changes in the distribution of fluorescently labeled microtubules have been studied by J. Sabry et al (personal communication). They have seen that bundling of microtubules on one side of the growth cone and the invasion of such bundles into particular growth cone branches can predict the direction of turning. It has been argued that signals received on one side of a growth cone can be amplified by a transduction cascade to create a local concentration gradient of an intermediate across the growth cone that is ultimately responsible for this asymmetric extension (Gierer 1987; Walter et al 1990). As noted above, src could conceivably play a role in such a process.

The factors that have been implicated in guiding axons are listed in Table 2. Because it is difficult to establish unequivocally a guidance func-
### Table 2  Putative guidance factors

<table>
<thead>
<tr>
<th>Factors</th>
<th>References</th>
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<tbody>
<tr>
<td><strong>Cell surface</strong></td>
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<tr>
<td>Ig superfamily</td>
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<tr>
<td>NCAM</td>
<td>Silver &amp; Rutishauser (1984)</td>
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<td></td>
<td>Fraser et al (1988)</td>
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<tr>
<td>Olfactory NCAM</td>
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<tr>
<td>Fasciclin II</td>
<td>Harrelson &amp; Goodman (1988)</td>
</tr>
<tr>
<td>L1</td>
<td>Dodd &amp; Jessell (1990)</td>
</tr>
<tr>
<td>TAG-1</td>
<td>Dodd &amp; Jessell (1990)</td>
</tr>
<tr>
<td>Contactin</td>
<td>Ranscht (1988)</td>
</tr>
<tr>
<td><strong>Cadherins</strong></td>
<td></td>
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<tr>
<td>N-cadherin</td>
<td>Takeichi (1990)</td>
</tr>
<tr>
<td>T-cadherin</td>
<td>Ranscht &amp; Dours-Zimmerman (1991)</td>
</tr>
<tr>
<td><strong>Others</strong></td>
<td></td>
</tr>
<tr>
<td>Fasciclin I</td>
<td>Elkins et al (1990)</td>
</tr>
<tr>
<td><strong>ECM</strong></td>
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<tr>
<td>Laminin</td>
<td>Hammarback &amp; Letourneau (1986)</td>
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<tr>
<td>Tenascin/J1</td>
<td>Steindler et al (1990)</td>
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<td></td>
<td>Snow et al (1990)</td>
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<tr>
<td>Proteoglycans and glycoconjugates</td>
<td>Lander (1989)</td>
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<tr>
<td></td>
<td>Dodd &amp; Jessell (1987)</td>
</tr>
<tr>
<td><strong>Diffusible</strong></td>
<td></td>
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<tr>
<td>NGF</td>
<td>Gunderson &amp; Barrett (1990)</td>
</tr>
<tr>
<td>Max factor</td>
<td>Davies &amp; Lumsden (1990)</td>
</tr>
<tr>
<td>Pons factor</td>
<td>Heffner et al (1990)</td>
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<tr>
<td>Floor plate factor</td>
<td>Tessier-Lavigne et al (1988)</td>
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<tr>
<td></td>
<td>Placzek et al (1990)</td>
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<tr>
<td><strong>Inhibitory</strong></td>
<td></td>
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<tr>
<td>Central</td>
<td>Kaplhammer &amp; Raper (1987b)</td>
</tr>
<tr>
<td>Peripheral</td>
<td>Kaplhammer &amp; Raper (1987b)</td>
</tr>
<tr>
<td>Nasal</td>
<td>Raper &amp; Grunewald (1990)</td>
</tr>
<tr>
<td>Posterior sclerotome</td>
<td>Davies et al (1990)</td>
</tr>
<tr>
<td>Posterior tectal</td>
<td>Stahl et al (1990)</td>
</tr>
<tr>
<td>NI-35 and NI-250</td>
<td>Schwab (1990)</td>
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<tr>
<td>Serotonin and dopamine</td>
<td>McCobb (1990)</td>
</tr>
</tbody>
</table>
Table 2 (continued)

<table>
<thead>
<tr>
<th>Factors</th>
<th>References</th>
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<tbody>
<tr>
<td>Topographic</td>
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<tr>
<td>TRAP</td>
<td>McLoon (1991)</td>
</tr>
<tr>
<td>TOP Av</td>
<td>Trisler &amp; Collins (1987)</td>
</tr>
<tr>
<td>&quot;68K-Laminin Receptor&quot;</td>
<td>Rabacchi et al (1990)</td>
</tr>
<tr>
<td>JONES</td>
<td>Constantine-Paton et al (1986)</td>
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<tr>
<td>ROCA-1</td>
<td>Suzue et al (1990)</td>
</tr>
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</table>

