**Neuronal Growth Cone Responses to Extracellular Guidance Cues:**

**an *In Vitro* Study**

by

Rachel Mary Kindt

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Submitted to the Department of Biology
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November, 1995



Signature of Author: _____
Rachel M. Kindt
Department of Biology
November 3, 1995

Certified by: _____
Arthur D. Lander
Thesis Supervisor

Accepted by: _____
Frank Solomon
Chairman of the Graduate Committee
Department of Biology

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**Abstract**

Specific interactions of axons with molecules in their environment are crucial to proper pathfinding. In this study, the cellular mechanisms of two effects of the extracellular matrix protein laminin were investigated. First, laminin reduces the adhesion of neurons to fibronectin. The current study demonstrates that this anti-adhesive effect of laminin is due not to intracellular signaling events but possibly to topographic effects of laminin on the substratum.

Second, patterns of laminin can guide growth cones *in vitro*. Here, an assay was developed using patterned laminin to screen for pharmacological treatments that disrupt growth cone guidance. Pertussis toxin caused neurites from chick dorsal root ganglia (DRG) to wander from paths that normally guided them. That pertussis toxin had no effect on laminin-mediated neurite extension but disrupted the interaction of growth cones with a putative inhibitory guidance cue suggests that the cellular mechanisms of growth cone guidance and neurite elongation are different and distinguishable, yet common mechanisms exist for the neuronal response to disparate types of guidance cues. Pertussis toxin's effects here are independent of direct G protein inactivation and mediated by cellular N-linked complex-type carbohydrates. Lectin from *Maackia amurensis* but not *Sambucus nigra*, sialic acid-binding lectins of distinct specificities, mimicked the effect of pertussis toxin.

A soluble B-galactoside-binding lectin, galectin-1, caused neurites to extend on discontinuous laminin paths that normally halted them. This observation led the finding that galectin-1 enhances neurite extension in a cellular carbohydrate- and β1 integrin-dependent manner. As galectin-1 is expressed *in vivo* by neurons bearing carbohydrates to which it binds, the lectin may mediate axonal extension by an autocrine mechanism.

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# Chapter 1

# An Introduction to Growth Cone Guidance

**Overview**

The connections that form the basis for a functioning nervous system are initially established by neurons projecting axons to their synaptic targets. The ability of axons to find their targets with a high success rate, both during normal development and when their cell bodies have been relocated or their targets reoriented experimentally, is a testament to a robust pathfinding ability. The diversity of interactions underlying successful axon guidance is illustrated by the observation that axons from different types of neurons can migrate through the same space but choose different ways.

Specific interactions between axons and their environment, both along pathways and within targets, are believed to mediate axonal guidance. Axons and their growth cones--specialized structures at the tip of an the axon--undergo characteristic changes in morphology and behavior in response to certain tissues, cells or molecules. As an axon passes through different terrain, it will extend at different speeds, exhibit different patterns of extension and retraction, and their growth cone will alter size, shape and number and length of filopodial extensions.

The molecular mechanism of these axon and growth cone behaviors that are the basis for growth cone guidance is not well understood. Many molecules have been identified that by virtue of their distribution in the nervous system and their effects on neurons *in vivo* or *in vitro* are candidate guidance molecules. Cell surface receptors have also been identified for many of these molecules, some of which could convey guidance information via intracellular signals. Yet, it is still not understood how these molecules or signals could act in axons to generate the behaviors that are observed *in vivo*.

This chapter is intended to provide an overview of the current state of the axon guidance field. First, general models for axon guidance are described. Second, examples of *in vivo* pathfinding are described in the context of these models. Third, candidate guidance molecules are described. And last, events inside the growth cone that may be involved in the cellular response to guidance cues are discussed.

**Part I. Models of axon guidance**

For some time, axonal pathfinding was explained by reference to the adhesive interactions of axons and their molecules in their environment. According to what I will call the differential adhesion theory, axons choose to grow on the surface to which they adhere best. The adhesive interactions provide the mechanism for growth and the hierarchy of adhesive molecules in the brain provides the means for choice of path. This theory stemmed in part from *in vitro* observations that neurites, given a choice of non-physiological substrata, such as polyornithine and palladium, often chose to grow on the more adhesive substrata (Letourneau, 1975). That molecules, such as laminin-1, enhance both neurite outgrowth and neuronal cell adhesion appeared to support this theory. However, other studies have demonstrated that neurites do not always exhibit a preference when confronted with substrata of different adhesivity (Gomez and Letourneau, 1994; Lemmon et al., 1992), and when they do exhibit a preference, do not always choose the most adhesive substratum (Calof and Lander, 1991; Gundersen, 1987).

A different model for axon guidance can be derived from studies of migrating cells. Some eukaryotic cells such as leukocytes, respond to gradients of soluble chemoattractants (or chemorepellants). This phenomenon has been extensively studied and is understood in some detail (for review see Devreotes and Zigmond, 1988). Migrating cells can be guided by a molecule in a gradient by one of two mechanisms: chemotaxis--guidance due to orientation as a result of the local detection of a gradient, or chemokinesis--guidance due to increased random motility as a function of the concentration of a molecule. Analogous models can be proposed for how axons are guided. Figure 1 shows a dorsal root ganglion extending axons in a hypothetical gradient. If the molecule has a chemotactic effect, axons will orient to the gradient, as shown in part A. If the molecule only has a chemokinetic effect on the axon, the rate of outgrowth will be higher where the absolute concentration of the molecule is higher, but the axons will not orient in response to the gradient (part B). It is conceivable that a chemorepellant molecule could orient axons *down* a

concentration gradient--negative chemotaxis--or decrease axons' rate of extension and thereby favor growth away from high concentrations--negative chemokinesis. Just as eukaryotic cells can sense the gradient of a chemoattractant by measuring very small local changes in concentration using protrusions called pseudopods. so growth cones and their filopodia could be used to sample the environment and detect changes in concentration of a guidance molecule. Though chemotaxis usually refers to guidance by soluble molecules, there is no *a priori* reason that the same mechanisms could not be used for the response to immobilized gradients, so I will use the terms chemotaxis or chemokinesis to refer to directed guidance by any molecule distributed in a non-uniform fashion.[1]

Gradients are not the only spatial configuration of molecules that could convey directional information. Contact with localized cues could also guide axons. The localized cue could be a point source, like a cell, that, though contact, was able to convey one-time directional information to a growth cone, i.e., to retract, to move in the direction of the cue, or to turn. A striking example of a localized guidance cue is found in the developing limb bud of the grasshopper, where a single contact by the growth cone of a pioneering neuron with one specific cell can redirect the entire axon (O'Connor et al., 1990). Given the larger scale of the developing vertebrate nervous system, perhaps a "guidepost tissue"--cells with the ability to alter the direction of the axons contacting them--could serve the same function (see Dodd and Jessell, 1988). In another model, guidance information could be arranged in a repetitive fashion, such that growth cones follow discreet, spaced cues, like a trail of bread crumbs. The ability of growth cones to cross gaps between areas of preferred substratum has been presented as evidence for growth cone's ability to navigate using discontinuous cues (Hammarback and Letourneau, 1986).

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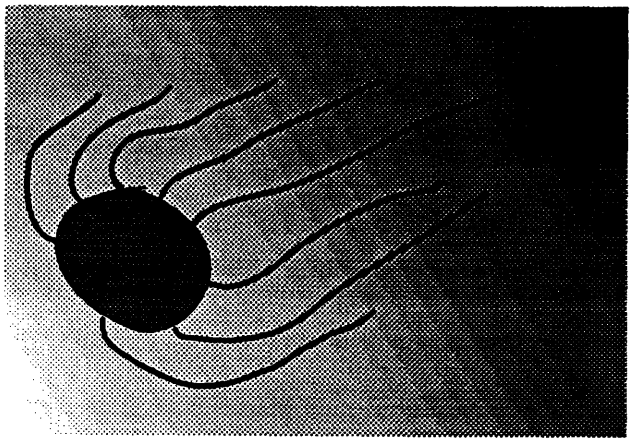
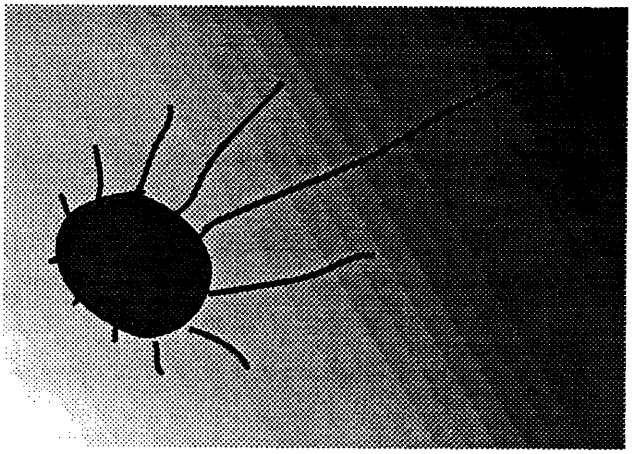
[1]The term "haptotaxis " has been used to refer to guidance of growth cones by an immobilized gradient (c.f. Tessier-Lavigne, 1992). I will ignore this term for two reasons. First, haptotaxis initially meant "guidance by adhesion" which is a limited if not entirely incorrect way of considering the function of such a gradient. Second, many molecules that are classified as soluble have the capacity to interact with cell surfaces or matrix molecules via heparin-binding domains. This includes some growth factors and a well-known recently identified family of guidance molecules.

**Figure 1: Models of Axon Guidance: Chemotaxis vs. Chemokinesis**
Diagram of a dorsal root ganglia extending axons in a gradient of a hypothetical chemoattractant (darker = higher concentration). **(A)** Chemotaxis. The axons orient and grow according to the local slope of the gradient. **(B)** Chemokinesis. Axons grow longer where the concentrations are higher, but do not orient to the gradient. See text for more explanation.
This figure was adapted from a slide by A.D. Lander.

A



B



How could contact-mediated cues guide axons? Encountering a localized signal such as a cell or a molecule with a delimited expression, the axon's growth cone will, at a certain point in the encounter, experience the external factor asymmetrically. Perhaps this asymmetry is "measured" by the growth cone in the same way that it would "measure" a gradual concentration change and the growth cone orients accordingly. Or, perhaps the encounter with the external factor is not integrated across the growth cone, but causes a local change in the growth cone that has the effect of biasing movement in the direction of the cue or away from it. Analogous models have been proposed to explain chemotaxis in eukaryotic cells (Devreotes and Zigmond, 1988). Alternatively, contact with the cue could increase or decrease the rate of extension of the axon. This would have the overall effect of biasing growth cone movement, as described for chemokinesis.

**Part II: *In vivo* guidance activities seeking molecules**

In this section I will discuss several examples of *in vivo* pathfinding in the developing nervous system, including a choice point where contact-mediated guidance cues may be important, and examples of soluble and target derived chemotaxis or chemokinesis. Though there is a growing appreciation of the similarity of molecules and mechanisms involved in vertebrate- and non-vertebrate axon guidance (see Goodman, 1994), this discussion will be limited to vertebrates.

*A choice point: the optic chiasm*

There are places in the developing nervous system where axons traveling on the same path diverge and follow different paths. These "choice points" are valuable for the study of axon guidance, first because molecules that mediate growth cone choicemaking may be preferentially expressed here, and second, because the differential behaviors of axons that act differently in the choice point region can be used to test specific hypotheses about guidance mechanisms.

One well-studied example is the optic chiasm. Mammalian retinal ganglion neurons innervate targets on both sides of the brain,

so some axons must cross the midline to reach their targets. All retinal axons follow the same path down the optic nerve. At the optic chiasm, one group of axons turns to grow ipsilaterally (i.e. stays on the same side of the brain) and the rest project contralaterally (i.e. cross to the opposite side) (see Holt and Harris, 1993). A number of observations indicate that guidance cues at the optic chiasm induce the broad, wide turn that redirects the ipsilaterally--projecting neurons (Godement et al., 1990; Sretavan and Reichardt, 1993). First, the axons that will eventually project ipsi- or contralaterally are mixed, not presorted, as they enter the chiasm (Sretavan, 1990). Second, the number of pathfinding errors is small, indicating that the projections are not made randomly and corrected later by another process. Third, axon-axon interactions in the chiasm are not causal to the direction eventually taken by the axons. Likewise, decreasing the number of axons in the chiasm (by eliminating one eye) does not alter the pathfinding ability of axons from the remaining eye (Sretavan and Reichardt, 1993). Finally, growth cones are more elaborated--larger, bearing more filopodia--in the optic chiasm than when passing through the optic nerve earlier (Bovolenta and Mason, 1987). This change in morphology could be an aide to or a result of the interaction of the growth cones with the potentially more complex molecular environment of the chiasm.

What types of guidance cues are the axons responding to in this region? One possibility is that there is a factor(s) at the optic chiasm midline that is inhibitory for ipsilaterally-projecting axonal growth. Consistently, retinal axons that do not normally cross the midline will not grow on membrane material prepared from the midline at the optic chiasm (Wizenmann et al., 1993). Probing the optic chiasm for known molecules revealed that L1 and CD44 are expressed on neuronal cell bodies in the optic chiasm (Sretavan et al., 1994). L1 is known to promote neurite outgrowth *in vitro* (Lagenaur and Lemmon, 1987) and CD44 was demonstrated to inhibit neurite outgrowth from retinal ganglion cells (Sretavan et al., 1994). It was postulated that L1 and CD44 could act to steer the growth cones in a balance of positive and negative cues. However, the *in vitro* responses to these molecules are indistinguishable for all retinal axons, suggesting no distinction

that could explain the different behavior of the two groups of axons at the chiasm. If L1 and CD44 are involved in pathfinding at the optic chiasm, it could be explained by their effects on axons that might not be revealed in straightforward *in vitro* assays, or that there are other as yet unidentified molecules acting in concert to generate the requisite specificity. In all there are likely to be contact-mediated guidance cues in the optic chiasm, and the cues that are responsible for the distinctive behavior of the two sets of axons may include L1 and CD44.

*Chemoattractants and chemorepulsants*

Studies in other parts of the nervous system test a different model for axon guidance. As mentioned in the first section, gradients may be an effective means of organizing guidance information. Secreted molecules could diffuse from target cells, creating a soluble gradient to guide axons to their targets. Several studies made neurotrophic factors such as nerve growth factor (NGF) early candidates for such factors. For example, local asymmetric application of NGF to a growth cone in culture will cause it to turn (Gundersen and Barrett, 1980). However, the timing of NGF expression does not support a role in the guidance of the same axons to their target *in vivo* (Davies et al., 1987). More recently, *in vivo* studies have identified at least three potential sources of chemoattractants in the developing vertebrate nervous system. First, axons from neurons in the trigeminal ganglia in the peripheral nervous system (PNS) are attracted by their target epithelium (Lumsden and Davies, 1983). Second, axons that project from the neocortex to the spinal cord send collaterals and innervate the pons and other subcortical tissues. The pons appears to release a diffusible factor that induces de novo branch formation along the axons, allowing these collaterals to form (Heffner et al., 1990; Sato et al., 1994). Third, commissural neurons in the spinal cord send axons dorsoventrally to the floorplate, where they cross the midline and turn. *In vitro*, commissural axons grow towards the floorplate (Placzek et al., 1990; Tessier-Lavigne et al., 1988). In all of these cases, the existence of a diffusible chemattractant was confirmed by co-cultures of the neuronal tissue and the target tissue in

collagen gels; the target tissue induced directed neurite extension (or in the case of the cortex and pons, branching) and turning at a distance, suggesting a diffusible chemoattractant.

Candidate floor-plate derived chemoattractants have been identified. Netrin-1 and netrin-2 were biochemically purified from embryonic chick brain based on their ability to promote outgrowth of the commisural axons (Serafini et al., 1994). Are these molecules the diffusible chemattractants that were sought? Netrin-1 is expressed strongly in the floorplate, as might be expected, while netrin-2 has a more diffuse expression pattern in the spinal cord (Kennedy et al., 1994). An aggregate of COS cells secreting either of the netrins can, from a distance, cause commisural axons to turn slightly toward the netrin source, suggesting that these molecules could be soluble chemoattractants. The netrins are indeed secreted molecules according to predicted amino acid sequence and are similar to a *C. elegans*, protein, Unc-6, mutations in which disrupt pathfinding of some axons (Ishii et al., 1992) and to domains V and VI of the ECM molecule laminin. Interestingly, these are regions required for laminin self-assembly (Yurchenco and Cheng, 1993). The netrin family may play a role in directed outgrowth in many parts of the brain: Netrin-1 induces oriented growth of cerebellofugal axons which grow toward the floorplate in the hindbrain (Shirasaki et al., 1995). It remains to be determined whether the netrins act as soluble, freely diffusible molecules, and whether they are expressed in a gradient as originally predicted.

Negative chemotaxis has also been demonstrated in the nervous system. Pini (1993) demonstrated that olfactory neuronal axons in co-cultures do not extend axons in the direction of tissue from which they turn *in vivo*. The repellent activity is developmentally regulated, acts at a distance, and causes axons to orient away from the avoided tissue. As presented, this phenomenon is an example of negative chemotaxis. The identity of this chemorepellent molecule is not yet known. Using a similar *in vitro* assay, other tissues have been described as "chemorepulsive" for other neurons. For example, ventral spinal cord inhibits outgrowth of neurites from co-cultured dorsal root ganglia (Fitzgerald et al., 1993). In this and other cases,

certain molecules that were candidates for the repulsive activity were tested, secreted from aggregates of non-neuronal cells. For example, semaphorin III (sema III), a candidate repulsive guidance molecule, is expressed in the ventral spinal cord. Cells secreting sema III selectively inhibited the subset of dorsal root ganglion neurons that do not project to the ventral spinal cord *in vivo* (Messersmith et al., 1995). In the latter study it was suggested that the spinal cord explants not only inhibit directed outgrowth but reorient the axons; however, the difference between axons turning and not turning in these assays can be very subtle.[2] In other studies of inhibitory activities in the spinal cord, netrin-1, a putative chemoattractant for some neurons as described above, was identified as a putative chemorepellant: The floor plate and netrin-1-expressing cells inhibit directional axonal growth of axons that grow away from the floorplate in vivo: axons of the trochlear nerve (Colamarino and Tessier-Lavigne, 1995) and motor axons (Guthrie and Pini, 1995) While these activities are certainly inhibitory, it appears that more stringent assays may be required to determine whether they can be explained by negative chemotaxis or negative chemokinesis.

*Target derived guidance cues in the optic tectum*

Theoretically, a guidance molecule in a gradient could be soluble or associated with cell surfaces. An example of the latter case is found in the chick retinotectal system. The spatial organization of retinal projections onto the tectum mimics the arrangement of the neurons in the retina. The general pattern is established as the axons grow into the target tissue (Holt and Harris, 1993): axons emanating from neurons in the nasal portion of the retina grow through to the posterior part of the tectum and axons from neurons in the temporal side of the retina remain in the anterior tectum.

One group took an *in vitro* approach to determine why axons behave differently in the tectum. Exploring the hypothesis that these

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[2]Another group has cloned the murine collapsin homologue (Semaphorin D) and demonstrated a striking inhibitory response of DRG neurons to transfected cells secreting this molecule in co-culture (Puschel et al., 1995). By the author's description (and this reader's eyes) Sema D does not appear to orient growth cones.

differences might be due to variations in cell surface protein expression in different parts of the tectum, they prepared membrane material from posterior and anterior tectum and presented it, deposited on a substratum in adjacent stripes, to retinal explants. Neurites from temporal retina remained confined to the stripes of anterior tectal material, while neurites from nasal retina (Walter et al., 1987) crossed freely between the stripes.[3] This observation could be interpreted either as a preference by the temporal axons for the anterior membranes or a distaste for the posterior membranes. Another result suggested that this preference could be due to an interaction between the posterior membranes and the temporal axons: acutely applied posterior membranes caused temporal axon growth cones to collapse (Cox et al., 1990). That this effect was related to growth cone guidance was demonstrated very elegantly by preparing gradients of posterior tectal membranes and showing that distance traveled by temporal axons was inversely related to the steepness of the slope of the gradient (Baier and Bonhoeffer, 1992). Thus, a growth cone collapsing activity can also guide axons.

Several proteins have been identified that could play a role in this repulsive activity. Since the repulsive activity is sensitive to an enzyme that cleaves phosphoinositol-linked proteins (Walter et al., 1990), some searches have concentrated on GPI-anchored proteins. A candidate 33 kD GPI-linked protein has been partially purified (Stahl et al., 1990). A 25 Kd protein called RAGS was purified from tectum on the basis of differential anterior-posterior expression and cloned and sequenced (Drescher et al., 1995). RAGS is expressed in a posterior-anterior gradient in the tectum, and is a member of a family of ligands for receptor tyrosine kinases. However, RAGS restricts the outgrowth and collapses the growth cones of both temporal and nasal axons. Therefore, it is unlikely to be the sole component of the inhibitory activity. Another member of this ligand family, Elf-1, is also found in a similar gradient in the tectum, and strikingly, a

[3]Interestingly, when explants from temporal retina are given only posterior tectal membranes as a substratum, they can extend neurites as vigorously as on anterior tectal membranes alone.

complementary receptor is expressed in the retina in a gradient (Cheng et al., 1995).

Interestingly, a gradient of GPI anchored molecules in rat superior colliculus (the equivalent of the chick tectum) may play a role in the patterning of the retinal projections, but by a different mechanism. In the rat, retinal axons show no topographic specificity in their projections: the specificity is determined later by branch formation (Simon and O'Leary, 1992). When rostral and caudal membranes are presented in stripes, temporal retinal axons branch preferentially on rostral membranes (Roskies and O'Leary, 1994). Treatment with PI-PLC abolishes the preference. Thus, it is possible that the same or related inhibitory molecules can insure the correct axonal patterning by modulating two different neuronal activities, extension and branching.

In summary, these examples suggest that the trend in axon guidance is to move from in vivo observations to the molecular definition of the factors involved, often with the use of *in vitro* assays. This type of approach will yield many new reagents to study axon guidance, and will undoubtably also reveal a new level of complexity of axon guidance mechanisms.

**Part III: Putative guidance molecules seeking function**

An elongating axon will be in contact with matrix molecules, other axons, and other cells. Guidance information could come from any of these sources. There are many molecules expressed in the nervous system during the time of axonal extension that have some effect on neurites *in vitro* and so are *de facto* candidates for a role in axon guidance. These molecules can be generally divided into extracellular and cell surface molecules. Many were first recognized for their ability to mediated adhesion of neuronal (as well as nonneuronal) cells and/or for their ability to serve as a favorable substratum for *in vitro* neurite extension. However, given the complexity of axon behaviors described in the last section, it is not altogether clear how the ability to induce cell adhesion and neurite extension is related to the ability to guide axons. This discussion will

focus on molecule for which there is some evidence for actual guidance activity.

*Extracellular matrix molecules*

Extracellular matrix (ECM), far from simply providing an inert scaffolding, has been recognized for its important role in many important cellular processes. In non-neuronal cells, ECM molecules promote migration, differentiation, and division in the cells that they contact (reviewed in Adams and Watt, 1993). Though the structural arrangement of ECM molecules is not as well understood in the nervous system, the molecules of the ECM have been found to have profound effects on neuronal behavior. ECM molecules found in the developing brain include the laminins, the tenascins, fibronectin, proteoglycans, some collagens, thrombospondins, and vitronectin (for review see Reichardt and Tomaselli, 1991). This discussion will focus on the first four of this list.

*The laminin family*

The laminins are large modular proteins composed of three amino acid chains ($\alpha$, $\beta$ and $\gamma$) mixed and matched to form different members of an increasing large family of (Timpl and Brown, 1994). The general structure of the members of this family consists of a "long arm" of the coiled-coil N terminals of the three chains with up to three short arms (depending on the length of the chains) composed of the C terminals of the chains. Of the eight currently identified members of the family, at least four are found in the nervous system. Laminin-1, or laminin purified from the Engelbreth-Holm Swarm sarcoma, is the most widely studied in the nervous system to date. Laminin-1 has a wide range of effects on neurons. It alters the requirement of neurons for neurotropic factors (Edgar et al., 1984), affects the activity of enzymes involved in neurotransmitter synthesis (Acheson et al., 1986) and enhances neuronal polarity (Lein et al., 1992). Substratum-bound laminin is adhesive for some neuronal cell types, such as retinal neurons (Hall et al., 1987) while for others, like olfactory neurons, laminin interferes with adhesion to other ECM molecules (Calof and Lander, 1991). Laminin also promotes neuronal

cell migration *in vivo* and *in vitro* (Bronner-Fraser and Lallier, 1988; Calof and Lander, 1991). These varied responses may be due in part to the multiple laminin receptors found on neurons, including several classes of integrins (Cohen and Johnson, 1991; Tawil et al., 1990; Tomaselli et al., 1993), α-dystroglycan (Gee et al., 1992), heparan sulfate proteoglycans (C.S. Stipp, pers. commun.), and cell surface galactosyltransferase (Begovac and Shur, 1990). Additionally, different activities have been mapped to distinct parts of the laminin molecule using peptides and proteolytic fragments (e.g., Calof et al., 1994)

Laminin, when applied uniformly to a substratum, is a potent inducer of neurite outgrowth for many types of neurons (Baron-van-Evercooren et al., 1982; Rogers et al., 1983) increasing both the frequency of neurite initiation and the rate of neurite elongation (Buettner and Pittman, 1991). When laminin is applied nonuniformly to a substratum, e.g., in stripes or dots, neurites remain confined to the areas of laminin (Gundersen, 1987; Hammarback et al., 1985). Growth cones can cross gaps of approximately filopodial size from one area of laminin to another (Buettner et al., 1994; Hammarback and Letourneau, 1986). These observations suggest that laminin could play a role in guiding axons *in vivo*, perhaps as a contact-mediated guidance cue in axonal pathways.

Studies of laminin-like immunoreactivity in the brain indicate that laminin expression coincides with axonal extension. In the central nervous system (CNS) of the mouse, a laminin-like molecule is present when and where the first long axon tract of the CNS forms (c. embryonic day 9 (E9)) (Letourneau et al., 1987). Several studies have found laminin immunoreactivity in both CNS tracts such as the optic nerve (Cohen et al., 1987; Liesi and Silver, 1988) and PNS tracts such as trigeminal nerve (Riggott and Moody, 1987; Rogers et al., 1986). In many of these studies, the laminin immunoreactivity is described as "punctate." It is not clear if this observation is a result of fixing and staining procedures or reflects a non-uniform distribution of laminin *in vivo*.

While laminin expression patterns as determined by immunoreactivity were originally attributed to the isoform now known as laminin-1, these expression patterns could actually be combinatorial

representations of several members of the laminin family, due to cross-reactivity. Specifically, merosin (laminin-2)[4], an isoform of laminin-1 bearing a distinct, truncated α chain, is known to be concentrated in the basement membrane of peripheral nerve (Sanes et al., 1990). When merosin is applied to a substratum *in vitro*, it promotes neurite outgrowth from several cell types including retinal neurons, and ciliary ganglion cells as well as the migration of some cell bodies (Calof and Lander, 1991; Cohen and Johnson, 1991; Engvall et al., 1992). To my knowledge it has not been tested if merosin has the same *in vitro* guidance activity as laminin-1. A recent report has demonstrated that kalinin (laminin-5) is also detected in the nervous system (Galliano et al., 1995).

Another member of the laminin family, S-laminin (laminin-3) has quite different effects on neurons. S-laminin, a β chain homologue of laminin-1, was discovered in a search for molecules specifically expressed at the neuromuscular junction. Its restricted expression and ability to promote adhesion of ciliary motoneurons via a specific tripeptide sequence LRE (leu-arg-glu)[5] suggested that s-laminin might play a role in nerve-muscle interactions (Hunter et al., 1991; Hunter et al., 1989). Interestingly, neurites from chick ciliary ganglion neurons do not cross from a laminin-1 substratum onto an area of laminin-1 and LRE-bearing fusion protein. Instead, the growth cones of these neurites appear to stop at the border, without collapsing, and most eventually retract (Porter et al., 1995). If the activity of the tripeptide is representative of the activity of the whole molecule, these results suggest that s-laminin could modulate motoneuron axon-target interactions, perhaps facilitating synapse formation by arresting growth of the axon at the proper location.

*Fibronectin*

Fibronectin is a well-dissected molecule. Cell binding sites are well characterized and its role in differentiation and development in

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[4]Commercially available merosin (from placenta) is a mixture of laminin-2 and laminin-4.

[5]Oddly, LRE-mediated adhesion is inhibited by physiological concentrations of calcium (Hunter et al., 1991).

many systems has been extensively studied (for reviews see Adams and Watt, 1993; Hynes, 1990). Fibronectin is expressed along pathways on which peripheral axons grow (Lefcort et al., 1992) and in the subplate of the developing cortex at the time cortical axons pass through (Sheppard et al., 1991). When applied to a substratum *in vitro*, fibronectin promotes neurite extension from peripheral neurons, and a fragment of fibronectin, though not the intact molecule, promotes neurite extension of CNS neurons (Rogers et al., 1983). Interestingly, although immobilized fibronectin is a potent substratum for neurites from dorsal root ganglia (DRG), these neurites are not spatially restricted by fibronectin patterns (Gundersen, 1987). Interaction of DRG neurites with fibronectin-coated beads has been reported to cause a sustained decrease in the rate of extension, while laminin-coated beads caused an increase in rate (Kuhn and Schmidt, 1995). These results suggest that fibronectin could as a negative chemokinetic factor, i.e., it could act as an inhibitory molecule by reducing the rate of outgrowth of axons it contacted. That fibronectin could be inhibitory for some growth cones is also suggested by observations that motoneurons in the zebrafish avoid naturally fibronectin-rich areas or areas in which fibronectin has been ectopically introduced (Eisen, 1991). In summary, the effects of fibronectin on axons may be more complex than simple promotion of neurite outgrowth, and may warrent further investigation.

*Tenascins*

The tenascins are a family of large, multidomain proteins (for review see Erickson, 1993). Their modular structure contains domains common to other ECM proteins: EGF-like domains, fibronectin type III domains and fibrinogen-like domains. More specific to tenascin are its spider-like hexamers (tenascin-C tenascin-R) or trimers (tenascin-X) structure. The founding member of the tenascin family (otherwise known as cytotactin, hexabrachion, et al., and now called tenascin-C) has a variety of effects on neurons and their neurites *in vitro*. It promotes adhesion for some cells, and for others disrupts adhesion to other ECM molecules (Faissner and Kruse, 1990; Spring et al., 1989). Similarly, tenascin-C, when bound to a

substratum, can stimulate neurite outgrowth for some neurons (e.g. Wehrle and Chiquet, 1990) yet when added to the culture medium inhibits neurite outgrowth on a variety of substratum-bound molecules including tenascin-C itself (Lochter et al., 1991). Interestingly, for some neurons (such as cerebellar neurons) the neuronal cell body does not attach well on a tenascin substratum, yet these neurons extend neurites. This obsrvation suggests that, as discussed in part 1 of this introduction, cellular adhesion and neurite outgrowth are distinct activities. Indeed, all of tenascin-C's activities that have been mapped are found in distinct domains on the tenascin molecule (Lochter et al., 1991; Prieto et al., 1992). Tenascin-R has a resumé of biological activities similar to that of tenascin-C. It inhibits fibronectin-mediated adhesion by a variety of cell types (Pesheva et al., 1994), and either allows or inhibits neurite outgrowth dependent on its presentation and the type of neuron (Lochter et al., 1994; Pesheva et al., 1993).

Though a number of groups have worked very hard to find a role for tenascins in the nervous system, their function is still elusive. One theory is that tenascin-C demarcates boundaries of axonal extension by acting as an non-preferred or inhibitory molecule for axonal extension. The observation that growth cones *in vitro* avoid growing onto areas of tenascin-R or tenascin-C is consistent with such a model (Taylor et al., 1993). Tenascin-C is expressed in boundary structures such as the barrel fields of the somatosensory cortex (Steindler et al., 1989). However, examination of mice with a disruption in the tenascin-C gene (Saga et al., 1992) specifically for any defects in this region revealed no obvious differences with normal mice (Steindler et al., 1995), so the role of tenascin as a "boundary molecule" is still unclear.

*Proteoglycans*

Proteoglycans (PG's) are protein bearing glycosaminoglycan chains. The chains are based on repeating units of heparan sulfate, chondroitin sulfate or keratan sulfate (for review see Ruoslahti, 1988). There are a number of distinct PG cores in the brain, many of them developmentally regulated (Herndon and Lander, 1990). They can be soluble, transmembrane, GPI-anchored or extracellular. Their activities can be mediated by the core protein, the glycosaminoglycan

(GAG) chains or both. They could thus function as cell surface receptors, "co-receptors" (see (Saunders and Bernfield, 1988), extracellular ligands, or extracellular modulators of other ligand-receptor interactions.

The continuing identification and characterization of individual PG cores is certain to reveal a diverse range of functions in the nervous system. For example, many proteins believed to regulate axon behavior are known to bind PG's, including but not limited to laminin, tenascin, N-CAM, L1 and thrombin (for a review of PG-binding proteins see Lander, 1994). Many studies of the effects of PG's in the nervous system have effectively tested all of one class, e.g. heparan sulfate proteoglycans (HSPG's) or chondroitin sulfate proteoglycans (CSPG's) for a given biological activity. Such studies have demonstrated that PG's can both enhance and inhibit cell adhesion and neurite outgrowth (for a review see Letourneau, 1992). For example, there are many reports of CSPG interference with neurite outgrowth on a variety of substrata, including laminin (e.g. Brittis et al., 1992; Oohira et al., 1991; Snow et al., 1990b). These results are potentially interesting because CSPG's are found frequently, though not exclusively, in areas where axons do not grow (Oakley and Tosney, 1991; Pindzola et al., 1993). While it is suggested in some cases that this inhibition might occur via a cell surface receptor for the proteoglycans, the concentrations of applied CSPG required for these effects is large, the CSPG's themselves are large in size, and the studies generally do not rule out a non-specific steric mechanism. In some cases, however, demonstration of specific interactions between PG's and neurite-enhancing molecules suggest that these effects are real. The large CSPG's phosphocan and neurocan have been shown to interfere with NCAM and L1/Ng-CAM mediated homophilic adhesion and neurite outgrowth (Friedlander et al., 1994; Milev et al., 1994). The inhibition appears to be mediated by the core proteins, as enzymatic digestion of the GAG chains did not affect it. That this interaction might have *in vivo* relevance is suggested by the fact that neurocan (and phosphocan) colocalize in the developing nervous system with the immunoglobulin family members (Friedlander et al., 1994).

Continued identification and functional analysis of individual proteoglycan cores will be required to precisely define their roles in axonal elongation or guidance.

*Cell surface molecules*

Cell surface molecules can act as receptors for extracellular matrix proteins or mediate cell-cell or cell-axon interactions. The former category will not be discussed here except to mention the integrins. The integrin family of heterodimeric receptors are required for neurite extension by many types of neurons on most ECM molecules, and are expressed in the nervous system in a pattern consistent with a role in axonal elongation (for review see Reichardt and Tomaselli, 1991). It is not known if integrins also play a role in axon guidance.

One possible role for molecules mediating axon-axon or axon-cell interactions would be to specify a pathway amid the myriad of possibilities facing a growth cone and axon. Some cell surface molecules are expressed in the time and place to perform such a function, and in some cases have demonstrated relevant *in vitro* activities. One simple model for these molecules would be guidance by recognition of self, i.e. axons would selectively grow on cells (or other axons, promoting fasciculation) because they shared the same cell surface marker. However, as described below, the variety of heterophilic interactions amongst cell surface molecules precludes such a simple interpretation of their function.

*The cadherin family*

One family of cell adhesion molecules, the cadherins, are well represented in the nervous system, with many different isoforms and a generally broad pattern of expression (Takeichi, 1988). While N-cadherin is one of the major proteins mediating neurite outgrowth on Schwann cells *in vitro* (Bixby et al., 1988), there is no clear indication yet of a role for the family in axon guidance.

*The immunoglobulin superfamily*

The structural distinction of having immunoglobulin (Ig) like domains assigns many cell surface molecules in the brain to the Ig superfamily. The founding member of the Ig family in the brain is N-CAM. N-CAM established the precedent for the designation of Ig family members as homophilic cell adhesion molecules, though, like many other Ig family members many of N-CAM's functional interactions appear to be heterophilic. N-CAM is widely expressed in the nervous system, and has many different effects on neurons, including the enhancement of neurite outgrowth (Doherty et al., 1990).

One study suggests that N-CAM-associated polysialic acid is important for axonal pathfinding. This polysialic acid (PSA) is known to modulate cell interactions *in vitro* (Acheson et al., 1991; Zhang et al., 1992). Motoneuron projection errors result from enzymatic removal of PSA at the time that these axons pass through the plexus, a "choice point" (Tang et al., 1992). These projection errors were reversed by anti-L1 antibodies and not anti-PSA antibodies (Tang et al., 1994). These results suggest a role for N-CAM in modulating via PSA L1-mediated interactions such as fasciculation. Notably, this result would not have been predicted from *in vitro* studies in which the effect of removing PSA was to interfere with L1-mediated neurite outgrowth (Zhang et al., 1992).

Members of the Ig/FNIII subfamily are particulary noteworthy candidates for molecules involved in axon behavior. This subfamily is named for the immunoglobulin-like fibronectin type III repeats. Given the confusing nomenclature in this family, it might appear that another requirement is having at least two names. Two of the members, F3/F11 and axonin-1/TAG-1, are found in both soluble and GPI (glycosyl-phosphatidylinsitol)-linked form; others (e.g. Bravo/NrCAM and L1/NgCAM) have transmembrane and cytoplasmic domains (for review see Sonderegger and Rathjen, 1992). Many of these molecules promote neurite extension if applied to a substratum or presented on a cell surface: axonin-1/TAG-1 (Stoeckli et al., 1991), F3/F11 (Durbec et al., 1992; Gennarini et al., 1991), and L1/Ng-CAM (Lagenaur and Lemmon, 1987). Antibodies against most of these proteins have been shown to defasciculate neurites (e.g. Rathjen et al.,

1987), which suggests that the proteins could be involved in axon-axon interactions. Expression patterns are consistent with a role in axon behavior. For example, axonin-1 expression is neuron specific and is strong in many fiber tracts (Wolfer et al., 1994).

Heterophilic interactions with other Ig superfamily members as well as ECM molecules have been demonstrated for almost all Ig/FN III family members In some cases, the interactions, usually initially defined using bead-coated proteins, have been shown to be physiologically relevant. For example, axonin-1 stimulation of neurite outgrowth is completely blocked by anti-L1 antibodies (Kuhn et al., 1991). Likewise, antibodies to Nr-CAM inhibit substratum-bound F11-mediated neurite outgrowth (Morales et al., 1993). The complexity of interactions can be very high; for example, F11 also binds to Ng-CAM and tenascin-R. The binding maps to two different sites on the F11 molecule, distinct also from the Nr-CAM mediated neurite outgrowth domain (Brummendorf et al., 1993). Moreover, in the presence of antibodies to F11, neurite extension is increased on tenascin-R (Pesheva et al., 1993), suggesting that F3/F11 might also mediate the inhibitory effect of the matrix molecule.

Direct tests of the functions of these interactions *in vivo* will be necessary to determine which interactions are actually important for axon guidance. For example, Stoeckli and Landmesser (1995) have examined the role of several Ig superfamily members in the pathfinding of commissural axons in the spinal cord. Commisural neurons grow ventrally in the spinal cord to the floorplate. They then grow into the floorplate, cross the midline and turn longitudinally. When whole or explanted embryos are treated with soluble axonin-1 or antibodies to axonin-1 during the time of this outgrowth, a small percentage of the commissural axons fail to cross the midline and instead make their longitudinal turn on the same (ipsilateral) side of the midline. This effect was also observed with antibodies to Nr-CAM but not by antibodies to Ng-CAM. At that stage the axons, but not the floorplate cells, express axonin-1, and the floorplate, but not the axons, express Nr-CAM. It is thus possible that the heterophilic interaction of Nr-CAM and axonin-1 is required for appropriate pathfinding of these axons across the midline.

For some Ig superfamily members, the simple model of homophilic interactions mediating specific neural projections might be valid. LAMP (limbic-system associated membrane protein) is a member of the Ig superfamily preferentially expressed in subsets of cortical and subcortical neurons that are associated with the limbic system (Levitt, 1984; Pimenta et al., 1995). This molecule, when present on the surface of cells, promotes the outgrowth *in vitro* of limbic system-associated cortical neurons. And, *in vivo* antibodies to LAMP alter a cortical projection to the hippocampus such that fibers are unusually diffuse and occasionally found in inappropriate tracts (Pimenta et al., 1995). LAMP could thus be an example of a cell surface molecule that guides via homophilic interactions. Or, further experiments will reveal the same level of complexity in the interactions of this molecule as for its relatives.

**Part IV: The cellular basis of growth cone motility and guidance**

How external cues can change growth cone behavior has been discussed. The topic of this section will be the events inside the growth cone that may be involved in the response to these cues. I will begin with a description of a growth cone's cytoskeletal components and their regulation during outgrowth and guidance, and conclude with other signaling events implicated in axon outgrowth and guidance.

*Cytoskeleton in growth cones*

The growth cone is a specialized cellular structure that can be divided into two parts: a thicker cytoplasm-rich base area, densely populated with microtubules and vesicles, and the distal actin-rich area in which actin is visibly organized into protruding bundles (filopodia) and more randomly oriented networks (lamellipodia) (see Lewis and Bridgman, 1992). Growth cones visualized by time lapse microscopy move constantly: filopodia advance and retract, and lamellipodial ruffles propagate over the growth cone's dorsal surface. Some of this movement translates into net forward displacement: that the growth cone is intrinsically motile is demonstrated by the observation that a growth cone transected from its axon continues to

advance for some time. Longer term advance requires a connected axon, apparently for transport of materials from the cell body (Martenson et al., 1993). Growth cones do not need to be "pushed" along by the axon extending behind them: growth cones, and even individual filopodia, generate surprisingly forceful tension that correlates with periods of growth cone advance. This was demonstrated by direct measurement of force generated by growth cones attaching to glass needles (Lamoureux et al., 1989).

Actin dynamics play a crucial role in the motility of the growth cone. In a popular model (Mitchison and Kirschner, 1988; Smith, 1988), the forward protrusion of the growth cone is caused by the addition of new actin polymers at the leading edge of the growth cone coupled with retrograde (i.e., towards the axon) movement of the actin filaments. Depolymerization of actin completes the cycle. This model is supported by observations that when actin polymerization is blocked by cytochalasin treatment the rearward movement of filaments continues but forward protrusion stops (Forscher and Smith, 1988). Also, some experiments have determined that the rate of actin filament retrograde movement is inversely proportional to the rate of growth cone advance in the same axis (Lin and Forscher, 1995).[6] To actually generate force and move the growth cone forward, this actin cycle also requires anchorage to the substratum and a motor to move the actin filaments. Anchorage could be generated by cell-substratum receptors that can associate directly or indirectly with the cytoskeleton, such as integrins (Hynes, 1992). Ideally, a motor molecule would be able to attach and detach readily from the actin filaments and/or the membrane to smoothly move the actin relative to the substratum. Non-muscle myosins are attractive candidates for this motor molecule, as they have been shown to associate with both actin and the plasma membrane and can translocate isolated actin filaments (e.g. Zot et al., 1992). While a number of non-muscle myosins have been found in growth cones (Cheng et al., 1993; Miller et al., 1992), it

[6]In contrast, Thierot and Mitchison (1992) found no correlation between the rate of retrograde actin movement and forward protrusion at the leading edge of motile fibroblasts.

is not known which if any of these could be this putative motor molecule.

One way of addressing the mechanism of guidance is to ask how the growth cone's motile machinery is regulated. If actin dynamics move the growth cone, limiting where in the growth cone polymerization occurs can restrict the direction of movement of the growth cone moves. If external cues can do this, they should be able to produce biased axonal extension and thus guide. Growth cone movement in response to external guidance cues have been divided into three parts: sampling, orientation, and consolidation (O'Connor and Bentley, 1993). Visualization of actin and tubulin structures in live growth cones has provided some basis for understanding what happens in the growth cone during these steps.

The growth cone's filopodia are believed to play a major role in the first stage of growth cone detection of cues: sampling. Filopodia are actin rich, dynamic structures whose existence is dependent on actin polymerization, as growth cones treated with cytochalasins, drugs that blocks the polymerization of actin, lack filopodia. That these growth cones still elongate indicates that filopodia are not required for motility *per se* (Marsh and Letourneau, 1984). The role of the other major dynamic form at the edge of the growth cone, lamellipodia, is less clear. The contribution of filopodia to the guidance of the whole growth cone has been repeatedly demonstrated. Filopodial extension does not prefigure the eventual direction of growth cone movement; it seems to occur in all directions. Instead, the specific events that control guidance seem to occur after a given filopodium has made contact with something in the environment. The most striking example of this is found in the grasshopper limb bud. During the trajectory of the pioneer T1 axons, several turns are made, one of which is dependent on the presence of a differentiated neuron (Bentley and Caudy, 1983). Contact of this "guidepost cell" by a single filopodium is sufficient to reorient the entire growth cone (O'Connor et al., 1990). Growth cones that lack filopodia due to cytochalasin treatment have been observed to make aberrant pathfinding decisions *in vivo* (Bentley and Toroian-Raymond, 1986; Chien et al., 1993). Though blocking actin polymerization could have other effects on both

the elongating axon and/or the cells in its environment, these results support an important role for filopodia and the rest of the actin cytoskeleton in growth cone guidance.

Actin also plays a key role in the following step of the growth cone response: orientation. Fluorescently labeled actin and actin analogs were used to follow actin dynamics in the trajectory of the grasshopper T1 axon mentioned above (O'Connor and Bentley, 1993). In a variety of extracellular environments, actin accumulated where the growth cone would grow and was depleted when the growth cone was retracting. During a time of growth in which branching occurred randomly, i.e. did not result in net changes in direction of the growth cone, the branch that would persist was more actin dense than the branch that would be eliminated. The increase in actin density occurred before any obvious morphological changes differentiating the branches. Also, when a filopodium contacted the guidance cell described above, its actin core thickened.[7] Similarly, Lin and Forscher (1993) observed f-actin accumulation at the site of contact between growth cones and physiological targets in culture.

Consolidation of the growth cone in response to a guidance cues involves the movement of microtubules in the eventual direction of axonal extension. Studies of microtubule behavior in the grasshopper guidance system have demonstrated microtubule movements associated with many morphological changes involved in pathfinding such as branching and filopodial engorgement, i.e. the conversion of a filopodium into an axon (Sabry et al., 1991). These studies suggested that microtubules can be directed either by selective elongation or selective retention: i.e., in some cases microtubules were found to move selectively into branches that were retained, while at other times they were present in more than one branch and then persisted in the surviving branch. Importantly, the arrangement of the

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[7] In other observations of filopodial dynamics at this guidance cell, growth cone advance was shown to occur by filopodial engorgement, i.e. thickening of the filopodia until it became the axon. In these studies, several filopodia sequentially contact the guidepost cell and all become engorged. Only one will form the axon; unfortunately, the study does not address if that choice is accompanied by a decrease in the actin density of the others.

microtubules did not predict the movement of the growth cone, but instead seemed to be a causal effect.

The general impression from these experiments is that regulation of actin dynamics is a key step in a growth cone's choice of direction. The polymerization and depolymerization of actin is regulated in non-neuronal cells by a number of actin binding proteins. Not surprisingly, many of these proteins are expressed in neurons during the time of axon extension, and some have been localized to growth cones by immunocytochemistry. These proteins regulate the assembly and disassembly of actin filaments, such as profilin and gelsolin, or anchoractin filaments to the membrane or to membrane associated receptors, such as alpha-actinin, vinculin, and the ERM family of proteins (ezrin, radixin and moesin) (Amieva and Furthmayr, 1995; Birgbauer et al., 1991; Faivesarrailh et al., 1993; Letourneau and Shattuck, 1989; Sobue and Kanda, 1989; Tanaka et al., 1993). In most cases the functions of these molecules in the axon and growth cone has not been directly tested. One exception is vinculin, which when depleted in neurons by administration of antisense RNA leads to a decrease rate of neurite extension (Varnum-Finney and Reichardt, 1994). A decrease was found in filopodial and lamellipodial lifetime, but not in the rate of advance of filopodia, suggesting that vinculin plays a specific role in stabilizing filopodia. It would be informative to repeat this type of experiment for other actin-binding proteins.

*Calcium in the growth cone*

The activities of many of these actin binding proteins are calcium dependent (Forscher, 1989). Indeed, changes in neurite calcium levels results in actin filament depolymerization (e.g. Lankford and Letourneau, 1989). Because calcium has so many functions in a neuron (see Ghosh and Greenberg, 1995), it is difficult to interpret the results of experiments that alter calcium levels. Nonetheless, many studies have used a variety of perturbations to alter overall calcium in the growth cone and assay effects on neurite behavior (reviewed in Kater and Mills, 1991). One general conclusion from these experiments is that growth cones appear to have a "set-point" optimal for neurite extension, any increase or decrease from which

will slow neurite extension. This suggests that the calcium levels in growth cones are highly regulated. Some attention has also focused on the effects of calcium changes on filopodia: they elongate and retract when calcium levels are changed, and can respond to extracellular stimuli by changes in calcium levels even if severed from the growth cone (Davenport et al., 1993; Rehder and Kater, 1992). These experiments do indicate that filopodia may respond to some extracellular cues by changes in local calcium concentration, which could lead to cytoskeletal remodeling.

Perhaps a better approach to understanding calcium dynamics in the growth cone is to look at specific molecules in the growth cone that regulate calcium or that are regulated by calcium. Calcineurin is a serine/threonine phosphatase regulated by calcium that is strongly expressed in neurons (Ferreira et al., 1993). A general inhibition of calcineurin function (i.e. bath-applied drug) inhibits neurite outgrowth. A localized inhibition of calcineurin in filopodia made them retract (Chang et al., 1995). The local inactivation also biased growth cone movement away from the site of retraction. Targets of calcineurin include tau, a microtubule-associated protein and GAP-43 (see below).

*Other second messenger systems in the growth cone*

In addition to calcium, other second messenger and signaling systems have been proposed to act in the growth cone and neurite to regulate axon extension and axon guidance. These include cAMP, phosphoinositides, arachidonic acid metabolites, serine/threonine kinases, tyrosine kinases, and phosphatases. (Lankford and Letourneau, 1991; Miller et al., 1993; Schuch et al., 1989; see Doherty and Walsh, 1994 for review). In many cases these effects were determined by addition of pharmacological agents to cultured neurons plated on neurite-outgrowth promoting molecules, such as laminin, N-CAM or N-cadherin, and the effect on neurite length recorded. In some cases the effects of the agents varied with substratum (e.g., Williams et al., 1994). While these experiments implicate many classes of intracellular regulators in neurite behavior, it is difficult to extract a clear picture of their actions. The rest of this section will

discuss some cases where a clearer picture of the importance of certain signalling molecules is emerging.

Protein tyrosine kinases and phosphatases represent one large class of signalling molecules for which there is growing evidence from a variety of experiments for an involvement in neurite outgrowth and neurite guidance. A number of tyrosine kinases and phosphatases are enriched in growth cone preparations or localized to growth cones (e.g., Bixby and Jhabvala, 1993; Sahin and Hockfield, 1993; Stoker et al., 1995). Staining of *Aplysia* growth cones with an antibody to phosphotyrosine detected strong staining in the tips of filopodia that was responsive to changes in the substratum (Wu and Goldberg, 1993). Application of genistein, a broad-based tyrosine kinase inhibitor, caused a lengthening of the filopodia. Genistein also blocks neurite retraction induced by some agents (Smallheiser, 1993). A number of tyrosine kinase inhibitors alter the degree of neurite extension on different substrata (Miller et al., 1993). Consistently, some kinases, such as c-src are activated in response to cell adhesion molecules: soluble fragments of L1, N-CAM or MAG have been shown to alter tyrosine phosphorylation of tubulin in a c-src dependent manner (Atashi et al., 1992). These results indicate that phosphoproteins may be involved in the regulation of cytoskeletal dynamics in response to external cues. Additionally, there are several examples in *Drosophila* of tyrosine kinases mutations in which contribute to pathfinding defects: the receptor protein tyrosine kinase *derailed*, and *abl* (when deleted in conjunction with another gene, *fasciclin I*) (Callahan et al., 1995; Elkins et al., 1990). RAGS and Elf-1, putative guidance molecules in the chick tectum, are ligands for receptor tyrosine kinases (Drescher et al., 1995). Further examination of the actions of these tyrosine kinases and tyrosine phosphatases might yield very useful information about growth cone guidance.

While many receptors and signaling systems identified in the growth cone are common to many different cells types, some signaling molecules are noteworthy for their enrichment in the neuronal growth cone. For example, a G protein, $G_O$, is one of the most abundant proteins in growth cone preparations (Strittmatter et al., 1990). G proteins and G protein-coupled receptors in other systems are used to

amplify signals; this is an intriguing possibility for their action in the growth cone, given that events in one filopodium can alter the behavior of the whole growth cone. Unfortunately, no clear tests of Go function have been conducted. The interpretation of studies using pertussis toxin as an inhibitor of Go activity (e.g., Doherty et al., 1991) is difficult because pertussis toxin has G protein-independent activities as well (Tamura et al., 1983).

GAP-43 is a cytosolic protein also noteworthy for its strong expression in growth cones. It is billed as an important player in signal transduction in the growth cone, based on its ability to activate Go and to interact with the calcium-binding protein calmodulin. GAP-43 does not seem to be required for neurite outgrowth (Aigner and Caroni, 1993; Strittmatter et al., 1995). However, axonal behavior at the optic chiasm (see section 2) is perturbed in GAP-43 mutant mice; axons extend normally to the chiasm and then stall and do not continue on their appropriate tracts (Strittmatter et al., 1995). If this phenotype is confirmed as a guidance defect and not as an elongation defect, GAP-43 may actually be a transducer of guidance signals.

*In vitro assays of neurite behavior*

Most *in vitro* studies of growth cone intracellular mechanisms have concentrated on neurite outgrowth. However, there are other *in vitro* growth cone behaviors that could provide clues to the mechanism of growth cone guidance. For example, growth cones will turn in response to a soluble gradient of NGF, membrane permeable analogs of cyclic AMP, or a neurotransmitter, acetylcholine (Gundersen and Barrett, 1980; Lohof et al., 1992; Zheng et al., 1994). While these experiments are difficult to interpret, they do provide *in vitro* evidence that a growth cone can orient to an external chemical source. Growth cones and their filopodia can also orient to an electric field, a phenomenon known as galvanotaxis (Patel and Poo, 1982). Like man other guidance-related phenomena, this effect is sensitive to calcium perturbation and actin depolymerization (Bedlack et al., 1992).

Molecules that, when added acutely to growth cones, cause rapid loss of growth cone structure have become very important in the field

of growth cone guidance because of the correlations between collapsing activity and the ability to guide neurites and axons by inhibition (Baier and Bonhoeffer, 1992; Messersmith et al., 1995). Some downstream effectors of different types of collapsing factors are known. Inhibitors of the small GTP-binding protein rho block thrombin-induced growth cone collapse; rho is believed to regulate the actin cytoskeleton (Jalink et al., 1994). In fact, local depletion of actin is observed *in vitro* when growth cones are exposed to a the collapsing factor collapsin (Fan et al., 1993). A putative signal-transducing molecule was recently identified by its ability to mediate calcium currents induced by collapsin. Microinjected antibodies to this molecule, CRMP-62, block collapsin-induced growth collapse with no other obvious effects on neurons (Goshima et al., 1995). The growth cone collapse assay has been a powerful tool for identifying both extracellular and intracellular molecules potentially involved in axon guidance.

**Conclusions**

Many basic questions about axon guidance remain unanswered. Of the many molecules that are expressed in the nervous system and have *in vitro* effects on neurons which actually act on neurons and axons *in vivo* and how are their effects integrated? How are neurite outgrowth and choicemaking related on the intracellular level? How are growth cone collapse and neurite outgrowth related? How are guidance by positive and inhibitory factors related?

My thesis addresses several of these issues. In chapter 2, I analyze how one extracellular matrix molecule, laminin, regulates the adhesive response of cells to another, fibronectin. In chapter 3, I describe a novel assay to test for treatments that specifically affect growth cone guidance, the discovery of such a treatment, pertussis toxin, and its unexpectedly G protein independent, cellular carbohydrate-dependent actions on neurite guidance but not neurite outgrowth. The beginnings of a characterization of pertussis toxin-binding proteins on neurons is described in chapter 4. And in chapter 5, I discuss a naturally occurring lectin, galectin-1, that stimulates neurite extension via cellular carbohydrates.

## References


Acheson, A., Edgar, D., Timpl, R., and Thoenen, H. (1986). Laminin increases both levels and activity of tyrosine hydroxylase in calf adrenal chromaffin cells. J. Cell Biol. *102*, 151-159.

Acheson, A., Sunshine, J. L., and Rutishauser, U. (1991). NCAM polysialic acid can regulate both cell-cell and cell-substrate interactions. J. Cell Biol. *114*,

Adams, J. C., and Watt, F. M. (1993). Regulation of development and differentiation by the extracellular matrix. Development *117*, 1183-1198.

Aigner, L., and Caroni, P. (1993). Depletion of 43-kD growth-associated protein in primary sensory neurons leads to diminished formation and spreading of growth cones. J. Cell Biol. *123*, 417-429.

Amieva, M. R., and Furthmayr, H. (1995). Subcellular localization of moesin in dynamic filopodia, retraction fibers, and other structures involved in substrate exploration, attachment, and cell-cell contacts. Exp. Cell Res. *219*, 180-196.

Atashi, J. R., Klinz, S. G., Ingrham, C. A., Matten, W. T., Schachner, M., and Maness, P. F. (1992). Neural cell adhesion molecules modulate tyrpsine phosphorylation of tubulin in nerve growth cone membranes. Neuron *8*, 831-842.

Baier, H., and Bonhoeffer, F. (1992). Axon guidance by gradients of a target-derived component. Science *255*, 472-475.

Baron-van-Evercooren, A., Kleinman, H. D., Ohno, S., Marangos, P., Schwartz, J. P., and Dubois-Dalcq, M. E. (1982). Nerve growth factor, laminin and fibronectin promote nerve growth in human fetal sensory ganglia cultures. J. Neursci. Res. *8*, 179-183.

Bedlack, R. S., Wei, M.-D., and Loew, L. M. (1992). Localized membrane depolarizations and localized calcium influx during electric field-guided neurite growth. Neuron *9*, 393-403.

Begovac, P. C., and Shur, B. D. (1990). Cell surface galactosyltransferase mediates the initiation of neurite outgrowth from PC12 cells on laminin. J. Cell Biol. *110*, 461-470.

Bentley, D., and Caudy, M. (1983). Pioneer axons lose directed growth after selective killing of guidepost cells. Nature *304*, 62-65.

Bentley, D., and Toroian-Raymond, A. (1986). Disoriented pathfinding by pioneer neurone growth cones deprived of filopodia by cytochalasin treatment. Nature *323*, 712-715.

Birgbauer, E. J., Dinsmore, J. H., Winckler, B., Lander, A. D., and Solomon, F. (1991). Association of ezrin isoforms with the neuronal cytoskeleton. J. Neurosci. Res, *30*, 232-241.

Bixby, J. A., Likien, J., and Reichardt, L. F. (1988). Identification of the major proteins that promote neuronal process outgrowth on Schwann cells *in vitro*. J. Cell Biol. *107*, 353-361.

Bixby, J. L., and Jhabvala, P. (1993). Tyrosine phosphorylation in early embryonic growth cones. J. Neurosci. *13*, 3421-3432.

Bovolenta, P., and Mason, C. (1987). Growth cone morphology varies with position in the developing mouse visual pathway from retina to first targets. J. Neurosci. *7*, 1447-1460.

Brittis, P. A., Canning, D. R., and Silber, J. (1992). Chondroitin sulfate as a regulator of neuronal patterning in the retina. Science *255*, 733-736.

Bronner-Fraser, M., and Lallier, T. (1988). A monoclonal antibody against a laminin-heparan sulfate proteoglycan complex perturbs cranial neural crest migration *in vivo*. J. Cell Biol. *106*, 507-515.

Brummendorf, T., Hubert, M., Treubert, U., Leuschner, R., Tarnok, A., and Rathjen, F. G. (1993). The axonal recognition molecule F11 is a multifunctional protein: specific domains mediate interactions with Ng-CAM and restrictin. Neuron *10*, 711-727.

Buettner, H. M., and Pittman, R. N. (1991). Quantitative effects of laminin concentration on neurite outgrowth *in vitro*. Dev. Biol. *145*, 266-276.

Buettner, H. M., Pittman, R. N., and Ivins, J. K. (1994). A model of neurite extension across regions of nonpermissive substrate: simulations based on experimental measurement of growth cone motility and filopodial dynamics. Dev. Biol. *163*, 407-422.

Callahan, C. A., Muralidhar, M., Lundgren, S. E., Scully, A. L., and Thomas, J. B. (1995). Control of neuronal pathway selection by a *Drosophila* receptor protein-tyrosine kinase family member. Nature *376*, 171-174.

Calof, A. L., Campanero, M. R., O'Rear, J. J., Yurchenco, P. D., and Lander, A. D. (1994). Domain-specific activation of neuronal migration and neurite outgrowth-promoting activities of laminin. Neuron *13*, 117-130.

Calof, A. L., and Lander, A. D. (1991). Relationship between neuronal migration and cell-substratum adhesion: laminin and merosin promote olfactory neuronal migration but are anti-adhesive. J. Cell Biol. *115*, 779-794.

Chang, H. Y., Takel, K., Sydor, A. M., Born, T., Rusnak, F., and Jay, D. G. (1995). Asymmetric retraction of growth cone filopodia following focal inactivation of calcineurin. Nature *376*, 686-690.

Cheng, H.-J., Nakamoto, M., Bergemann, A. D., and Flanagan, J. G. (1995). Complementary gradients in expression and binding of ELF-1 and Mek4 in development of the topographic retinotectal projection map. Cell *82*, 371-381.

Cheng, T. P. O., Murakami, N., and Elizinga, M. (1993). Localization of myosin IIB at the leading edge of growth cones from rat dorsal root ganglion cells. FEBS Lett. *311*, 91-94.

Chien, C.-B., Rosenthal, D. I., Harris, W. A., and Holt, C. E. (1993). Navigational errors made by growth cones without filopodia in the embryonic *Xenopus* brain. Neuron *11*, 237-251.

Cohen, J., Burne, J. F., McKinlay, C., and Winter, J. (1987). The role of laminin and the laminin/fibronectin receptor complex in the outgrowth of retinal ganglion cell axons. Dev. Biol. *122*, 407-418.

Cohen, J., and Johnson, A. R. (1991). Differential effects of laminin and merosin on neurite outgrowth by developing retinal ganglion cells. J. Cell Sci. Suppl. *15*, 1-7.
Colamarino, S. A., and Tessier-Lavigne, M. (1995). The axonal chemoattractant netrin-1 is also a chemorepellent for trochlear motor axons. Neuron *81*, 621-630.
Cox, E. C., Muller, B., and Bonhoeffer, F. (1990). Axonal guidance in the chick visual system: posterior tectal membranes induce collapse of growth cones from the temporal retina. Neuron *4*, 31-37.
Davenport, R. W., Dou, P., Rehder, V., and Kater, S. B. (1993). A sensory role for neuronal growth cone filopodia. Nature *361*, 721-723.
Davies, A. M., Bandtlow, C., Heumann, R., Korsching, S., Rohrer, H., and Thoenen, H. (1987). Timing and site of nerve growth factor synthesis in developing skin in relation to innervation and expression of the receptor. Nature *326*, 353-358.
Devreotes, P. N., and Zigmond, S. H. (1988). Chemotaxis in eukaryotic cells. Ann. Rev. Cell Biol. *4*, 649-686.
Dodd, J., and Jessell, T. M. (1988). Axon guidance and the patterning of neuronal projection in vertebrates. Science *242*, 692-699.
Doherty, P., Ashton, S. V., Moore, S. E., and Walsh, F. S. (1991). Morphoregulatory activities of NCAM and N-Cadherin can be accounted for by a G protein dependent activation of L- and N-type neuronal Ca2+ channels. Cell *67*, 21-33.
Doherty, P., Fruns, M., Seaton, P., Dickson, G., Barton, C. H., Sears, T. A., and Walsh, F. S. (1990). A threshold effect of the major isoforms of NCAM on neurite outgrowth. Nature *343*, 464-466.
Doherty, P., and Walsh, F. S. (1994). Signal transduction events underlying neurite outgrowth stimulated by cell adhesion molecules. Curr. Opin. Neurobiol. *4*, 322-336.
Drescher, U., Kremoser, C., Handwerker, C., Loschinger, J., Noda, M., and Bonhoeffer, F. (1995). *In vitro* guidance of retinal ganglion cell axons by RAGS, a 25 kDa tectal protein related to ligands for Eph receptor typrosine kinases. Cell *82*, 359-370.
Durbec, P., Gennarini, G., Goridis, G., and Rougon, G. (1992). A soluble form of the F3 neuronal cell adhesion molecule promotes neurite outgrowth. J. Cell Biol. *117*, 877-887.
Edgar, D., Timpl, R., and Thoenen, H. (1984). The heparin-binding domain of laminin is responsible for its effects on neurite outgrowth and neuronal survival. EMBO J. *3*, 1463-1468.
Eisen, J. S. (1991). Developmental neurobiology of the zebrafish. J. Neurosci. *11*, 311-317.
Elkins, T., Zinn, K., McAllister, L., Hoffman, F. M., and Goodman, C. S. (1990). Genetic analysis of a Drosophila neural cell adhesion molecule: interaction of fasciclin I and Abelson tyrosine kinase mutations. Cell *60*, 565-575.
Engvall, E., Earwicker, D., Day, A., Muir, D., Manthorpe, M., and Paulsson, M. (1992). Merosin promotes cell attachment and neurite

outgrowth and is a component of the neurite-promoting factor of RN22 schwannoma cells. Exp. Cell Res. *198*, 115-123.
Erickson, H. P. (1993). Tenascin-C, tenascin-R and tenascin-X: a family of talented proteins in search of functions. Curr. Opin. Cell Biol. *5*, 869-876.
Faissner, A., and Kruse, J. (1990). J1/tenascin is a repulsive substrate for central nervous system neurons. Neuron *5*, 627-637.
Faivesarrailh, C., Lena, J. Y., Had, L., Vignes, M., and Londberg, U. (1993). Location of profilin at presynaptic sites in the cerebellar cortex--implication for the regulation of the actin polymerization state during axonal elongation and synaptogenesis. J. Neurocytol. *22*, 1060-1072.
Fan, J., Mansfield, S. G., Redmond, T., Gordon-Weeks, P. R., and Raper, J. A. (1993). The organization of f-actin and microtubules in growth cones exposed to a brain-derived collapsing factor. J. Cell Biol. *121*, 867-878.
Ferreira, A., Kincaid, R., and Kosik, K. S. (1993). Calcineurin is associated with the cytoskeleton of cultured neurons and has a role in the acquisition of polarity. Mol. Biol. Cell *4*, 1225-1238.
Fitzgerald, M., Kwait, G. C., Middleton, J., and Pini, A. (1993). Ventral spinal cord inhibition of neurite outgrowth from embryonic rat dorsal root ganglia. Development *117*, 1377-1384.
Forscher, P. (1989). Calcium and phosphoinositide control of cytoskeletal dynamics. Trends Neurosci *12*, 468-474.
Forscher, P., and Smith, S. J. (1988). Actions of cytochalasins on the organization of actin filaments and microtubules in a neuronal growth cone. J. Cell Biol. *107*, 1505-1516.
Friedlander, D. R., Milev, P., Karthikeyan, L., Margolis, R. K., Margolis, R. U., and Grumet, M. (1994). The neuronal chondroitin surfate proteoglycan neurocan binds to the neural cell adhesion molecules Ng-CAM/L1/NILE and N-CAM, and inhibits neuronal adhesion and neurite outgrowth. J. Cell Biol. *125*, 300-307.
Galliano, M. F., Aberdam, D., Aguzzi, A., Ortonne, J. P., and Meneguzzi, G. (1995). Cloning and complete primary structure of the mouse laminin alpha 3 chain. Distinct expression pattern of the laminin alpha 3A and alpha 3B chain isoforms. J. Biol. Chem. *270*, 21820-21826.
Gee, S. H., Blacher, R. W., Douville, P. J., Provost, P. R., Yurchenco, P. D., and Carbonetto, S. (1992). Laminin-binding protein 120 from brain is closely related to the dystrophin-associated glycoprotein, dystroglycan, and binds with high affinity to the major heparin binding domain of laminin. J. Biol. Chem. *268*, 14972-14980.
Gennarini, G., Durbec, P., Boned, A., Rougon, G., and Groidis, C. (1991). Transfected F3/F11 neuronal cell surface protein mediates intercellular adhesion and promotes neurite outgrowth. Neuron *6*, 595-606.
Ghosh, A., and Greenberg, M. E. (1995). Calcium signaling in neurons: molecular mechanisms and cellular consequences. Science *268*, 239-247.

Godement, P., Salaun, J., and Mason, C. A. (1990). Retinal axon pathfinding in the optic chiasm: divergence of crossed and uncrossed fibers. Neuron *5*, 173-186.
Gomez, T. M., and Letourneau, P. C. (1994). Filopodia initiate choices made by sensory neuron growth cones at laminin/fibronectin borders *in vitro*. J. Neurosci. *14*, 5959-5972.
Goodman, C. S. (1994). The likeness of being: phylogenetically conserved molecular mechanisms of growth cone guidance. Cell *78*, 353-356.
Goshima, Y., Nakamura, F., Strittmatter, P., and Strittmatter, S. M. (1995). Collapsin-induced growth cone collapse mediated by an intracellular protein related to UNC-33. Nature *376*, 509-514.
Gundersen, R. W. (1987). Response of sensory neurites and growth cones to patterned substrata of laminin and fibronectin *in vitro*. Dev. Biol. *121*, 423-431.
Gundersen, R. W., and Barrett, J. N. (1980). Characterization of the turning response of dorsal root neurites toward nerve growth factor. J. Cell Biol. *87*, 546-554.
Guthrie, S., and Pini, A. (1995). Chemorepulsion of developing motor axons by the floor plate. Neuron *14*, 1117-1130.
Hall, D. E., Neugebauer, K. M., and Reichardt, L. F. (1987). Embryonic neural retina cell response to extracellular matrix proteins: developmental changes and effects of the cell substratum attachment antibody CSAT. J. Cell Biol. *104*, 623-634.
Hammarback, J. A., and Letourneau, P. C. (1986). Neurite extension across regions of low cell-substratum adhesivity: implications for the guidepost hypothesis of axonal pathfinding. Dev. Biol. *117*, 655-662.
Hammarback, J. A., Palm, S. L., Furcht, L. T., and Letourneau, P. C. (1985). Guidance of neurite outgrowth by pathways of substratum-adsorbed laminin. J. Neurosci. Res. *13*, 213-220.
Heffner, C. D., Lumsden, A. G. S., and O'Leary, D. D. M. (1990). Target control of collateral extension and directional axon growth in the mammalian brain. Science *247*, 217-220.
Herndon, M. E., and Lander, A. D. (1990). A diverse set of developmentally regulated proteoglycans is expressed in the rat central nervous system. Neuron *4*, 949-961.
Holt, C. E., and Harris, W. A. (1993). Position, guidance and mapping in the developing visual system. J. Neurobiol. *24*, 1400-1422.
Hunter, D. D., Cashman, N., Morris-Valero, R., Bulock, J. W., Adams, S. P., and Sanes, J. R. (1991). An LRE (leucine-arginine-glutamate)-dependent mechanism for adhesion of neurons to S-laminin. J. Neurosci. *11*, 3960-3971.
Hunter, D. D., Shah, V., Merlie, J. P., and Sanes, J. R. (1989). A laminin-like adhesive protein concentrated in the synaptic cleft of the neuromuscular junction. Nature *338*, 229-234.
Hynes, R. O. (1990). Fibronectins. (New York: Springer-Verlag).
Hynes, R. O. (1992). Integrins: versatility, modulation, and signaling in cell adhesion. Cell *68*, 303-322.

Ishii, N., Wadsworth, W. G., Stern, B. D., Culotti, J. G., and Hedgecock, E. M. (1992). UNC-6, a laminin-related protein, guides cell and pioneer axon migrations in *C. elegans*. Neuron *9*, 873-881.

Jalink, K., Corven, E. J. v., Hengeveld, T., Morii, N., Narumiya, S., and Moolenaar, W. H. (1994). Inhibition of lysophosphatidate- and thrombin-induced neurite retraction and neuronal cell rounding by ADP ribosylation of the small GTP-binding protein rho. J. Cell Biol. *126*, 801-810.

Kater, S. B., and Mills, L. R. (1991). Regulation of growth cone behavior by calcium. J. Neurosci. *11*, 891-899.

Kennedy, T. E., Serafini, T., Torre, J. R. d. l., and Tessier-Lavigne, M. (1994). Netrins are diffusible chemotropic factors for commissural axons in the embryonic spinal cord. Cell *78*, 425-435.

Kuhn, T. B., and Schmidt, M. F. (1995). Laminin and fibronectin guideposts signal sustained but opposite effects to passing growth cones. Neuron *14*, 275-285.

Kuhn, T. B., Stoeckli, E. T., Condrau, M. A., Rathjen, F. G., and Sonderegger, P. (1991). Neurite outgrowth on immobilized axonin-1 is mediated by a heterophilic interaction with L1(G4). J. Biol. Chem. *115*, 1113-1126.

Lagenaur, C., and Lemmon, V. (1987). An L1-like molecule, the 8D9 antigen, is a potent substrate for neurite extension. Proc. Natl. Acad. Sci *84*, 7753-7757.

Lamoureux, P., Buxbaum, R. E., and Heidemann, S. R. (1989). Direct evidence that growth cones pull. Nature *340*, 159-162.

Lander, A. (1994). Targeting the glycosaminoglycan-binding sites of proteins. Chem. and Biol. *1*, 73-78.

Lankford, K. L., and Letourneau, P. C. (1989). Evidence that calcium may control neurite outgrowth by regulating the stability of actin filaments. J. Cell Biol. *109*, 1229-1243.

Lankford, K. L., and Letourneau, P. C. (1991). Roles of actin filaments and three second-messenger systems in short-term regulation of chick dorsal root ganglion neurite outgrowth. Cell Motil Cytoskeleton *20*, 7-29.

Lefcort, F., Venstrom, K., McDonald, J. A., and Reichardt, L. F. (1992). Regulation of expression of fibronectin and its receptor, alpha 5 beta 1, during development and regeneration of peripheral nerve. Development *116*, 767-782.

Lein, P. J., Banker, G. A., and Higgens, D. (1992). Laminin selectively enhances axonal growth and accelerates the development of polarity by hippocampal neurons in culture. Dev. Brain Res. *69*, 191-197.

Lemmon, V., Burden, S. M., Payne, H. R., Elmslie, G. J., and Hlavin, M. L. (1992). Neurite growth on different substrates: permissive versus instructive influences and the role of adhesive strength. J. Neurosci. *12*, 818-826.

Letourneau, P. C. (1992). Extracellular matrix and neurite outgrowth. Curr. Opin. Genet. Dev. *2*, 625-34.

Letourneau, P. C., Madsen, A. M., Palm, S. L., and Furcht, L. T. (1987). Immunoreactivity for laminin in the developing ventral longitudinal pathway of the brain. Dev. Biol. *125*, 135-144.
Letourneau, P. C., and Shattuck, T. A. (1989). Distribution and possible interactions of actin-associated proteins and cell adhesion molecules of nerve growth cones. Development *105*, 505-519.
Levitt, P. (1984). A monoclonal antibody to limbic system neurons. Science *223*, 229-301.
Lewis, A. K., and Bridgman, P. C. (1992). Nerve growth cone lamellipodia contain two populations of actin filaments that differ in organization and polarity. J. Cell Biol. *119*, 1219-1243.
Liesi, P., and Silver, J. (1988). Is astrocyte laminin involved in axon guidance in the mammalian CNS? Dev. Biol. *130*, 774-785.
Lin, C.-H., and Forscher, P. (1993). Cytoskeletal remodeling during growth cone-target interactions. J. Cell Biol. *121*, 1369-1383.
Lin, C.-H., and Forscher, P. (1995). Growth cone advance is inversely proportional to retrograde f-actin flow. Neuron *14*, 763-761.
Lochter, A., Taylor, J., Fuss, B., and Schachner, M. (1994). The extracellular matrix molecule janusin regulates neuronal morphology in a substrate- and culture time- dependent manner. Eur. J. Neurosci. *6*, 597-606.
Lochter, A., Vaughan, L., Kaplony, A., Prochiantz, A., Schachner, M., and Faissner, A. (1991). J1/tenascin in substrate-bound and soluble form displays contrary effects on neurite outgrowth. J. Cell Biol. *113*, 1159-1171.
Lohof, A. M., Quillan, M., Dan, Y., and Poo, M.-M. (1992). Asymmetric modulation of cytosolic cAMP activity induces growth cone turning. J. Neurosci. *12*, 1253-1261.
Lumsden, A. G. S., and Davies, A. M. (1983). Earliest sensory nerve fibres are guided to peripheral targets by attractants other than nerve growth factor. Nature *306*, 786-788.
Marsh, L., and Letourneau, P. C. (1984). Growth of neurites without filopodial or lamellipodial activity in the presence of cytochalasin B. J. Cell Biol. *99*, 2041-2047.
Martenson, C., Stone, K., Reedy, M., and Sheetz, M. (1993). Fast axonal transport is required for growth cone advance. Nature *366*, 66-69.
Messersmith, E. K., Leonardo, E. D., Shatz, C. J., Tessier-Lavigne, M., Goodman, C. S., and Kolodkin, A. L. (1995). Semaphorin III can function as a selective chemorepellent to pattern sensory projections in the spinal cord. Neuron *14*, 949-959.
Milev, P., Friedlander, D. R., Sakurai, T., Karthikeyan, L., Flad, M., Margolis, R. K., Grumet, M., and Margolis, R. U. (1994). Interactions of the chondroitin sulfate proteoglycan phosphocan, the extracellular domain of a receptor-type protein tyrosine phosphatase, with neurons, glia, and neural cell adhesion molecules. J. Cell Biol. *127*, 1703-1715.
Miller, D. R., Lee, G. M., and Maness, P. F. (1993). Increased neurite outgrowth induced by inhibition of protein tyrosine kinase activity in PC12 pheochromocytoma cells. J. Neurochem. *60*, 2134-2144.

Miller, M., Bower, E., Levitt, P., Li, D., and Chantler, P. D. (1992). Myosin II distribution in neurons is consistent with a role in growth cone motility but not synaptic vesicle mobilization. Neuron *8*, 25-44.

Mitchison, T., and Kirschner, M. (1988). Cytoskeletal dynamics and nerve growth. Neuron *1*, 761-772.

Morales, G., Hubert, M., Brummendorf, T., Treubert, U., Tarnok, A., Schwarz, U., and Rathjen, F. G. (1993). Induction of axonal growth by heterophilic interactions between the cell surface recognition proteins F11 and Nr-CAM/Bravo. Neuron *11*, 1113-1122.

O'Connor, T. P., and Bentley, D. (1993). Accumulation of actin in subsets of pioneer growth cone filopodia in response to neural and epithelial guidance cues *in situ*. J. Cell Biol. *123*, 935-948.

O'Connor, T. P., Duerr, J. S., and Bentley, D. (1990). Pioneer growth cone steering decisions mediated by single filopodial contacts *in situ*. J. Neurosci. *10*, 3935-3946.

Oakley, R. A., and Tosney, K. W. (1991). Peanut agglutinin and chondroitin-6-sulfate are molecular markers for tissues that act as barriers to axon advance in the avain embryo. Dev. Biol. *147*, 187-206.

Oohira, A., Matsui, F., and Katoh-Semba, R. (1991). Inhibitory effects of brain chondroitin sulfate proteoglycans on neurite outgrowth from PC12D cells. J. Neurosci. *11*, 822-827.

Patel, N., and Poo, M. (1982). Orientation of neurite growth by extracellular electric fields. J. Neurosci. *2*, 483-496.

Pesheva, P., Gennarini, G., Goridis, C., and Schachner, M. (1993). The F3/F11 cell adhesion molecule mediates the repulsion of neurons by the extracellular matrix glycoprotein J1-160/180. Neuron *10*, 69-82.

Pesheva, P., Probstmeier, R., Shubitz, A. P. N., McCarthy, J. B., Furcht, L. T., and Schachner, M. (1994). Tenascin-R (J1 160/180) inhibits fibronectin-mediated cell adhesion - functional relatedness to tenascin-C. J. Cell Sci. *107*, 2323-2333.

Pimenta, A. F., Zhukareva, V., Barbe, M. F., Reinoso, B. S., Grimley, C., Henzel, W., Fischer, I., and Levitt, P. (1995). The limbic system-associated membrane protein is an Ig superfamily member that mediates selective neuronal growth and axon targeting. Neuron *15*, 287-297.

Pindzola, R. R., Doller, C., and Silver, J. (1993). Putative inhibitory extracellular matrix molecules at the dorsal root entry zone of the spinal cord during development and after root and sciatic nerve lesions. Dev. Biol. *156*, 34-48.

Pini, A. (1993). Chemorepulsion of axons in the developing mammalian central nervous system. Science *261*, 95-98.

Placzek, M., Tessier-Lavigne, M., Jessell, T., and Dodd, J. (1990). Orientation of commissural axons *in vitro* in response to a floor plate-derived chemoattractant. Development *110*, 19-30.

Porter, B. E., Weis, J., and Sanes, J. R. (1995). A motorneuron-selective stop signal in the synaptic protein S-laminin. Neuron *14*, 549-559.

Prieto, A. L., Andersson-Fisone, C., and Crossin, K. L. (1992). Characterization of multiple adhesive and counteradhesive domains in the extracellular matrix protein cytotactin. J. Cell Biol. *119*, 663-678.

Puschel, A. W., Adams, R. H., and Betz, H. (1995). Murine semaphorin D/collapsin is a member of a diverse gene family and creates domains inhibitory for axonal extension. Neuron *14*, 941-948.

Rathjen, F. G., Wolff, J. M., Chang, S., Bonhoeffer, F., and Raper, J. A. (1987). Neurofascin: a novel chick cell-surface glycoprotein involved in neurite-neurite interactions. Cell *51*, 841-849.

Rehder, V., and Kater, S. B. (1992). Regulation of neuronal growth cone filopodia by intracellular calcium. J. Neurosci. *12*, 3178-3186.

Reichardt, L. F., and Tomaselli, K. J. (1991). Extracellular matrix molecules and their receptors: functions in neural development. Annu. Rev. Neurosci. *14*, 531-570.

Riggott, M. J., and Moody, S. A. (1987). Distribution of laminin and fibronectin along peripheral trigeminal axon pathways in the developing chick. J. Comp. Neurol. *258*, 580-598.

Rogers, S., Letourneau, P., Palm, S., McCarthy, J., and Furcht, L. (1986). Distribution of laminin in the developing peripheral nervous system of the chick. Dev. Biol. *98*, 212-220.

Rogers, S. L., Letourneau, P. C., Palm, S. L., McCarthy, J., and Furcht, L. T. (1983). Neurite extension by peripheral and central nervous system neurons in response to substratum-bound fibronectin and laminin. Dev. Biol. *98*, 212-220.

Roskies, A. L., and O'Leary, D. D. M. (1994). Control of topographic retinal axon branching by inhibitory membrane-bound molecules. Science *265*, 799-803.

Ruoslahti, E. (1988). Structure and biology of proteoglycans. Annu. Rev. Cell Biol. *4*, 229-255.

Sabry, J. H., O'Connor, T. P., Evans, L., Toroian-Raymond, A., Kirschner, M., and Bentley, D. (1991). Microtubule behavior during guidance of pioneer neuron growth cones in situ. J. Cell Biol. *115*, 381-395.

Saga, Y., Yagi, T., Ikawa, Y., Sakakura, T., and Aizawa, S. (1992). Mice develop normally without tenascin. Genes Dev. *6*, 1821-1831.

Sahin, M., and Hockfield, S. (1993). Protein tyrosine phosphatases in the developing rat brain. J. Neurosci. *13*, 4968-4978.

Sanes, J. R., Engvall, E., Butkowski, R., and Hunter, D. D. (1990). Molecular heterogeneity of basal laminae: isoforms of laminin and collagen IV at the neuromuscular junction and elsewhere. J. Cell Biol. *111*, 1685-1699.

Sato, M., Lopez-Mascaraque, L., Heffner, C. D., and O'Leary, D. D. M. (1994). Action of a diffusible target-derived chemoattractant on cortical axon branch induction and directed growth. Neuron *13*, 791-803.

Saunders, S., and Bernfield, M. (1988). Cell surface proteoglycan binds mouse mammary epithelial cells to fibronectin and behaves as a receptor for interstitial matrix. J. Cell Biol. *106*, 423-430.
Schuch, U., Lohse, M. J., and Schachner, M. (1989). Neural cell adhesion molecules influence second messenger systems. Neuron *3*, 13-20.
Serafini, T., Kennedy, T. E., Galko, M. J., Mirzayan, C., Jessell, T. M., and Tessier-Lavigne, M. (1994). The netrins define a family of axon outgrowth-promoting proteins homologous to *C. elegans* UNC-6. Cell *78*, 409-424.
Sheppard, A. M., Hamiliton, S. K., and Pearlman, A. L. (1991). Changes in the distribution of extracellular matrix components accompany early morphogenetic events of mammalian cortical development. J. Neurosci. *11*, 3928-3942.
Shirasaki, R., Tamada, A., Katsumata, R., and Murakami, F. (1995). Guidance of cerebellofugal axons in the rat embryo: directed growth toward the floor plate and subsequent elongation along the longitudinal axis. Neuron *14*, 961-972.
Simon, D. K., and O'Leary, D. D. M. (1992). Responses of retinal axons *in vivo* and *in vitro* to position-encoding molecules in the embryonic superior colliculus. Neuron *9*,
Smallheiser, N. R. (1993). Acute neurite retraction elicited by diverse agents is prevented by genistein, a tyrosine kinase inhibitor. J. Neurochem. *61*, 340-343.
Smith, S. J. (1988). Neuronal cytomechanics: the actin-based motility of growth cones. Science *242*, 708-715.
Snow, D. M., Lemmon, V., Carrino, D. A., Caplan, A. I., and Silver, J. (1990). Sulfated proteoglycans in astroglial barriers inhibit neurite outgrowth *in vitro*. Exp. Neurol. *109*, 111-130.
Sobue, K., and Kanda, K. (1989). Alpha-actinins, calspectin brain spectrin or fodrin, and actin participate in adhesion and movement of growth cones. Neuron *3*, 311-319.
Sonderegger, P., and Rathjen, F. G. (1992). Regulation of axonal growth in the vertebrate nervous system by interactions between glycoproteins belonging to two subgroups of the immunoglobulin superfamily. J. Cell Biol. *119*, 1387-1394.
Spring, J., Beck, K., and Chiquet-Ehrismann, R. (1989). Two contrary functions of tenascin: dissection of the active sites by recombinant tenascin fragments. Cell *59*, 325-334.
Sretavan, D. W. (1990). Specific routing of retinal ganglion cell axons at the mammalian optic chiasm during embryonic development. J. Neurosci. *10*, 1995-2007.
Sretavan, D. W., Feng, L., Pure, E., and Reichardt, L. F. (1994). Embryonic nerons of the developing optic chiasm express L1 and CD44, cell surface molecules with oposing effects on retinal axon growth. Neuron *12*, 957-975.
Sretavan, D. W., and Reichardt, L. F. (1993). Time-lapse video analysis of retinal ganglion cell axon pathfinding at the mammalian optic

chiasm: growth cone guidance using intrinsic chiasm cues. Neuron *10*, 761-777.
Stahl, B., Muller, B., von-Boxberg, Y., Cox, E. C., and Bonhoeffer, F. (1990). Biochemical characterization of a putative axonal guidance molecule of the chick visual system. Neuron 5, 773-743.
Steindler, D. A., Cooper, N. G. F., Faissner, A., and Schachner, M. (1989). Boundaries defined by adhesion molecules during development of the cerebral cortex: the J1/tenascin glycoprotein in the mouse somatosensory cortical barrel field. Dev. Biol. *131*, 243-260.
Steindler, D. A., Settles, D., Erickson, H. P., Laywell, E. D., Yoshiki, A., Faissner, A., and Kusakabe, M. (1995). Tenascin knockout mice: barrels, boundary molecules and glial scars. J. Neurosci. *25*, 1971-83.
Stoeckli, E. T., Kuhn, T. B., Duc, C. O., Ruegg, M. A., and Sonderegger, P. (1991). The axonally secreted protein axonin-1 is a potent substratum for neurite growth. J. Cell Biol. *112*, 449-455.
Stoeckli, E. T., and Landmesser, L. T. (1995). Axonin-1, Nr-CAM and Ng-CAM play different roles in the *in vivo* guidance of chick commissural neurons. Neuron *14*, 1165.
Stoker, A. W., Gehrig, B., Haj, F., and Bay, B.-H. (1995). Axonal localisation of the CAM-like tyrosine phosphatase CRYPa: a signalling molecule of embryonic growth cones. Development *121*, 1833-1834.
Strittmatter, S. M., Fankhauser, C., Huang, P. L., Mashimo, H., and Fishman, M. C. (1995). Neuronal pathfinding is abnormal in mice lacking the neuronal growth cone protein GAP-43. Cell *80*, 445-452.
Strittmatter, S. M., Valenzuela, D., Kennedy, T. E., Neer, E. J., and Fishman, M. C. (1990). Go is a major growth cone protein subject to regulation by GAP-43. Nature *344*, 836-841.
Takeichi, M. (1988). The cadherins: cell-cell adhesion molecules controlling animal morphogenesis. Development *102*, 639-655.
Tamura, M., Nogimori, K., Yajima, M., Ase, K., and Ui, M. (1983). A role of the B-oligomer moiety of islet-activating protein, pertussis toxin, in development of the biological effects on intact cells. J. Biol. Chem. *258*, 6756-6761.
Tanaka, J., Kira, M., and Sobue, K. (1993). Gelsolin is localized in neuronal growth cones. Dev. Brain Res. *76*, 268-271.
Tang, J., Landmesser, L., and Rutishauser, U. (1992). Polysialic acid influences specific pathfinding by avian motoneurons. Neuron *8*, 1031-1044.
Tang, J., Rutishauser, U., and Landmesser, L. (1994). Polysialic acid regulates growth cone behavior during sorting of motor axons in the plexus region. Neuron *13*, 405-414.
Tawil, N. J., Houde, M., Blacher, R., Esch, F., Reichardt, L. F., Turner, D. C., and Carbonetto, S. (1990). Alpha 1 beta 1 integrin heterodimer functions as a dual laminin/collagen receptor in neural cells. Biochem. *29*, 6540-6544.

Taylor, J., Pesheva, P., and Schachner, M. (1993). Influcence of janusin and tenascin on growth cone behavior *in vitro*. J. Neurosci. Res. *35*, 347-362.

Tessier-Lavigne, M. (1992). Axon guidance by molecular gradients. Curr. Opin. Neurobiol. *2*, 60-65.

Tessier-Lavigne, M., Placzek, M., Lumsden, A. G. S., Dodd, J., and Jessell, T. M. (1988). Chemotropic guidance of developing axons in the mammalian central nervous system. Nature *336*, 775-778.

Theriot, J. A., and Mitchison, T. J. (1992). Comparison of actin and cell surface dynamics in motile fibroblasts. J. Cell Biol. *118*, 367-377.

Timpl, R., and Brown, J. C. (1994). The laminins. Matrix Biol. *14*, 275-281.

Tomaselli, K. J., Doherty, P., Emmett, C. J., Damsky, C. H., Walsh, F. S., and Reichardt, L. F. (1993). Expression of β1 integrins in sensory neurons of the dorsal root ganglion and their functions in neurite outgrowth on two laminin isoforms. J. Neurosci. *13*, 4880-4888.

Varnum-Finney, B., and Reichardt, L. F. (1994). Vinculin-deficient PC12 lines extend unstable lamellipodia and filopodia and have a reduced rate of neurite outgrowth. J. Cell Biol. *127*, 1071-1084.

Walter, J., Heinke-Fahle, S., and Bonhoeffer, F. (1987). Avoidance of posterior tectal membranes by temporal retinal axons. Devel. *101*,

Walter, J., Muller, B., and Bonhoeffer, F. (1990). Axonal guidance by an avoidance mechanism. J. Physiol. *84*, 104-110.

Wehrle, B., and Chiquet, M. (1990). Tenascin is accumulated along developing peripheral nerves and allows neurite outgrowth *in vitro*. Development *110*, 405-415.

Williams, E. J., Walsh, F. S., and Doherty, P. (1994). Tyrosine kinase inhibitors can differentially inhibit integrin-dependent and CAM-stimulated neurite outgrowth. J. Cell Biol. *124*, 1029-1037.

Wizenmann, A., Thanos, S., Boxberg, Y. V., and Bonhoeffer, F. (1993). Differential reaction of crossing and non-crossing rat retinal axons on cell membranes preparation from the chiasm midline: an *in vitro* study. Development *117*, 725-735.

Wolfer, D. P., Henehan-Beatty, A., Stoeckli, E. T., Sonderegger, P., and Lipp, H. P. (1994). Distribution of TAG-1/axonin in fibre tracts and migratory streams of the developing mouse nervous system. J. Comp Neurol. *345*, 1-32.

Wu, D. Y., and Goldberg, D. J. (1993). Regulated tyrosine phosphorylation at the tips of growth cone filopodia. J. Cell Biol. *123*, 653-664.

Yurchenco, P. D., and Cheng, Y. S. (1993). Self-assembly and calcium-binding sites in laminin: a three-arm interaction model. J. Biol. Chem. *268*, 17286-17299.

Zhang, H., Miller, R. H., and Rutishauser, U. (1992). Polysialic acid is required for optimal growth of axons on a neuronal substrate. J. Neurosci. *12*, 3107-3114.

Zheng, J. Q., Felder, M., Connor, J. A., and Poo, M.-M. (1994). Turning of nerve growth cones induced by neurotransmitters. Nature *368*, 140-144.

Zot, H. G., Doberstein, S. K., and Pollard, T. D. (1992). Myosin-I moves actin filaments on a phospholipid substrate: implications for membrane targeting. J. Cell Biol. *116*, 367-376.

# Chapter 2

## An Investigation of the Mechanism of Laminin-Mediated Anti-Adhesion

## Introduction

Cell interaction with extracellular matrix (ECM) is thought to regulate developmental events such as tissue morphogenesis, cell migration, and cell differentiation (for reviews see Adams and Watt, 1993; DeSimone, 1994; Reichardt and Tomaselli, 1991). ECM molecules are present in the embryo from the earliest stages of development and particular ECM proteins are often present at sites of cell migration and tissue rearrangement. *In vivo*, treatments that interfere with ECM protein function or inhibit ECM receptors can disrupt developmental processes (Boucaut et al., 1984; Bronner-Fraser, 1986; Galileo et al., 1992). However, much of what is known about the function of ECM molecules has been learned by observing the effects of individual purified matrix molecules on cell behavior *in vitro*.

A number of studies have shown that mixtures of certain purified ECM molecules have effects on cell behavior that would not be predicted from cellular responses to the individual components of that mixture (for review see Chiquet-Ehrismann, 1995). Thrombospondin, tenascin-C and tenascin-R, when present on the substratum, reduce the binding of cells to substratum-bound fibronectin (Chiquet-Ehrismann et al., 1988; Lahav, 1988; Pesheva et al., 1994). Addition of soluble thrombospondin or tenascin to cells previously spread on serum or fibronectin reduces the number of focal contacts (Murphy-Ullrich and Hook, 1989; Murphy-Ullrich et al., 1991). Another matrix protein, SPARC prevents spreading--but not attachment--of several cell types to collagen substrata (Sage et al., 1989) Proteoglycans have been proposed to have similar activities: a chondroitin sulfate proteoglycan isolated from chick embryo fibroblasts blocks cell adhesion to fibronectin, laminin, vitronectin and GRGDS peptide-coated substrata (Yamagata et al., 1989), and a chondroitin sulfate proteoglycan isolated from cartilage blocks cell adhesion and neurite outgrowth (Snow et al., 1990). These varied phenomena have in common an interference with adhesive protein function that has been called either "anti-adhesion" or "counter-adhesion".

The ECM protein laminin, although usually thought to function as an adhesive molecule--it promotes the attachment of many cell types--apparently has anti-adhesive activity as well. Calof and Lander (1991) demonstrated that, when present on the substratum, laminin dramatically reduced the adhesion of mouse olfactory epithelial neurons to fibronectin. Those cells that did adhere to the mixed substrata formed fewer close cell-substratum contacts and migrated more actively than did cells plated on fibronectin alone.

The mechanism of anti-adhesion, whether mediated by laminin or any other molecule, is unknown. Fibronectin interacts with cells via several classes of cell-surface molecules including the integrin family of heterodimeric transmembrane proteins (Hynes, 1992), proteoglycans (Saunders and Bernfield, 1988), and other receptors such as the N-terminal matrix assembly site receptor (McKoewn-Longo and Mosher, 1985). Given the complexity of cell-matrix interactions, any of several types of mechanism could explain anti-adhesion: An anti-adhesive molecule might bind to fibronectin on the substratum in a way that blocks fibronectin's interaction with particular cell surface receptors. An anti-adhesive molecule might interact with cell surface fibronectin receptors but in a manner that does not promote cell adhesion. Alternatively, an anti-adhesive molecule might interact with a specific cell surface receptor and generate intracellular signals that inhibit the function of cell surface fibronectin receptors. The experiments presented below address the mechanism of laminin-mediated anti-adhesion. Our results argue against a role for intracellular signaling in anti-adhesion, but could support two alternative models.

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## Materials and Methods

*Materials*

Human plasma fibronectin was purchased from the New York Blood Center (New York, NY). Mouse laminin was purified from the Engelbreth-Holm-Swarm tumor according to published procedures (Kleinman et al., 1982; Timpl et al., 1982). Concentration of purified laminin was determined by amido black dye binding assay (Schaffner and Weissman, 1973) and confirmed by amino acid analysis. For the preparation of proteolytic fragments of laminin (E1', E3, E4 and E8), laminin and laminin/entactin were isolated from lathyritic Engelbreth-Holm-Swarm tumor based on the EDTA-extraction method of Paulsson (Paulsson et al., 1987) and purified as described (Yurchenco et al., 1992). Elastase fragments E1', E4, E8, and E3 of laminin were generated at an enzyme-substrate ratio of about 1:250 at 0°C. for one hr. followed by 25°C. for 23 hrs. The fragments were purified by gel filtration on Sepharose CL-6B (Pharmacia, Piscataway, NJ) and by HPLC DEAE-5PW ion exchange chromatography (15 cm x 2 cm I.D.: Toso-Haas, Philadelphia, PA) as previously detailed (Yurchenco et al., 1990) except that only the peak fractions of E1' were pooled after ion exchange for higher purity. Human vitronectin, the 120kD cell binding proteolytic fragment of fibronectin, synthetic peptides, anti-human fibronectin receptor antibody and monoclonal antibodies to the fibronectin cell attachment site (clone II) and the fibronectin carboxy-terminal heparin-binding site (clone I) were purchased from Telios Pharmaceuticals (San Diego, CA). Porcine intestinal mucosal heparin was from Sigma Chemical Co (St. Louis, MO) and chondroitin sulfate from Fluka Chemika (Ronkonkoma, NY). Polystyrene for adhesion assay plates was purchased from Altec Plastics (Boston, MA) and silicon rubber Biodot gaskets from BioRad (Richmond, CA). Other reagents, unless noted, were purchased from Sigma Chemical Co. (St. Louis, MO).