For any factor, and because we believe some factors have both guidance and growth-promoting functions, we have included many of the factors from Table 1 in Table 2. We used a few simple criteria for assigning a factor a possible role in guidance. These criteria are reflected in the following questions. (a) Is the spatio-temporal expression of the activity in vivo strongly suggestive of a guidance function? (b) Does the block or (c) misexpression of the putative guidance molecule cause predictable misrouting, especially if blocking it does not inhibit further neurite extension? (d) In culture, is the patterned or localized application of the factor able to cause growth cones growing on a permissive substrate to change their direction? (e) Can this response be blocked with specific reagents? An answer in the affirmative to one or more of these questions was sufficient for inclusion in Table 2 as a putative guidance factor. Of course, a negative answer to any of these questions does not rule out a guidance function. In most of the cases listed, a definitive role in guidance has not been established.

Putative guidance factors come in several different categories. First there are the growth promoters, which are cell surface bound, extracellular matrix bound, or diffusible. Second are a class of factors that are inhibitory for axon growth in vitro; i.e. factors that cause axon collapse or growth cone paralysis. These are obviously not growth promoting. Third, there are a few factors that should be considered as putative guidance molecules solely because of their topographic distribution, which matches the topographic distribution of certain sets of axonal projections. Finally, there is a set of nonchemical factors that has been proposed to have guidance activity. Chief among these are natural electric fields, and the strategic placement or development of physical or morphological barriers. We refer readers to other reviews for these topics, as they will not be discussed.
further here (Lockerbie 1987; Kater & Letourneau 1985; Letourneau 1982).

**Cell Surface Proteins**

**NCAM** NCAM was originally shown to have a role in axon fasciculation in vitro; a similar role in vivo might well argue for a guidance function of NCAM (Edelman 1986). Evidence that NCAM does have a role in axon guidance in vivo comes from experiments in which functionally effective NCAM antibodies were injected into different regions of the developing and regenerating visual and motor system. Injections into the eye caused aberrations of optic fiber growth, particularly at the optic nerve head (Silver & Rutishauser 1984), applications to the tectum caused optic axons to avoid the area of high antibody concentration (Fraser et al 1988), and injections into the egg led to decreased branching in particular leg muscles in the chick (Landmesser et al 1988). The early, sustained, and widespread spatial distribution of NCAM in the CNS in vivo does not give much of an indication that it can act as a guidance molecule, and it may be argued that the in vivo effect of antibodies was nonspecific in terms of axon guidance because the entire tissue exposed to the antibody was compromised. Silver et al (1987), however, suggest that optic axons may avoid entering the telencephalon by encountering and being blocked by an NCAM-free “glial knot.” The knot may also present physical barriers to axon growth so the relevance of NCAM to steering in this case is questionable. Furthermore, such NCAM-free zones do not seem to be common in the CNS.

Since NCAM can be found in various states of sialylation, which changes developmentally in the brain, and which shows differential adhesivity (Edelman 1986), it has been proposed that the changing state of this molecule rather than the distribution of the protein core can guide axons. Indeed, reductions in the sialylation state of NCAM can be correlated with innervation of target structures (Landmesser et al 1990; Schlosshauer et al 1984). This argues that NCAM may be influencing target choice or branching pattern, for example, by making the target particularly adhesive and unsupportive of further growth; in a sense gluing the innervating axons into place. In line with this idea, enzymatic removal of the polysialic acid of NCAM led to increased fasciculation and decreased branching of ingrowing motor nerve terminals onto their target muscles (Landmesser et al 1990). In addition, while reducing the sialylation state of NCAM increases cell adhesion (Rutishauser et al 1988), fully unsialylated NCAM has almost no neurite outgrowth-promoting activity in culture (Doherty et al 1990).

Key & Akeson (1990) recently reported that there is a uniquely glyco-
sylated form of NCAM, recognized by a panel of monoclonal antibodies, that is expressed solely on the olfactory pathway. This highlights the question of whether or not there is a pattern of particular forms of the molecule present that correlates with and may guide specific axons, but at present reagents are too crude to resolve this matter.