*Cell Culture and Metabolic Labelling*

WEHI 231 mouse lymphoid cells were maintained as non-adherent cultures in DME containing 10% iron supplemented calf

serum (Hyclone Laboratories, Logan, UT). MG63 human osteosarcoma cells and human fibroblasts were maintained in DMEM containing 10% FCS (Hyclone Laboratories). Dissociated retinal neurons from embryonic day 7 chick (obtained from Spafas) were dissected and prepared as described (Hall et al., 1987). For metabolic labelling, cells were incubated in 25 μCi/ml $^{35}$S-EXPRESS amino acid mixture (New England Nuclear, Boston, MA) in methionine-free medium supplemented with 10% calf serum for 2 to 6 hours, except for retinal neurons which were labelled in methioninine-free media supplemented with N2 Botts additives (Bottenstein and Sato, 1979). MG63 cells were harvested for adhesion assays either by trypsinization with 0.5 mg/ml trypsin + 5 mM EDTA in HBSS followed by addition of 0.25 mg/ml soybean trypsin inhibitor or by trituration in 5 mM EDTA in HBSS. Cells were resuspended in 10 mg/ml heat-inactivated BSA (Sigma catalog #A-7030; [Goodman et al., 1991]) in DME at a final concentration of $5x10^4$ to $2x10^5$ cells/ml.

*Cell Adhesion Assay*

Cell adhesion assays were performed essentially as described by Calof and Lander (1991) except as noted below and in figure legends. In some experiments, peptides or antibodies were mixed with the cells before plating. For initial (undeveloped) adhesion measurements, cells were treated with 10 μg/ml cytochalasin B for 1 hour prior, harvested, and chilled to 4°C. Adhesion was measured by rapidly centrifuging the cells onto the substratum (using a refrigerated centrifuge), immediately covering and inverting the assay plate and then centrifuging the cells off the substratum. Bound radioactivity was detected using a Phosphorimager (Molecular Dynamics, Sunnyvale, CA). In the experiments with MG63 cells and chick retinal neurons, adhesion to a polylysine-coated substratum (0.1 mg/ml coating concentration) in the absence of any dislodging force was used as a measure of maximum possible cell adhesion. Maximum WEHI 231 cell adhesion as a fraction of input cell number was determined by direct counting: after a standard adhesion assay, adherent cells were stained with Hoechst 33258 (bisbenzamid, Sigma) and the cells that adhered to wells coated with 25 μg/ml fibronectin were visualized using a

fluorescence microscope. All cells present in the well were counted. The number of input cells was determined by hemacytometer counting of the initial cell suspension.

*Radioiodinated Protein Binding Assay*

Fibronectin, vitronectin, laminin, and laminin fragments were iodinated by the Iodogen method (Pierce Chemical Co., Rockford, IL). Concentrations of radioiodinated proteins were determined by the Quantigold protein assay (Diversified Biotech, MA) and specific activities calculated. Observed specific activities ranged from $2X10^7$ to $10^8$ cpm per μg protein. Known concentrations of $^{125}$I-proteins were combined with unlabeled proteins and the resulting mixture applied to polystyrene assay plates under the conditions used for coating of adhesion assay substrata. Protein bound to the plastic was detected by direct counting of the assay plate.

*Anti-Fibronectin Antibody Binding*

Substrata coated with fibronectin, laminin, or mixtures of the two were prepared as for adhesion assays. After blocking the substratum overnight with 10 mg/ml BSA, antibodies directed against the heparin binding domain or cell binding domain of fibronectin were applied at 1/500 dilution of ascites fluid in HBSS containing 10 mg/ml BSA for 4 hours at 37°. The plates were washed with HBSS, $^{125}$I-goat anti-mouse IgG (New England Nuclear, Boston, MA) was added at 0.5 mCi/ml and the plates were incubated overnight at 4°C. After washing, radioactivity associated with the plastic was determined by gamma counting.

*Liposome Binding Assay*

Integrin-containing, radiolabelled liposomes were prepared essentially as described by Pytela et al, (1985). Integrins were isolated from $2x10^8$ cell surface-radioiodinated MG63 cells by affinity chromatography on a fibronectin-Sepharose column (prepared by coupling plasma fibronectin to CNBr-activated Sepharose CL2B). The integrins were eluted with 1 mg/ml GRGDSP peptide. The fractions obtained were analyzed by SDS PAGE. Under non-reducing

conditions, two bands were observed by autoradiography. Both bands were immunopreciptated by an antiserum directed against the cytoplasmic domain of the α5 integrin subunit (provided by Richard Hynes, MIT), indicating that αβ heterodimers were intact in the column eluate. Integrin protein concentrations were estimated from the specific activity of total cell surface proteins as follows: Iodinated cell surface proteins were isolated from cell extracts by binding to a Wheat Germ Agglutinin-Sepharose column. Protein concentration of the bound fraction was determined by Quantigold assay and the specific activity of $^{125}$I-protein calculated. Using this value, the concentration of $^{125}$I in the fractions eluted from the fibronectin column was converted to an estimated integrin protein concentration. Liposomes were prepared by dialysis of mixtures of fibronectin column fractions (containing approximately 7 μg protein) and $^{3}$H-phosphatidylcholine (New England Nuclear, Boston, MA;10 μCi/200 μg lipid) against PBS (containing 138 mM NaCl, 3 mM KCl, 8 mM $Na_2HPO_4$, 1.5 mM $KH_2PO_4$, 1 mM $CaCl_2$ and 1 mM $MgCl_2$). The resulting liposomes were purified by sucrose gradient centrifugation.

Liposome binding was assessed using substrata prepared exactly as for cell adhesion measurements. Liposomes, suspended in 10 mg/ml heat-inactivated BSA in PBS, were applied to the protein-coated and BSA-blocked wells and incubated 5 hours at 4°C. Unbound liposomes were removed by washing twice with PBS. Assay plates were dried and bound radioactivity determined by liquid scintillation counting. Machine background, determined by counting scintillation fluid alone, was 20 cpm. This value was subtracted from the experimental data points.

*Solution Binding of Integrins*

Radiolabelled fibronectin-binding integrins were purified from MG63 cells as described above and dialyzed into Tris buffered saline (pH 7.5) containing 1 mM $MgCl_2$ and 50 mM B-octoglucoside (TBS-BOG). Laminin was concentrated with a Centricon-30 microconcentrator (Amicon) to a final concentration of 2 mg/ml and resuspended in TBS-BOG. Approximately 1.1 ng (375 cpm) integrin was added to 50 μl fibronectin-Sepharose in a total volume of 120 μl

TBS-BOG with 1 mg/ml crystalline BSA in a long, thin micrcentrifuge tube. Laminin or GRGDS peptide were included as indicated in the reaction mixture. The integrins were allowed to bind for four hours at 4°C. The solution was underlayered with 100 µl TBS-BOG containing 5 % sucrose and 0.05% phenol red and spun at 10,000 g for 1 min. The fibronectin-sepharose-associated and-nonassociated fractions were physically separated by freezing the tube in dry ice-ethanol and cutting it in the sucrose-containing interface. The tube pieces were allowed to thaw in gamma counter tubes and the associated radioactivity determined by gamma counting. Machine backround was subtracted from all measurements.

## Results

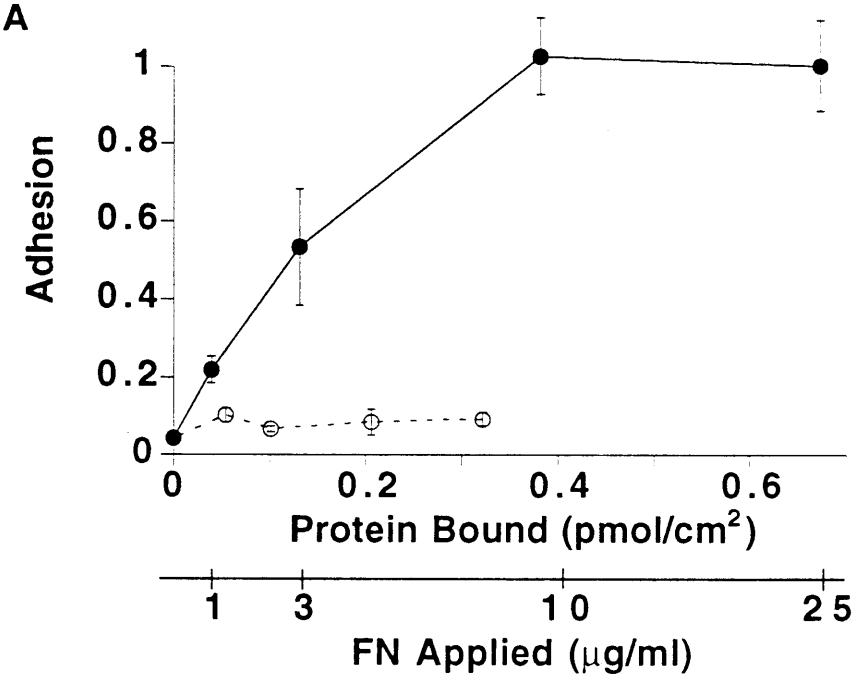
### Adhesion of WEHI 231 lymphocytes to fibronectin is lowered by laminin

To demonstrate that laminin-mediated reduction in cell adhesion is not unique to olfactory epithelial neuronal cells but can be observed in other cells as well, the adhesion of WEHI 231 cells, a mouse B lymphoma cell line, was measured using a centrifugal adhesion assay. WEHI cell adhesion to substrata coated with either fibronectin or laminin is shown in Figure 1A. At the removal force used in this assay (50g), the maximum number of WEHI cells adhering to fibronectin was approximately 25% of the input cells. In contrast, cell adhesion to laminin was no greater than adhesion to substrata coated only with BSA (1-2% of input cells). However, in the presence of laminin, cell adhesion to fibronectin was reduced, in a dose-dependent manner, to the level observed for laminin alone, i.e. the background level (Figure 1B).

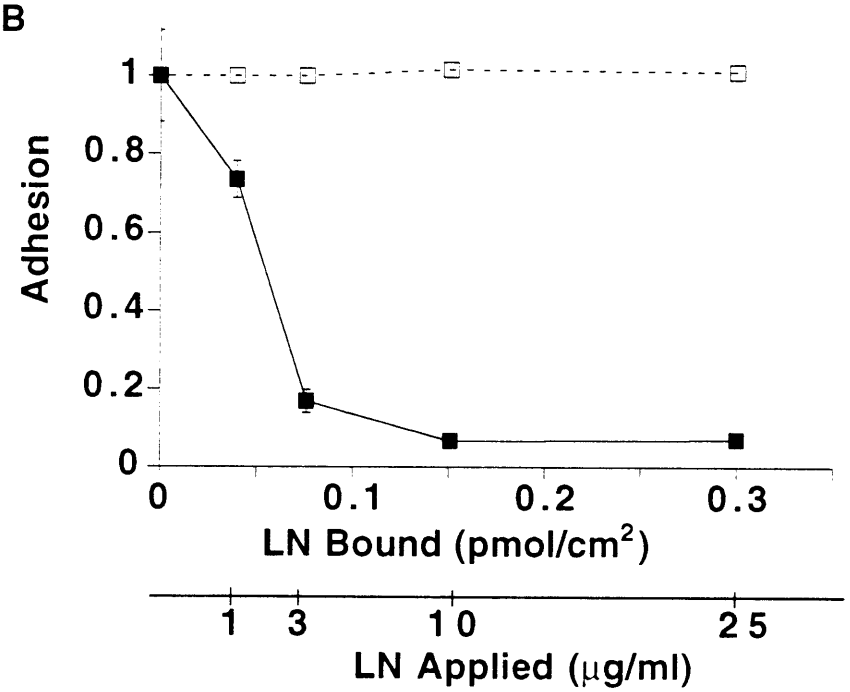
A reduction of adhesion to fibronectin in the presence of laminin could be due to a decrease in the amount of fibronectin deposited on the substratum when fibronectin and laminin are applied together. Radioiodinated protein binding assays were performed to determine the actual decrease in iodinated fibronectin bound to the substratum in the presence of several concentrations of unlabeled laminin (data not shown). Laminin did interfere measurably with the binding of fibronectin to the plastic substratum: when the maximum concentration of laminin (25 μg/ml) was added to 25 μg/ml fibronectin in the coating solution, the amount of fibronectin bound to the substratum was decreased by 25%; in contrast, for 3 μg/ml laminin mixed with fibronectin the decrease was only 2%. However, the decrease in fibronectin binding by any laminin concentration tested was insufficient to account for the observed decrease in cell adhesion to fibronectin. The "predicted adhesion" of the cells to mixtures of fibronectin and laminin was obtained as follows: The actual amount of fibronectin bound in the presence of each concentration of laminin was determined from the radioiodinated protein binding assay. and the level of adhesion which would occur if

**Figure 1. Adhesion of WEHI cells to fibronectin, laminin, and mixtures of fibronectin and laminin.** WEHI 231 cells were applied to **(A)**: fibronectin (●) and laminin. (O) & **(B)**: a mixture of 25 μg/ml fibronectin and varied concentrations of laminin (—■—). Removal force applied to cells in these assays was 50g. Data are expressed as picomoles/$cm^2$ bound with corresponding μg/ml protein applied indicated for fibronectin in 1A and laminin in 1B. Predicted cell adhesion (- -□- -, fig. 1B) was determined as described in the text. The data represent the mean ± SEM (n=4) of a representative experiment. Cell adhesion is normalized to maximal adhesion observed in the experiment.

A


Adhesion
1
0.8
0.6
0.4
0.2
0
0
0.2
0.4
0.6
Protein Bound (pmol/cm²)
1
3
10
25
FN Applied (μg/ml)

B


Adhesion
1
0.8
0.6
0.4
0.2
0
0
0.1
0.2
0.3
LN Bound (pmol/cm²)
1
3
10
25
LN Applied (μg/ml)

this amount of fibronectin were present alone was calculated from the fibronectin binding curve in Fig. 1A. In Figure 1B, this predicted adhesion to mixtures of fibronectin and laminin is compared with the observed cell adhesion. No reduction in cell adhesion is predicted, while observed cell adhesion is decreased to background levels.

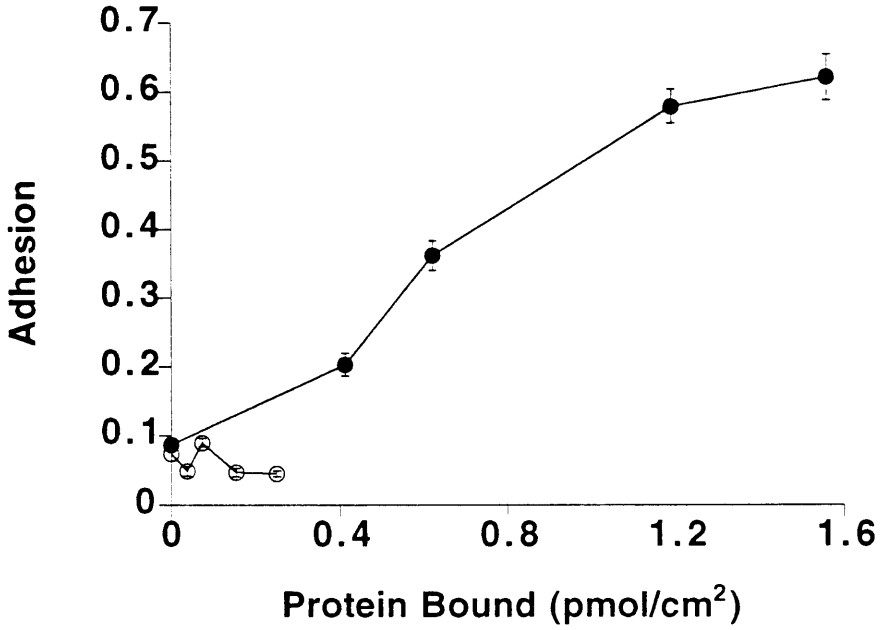
The density of laminin required for half maximal adhesion was calculated using the radioiodinated protein binding data. The average half maximally adhesive concentration of laminin, from several experiments, was found to be $0.08\pm0.03$ pmol/cm$^2$ ($2.4\pm 0.8$ μg/ml applied) or 1 pmol of laminin per 8 pmol of fibronectin bound to the substratum. This is close to the value of 0.04 pmol/cm$^2$ of laminin found by Calof and Lander to decrease by 50% the adhesion of olfactory epithelial neuronal cells to fibronectin.

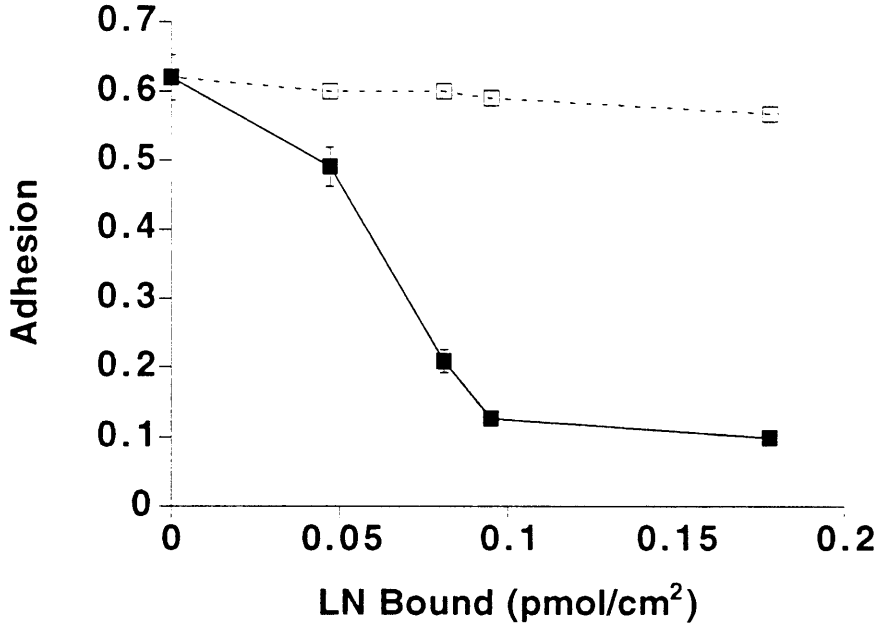
Laminin did not interfere with cell adhesion to all substrata: WEHI 231 cell adhesion to polylysine-coated substrata was unaltered by the presence of laminin. Additionally, not all large proteins bound to the substratum caused anti-adhesion; Mouse IgM (900 kD) and thyroglobulin (669 kD), when mixed with fibronectin in the coating solution at concentrations up to 20 μg/ml had no effect on WEHI cell adhesion to fibronectin (data not shown).

**Laminin interferes with retinal cell adhesion to vitronectin**

Retinal neurons isolated from embryonic day 7 chick adhere strongly to vitronectin (Figure 2A) as has been previously shown (Neugebauer et al., 1991). In order to determine if adhesion mediated by other extracellular matrix molecules was sensitive to laminin, adhesion of these neurons was assayed in the presence and absence of laminin. Laminin applied with the vitronectin decreased the adhesion to near backround levels (Figure 2B). Radioiodinated binding assays allowed the calculation of the predicted adhesion as described above for WEHI cell adhesion, and again, the decrease in adhesion could not be accounted for by the displacement of vitronectin from the substratum (Figure 2B). The density of laminin required for half-maximal adhesion to vitronectin was 0.06 pmol/cm$^2$ (2.8 μg/ml applied) which is similar to the density required for half maximal adhesion of WEHI cells to fibronectin (see above).

**Figure 2. Adhesion of embryonic chick retinal neurons to vitronectin, laminin and mixtures of vitronectin and laminin.** Retinal neurons were prepared from embryonic day 7 chick as described in Materials and Methods. They were applied to **(A)** (—●—) vitronectin and (—○—) laminin & **(B)**: (—■—) a mixture of 10 μg/ml vitronectin and 20, 10, 5 or 1 μg/ml laminin. Removal force was 50g. Data are expressed as picomoles/$cm^2$ bound. Predicted cell adhesion (- -□- -; fig. 2B) was determined as described for figure 1B. The data represent mean ± SEM (n=4). Cell adhesion is normalized to adhesion to polylysine coated substrata.


0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Adhesion
0
0.4
0.8
1.2
1.6
Protein Bound (pmol/cm2)


0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Adhesion
0
0.05
0.1
0.15
0.2
LN Bound (pmol/cm2)

**Domains of laminin involved in anti-adhesion**

To define the roles of particular structural features of laminin in mediating anti-adhesion, four proteolytic fragments, E1', E3, E4. and E8 were tested for anti-adhesive activity. When the 450 kD E1' proteolytic fragment of laminin, which contains the N-terminal sequences of all three laminin subunits, was combined with fibronectin on the substratum, cell adhesion was reduced to background levels (Fig 3). E1' also reduced retinal neuron adhesion to vitronectin (not shown). This fragment is slightly less active than laminin in reducing adhesion to fibronectin, as the concentration of bound protein required to achieve half maximal activity is $0.21\pm0.05$ pmol/cm$^2$ ($5.4\pm1.3$ μg/ml applied) as compared to $0.08\pm0.03$ pmol/cm$^2$ ($2.4\pm 0.8$ μg/ml) for laminin. The E8 fragment of laminin also reduced adhesion to fibronectin but the maximum decrease in adhesion observed with E8 was never as great as with laminin or the E1' fragment. The other laminin proteolytic fragments caused little reduction in WEHI cell adhesion to fibronectin at the concentrations tested (Fig. 3).

E1' has a number of sites that can mediate cell-matrix interactions. Experiments were carried out to determine if any of these domains were involved in anti-adhesion.

Two amino acid sequences, YIGSR and GRGDSP, that have been reported to be involved in cell adhesion (Goodman et al., 1991; Graf et al., 1987) are located in the E1' fragment. Synthetic peptides corresponding to these amino acid sequences were included with WEHI cells in an adhesion assay at concentrations up to 1 mg/ml. They did not significantly alter either the adhesion of the cells to fibronectin or the decrease in adhesion caused by laminin (data not shown).

The E1' fragment of laminin is known to bind heparin (Colognato-Pyke et al., 1995). To determine whether cell surface proteoglycan binding is involved in anti-adhesion, the potential glycosaminoglycan binding sites of laminin and fibronectin were blocked by preincubation of the substratum with heparin (Fig. 4A) or chondroitin sulfate (Fig. 4B) and by inclusion of either compound with

**Figure 3. Effect of laminin fragments on adhesion of WEHI 231 cells to fibronectin.** WEHI 231 cells were applied to substrata coated with 25 μg/ml fibronectin mixed with varying concentrations of whole laminin (—■—) or the E1' (--○--), E3 (●), E4 (□), or E8 (--▲--) fragments. None of these fragments plated alone at these concentrations shows adhesion to WEHI cells above backround level. Removal force applied to the cells was 50g. Data are expressed as picomoles/$cm^2$ fragment bound. The data represent the mean ± SEM (n=4). Cell adhesion is normalized to the level of adhesion to fibronectin alone.

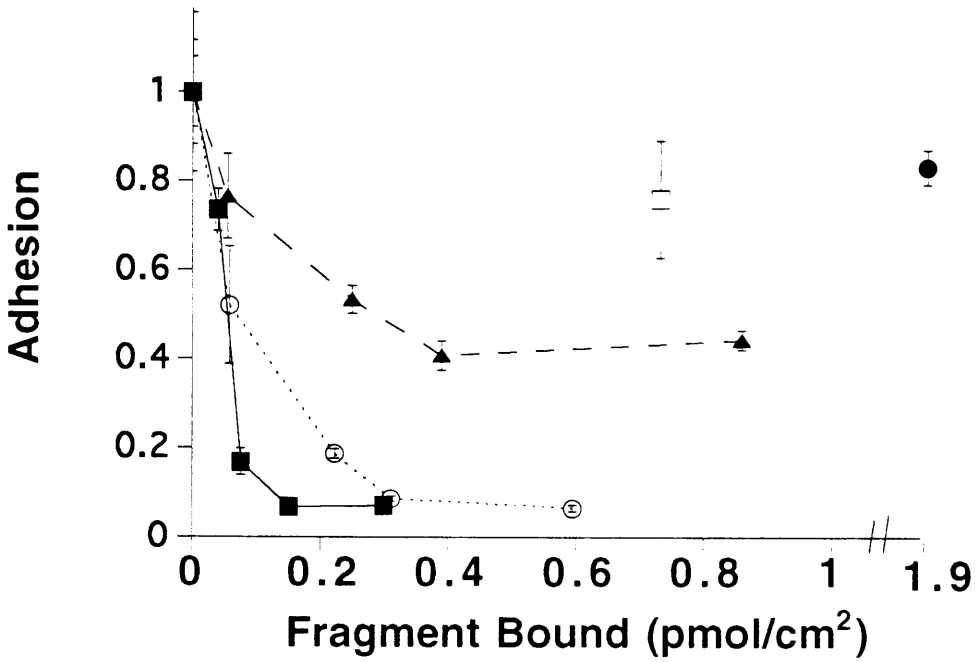
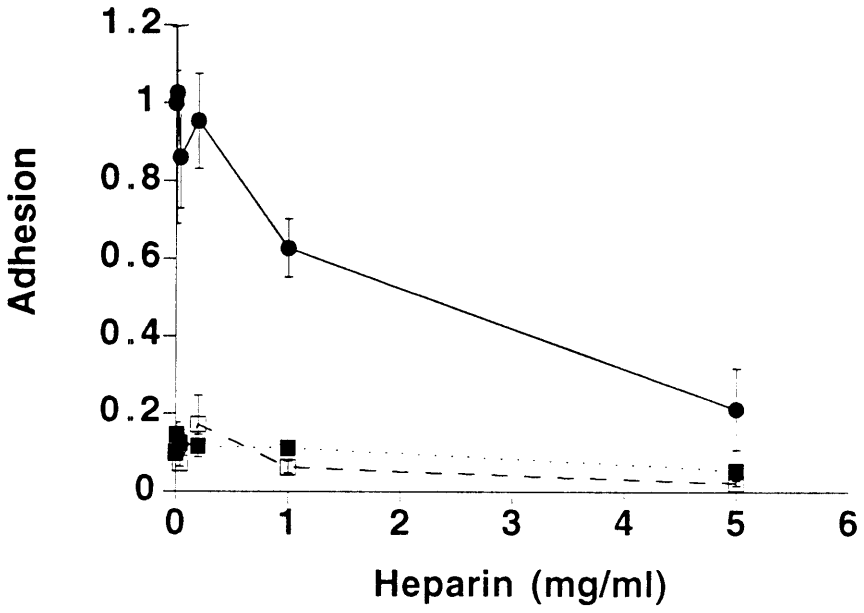
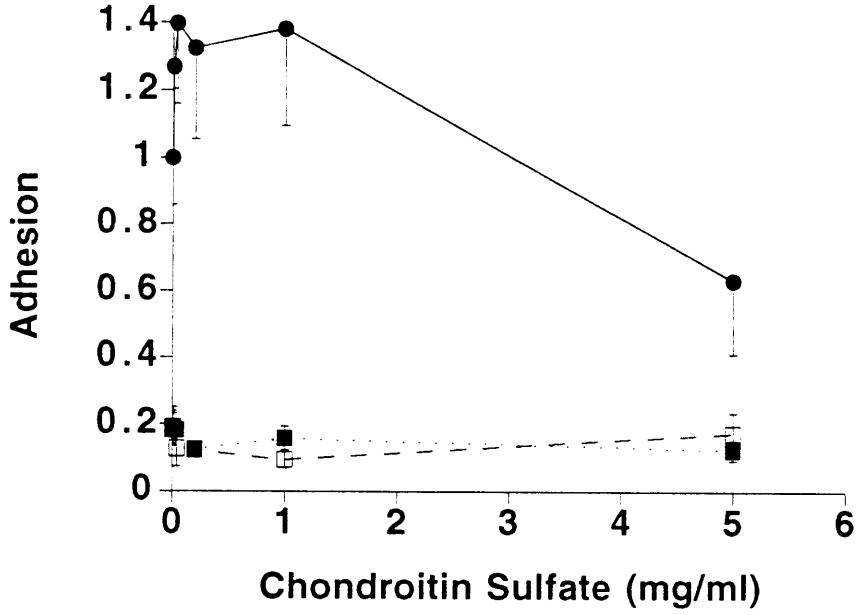

Adhesion
1
0.8
0.6
0.4
0.2
0
0
0.2
0.4
0.6
0.8
1
1.9
Fragment Bound (pmol/cm$^2$)

**Figure 4. Effect of putative blocking reagents on cell adhesion and anti-adhesion.** Substrata were coated with (—●—) 25 μg/ml fibronectin, (--□--) 25 μg/ml fibronectin & laminin, or (--■--) 25 μg/ml laminin then preincubated with the indicated heparin **(4A)** or chondroitin sulfate **(4B)** solution. The same concentration of GAG was included with the WEHI cells during the adhesion assay which was otherwise performed as in Figure 1. Data and are normalized to the adhesion observed to fibronectin alone.

A


Adhesion
1.2
1
0.8
0.6
0.4
0.2
0
0
1
2
3
4
5
6
Heparin (mg/ml)

B


Adhesion
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
1
2
3
4
5
6
Chondroitin Sulfate (mg/ml)

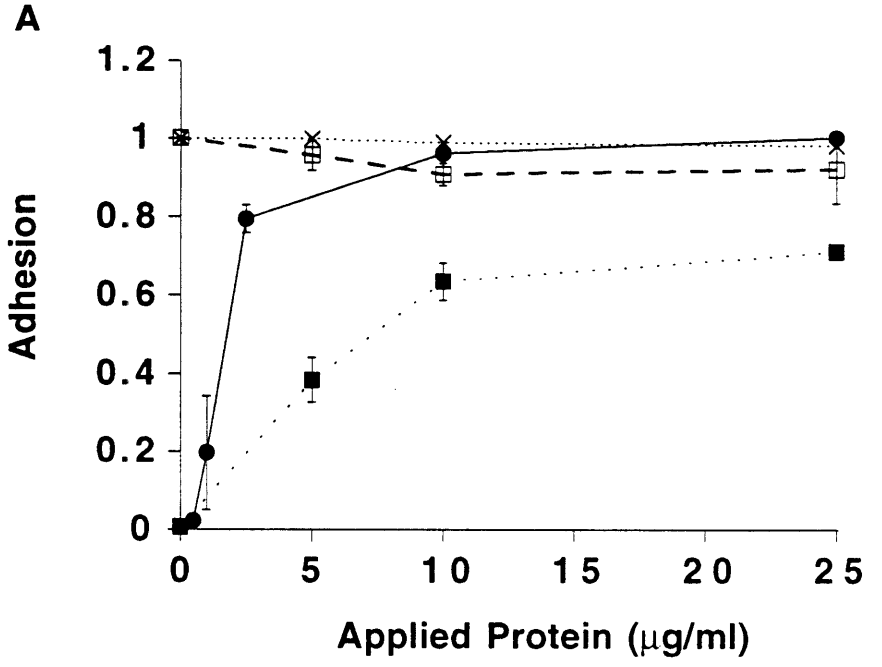
WEHI cells during the adhesion assay. The glycosaminoglycans decreased cell adhesion to fibronectin only at very high concentrations (> 1 mg/ml) and had no effect on the decrease in adhesion caused by laminin.

**Cell susceptibility to anti-adhesion correlates inversely with strength of adhesion to fibronectin**

WEHI cells adhere relatively weakly to fibronectin and express the $\alpha_4\beta_1$ integrin rather than the higher affinity fibronectin-binding integrin $\alpha_5\beta_1$ (Guan and Hynes, 1990). The effects of laminin were also tested using cells that adhere strongly to fibronectin. MG63 osteosarcoma cells, which express several integrin receptors known to bind to fibronectin ($\alpha_5\beta_1$, $\alpha_3\beta_1$, and $\alpha_v\beta_3$ Pytela et al., 1985), adhere to fibronectin strongly: at saturating concentrations of fibronectin approximately 60% of input cells were resistant to removal forces up to 1600g (Fig. 5A and data not shown). When laminin is included with fibronectin on the substratum, little decrease in adhesion is seen, but MG63 cells adhere to laminin alone (Figure 5A). However LN's E1' fragment was very poorly adhesive for MG63 cells and, when mixed with fibronectin, caused a small but significant decrease in cell adhesion (Figure 5B). Similar results were obtained using two fibroblast cell lines, human diploid fibroblasts and F2408 rat fibroblasts, both of which also adhere strongly to fibronectin (data not shown).

To determine whether strength of adhesion to fibronectin actually plays a causal role in determining a cell's susceptibility to anti-adhesion, MG63 cells were treated in ways that would reduce their adhesive strength. When either GRGDSP peptide (Fig. 6A) or low levels of an anti-human fibronectin receptor antibody (Fig. 6B) was included during adhesion assays, the E1' fragment of laminin caused a much greater decrease in adhesion of MG63 cells to fibronectin than had been observed with untreated cells (see Fig. 5B). These data suggest that conditions that weaken adhesion to fibronectin increase the cells' susceptibility to anti-adhesion. The converse also appears to be true. When WEHI cells were treated with the phorbol ester PMA,

**Figure 5. Effect of laminin and E1' fragment on adhesion of MG63 cells to fibronectin.** **(A)** MG63 cell adhesion to (—●—) fibronectin, (--■--) laminin, and (--□--) 25 μg/ml fibronectin + varied concentrations of laminin. **(B)** MG63 cell adhesion to (—●—) fibronectin, (--■--) E1' fragment, and (--□--) 25 μg/ml fibronectin + varied concentrations of E1' fragment (concentrations plated are expressed as equivalent molar amount of laminin plated for comparison to **(A)**). MG63 cells were incubated at 37° C. for 30 min. prior to being subjected to a 150g removal force. Observed adhesion is expressed as the fraction of cells bound to a polylysine control and is the mean ± SEM (n=4). Predicted adhesion (--X--) was calculated based on the measured decreases in $^{125}$I-fibronectin binding to plastic in the presence of laminin or E1' fragment.


A
Adhesion
1.2
1
0.8
0.6
0.4
0.2
0
0
5
10
15
20
25
Applied Protein (μg/ml)

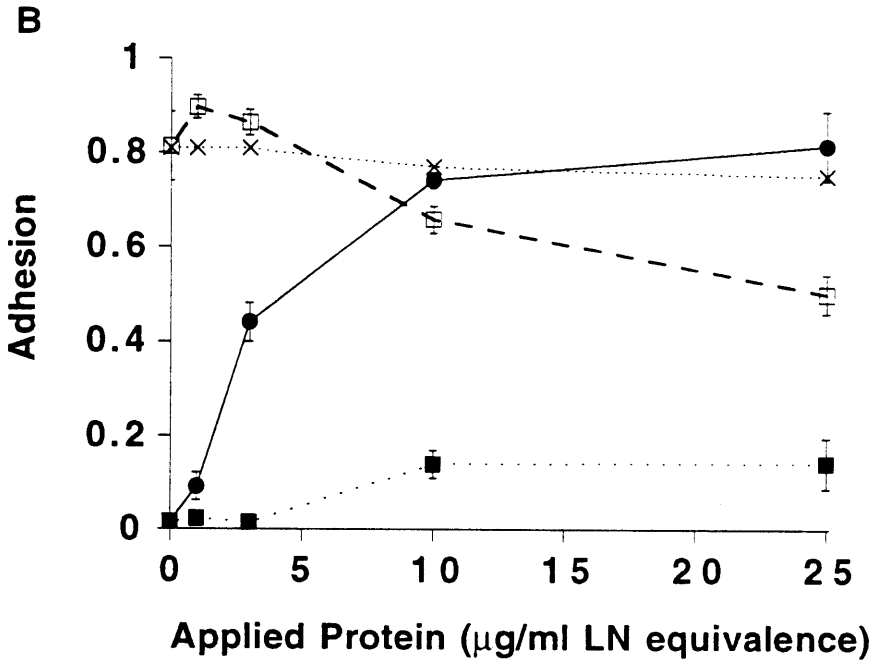
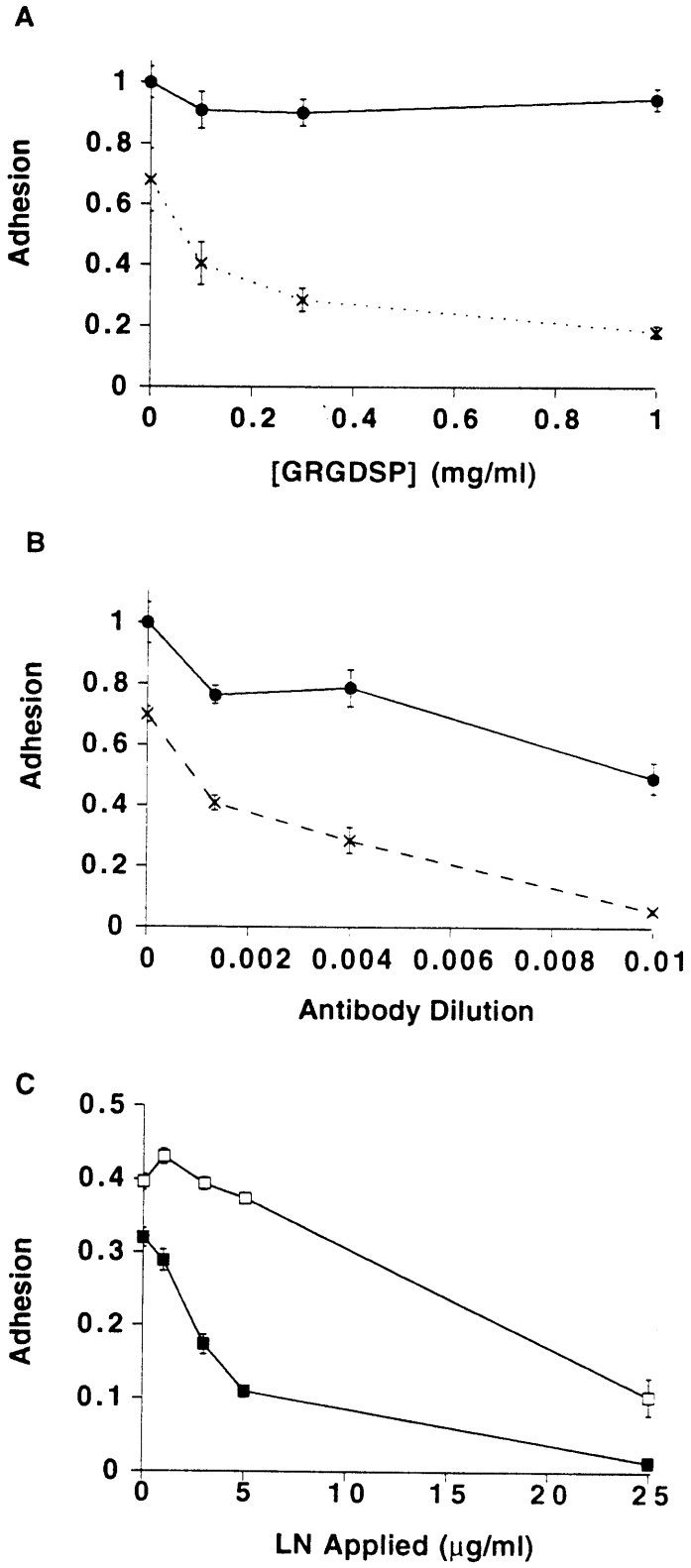

B
Adhesion
1
0.8
0.6
0.4
0.2
0
0
5
10
15
20
25
Applied Protein (μg/ml LN equivalence)

**Figure 6. Effect of altered adhesive strength on E1'- and laminin-mediated anti-adhesion.** MG63 cell adhesion to (—●—) 25 μg/ml fibronectin or (--X--) 25 μg/ml fibronectin mixed with 25 μg/ml E1' fragment of laminin was measured in the presence of (A) synthetic RGD peptide or (B) anti-fibronectin receptor antibody. Cells were mixed with the indicated concentration of peptide or antibody, applied to the assay plate, incubated at 37° for 30 min. and subjected to a 150g removal force. Observed adhesion was normalized to the maximum adhesion observed in each experiment and is the mean ± SEM (n=4). (C) WEHI cells were treated with (—□—) 50 nM PMA in DMSO or (—■—) DMSO alone for 15 min. prior to harvest for the adhesion assay. Adhesion to substrata coated with 25 μg/ml fibronectin mixed with varying concentrations of laminin was measured using a 50g removal force. The data are presented as the fraction of input $^{35}S$ bound to the substratum and are the mean ± SEM (n=4).


A
Adhesion
1
0.8
0.6
0.4
0.2
0
0
0.2
0.4
0.6
0.8
1
[GRGDSP] (mg/ml)
B
Adhesion
1
0.8
0.6
0.4
0.2
0
0
0.002
0.004
0.006
0.008
0.01
Antibody Dilution
C
Adhesion
0.5
0.4
0.3
0.2
0.1
0
0
5
10
15
20
25
LN Applied (μg/ml)

their adhesion to fibronectin was increased, and the ability of laminin to block adhesion was reduced (Fig. 6C).

**Adhesion to at least two sites in fibronectin is susceptible to the anti-adhesive effects of laminin**

Since two cell lines that adhere to fibronectin via different integrin receptors both respond to the anti-adhesive effects of laminin, it was possible to test directly whether adhesion mediated through different fibronectin cell binding sites could be affected. To this end, the binding sites of MG63 cells and WEHI cells were mapped (Table 1). MG63 cells bound to the 120 kD fragment of fibronectin, which contains the GRGDSP cell binding domain, but not the C-terminal heparin binding region. As would be expected for some integrin-mediated interactions, adhesion to the 120 kD fragment was inhibited by the GRGDSP peptide. Addition of 25 μg/ml of the E1' fragment of laminin reduced MG63 cell adhesion to this fragment 5-fold. In contrast, WEHI cells did not adhere to the 120 kD proteolytic fragment of fibronectin (Table 1). Thus WEHI and MG63 cells adhere to different sites on fibronectin, and consequently adhesion to at least two sites in fibronectin is susceptible to the anti-adhesive effects of laminin. Interestingly, MG63 cells also appear to adhere to a site outside the 120kD fragment; adhesion to intact fibronectin is not blocked by concentrations of GRGDSP that do block binding to the 120 kD fragment (compare Figure 6A and Table 1), and, this GRGDSP-resistant adhesion is also susceptible to the anti-adhesive effect of the E1' fragment (see Figure 6A).

**Anti-adhesion is observed at early stages of cell adhesion to fibronectin**

Cell adhesion has been proposed to occur in distinct phases (McClay et al., 1981). Initial contact with the substratum permits binding of some cell surface receptors to their ligands. At this stage, adhesion is usually weak. Subsequently, additional receptors and cytoskeletal proteins are recruited to the site of contact, stress fibers may be formed and a larger close contact between cell and substratum develops. At this stage, the cell is more resistant to removal from the substratum. It has been observed that the development of strong adhesive contacts requires 10-30 minutes,

| | Cell Type | Substratum | Peptide | Adhesion |
|---|---|---|---|---|
| Experiment 1: | MG63 | FN | - | 100 ± 2.4 |
| | | 120kD | - | 88 ± 6.3 |
| | | 120kD | GRGDS | 29 ± 5.7 |
| | | BSA | - | 6 ± 1.1 |
| Experiment 2: | MG63 | 120kD | - | 100 ± 3.7 |
| | | 120 kD & E1' | - | 20 ± 0.6 |
| Experiment 3: | WEHI | FN | - | 100 ± 13 |
| | | 120 kD | - | 4 ± 1.5 |

**Table 1: Characterization of fibronectin binding sites that support anti-adhesion.** The concentration of each protein in the coating solutions was 25 mg/ml. Peptides as indicated were added to the cells just prior to plating for the adhesion assay. After incubation, WEHI cells were subjected to a 50g removal force and MG63 to a 150g removal force. Adhesion was normalized to the maximum adhesion observed in each experiment.

and can be prevented by lowering the temperature to 4°C, blocking cell metabolism, or disrupting cytoskeletal structures. Since the adhesion measurements presented above were all made 30 minutes after cell-substratum contact, the anti-adhesive action of laminin could potentially reflect a block at either an early or late stage in the development of cell adhesion. To test whether laminin affects cell adhesion to fibronectin at an early stage of adhesion, initial or "undeveloped" adhesion of WEHI cells was assayed. Cells were treated with cytochalasin B, chilled to 4°C. and, after a quick centrifugation to bring the cells into contact with the substratum, the plates were immediately covered, inverted and centrifuged as usual. Under these conditions, in which maximum cell adhesion to fibronectin alone was decreased by approximately 30%, laminin was still effective at inhibiting cell adhesion (Fig. 7A). Indeed, no change was observed in the concentrations of laminin required to inhibit adhesion. In addition, when MG63 cells were treated with cytochalasin and initial adhesion measured, laminin prevented adhesion of these cells to fibronectin as effectively as it blocked WEHI cell adhesion (Figure 7B).

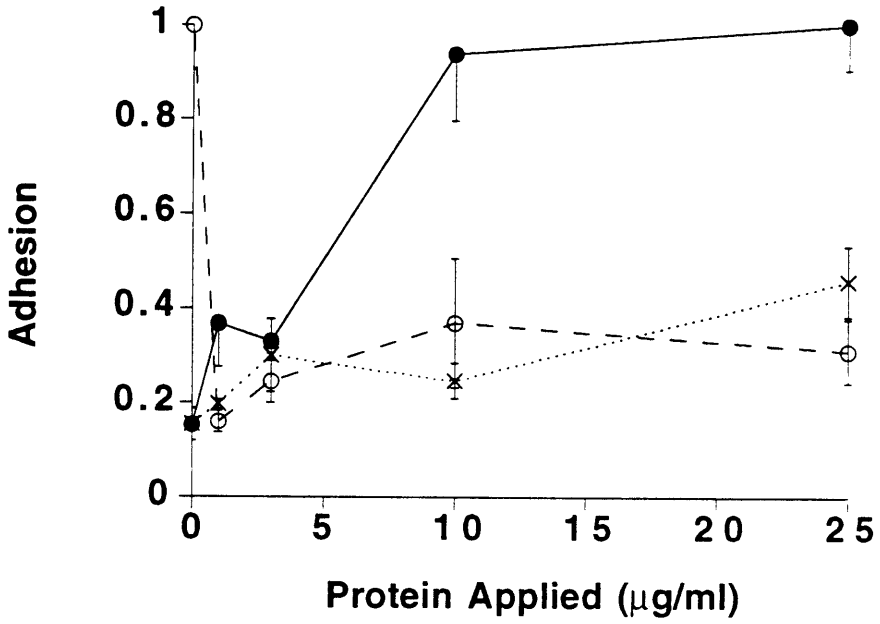
Cell adhesion was also tested under conditions that inhibit cellular processes other than the direct interaction of cell-surface receptors with their ligands. WEHI cells were treated with 0.05% formalin and 0.01% digitonin, conditions that rendered them permeable to trypan blue and reduced incorporation of $^{35}S$-methionine into TCA-precipitable protein to background levels. Adhesion of these cells to fibronectin, though weak, could still be detected, and was blocked by the same concentrations of laminin that blocked the adhesion of untreated cells (data not shown).

**Binding of liposomes containing purified integrins to fibronectin is reduced in the presence of laminin**

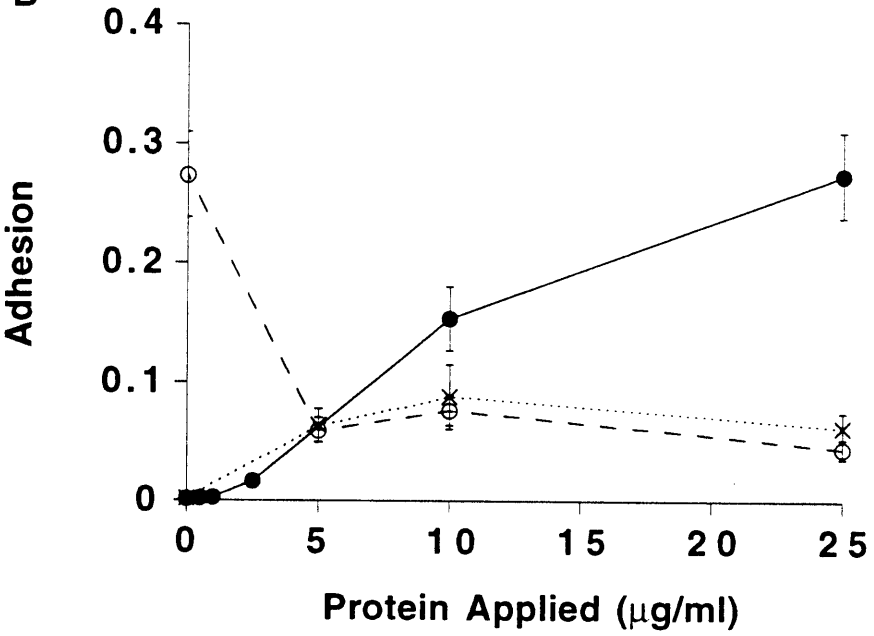
The ability of laminin to inhibit adhesion to fibronectin at even the earliest stages of the adhesion process and in the absence of energy-dependent cellular events suggests that laminin might alter adhesion to fibronectin by interfering directly with functions of cell surface fibronectin receptors. To test the effect of laminin on receptor-fibronectin interactions, integrin $\alpha_5\beta_1$ complexes from

**Figure 7. Effect of laminin on initial adhesion of WEHI and MG63 cells to fibronectin.** Plates were coated with (—●—) fibronectin, (--X--) laminin, or (--O--) 25 μg/ml fibronectin mixed with varying concentrations of laminin. WEHI cells **(A)** and MG63 cells **(B)** were cytochalasin B treated and subjected to a 50 g removal force immediately after plating onto the substrata, as described in Materials and Methods for the undeveloped adhesion assay. Data are expressed as mean ± SEM's of quadruplicate points and normalized to maximal adhesion **(A)** or to polylysine **(B)**.