**LI, TAG-1, F11/CONTACTIN, FASCICLIN II, NEUROGLIAN**  Several other members of the Ig superfamily such as L1 (Chang et al. 1987), F11/contactin (Brummendorf et al. 1989; Ranscht 1988), and TAG-1 (Furley et al. 1990), as well as fasciclin II (Harrelson & Goodman 1988) and neuroglian (Bieber et al. 1989) in the fly, have a more limited distribution on specific axon bundles and thus may be more likely to have guidance functions. Few have been explicitly tested for guidance activity. In the mammalian embryo, TAG-1 is expressed on the lateral edges of the spinal cord on the ventral-going segments of the axons of commissural descending interneurons, while L1 is expressed on the posterior-going or descending segments of these neurons (Dodd & Jessell 1988). The regional expression of different surface proteins along the length of a single axon (Dodd et al. 1988), when the transition from one factor to another occurs at a point on the axon where the trajectory changes dramatically (Bovolenta & Dodd 1990), strongly suggests that the change in expression of these molecules has a causal connection with the change in direction, especially when these proteins can be demonstrated in vitro to have growth-promoting activity. The challenge now is to demonstrate a guidance function for TAG-1 or L1 in an in vivo or near in vivo situation.

**FASCICLIN I**  The same considerations have led Goodman and colleagues to be particularly interested in axonal surface antigens that are expressed along part of an axonal trajectory of embryonic grasshopper neurons (Goodman et al. 1986). Such criteria helped focus attention on the fasciclin. In the case of *Drosophila* fasciclin, powerful experiments are possible because of mutational analysis. It has, nevertheless, been difficult to demonstrate the in vivo significance of these molecules by mutational deletion analyses. The *fas I* story is furthest advanced, with the *fas I* mutants have a normal looking CNS at all stages examined, which indicates that the molecule is not essential for either growth or guidance of any axons of the CNS (Elkins et al. 1990). Of course, this does not mean that *fas I* is not a guidance molecule, but it does suggest that *fas I* might be part of a redundant guidance system. To test this possibility, a series of double mutants was made with *fas I* and several other mutants in putative guidance or growth cone function, each of which also had no phenotype on its own. Only one of the several tested showed neural defects in combination with *fas I*. This was the *fas I; abl* double mutant. *abl*
codes for a tyrosine kinase (see above), which may function in a signal transduction pathway in the growth cone. That both genes had to be knocked out to achieve axon disorientation suggests that the two genes are part of two separate pathways, either of which may suffice for guidance (Elkins et al. 1990).

In the grasshopper limb, Jay & Keshishian (1990) recently were able to knock out fas I in vivo by a new and potentially powerful technique called chromophore-assisted laser inactivation (CALI). In contrast to the mutational analysis, however, they found that the inactivation of fas I alone disturbed axon guidance. It caused pioneer axons to wander and defasciculate, although in the end they were able to find their way into the CNS. Why could CALI reveal an effect where mutational analysis did not? First, CALI might inactivate entire molecular complexes while mutations knock out one molecule at a time. Second, mutants might have time to compensate for the loss of fas I, whereas the CALI knock-out immediately preceded axon growth. Finally, there is the possibility that the fas I mutants might have shown an effect in the fly leg disc that was not examined, as the CNS of the CALI-treated grasshopper was also not examined. While these details have to be sorted out, it is clear from both of these studies that fas I meets sufficient conditions to call it a true guidance molecule in vivo. Indeed, it may be the first to achieve such status. The ultimate experiment, of expressing fas I in inappropriate tracts, for instance, by putting it under the control of a different fasciclin promoter has yet to be done. Predictable misdirecting of particular axons should occur in this situation.

Fasciclin II and III The proteins fasciclin II and fasciclin III were isolated and cloned from Drosophila on the basis of a characteristic distribution on subsets of developing axons (Patel et al. 1987; Harrelson & Goodman 1989; Snow et al. 1989; Elkins et al. 1990). DNA transfection studies have shown that fasciclin III, like fasciclin I, can function as a homophilic cell adhesion molecule, similar to members of the Ig superfamily or the cadherins (Elkins et al. 1990). The localization and CAM structure (fas II) or function (fas III) strongly suggest that they too will be involved with axonal growth and guidance.