A


Adhesion
1
0.8
0.6
0.4
0.2
0
0
5
10
15
20
25
Protein Applied (μg/ml)

B


Adhesion
0.4
0.3
0.2
0.1
0
0
5
10
15
20
25
Protein Applied (μg/ml)

MG63 cells were isolated by affinity chromatography. These molecules were reconstituted into radiolabelled liposomes and their binding to fibronectin-coated or fibronectin- and laminin-coated substrata was measured (Fig. 8A). The liposomes bound to fibronectin in a dose-dependent manner similar to that observed in cell adhesion assays. Binding was specific: liposomes did not bind to laminin, and binding to fibronectin was abolished by the presence of 1 mg/ml GRGDSP peptide. When laminin was mixed with the fibronectin on the substratum, however, liposome binding was reduced to background levels. The dose-dependence of this effect was strikingly similar to that observed for laminin-mediated reduction in cell adhesion (cf. Fig. 1A). Thus, it appears that the cellular phenomenon of anti-adhesion can be mimicked using a simple non-cellular system--binding of integrin-containing liposomes to substratum-bound fibronectin.

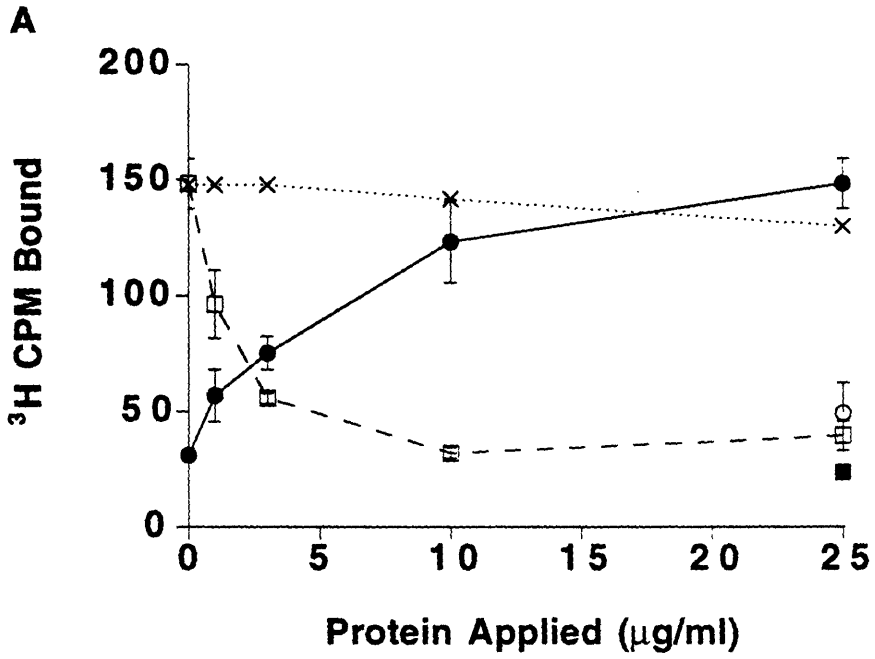
**Laminin in solution does not block binding of purified integrins to fibronectin**

The above data indicate that laminin can modulate the interaction of integrins in a plasma membrane with fibronectin. This led to the possibility that laminin could block the binding of soluble integrins to fibronectin. Radiolabelled integrins purified from MG63 cells were mixed with immobilized fibronectin in the presence or absence of laminin and the radioactivity associated with the fibronectin measured. While under these conditions a specific inhibitor of the integrin-fibronectin interaction, the peptide GRGDSP (1 mg/ml), significantly blocked binding of integrins to fibronectin in solution, soluble laminin (1 mg/ml) had no effect on this binding (Figure 8B). Thus, under these conditions, laminin does not interfere with the interaction of integrins and fibronectin.

**Antibody binding to fibronectin in the presence of laminin**

It is also possible that laminin interacts with fibronectin, and not the cell, to effect anti-adhesion. In one model, laminin could specifically bind substratum-bound fibronectin and block cell access to FN's adhesive sites. To test this hypothesis, anti-fibronectin monoclonal antibodies were used to measure the accessibility of

**Figure 8. Binding of integrin-containing liposomes and soluble integrins to fibronectin in the presence of laminin. (A)** Liposomes were prepared as described in Materials and Methods. They were applied to substrata coated with (—●—) fibronectin, (O) 25 μg/ml after being mixed with 1 mg/ml GRGDS. (■) 25 μg/ml laminin, or (--□--) 25 μg/ml fibronectin mixed with laminin. Data are expressed as mean ± SEM (n=4). Predicted binding of liposomes to fibronectin in the presence of laminin (x) was calculated as for predicted binding of cells (see fig. 1). **(B)** Binding of soluble integrins to fibronectin-sepharose. Radioiodinated integrins were mixed with fibronectin-sepharose alone (column1) with 1 mg/ml laminin (column 2) or with 1 mg/ml GRGDSP (column 3). Data are expressed as mean + SEM (n=3) of percent of input counts. The change in fraction bound in the presence of GRGDSP is significant ($p<.001$) by Students t-test, while the change in the presence of laminin is not ($p>0.5$).


A
200
150
100
50
0
$^3$H CPM Bound
0
5
10
15
20
25
Protein Applied (μg/ml)

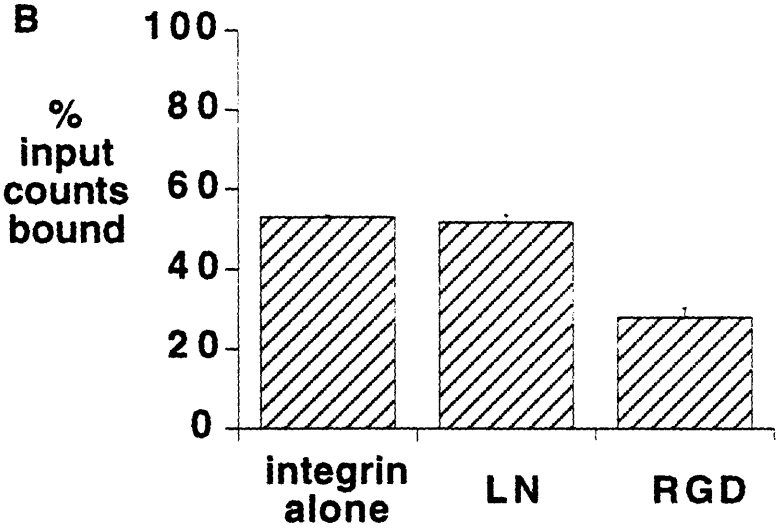
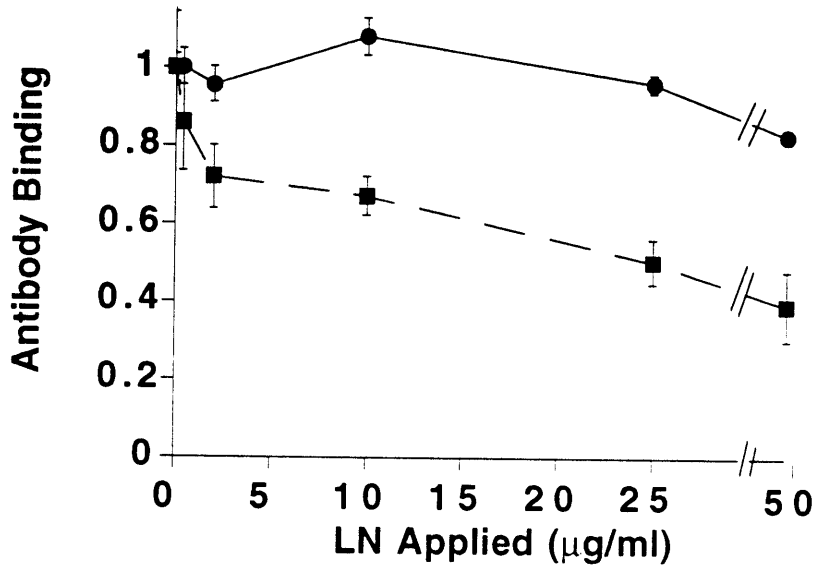
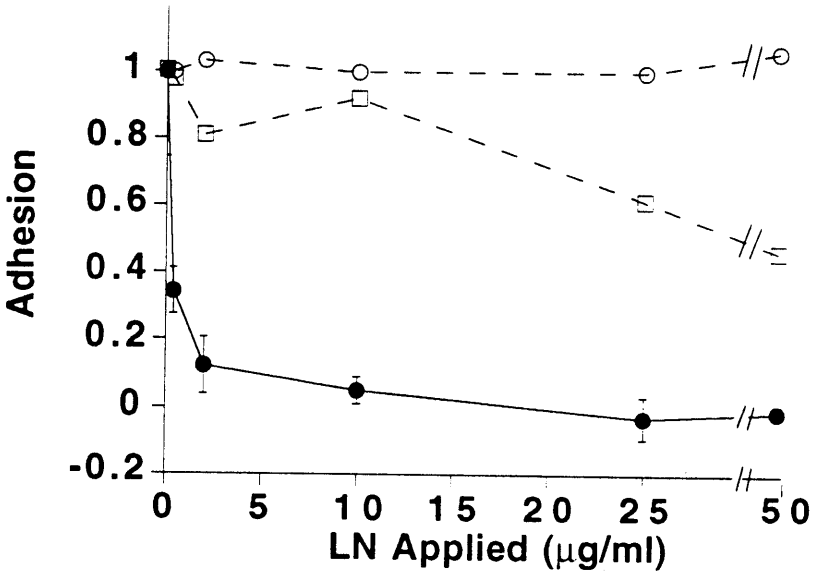

B
100
80
60
40
20
0
% input counts bound
integrin alone
LN
RGD

**Figure 9. Binding of anti-fibronectin monoclonal antibodies to fibronectin/laminin mixtures compared to cell adhesion. (A)** (--■--) Anti-heparin binding site monoclonal antibody and (—●—) anti-cell binding site monoclonal antibody followed by $^{125}$I-goat anti-mouse IgG antibody were applied to plates coated with 25 μg/ml fibronectin mixed with varying concentrations of laminin. **(B)** (—●—) WEHI cell adhesion to the same laminin/fibronectin mixtures was determined as in figure 1, and compared to the level of cell binding predicted by the binding of the (--□--) HBS and (--○--) CBS antibodies (see text). The reported binding of antibodies and cells was corrected by subtraction of binding to laminin in the absence of fibronectin and normalized to the value observed on 25 μg/ml fibronectin in the absence of laminin. Data presented are the mean± SEM of a representative experiment.

A


Antibody Binding
1
0.8
0.6
0.4
0.2
0
0
5
10
15
20
25
50
LN Applied (μg/ml)

B


Adhesion
1
0.8
0.6
0.4
0.2
0
-0.2
0
5
10
15
20
25
50
LN Applied (μg/ml)

## Discussion

We have investigated the mechanism by which substratum-bound laminin reduces the adhesion of some cells to fibronectin. A lymphoid cell line was found to respond to laminin in this way, and did so at substratum-bound concentrations of laminin similar to those previously found to suppress the adhesion of neuronal cells (Calof and Lander, 1991). Similarly, the adhesion of retinal neurons to another ECM molecule, vitronectin, was reduced in the presence of laminin. A single proteolytic fragment of laminin, E1', suppressed WEHI 231 cell adhesion to fibronectin to the same extent as did laminin, although with a somewhat lower specific activity (Fig 3). Using this proteolytic fragment, it was shown that an additional cell type (MG63 osteosarcoma cells) was sensitive to anti-adhesion, although the reduction in developed cell adhesion was not as large as observed with the WEHI 231 cells (Fig. 5A and 5B).

At least two fibronectin receptors and two sites on fibronectin are susceptible to the effects of anti-adhesion. The predominant integrin receptor expressed by WEHI cells, the $\alpha_4\beta_1$ integrin dimer (Guan and Hynes, 1990). can bind fibronectin at a site in the C terminus of the molecule (Mould and Humphries, 1991). Consistently, WEHI cells did not adhere to an amino terminal fragment (Table 1). Instead, MG63 cells bound to the 120 kD fragment of fibronectin in a GRGDSP sensitive manner, indicating the use of an integrin other than $\alpha_4\beta_1$ (Hayman et al., 1985; Pierschbacher et al., 1981), and this adhesion was sensitive to the E1' fragment (Table 1).

When the strength of the MG63 cells' adhesion to fibronectin was artificially lowered (Fig 6A and B), the E1' fragment suppressed cell adhesion more efficiently. Similarly, artificially increasing the strength of WEHI 231 cell adhesion to fibronectin rendered these cells less sensitive to the anti-adhesive effects of laminin (Fig. 6C). Both cell types are equally susceptible to anti-adhesion at early timepoints, when cell adhesion is weaker (compare axes on figure 7A and 5A). It is interesting that the more strongly adhering cells, MG63 cells, appear to overcome the anti-adhesive effects of laminin in a time-dependent manner: adhesion was lowered by laminin when

measured immediately after plating, but not thirty minutes later. These data together imply that anti-adhesion is a graded (i.e. not all-or none) phenomenon and that dynamic cell properties (e.g. adhesive strength) regulate the level to which it occurs.

How does laminin act to modulate cell adhesion? Potentially, anti-adhesion could involve an interaction of laminin with cellular components. Laminin does suppress adhesion to fibronectin at a low stoichiometry: As shown in Fig. 1, half-maximal anti-adhesion required approximately 0.1 molecule of laminin per molecule of fibronectin, or 0.05 per fibronectin monomer. Maximal anti-adhesion required no more than 0.3 molecules of laminin per fibronectin dimer. However, laminin is unlikely to exert its effects through the production of intracellular signals. Neither extended time, physiological temperature, cytoskeletal integrity, or cellular metabolism were necessary for cells to exhibit anti-adhesion in response to laminin (Figs. 7A and B). Most significantly, phospholipid vesicles containing only integrin $\alpha5\beta1$ could substitute for cells in exhibiting both adhesion to fibronectin, and anti-adhesion in response to laminin (Fig 8A).

These data suggest that no cellular machinery other than fibronectin receptors and a plasma membrane is required for anti-adhesion. Thus, if laminin suppresses cell adhesion through a *specific* interaction with cells, that interaction is apparently a direct one between laminin and fibronectin (or vitronectin) receptors. A solution binding experiment suggests that laminin could not interact directly with integrin receptors for fibronectin (figure 8B). However, the concentration of laminin in this experiment may be effectively lower than the concentration when laminin and fibronectin are both confined to a substratum. Or, the effects of laminin on integrins may only be significant when the integrins are confined in a plasma membrane and are not acting individually, in solution.

If, instead, laminin were to interact with the substratum, it is unlikely that it specifically blocks cell binding sites on the fibronectin molecule. When antibodies directed against the fibronectin domains that support cell adhesion are used to probe fibronectin substrata that contain maximally anti-adhesive amounts of laminin, either no

reduction in antibody binding is seen, or the reduction that is seen is too small to predict the observed decline in cell adhesion (Fig. 9). The antibody whose binding to fibronectin was unaffected by the presence of laminin, binds at or near the RGD-containing binding site for integrin $\alpha_5\beta_1$, as it blocks adhesion to this site (Pierschbacher et al., 1981). This is one site that MG63 cells recognize. The other antibody used in this study recognizes the C-terminal heparin binding domain of fibronectin, which could be used by WEHI cells (Guan and Hynes, 1990; Mould and Humphries, 1991). While the binding of this antibody was partially inhibited by the presence of laminin, the decrease in the antibody binding led to a predicted decrease in WEHI adhesion that was much less than what was observed. Interestingly, Lightner and Erickson (1990) have reported that laminin can block the binding of anti-fibronectin antibodies to substratum-bound fibronectin, with half maximal block seen at about 8 μg/ml laminin. However, in their study, different assay conditions were used; low amounts of fibronectin (e.g. 1.5 μg/ml) were applied, and the laminin was applied after fibronectin had been adsorbed.

**Models of Anti-adhesion**

These data support two models for laminin mediated anti-adhesion. In the first, laminin interacts with the cell adhesion receptor to modulate binding. In the second, laminin alters the substratum in a non-specific manner to block cell access to the adhesive molecule.

There are several constraints on the model that laminin interacts directly with the cellular receptor. First, such an interaction must be fairly promiscuous: laminin would have to interact with several different classes of integrin receptors including $\alpha4\beta1$ (i.e. the WEHI cell receptor), $\alpha5\beta1$ (on MG63 cells), and at least one other integrin or other type of cell receptor, as laminin also reduces the non-$\alpha5\beta1$-dependent (i.e. the RGD-insensitive--Figures 6 and 8) adhesion of MG63 cells to fibronectin. Retinal cell adhesion to vitronectin is mediated by at least one other integrin, $\alpha v\beta5$, and is heparin sensitive (Neugebauer et al., 1991) It was not determined if the integrin- or heparin- dependent components of vitronectin-

mediated adhesion (or both) is susceptible to anti-adhesion. Moreover, studies by others have demonstrated that anti-adhesion is not limited to integrin-mediated adhesion: adhesion of olfactory epithelial neurons to a recombinant fragment of laminin, G (Yurchenco et al., 1993) is both heparin sensitive and inhibited by the E1' fragment of laminin (A.L. Calof and A.D. Lander, pers. comm.).

As another constraint, the interaction of laminin with the adhesive receptor would be expected to be low affinity, so as not to be adhesive itself. How could a weak interaction interfere with the stronger interaction of those same integrins with fibronectin? One possibility is that when laminin interacts with fibronectin-binding receptors it induces a relatively stable conformational change that reduces or eliminates their affinity for fibronectin. This model is particularly appealing when considering integrin-mediated cell adhesion, as integrins are known exist in conformationally active and inactive states that can be switched by extracellular factors, such as antibodies (Chan and Hemler, 1993; Hynes, 1992; Neugebauer and Reichardt, 1991). Another possibility is that weak interactions with laminin retard the rate at which integrins diffuse in the plasma membrane and encounter fibronectin molecules. If the initiation of cell adhesion requires the simultaneous binding of many integrins to fibronectin, it is possible that rapid rates of integrin-fibronectin encounter are essential for adhesion.

As an alternative to laminin-receptor interactions, laminin could inhibit cell adhesion by altering the physical topography of the substratum. Cell (or liposome) attachment requires that the cell membrane come within a certain small distance of the substratum. This distance must be less than or equal to the distance that receptors (integrins) protrude from the cell membrane plus the average distance that fibronectin's cell binding sites protrude above the surface of the substratum. If a large molecule, such as laminin, protrudes a great enough distance above the substratum, and if it is present on the substratum in sufficient abundance, it could physically block large, membrane bound structures (cells or liposomes) from approaching the substratum closely enough, and yet pose no barrier to the approach of small macromolecules, such as fibronectin-binding antibodies.

The feasibility of this model depends on how the laminin and fibronectin are arranged on the substratum. In order for adhesion to be blocked, the laminin molecules must be spaced often enough to prevent the approach of a cell or liposome and must protrude far enough above the fibronectin cell binding site to block access of the downwardly protruding cell receptor (20 nm beyond the cell surface for an integrin receptor; Nermut et al., 1988). The first of these conditions is likely to be fulfilled under the conditions used in this study. According to Figure 1, laminin is half-maximally anti-adhesive at a bound concentration of 0.06 pmol/cm$^2$. This corresponds to an average spacing of roughly 65 nm between laminin molecules, if laminin molecules deposit only as monomers and the substratum has no irregularities. This spacing is smaller than the average size of liposomes prepared by detergent dialysis (~250 nm in diameter: (Mimms et al., 1981).

Whether laminin protrudes sufficiently above the fibronectin-binding sites depends on how the two molecules are associated with the substratum. Fibronectin molecules as seen by electron microscopy are only 50 nm across and appear flat and spread (Engel et al., 1981). If laminin molecules are also spread flat upon the substratum (as they appear in electron micrographs), they are unlikely to protrude upwards more than width of LN's individual globular domains (5-7 nm; (Mimms et al., 1981)). Yet a globular molecule, thyroglobulin, of considerably larger size (diameter 17 nm: Heidelberger and Pedersen, 1935) was not observed to be anti-adhesive. Thus, in this model, laminin molecules apparently cannot lie flat upon the substratum. On the other hand, were laminin to "stand erect," it could easily extend 80 nm from the substratum, four times the length of the extracellular domain of integrins, and presumably adequate to interfere non-specifically with adhesion. In this case, the point of association of each laminin molecule with the polystyrene substratum must be limited to one small region of the protein. If the laminin touches touches the substratum in several places the height it protrudes above the substratum would be dramatically reduced, especially given that laminin is apparently a relatively rigid molecule, tolerant of only limited bending, even in solution (Engel et al., 1981). Electron

microscopy of substratum-bound laminin could provide valuable information about the actual arrangement of the molecule under the conditions in which adhesion is measured.

In short, if non-specific, steric effects are to explain laminin-mediated anti-adhesion, considerable constraints must be placed on the way laminin molecules must be configured on the substratum. It is unlikely that the chemical properties of the substratum influences laminin orientation, since the interaction of polypeptides with polystyrene surfaces appears to be highly non-selective (Pesce et al., 1977), and because laminin-mediated anti-adhesion has been observed on glass substrata as well as plastic (Calof and Lander, 1991, and unpublished observations). If the above model is correct, the deposition of laminin onto solid substrata may be a highly ordered process, a point of likely *in vivo* signficance.

**Relationship to Other Anti-adhesive Molecules**

It may be useful to compare these observations on laminin mediated adhesion to what is known for other ECM molecules that inhibit cell adhesion and/or spreading. Both thrombospondin and tenascin have been shown to reduce cell adhesion to fibronectin-coated substrata under conditions similar to those used in our experiments (Lahav, 1988; Lightner and Erickson, 1990). The tenascin molecule can be divided into functional domains and, as with laminin, anti-adhesive activity maps to a region containing EGF-like repeats (Prieto et al., 1992; Spring et al., 1989). Although Lightner and Erickson (1990) reported only the concentration of protein applied to the substratum (instead of the amount of protein bound), they found that the amount of intact tenascin required to reduce cell adhesion to half-maximal levels was approximately 3 μg/ml, quite close to the value obtained here for laminin.

However, no consensus exists for the mechanism of action of anti-adhesive ECM molecules. The reduction of focal contacts by thrombospondin and tenascin can be blocked by glycosaminoglycans--chondroitin sulfate in the case of tenascin (Murphy-Ullrich et al., 1991) and heparin for thrombospondin (Murphy-Ullrich and Hook, 1989). The focal adhesion labilizing activity of two members of the

thrombospondin family has in fact been localized to a 19 amino acid sequence in the heparin-binding region of the protein (Murphy-Ullrich et al., 1993). Although laminin can also decrease the closeness of cell-subtratum apposition (Calof and Lander, 1991), glycosaminoglycans apparently have no effect on laminin's anti-adhesive activity as assayed by cell adhesion (figure 4).

Disparate models are found in the literature for the mechanism of tenascin mediated anti-adhesion. On the one hand, there are reports that fragments of tenascin that possess anti-adhesive activity can lower cellular pH when exposed to cells alone and can inhibit the rise of pH cause by fibronectin (Krushel et al., 1994). This could be evidence for a direct signalling mechanism for the anti-adhesive activity of tenascin, or the changes in pH may be a secondary response of the physiological changes effected by the molecules. In another report, adhesion of glutaraldehyde-treated cells to fibronectin still decreased in the presence of one isoform of tenascin-R (JI/160) but not in the presence of JI 180 or tenascin-C, indicating that the latter may require cell surface protein interactions to affect cell adhesion (Pesheva et al., 1994). On the other hand, Lightner and Erickson (1990) have demonstrated that, under the conditions of their assay (see above), tenascin can block access of fibronectin to antibodies. From these and other data they propose that tenascin reduces cell adhesion to fibronectin by forming a continuous scaffold or cage of large multi-armed tenascin molecules overlying substratum-bound fibronectin molecules. This model is not unlike the one proposed above for laminin. Whether the sterically hindering structure is called a scaffold or a protrusion may simply depend on whether one has tenascin (with its six-armed structure) or laminin (with a cross-like structure) in mind.

A cell surface molecule that has anti-adhesive effects provides an interesting parallel to laminin's effects. Episialin (the MUC1 gene product), if overexpressed on the cell surface, has been found to interfere with cellular aggregation (Ligtenberg et al., 1992) and, recently, with integrin-mediated adhesion (Wesseling et al., 1995). Episialin blocks integrin-dependent binding of a variety of cell types to fibronectin, laminin, collagen I and IV, as well as to immobilized

antibodies to integrins. This effect, like for laminin, is inversely dependent on the strength of the adhesion interaction; a stimulatory anti-integrin antibody that increased cell adhesion decreased the anti-adhesion. The extracellular domain of this protein is composed of 30-90 heavily O-glycosylated proline-rich repeats (Hilkens et al., 1992), forming a rigid, extended structure that electron micrographic studies show protruding 200-500 nm above the plasma membrane (Bramwell et al., 1986). One could imagine that laminin, protruding from the substratum as described above, could have the same effect as episialin protruding from the cell, both blocking access of the adhesive substratum to the cell surface receptors.

**Biological Significance of Anti-adhesion**

Historically, studies of cell adhesion to defined *in vitro* substrata have been invaluable in elucidating interactions that can occur between cells and their surrounding ECM *in vivo*. One question raised by all studies of anti-adhesion is whether the existence of *in vitro* anti-adhesion also provides insights into biologically relevant phenomena. How this question should be approached will depend on which of the two models proposed here correctly explains anti-adhesion.

For example, if laminin-mediated anti-adhesion reflects an interaction of laminin with fibronectin-binding integrins, then anti-adhesion may represent an example of extracellular regulation of integrin function. It is known from other studies that the activity of cell-surface integrins on neutrophils, monocytes, T lymphocytes and platelets is modulated by interactions of the cells with molecules present in their environment, and that in some cases this activation can be mimicked by binding of molecules (e.g. antibodies) directly to the integrin (cf. Hynes and Lander, 1992 for review). In contrast, if laminin mediates anti-adhesion by affecting how closely cells can approach their substratum, then anti-adhesion may provide insights into the interactions of cells *in vivo* with organized ECMs, such as basement membranes, in which the three-dimensional arrangement of molecules such as laminin may be highly ordered (Yurchenco et al., 1992).

## References


Adams, J. C., and Watt, F. M. (1993). Regulation of development and differentiation by the extracellular matrix. Development *117*, 1183-1198.

Bottenstein, J. E., and Sato, G. H. (1979). Growth of a rat neuroblastoma cell line in serum-free supplemented medium. Proc. Natl. Acad. Sci. *76*, 514-517.

Boucaut, J. C., Darribere, T., Boulekbache, H., and Thiery, J. P. (1984). Prevention of gastrulation but not neurulation by antibodies to fibronectin in amphibian embryos. Nature *307*, 364-367.

Bramwell, M. E., Wiseman, G., and Shotton, D. M. (1986). Electron-microscopic studies of the CA antigen, epitectin. J. Cell Sci. *86*, 249-261.

Bronner-Fraser, M. (1986). An antibody to a receptor for fibronectin and laminin perturbs cranial neural crest development *in vivo*. Dev. Biol. *117*,

Calof, A. L., and Lander, A. D. (1991). Relationship between neuronal migration and cell-substratum adhesion: laminin and merosin promote olfactory neuronal migration but are anti-adhesive. J. Cell Biol. *115*, 779-794.

Chan, B. M. C., and Hemler, M. E. (1993). Multiple function forms of the integrin VLA-2 can be derived from a single α2 cDNA clone: interconversion of forms induced by an anti-β1 antibody. J. Cell Biol. *120*, 537-543.

Chiquet-Ehrismann, R. (1995). Inhibition of cell adhesion by anti-adhesive molecules. Curr. Opin. Cell Biol. *7*, 715-719.

Chiquet-Ehrismann, R., Kalla, P., Pearson, C. A., Beck, K., and Chiquet, M. (1988). Tenascin interferes with fibronectin action. Cell *53*, 383-390.

Colognato-Pyke, H., O'Rear, J. J., Yamada, Y., Carbonetto, S., Cheng, Y. S., and Yurchenco, P. D. (1995). Mapping of network-forming, heparin-binding, and alpha 1 beta 1 integrin-recognition sites within the alpha-chain short arm of laminin-1. J. Biol. Chem. *270*, 9398-9406.

DeSimone, D. (1994). Adhesion and matrix in vertebrate development. Curr. Opin. in Cell Biol. *6*, 747-751.

Engel, J., Odermatt, E., Engel, A., Madri, J. A., Furthmayr, H., Rohde, H., and Timpl, R. (1981). Shapes, domain organization and flexibility of laminin and fibronectin, two multifunction proteins of the extracellular matrix. J. Mol. Biol. *150*, 97-120.

Galileo, D. S., Majors, J., Horwitz, A. F., and Sanes, J. R. (1992). Retrovirally introduced antisense integrin RNA inhibits neuroblast migration *in vivo*. Neuron *9*, 117-1131.

Goodman, S. L., Aumailley, M., and Mark, H. v. d. (1991). Multiple cell surface receptors for the short arms of laminin: α1β1 integrin and RGD-dependent proteins mediate cell attachment only to domains III in murine tumor laminin. J. Cell Biol. *113*, 931-941.

Graf, J., Iwamoto, Y., Sasaki, M., Martin, G. R., Kleinman, H. K., Robey, F. A., and Yamada, Y. (1987). Identification of an amino acid sequence in laminin mediating cell attachment, chemotaxis and receptor binding. Cell *48*, 989-966.

Guan, J.-L., and Hynes, R. O. (1990). Lymphoid cells recognize an alternatively spliced segment of fibronectin via the integrin receptor α4β1. Cell *60*, 53-61.

Hall, D. E., Neugebauer, K. M., and Reichardt, L. F. (1987). Embryonic neural retina cell response to extracellular matrix proteins: developmental changes and effects of the cell substratum attachment antibody CSAT. J. Cell Biol. *104*, 623-634.

Hayman, E. G., Pierschbacher, M. D., and Ruoslahti, E. (1985). Detachment of cells from culture substrate by soluble fibronectin peptides. J. Cell Biol. *100*, 1948-1954.

Heidelberger, M., and Pedersen, K. O. (1935). The molecular weight and isoelectric point of thyroglobulin. J. General Physiol. *19*, 95-109.

Hilkens, J., Ligtenberg, M. J. L., Vos, H. L., and Litvinov, S. V. (1992). Cell membrane-associated mucins and their adhesion-modulating property. Trends Biochem. Sci. *17*, 359-363.

Hynes, R. O. (1992). Integrins: versatility, modulation, and signaling in cell adhesion. Cell *68*, 303-322.

Kleinman, H. K., McGarvey, M. L., Liotta, L. A., Robey, P. G., Tryggvason, K., and Martin, G. R. (1982). Isolation and characterization of type IV procollagen, laminin, and heparan sulfate proteoglycan from the EHS sarcoma. Biochemistry *21*, 6188-6193.

Krushel, L., Prieto, A. L., Edelman, G. M., and Crossin, K. L. (1994). Differential effects of cytotactin-tenascin fusion proteins on intracellular pH and cell morphology. J. Cell. Physiol. *161*, 508-518.

Lahav, J. (1988). Thrombospondin inhibits adhesion of endothelial cells. Exp. Cell Res. *177*, 199-204.

Lightner, V. A., and Erickson, H. P. (1990). Binding of hexabrachion (tenascin) to the extracellular matrix and substratum and its effect on cell adhesion. J. Cell Sci. *95*, 263-277.

Ligtenberg, M. J. L., Buijs, F., Vos, H. L., and Hilkens, J. (1992). Suppression of cellular aggregation by high levels of episialin. Cancer Res. *52*, 2318-2324.

McClay, D. R., Wesse, G. M., and Marchase, R. B. (1981). Intercellular recognition: quantitation of initial binding events. Proc. Natl. Acad. Sci. USA *78*, 4975-4979.

McKoewn-Longo, P. J., and Mosher, D. F. (1985). Interaction of the 70,000 molecular-weight amino-terminal fragment of fibronectin with the matrix assembly receptor of fibroblasts. J. Cell Biol. *100*, 364-370.

Mimms, L. T., Zampighi, G., Nozaki, Y., Tanford, C., and Reynolds, J. A. (1981). Phospholipid vesicle formation and transmembrane protein incorporation using octyl glucoside. Biochem. *20*, 833-840.

Mould, A. P., and Humphries, M. J. (1991). Identification of a novel recognition sequence for the integrin α4β1 in the COOH-terminal heparin-binding domain of fibronectin. EMBO J. *10*, 4093-4099.
Murphy-Ullrich, J., and Hook, M. (1989). Thrombospondin modulates focal adhesions in endothelial cells. J. Cell Biol. *109*, 1309-1319.
Murphy-Ullrich, J. E., Gurusiddappa, S., Frazier, W. A., and Hook, M. (1993). Heparin-binding peptides from thrombospondins 1 and 2 contain focal adhesion-labilizing activity. J. Biol. Chem. *268*, 26784-26789.
Murphy-Ullrich, J. E., Lightner, V. A., Aukhil, I., Yan, Y. Z., Erickson, H. P., and Hook, M. (1991). Focal adhesion integrity is downregulated by the alternatively spliced domain of human tenascin. J. Cell Biol. *115*, 1127-1136.
Nermut, M. V., Green, N. M., Eason, P., Yamada, S. S., and Yamada, K. M. (1988). Electron microscopy and structural model of human fibronectin receptor. EMBO J. *7*, 4093-4099.
Neugebauer, K. M., Emmett, C. J., Venstrom, K. A., and Reichardt, L. F. (1991). Vitronectin and thrombospondin promote retinal neurite outgrowth: developmental regulation and role of integrins. Neuron *6*, 348-358.
Neugebauer, K. M., and Reichardt, L. F. (1991). Cell-surface regulation of β1-integrin activity on developing retinal neurons. Nature *250*, 68-71.
Paulsson, M., Aumailley, M., Deutzmann, R., Timpl, R., Beck, K., and Engel, J. (1987). Laminin-nidogen complex: extraction with chelating agents and structural characterization. EMBO J. *7*, 4093-4099.
Pesheva, P., Probstmeier, R., Shubitz, A. P. N., McCarthy, J. B., Furcht, L. T., and Schachner, M. (1994). Tenascin-R (J1 160/180) inhibits fibronectin-mediated cell adhesion - functional relatedness to tenascin-C. J. Cell Sci. *107*, 2323-2333.
Pierschbacher, M. D., Hayman, E. G., and Ruoslahti, E. (1981). Location of the cell attachment site in fibronectin with monoclonal antibodies and proteolytic fragments of the molecule. Cell *26*, 259-267.
Prieto, A. L., Andersson-Fisone, C., and Crossin, K. L. (1992). Characterization of multiple adhesive and counteradhesive domains in the extracellular matrix protein cytotactin. J. Cell Biol. *119*, 663-678.
Pytela, R., Pierschbacher, M. D., and Ruoslahti, E. (1985). Identification and isolation of a 140 kD cell surface glycoprotein with properties expected of a fibronectin receptor. Cell *40*, 191-198.
Reichardt, L. F., and Tomaselli, K. J. (1991). Extracellular matrix molecules and their receptors: functions in neural development. Annu. Rev. Neurosci. *14*, 531-570.
Sage, H., Vernon, R. B., Funk, S. E., Everitt, E. A., and Angello, J. (1989). SPARC, a secreted protein associated with cellular proliferation, inhibits cell spreading *in vitro* and exhibits Ca-

dependent binding to the extracellular matrix. J. Cell Biol. *109*, 341-356.
Saunders, S., and Bernfield, M. (1988). Cell surface proteoglycan binds mouse mammary epithelial cells to fibronectin and behaves as a receptor for interstitial matrix. J. Cell Biol. *106*, 423-430.
Schaffner, W., and Weissman, C. (1973). A rapid, sensitive and specific method for the determination of protein in dilute solution. Anal. Biochem. *56*, 502-514.
Snow, D. M., Lemmon, V., Carrino, D. A., Caplan, A. I., and Silver, J. (1990). Sulfated proteoglycans in astroglial barriers inhibit neurite outgrowth *in vitro*. Exp. Neurol. *109*, 111-130.
Spring, J., Beck, K., and Chiquet-Ehrismann, R. (1989). Two contrary functions of tenascin: dissection of the active sites by recombinant tenascin fragments. Cell *59*, 325-334.
Timpl, R., Rohde, H., Risteli, L., Ott, U., Robey, P. G., and Martin, G. R. (1982). Laminin. Meth. Enzymol. *82*, 831-338.
Wesseling, J., Valk, S. W. v. d., vos, H. L., Sonnenberg, A., and Hilkens, J. (1995). Episialin (MUC1) overexpression inhibits integrin-mediated cell adhesion to extracellular matrix components. J. Cell Biol. *129*, 255-265.
Yamagata, M., Suzuki, S., Akiyama, S. K., Yamada, K. M., and Kimata, K. (1989). Regulation of cell-substrate adhesion by proteoglycans immobilized on extracellular substrates. J. Biol. Chem. *264*, 8012-8018.
Yurchenco, P. D., Cheng, Y.-S., and Colognato, H. (1992). Laminin forms an independent network in basement membranes. J. Cell Biol. *117*, 1119-1133.
Yurchenco, P. D., Cheng, Y.-S., and Schittny, J. C. (1990). Heparin modulation of laminin polymerization. J. Biol. Chem. *265*, 3981-3991.
Yurchenco, P. D., Sung, U., Ward, M. D., Yamada, Y., and O'Rear, J. J. (1993). Recombinant laminin G domain mediates myoblast adhesion and heparin binding. J. Biol. Chem. *268*, 8356-8365.

# Chapter 3

# G Protein-Independent Effects of Pertussis Toxin on Growth Cone Guidance

## Introduction

Axon pathfinding results from the navigation of growth cones in response to specific guidance cues. Such cues are thought to be provided by cell-surface and secreted molecules, some of which stimulate neurite growth; others mediate or regulate fasciculation of neurites; others act as soluble chemoattractants; others influence rates of neurite elongation; others render substrata non-permissive for neurite growth; and others induce growth cone paralysis or collapse (for reviews see Lander, 1987; Monard, 1988; Grumet, 1991; Tessier-Lavigne, 1992; Keynes and Cook, 1992; Letourneau, 1992, 1994; also see Pittman et al., 1989; O'Leary et al., 1991; Oohira et al., 1991; Luo et al., 1993; Kennedy et al., 1994; Tang et al., 1994). Little is known at the molecular level about how such molecules guide. In some cases, information about possible initial signaling events exists (Schuch et al., 1989; Bixby and Jhabvala, 1992; for review see Doherty and Walsh, 1994) but in no case is it yet understood how those or other signals are transduced into changes in growth cone behavior.

Among the best studied guidance molecules are the laminins, a family of large, multi-domain extracellular matrix proteins that are present *in vivo* in certain pathways along which axons are guided (e.g. Cohen et al., 1987; Riggott and Moody, 1987). *In vitro*, substratum-bound laminin-1 both promotes neurite outgrowth (i.e., it increases neurite initiation and/or rates of neurite elongation) and, if presented in a pattern such as stripes, guides growth cones (Hammarback et al., 1985; Gundersen, 1987; Buettner and Pittman, 1991; Clark et al., 1993).

The present study was undertaken to test for the existence of signaling events or cellular machinery specifically involved in the *guidance* of growth cones by laminin. An assay developed to search for reagents--drugs, antibodies, or enzymes--that interfere with neurite guidance by laminin patterns, but have little or no effect on the promotion of neurite outgrowth by laminin, yielded one molecule: pertussis toxin. Surprisingly, the subunit of pertussis toxin that covalently modifies and inactivates cellular G proteins was not required for this effect. Further investigation showed that the recently reported ability of pertussis toxin to block the effects of a growth cone collapsing

factor (Igarashi et al., 1993) is also not mediated by G protein inactivation. As such collapsing factors are believed to be inhibitory guidance cues *in vivo* (Walter et al., 1990), the results suggest that pertussis toxin, acting via G protein independent pathways, may disrupt cellular mechanisms common to the guidance of growth cones by disparate types of guidance cues.

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## Materials and Methods

### *Materials*

Chrome-on-quartz photomasks (thickness, 0.09 inch) were fabricated by Advance Reproductions Co. (North Andover, MA). Laminin was purified from the mouse Engelbreth-Holm Swarm sarcoma (Kleinman et al., 1982; Timpl et al., 1982). Rabbit anti-mouse laminin serum was purchased from Polysciences, Inc. (Warrington, PA). Pertussis toxin was obtained from List Pharmaceuticals (Campbell, CA) or Sigma and the B oligomer of pertussis toxin from Calbiochem. Absence of the S1 subunit from the B oligomer was confirmed by SDS-gel electrophoresis. Tissue culture media were purchased from GIBCO-BRL or Cellgro-Mediatech. Unless noted, all other reagents were from Sigma.

### *Preparation of Substrata*

Laminin (20 μg/ml) was applied to sterile, acid washed 12 mm round coverslips (#1 thickness, Propper Mfg., Long Island City, NY) in calcium- and magnesium-free Hanks Balanced Salt Solution (CMF-HBSS) for 3 hr at 37°, then washed 5 times with CMF-HBSS. To generate patterned substrata, laminin coverslips were washed 5 times with sterile distilled water, air dried, placed protein side down on a quartz photomask resting 0.5 cm above a short wave ultraviolet light source (UVP Inc. model UVG-54; VWR Scientific), and irradiated for 10 min. In some cases, marks were made on the backs of coverslips with an indelible lab marker to record pattern orientation.

### *Cell Isolation and Preparation*

Fertile chicken eggs were obtained from Spafas Inc. (Storrs, CT). Dorsal root ganglia (DRG) from embryonic day 7 or 8 (E7-E8) embryos were removed in sterile calcium and magnesium-free Dulbecco's phosphate buffered saline (PBS) and cleaned in holding medium (Leibovitz' L15 medium with 0.5 % glucose, 100 U/ml penicillin and 100 μg/ml streptomycin). For experiments using whole ganglia, groups of 8-10 intact ganglia were threaded onto ~13 mm pieces of 8-0 nylon suture, and affixed, by means of small amounts of silicone grease at both ends of the suture, to patterned laminin coverslips immersed in culture

medium. In experiments using dissociated neurons, cleaned ganglia were incubated in 0.25% trypsin (Sigma type III) in holding medium for 25 min at 37°C. They were then treated with DNase I (1 mg/ml) and soybean trypsin inhibitor (0.25 mg/ml) for 2 min, washed with complete medium (see below), and dissociated by trituration (15-20 strokes using a flame polished Pasteur pipet). Non-neuronal cells were removed by preplating in a 60 mm tissue culture dish for 4.5 hr at 37°C. Weakly adherent cells were harvested by gentle washing, collected by centrifugation, and resuspended to 5 x $10^4$ cells/ml. 100 µl of cell suspension was applied to prepared coverslips in a parafilm-lined dish. The cells were 70-80% neurons, as judged by morphology.

*Cell Culture and Pharmacological Treatment*

Ganglia and dissociated neurons were cultured at 37°C in serum free medium consisting of a basal medium (50:50 DMEM:F12 for intact ganglia; F12 for dissociated neurons), supplemented with N2 additives (Bottenstein and Sato, 1979), 1 mg/ml crystalline bovine serum albumin (ICN Biochemicals), 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 20 ng/ml 2.5S NGF (Collaborative Research). Ganglia were cultured in an 8% $CO_2$ atmosphere; dissociated neurons in a 5% $CO_2$ atmosphere. After 24 hr, ganglion explant cultures were supplemented with 10 µM 5-fluoro-2-deoxyuridine to inhibit non-neuronal cell growth. Compounds (e.g. pertussis toxin) were added to ganglion explant cultures after 20-24 hr, a time at which neurite bundles had appeared, but had not extended beyond the central zone of the pattern shown in Figure 1. Compounds were added to cultures of dissociated neurons at the time of plating unless otherwise indicated. Cultures were fixed in 4% formaldehyde 5% sucrose in PBS. In most experiments CMFDA Cell Tracker dye (Molecular Probes, Eugene, OR) was added to 10 µM to ganglion explant cultures 30 min before fixation.

*Visualization of Laminin and Neurite Patterns*

To detect patterns of laminin inactivation, fixed cultures were blocked with 10% goat serum in PBS and stained with anti-laminin serum (Hammarback et al., 1985) at 1:1000 in PBS-BSA (PBS with 1% BSA) for ≥30 min at 37°. Coverslips were washed three times in PBS, followed by either rhodamine-conjugated goat anti-rabbit IgG (Cappel; 1:100 dilution in PBS-BSA) or a mixture of fluorescein-conjugated goat anti-rabbit IgG (Cappel; 1:1000 dilution in PBS-BSA) and rhodamine-phalloidin (0.2 μg/ml; Molecular Probes) applied for 30 min at 37°C. Coverslips were washed 3 times in PBS prior to observation. With ganglion explant cultures, care was taken to minimize detachment of neurite bundles from the substratum during washing.

Coverslips containing ganglion explants were placed on a glass Titertek slide (Fisher) in PBS and viewed using a Bio-Rad MRC-600 confocal microscope equipped with a krypton/argon laser. Coverslips with dissociated neurons were mounted onto glass slides in 50:50 PBS/glycerol and viewed on a Zeiss Axiophot microscope.

*Analysis of Neurite Outgrowth*

For analysis of ganglion explant cultures plated on the pattern shown in Figure 1A, images of both neurite bundles and laminin patterns were collected on the confocal microscope. To measure distances travelled by neurites along laminin stripes the length of the longest neurite, measured from the beginning of the outer zone, was determined for at least one side of at least 20 of each of the four types of stripes (i.e. with gap widths of 0, 5, 10, and 20 μm). To quantify neurite wandering, a variation on the pattern in Figure 1A was used in which the type of stripe from which a neurite had wandered could be determined unambiguously. Briefly, sets of 4 stripes, all of the same gap width and spaced 60 μm from one another, were grouped together, and were separated from other groups by 120 μm. The percentage of neurite mass "on" and "off" stripes was determined for each set of four similar stripes by using the confocal microscope to measure total, background-subtracted fluorescence in the fluorescein channel (which detects the cytoplasmic dye CMFDA) in the 4 rectangular areas (starting

from the beginning of the outer zone of the pattern) representing the stripes themselves ("on"), as well as the 5 rectangular areas constituting the lanes between the stripes ("off"). These areas were identified by reference to images of laminin immunostaining.

For analysis of dissociated neuron cultures plated on the pattern shown in Figure 4A, cells were examined using fluorescence and phase contrast optics simultaneously, and neurites scored for wandering as described in the figure legend. For analysis of neurite lengths on unpatterned substrata, phase contrast images of neurites viewed on a Zeiss Axiovert microscope were collected using a neuvicon video camera (Hamamatsu Photonics) connected to a series 151 Image Processor (Imaging Technologies, Woburn, MA).

For analysis of neurite growth by time-lapse microscopy, dissociated neurons were plated on laminin-coated coverslips (22 mm diameter) attached to the bottom of 35 mm dishes from which a 15 mm circle had been drilled out. Cells were plated into 4 ml of serum free medium in which F12 was replaced by L15 medium containing 20 mM Hepes pH 7.4 and 0.5% glucose. The dish was overlayed with light mineral oil (Sigma) to prevent evaporation, and maintained at 37±0.2° using a PDMI-2 culture chamber (Medical Systems). Neurites were visualized by phase contrast optics using a Zeiss Axiovert microscope and images were collected with a video camera connected to an image processor (see above). Images were processed by correction for background inhomogeneities using Vidim software (version 2.86, written by Drs. G. Belford, J. Stollberg, and S. Fraser, California Institute of Technology) and stored on a Panasonic 2026 optical disc recorder. Pertussis toxin (0.5 μg/ml) was added to cultures 1 hr after plating and outgrowth measured 3-5 hr later. Treated and untreated cultures were observed alternately, with neurite growth recorded for 30-40 min per neurite. The x-y coordinates of the approximate centroid of the growth cone were used to determine distance travelled during 4 min intervals. Plotting the running total of distance against the total time gave the rate of outgrowth as the slope.

*Preparation of complex-type N-linked carbohydrate-deficient laminin*

Laminin was isolated from deoxymannojirimycin (DMN)-treated M15 cells by a modification of published procedures (Chung et al., 1977; Yurchenco et al., 1992) Briefly, 1 mM DMN (Boeringer Mannheim) was added every 2-3 days to M15 cells that were maintained for 2 days in monolayer culture followed by 11 days in suspension culture. Laminin-containing "cores" were isolated as described and solubilized in 2 mM EDTA in PBS overnight at 4° C. The yield of protein (from two 100 mm dishes) was 60 μg as determined by amido black binding (Schaffner and Weissman, 1973). To remove protein with any residual complex-type N-linked carbohydrates, the laminin from DMN-treated cells (D-laminin) was passed over wheat germ agglutinin-Sepharose. 97% of the recoverable protein was found in the flow through fraction.

*Measurement of Pertussis Toxin Binding to Laminin Coverslips*

Laminin- and D-laminin-treated coverslips were prepared, patterned, and incubated with pertussis toxin as indicated in the text. After binding for the indicated amounts of time, coverslips were fixed in the same manner as coverslips used for cell culture. After washing with PBS, coverslips were blocked with 10% goat serum in PBS, incubated with monoclonal antibody 6DX3 (a 1:1000 dilution of ascites fluid) for 1 hour at 37°C, washed 3 times with PBS, and then incubated with $^{125}$I-labeled goat anti-rabbit IgG (Dupont-New England Nuclear; 12 μCi/ml in PBS-BSA) for 1 hour at 37° C. These antibody concentrations were determined to be saturating (data not shown). Coverslips were then washed 5 times with PBS, air-dried, and bound radioactivity determined using a Molecular Dynamics Phosphorimager (Sunnyvale, CA). Background values (obtained from identically-processed coverslips that had not been treated with pertussis toxin) were subtracted from the data.