Cadherins The cadherins form another set of putative neuronal guidance proteins. N-cadherin, which in early stages of development seems widely distributed throughout the nervous system, becomes restricted to certain pathways such as the retina and optic tract during the differentiation of axonal tracts (Takeichi 1988). This restriction is not really sharp enough to suggest that N-cadherin is a guidance molecule. There is another neural cadherin, however, called T-cadherin, which is expressed in a very restric-
tacted pattern, i.e. on motor neurons, on the floor plate, and on the posterior
trophontomes of chick embryos (Ranscht & Dours 1989; Ranscht & Bronner-
Fraser 1991). This distribution makes it a candidate for a guidance
molecule, perhaps inhibitory in nature (see below). Finally, there is R-
cadherin, which is expressed solely on the vitreal surface of the retina, and
may promote or guide the intraretinal growth of optic fibers (M. Takeichi,
personal communication). Whereas the function of N-cadherin in axon
growth has been established, we emphasize that there is no evidence to
support the notion that T- and R-cadherin play a role in this process, other
than this patterned expression.

**LEECH DEFASCICULATION FACTOR** While many of the cell surface molecules
discussed above can be thought of as fasciculation-inducing molecules,
Zipser et al (1989) reported a carbohydrate determinant that is expressed
on 130-kd glycoproteins in sensory afferents of leech neurons, and which
appears to be involved in the defasciculation of these fibers as they enter
the CNS. A monoclonal agent against this determinant prevented the
defasciculation in organ culture, and the sensory afferents failed to expand
their axonal arbors when they entered the CNS. Whether this is a dedicated
defasciculation factor or a molecule like NCAM, with the adhesive state
modified by the carbohydrate moiety, is unclear.

**ECM Molecules**

**LN** In vitro LN clearly has promoting activity (see above). When applied
as a patterned substrate, in vitro growth cones can follow LN trails,
preferring LN to other growth-promoting substrates, such as FN, when
given a choice (Vielmetter et al 1990). Axons can skip from one region of
LN to another over areas of low adhesivity (Hammarback & Letourneau
1986). These culture results show that it is indeed feasible for LN to act
as a guidance molecule. Concentration gradients of LN have also been
tested in vitro to see if it is possible that an adhesive gradient might guide
axons. Even though the gradients studied were steep and extended through
the physiological range of activity, they did not seem to be capable of
steering growth cones (McKenna & Raper 1988). These results make it
likely that if LN is acting as a guidance molecule in vivo, it is doing so by
its presence or absence, rather than by a graded distribution. Interestingly,
however, the unc-6 mutant of *C. elegans* has a defect in dorso-ventral
guidance and is mutated in the B2 chain of laminin, which suggests that
laminin is somehow responsible for topographic guidance in this system
(reviewed in Hedgecock & Hall 1990). The problem in the vertebrate is
that LN seems to be all over the basal lamina and the endfeet of glial cells
in the CNS and thus does not form any patterns reminiscent of particular
pathways, except that the pial surface of the brain seems to be where most long projecting axons of the CNS grow during development. Moreover, LN appears to be an excellent substrate for most, if not all, central neurons (Lander 1987). These two facts argue that LN is largely playing a permissive rather than an instructive role in axon navigation, at least in the CNS. However, until more is learned about the distribution and exposure of the different forms of laminin, the different binding sites on LN, and the expression of different integrin complexes and LN receptors (see above), we cannot be certain of this conclusion.

FN, TENASCIN, PROTEOGLYCANS, GLYCOCONJUGATES FN is clearly a growth-promoting ECM factor, but its rather general immunohistochemical localization in the nervous system suggests that it too is not an important guidance molecule (Rogers et al 1989), and experiments in vivo do not suggest that FN has guidance activity. Tenascin is another transiently expressed ECM molecule in the brain with a more restricted pattern, particularly around central glia (Snow et al 1990). Like T-cadherin, it is restricted to half somites, but does not seem to influence the growth of motorneurons (Stern et al 1989). It has been argued that the distribution of tenascin surrounding the barrel columns in the developing somatosensory cortex serves a role in restricting afferents or otherwise limiting horizontal connections within individual barrels (Steindler et al 1990). In vitro studies of tenascin show it to be a complicated multifunctional molecule with both adhesive and anti-adhesive activities, depending perhaps on the functional domain exposed (Spring et al 1989; Faissner & Kruse 1990). While growth cones respond to tenascin, there is no evidence to date that it can support or inhibit neurite outgrowth. Proteoglycans (PGs) are an extremely diverse group of molecules composed of glycosaminoglycan (GAG) chains and protein cores. These PGs, such as heparin sulfate PG, can by themselves promote neurite outgrowth (Hantaz-Ambroise et al 1987), but they are often found in combination with ECM molecules (Lander 1989). The potentially vast array of PG-ECM molecule complexes makes this an intriguing possibility for a combinatorial molecular system with enough complexity to guide a variety of axons along different pathways (Herndon & Lander 1990). There is also a large variety of glycoconjugates on the surfaces of subsets of neurons (Dodd & Jessell 1986; Jessell et al 1990), which may also have a role in axon growth and guidance. Before we take these ideas too seriously, it is critical to know much more about the distribution and activities of these complexes.

**Diffusible Neurotropic Factors**

NGF, MAX FACTOR The only identified protein demonstrated to be chemo-tropic to axons is NGF. In vitro, NGF can keep the growth cone-tipped...
axons of sympathetic cells from retracting (Campenot 1982a,b); moreover such growth cones, while growing on a permissive substrate, will turn up concentration gradients of NGF (Gunderson & Barrett 1980). Gunderson & Barrett (1980) also demonstrated that growth cones would turn toward dB cAMP released from a nearby micropipette, which indicates a possible role of cAMP as an intermediate in NGF-directed guidance. Intracellular Ca$^{2+}$ release was also implicated in their experiments, since growth cones would turn up concentration gradients of Ca$^{2+}$ in the presence of the Ca$^{2+}$-ionophore, A23187, while dantrolene, which blocks the intracellular release of Ca$^{2+}$, blocks turning toward either NGF or dB cAMP (Gunderson & Barrett 1980).

In vivo we know that NGF is manufactured and secreted from targets (Davies et al 1987). Further, when NGF is injected into the brain, it leads to unusual central projections of sympathetic fibers toward the source of injected NGF (Menesini-Chen et al 1978), and when it is expressed from a transgene in fibroblasts injected into the brain, it can lead to the sprouting of cholinergic neurons towards the transplanted cells (Rosenberg et al 1988). Edwards et al (1989) showed in transgenic mice that pancreatic beta cells expressing NGF from a heterologous promoter attract extra sympathetic innervation. In spite of this extremely powerful evidence, it is still uncertain whether NGF has a real tropic role in vivo. It can be argued that the levels of NGF needed to make axons turn in vitro and in vivo are not physiological. Moreover, NGF has more or less been ruled out to be a guidance molecule in the trigeminal sensory system, where sensory axons are chemotropically guided to the maxillary whisker pad of the epithelium (Davies & Lumsden 1990). In this case it appears that the NGF receptor is up-regulated in the sensory neurons, but only after the neurons arrive at their target, consistent with a role for NGF as a trophic factor, shaping innervation by restricting neuronal survival rather than guiding axons (Wyatt et al 1990). This strongly suggests the existence of an as yet unidentified chemotropic factor (tentatively called Max factor) that is responsible for this attraction (Davies & Lumsden 1990). Chemotropic studies have not yet been reported for the more recently discovered members of the NGF family of neurotrophic molecules.

**FLOOR PLATE AND PONS FACTORS** Recently, other instances of tropic guidance have been demonstrated in the CNS. For example, cortical fibers are attracted to the pons (Heffner et al 1990), and commissural interneurons of the spinal cord are attracted to the floor plate (Tessier-Lavigne et al 1988; Placzek et al 1990). That these are instances of chemotropism has been established in co-cultures imbedded in three-dimensional collagen gels. The floor plate factor appears to be a large protein, about 90 kd, that
may possibly bind to the ECM (T. Jessell & M. Tessier-Lavigne, personal communication), characteristics which distinguish it from NGF. The actual diffusible factors released from the maxillary pad, the floor plate, and the pons have not yet been identified, and it will be intriguing to learn more about their biochemical characteristics. Are they, for instance, related to each other? At the moment, we can only say that the existence of such chemotropic guidance mechanisms in the nervous system seems certain.