*Assay of Growth Cone Collapse*

A crude fraction from E10 chick brain membranes was prepared as described (Raper and Kapfhammer, 1990). Briefly, brains from E10 chick were dissected and homogenized in a Dounce homgenizer in

CMF-Hanks. The crude membranes were pelleted by spinning at 14,000 g and resuspended three times in CMF-Hanks, and stored at -80° C until use. Before use in the assay, the crude membrane preparation was dialyzed against PBS overnight and for an additional 2-4 hr against basal F12 medium. The material was added directly to cultures at a ten-fold final dilution (final protein concentration: 100-150 µg/ml). The collapse assay was as described by Raper and Kapfhammer (1990) with the following modifications: E7 dorsal root ganglion explants were maintained continuously in serum-free F12-based medium in 96 well plates and pertussis toxin or its B oligomer added 2-3 hr before application of the collapsing activity. After 30 min, cultures were fixed, images collected by video camera as described above, and growth cones visually scored for a collapsed morphology.

## Results

### Neurite guidance by continuous and interrupted paths of laminin

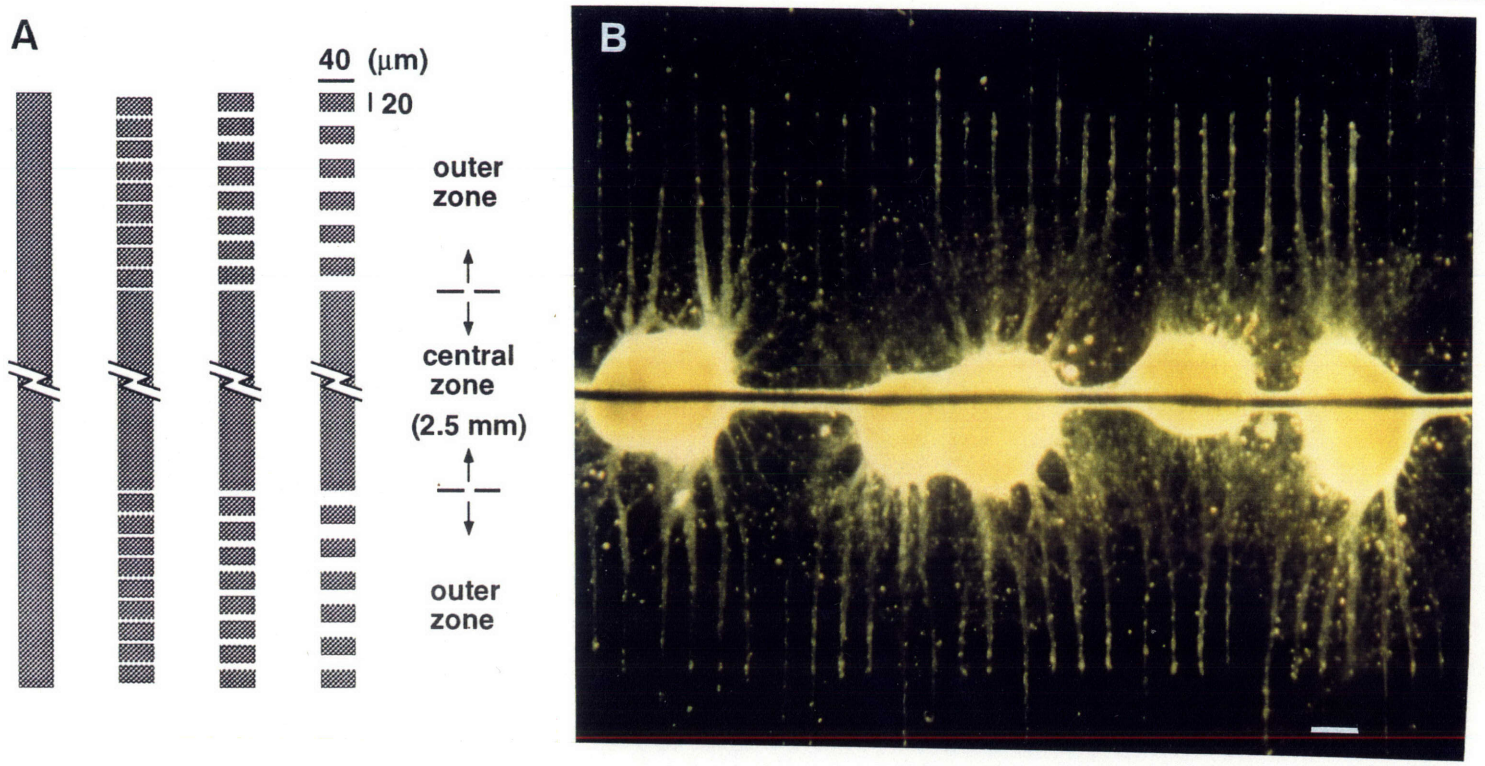
Contact photolithography was used to generate a patterned laminin substratum on which the guidance of large numbers of neurites could be assayed. Briefly, a laminin-coated glass coverslip is exposed to ultraviolet light shone through a chrome-on-quartz photomask. Laminin not protected by the chrome pattern is inactivated by the ultraviolet light, losing both its neurite outgrowth-promoting activity and much of its reactivity with anti-laminin antibodies (Hammarback et al., 1985). In this manner, sharp, micron-resolution patterns of active laminin can be created on a background of inactivated laminin, and the patterns revealed by anti-laminin immunostaining.

Patterned substrata used in the first sets of experiments consisted of a 10 mm square area containing four types of parallel stripes of active (unirradiated) laminin. The photomask was designed to produce stripes 40 μm wide and separated from each other by 80 μm of UV-inactivated laminin. The four kinds of stripes were grouped together in sets (Figure 1A): The first stripe in each set was unbroken over its entire 10 mm length, and the remaining three stripes were unbroken only in their central 2.5 mm ("central zone"). On either side of this zone the second, third and fourth stripes had periodic gaps of inactivated laminin 5, 10 or 20 μm in length, respectively. The length of active laminin between each gap was 20 μm.

Patterned laminin coverslips were immersed in culture medium, and a row of embryonic chick dorsal root ganglia was immobilized within the central zone of each coverslip, perpendicular to the laminin stripes (see Materials and Methods). Parallel bundles of neurites were readily apparent within a day, and within 48 hr had reached the ends of the central zone and grown onto the interrupted portions of the laminin stripes. At this time, it could be seen that the degree of neurite bundle extension differed markedly from one stripe to another (Figure 1B).

When patterns of neurite outgrowth after 48 hr were compared with patterns of laminin inactivation, neurites were found to have traveled farther on uninterrupted laminin stripes than on stripes with 5 μm or 10 μm gaps, and often halted entirely when confronted with

**Figure 1. Culture of dorsal root ganglion explants on patterned laminin.** **(A)** Expanded diagram of the repeated unit of the photomask used in the experiment shown in panel B. Shaded areas depict chrome coating on a quartz mask and correspond to areas in which laminin is protected from ultraviolet irradiation. Each of the four stripes shown extends a total distance of 10 mm (3.75 mm on either side of the central zone), and the pattern shown is repeated 20 times across the width of the photomask, with each group of four stripes separated by 120 μm. **(B)** Neurite outgrowth on laminin patterned using the photomask shown in panel A. A glass coverslip coated with 20 μg/ml laminin (3 hr, 37°C) was placed in contact with the photomask and exposed to ultraviolet light as described in Methods. Embryonic day 8 chick dorsal root ganglia were immobilized in an area corresponding to the central zone of the pattern. Cultures were incubated for 40 hr in NGF-containing serum-free medium, then fixed and visualized using oblique illumination. Arrows demarcate the beginning of the outer zones. Systematic variations in neurite length, that repeat in groups of four, can be seen in the outer zones at the top and bottom of the image. It can also be seen that the only neurites not confined to parallel bundles are those associated with small halos of non-neuronal cells surrounding each ganglion. Bar = 100 μm.


A
40 (μm)
20
outer zone
central zone
(2.5 mm)
outer zone
B

20 μm gaps. This behavior is shown for a single set of four stripes in Figure 2A, and was quantified by collecting measurements of neurite bundle lengths (starting from the beginning of the outer zone) and pooling the data from each kind of stripe. As confirmed by Figure 2B (open symbols), mean neurite progression in the outer zone falls steadily as a function of gap size.

These behaviors of neurites are consistent with previous observations: Neurites follow stripes of laminin (Hammarback et al., 1985), and once on laminin do not readily extend onto less preferred substrata (Gundersen, 1987). However, when confronted with a short gap of a less preferred substratum, a growth cone can extend filopodia, recognize the laminin on the other side, and subsequently grow across the gap (Hammarback and Letourneau, 1986).

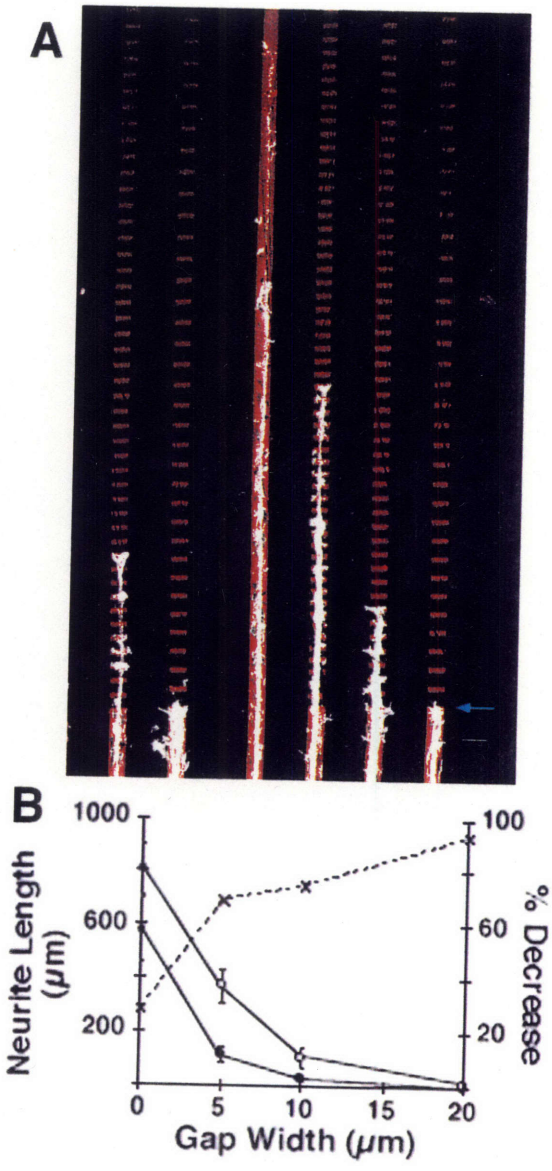
**Pertussis toxin causes neurites to wander from laminin stripes**

Initially, it was predicted that a drug or enzyme that selectively interfered with neurite guidance would have the following effect in the assay described above: Neurite progression along uninterrupted laminin stripes would be relatively undisturbed, while neurite progression along interrupted stripes would be inhibited. This prediction was based on the idea that growth cones crossing repeated interruptions would have to make more frequent guidance decisions in response to laminin than growth cones progressing along a continuous, relatively wide stripe.

The drug cytochalasin B, an inhibitor of actin polymerization, was used to test this prediction. In the presence of moderate doses of this drug, neurites lack filopodia and lamellipodia and make incorrect guidance decisions (Marsh and Letourneau, 1984; Bentley and Toroian-Raymond, 1986; Chien et al., 1993) yet still elongate, albeit slowly, (Marsh and Letourneau, 1984). As shown in Figure 2B (filled symbols), the effects of cytochalasin B (50 ng/ml) on neurite outgrowth on patterned laminin fit the predictions: elongation of neurite bundles on uninterrupted laminin stripes fell by only 28%, whereas on the same coverslips, mean outgrowth on interrupted laminin stripes was reduced between 68% (5 μm gaps) and 93% (20 μm gaps).

Several other reagents suspected to affect signaling processes within growth cones were also tested. Most had no detectable effect on

**Figure 2. Neurite guidance by interrupted laminin stripes.** **(A)** Detail of neurite outgrowth at the start of the outer zone of a patterned laminin coverslip. Explants were cultured as in B for 48 hr, fixed, and stained with an anti-laminin antibody followed by a fluorescein-conjugated second antibody. Neurites were visualized by counterstaining with rhodamine phalloidin. Fluorescent images were collected using a confocal microscope and were superimposed. The immunoreactive (unirradiated) laminin has been pseudocolored red; the phalloidin-stained neurites white. Laminin stripes with no gaps, and gap widths of 5, 10 and 20 μm are shown from left to right. An arrow marks the beginning of the outer zone. Phalloidin staining, which reveals F-actin, emphasizes growth cones and their filopodia, showing that these structures, and not just neurite shafts, are confined to the laminin paths. Bar=50 μm. Note that the dimensions of regions of immunoreactive laminin are somewhat smaller than the dimensions of the chrome-coated areas of the photomask (see panel A). This was presumably due to refraction and reflection of ultraviolet light during irradiation. **(B)** Explants cultured as in panel A were stained for laminin, and neurites were visualized using the cytoplasmic dye CMFDA (see Experimental Procedures). Mean distances traveled by neurite bundles, starting from the beginning of the outer zone, are plotted as a function of the sizes of gaps in the laminin stripes (uninterrupted stripes are plotted as having a gap width of zero). Data were obtained by averaging the lengths of the longest neurite on each of 20 stripes of each gap width, and are presented ± SEM. Explants were either grown in normal culture medium for 44 hours (O), or in normal culture medium for 20 hours, followed by an additional 24 hours in medium containing 50 ng/ml cytochalasin B and 0.5% DMSO (the cytochalasin B had been diluted from a stock dissolved in DMSO) (●). The percent decrease in mean neurite length caused by cytochalasin B treatment has been calculated (X). Cultures treated with 0.5% DMSO alone were indistinguishable from untreated cultures (not shown).


A
B
1000
600
200
Neurite Length (μm)
100
60
20
% Decrease
0
5
10
15
20
Gap Width (μm)

neurite outgrowth on either interrupted or continuous stripes, and others strongly inhibited neurite outgrowth on all stripes. The agents tested are summarized in Table 1.

One compound--pertussis toxin--did selectively disrupt neurite outgrowth on interrupted laminin stripes, but in a manner qualitatively different from cytochalasin B. Instead of simply halting prematurely on interrupted stripes, neurites elongating in the presence of pertussis toxin left those stripes altogether (Figure 3A-B).

To quantify this effect, a fluorescent cytoplasmic dye was used to stain neurites extending in the presence and absence of pertussis toxin. Quantitative fluorescence microscopy was used to compare the amounts of neurite mass in the lanes *between* the stripes with the amounts on the stripes themselves, including the gaps in the stripes. This was measured separately for each of the four categories of stripes by using coverslips patterned with a photomask somewhat different from that shown in Figure 1A, in which stripes of the same gap width were grouped together (see Methods).

As shown in Figure 3C, pertussis toxin caused a dose-dependent, as well as a gap width-dependent, departure of neurites from laminin stripes. At higher doses of the toxin (0.5 μg/ml), as much as 30-40% of total neurite mass left laminin stripes with 20 μm gaps; at the same dose, only 5% of neurite mass left uninterrupted laminin stripes (Figure 3C, circles). It is noteworthy that no other reagent tested, including those that inhibited neurite outgrowth, was ever observed to cause neurites to wander from stripes of active laminin (data not shown).

**Pertussis toxin interferes with the guidance of individual neurites**

Although the above data suggest that pertussis toxin specifically inhibits the guidance of growth cones by laminin, other explanations are possible. Pertussis toxin might induce defasciculation of the bundled neurites, forcing growth cones to encounter laminin borders more frequently and thus, by chance, wander across those borders. Or, pertussis toxin might increase spontaneous growth cone turning, so that, again, borders are encountered more frequently.

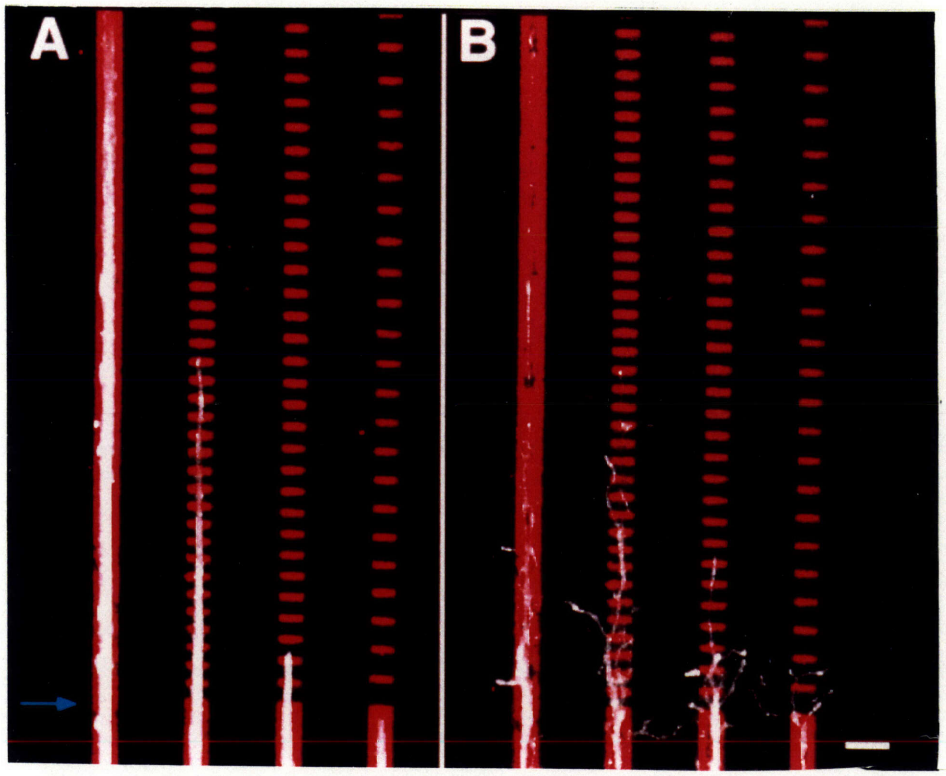
To address these possibilities, dissociated, purified dorsal root ganglion neurons were plated at low density onto coverslips patterned so

| | Concentration | Target | References |
|---|---|---|---|
| **NO EFFECT:** | | | |
| 8-bromo cyclic GMP | 1 mM | cGMP analog | (Weeks et al., 1991) |
| cholera toxin | 1-500 ng/ml | gangliosides; G proteins ($G_S$) | (Mann et al., 1989) |
| chondroitinase ABC | 0.1 U/ml | cellular ChS proteoglycans | |
| heparin | 0.5-1.0 mg/ml | cellular HeS proteoglycans | (Carri et al., 1988) |
| heparatinase | 10-20 µg/ml | " " | (Dow et al., 1991) |
| PI specific-phospholipase C | 0.05 U/ml | GPI-linked proteins | (Mahanthappa and Patterson, 1992) |
| **BLOCKS NEURITE OUTGROWTH:** | | | |
| anti-laminin antibody (rabbit polyclonal) | 1:100-1:500 | | |
| genistein | 4, 40 µg/ml | tyrosine kinases | (Wu and Goldberg, 1993) |
| phorbol 12-myristate 13-acetate | 1 mM | protein kinase C | (Campenot et al., 1991) |
| staurosporine | 10 mM | $Ca^{2+}$-dependent PKA, PKC, PKG | (Campenot et al., 1991) |
| **BLOCKS GAP CROSSING** | | | |
| cytochalasin B | 0.01-1.0 µg/ml | f-actin | (Marsh and Letourneau, 1984) |

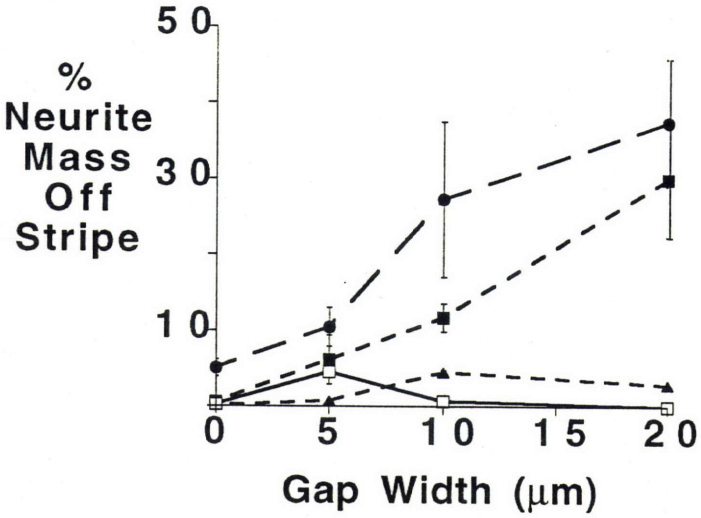
**Table 1: Reagents Tested In Discontinuous Laminin Assay.** Dorsal root ganglion explants were cultured on patterned laminin substrata as described in the legend to Figure 3. Treatments were added at 20-24 hours. Ganglia were stained with CMFDA and fixed after 44-48 hours in culture. Neurite lengths in the discontinuous pattern area were determined as described in Methods. "No effect" indicates that the mean neurite length for stripes of any gap width was not significantly different between treated and untreated cultures. "Blocks neurite outgrowth" indicates that most neurites did not reach the outer zone of

the coverslip. When possible, concentrations tested were at or below levels shown by others to affect neurite behaviors. For an explanation of "Blocks gap crossing," see Figure 2 and Results.

**Figure 3. Effect of pertussis toxin on neurite outgrowth on patterned laminin.** **(A-B)** Explants were cultured on patterned laminin as in figure 1. Pertussis toxin was added to some cultures after 22 hr. After 46 hr, all cultures were treated with CMFDA, fixed, immunostained for laminin, and viewed by confocal microscopy. Active (unirradiated) laminin has been pseudocolored red and CMFDA-stained neurites white. **(A)** Representative neurites growing in the absence of drug. **(B)** Representative neurites treated with pertussis toxin (0.5 μg/ml). The arrow marks the beginning of the outer zone. As shown by panel B, pertussis toxin induces wandering of neurites from laminin stripes. Unlike rhodamine phalloidin, CMFDA stains neurites evenly, demonstrating that whole neurites, not only growth cones and filopodia, leave the laminin stripes. Bar=50 μm. **(C)** From patterned coverslips in which stripes of like gap width were grouped together, amounts of neurite mass on and off each type of stripe were measured and averaged (see Methods). Each data point represents the mean ± SEM of values obtained from at least 3 groups of 4 stripes. The data show that the degree of neurite wandering is dependent on both the dose of pertussis toxin and on the widths of the gaps encountered by neurites. Concentrations of pertussis toxin were zero (□), 50 ng/ml (▲), 250 ng/ml (■), or 500 ng/ml (●).




C



that multiple 40 μm squares of active laminin were separated by 25 μm gaps of inactivated laminin (Figure 4A). Plating sparsely made it possible to score the behaviors of individual, unfasciculated neurites, and by using small repeating squares, rather than stripes, any increase in growth cone turning would not increase the frequency with which borders were encountered.

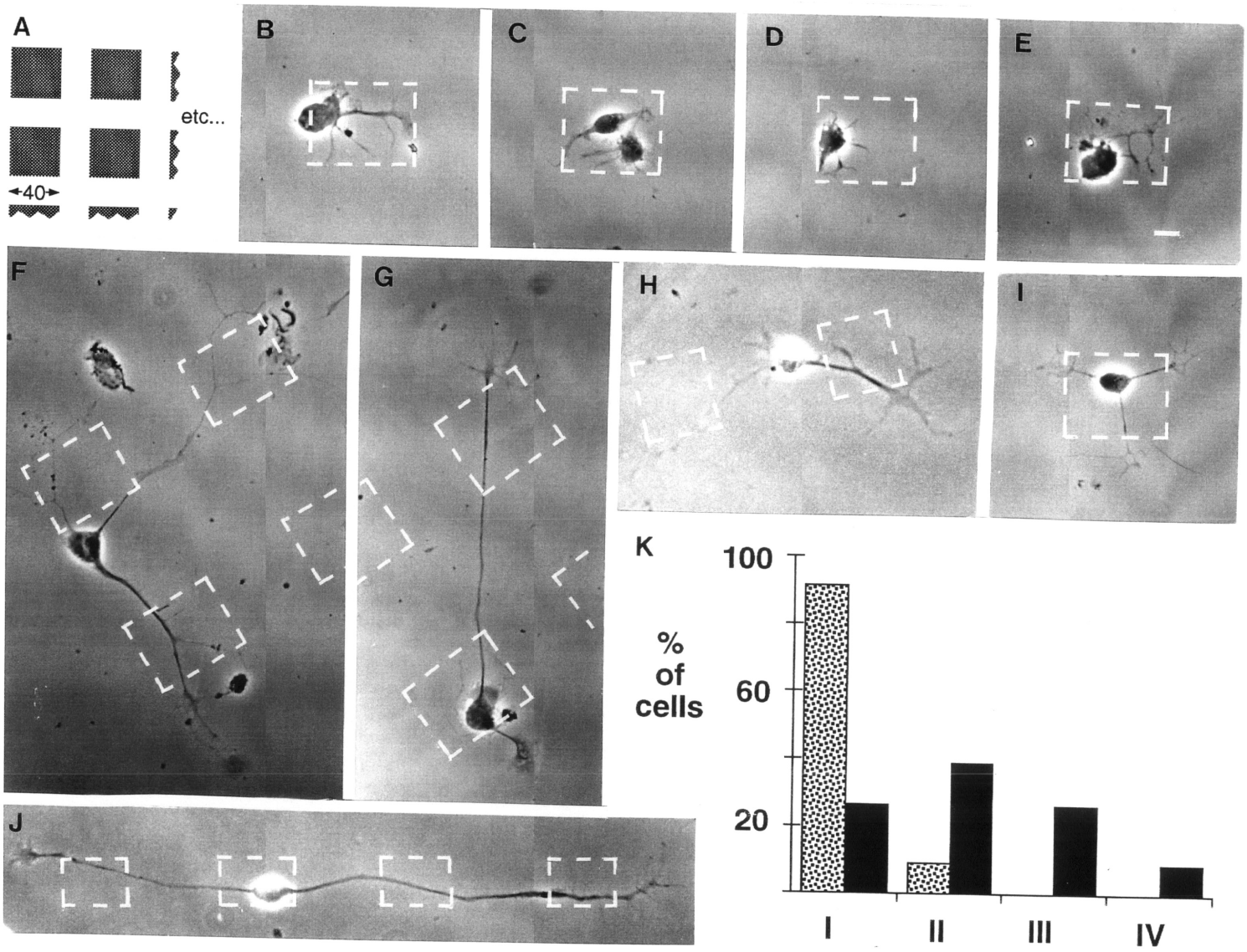
Examples of neurons cultured for 16 hr on this pattern in the absence of pertussis toxin are shown in Figure 4B-E. Most neurites remained confined to the laminin square on which they originated, with only the occasional filopodium leaving the squares (e.g. Figure 4E). This is not surprising, given that the gaps between the squares exceed what these neurites seem to be able to cross (cf. Figure 2; also see Hammarback and Letourneau, 1986). In contrast, in the presence of 0.8 μg/ml pertussis toxin, neurites frequently crossed laminin boundaries, sometimes extending over one or several squares (Figures 4F-J). This occurred regardless of whether pertussis toxin was added at the time of cell plating or 1 hr later, when cells had already attached (not shown). Interestingly, growth cones in pertussis toxin-treated cultures bore filopodia and appeared morphologically similar to growth cones in untreated cultures.

These observations are quantified in Figure 4K, in which neurite-bearing neurons were placed into categories based on whether their longest neurite remained on its square of origin (category I), or extended beyond that square (categories II-IV; see figure legend). In untreated cultures, 90% of neurons were in category I, whereas in pertussis toxin-treated cultures, only 30% of neurons were in category I. Clearly, pertussis toxin interferes with the ability of individual neurites to be guided by laminin boundaries.

**Pertussis toxin does not alter laminin's ability to promote neurite outgrowth**

In the above experiment, >95% of neurons with cell bodies that attached to regions of inactivated laminin between the laminin squares had no discernible neurites (not shown). This result, combined with the observation that pertussis toxin-treated neurites sometimes appeared to travel preferentially from one laminin square to another

**Figure 4. Effect of pertussis toxin on the guidance of neurites from dissociated cells.** **(A)** Diagram of the repeated unit of the photomask used in the experiments shown in panels B-K. Shaded areas depict chrome coating on the quartz mask, and correspond to areas in which laminin is protected from irradiation. The pattern is designed to produce 40 x 40 μm squares of active laminin, spaced 25 μm apart. **(B-E)** Laminin-coated coverslips were patterned using the photomask depicted in panel A. Dissociated dorsal root ganglion neurons were plated at 5000 cells/coverslip and cultured for 16 hr in NGF-containing serum-free media. Fixed cultures were immunostained for laminin. Phase contrast micrographs of representative neurons are shown. From matched phase contrast and fluorescence photomicrographs, the boundaries of the laminin squares were determined and marked on the photographs with dotted white lines. Bar = 10 μm. Note that the actual dimensions of the laminin squares were somewhat smaller than the chrome squares on the photomask; as in Figures 1-2, this was presumably due to refraction and reflection of ultraviolet light during irradiation. **(F-J)** Phase contrast micrographs of representative neurons from cultures prepared as in panels B-E, except that pertussis toxin (0.8 μg/ml) was included in the culture medium at the time of plating. **(K)** Neurons cultured as in panels B-J were scored with respect to the confinement of their neurites to laminin squares. Data are shown from parallel cultures grown in the absence (stippled bars) or presence (filled bars) of pertussis toxin (0.8 μg/ml). Only solitary neurons with cell bodies on or partially on laminin squares were scored. Neurite-bearing neurons were placed into one of four categories depending on the behavior of their longest neurite, ($n \geq 100$ for each treatment). Category I: the neurite did not extend beyond the boundary of the laminin square (e.g. panel B). Category II: the neurite left the laminin square but did not contact any adjacent laminin square (e.g. panel I). Category III: the neurite reached one but not two adjacent squares (e.g. panel G). Category IV: the neurite extended at least two squares beyond its square of origin (e.g. panel J). The normal confinement of neurites to laminin squares is markedly disrupted by pertussis toxin ($P < 0.005$ by $\chi^2$ analysis).


A
etc...
40
B
C
D
E
F
G
H
I
J
K
100
60
20
% of cells
I
II
III
IV

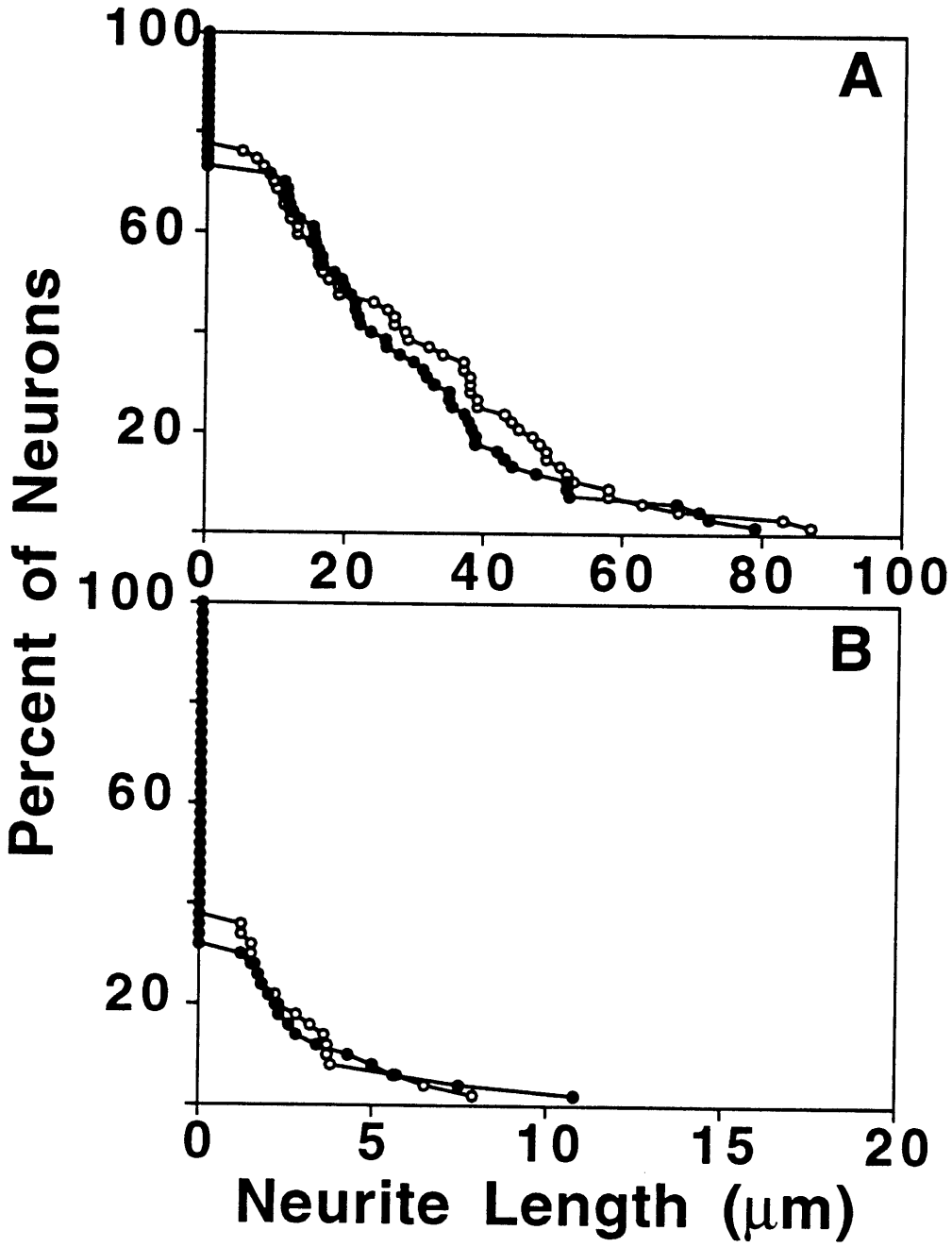
(e.g. Figure 4J), suggested that neurites growing in the presence of pertussis toxin do not lose the ability to recognize active laminin, nor do they gain an enhanced ability to extend on UV-inactivated laminin.

To address this issue more directly, dissociated neurons were cultured for 16 hr in the presence or absence of pertussis toxin on substrata consisting of either uniform (i.e., unpatterned) laminin, or uniformly UV-inactivated laminin. As shown in Figure 5, neither neurite initiation nor outgrowth on active laminin (panel A) or inactivated laminin (panel B; note different scale) was significantly affected by pertussis toxin. Further observations made by time-lapse microscopy confirmed that pertussis toxin does not significantly affect rates of neurite elongation: When either pertussis toxin (0.5 μg/ml) or vehicle alone was added to cultures of dissociated neurons plated on uniform, active laminin and rates of neurite elongation measured 3 to 5 hr later, the mean values obtained were not statistically different (111±25 [SD] vs. 100±27 μm/hr respectively; N = 15; P>0.2). Pertussis toxin did appear, however, to have a transient effect on outgrowth rate: Growth cones in explant cultures observed immediately after the addition of pertussis toxin (0.5 μg/ml) exhibited a small but statistically significant drop in growth rate (~25%) for about 1 hr (data not shown). Interestingly, time lapse observations failed to reveal any obvious effect of pertussis toxin on the extension and movements of growth cone filopodia (data not shown).

**Pertussis toxin affects neurite guidance by interacting with cellular carbohydrates, not by ADP-ribosylating G proteins**

Pertussis toxin is well known to inactivate certain heterotrimeric G proteins, a direct result of ADP-ribosylation catalyzed by the toxin's S1 subunit (Tamura et al., 1982). Some effects of pertussis toxin, however, do not involve ADP-ribosylation of G proteins and can be mimicked by a form of the toxin, the B oligomer, that lacks the S1 subunit (Tamura et al., 1983; Gray et al., 1989; for review see Kaslow and Burns, 1992). Apparently, pertussis toxin's effect on neurite guidance falls into this latter category: In assays of neurite growth on continuous and interrupted laminin stripes, the B oligomer of pertussis toxin was as effective as the holotoxin at causing neurite

**Figure 5. Lack of effect of pertussis toxin on neurite outgrowth on unpatterned substrata.** Laminin coverslips were prepared as described (Methods), and either not irradiated (**A**), or uniformly irradiated for 10 min by ultraviolet light shone through clear, 0.09 inch thick quartz (**B**). Dissociated neurons were prepared and plated as in figure 4, and cultured for 16 hr in the presence (●) or absence (○) of pertussis toxin (0.8 μg/ml). The length of the longest neurite was measured for ≥100 solitary neurons in each culture condition. Neurite lengths were taken to be the straight-line distance from the neurite's point of origin on the soma to the approximate centroid of the growth cone. For neurons without neurites, a value of zero was recorded. Neurite lengths were plotted as a running total histogram (cf. Neugebauer et al., 1988) and reveal no significant effect of pertussis toxin on either substratum (for mean neurite length, $p>0.5$ in both cases by Student's t-test).


100
60
20
A
0
20
40
60
80
100
100
60
20
B
0
5
10
15
20
Percent of Neurons
Neurite Length (μm)

wandering (Figure 6A).

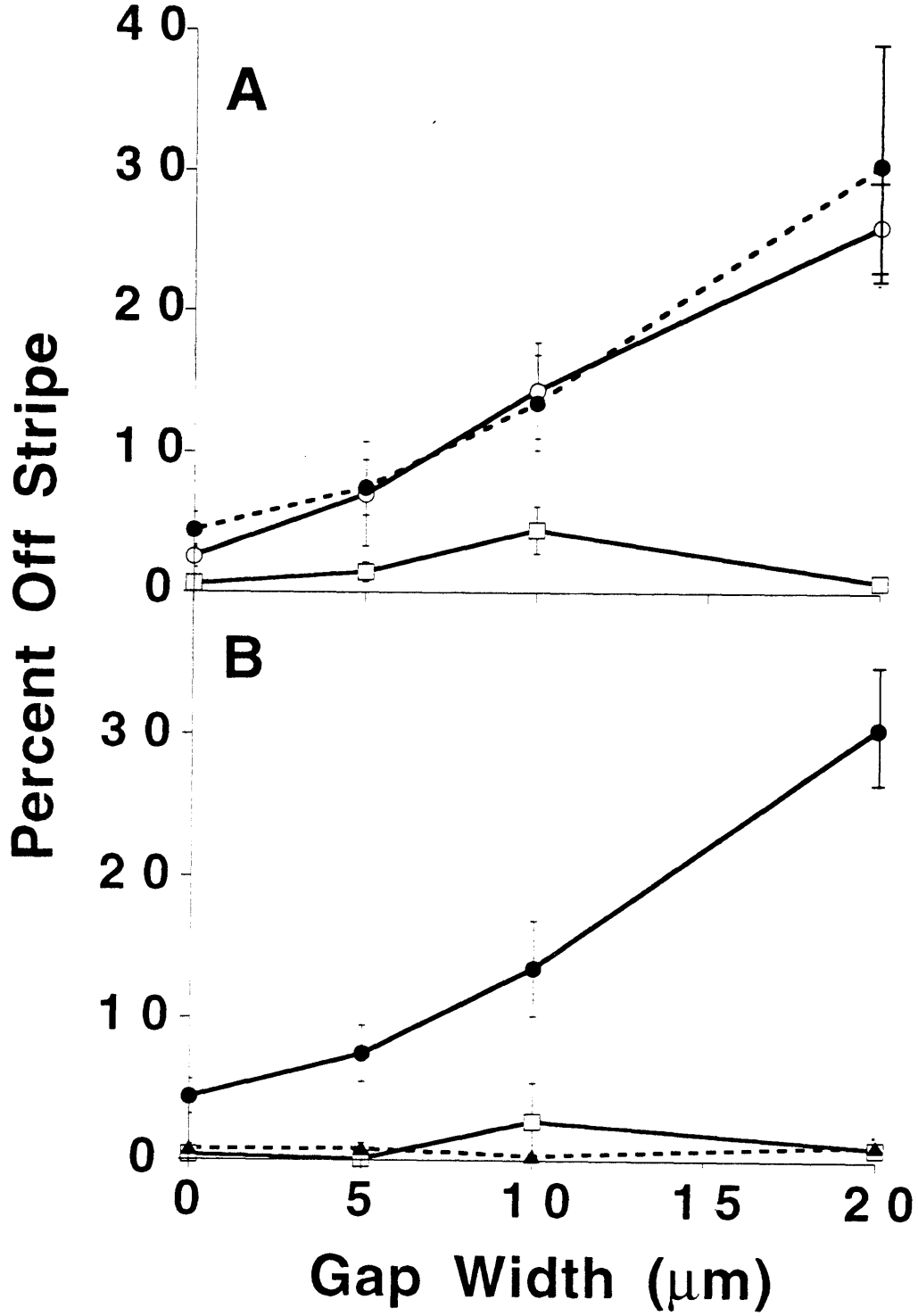
Two of the four polypeptides that comprise the B oligomer bind oligosaccharides, especially oligosaccharides with terminal sialic acid residues, and it is generally believed that biological activities of the B oligomer require binding via such carbohydrate structures to cell surface glycoproteins (Armstrong et al., 1988; Witvliet et al., 1989). However, pertussis toxin has also been shown to bind to laminin (Table 2 and Witvliet, et al., 1989). It was therefore necessary to determine whether the effects of pertussis toxin on neurite guidance result from binding of the toxin to cells, or binding of the toxin to laminin.

To address this question, dorsal root ganglia were plated onto patterned laminin substrata (as in Figure 1) and cultured in the presence of deoxymannojirimycin (DMN), an inhibitor of the Golgi enzyme mannosidase I. DMN arrests the processing of N-linked carbohydrates in the high mannose form, preventing the formation of complex-type chains--the type expected to bind the toxin--on newly synthesized protein (Elbein, 1987). Ganglia cultured in DMN extended neurites that appeared normal and were guided appropriately by the laminin pattern (not shown). However, when pertussis toxin (0.5 μg/ml) was added to DMN-treated cultures, neurites failed to wander from the laminin stripes, even though the same amount of the toxin caused extensive wandering of neurites from non-DMN-treated ganglia (Figure 6B).

These data indicate that interaction of pertussis toxin with cellular N-linked carbohydrates is necessary for pertussis toxin's effects on neurite guidance. However, they do not rule out the possibility that binding of pertussis toxin to laminin might *also* be necessary for these effects. For example, pertussis toxin molecules might need to bind both neurites and laminin, possibly even crosslinking them. For example, pertussis toxin is known to cause the agglutination of erythrocytes (Tamura et al., 1983).

Two approaches were taken to address this possibility. The first was to obtain laminin that was free of complex N-linked carbohydrates (with the expectation that it would not bind pertussis toxin) and determine whether the toxin inhibited neurite guidance on patterns of this modified laminin. The second approach was to pre-bind pertussis toxin to patterned laminin substrata, and determine whether the binding

**Figure 6. The effect of pertussis toxin on neurite guidance does not require direct inactivation of G proteins but does require the presence of cellular complex-type N-linked carbohydrates.** Dorsal root ganglion explants were prepared and cultured on patterned laminin substrata as in Figure 1, and wandering of neurite bundles from laminin stripes was quantified as in Figure 3C. **(A)** Data from cultures treated with 0.5 μg/ml pertussis toxin (--●--); an equimolar amount (0.66 μg/ml) of the B oligomer of pertussis toxin (—○—); or no drug (—□—). Drugs were applied after 20 hr and cultures were fixed 24 hr later. The data indicate that the B oligomer of pertussis toxin is equipotent at inducing neurite wandering. **(B)** Results from cultures treated with no drug (—□—); 0.5 μg/ml pertussis toxin (—●—); or 1 mM deoxymannojirimycin (DMN) and 0.5 μg/ml pertussis toxin (--▲--). Pertussis toxin was added after 20 hr in culture, whereas DMN was added at the time of plating. Cultures were fixed after a total of 44 hr. Cultures treated with DMN alone were indistinguishable from cultures treated with no drug (not shown). The data indicate that treatment of ganglia with DMN renders them insensitive to the effect of pertussis toxin on neurite guidance.

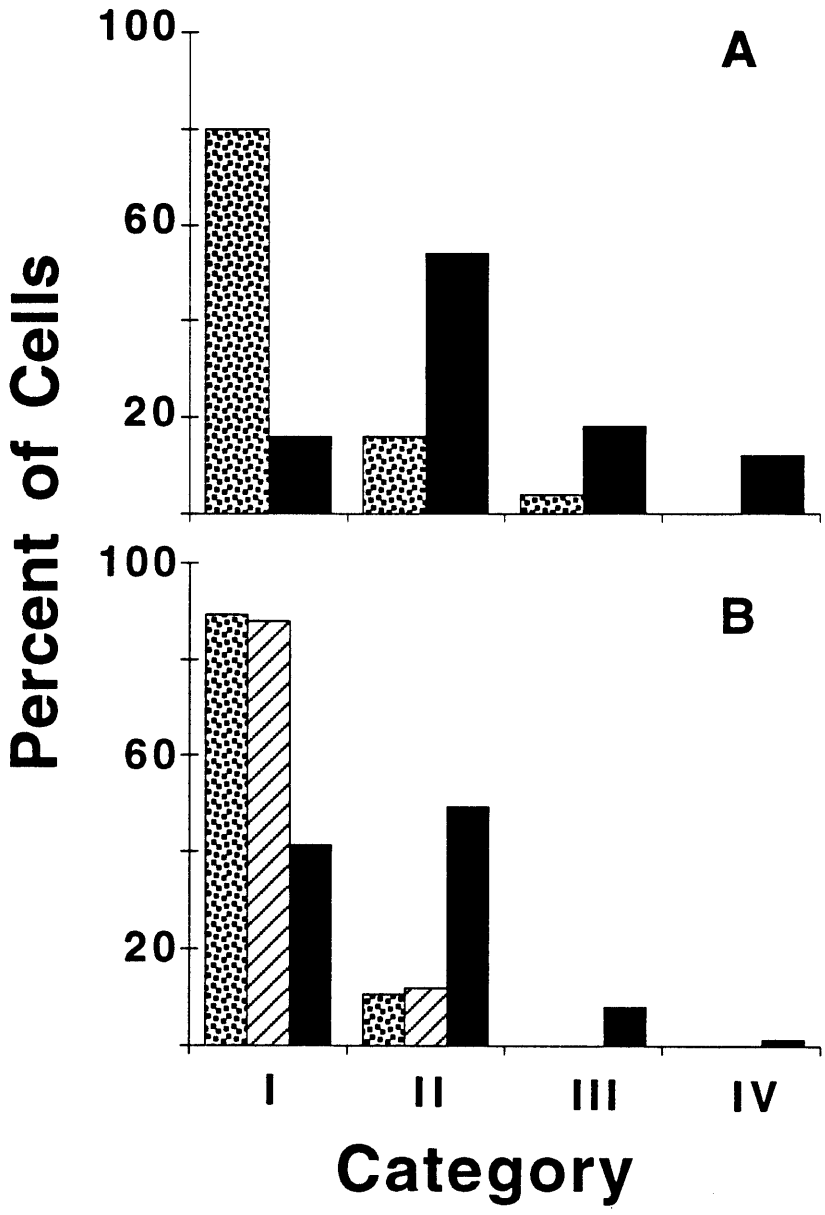

A
B
40
30
20
10
0
30
20
10
0
0
5
10
15
20
Percent Off Stripe
Gap Width (μm)

of pertussis toxin to laminin was sufficient to interfere with neurite guidance.

In the first set of experiments, laminin was isolated from M15 embryonal carcinoma cells (Yurchenco et al., 1992) chronically grown in DMN. This material, termed D-laminin, was passed over a wheat germ agglutinin column to remove any residual protein containing complex N-linked carbohydrates. The unbound material adsorbed to coverslips, promoted neurite outgrowth (not shown), could be patterned by ultraviolet light, and guided neurites (cf. Figure 7A stippled bars). Furthermore, pertussis toxin was able to induce neurite wandering on D-laminin (Figure 7A). Although this experiment suggests that binding to laminin might not be required for pertussis toxin's effects on neurite guidance, it turns out not to be definitive. As shown in table 2 (parts A-B), making laminin deficient in complex-type N-linked carbohydrates reduces the binding of pertussis toxin, but does not completely abolish it. It is noteworthy that more pertussis toxin binds inactivated laminin than active laminin. It is also interesting that the decrease in toxin binding to patterned D-laminin coverslips appears to be due solely to decreased binding to UV-inactivated regions: Experiment B indicates that pertussis toxin binds active D-laminin about as well as active laminin, but binds inactivated D-laminin much more weakly than inactivated laminin. One simple interpretation is that active laminin binds pertussis toxin partly through a UV-sensitive non-N-linked complex-type carbohydrate site(s).