**Inhibitory Factors**

**Collapsing Factors** The notion that axons could be guided by inhibitory rather than growth-promoting factors was rekindled in our consciousness only a few years ago when Kapfhammer & Raper (1987a) demonstrated that the growth cones of retinal cells collapse when they encounter the axons of sympathetic cells and vice-versa. By testing a variety of different types of axons against each other, Kapfhammer & Raper (1987b) demonstrated that there are a minimum of three collapsing factors among the several classes of neurons tested: a central collapsing factor, a peripheral one, and one on nasal, but not temporal, retinal axons (Raper & Grunewald 1990). The actual number of distinct collapsing factors in the brain may be considerably larger. Collapsing activity can be easily demonstrated in vitro by sprinkling membranous vesicles of test material onto growing axons (Raper & Kapfhammer 1990). Using this assay, two collapsing activities, one from posterior sclerotome tissues (Davies et al 1990) and one from posterior tectum, have been identified (Stahl et al 1990), and another from brain has been greatly enriched (J. Raper, personal communication). All these factors are different in terms of their molecular weights, lectin-binding capacity, and susceptibility to urea (Walter et al 1990). Whether there are families of such factors will be revealed only after these and others are identified and sequenced.

The reason for thinking that inhibitory factors are true guidance molecules has to do first with their distribution in vivo. For instance, both the posterior tectal factor and posterior sclerotome factors are expressed in a pattern that by itself can account for the absence of particular axons entering these regions. A first test to see whether these factors truly have guidance function in vivo will be to block the activity with antibodies. This should lead to the invasion of new areas by spinal axons and temporal retinal fibers.

Ca\(^{2+}\) has been measured using fura-2 as a possible intermediate in growth cone collapse (Ivins et al 1990). However, Ca\(^{2+}\) levels were relatively constant through this dramatic change in growth cone morphology.

T-Cadherin, Ni-35, Ni-250 T-cadherin also may have an activity in repelling
spinal axons from entering the posterior somite. T-cadherin is GPI-linked (Ranscht & Dours-Zimmerman 1991) and thus lacks an intracellular domain that is critical for the adhesive functions of cadherins (Nose et al 1990). This may be the basis of its putative avoidance function. Schwab (1990) identified two factors (NI-35 and NI-250) on oligodendrocytes that inhibit or paralyze growth cone advance. These factors have not been postulated to have a role in axonal guidance, and their normal function in vivo is still a mystery. It seems likely, however, that their activity is partially responsible for preventing the regeneration of axonal tracts in the mammalian CNS.

**TRANSMITTERS** Another class of growth-inhibiting factor is neurotransmitters, which when applied to the growth cones of specific growing *Helisoma* neurons in culture, paralyzes them (McCobb et al 1990), perhaps by opening Ca\(^{2+}\) channels (see above). The growth cones paralyzed by serotonin come from neurons that normally make contact with serotonergic postsynaptic cells and can, therefore, be expected to receive stop and synapse cues from these postsynaptic partners. Few would have guessed that the growth cones of the presynaptic cell would be sensitive to the synaptic transmitter of its postsynaptic partner, but these findings suggest the possibility that such a feedback system may indeed be used in the nervous system. Low concentrations of serotonin can admit Ca\(^{2+}\) and block growth cone advance in organ culture (Murrain et al 1990). Moreover, serotonin is expressed in the developing nervous system at a time when it could participate in growth cone guidance, and 5,7-dihydroxytryptamine treatment of the embryo leads to aberrations in the growth pattern of the specific neurons whose growth cones are stopped by serotonin in vitro (Goldberg & Kater 1989).

s-LN  s-LN is an ECM protein localized selectively at motor end plates in vivo. In vitro, it can promote cell adhesion, and its localization in vivo suggests that it too may be a cue to stop and synapse (Hunter et al 1989).

**Topographic Guidance Molecules**

**POSTERIOR TECTAL FACTOR, TRAP** A number of molecules are good candidates for guidance factors not because of their presence on certain fascicles or tracts in the brain, but solely because of their patterned distribution. If a neural tissue appears histologically uniform and yet it displays a gradient of a cell surface marker that in some way parallels the distribution of axonal projections, we must consider the possibility that the molecule is a guidance factor. We have already discussed one of these, the inhibitory factor of the posterior tectum. Interestingly, the choice of temporal retinal cells not to grow on posterior tectal membranes is not graded in the retina,
but sharp. At approximately the center of the eye, there is a clear boundary between nasal and temporal retinal axons, and it is only the temporal retinal axons that avoid the posterior tectal membranes (Walter et al 1990). Until the distribution of the avoidance factor is demonstrated in vivo as a gradient, we must consider the possibility that it too is discontinuously distributed, perhaps along a curved or slanted line that separates anterior and posterior tectum. This avoidance protein was found (Stahl et al 1990) using the growth cone collapsing bioassay of Raper & Kapfhammer (1990). More often, however, topographically distributed molecules in the nervous system have been found not by a functional assay, but by monoclonal antibodies. Along with the functional dichotomy of the retina into nasal and temporal halves, McLoon (1991) recently described a protein called TRAP, which is distributed almost in a step function across the chick retina and mainly expressed in the temporal retina.

**TOP\textsubscript{DV}, TOP\textsubscript{AP}** Trisler and colleagues (Trisler et al 1981; Trisler 1990) used selective immunization and screening of monoclonal antibodies to identify two antigens that are expressed as orthogonal gradients on cell surfaces and ganglion cell axons of the chick retina. The first, called TOP\textsubscript{DV}, a 47-kd protein, is 35-fold higher in the dorsal than ventral retina. The second, called TOP\textsubscript{AP}, a 40-kd protein, is 16-fold higher in the anterior (nasal) than posterior (temporal) retina. The concentration of these TOP molecules varies continuously and logarithmically across the retina (Trisler 1990). Both of these antigens are present in the tectum as well as the retina (Trisler & Collins 1987; Trisler 1990). In the tectum they are present as gradients that are complementary to the retinal gradients. TOP\textsubscript{DV} is 10-fold higher in the ventral (lateral) tectum, and TOP\textsubscript{AP} is 8-fold higher in the anterior tectum. The retinal gradients persist through the development and maturation of the retina, while the tectal gradients become visible just prior to retinal innervation (Trisler 1990). The finding of reciprocally graded, orthogonally expressed molecules in the retina and tectum is a prediction of Sperry's (1963) hypothesis of cytochemical chemospecificity, particularly as modified by proponents of antagonistic gradients of adhesion molecules and their receptors (e.g. Fraser & Hunt 1980; Bonhoeffer & Gierer 1984). Whether or not these molecules actually serve the roles suggested by their distribution has yet to be tested, but functional studies of TOP\textsubscript{DV} within the retina, obtained by injecting antibodies intraocularly, suggest that TOP\textsubscript{DV} has an effect on growth cone persistence and synaptogenesis (Trisler et al 1986; Trisler 1990).

**68K-LN RECEPTOR** Rabacchi et al (1990) recently reported two antibodies that stain the embryonic retina and tectum in a strongly graded fashion, from dorsal to ventral in the retina and lateral to medial in the tectum.
These antibodies recognize a molecule previously identified as a "68K-LN receptor" (Wewer et al 1987), but which, as indicated by the quotation marks, is likely to have other functions. Interestingly, the protein recognized by the antibodies and its mRNA are equally distributed from dorsal to ventral. It is the conformation of the antigen that appears graded. While TOPDv with its apparent relative molecular mass of a 47 kd would seem biochemically distinct from the "68K-LN receptor," Western blots of the "68K-LN receptor" using antibodies that recognize the topographically graded antigen reveal a predominant band of about 44 kd, which suggests that the mRNA might be spliced or that the 68 K form of the protein is post-translationally processed. The similarity of the molecular weights of TOPDv and the graded "68K-LN receptor" thus leave open the question of whether the two antigens are related.

JONES JONES is the name given to another dorsal to ventral-graded antigen in the developing retina (Constantine-Paton et al 1986). JONES is a ganglioside. Its temporal as well as its spatial pattern of expression on axons is again suggestive of a chemoaffinity molecule, but it may instead have a role in the sequential maturation of the retina. Again, functional studies are needed to define its biological role.

ROCA-1 Suzue et al (1990) recently described an antigen called ROCA-1, a 65-kd protein on the surfaces of glial cells of the sympathetic chain and on the Schwann cells of the intercostal spinal nerves of the rat. ROCA-1's abundance is strongly graded from rostral to caudal along the length of the animal. It has long been known that there is a reproducible rostrocaudal topography of sympathetic inputs, which is reestablished after regeneration, suggestive of graded chemospecificity. The expression pattern of ROCA-1 engenders some hope that it is in fact a guidance factor that helps establish the topographically appropriate connections of the sympathetic chain.