In the second set of experiments, patterned laminin coverslips were pre-incubated for 5 hours in pertussis toxin (2.5 μg/ml) or medium alone, washed, and seeded with dissociated neurons. Pertussis toxin (0.5 μg/ml) was added with the cells to some coverslips that had been preincubated with medium alone. As shown in Figure 7B, neurites growing on pertussis-toxin pretreated laminin showed no evidence of wandering: they behaved like neurites growing on untreated laminin in the absence of pertussis toxin. The concentration of toxin for pretreatment was chosen so that the amount remaining bound to the substratum at the end of the experiment was at least as great as the amount bound at the end of the parallel experiment in which pertussis toxin was added only with the neurons. This was verified by measuring toxin binding to patterned laminin coverslips, as well as to active and

**Figure 7. Inhibition of neurite guidance by pertussis toxin is not blocked by rendering laminin deficient in complex N-linked carbohydrates.** Coverslips were coated with D-laminin or normally glycosylated laminin at 20 μg/ml for 3 hours at 37° C and patterned as in figure 4. Dissociated neurons were seeded (5000 cells/ coverslip) in the presence or absence of pertussis toxin and allowed to extend neurites. The wandering of neurites was scored as described in figure 4K, with ≥100 neurite-bearing neurons counted for each condition. **(A)** Neurite behavior on squares of patterned D-laminin. Stippled bars: no drug. Filled bars: pertussis toxin (0.5 μg/ml) at the time of plating. Cultures were fixed after 16 hours. The data are not noticeably different from those obtained with normally glycosylated laminin (cf. Figure 4K), i.e. most neurites were confined by D-laminin squares, and most wandered off the squares in the presence of pertussis toxin (by $\chi^2$ analysis, $P<0.005$ for pertussis toxin vs. no drug; $P>0.05$ for pertussis toxin and D-laminin versus pertusis toxin and normally glycosylated laminin [figure 4]). **(B)** Effect of pre-treatment of normally glycosylated laminin coverslips with pertussis toxin. Stippled bars: cultures plated in the presence of no drug. Striped bars: cultures plated in the presence of no drug, but onto coverslips that had been pre-treated with pertussis toxin. Filled bars: cultures plated in the presence of pertussis toxin (0.5 μg/ml), but onto coverslips that had not been pre-treated with the toxin. Pre-treatment was accomplished by incubating washed, patterned coverslips with 2.5 μg/ml pertussis toxin in CMF-HBSS for 6 hrs at 37°C, followed by 5 washes with CMF-HBSS just before use. Coverslips that were not pre-treated with the toxin were mock-treated with CMF-HBSS to control for the repeated washing. Cultures were fixed after 12 hours. The selection of 2.5 μg/ml as the toxin concentration used for pre-treatment was empirical: It was found that, after maintaining pre-treated coverslips for 12 hours in culture medium at 37°C, significant pre-bound toxin washed away (not shown), but that pre-treatment with 2.5 μg/ml was sufficient to ensure a level of bound toxin at the end of 12 hours that matched or exceeded the level of bound toxin that accumulated when 0.5 μg/ml was applied in culture medium for 12 hours at 37°C (cf. Table 2).


100
60
20
A
100
60
20
B
Percent of Cells
I
II
III
IV
Category

| | Laminin Substratum | | | |
|---|---|---|---|---|
| | patterned | | uniform | |
| **Experiment** | | | active | inactivated |
| **A.** Laminin + PTX | 7.9 | ±2.1* | | |
| D-laminin + PTX | 4.4 | ± 1.4 | | |
| **B.** Laminin + PTX | | | 31.6 ± 1.9 | 79.0 ±17.0* |
| D-laminin + PTX | | | 29.9 ± 0.9 | 12.1 ± 4.2 |
| **C.** PTX preapplied, washed, incubated | 34.1 | ± 2.3 | | |
| PTX present during incubation | 34.6 | ± 1.8 | | |
| **D.** PTX preapplied, washed, incubated | | | 32.3 ± 0.4 | 95.6 ± 5.2 |
| PTX present during incubation | | | 31.6 ± 1.9 | 79.0 ±17.0 |

**Table 2. Binding of Pertussis Toxin to Substratum-Bound Laminin.** Coverslips were treated with normally glycosylated laminin or D-laminin, and were either patterned (as in figure 4), left unirradiated ("active") or uniformly irradiated as in figure 5 ("inactivated"). In experiments A and B, coverslips were incubated in culture medium containing 0.5 μg/ml pertussis toxin (PTX) for 16 hours at 37°C (conditions identical to those used in Figure 7A). In experiments C and D, coverslips were either pre-treated with 2.5 μg/ml pertussis toxin, washed, and maintained in the absence of toxin for 12 hours at 37°C, or not pre-treated, washed, and maintained in the presence of 0.5 μg/ml pertussis toxin for 12 hours at 37°C (conditions identical to those used in Figure 7B). Toxin binding was measured as described in Methods. Values (mean of 3 coverslips ± SEM) are in arbitrary units that can only be compared within an experiment (A, B, C, or D). Every value significantly different ($p<0.05$) from the value directly below it is marked with an asterisk.

inactivated laminin separately (Table 2 parts C-D).

Together, the data in Figure 7 and Table 2 indicate that a reduction in the ability of laminin to bind pertussis toxin does not prevent the toxin from inhibiting neurite guidance; nor is pre-binding of pertussis toxin to laminin sufficient to disrupt guidance by that laminin. Along with the data in Figure 6, these results strongly imply that it is the interaction of pertussis toxin with neurons, and not with laminin, that inhibits neurite guidance.

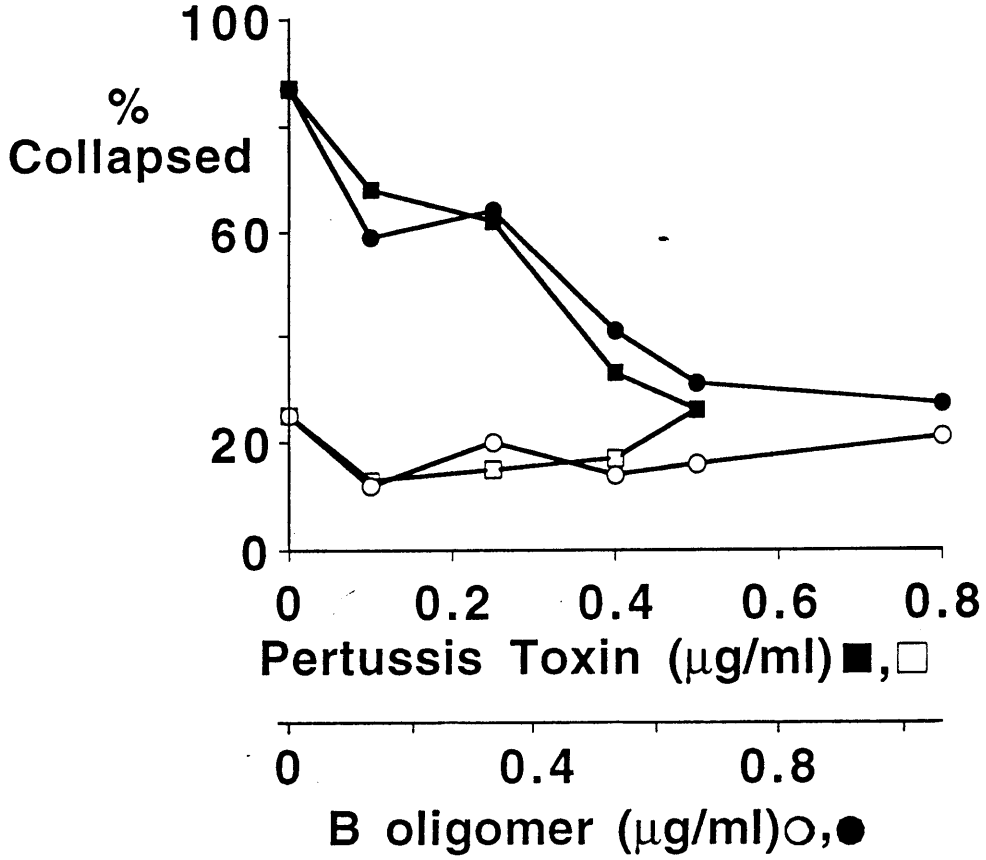
**Inhibition of growth cone collapse by pertussis toxin is not mediated by ADP-ribosylation of G proteins**

Molecules that, when added abruptly to neuronal cultures, cause the collapse of certain classes of growth cones have been isolated from a variety of tissues, and are thought to play inhibitory roles in axonal pathfinding (Keynes and Cook, 1992). It has been suggested that the mechanism of action of collapsing factors *in vivo* is to selectively destabilize parts of growth cones, thereby inhibiting their progression in a given direction (cf. Fan and Raper, 1995; Walter et al., 1990).

Recently, Igarashi et al. (1993) reported that pertussis toxin blocks the ability of a brain-derived collapsing factor to cause the collapse of sensory neuron growth cones. In view of the evidence presented here that the neurite guidance activity of laminin is disrupted by pertussis toxin, and the emerging view that collapsing factors are also neurite guidance molecules, it seemed possible that pertussis toxin might exert its effects on a physiological process that is common to both the mechanism of action of laminin and the mechanism of action of collapsing factors. Consistent with this possibility, it was found that the collapse of growth cones from chick dorsal root ganglia by a crude collapsing activity from embryonic chick brain membranes was not only blocked by pertussis toxin (as previously shown by Igarashi et al. [1993]), but--like neurite guidance by laminin--was blocked equally well by the B oligomer of pertussis toxin (Figure 8).

Thus, it appears that the binding of pertussis toxin to one or more cell surface glycoproteins is sufficient to block the responses of growth cones of two very different guidance cues.

**Figure 8. Pertussis toxin and its B oligomer block growth cone collapse.** Embryonic day 7 chick dorsal root ganglia cultured for 12 hr on laminin-coated 96 well plates were treated with various concentrations of pertussis toxin (squares) or its B oligomer (circles) for an additional 3 hr. Cultures were exposed for 30 min to a fraction from E10 chick brain (filled symbols) or basal F12 medium (open symbols), and fixed. Growth cones were then visually scored for a collapsed morphology. Greater than 70 growth cones were examined for each data point. Concentrations of pertussis toxin and B oligomer are plotted on separate axes so that equivalent molar concentrations of the two reagents occur at the same distance from the origin. The data indicate that pertussis toxin and its B oligomer are equipotent at blocking membrane-induced growth cone collapse.


100
%
Collapsed
60
20
0
0
0.2
0.4
0.6
0.8
Pertussis Toxin (μg/ml)■,□
0
0.4
0.8
B oligomer (μg/ml)○,●

## Discussion

With the goal of identifying molecules that specifically disrupt growth cone responses to axon guidance cues, *in vitro* assays were developed in which large numbers of dorsal root ganglion growth cones confronted discontinuous patterns of laminin. The patterns consisted of laminin stripes, some of which contained periodic small gaps in them (Figure 1), or widely spaced laminin squares (Figure 4). It was initially predicted that substances that specifically inhibited growth cone guidance would behave like cytochalasin B: they would impair the progression of neurites along gapped laminin stripes to a greater extent than on uninterrupted laminin stripes (Figure 2B).

Of the additional compounds and enzymes tested, only pertussis toxin preferentially affected neurites on interrupted stripes, but in an unexpected manner. Pertussis toxin caused neurites to wander from laminin stripes, particularly from those stripes with large gaps (Figure 3). The wandering caused by pertussis toxin was not the result of an effect on neurite-neurite interactions, because it could be observed with sparsely plated dissociated neurons (Figure 4). The effect was not due to a decrease in the tendency of neurites to grow straight, because it could be observed on laminin patterns designed to confine neurites to squares, rather than channel them into straight lanes (Figure 4). The effect did not depend upon the addition of pertussis toxin at the time of cell or ganglion plating (see Methods), nor was the effect limited to a short time after toxin addition, since neurites wandering across patterns of laminin squares often wandered across several squares without being "re-captured" by the pattern (cf. Fig 4J).

### Pertussis Toxin Uncouples Promotion of Neurite Outgrowth from Neurite Guidance.

Until recently, it was widely believed that the guiding effects, and possibly also the outgrowth-promoting effects of laminin, were mediated through increases in growth cone-substratum adhesion (e.g. Hammarback et al., 1988). This explanation, while plausible, has been largely discredited, not only for laminin but for other guidance molecules (see Ch. 1).

An alternative is that the guiding effects of laminin are simply a consequence of its potent neurite outgrowth-promoting activity. One could imagine that growth cones grow *preferentially* on laminin because they grow *faster* on laminin, and therefore spend more time on it. Indeed, dorsal root ganglion neurites do grow considerably faster on laminin than on UV-inactivated laminin (cf. Figure 5). Such a mechanism of neurite guidance can be seen as a special case of chemokinesis, the phenomenon in which motile cells are guided toward to a chemoattractant simply because their rate of random motility is increased by the chemoattractant (cf. Devreotes and Zigmond, 1988).

The observation that pertussis toxin interferes with neurite guidance by patterned laminin substrata provided an approach that could potentially distinguish between guidance mediated by chemotaxis and guidance mediated by chemokinesis. If pertussis toxin had narrowed the gap between the outgrowth-promoting abilities of active and inactivated laminin (i.e. by slowing down neurite outgrowth on active laminin, or speeding it up on inactivated laminin), then the data would have favored a model in which outgrowth-promoting activities were the determining factor in guiding neurites. Instead, pertussis toxin did not potentiate neurite outgrowth on inactivated LN and did not affect the ability of active LN to induce rapid outgrowth.

The data presented above argue strongly against the idea that laminin guides growth cones by chemokinesis, since rates of growth cone motility on active and inactive laminin were not affected by pertussis toxin, even at concentrations that dramatically impaired growth cone guidance. If growth cone guidance by laminin is similar in this regard to chemotaxis, then it raises the intriguing possibility that laminin and more conventional chemoattractants may share some of the same intracellular signaling pathways.

**How is Pertussis Toxin Working?**

When pertussis toxin disrupts neurite guidance by laminin, it does not do so by inactivating G proteins (cf. Figure 6A), at least not directly. Although the toxin happens to bind laminin (Witvliet, et al., 1989), observations that the neurites of ganglia cultured in DMN are insensitive

to the effects of pertussis toxin (Figure 6B), and that pre-binding of pertussis toxin to laminin substrata fails to perturb neurite guidance (Figure 7A), strongly suggest that the toxin inhibits neurite guidance by binding to neuronal glycoproteins via their N-linked complex-type carbohydrate chains.

Although effects of pertussis toxin on neurons that are mediated through the B oligomer have not previously been described, in other cell types the toxin typically recognizes only one or a few surface proteins (e.g. Brennan et al., 1988; Clark and Armstrong, 1990). Such a high degree of specificity may reflect the fact that the B-oligomer contains several widely separated sugar binding sites that bind sialylated oligosaccharides, but apparently exhibit different preferences for specific carbohydrate structures (Witvliet, et al., 1989, 1992; Saukkonen et al., 1992; Stein et al., 1994; for review see Kaslow and Burns, 1992). Perhaps high affinity binding requires multivalent interactions that only a small number of glycoproteins can achieve.

How might the binding of pertussis toxin to one or a few glycoproteins on neurons lead to a disruption in neurite guidance by laminin? One possibility is that pertussis toxin binds receptors for laminin, inhibiting their function. Several integrin receptors for laminin are expressed by dorsal root ganglion neurons (Tomaselli et al., 1993), although blockade of their function typically results in decreased or absent neurite outgrowth (Bozyczko and Horwitz, 1986; Hall et al., 1987). The fact that pertussis toxin disrupts neurite guidance by laminin without affecting neurite outgrowth (Figure 5) suggests that integrins might not be among the toxin's targets, and that other less well understood neuronal receptors for laminin (e.g. α-dystroglycan, proteoglycans) may be better candidates.

Alternatively, pertussis toxin or its B oligomer may itself elicit intracellular signals that disrupt biochemical steps that lie downstream of the interaction of laminin with its receptor(s), but that are important for guidance. Several studies document the ability of the B oligomer of pertussis toxin to trigger intracellular events: For example, in T lymphocytes, the B oligomer (or the holotoxin) can induce cell division (Strnad and Carchman, 1987) and mimic early signaling events in T cell activation (Rosoff et al., 1987; Strnad and Carchman, 1987). Evidence

to date suggests that the cell-surface pertussis toxin-binding proteins that mediate such effects are different in different cell types (see Kaslow and Burns, 1992). In some cases, pertussis toxin "receptors" are proteins that normally send biologically important signals (e.g. platelet glycoprotein Ib), and the binding of pertussis toxin to these proteins can cause their activation (Sindt et al., 1994). Thus, characterization of pertussis toxin's receptors on neuronal cells may lead to the identification of novel growth cone signaling molecules.

The observation that the B oligomer inhibits growth cone collapse (Figure 8) has several potentially important implications. Although receptors that mediate collapse are not known, there is little reason to think they are the same as laminin receptors. Moreover, if collapsing factors are indeed guidance molecules (cf. Fan and Raper, 1995) their effects are essentially opposite in direction to those of laminin: i.e., growth cones prefer to grow where collapsing factors aren't. The fact that the B oligomer of pertussis toxin blocks both the guidance effects of laminin and growth cone collapse raises the possibility of a signaling pathway that plays a central role in guidance and is modulated both by laminin and collapsing factors (presumably in opposite directions). Perhaps the B oligomer affects this signaling pathway so that it cannot be correctly modulated in either direction.

The idea of different classes of guidance molecules converging on a common signaling pathway is appealing, especially as it provides a means by which growth cones might integrate information coming from multiple, disparate guidance cues (something they clearly must do *in vivo*). Identification of the molecules that pertussis toxin binds on dorsal root ganglion neurons and determination of the intracellular signals, if any, that are elicited by binding of the B oligomer may together provide some of the tools needed to test this idea.

## References


Armstrong, G. D., Howard, L. A., and Peppler, M. S. (1988). Use of glycosyltransferases to restore pertussis toxin receptor activity to asialoagalactofetuin. J. Biol. Chem *263*, 8677-8684.

Bentley, D., and Toroian-Raymond, A. (1986). Disoriented pathfinding by pioneer neurone growth cones deprived of filopodia by cytochalasin treatment. Nature *323*, 712-715.

Bixby, J. L., and Jhabvala, P. (1992). Inhibition of tyrosine phosphorylation potentiates substrate-induced neurite growth. J. Neurobiol. *23*, 468-480.

Bottenstein, J. E., and Sato, G. H. (1979). Growth of a rat neuroblastoma cell line in serum-free supplemented medium. Proc. Natl. Acad. Sci. *76*, 514-517.

Bozyczko, D., and Horwitz, A. F. (1986). The participation of a putative cell surface receptor for laminin and fibronectin in peripheral neurite extension. J. Neurosci. *6*, 1241-1251.

Brennan, M. J., David, J. L., Kenimer, J. G., and Manclark, C. R. (1988). Lectin-like binding of pertussis toxin to a 165-kilodalton chinese hamster ovary cell glycoprotein. J. Biol. Chem. *263*, 4895-4899.

Buettner, H. M., and Pittman, R. N. (1991). Quantitative effects of laminin concentration on neurite outgrowth *in vitro*. Dev. Biol. *145*, 266-276.

Campenot, R. B., Walji, A. H., and Draker, D. D. (1991). Effects of sphingosine, staurosporine, and phorbol ester on neurites of rat sympathetic neurons growing in compartmented cultures. J. Neurosci. *11*, 1126-1139.

Carri, N. G., Perris, R., Johansson, S., and Ebendal, T. (1988). Differential outgrowth of retinal neurites on purified extracellular matrix molecules. J. Neurosci. Res. *19*, 428-439.

Chien, C.-B., Rosenthal, D. I., Harris, W. A., and Holt, C. E. (1993). Navigational errors made by growth cones without filopodia in the embryonic *Xenopus* brain. Neuron *11*, 237-251.

Chung, A. E., Freeman, I. L., and Braginski, J. E. (1977). A novel extracellular membrane elaborated by a mouse embryonal carcinoma-derived cell line. Biochem. Biophys. Res. Commun. *79*, 859-867.

Clark, C. G., and Armstrong, G. D. (1990). Lymphocyte receptors for pertussis toxin. Infect. Immun. *58*, 3840-3846.

Clark, P., Britland, S., and Connolly, P. (1993). Growth cone guidance and neuron morphology on micropatterned laminin surfaces. J. Cell Sci. *105*, 203-212.

Cohen, J., Burne, J. F., McKinlay, C., and Winter, J. (1987). The role of laminin and the laminin/fibronectin receptor complex in the outgrowth of retinal ganglion cell axons. Dev. Biol. *122*, 407-418.

Devreotes, P. N., and Zigmond, S. H. (1988). Chemotaxis in eukaryotic cells. Ann. Rev. Cell Biol. *4*, 649-686.

Doherty, P., and Walsh, F. S. (1994). Signal transduction events underlying neurite outgrowth stimulated by cell adhesion molecules. Curr. Opin. Neurobiol. *4*, 322-336.

Dow, K. E., Riopelle, R. J., and Kisilevsky, R. (1991). Domains of neuronal heparan sulphate proteoglycans involved in neurite growth on laminin. Cell Tissue Res. *265*, 345-351.
Elbein, A. D. (1987). Inhibitors of the biosynthesis and processing of N-linked oligosaccharide chains. Ann. Rev. Biochem. *56*, 497-534.
Fan, J., and Raper, J. A. (1995). Localized collapsing cues can steer growth cones without inducing their full collapse. Neuron *14*, 263-274.
Gray, L. S., Huber, K. S., Gray, M. C., Hewlett, E. L., and Engelhard, V. H. (1989). Pertussis toxin effects on T lymphocytes are mediated through CD3 and not by pertussis toxin catalyzed modification of a G protein. J. Immunol. *142*, 1631-1638.
Grumet, M. (1991). Cell adhesion molecules and their subgroups in the nervous system. Curr. Opin Neurobiol. *1*, 370-376.
Gundersen, R. W. (1987). Response of sensory neurites and growth cones to patterned substrata of laminin and fibronectin *in vitro*. Dev. Biol. *121*, 423-431.
Hall, D. E., Neugebauer, K. M., and Reichardt, L. F. (1987). Embryonic neural retina cell response to extracellular matrix proteins: developmental changes and effects of the cell substratum attachment antibody CSAT. J. Cell Biol. *104*, 623-634.
Hammarback, J. A., and Letourneau, P. C. (1986). Neurite extension across regions of low cell-substratum adhesivity: implications for the guidepost hypothesis of axonal pathfinding. Dev. Biol. *117*, 655-662.
Hammarback, J. A., McCarthy, J. B., Palm, S. L., Furcht, L. T., and Letourneau, P. C. (1988). Growth cone guidance by substrate-bound laminin pathways is correlated with neuron-to-pathway adhesivity. Dev. Biol. *126*, 29-39.
Hammarback, J. A., Palm, S. L., Furcht, L. T., and Letourneau, P. C. (1985). Guidance of neurite outgrowth by pathways of substratum-adsorbed laminin. J. Neurosci. Res. *13*, 213-220.
Igarashi, M., Strittmatter, S. M., Vartanian, T., and Fishman, M. C. (1993). Mediation by G proteins of signals that cause collapse of growth cones. Science *259*, 77-79.
Kaslow, H. R., and Burns, D. L. (1992). Pertussis toxin and target eukaryotic cells: binding, entry, and activation. FASEB J. *9*, 2684-2690.
Kennedy, T. E., Serafini, T., Torre, J. R. d. l., and Tessier-Lavigne, M. (1994). Netrins are diffusible chemotropic factors for commissural axons in the embryonic spinal cord. Cell *78*, 425-435.
Keynes, R. J., and Cook, G. M. W. (1992). Repellent cues in axon guidance. Curr. Opin. Neurobiol. *2*, 55-59.
Kleinman, H. K., McGarvey, M. L., Liotta, L. A., Robey, P. G., Tryggvason, K., and Martin, G. R. (1982). Isolation and characterization of type IV procollagen, laminin, and heparan sulfate proteoglycan from the EHS sarcoma. Biochemistry *21*, 6188-6193.
Lander, A. D. (1987). Molecules that make axons grow. Mol. Neurobiol. *1*, 213-245.

Letourneau, P. C. (1992). Extracellular matrix and neurite outgrowth. Curr. Opin. Genet. Dev. *2*, 625-34.
Letourneau, P. C., Condic, M. L., and Snow, D. M. (1994). Interactions of developing neurons with the extracellular matrix. J. Neurosci. *14*, 915-928.
Luo, Y., Raible, D., and Raper, J. A. (1993). Collapsin: a protein in brain that induces the collapse and paralysis of neuronal growth cones. Cell *75*, 217-227.
Mahanthappa, N. K., and Patterson, P. H. (1992). Thy-1 involvement in neurite outgrowth: perturbation by antibodies, phospholipase C, and mutation. Dev. Biol. *150*, 47-59.
Mann, D. A., Doherty, P., and Walsh, F. S. (1989). Increased intracellular cyclic AMP differentially modulates nerve growth factor induction of three neuronal recognition molecules involved in neurite outgrowth. J. Neurochem. *53*, 1581-1588.
Marsh, L., and Letourneau, P. C. (1984). Growth of neurites without filopodial or lamellipodial activity in the presence of cytochalasin B. J. Cell Biol. *99*, 2041-2047.
Monard, D. (1988). Cell-derived proteases and protease inhibitors as regulators of neurite outgrowth. Trends Neurosci. *11*, 541-544.
Neugebauer, K. M., Tomaselli, K. J., Lilien, J., and Reichardt, L. F. (1988). N-cadherin, NCAM, and integrins promote retinal neurite outgrowth on astrocytes *in vitro*. J. Cell Biol. *107*, 1177-1187.
O'Leary, D. D., Heffner, C. D., Kutka, L., Lopez-Mascaraque, L., Missias, A., and Reinoso, B. S. (1991). A target-derived chemoattractant controls the development of the corticopontine projection by a novel mechanism of axon targeting. Development Suppl. *2*, 123-130.
Oohira, A., Matsui, F., and Katoh-Semba, R. (1991). Inhibitory effects of brain chondroitin sulfate proteoglycans on neurite outgrowth from PC12D cells. J. Neurosci. *11*, 822-827.
Pittman, R. N., Ivins, J. K., and Buettner, H. M. (1989). Neuronal plasminogen activators: cell surface binding sites and involvement in neurite outgrowth. J. Neurosci. *9*, 4269-4289.
Raper, J. A., and Kapfhammer, J. P. (1990). The enrichment of a neuronal growth cone collapsing activity from embryonic chick brain. Neuron *4*, 21-29.
Riggott, M. J., and Moody, S. A. (1987). Distribution of laminin and fibronectin along peripheral trigeminal axon pathways in the developing chick. J. Comp. Neurol. *258*, 580-598.
Rosoff, P. M., Walker, R., and Winberry, L. (1987). Pertussis toxin triggers rapid second messenger production in human T lymphocytes. J. Immunol. *139*, 2419-2423.
Saukkonen, K., Burnette, W. N., Mar, V. L., Masure, H. R., and Tuomanen, E. I. (1992). Pertussis toxin has eukaryotic-like carbohydrate recognition domains. Proc. Natl. Acad. Sci. USA *89*, 118-122.
Schaffner, W., and Weissman, C. (1973). A rapid, sensitive and specific method for the determination of protein in dilute solution. Anal. Biochem. *56*, 502-514.

Schuch, U., Lohse, M. J., and Schachner, M. (1989). Neural cell adhesion molecules influence second messenger systems. Neuron *3*, 13-20.

Sindt, K. A., Hewlett, E. L., Redpath, G. T., Rappuoli, R., Gray, L. S., and Vandenberg, S. R. (1994). Pertussis toxin activates platelets through an interaction with platelet glycoprotein Ib. Infect. Immun. *62*, 3108-3114.

Stein, P. E., Boodhoo, A., Armstrong, G. D., Heerze, L. D., Cockle, S. A., Klein, M. H., and Read, R. J. (1994). Structure of a pertussis toxin-sugar complex as a model for receptor binding. Nature Struct. Biol. *1*, 591-596.

Strnad, C. F., and Carchman, R. A. (1987). Human T lymphocyte mitogenesis in response to the B oligomer of pertussis toxin is associated with an early elevation in cytosolic calcium concentrations. FEBS Letters *225*, 16-20.

Tamura, M., Nogimori, K., Murai, S., Yajima, M., Ito, K., Katada, T., Ui, M., and Ishii, S. (1982). Subunit structure of islet-activating protein, pertussis toxin, in conformity with the A-B model. Biochem. *21*, 5516-5522.

Tamura, M., Nogimori, K., Yajima, M., Ase, K., and Ui, M. (1983). A role of the B-oligomer moiety of islet-activating protein, pertussis toxin, in development of the biological effects on intact cells. J. Biol. Chem. *258*, 6756-6761.

Tang, J., Rutishauser, U., and Landmesser, L. (1994). Polysialic acid regulates growth cone behavior during sorting of motor axons in the plexus region. Neuron *13*, 405-414.

Tessier-Lavigne, M. (1992). Axon guidance by molecular gradients. Curr. Opin. Neurobiol. *2*, 60-65.

Timpl, R., Rohde, H., Risteli, L., Ott, U., Robey, P. G., and Martin, G. R. (1982). Laminin. Meth. Enzymol. *82*, 831-338.

Tomaselli, K. J., Doherty, P., Emmett, C. J., Damsky, C. H., Walsh, F. S., and Reichardt, L. F. (1993). Expression of β1 integrins in sensory neurons of the dorsal root ganglion and their functions in neurite outgrowth on two laminin isoforms. J. Neurosci. *13*, 4880-4888.

Walter, J., Appsopp, T. E., and Bonhoeffer, F. (1990). A common denominator of growth cone guidance and collapse? Trends Neurosci. *13*, 447-452.

Weeks, B. S., Papadopoulos, V., Dym, M., and Kleinman, H. K. (1991). cAMP promotes branching of laminin-induced neuronal processes. J. Cell Physiol. *147*, 62-67.

Witvliet, M. H., Burns, D. L., Brennan, M. J., Poolman, J. T., and Manclark, C. R. (1989). Binding of pertussis toxin to eukaryotic cells and glycoproteins. Infect. Immun. *57*, 3324-3330.

Witvliet, M. H., Vogel, M. L., Wiertz, E. J. H. J., and Poolman, J. T. (1992). Interaction of pertussis toxin with human T lymphocytes. Infect. Immun. *60*, 5085-5090.

Wu, D. Y., and Goldberg, D. J. (1993). Regulated tyrosine phosphorylation at the tips of growth cone filopodia. J. Cell Biol. *123*, 653-664.

Yurchenco, P. D., Cheng, Y.-S., and Colognato, H. (1992). Laminin forms an independent network in basement membranes. J. Cell Biol. *117*, 1119-1133.

# Chapter 4

# Further Characterization

# of Pertussis Toxin's Effects on Neurons

## Introduction

Despite increased identification of and growing appreciation for molecules that guide axons, little is known about the mechanisms in the growth cone that mediate the response to these molecules. While many signaling pathways have been invoked to explain neurite extension (see Doherty and Walsh, 1994), it is not clear which, if any, of these are important for the guidance of growth cones. Studies are hampered by the fact that most molecules that are believed to provide directional information, i.e., guide neurites, also enhance (or inhibit) non-directed motility, i.e., neurite extension.

The study of the mode of action of pertussis toxin presents an opportunity to specifically approach how growth cones choose their path. Pertussis toxin has been shown to affect neurite guidance and not neurite elongation: it interferes with the ability of patterns of laminin to guide, and the ability of a putative inhibitory guidance factor(s) to effect growth cone collapse (Kindt and Lander, 1995). Yet, pertussis toxin has no effect on the ability of laminin to promote neurite elongation. Pertussis toxin was found to alter neurite behavior not through direct G protein inactivation but by binding cellular N-linked carbohydrates. Since the absence of these carbohydrates does not interfere with neurite guidance (Kindt and Lander, 1995), it is possible that pertussis toxin-binding glycoproteins themselves are involved in growth cone guidance or are capable of transmitting signals that interfere with normal guidance. A series of experiments were undertaken to identify proteins in DRG neurons with which pertussis toxin interacts. In these experiments, plant lectins were used both to define the specificity of pertussis toxin's interaction with cellular carbohydrates and to identify biochemical reagents useful for the purification of the pertussis toxin "receptor."

Also, it is worth considering whether pertussis toxin is more than just a pharmacological tool, but instead mimics an *in vivo* lectin-like activity involved in axonal guidance. With this possibility in mind, several carbohydrate-binding proteins of biological significance were tested in the guidance assay in which the activity of pertussis toxin was discovered.

Finally, the repertoire of pertussis toxin's carbohydrate-mediated effects on neurons was expanded by one. Neurites treated with thrombin normally undergo growth cone collapse and withdrawal (Hawkins and Seeds, 1986; Jalink and Moolenaar, 1992). This collapse was found to be blocked by the B oligomer of pertussis toxin. In contrast to the brain membrane-derived collapsins used in early experiments, thrombin's effects on neurons are mediated by a known cell surface receptor and downstream signaling events have been identified. With this new finding, these are now candidate signaling events for pertussis toxin-mediated disruption of neurite guidance.

**Acknowledgments**

The Fc chimeras were generously donated by Paul Crocker (ICRF Labs, University. of Oxford, England), Marie Filbin (Hunter College, NY, NY) and Ajit Varki (UCSD, San Diego, CA). I thank Hidde Ploegh (MIT) and Phil Rosoff (Tufts University School of Medicine, Boston, MA) for their gifts of pertussis toxin, and Jon Ivins for the brain membrane preparation.

**Materials and Methods**

*Materials*

Chrome-on-quartz photomasks (thickness, 0.09 inch) were fabricated by Advance Reproductions Co. (North Andover, MA). Laminin was purified from the mouse Engelbreth-Holm Swarm sarcoma (Kleinman et al., 1982; Timpl et al., 1982). Rabbit anti-mouse laminin serum was purchased from Polysciences, Inc. (Warrington, PA). Pertussis toxin was obtained from List Phamaceuticals (Campbell, CA) and donated by Hidde Ploegh and Phil Rosoff. CHAPS (3-((3-cholamidopropyl)-dimethyammonio)-1-propanesulfonate) and the B oligomer of pertussis toxin were from Calbiochem. Lectins from *Maackia amurensis* (MAL) and *Sambucus nigra* (SNA) were obtained from Vector Laboratories (Burlingame, CA). Human thrombin (from serum: 31.8 NIH units/mg) was obtained from Enzyme Research Laboratories, Inc. (South Bend, IN). The mouse Fc-human CD22β protein was provided by Ajit Varki (UCSD), the human Fc-murine MAG protein by Marie Filbin (Hunter College), and the human Fc-murine sialoadhesin and human Fc-human CD33 by Paul Crocker (ICRF Labs, U. of Oxford, England).

*Cell Isolation and Culture*

Isolation and culture of chick dorsal root ganglia and dissociated chick DRG neurons were performed essentially as described (Kindt and Lander, 1995, and chapter 3 Methods). Ganglia or dissociated neurons were cultured in serum-free media on uniformly-coated or patterned coverslips (see Chapter 3) as indicated in the figure legends, and fixed at the times indicated with 4% formaldehyde/5% sucrose. For detection of neurites, CMFDA Cell Tracker dye (Molecular Probes) was added (10 μM final) to ganglion explant cultures 30 min before fixation.

*Quantification of Neurite Outgrowth*

Patterned laminin was visualized by immunostaining with polyclonal anti-laminin antibodies as previously described (Kindt and Lander, 1995). Analysis of neurite wandering in whole ganglion

cultures plated onto patterned laminin was as follows: coverslips containing ganglion explants were placed on a glass slide in phosphate buffered saline and images of both neurite bundles and laminin patterns were collected using a confocal microscope. The pattern consisted of sets of 4 stripes, all of the same gap width and spaced 60 μm from one another, grouped together and separated from other groups by 120 μm. The percentage of neurite mass "on" and "off" stripes was determined for each set of four similar stripes using the confocal microscope to measure total, background-subtracted fluorescence in the fluorescein channel (which detects the cytoplasmic dye CMFDA) in the 4 rectangular areas representing the stripes themselves ("on"), and in the 5 rectangular areas constituting the lanes between the stripes ("off"). These areas were identified by reference to images of laminin immunostaining.

For analysis of dissociated neuron cultures plated on patterned laminin, cells were examined using fluorescence and phase contrast optics simultaneously, and neurites scored for wandering as follows: Solitary neurite-bearing neurons with cell bodies on or partially on laminin squares were placed into one of four categories depending on the behavior of their longest neurite ($n \geq 60$ for each treatment). Category I: the neurite did not extend beyond the boundary of the laminin square. Category II: the neurite left the laminin square but did not contact any adjacent laminin square. Category III: the neurite reached one but not two adjacent squares. Category IV: the neurite extended at least two squares beyond its square of origin.

For analysis of neurite lengths on unpatterned substrata, phase contrast images of neurites viewed on a Zeiss Axiovert microscope were collected using a neuvicon video camera (Hamamatsu Photonics) connected to a series 151 Image Processor (Imaging Technologies, Woburn, MA). Neurite lengths were measured as the straight-line distance from the edge of the soma to the tipe of the neurite.

*Assay of Growth Cone Collapse*

For assays using brain derived growth cone collapsing factor, a crude fraction from E10 chick brain membranes was prepared as described (Raper and Kapfhammer, 1990, and see Chapter 3) and the

assay was performed essentially as described (Kindt and Lander, 1995).

For thrombin-induced collapse assays, ganglia cultured in serum free F12 medium in laminin-coated 96 well plates were treated with B oligomer or media after 12 hours; 3 hours later thrombin or media was added. Cultures were fixed approximately 3 or 8 minutes after the plate was returned to the incubator.

*Lectin and Pertussis Toxin Affinity Chromatography*

All protein concentrations were determined by amido black binding (Schaffner and Weissman, 1973).

*Columns*

For the pertussis toxin affinity column, pertussis toxin was dialyzed into 20 mM HEPES (pH 7.2). Pertussis toxin (1.4 ml) at 190 µg/ml (concentration after dialysis) was coupled to ~800 µl packed volumne Affigel 10 beads (BioRad) at 4° according to the manufacturer's instructions. 90% of pertussis toxin protein coupled to the column according to measurement of protein in the washes after the conjugation (2 x 800 µl 20 mM HEPES followed by 2 x 50 mM ethylenediamine), yielding 300 µg pertussis toxin/ml column. MAL- and SNA-conjugated agarose (2-3 mg lectin/ml gel according to the manufacturer) were obtained from E-Y Laboratories, Inc. (San Mateo, CA).

*Preparation of DRG Neuron Lysates*

Purified DRG neurons were grown 12 hours on laminin-coated 35 mm tissue culture dishes (about 2 x $10^5$ cells/dish) in serum free F12 media prepared as described (see chapter 3 methods) except with only 20 µg/ml BSA. To homogenize the cells, the media was gently removed, the dishes washed with warm F12 basal medium, and 120 µl ice cold lysate buffer (50 mM Tris pH 7.5, 0.15 M NaCl, 2 mM EDTA, 1% Triton X-100) with freshly added protease inhibitor mix (1 µg/ml pepstatin, 1 mM PMSF and 0.25 mg/ml N-ethyl maleimide) was added. Triton X-100 was chosen as a solubilizing detergent because it had been used in a previous study of pertussis toxin-binding proteins

(Clark and Armstrong, 1990). The cells were incubated in lysis buffer for 10 minutes on ice and removed by scraping. This material was further homogenized by repeated pipetting with a yellow tip and spun 10 minutes at 10,000 g to remove insoluble material. The supernatant was frozen and stored at -80° C. The yield from 1 35 mm dish was 30-50 µg (~0.3 µg/ganglia). Material from several preparations was pooled for the next step.

*WGA chromotography*

DRG neuron extract (133 µg) was applied at 4° C to 400 µl packed volume of wheat germ agglutinin-Sepharose (WGA-Sephaorse) equilibrated in 50 mM Tris pH 7.5, 0.15 M NaCl, 2 mM EDTA, 0.5% Triton X-100. The material was repeatedly passed over the column to enhance binding. The column was washed with TBS (pH 7.5) with 0.5% CHAPS, and eluted with 5 x 250 µl 0.2 M N-acetylglucosamine (GlcNAc) and 0.3% CHAPS in diluted iodination buffer (a 1:3 dilution of 0.25 M phosphate buffer pH 7.5). The eluate fractions were pooled and concentrated to 1/3 original volume. The yield from the WGA column was 11 µg (8.5 % of input protein).

The pooled, concentrated eluate was then iodinated by the Iodogen method (Pierce). It was immediately passed over a Quik-Sep 2 ml desalting column (Pierce) previously blocked with 10 mg/ml crystalline BSA (ICN Biochemicals) and equilibrated in TBS with 2 mM EDTA and 0.5% CHAPS. Note that the desalting column removes the GlcNAc from the eluate. The specific activity of the iodinated WGA eluate was estimated at 2 X $10^9$ cpm/µg protein. This material was radioprotected by addition of BSA to 1 mg/ml and stored at -80° C until use.

*Pertussis Toxin and Lectin Chromatography*

The procedure was essentially the same for the pertussis toxin, MAL, and SNA affinity columns. About 1.4 X $10^8$ cpm (90 µl) iodinated WGA eluate in a total volume of 250 µl TBS-CHAPS (50 mM TBS (pH 7.5 at 4°), 2 mM EDTA and 0.5% CHAPS) was combined with 400 µl packed volume lectin column and incubated with agitation for 5 hours at 4° C. The material was transferred to a column bed and

washed 8 X with 400 µl TBS-CHAPS with 1M NaCl, 1 minute incubation per wash, followed by 1 X 400 µl TBS-CHAPS. The column material was then mixed with 4 X 250 µl 100 mM sialyllactose (N-acetylneuramin-lactose from bovine colostrum: 66% 2-3 isomer and 28% 2-6 isomer according to the manufacturer. or 100 mM lactose (SNA column) and incubated with agitation 1 hour, 16 hours (overnight), 2 hours and 2 hours for the four elution steps respectively. Protease inhibitors (see above) were included in all incubations. The columns were then washed with 400 µl TBS-CHAPS, and (for the pertussis toxin column only) followed by 100 µl 50 mM ethanolamine in 0.15 M NaCl, pH 11.5, followed by TBS-CHAPS. The percent of counts eluted with saccharide from the pertussis toxin, MAL and SNA columns were 0.006, 0.0045 and 0.0064% respectively.

The flow through (250 µl: 6.5 x $10^7$ cpm) from the SNA column was passed over pertussis toxin-Affigel 10 using the same protocol as for the iodinated WGA eluate. 0.002% of the counts eluted with the saccharide.

To detect proteins in the saccharide-eluted fractions, 40 µl of the fractions were electrophoresed on 5-15% gradient SDS gels. Gels were exposed on Hyperfilm-MP autoradiography film (Amersham) and scanned to an image file with a flatbed scanner. Light/dark levels were adjusted using Adobe Photoshop 2.5 computer software.

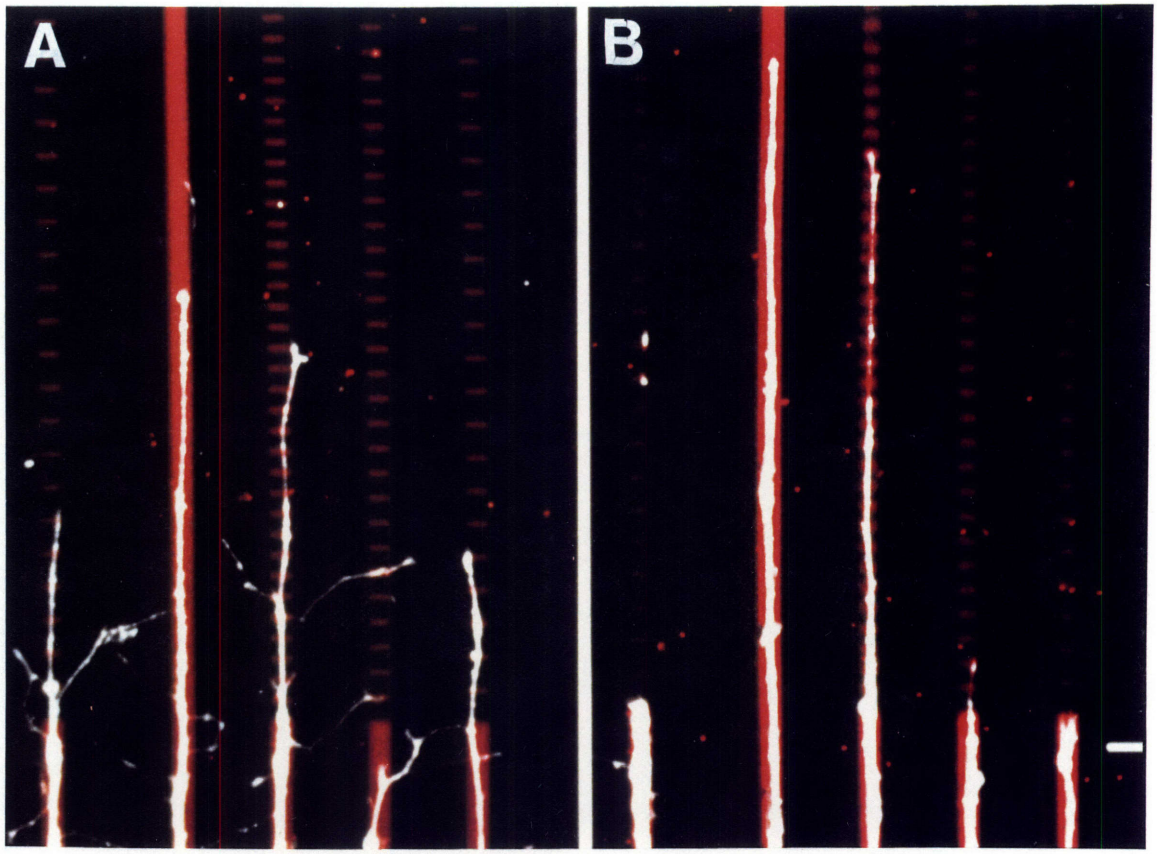
## Results

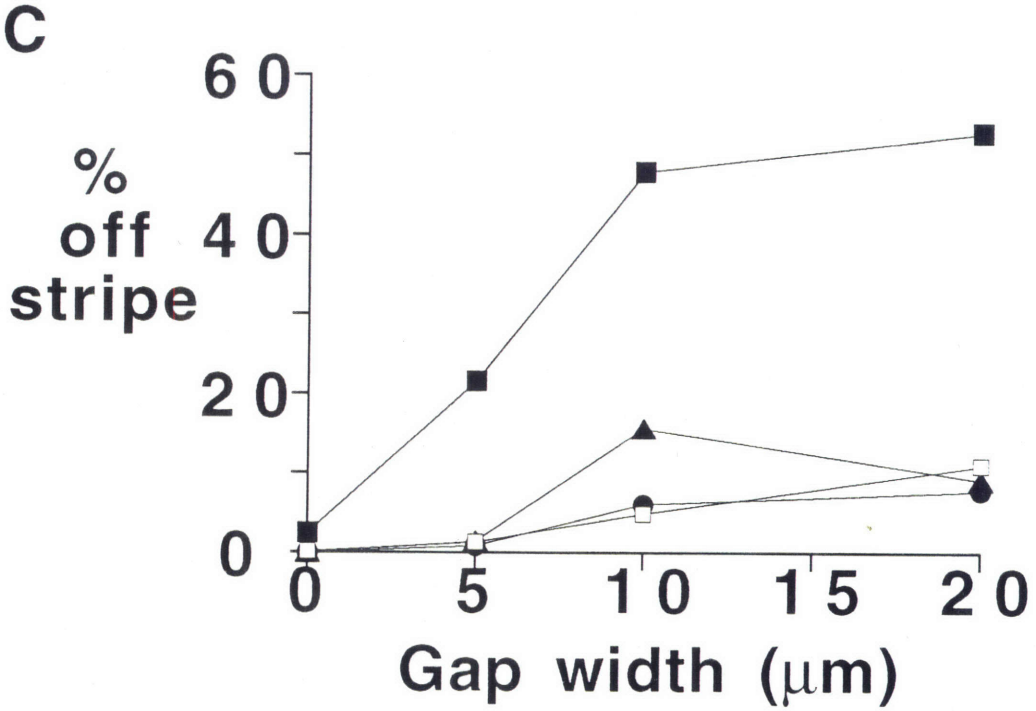
### A plant lectin causes neurite wandering

Pertussis toxin binds to sialic acid-containing glycoproteins, with high affinity for branched sialyllactose containing sequences (Armstrong et al., 1988; Witvliet et al., 1989). To test if other sialic acid-binding lectins could also cause neurite misguidance, neurites extending on patterned laminin were treated with lectins from *Maackia amurensis* (MAL) and *Sambucus nigra* (SNA). MAL binds with high affinity to carbohydrates containing the sequence Siaα (2->3)-lactose (sialic acid α (2->3)Galβ1-4Glc) while SNA lectin prefers sequences with Siaα (2->6)-galactose or -galactosamine (sialic acid α (2->6)Gal or sialic acid α (2->6)GalNAc) (Knibbs et al., 1991; Shibuya et al., 1987). In the presence of SNA lectin at 1 μg/ml, neurons extended neurites of normal length that were guided appropriately by the laminin pattern (Figure 1B). Neither higher concentrations (5 μg/ml) nor SNA obtained from other manufacturers (Sigma and E-Y Labs) had any other effect in the stripe assay (data not shown). In contrast, in the presence of MAL (0.4 μg/ml) neurites left the laminin stripes and were found on the UV-inactivated laminin between the stripes (Figure 1A). At concentrations $\geq$0.8 μg/ml, the lectin had apparent toxic effects (not shown). That this neurite wandering varied with gap width in the stripe was confirmed by quantifying the percent of neurite mass on and off the laminin stripe area (Figure 1C). Thus, one of two sialic acid-binding plant lectins can alter neurite behavior.

Vertebrate glycoproteins have also been identified as sialic acid-binding proteins (for reviews see Powell and Varki, 1995; Varki, 1992b). Like plant lectins, their specificity for sialic acid linkage and other carbohydrate residues differs. For some of these proteins, chimeric molecules have been constructed consisting of the carbohydrate binding domain attached to the "arms" of the Fc domain of an antibody molecule creating a dimeric molecule that can be used to specifically test the carbohydrate binding activities of these proteins (Kelm et al., 1994; Powell et al., 1993). Four Fc chimeras were obtained and tested in the neurite guidance assay.

**Figure 1. Effect of plant lectins on neurite outgrowth on patterned laminin.** Explants were cultured on the laminin pattern shown in chapter 2 Figure 1. Lectins were added to some cultures after 22 hr. After 44 hr, cultures were fixed, immunostained for laminin, and viewed by confocal microscopy. Active (unirradiated) laminin has been pseudocolored red and CMFDA-stained neurites white. **(A)** Neurites treated with MAL (0.4 μg/ml). **(B)** Neurites treated with SNA (1.0 μg/ml). Cultures with no lectin added were indistinguishable from cultures treated with SNA (not shown). Bar= 50 μm. **(C)** From patterned coverslips in which stripes of like gap width were grouped together, amounts of neurite mass on and off each type of stripe were measured and averaged (see Methods). Each data point represents the mean of values obtained from at least 3 groups of 4 stripes. Data are shown for cultures treated with MAL at 0.4 μg/ml (—■—) and at 0.25 μg/ml (—▲—), SNA at 1.0 μg/ml (—●—), or no treatment (- - O - -).