If axons are using positionally graded cues to navigate, perhaps the genes that code for molecules like ROCA-1 are under the direct control of spatially orchestrated patterns of transcription factors, like the homeobox-containing genes that seem to be responsible for segmentation or anterior-posterior axis formation. If so, this would mean that the positional information, which guides the fate of cells in the nervous system could also be used to produce topographic displays of a guidance factors (Patel et al 1989). Axons could then navigate through the brain to distant targets using these topographic cues like a map. Axons with different final destinations could use the same map but, by dint of their own particular topographic origin (and hence receptor types), could be guided to different targets with different positional values (Harris 1989).
Perspectives

Unlike the situation for growth-promoting factors discussed above, the number of proven guidance molecules is very small, so it is unclear how many families exist and impossible to hazard even a wild guess as to their total numbers. It is also unclear how much overlap there is between guidance and growth-promoting factors. We know even less about the transduction pathways that lead to growth cone steering. It may be based on a receptor-mediated transduction cascade or even a stretch-activated mechanism. The number or different categories of putative guidance factors suggest, however, that the molecules involved may be a more varied and complex group than are the growth promoters. Unfortunately, because the assays for guidance often require in vivo analysis, the going will be harder. One way investigators are trying to improve their ability to deal with this difficulty is to set up in vitro systems that preserve natural guidance.

The establishment of manipulatable in vivo systems or in vitro systems where guidance is separable from growth is the basis for establishing guidance function. Experimentally, the situation is a difficult one. A simple culture system, with neurons growing on plastic or LN in a defined chemical medium is easy to manipulate experimentally, but it does not come close to simulating the way axons grow and normally respond in vivo. In addition, what guides axons in a dish of dissociated cells may not be relevant to axon growth or guidance in vivo. Moreover, real axonal guidance might rely on complex spatial and temporal arrays of guidance factors that are difficult to recreate in vitro. The embryo obviously provides real guidance cues, but is not as accessible experimentally. The embryo provides barriers to visibility, antibody penetration, and chemical manipulation, although there was stunning work done on visualizing growing neurons in the amphibian tail half a century ago (Speidel 1933). In some cases manipulations would be lethal to the embryo and thus compromise the experiment. The arguments above are abstracted from Landmesser (1988), who is advocating the use of culture systems that on the one hand try to conserve the in vivo environment of the growing axon, and on the other, open the system up for visualization and various forms of experimentation not possible in vivo. Of course, the more the culture system takes on aspects of the true in vivo situation, the more confident one is that the guidance cues one is investigating are physiological, but the more difficult the system is to characterize chemically. Nevertheless, these halfway systems like the cultured whole retina (Halfter & Deiss 1984), the developing live slices of the chick limb bud (Landmesser 1988), or thalamocortex (Bolz et al 1990), as well as the filleted grasshopper limb bud in culture
and the exposed amphibian brain (Harris et al. 1987), have begun to provide us with manipulatable systems in which real guidance is demonstrable.

Molecular and genetic technology provide other powerful ways of testing growth and guidance factors in vivo. In flies and worms, various mutants have been found or constructed that seem to perturb axon growth, fasciculation or guidance (e.g. Hedgecock & Hall 1990; Boschert et al. 1990). Such genetic approaches may define new components of guidance. They may also provide a strategy for testing already identified factors in vivo, as was done with fasciclin I (Elkins et al. 1990). Homologous recombination techniques with embryonic stem cells may make the latter approach feasible in the mouse. In many species it may be possible to express a genetically engineered mutant form of the putative guidance molecule transgenetically for in vivo analysis. While the latter technique has been successfully applied to study other cellular interactions, particularly developmental ones in mice and frogs, such studies are only beginning with a view to axon growth and guidance in vivo (Holt et al. 1990; Edwards et al. 1990).

CONCLUSIONS

In the last ten years, we have learned a great deal about various molecular factors that play roles in neurite outgrowth and guidance. We have identified several families of molecules that promote neurite outgrowth. We have made inroads into the transduction cascades of the growth cone, identifying receptors, second messenger systems, and motile cytoskeletal elements. We have also begun to classify the types of guidance cues that exist in the embryonic nervous system. What can we expect in the next ten years? Perhaps we shall come close to completing a list of the general growth-promoting molecules in at least the best-studied systems. We should find the missing links in the chain of sensory-motor activation in the growth cone, at least to a first approximation. And we shall, using in vivo or near in vivo systems, like the ones described above, identify a great many more authentic guidance factors and begin to understand the logic of their deployment in the embryonic nervous system.

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