A
B


C
60
%
off
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40
20
0
0
5
10
15
20
Gap width (μm)

| I-type lectin | Known expression | Minimum binding sequence | Conc. tested (μg/ml) | References |
|---|---|---|---|---|
| CD22β | B cells | Siaα2-6->Gal β1->4 GlcNAc | 1-10 | (Powell and Varki, 1994) |
| myelin-associated glycoprotein (MAG) | peripheral nerve | Siaα2-3->Gal β1-> 3 GalNAc | 0.4-1.6 * | (Kelm et al., 1994) |
| sialoadhesin | macro-phages | Siaα2-3->Gal β1->4GlcNAc ->3GalNAc | 5<br>2-5 * | (Kelm et al., 1994) |
| CD33 | myeloid cells | Siaα2-3->Gal β1->4 GlcNAc | 5<br>2-5 * | (Freeman et al., 1995) |

**Table 1: Carbohydrate binding specificities of Fc chimeras tested in the neurite guidance assay.** Fc chimeras were added at the indicated concentrations to chick DRG cultured on patterned laminin (see figure 1 legend). The different concentrations tested reflected variably limiting amounts of protein available. In some cases (indicated by an asterisk) the chimeras were preincubated with an antibody to the Fc domain to create complexes of higher valency (Kelm et al., 1994): briefly, chimeras at 11x of the final concentration tested were incubated with rabbit anti-human IgG specific for the Fc domain at a ratio of 1:10 chimera:anti-Fc antibody for 1 hour at room temperature. Neurite wandering was analyzed as described in Methods. The anti-Fc antibody alone (<50 μg/ml) had no effect on neurite behavior.

This table was partially adapted from Powell and Varki, (1995).

The proteins, carbohydrate binding specificities and concentrations tested are shown in Table 1. No effect on neurite behavior was observed for any of the molecules at any of the concentrations tested (data not shown).

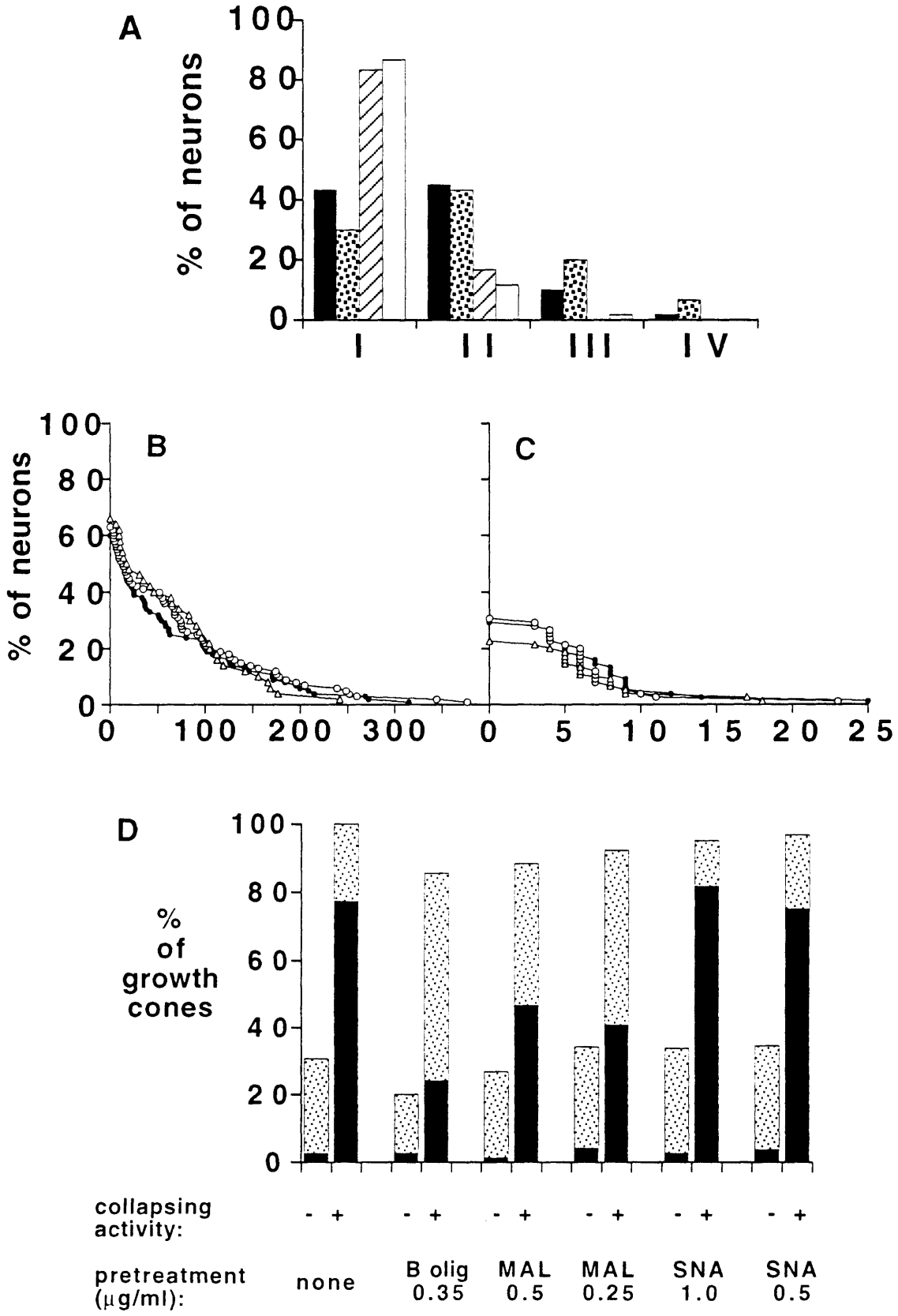
**MAL lectin affects neurite guidance and not neurite elongation.**

To determine if the effect of MAL in the stripe guidance assay was actually due to the disruption of individual neurites' ability to be guided by laminin, other bioassays used to define the activity of pertussis toxin were repeated with MAL and SNA. As shown in figure 2A, MAL, but not SNA, disrupted the confinement of single cell neurites from dissociated DRG neurons to laminin squares This effect could not be attributed to changes in the extent of neurite outgrowth. Neither lectin altered neurite growth of dissociated neurons on uniform laminin or UV-inactivated laminin (Figure 2B-C). These results indicate that the effects of MAL on neurite behavior result from the perturbation of guidance of individual neurites.

MAL was also assayed for inhibition of growth cone collapse induced by brain membranes, another effect of pertussis toxin and its B oligomer, Dorsal root ganglia pretreated with MAL or SNA lectin were exposed to a brain-derived collapsing factor. It was noticed that exposure to lectins and brain membranes together resulted in growth cones that were smaller, less spread, and darker as visualized by phase-contrast optics. For an accurate representation of growth cones in the different cultures, growth cones were binned into three categories: "full-sized", "small," and "collapsed." MAL (0.25 - 0.5 μg/ml) but not SNA (0.5 μg/ml - 1.0 μg/ml) significantly reduced growth cone collapse in the presence of a brain-derived collapsing factor (Figure 2D, black bars). More intact growth cones were in the "small" category for cultures treated with MAL (or B oligomer) and brain membranes than in untreated cultures. Lectin treatment alone did not increase the frequency of small growth cones (Figure 2D). In sum, MAL lectin blocks acute growth cone collapse, but growth cone morphology is altered relative to control cultures.

It is interesting that the effects of MAL in guidance and collapse

**Figure 2. MAL perturbs growth cone guidance, but not neurite outgrowth on uniform substrata. (A)** Effect of lectins on neurite behavior of dissociated cells on a laminin pattern. Laminin-coated coverslips were patterned using a photomask designed to produce 40 x 40 μm squares of active laminin, spaced 25 μm apart (see Ch. 3, Figure 4A). Dissociated dorsal root ganglion neurons were plated at 5000 cells/coverslip and cultured for 16 hr in NGF-containing serum-free media. Fixed cultures were immunostained for laminin and scored with respect to the confinement of their neurites to laminin squares (see Methods). Data are shown from parallel cultures grown in the presence of MAL (0.5 μg/ml: black bars), pertussis toxin (0.5 μg/ml: stippled bars), SNA1 (2.0 μg/ml: striped bars) or no treatment (open bars). **(B-C)** Neurite outgrowth on uniform substrata. Glass coverslips were coated with laminin (20 μg/ml) for 3 hours at 37° C, washed, dried, and either not irradiated **(B)**, or uniformly irradiated for 10 min by ultraviolet light shone through clear, 0.09 inch thick quartz **(C)**. Dissociated neurons were prepared and plated as in part A, and cultured for 16 hr in the presence of 0.5 μg/ml MAL1 lectin (●), SNA lectin (triangles) or no lectin (O). The length of the longest neurite was measured for ≥75 solitary neurons in each culture condition. Neurite lengths were plotted as a running total histogram (cf. Neugebauer et al., 1988) and reveal no significant effect of either lectin on either substratum (for mean neurite length, $p>0.5$ by Student's t-test). **(D)** Effect of lectins on brain membrane induced growth cone collapse. E7 chick dorsal root ganglia cultured for 12 hr on laminin-coated 96 well plates were pretreated with lectin for 3 hours. A fraction from E10 chick brain (+) or basal F12 medium (-) was then added and the cultures fixed after 30 min. Growth cones were visually scored and binned into 3 categories: large and spread, small and phase dark (stippled bars) or collapsed (black bars). ≥75 growth cones were scored for each treatment condition.


A
100
80
60
40
20
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% of neurons
I
II
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IV
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% of neurons
0
100
200
300
0
5
10
15
20
25
D
100
80
60
40
20
0
% of growth cones
collapsing activity:
- + - + - + - + - + - +
pretreatment (μg/ml):
none
B olig 0.35
MAL 0.5
MAL 0.25
SNA 1.0
SNA 0.5

assays, while significant, are not as pronounced as those of pertussis toxin and its B oligomer (see Discussion).

**Identification of pertussis toxin-binding proteins**

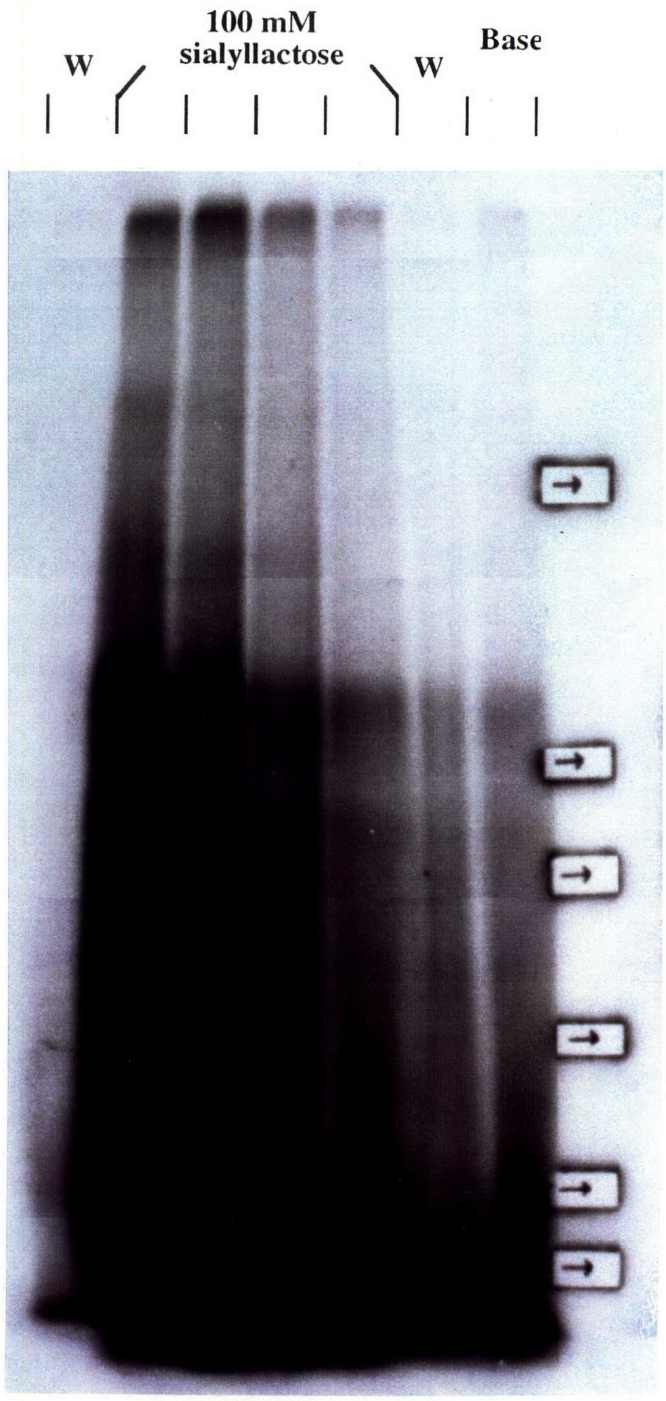
An important step in understanding the effects of pertussis toxin and MAL on growth cone guidance is to identify the cellular proteins to which they bind. Affinity chromatography was chosen as the means to identify such proteins. The overall scheme was to prepare neuronal cell extracts, enrich for glycoproteins using a wheat germ agglutinin column, and radioiodinate and test the WGA eluate for interaction with immobilized pertussis toxin, MAL, and SNA lectin.

Triton X-100 extracts of dissociated neurons were chosen as the starting material (instead of the more abundant whole dorsal root ganglia) in order to assay as pure a neuronal population as possible. Pooled cellular extracts from several preparations were bound to a WGA column and eluted with a competing sugar, N-acetylglucosamine (GlcNAc). The eluting fractions were pooled and radioiodinated to high specific activity. The radioiodinated material contained many proteins, as visualized by gel electrophoresis followed by autoradiography (data not shown). The material was passed over a pertussis toxin affinity column and the column eluted with sialyllactose (100 mM). The fractions were analyzed by gel electrophoresis followed by autoradiography.

The gel profile of the pertussis toxin column as visualized by non-reducing gel electrophoresis is shown in Figure 3, and estimated molecular weights of eluted proteins are shown in Table 2 column 1. At least 11 different bands specifically eluted from the column, an estimated 0.006% by counts of the starting material. Treatment with 50 mM ethanolamine (pH 11.5) did not elute any new bands (lane "B" and the following fractions, not shown). Thus, pertussis toxin can interact with a number of solubilized DRG neuronal proteins in a carbohydrate sensitive manner.

Since MAL but not SNA can mimic the biological activities of pertussis toxin, it was reasoned that a comparison between the pertussis toxin binding proteins and MAL- and SNA-binding proteins in the neuronal extracts might help identify which proteins actually

**Figure 3. Specific elution of DRG neuronal glycoproteins from immobilized pertussis toxin.** Iodinated WGA-binding DRG neuronal extracts were bound to pertussis toxin-Affigel 10 for 5 hours at 4°, washed, and eluted with 100 mM sialyllactose as described in Methods. 40 µl of the 250 µl fractions were analyzed by gradient gel electrophoresis followed by autoradiography. W=wash (tris buffered saline pH 7.5; 0.5% CHAPS, 1 mM EDTA); 100 mM sialyllactose= saccharide in wash buffer; Base= 50 mM ethanolamine in 0.15 M NaCl, pH 11.5. Arrows indicate molecular weight standards (kD): 216.8; 110.8; 71.5; 43.7; 27.8; 18.2.


W
100 mM
sialyllactose
W
Base

| MW range | PTX | | MAL | | SNA | |
|---|---|---|---|---|---|---|
| 245 | + | (245) | | | | |
| 182-190 | + | (190) | + | (182) | | |
| 167 | | | | | + | (167) |
| 133-140 | +++ | (smear 133-40) | + | (134) | ++ | (136) |
| 110-117 | ++ | (117) | | | ++ | (110) |
| 87 | ++ | (87) | ++ | (87) | ++ | (87) |
| 71-73 | + | (73) | | | ++ | (71) |
| 57-58 | ++ | (58) | ++ | (58) | ++ | (57) |
| 40-42 | ++ | (42) | +++ | (40) | ++ | (45) |
| 27-29 | +++ | (29) | +++ | (29) | +++ | (27) |
| 25 | +++ | (25) | +++ | (24) | +++ | (24) |

**Table 2: Apparent molecular weights of proteins eluted from lectin and pertussis toxin columns.** Iodinated, WGA binding proteins from chick DRG neuronal extracts were applied to pertussis toxin (PTX), MAL and SNA columns and eluted with saccharides as described in Materials and Methods. The eluted fractions were then run on a non-reducing 5-15% SDS gradient gel and iodinated proteins visualized by autoradiography. Apparent molecular weights were determined by motility relative to molecular weight standards in an adjacent lane for all distinguishable bands in all eluted fractions. Since fractions from different columns were analyzed on different gels, variable measurement of apparent molecule weight could occur for identical bands. So, identified bands were grouped into categories by molecular weight range. The molecular weight actually calculated from the gel is shown in parentheses for each band. The +'s indicate abundance of the band relative to other bands eluted from the same column.

mediate the biological effects of pertussis toxin on these cells. The iodinated WGA eluate was applied to immobilized MAL lectin and SNA lectin following the same protocol used for the pertussis toxin column, and bound protein eluted with 100 mM sialyllactose (MAL column) or 100 mM lactose (for the SNA column). The saccharides eluted a number of proteins from each column. Most of the counts eluted and all of the bands identified were found in the first two (of four) elution fractions, so these are shown for each gel in Figure 4. The major bands are shown in Table 2. The most abundant band eluting off of all the columns is at 24-25 kD.

It was reasoned that any proteins that did not attach to the SNA column but specifically bound to the pertussis toxin column could be a candidate for a protein mediating the specific effects of pertussis toxin. Therefore, the flow through from the SNA column was rechromatographed on the pertussis toxin column. The first two elution fractions from this column are shown in Figure 4 ("PTX*"). Several proteins bound specifically to the column; at least one (indicated by the middle asterix) may be absent from the eluted fractions of the SNA column though estimated molecular weights of adjacent proteins are very close (see Table 2).

**The B-oligomer of Pertussis Toxin Blocks Thrombin-Induced Neurite Retraction**

Thrombin is known to cause rapid growth cone collapse (Hawkins and Seeds, 1986; Jalink and Moolenaar, 1992) via a receptor-mediated mechanism. To determine if the B oligomer of pertussis toxin could block growth cone collapse induced by more than one type of collapsing factor, thrombin was added to explanted dorsal root ganglia, some of which had been pretreated with B oligomer. Thrombin (1 μg/ml) alone induced a rapid collapse and retraction of neurites. Typical appearance of the leading edge of neurites from the ganglia 8 minutes after thrombin treatment ranged from the field shown in Figure 5A, in which most of the growth cones are collapsed, to panel B, where the retraction is such that individual neurites are no longer visible. In contrast, in the presence of B oligomer (0.5 μg/ml), there was no evidence of neurite retraction, and

most of the growth cones were intact (figure 5C). These observations were quantified at two time points, showing that, as early as the ganglia were examined, B oligomer blocked thrombin-induced collapse (Figure 5D).

**Figure 4. Comparison of eluted fractions from pertussis toxin, MAL and SNA columns.** Iodinated WGA-binding DRG neuronal extracts were applied and eluted from pertussis toxin ("PTX"), MAL and SNA columns as described in Methods. "PTX*" indicates fraction from when the flow-through from the SNA column was re-applied to a pertussis toxin column (see Methods). The first two elution fractions of each column are shown in each case. Arrows indicate molecular weight standards (see Figure 3 legend). Variation in molecular weight standard position was <0.2 cm from gel to gel. Asterisks mark band locations for comparison.

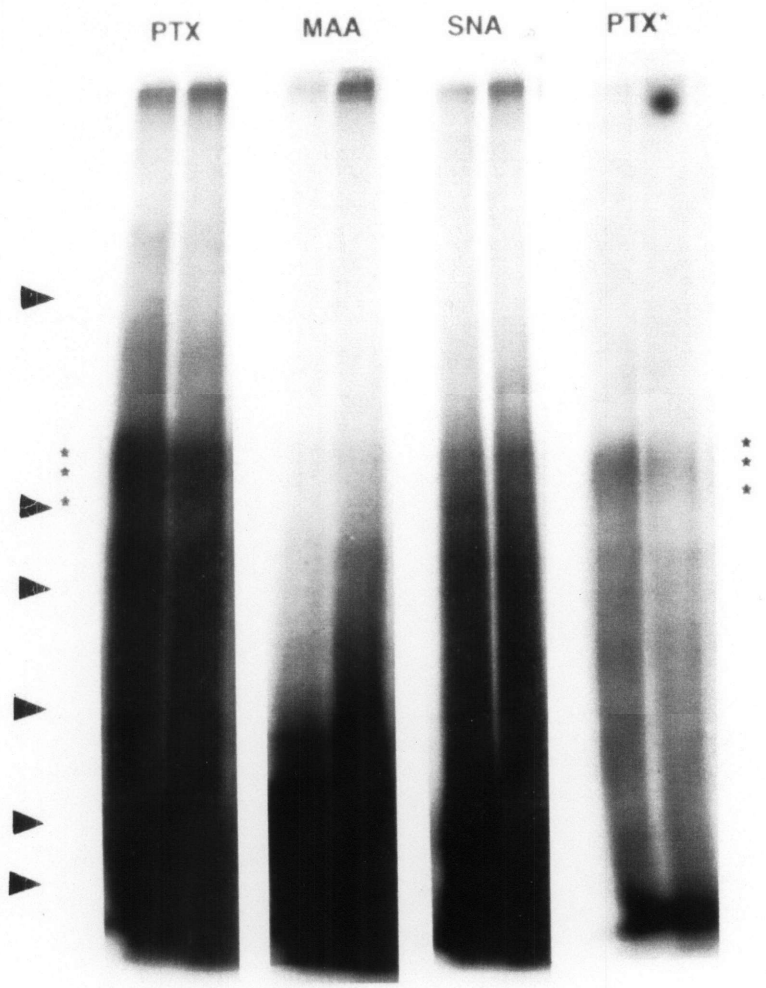
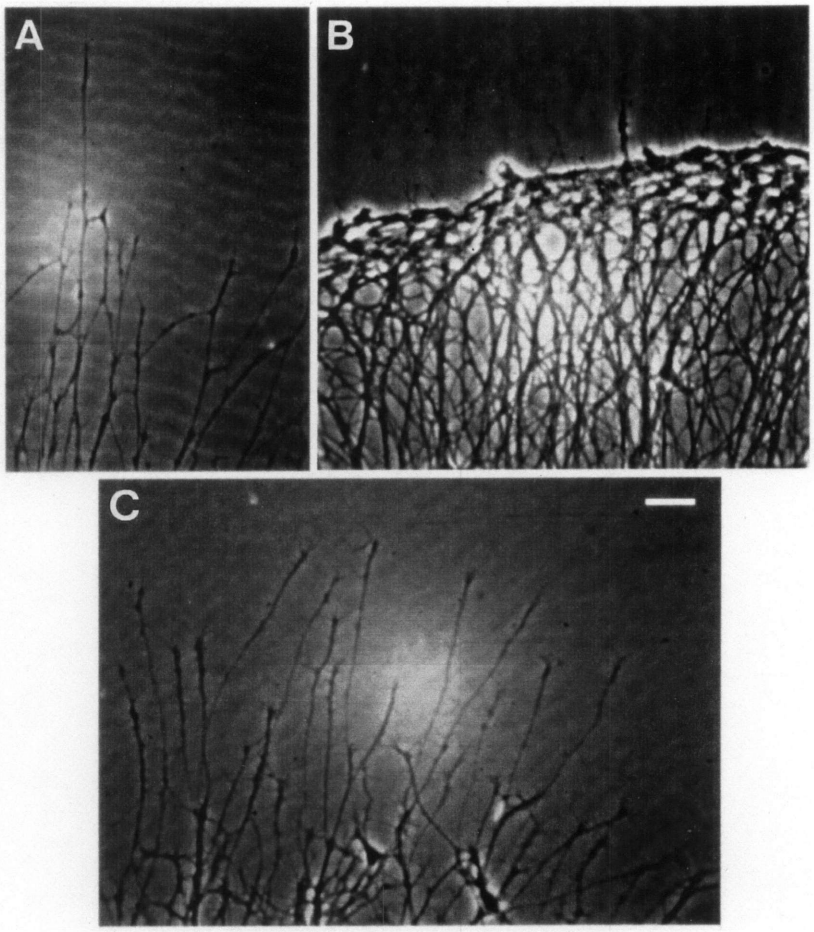
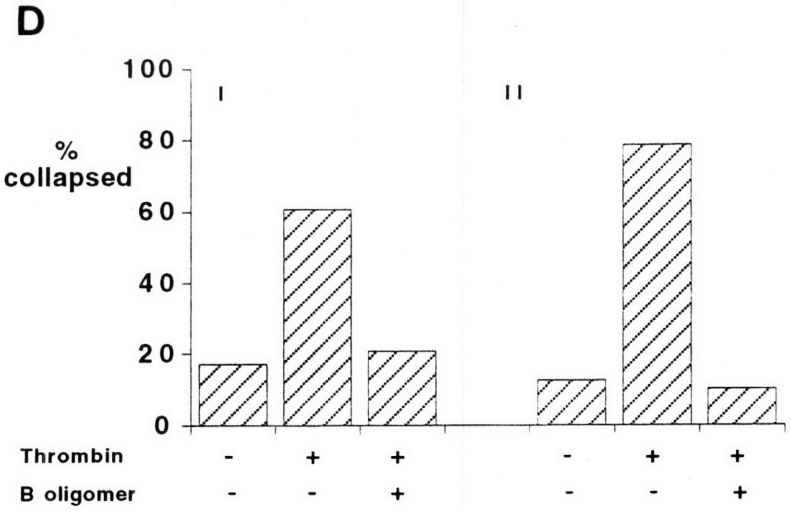

PTX
MAA
SNA
PTX*

**Figure 5. The B oligomer of pertussis toxin blocks thrombin-induced growth cone collapse.** 96-well tissue culture plastic was coated with laminin (20 μg/ml) for 3 hours at 37°C and washed. Cleaned E7 whole dorsal root ganglia were applied in serum-free F12 medium and allowed to extend neurites for 16 hours. Some cultures were treated with B oligomer (0.5 μg/ml) for the last three hours of this time. Thrombin (final concentration 1.0 μg/ml) was added to some wells, and cultures fixed 8 minutes after the plate was returned to the incubator. **(A-C)** Representative phase contrast images of neurites and fixed after 8 minutes from ganglia treated with thrombin alone **(A-B)** and thrombin and B oligomer **(C)**. **(D)** Quantification of growth cone collapse. Growth cones were visually scored as intact or collapsed (n=75 growth cones per treatment condition). Only solitary growth cones were counted (e.g. growth cones in fields such as panels A and C but not B). Part I: cultures fixed 3 minutes after thrombin treatment. Part II: cultures fixed 8 minutes after thrombin treatment.


A
B
C


D
100
80
60
40
20
0
% collapsed
I
II
Thrombin
B oligomer
- + + - + +
- - + - - +

## Discussion

### Functional Relationship of Pertussis Toxin to Other Lectins

In this study, experiments were carried out to identify the pertussis toxin binding proteins in DRG neurons. Many lines of evidence--structural studies, *in vitro* binding studies, and studies with cell lines defective in glycosylation--indicate that some if not all of the carbohydrate binding sites in the toxin recognize sialic acid-bearing carbohydrates (Saukkonen et al., 1992; Stein et al., 1994; Witvliet et al., 1989). Therefore, sialic acid binding lectins were tested for their effects on guidance. These experiments aimed both to confirm that the effects of pertussis toxin are due to a lectin-like activity and to identify reagents useful for the eventual purification of pertussis toxin binding proteins.

Lectin from *Maackia amurensis* (MAL) did mimic the effect of pertussis toxin in an *in vitro* guidance assay, causing neurites to deviate from paths that normally guided them (Figure 1). That this activity was due to an effect on individual neurites and not to any changes in the extent of neurite outgrowth was confirmed by experiments using dissociated cells (Figure 2A-C). Like pertussis toxin, MAL's effects were not limited to laminin-mediated guidance, as MAL blocked brain membrane-induced growth cone collapse (Figure 2D). Another sialic acid-binding lectin with a specificity complementary to that of MAL, SNA, had no effect in any of these bioassays. The observation that neurites on the continuous stripes in MAL-treated cultures were shorter than in SNA-treated cultures (Figure 1A vs. 1B) or untreated cultures (not shown) could be interpreted as the lectin perturbing neurite outgrowth as well as neurite guidance. Yet, MAL at concentrations $\leq$0.5 μg/ml does not block laminin-mediated neurite outgrowth, as demonstrated by direct measurements of neurite outgrowth in dissociated cell culture. It may be that in the longer time period of the whole ganglia assay (20 vs. 14 hours in the presence of the lectin) the toxic effect observed with higher concentration of the lectin is more noticeable at the concentrations that alter growth cone behavior.

These results would suggest that proteins bearing α(2-3) linked sialic acid residues are involved in pertussis toxin-mediated misguidance. It should be noted that laminin bears α2-3 linked sialic acid residues (Knibbs et al., 1989) and that MAL can bind to laminin (Knibbs et al., 1991). However, N-linked complex-type carbohydrates on laminin are not required for pertussis toxin's effect on neurons, as neurites cultured on patterns of laminin synthesized in the presence of DMN still responded to pertussis toxin (see Ch. 3 Figure 7).[1]

Other studies of pertussis toxin binding specificity have not demonstrated a preference of sialic acid linkage for the toxin. Several studies using proteins bearing only α2-6 linked sialic acids (Heerze and Armstrong, 1990) or glycoproteins reconstituted with α2-6 sialyltransferase (Armstrong et al., 1988) demonstrate that pertussis toxin can interact with α2-6 containing ligands in binding assays, but do not rule out that pertussis toxin also binds α2-3 linked sialic acid. Also, there are many variations of sialic acid structure (see Varki, 1992a) that have been implicated in the binding specificity of other sialic acid binding proteins such as CD22β; no studies have addressed these preferences for pertussis toxin.

A number of proteins with sialic-acid binding activity have been identified . The selectin family is characterized by calcium-dependent binding of primarily sialylated, fucosylated carbohydrate structures (Varki, 1992b). The I-type lectins, or sialoadhesins, have been named a family based on structural homology; they are members of the immunoglobulin superfamily, have transmembrane domains, and bind to sialic acid containing carbohydrates, as shown in Table 1 (Powell and Varki, 1995). CD22β, CD33 and sialoadhesin are believed to mediate specific cellular interactions in the immune system (Freeman et al., 1995; Stamenkovic and Seed, 1990; van-den-Berg et al., 1992). MAG, expressed in the nervous system, both enhances and inhibits

[1]The binding of pertussis toxin to DMN-treated laminin decreased by 50% relative to untreated laminin (Ch. 3, Table 2), indicating that there is also a protein- or high mannose carbohydrate- mediated component of the interaction of pertussis toxin and laminin. It would be interesting to test binding of MAL to DMN-treated laminin, as this component of binding might be absent. If MAL could then induce wandering on patterns made with DMN-treated laminin, it would show definitely that interactions with laminin are not necessary for misguidance.

neurite extension from embryonic and adult neurons, respectively (McKerracher et al., 1994; Mukhopadhyay et al., 1994). It is not known if any of MAG's effects on neurons are mediated by its carbohydrate binding activity. It is interesting that for both the I-type lectins and the selectins the carbohydrate binding specificity as defined biochemically is very broad, especially in cells types that are expected to be in contact with a large number of glycosylated proteins in milieus such as plasma. It is argued that the specificity and strength of the relevant biological interactions is generated by multiple interactions between the lectin and the carbohydrate residues (see Powell et al., 1995). Though none of these compounds turned out to have an effect on neurite behavior as assayed by the whole ganglia stripe assay,[2] it is possible that a related sialic-acid binding protein exists that can alter axonal behavior *in vivo* as pertussis toxin does *in vitro* (see chapter 5 for a different effect by a naturally occurring lectin).

**A Biochemical Approach**

Because pertussis toxin binding proteins differs among cell types, it seemed necessary to identify the pertussis toxin-binding proteins in DRG neurons. The purest population of cells that respond to pertussis toxin, dissociated DRG neurons, was used as starting material. The disadvantage of this approach was that the quantity of material was very small, but radioiodination aided detection of the proteins. As WGA strongly binds glycoproteins with GlcNAc (found in N-linked complex-type carbohydrates) as well as sialic acid residues (Peters et al., 1979), and can itself cause neurite wandering at certain concentrations (data not shown) it was expected to interact with any pertussis toxin-binding proteins. A WGA column was thus used as a prepurification step before application of material to the other columns.

The sialyllactose elute of the pertussis toxin column contained at least 11 distinct bands. It cannot be ruled out that some of these bands represent degradation products of others, or degradation

[2]Note that the lectins' activity was never confirmed in this lab by a separate assay.

products of laminin that might have contaminated the extract (intact laminin would not enter these gels under non-reducing conditions). Metabolic labelling with $^{35}S$ methonine would have eliminated the possibility of the latter source of bands. The most intense band was 24-25 kD. This band is probably not an integral membrane protein; when lectin column fractions were subjected to Triton X-114 partitioning (Bordier, 1981), this band was depleted in the detergent phase (data not shown).

The fact that pertussis toxin binds many proteins is inconvenient from the point of view of a biochemical purification of the physiologically active pertussis toxin receptor, but is not surprising. Affinity chromatography will identify proteins binding with a wide range of affinity. Not all of these interactions will occur in the concentration range in which pertussis toxin when applied to cells affects neurite guidance. In a study of pertussis toxin-binding proteins in a lymphoid cell line, at least six proteins from cell surface-iodinated detergent extracts bound to a pertussis toxin column. Yet pertussis toxin when coupled to a photoactivatable crosslinker and exposed to intact cells bound only one of these molecules, a 70 kD protein, later identified as a known protein, the LPS receptor (Armstrong et al., 1994; Clark and Armstrong, 1990). A 43-kD putative receptor was also identified via its crosslinking to pertussis toxin in similar experiments using a T cell line (Rogers et al., 1990). Crosslinking experiments were attempted in the DRG neuron system, but did not yield any cellular proteins (data not shown), perhaps because the number of cells used in the experiment was by necessity 100-fold less than the number used in the two other studies (Armstrong et al., 1994; Rogers et al., 1990).

It was hoped that comparison of the pertussis toxin- MAL- and SNA-binding proteins would identify proteins that might be relevant to the biological effects observed, specifically, proteins in the eluate of MAL and pertussis toxin columns but not the eluate of the SNA column. Distinct yet overlapping sets of bands were eluted using the same material applied to MAL and SNA columns.

It is interesting that SNA binds to many proteins in DRG neurons and yet when tested at the same concentrations as pertussis

toxin and MAL had no detectable effect on DRG neurons. In fact, the profiles of SNA- and pertussis toxin-binding proteins appear more alike than the profiles from the pertussis toxin and MAL columns. This may be due to the fact that the affinity of SNA for Siaα (2->6)-galactose is reported to be only 20-150 times greater than its affinity for Siaα (2->3)-galactose (Shibuya et al., 1987). It is possible that under the conditions of the affinity column, SNA interacts with Siaα(2->3)-containing glycoproteins but these interactions do not occur under the conditions of the biological assays. Passage of the flow-through from the SNA column over the pertussis toxin column yielded at least one band that may be absent or at low abundance in the SNA column eluates. It was not apparent from these gels if this band (~134 kD) was specifically eluted from the MAL column.

MAL eluates contained fewer proteins than the eluates from the other columns, and a large fraction of the eluted counts were associated with the common 25 kD band. The possibility that MAL binds even its preferred carbohydrate residues with low affinity is suggested by other studies in which immobilized MAL could retard, but not bind, Siaα(2->3)-containing glycopeptides and oligosaccharides (Wang and Cummings, 1988). Another study did observe lactose-sensitive binding of oligosaccharides to MAL-sepharose. It was suggested that the discrepancy between the studies was due a higher density of lectin on the column (>8 mg/ml) in the later study. In the present study (using MAL-agarose with 2-3 mg protein/ml column), the profile of counts washing off of the MAL column during the wash steps was not noticeably different from the profile of the SNA column run simultaneously with the same material (not shown). It would be worth examining if specifically eluted proteins are also found in high concentrations in the wash fractions, which would also indicate that other proteins that bound weakly were washed off before the specific elution steps. In any case, if MAL interactions with DRG neuronal glycoproteins were lower affinity than pertussis toxin's interactions with the same glycoprotein, this could explain why in some bioassays, the effects of MAL are not as pronounced as those of pertussis toxin (cf. Figures 2A and 2D).

The eventual goal of the biochemical experiments is to purify pertussis toxin-binding proteins in DRG neurons. It was hoped that MAL lectin, which mimics pertussis toxin's effects on neurons, could be used in the place of pertussis toxin for the biochemical purification, as it is cheaper and more plentiful. The results from these affinity chromatography experiments suggest that the best approach to finding the biologically active proteins might be to use these lectin columns in series.

**Insights on Growth Cone Guidance from Thrombin?**

The observation that the B oligomer of pertussis toxin blocks thrombin-induced growth cone collapse has important implications for how pertussis toxin acts on neurons. Thrombin has been shown to cause growth cone collapse, neurite retraction, and cell rounding by interacting with a cellular G-protein-coupled receptor. However, the observed effects on cell morphology are independent of G-protein mediated signaling pathways (Jalink and Moolenaar, 1992). Instead, thrombin (or a bioactive peptide of the thrombin receptor) appears to act via a member of the rho family of small GTP binding proteins, as pretreatment of neurons with the C3 exoenzyme from *C. botulinum*, which ADP-ribosylates and inactivates these proteins, blocks thrombin-induced neurite retraction (Jalink et al., 1994). Other studies using this inhibitor have demonstrated that the rho family can control cell shape by regulation of the actin cytoskeleton (Hall, 1992; Ridley and Hall, 1992) Indeed, C3 exoenzyme blocked thrombin-induced contraction of detached neurites (Jalink et al., 1994). Other signals that may act in the same pathway as rho in the neurite response to thrombin have been identified. For example, a broad based tyrosine kinase inhibitor, genistein, blocks thrombin-induced neurite retraction, and, $pp60^{src}$ kinase activity is reported to be upregulated after thrombin treatment (Jalink et al., 1993; Jalink and Moolenaar, 1992). The retraction is also blocked by a myosin light chain kinase inhibitor (Jalink et al., 1994).

As the B oligomer blocks thrombin-induced growth cone collapse, it could act by interfering with one of the above described events. If B oligomer blocks neurite guidance via the same

downstream intracellular actions as it blocks thrombin-induced neurite retraction, the pathway identified for the latter process may have significant overlap with intracellular events required for growth cone's response to extracellular guidance information. A logical next experiment would be to test if inactivation of rho by C3 exoenzyme interferes with growth cone guidance on laminin patterns.

## References


Armstrong, G. D., Clark, C. G., and Heerze, L. D. (1994). The 70-kildalton pertussis toxin-binding protein in Jurkat cells. Infect. Immun. *62*, 2236-2343.

Armstrong, G. D., Howard, L. A., and Peppler, M. S. (1988). Use of glycosyltransferases to restore pertussis toxin receptor activity to asialoagalactofetuin. J. Biol. Chem *263*, 8677-8684.

Bordier, C. (1981). Phase separation of integral membrane proteins in Triton X-114 solution. J. Biol. Chem. *256*, 1604-1607.

Clark, C. G., and Armstrong, G. D. (1990). Lymphocyte receptors for pertussis toxin. Infect. Immun. *58*, 3840-3846.

Doherty, P., and Walsh, F. S. (1994). Signal transduction events underlying neurite outgrowth stimulated by cell adhesion molecules. Curr. Opin. Neurobiol. *4*, 322-336.

Freeman, S. D., Kelm, S., Barber, E. K., and Crocker, P. R. (1995). Characterization of CD33 as a new member of the sialoadhesin family of cellular interaction molecules. Blood *85*, 2005-2012.

Hall, A. (1992). Ras-related GTPases and the cytoskeleton. Mol. Biol. Cell *3*, 475-479.

Hawkins, R. L., and Seeds, N. W. (1986). Effect of proteases and their inhibitors on neurite outgrowth from neonatal mouse sensory ganglia in culture. Brain Res. *398*, 63-70.

Heerze, L. D., and Armstrong, G. D. (1990). Comparison of the lectin-like activity of pertussis toxin with two plant lectins that have differential specificities for α(2-6) and α(2-3)-linked sialic acid. Biochem. Biophys. Res. Comm. *172*, 1224-1229.

Jalink, K., Corven, E. J. v., Hengeveld, T., Morii, N., Narumiya, S., and Moolenaar, W. H. (1994). Inhibition of lysophosphatidate- and thrombin-induced neurite retraction and neuronal cell rounding by ADP ribosylation of the small GTP-binding protein rho. J. Cell Biol. *126*, 801-810.

Jalink, K., Eichholtz, T., Stam, F. R. P., Croven, E. J. v., and Moolenaar, W. H. (1993). Lysophosphatidic acid induces neuronal shape changes via a novel, receptor-mediated pathway: similarity to thrombin action. Cell Growth Diff. *4*, 247-355.

Jalink, K., and Moolenaar, W. H. (1992). Thrombin receptor activation causes rapid neural cell rounding and neurite retraction independent of classic second messengers. J. Cell Biol. *118*, 411-419.

Kelm, S., Pelz, A., Schauer, R., Filbin, M. T., Tang, S., de-Bellard, M.-E., Schnaar, R. L., Mahoney, J. A., Hartnell, A., Bradfield, P., and Crocker, P. R. (1994). Sialoadhesin, myelin-associated glycoprotein and CD22 define a new family of sialic acid-dependent adhesion molecules of the immunoglobulin superfamily. Curr. Biol. *4*, 965-972.

Kindt, R. M., and Lander, A. D. (1995). Pertussis toxin specifically inhibits growth cone guidance by a mechanism independent of direct G protein inactivation. Neuron *15*, 79-88.

Kleinman, H. K., McGarvey, M. L., Liotta, L. A., Robey, P. G., Tryggvason, K., and Martin, G. R. (1982). Isolation and characterization of type IV procollagen, laminin, and heparan sulfate proteoglycan from the EHS sarcoma. Biochemistry *21*, 6188-6193.

Knibbs, R. N., Goldstein, I. J., Ratcliffe, R. M., and Shibuya, N. (1991). Characterization of the carbohydrate binding specificity of the leukoagglutinating lectin from *maackia amurensis*. J. Biol. Chem *266*, 83-88.

Knibbs, R. N., Perini, F., and Goldstein, I. J. (1989). Structure of the major concanavalin A reactive oligosaccharides of the extracellular matrix component laminin. Biochemistry *28*, 6379.

McKerracher, L., David, S., Jackson, D. L., Kottis, V., Dunn, R. J., and Braun, P. E. (1994). Identification of myelin-associated glycoprotein as a major myelin-derived inhibitor of neurite growth. Neuron *13*, 805-811.

Mukhopadhyay, G., Doherty, P., Walsh, F. S., Crocker, P. R., and Filbin, M. T. (1994). A novel role for myelin-associated glycoprotein as an inhibitor of axonal regeneration. Neuron *13*, 757-767.

Neugebauer, K. M., Tomaselli, K. J., Lilien, J., and Reichardt, L. F. (1988). N-cadherin, NCAM, and integrins promote retinal neurite outgrowth on astrocytes *in vitro*. J. Cell Biol. *107*, 1177-1187.

Peters, B. P., Ebisu, S., Goldstein, I. J., and Flasner, M. (1979). Interaction of wheat germ agglutinin with sialic acid. 18 5505-5511.

Powell, L. D., Jain, R. K., Matta, K. L., Sabesan, S., and Varki, A. (1995). Characterization of sialyloligosaccharide binding by recombinant soluble and native cell-associated CD22. Evidence for a minimal structural recognition motif and the potential importance of multisite binding. J. Biol. Chem. *270*, 7523-7532.

Powell, L. D., Sgroi, D., Sjoberg, E. R., Stamenkovic, I., and Varki, A. (1993). Natural ligands of the B cell adhesion molecule CD22β carry N-linked oligosaccharides with α-2-6-linked sialic acids that are required for recognition. J. Biol. Chem. *268*, 7019-7027.

Powell, L. D., and Varki, A. (1994). The oligosaccharide binding specificities of CD22β, a sialic acid-specific lectin of B cells. J. Biol. Chem. *269*, 10628-10636.

Powell, L. D., and Varki, A. (1995). I-type lectins. J. Biol. Chem. *270*, 14243-14246.

Raper, J. A., and Kapfhammer, J. P. (1990). The enrichment of a neuronal growth cone collapsing activity from embryonic chick brain. Neuron *4*, 21-29.

Ridley, A. J., and Hall, A. (1992). The small GTP-binding protein rho regulates the assembly of focal adhesion and actin stress fibers in response to growth factors. Cell *70*, 389-399.

Rogers, T. S., Corey, S., and Corey, P. M. (1990). Identification of a 43-kD human T lymphocyte membrane protein as a receptor for pertussis toxin. J. Immunol. *145*, 678-683.

Saukkonen, K., Burnette, W. N., Mar, V. L., Masure, H. R., and Tuomanen, E. I. (1992). Pertussis toxin has eukaryotic-like carbohydrate recognition domains. Proc. Natl. Acad. Sci. USA *89*, 118-122.

Schaffner, W., and Weissman, C. (1973). A rapid, sensitive and specific method for the determination of protein in dilute solution. Anal. Biochem. *56*, 502-514.

Shibuya, N., Goldstein, I. J., Broekaert, W. F., Nsimba-Lubaki, M., Peeters, B., and Peumans, W. J. (1987). The elderberry (*sambucus nigra* L.) bark lectin recognizes the Neu5Ac(α2-6)Gal/GalNAc sequence. J. Biol. Chem. *262*, 1596-1601.

Stamenkovic, I., and Seed, B. (1990). The B-cell antigen CD22 mediates monocyte and erythrocyte adhesion. Nature *345*, 74-77.

Stein, P. E., Boodhoo, A., Armstrong, G. D., Heerze, L. D., Cockle, S. A., Klein, M. H., and Read, R. J. (1994). Structure of a pertussis toxin-sugar complex as a model for receptor binding. Nature Struct. Biol. *1*, 591-596.

Timpl, R., Rohde, H., Risteli, L., Ott, U., Robey, P. G., and Martin, G. R. (1982). Laminin. Meth. Enzymol. *82*, 831-338.

van-den-Berg, T. K., Breve, J. J. P., Damoiseaux, J. G. M. C., Dopp, E. A., Kelm, S., Crocker, P. R., Dijkstra, C. D., and Kraal, G. (1992). Sialoadhesin on macrophages: its identification as a lymphocyte adhesion molecule. J. Exp. Med. *176*, 647-655.

Varki, A. (1992a). Diversity in the sialic acids. Glycobiol. *2*, 25-40.

Varki, A. (1992b). Selectins and other mammalian sialic acid-binding lectins. Curr. Opin. Cell Biol. *4*, 257-266.

Wang, W.-C., and Cummings, R. D. (1988). The immobilized leukoagglutinin from the seeds of *maackia amurensis* binds with high affinity to complex-type Asn-linked oligosaccharides containing terminal sialic acid-linked α-2-3 to penultimate galactose residues. J. Biol. Chem *263*, 4576-4585.

Witvliet, M. H., Burns, D. L., Brennan, M. J., Poolman, J. T., and Manclark, C. R. (1989). Binding of pertussis toxin to eukaryotic cells and glycoproteins. Infect. Immun. *57*, 3324-3330.

# Chapter 5

## Effects of Galectin-1, a Naturally Occurring Lectin, on Neurites from Dorsal Root Ganglion Neurons

## Introduction

Carbohydrate-binding proteins are found at all stages of vertebrate development (for review see Drickamer and Taylor, 1993). Their diversity and their spatially and temporally regulated expression are suggestive of functional importance, but these functions are only beginning to be elucidated.

The galectins, formerly known as S-lectins or S-lac lectins, are a family of soluble animal lectins defined by sequence similarity and affinity to B-galactoside sugars. The vertebrate members number at least eight (Barondes et al., 1994; Hadar et al., 1995), and relatives of this family are also found in invertebrates. All galectins of known structure bear two carbohydrate-binding domains, usually in a homodimer structure, and so, like lectins from other sources, they have the potential to crosslink ligands. The distributions and precise carbohydrate-binding specificities of members of the family are overlapping, yet distinct (Barondes, et al., 1994; Leffler and Barondes, 1986), suggesting related yet separate functions in a range of biological systems.

Galectin-1 (formerly known as L-14) is a good candidate for a role in axon extension or guidance in the nervous system. The 14 kDa secreted protein is expressed by sensory and motor neurons in rat (Hynes et al., 1990; Regan et al., 1986) and immunolocalizes to sensory neuronal fibers (Regan, et al., 1986). Galectin-1 is also expressed in other parts of the nervous system including certain brain stem nuclei and the olfactory nerve (Hynes, et al., 1990; Mahanthappa et al., 1994). Its function in the nervous system is not known but, provocatively, its expression in spinal cord coincides with the expression of β-galactoside-containing carbohydrates--potentially its own ligands (Dodd and Jessell, 1986). Outside the nervous system galectin-1 is found in a variety of tissue during development including all types of muscle (Poirer et al., 1992). Many of galectin-1's known functions are related to its association with the extracellular matrix molecule laminin. Galectin-1 has a high affinity for lactose- and lactosamine-containing carbohydrates (Leffler and Barondes, 1986) and is believed to interact with laminin via the latter's extensive array

of polyllactosamine chains (Zhou and Cummings, 1990). Galectin-1 has been shown to alter cellular response to laminin in interesting and sometimes opposing ways. For some cell types--rat olfactory neurons, melanoma cells, and CHO cells--the presence of galectin-1 enhances cellular adhesion to laminin (Brule et al., 1995; Mahanthappa, et al., 1994; Zhou and Cummings, 1993). Yet, addition of galectin-1 causes myoblast cells to detach from a laminin substratum (Cooper et al., 1991). Galectin-1, as a homodimer, has the potential to act as crosslinker between cell and substratum. Indeed, cell surface molecules such as a lactosamine-containing glycolipid in rat olfactory neurons (Mahanthappa, et al., 1994) and an integrin receptor ($\alpha 7\beta 1$) in muscle (Gu et al., 1994) have also been implicated in galectin-1's biological effects.

These diverse effects of galectin-1 suggest a complexity of interaction between galectin-1, extracellular matrix molecules like laminin, and the cell surface. Such interactions could modulate extracellular matrix-neuronal or neuronal-neuronal interactions important to axon guidance, elongation, or fasciculation. To investigate this hypothesis, galectin-1 was tested in an *in vitro* neurite guidance assay, in which two sialic-acid binding lectins, pertussis toxin and MAL lectin, had striking effects (Chs. 3 and 4; Kindt and Lander, 1995). Galectin-1 altered neurite behavior in the assay, but differently than other lectins, causing chick DRG neurites to extend on laminin paths on which they normally halt prematurely. Subsequent experiments assaying galectin-1's effect on neurite extension show that galectin-1 modulates neurite behavior by interacting with substratum-bound carbohydrates and cellular glycoproteins.

**Acknowledgments**

These experiments were carried out as a collaboration with Doug Cooper (UCSF) who provided the galectin-1 and many helpful suggestions.

**Materials and Methods**

*Materials.*

Recombinant rat galectin-1 was prepared as previously described (Cooper, et al., 1991) and stabilized against oxidative inactivation by treatment with iodoacetamide (Whitney et al., 1986). Laminin-1 was purified from the mouse Engelbreth-Holm Swarm sarcoma (Kleinman et al., 1982; Timpl et al., 1982). Ascites of the β1-integrin specific monoclonal antibody CSAT and the monoclonal antibody OKT8 were hydroxyapetite purified according to published procedures (Harlow and Lane, 1988). Chrome-on-quartz photomasks (thickness, 0.09 inch) were fabricated by Advance Reproductions Co. (North Andover, MA). Deoxymannojirimycin was from Boeringer Mannheim. Tissue culture media were purchased from GIBCO-BRL or Cellgro-Mediatech. Unless noted, all other reagents were from Sigma.

*Cell Isolation and Culture.*

Dorsal root ganglia were dissected from E7-E8 embryonic chicks and cultures prepared as described (Kindt and Lander, 1995 and chapter 2 methods). Briefly, for experiments using whole ganglia, groups of 8-10 intact ganglia were threaded onto pieces of 8-0 nylon suture and affixed to patterned laminin coverslips immersed in culture medium. For experiments using dissociated neuronal culture, cleaned ganglia were trypsinized, followed by brief treatment with DNase and soybean trypsin inhibitor, then 4.5 hours preplating in a tissue culture dish to remove the more adherent non-neuronal cells. Harvested cells were plated at 4000 cells/coverslip on substrata prepared as described in the figure legends.

Preparation of laminin-coated and patterned laminin coverslips was performed as described (Kindt and Lander, 1995 and chapter 2 methods). Ganglia and dissociated neurons were cultured at 37°C in serum free medium consisting of a basal medium (50:50 DMEM:F12 for intact ganglia; F12 for dissociated neurons), supplemented with N2 additives (Bottenstein and Sato, 1979), 1 mg/ml crystalline bovine serum albumin (ICN Biochemicals), 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 20 ng/ml 2.5S NGF

(Collaborative Research). Ganglia were cultured in an 8% $CO_2$ atmosphere; dissociated neurons in a 5% $CO_2$ atmosphere. After 24 hr, ganglion explant cultures were supplemented with 10 μM 5-fluoro-2-deoxyuridine to inhibit non-neuronal cell growth. Cultures were fixed with 4% formaldehyde 5% sucrose in PBS. Ganglion explant cultures were treated with 10 μM CMFDA Cell Tracker dye (Molecular Probes, Eugene, OR) 30 min before fixation.

For rat dorsal root ganglion cultures, 0 to 3 day old (P0-P3) Sprague-Dawley rat pups were anesthetized on ice and decapitated. The spinal cord was removed and dorsal root ganglia dissected in PBS and cleaned in holding medium (Leibovitz' L15 medium with 0.5 % glucose, 100 U/ml penicillin and 100 μg/ml streptomycin). The ganglia were treated at 37° with collagenase for 15 min followed by trypsin (0.25%: Sigma type III) for 15 minutes. They were then dissociated with a flame polished pasteur pipet, washed into serum-free NGF-containing 50:50 F12:DME media (see above) and plated at 4000 cells/coverslip or 1500 cells/96 well tissue culture dish.

*Quantification of Neurite Outgrowth.*

Patterned laminin was visualized by immunostaining with polyclonal anti-laminin antibodies as previously described (Kindt and Lander, 1995).

For analysis of neurite wandering, coverslips containing ganglion explants were placed on a glass slide in PBS, and images of both neurite bundles and laminin patterns were collected using a confocal microscope. Neurite wandering was quantified as described previously. Briefly, the pattern consisted of sets of laminin stripes, broken by gaps of 5, 10 or 20 μm of UV-inactivated laminin, and spaced 60 μm from one another. 4 stripes of like gap width were grouped together and separated from other groups by 120 μm. The percentage of neurite mass "on" and "off" stripes was determined for each set of four similar stripes by using the confocal microscope to measure total, background-subtracted fluorescence in the fluorescein channel (which detects the cytoplasmic dye CMFDA) in the 4 rectangular areas representing the stripes themselves ("on"), and the 5 rectangular areas constituting the lanes between the stripes ("off").

These areas were identified by reference to images of laminin immunostaining.

For analysis of neurite lengths on unpatterned substrata, phase contrast images of neurites viewed on a Zeiss Axiovert microscope were collected using a neuvicon video camera (Hamamatsu Photonics) connected to a series 151 Image Processor (Imaging Technologies, Woburn, MA). Neurite lengths were taken to be the straight-line distance from the neurite's point of origin on the soma to the approximate centroid of the growth cone. 75-100 neurites were measured unless otherwise indicated. For neurons without neurites, a value of zero was recorded. Neurite lengths $\leq$6 μm were discounted from the mean neurite length calculation.

## Results

### Neurite behavior on a discontinuous laminin pattern is altered in the presence of galectin-1

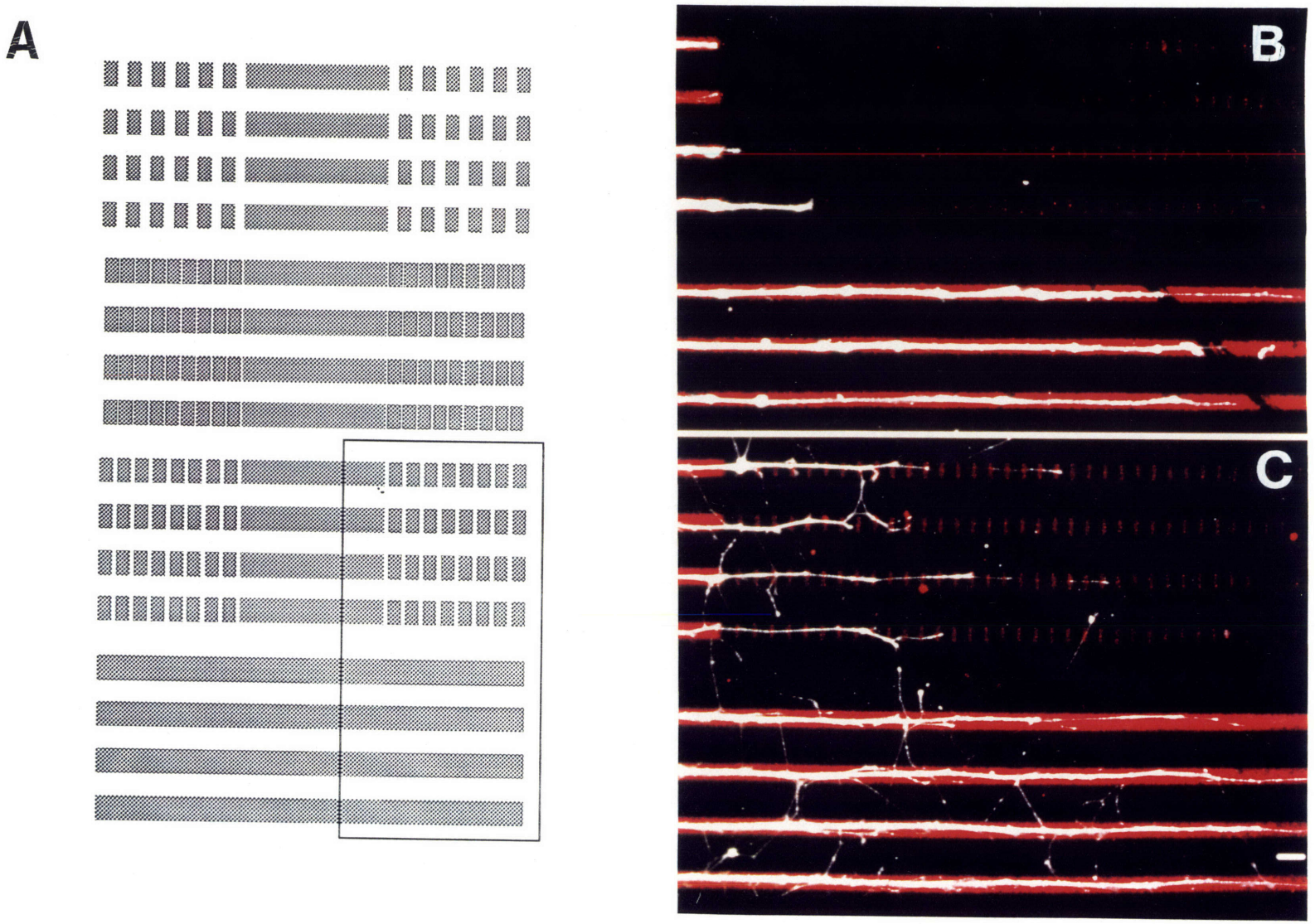
To determine the effect of an endogenous lectin on neurite behavior on a patterned substratum, galectin-1 was added to chick dorsal root ganglia extending neurites on a patterned laminin substratum. The pattern (shown in figure 1A) consisted of stripes of laminin 40 μm wide, some of which were broken by gaps of 5, 10 or 20 μm of UV-inactivated laminin. On this pattern, the distance traveled by DRG neurites on the discontinuous laminin stripes is normally shorter than on the continuous, unbroken stripes and varies inversely with the width of the gaps of inactivated laminin in the stripe. The neurites normally remain confined to the stripe area. Representative outgrowth on a set of continuous stripes and a set of stripes with 10 μm gaps is shown in Figure 1B.

In cultures treated with galectin-1, however, significant neurite outgrowth was observed along all stripes regardless of gap width. Additionally, neurites were observed in the 60 μm wide areas of inactivated laminin between the stripes, sometimes crossing to an adjacent stripe. A field of neurites from a culture treated with 100 μg/ml galectin is shown in Figure 1C. Both neurite length and neurite wandering measurements for stripes of different gap widths confirmed that galectin-1 significantly altered the behavior of neurites on the laminin pattern. In the presence of galectin-1 (100 μg/ml or higher) the distance traveled by the longest neurite on the discontinuous, gapped stripes was significantly increased, while the distance traveled on continuous stripes was not affected (Figure 1D). Neurite wandering, calculated as the percentage of neurite mass off the laminin stripe areas (see Methods), was also increased in the presence of galectin-1 for neurites on all types of stripes (Figure 1E). These effects were diminished or absent at lower concentrations of galectin-1 (i.e., 10 μg/ml).

### Galectin-1 induces neurite outgrowth in neurons cultured on UV-inactivated laminin

The above data show that galectin-1 improves the progress of

**Figure 1. Effect of galectin-1 on neurite outgrowth on patterned laminin.** Chick dorsal root ganglion explants were cultured on the discontinuous laminin pattern with four of the same type of stripes grouped together (see Methods). Galectin-1 (10, 100 or 200 μg/ml) was added to some cultures after 22 hr. After 46 hr, all cultures were treated with CMFDA, fixed, immunostained for laminin, and viewed by confocal microscopy. Active (unirradiated) laminin has been pseudocolored red and CMFDA-stained neurites white. **(A)** Diagram of repeating unit of laminin pattern, not drawn to scale. This pattern is a a variation on the pattern shown in Ch. 3 Figure 1 in which stripes of like gap width are grouped together. See Methods for more detail. Shaded areas indicate active laminin. The box represents region of the pattern depicted in B and C. **(B)** Representative neurites growing on laminin stripes with 10 μm gaps and continuous laminin stripes in the absence of lectin. **(C)** Representative neurites in a similar field treated with galectin-1 (100 μg/ml). Bar = 50 μm. **(D-E)** (overleaf). Concentrations of galectin-1 are 0 (○), 10 (■), 100 (●), and 200 (▲). **(D)** Mean distances traveled by neurite bundles, starting from the beginning of the outer zone, are plotted as a function of the sizes of gaps in the laminin stripes (uninterrupted stripes are plotted as having a gap width of zero). Data were obtained by averaging the lengths of the longest neurite on each of 20 stripes of each gap width, and are presented ± SEM. **(E)** The amounts of neurite mass on and off each type of stripe were measured and averaged as described in Materials and Methods. Each data point represents the mean ± SEM of values obtained from at least 3 groups of 4 stripes.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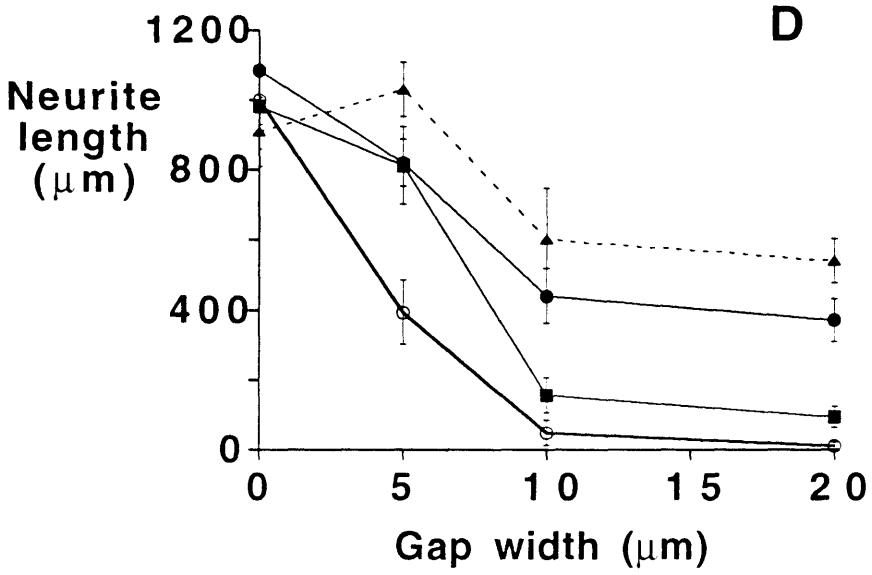
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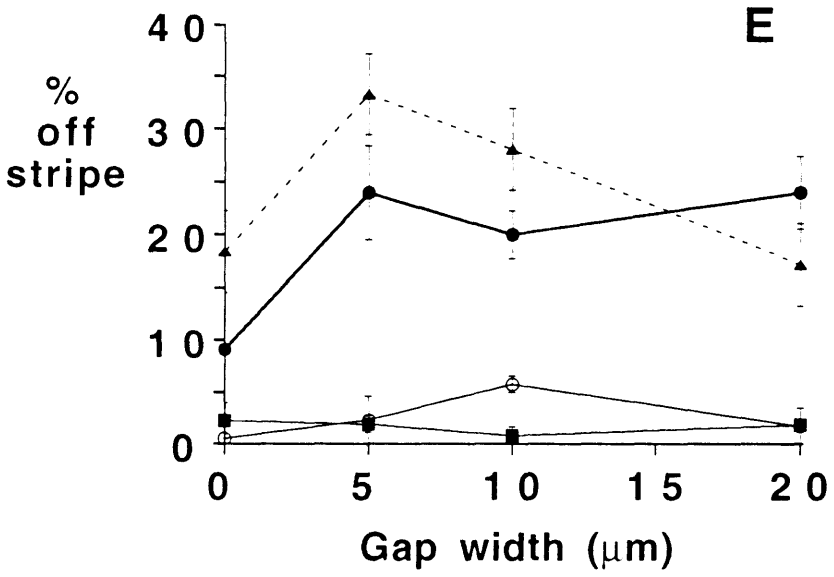
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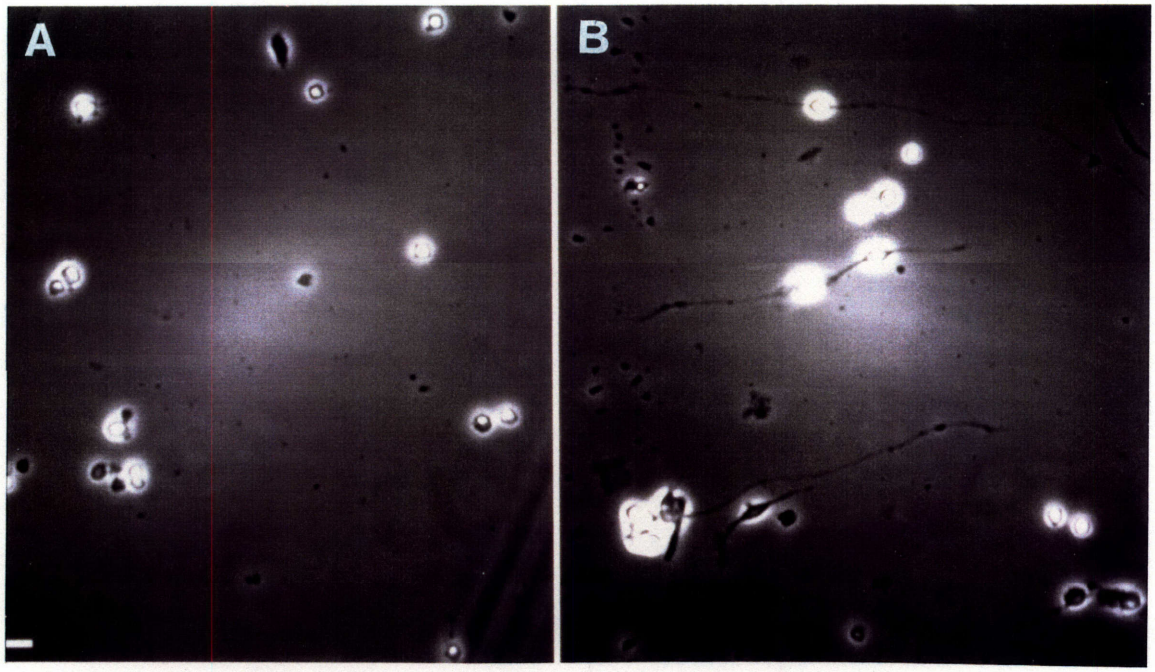
neurites along discontinuous paths of laminin. There are essentially two ways this facilitation could occur. The treatment could alter the growth cone in a way that enhanced its ability to cross gaps, for example by increasing the lengths of filopodia. Or, the treatment could alter neurite-substratum interactions such that all parts of the substratum were favorable for neurite outgrowth.

The latter possibility was tested by culturing dissociated chick DRG neurons on uniform UV-inactivated laminin, the material in the pattern's gaps, in the presence and absence of galectin-1. As previously shown, neurite outgrowth is normally very poor on on UV-inactivated laminin compared to a continuous laminin substratum, (Kindt and Lander, 1995 and chapter 3). A typical field is shown in figure 2A. Mean neurite length on UV inactivated laminin (averaged from three experiments) is 13 ± 4 μm with 14 ± 7 % (SEM) of neurons bearing neurites, compared to 106 ± 8 μm with 68 ± 6 % bearing neurite on uniform active laminin.[1] However, when galectin-1 (100 μg/ml) was added to the culture medium 0 to 15 minutes after cell plating, neurite extension increases dramatically (as shown for one field in figure 2B) to an average of 114 ± 2 μm with 40 ± 1 % of neurons bearing neurites (SEM: same three experiments as above). The distribution of neurite length for inactivated laminin treated with various concentrations of galectin-1 compared to laminin is shown in figure 1C. In all trials, mean neurite lengths of cells with neurites in cultures treated with ≥100 μg/ml galectin-1 were significantly different from untreated cultures on uniform inactive LN ($p<.05$ by Student's t test) and not significantly different from neurite lengths on uniform active laminin ($p>.5$). Interestingly, the percent of cells with neurites was significantly lower than on active laminin ($p<.05$).

Galectin-1 was also included with neurons cultured on coverslips coated with 20 μg/ml laminin, and had no significant effect on neurite length or percent of cells with neurites (Table 1). However, when coverslips were coated with lower concentrations of laminin, chosen for submaximal neurite outgrowth promoting activity (1-2 μg/ml), the

[1] Only 60-70% of DRG neurons survive 48 hours in NGF-containing media (see LoPresti and Scott, 1994).

**Figure 2: Galectin-1 on neurite outgrowth on unpatterned substrata.** Acid washed coverslips were coated with laminin (20 μg/ml) for 3 hours at 37°, washed and dried, and either not irradiated, or uniformly irradiated for 10 min. by ultraviolet light shone through clear, 0.09 inch thick quartz. Dissociated chick DRG neurons were prepared as described in methods and plated at 4000 cells/coverslip. Some cultures were treated with galectin-1 at the indicated concentrations 15 minutes after cell plating. **(A-B)** Phase contrast images of representative fields of neurons cultured on **(A)** UV-inactivated laminin alone and **(B)** UV-inactivated laminin with 100 μg/ml added with the cells. Bar = 25 μm. **(C)** Running histogram of neurite length (i.e., y-axis represents "% of neurons with a neurite greater than x") for neurons cultured on active, unirradiated laminin (—□—), UV-inactivated laminin alone (—**X**—) or UV inactivated laminin including 10 μg/ml (—·—), 50 μg/ml (open triangles) or 100 μg/ml (—○—) galectin-1.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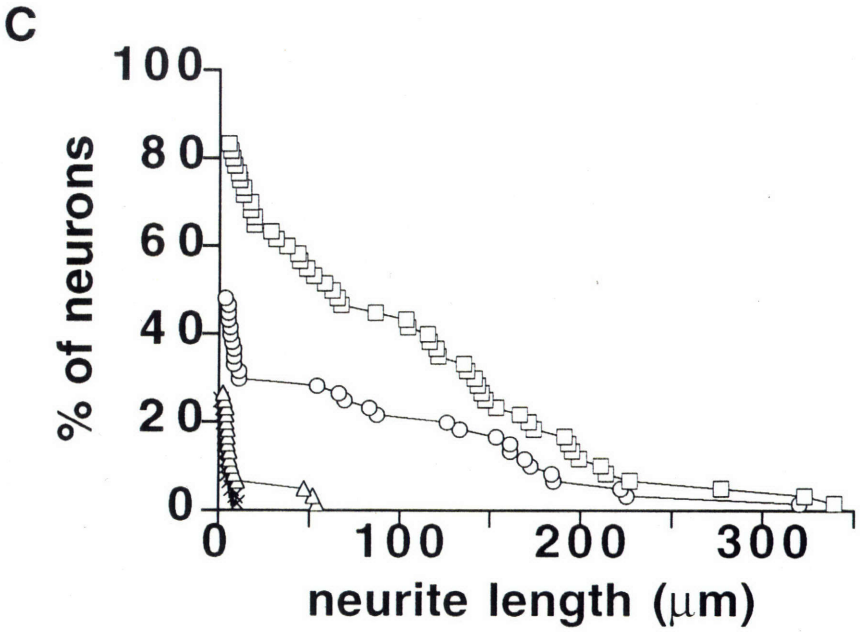
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presence of the lectin caused a small but significant increase in the mean neurite length (Table 1).

Galectin-1 did not improve neurite outgrowth under all conditions in which neurite outgrowth was normally submaximal. Neurons cultured on coverslips coated with bovine serum albumin showed no significant change in mean neurite length in the presence of the lectin (Table 1). Likewise, the presence of galectin-1 did not induce neurites in neurons cultured on tissue culture plastic, whether applied as a coating before or applied with the cells (data not shown).

**The neurite outgrowth-promoting activity of galectin-1 is carbohydrate mediated and can affect neuron cultured on asialofetuin**

Laminin is rich in carbohydrates, and galectin-1 has been shown to bind to laminin's N-linked polyllactosamine residues (Zhou and Cummings, 1990). It was likely that the outgrowth-enhancing effects of galectin-1 involved the lectin's ability to bind to carbohydrate residues on the substratum and/or the cells. Consistent with this possibility, inclusion of the simple non-metabolizable sugar thiodigalactoside (10 mM) in the culture medium blocked the neurite-enhancing properties of galectin-1 when neurons were plated on UV-inactivated laminin. A control saccharide, cellobiose, had no effect on galectin-1 activity in the assay (Table 1 part 2).

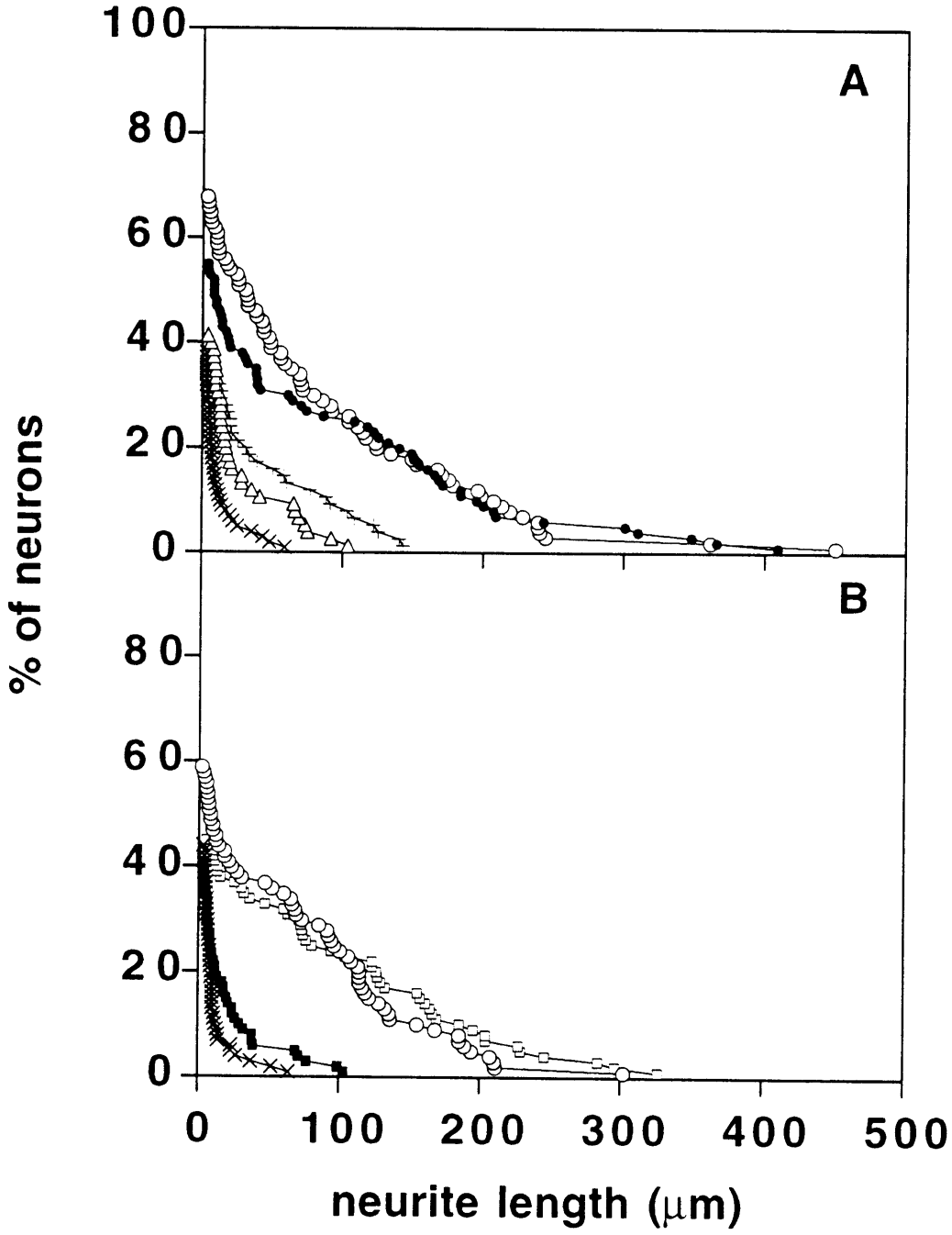
To determine if the neurite outgrowth promoting activity of galectin-1 was specific to laminin-based substrata or could be observed on any substratum bearing carbohydrates to which galectin-1 binds, DRG neurons were plated in the presence and absence of galectin-1 on tissue culture wells coated with asialofetuin, Asialofetuin (ASF) was chosen because of its numerous terminal N-acetyllactosamine residues, to which galectin-1 binds with high affinity (Gupta and Brewer, 1994). DRG neurons attached to asialofetuin-coated wells (not shown), and neurite outgrowth was very poor compared to laminin coated wells (figure 3A). Addition of galectin-1 at 100 μg/ml to the culture medium caused a dramatic increase in neurite outgrowth such that the cultures were nearly indistinguishable from those on laminin-coated wells ($p<0.5$ for mean neurite length). Interestingly, the fraction of cells with neurites was not significantly

| Substratum | Treatment | Range (μm) | % with neurites | Mean length ± SEM |
|---|---|---|---|---|
| Experiment 1: | | | | |
| LN (20 μg/ml) | none | 0-339 | 80.0 | 110.7 ± 12.3 |
| LN (20 μg/ml) | galectin-1 | 0-288 | 71.7 | 117.5 ± 12.4 |
| LN (2 μg/ml) | none | 0-354 | 62.0 | 86.4 ± 9.7 * |
| LN " | galectin-1 | 0-400 | 55.0 | 121.2 ± 13.7 |
| LN (1 μg/ml) | none | 0-155 | 37.3 | 40.3 ± 4.6 * |
| LN " | galectin-1 | 0-169 | 45.3 | 53.3 ± 5.8 |
| BSA (1 mg/ml) | none | 0-130 | 21.3 | 43.3 ± 6.8 |
| BSA " | galectin-1 | 0-169 | 30.0 | 46.3 ± 6.8 |
| Experiment 2: | | | | |
| inactive LN | none | 0-100 | 28.0 | 22.3 ± 3.9 * |
| " " | galectin-1, no sugar | 0-345 | 42.0 | 120.4 ± 12.8 |
| " " | galectin-1, + cellobiose | 0-405 | 42.0 | 137.1 ± 16.3 * |
| " " | galectin-1, TDG | 0-85 | 21.0 | 34.8 ± 5.1 |
| active LN | no sugar | 0-515 | 61.3 | 116.9 ± 15.8 |
| " " | + TDG | 0-397 | 68.0 | 103.6 ± 13.2 |
| from figure 3A: | | | | |
| active LN | none | 0-450 | 62.0 | 104.0 ± 11.7 * |
| asialofetuin | none | 0-59 | 20.0 | 19.0 ± 3.4 * |
| asialofetuin | galectin-1 | 0-409 | 52.0 | 112.0 ± 14.5 |

**Table 1: Effects of galectin-1 on chick DRG neurite outgrowth.**
Galectin-1 (100 μg/ml) was added to dissociated DRG neurons at the time of plating onto the indicated substrata (Experiment 1: glass coverslips coated with the indicated concentrations of coating protein; Experiment 2: glass coverslips prepared as in figure 2 legend; from figure 3A: 96 well tissue culture plates prepared as in figure 3 legend). Mean neurite length was determined as described in Methods. An asterix (*) indicates that the number is significantly different ($p<.05$) from the number below it.

**Figure 3: Galectin-1 and neurite extension on asialofetuin**

96 well tissue culture plates were coated with asialofetuin (200 μg/ml) or laminin (20 μg/ml) diluted in CMF-Hanks for 3 hours at 37 °C and washed 4x with CMF-Hanks. Dissociated chick DRG cells neurons were prepared as described in Methods and plated at 1500 cells/ well. Galectin-1 and sugar as indicated were added at the time of cell plating. After 16 hours, cultures were fixed and neurite lengths determined. Laminin alone (—●—); ASF alone (—**X**—). **(A)** Running histogram of neurite length for neurons cultured on ASF treated with 20 μg/ml (open triangles), 50 μg/ml (+), or 100 μg/ml (O) galectin-1. Mean neurite length values are reported in Table 1. **(B)** Sensitivity of galectin-1 mediated neurite outgrowth to TDG. Running histogram of neurite length for neurons cultured on ASF treated with galectin-1 (100 μg/ml) and no sugar (—O—), 10 mM cellobiose (—□—), or 10 mM thiodigalactoside (—■—). Mean neurite length on ASF with galectin-1 is significantly different in the presence of TDG ($p<.001$) but not cellobiose.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different over several experiments on laminin-coated tissue culture wells and asialofetuin coated wells with galectin-1 (100 μg/ml) added (51% versus 47%; p>.5; n=3 experiments), though both were slightly lower than the percent neurons with neurites on laminin-coated coverslips (i.e. 68%). At lower concentrations of galectin-1 (10 and 50 μg/ml) intermediate degrees of enhancement of neurite outgrowth were observed. As observed for cultures on UV-inactivated laminin, the effect of galectin-1 on neurons plated on asialofetuin was sensitive to thiodigalactoside (figure 3B).

**Cellular N-linked complex-type carbohydrates are required for the neuronal response to galectin-1**

The carbohydrates involved in the promotion of neurite outgrowth by galectin-1 could be associated with substratum-bound molecules, the cell, or both. Galectin-1 is homodimeric and so could bind matrix and cell-surface carbohydrates simultaneously. To test whether intact cellular N-linked complex-type oligosaccharides are required for the neuronal response to galectin-1, chick DRG neurons were treated with 1 mM deoxymannojirimycin (DMN), an inhibitor of the Golgi enzyme mannosidase I, for 4.5 hours (i.e., during the preplating step of the neuronal purification). They were then plated onto laminin and asialofetuin-coated wells in the presence and absence of galectin-1 and in the continuous presence of DMN. DMN-treated neurons cultured on laminin-coated plastic were indistinguishable from untreated neurons plated on laminin, extending long neurites (Figure 4). Neither was neurite outgrowth on ASF alone substantially affected by DMN treatment (p>.3 for mean neurite length). However, addition of galectin-1 to DMN-treated neurons on ASF did not cause any significant increase in neurite outgrowth compared to DMN-treated neurons on ASF alone (p>.5 for mean neurite length). Thus, intact N-linked complex-type carbohydrates on cellular glycoproteins are necessary for galectin-1's effect on neurite outgrowth.

**Galectin-1's effects on neurite outgrowth are sensitive to an antibody to β1 integrin**

It was of interest to find out whether the neurite outgrowth-promoting activity of galectin-1 was related to other receptor-ligand interactions that mediate neurite extension. Specifically, laminin-mediated DRG neurite extension is blocked by an antibody (CSAT) to the β1 integrin subunit, (Letourneau et al., 1988). It was reasoned that just as galectin-1 "restores" neurite outgrowth-promoting activity to UV-inactivated laminin, it might be able to induce neurites from CSAT treated neurons on a laminin substratum, However, the presence of galectin-1 did not have any effect on the inhibition of laminin-mediated neurite outgrowth by the CSAT antibody (figure 5A).

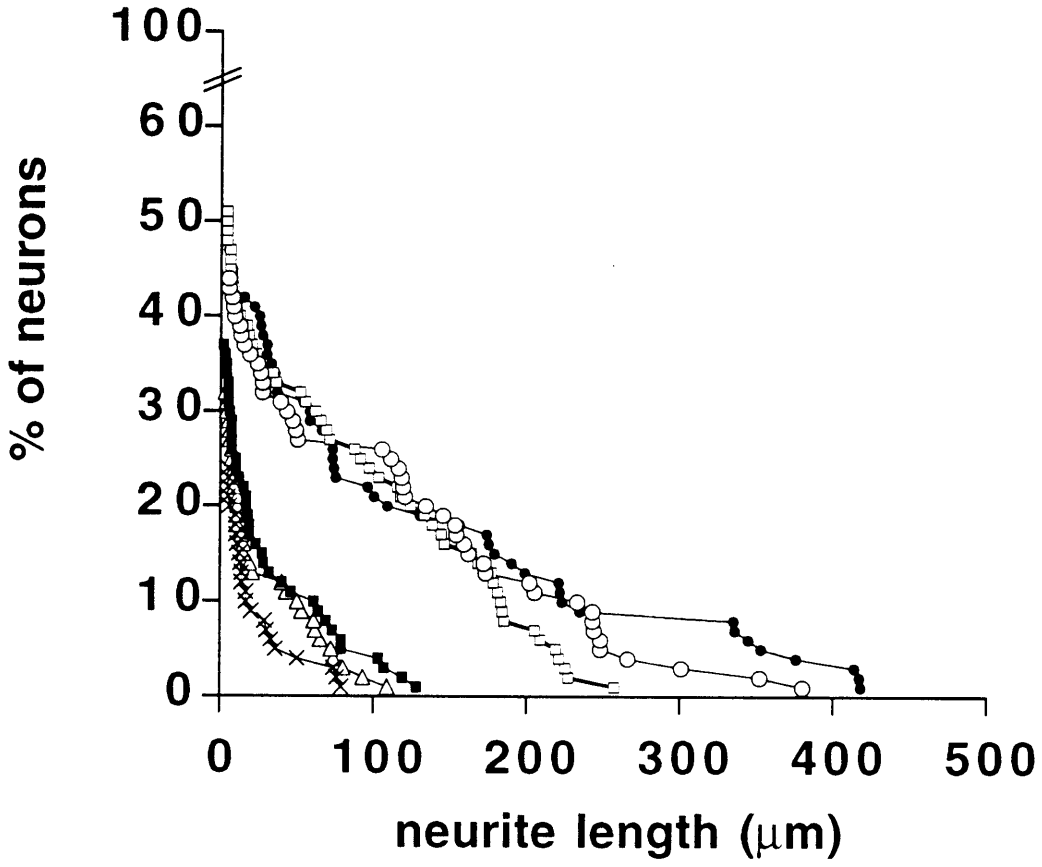
Given this result, it was possible that galectin-1 promoted neurite outgrowth by an integrin dependent mechanism. In that case, all galectin-1 mediated neurite outgrowth should be sensitive to β1 integrin perturbation. So, the effect of CSAT on galectin-1 induced neurite outgrowth on an asialofetuin substratum was tested. While a control antibody, OKT8, had no effect on the extensive neurite outgrowth induced by galectin-1, the anti-β1 integrin antibody CSAT blocked most of this neurite outgrowth, reducing neurite extension to the level of asialofetuin alone (Figure 5B). It appears that one or more integrins of the β1 family may be involved in galectin-1 mediated neurite extension.

**Rat dorsal root ganglion neurons also respond to galectin-1**

The distribution of galectin-1 and of cellular carbohydrate epitopes has been well characterized in dorsal root ganglia--but in rat, not chick. In order to take advantage of what is known about galectin-1 in the rat, and, additionally, to test rat galectin-1 on neurons of the same species, dissociated rat DRG neurons were tested for their response to galectin-1. Dorsal root ganglia were removed from 3 day old rat pups, the capsules removed, and the ganglia dissociated to a single cell suspension after enzyme treatment. They were then applied to laminin-and asialofetuin-coated tissue culture wells, on the latter with and without galectin-1, cultured for 16 hours, and their neurite lengths were counted.

As shown in figure 6, galectin-1 causes a small but significant increase ($p<.05$) in neurite extension on asialofetuin. Unlike in the

**Figure 4 Effect of deoxymannojirimycin treatment on galectin-1 mediated neurite outgrowth.** Running histogram of neurite lengths of DMN-treated chick DRG neurons plated on laminin (—O—), asialofetuin (open triangles), and ASF with 100 μg/ml galectin-1 added at time of plating (—□—); and untreated DRG neurons plated on laminin (—●—), ASF (—X—), and ASF with 100 μg/ml added at time of plating (—■—). DRG neurons were prepared as described in Methods except that during the 4.5 hour preplating step half of the cells were cultured in deoxymannojirimycin (1 mM). DMN-treated and untreated neurons were plated at the same concentration (1500 cells/well) on tissue culture wells prepared in parallel as described in figure 3 legend.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% of neurons
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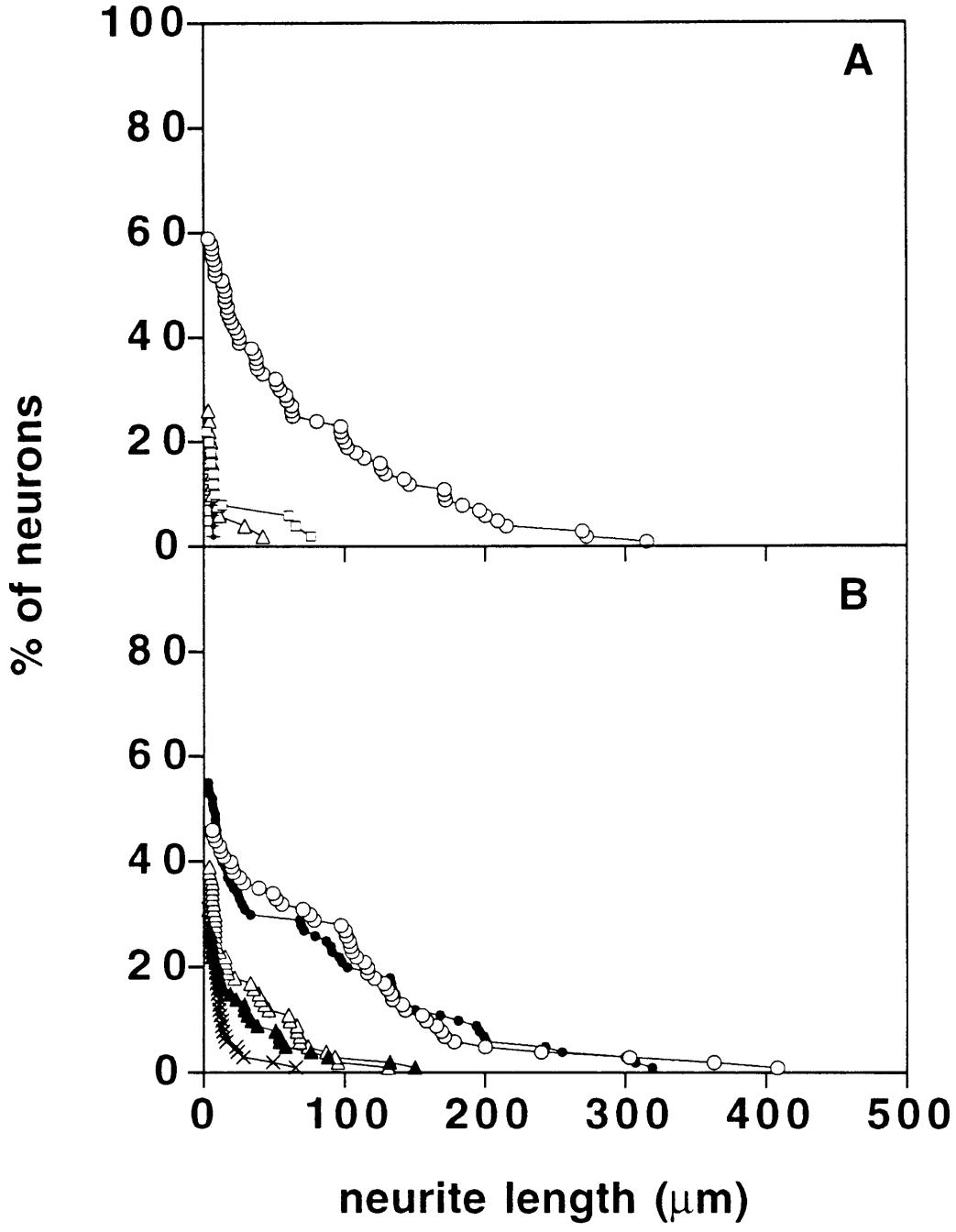
neurite length (μm)

**Figure 5: Galectin-1 mediated neurite outgrowth is sensitive to the CSAT antibody.** Dissociated chick DRG neurons were prepared and applied to 96 wells tissue culture plates coated with laminin (20 μg/ml) or ASF (200 μg/ml) as described in figure 3 legend. The antibodies CSAT or OKT8 were added with 10 minutes of plating at the dilutions indicated and galectin-1 (100 μg/ml) was added within 20 minutes of plating. **(A)** Neurons plated on a laminin substratum were treated with the antibody CSAT at 1:1000 dilution with (open triangles) and without (—•—) galectin-1, CSAT at 1:2500 dilution with (—□—) and without (—+—) galectin-1, or a control antibody OKT8 at 1:1000 dilution (—O—). These data indicate that galectin-1 cannot induce neurites from CSAT-treated neurons. **(B)** Neurons plated on ASF were left untreated (—**X**—) or treated with galectin-1 and: OKT8 (1:1000: —O—), CSAT (1:1000;—▲—) or CSAT (1:2500: open triangles), or . Neurite outgrowth on laminin (—●—) is shown for comparison. These data indicate that galectin-1 mediated outgrowth on ASF is sensitive to the CSAT antibody.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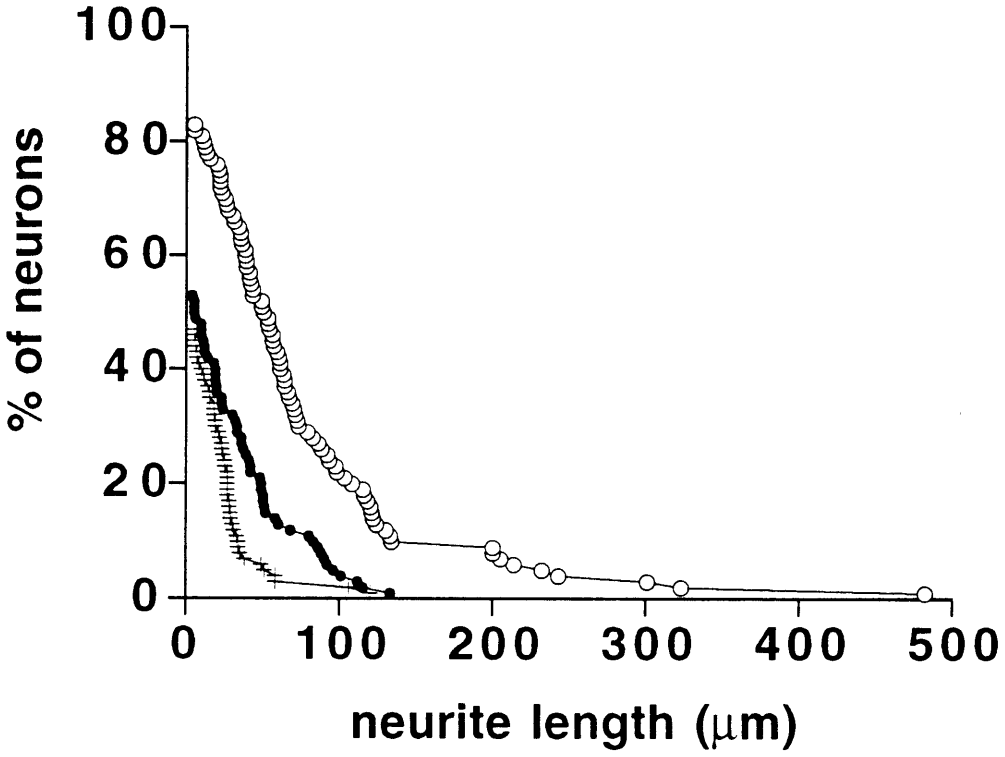
500
neurite length (μm)

chick, however, galectin-1-mediated neurite outgrowth was still significantly less than that on laminin. It is known that in rat dorsal root ganglia the cellular lactoseries carbohydrate epitopes that galectin-1 would be expected to bind to are found primarily on small and medium diameter neurons (Dodd and Jessell, 1986). To determine if the response to galectin-1 in cultured neurons corresponded to neuron size, neurite lengths were binned by soma size. Large diameter neurons (>~15 μm) extended long neurites on laminin but did not put out significantly longer neurites on asialofetuin in the presence of galectin-1. Small and medium diameter neurons, however, bore neurites of about equal length on laminin and asialofetuin-galectin, significantly longer than on asialofetuin (Table 2). Thus, the smaller, presumably lactoseries carbohydrate-bearing neurons, respond to galectin-1.

**Figure 6: Response of rat DRG neurons to galectin-1**. Dissociated rat DRG cells were plated on laminin alone (—O—); ASF alone (—**X**—): or ASF with galectin-1 (100 μg/ml) added at time of cell plating (—●—) Rat DRG cultures were prepared as described in Methods and plated (2000 phase-bright cells/well) onto 96 well tissue culture plated prepared as described in Figure 3 legend. 150 neurites were counted for each condition. See also Table 2.


100
80
60
40
20
0
% of neurons
0
100
200
300
400
500
neurite length (μm)

| Substratum | Size | Range (μm) | % with neurite | Mean length ± SEM |
|---|---|---|---|---|
| ASF | small | 0-106 | 42.3 | 30.2 ± 4.5 * |
| ASF + gal-1 | " | 0-115 | 59.7 | 54.3 ± 6.1 |
| LN | " | 0-134 | 85.0 | 55.7 ± 4.4 |
| ASF | large | 0-120 | 43.7 | 29.3 ± 5.2 |
| ASF + gal-1 | " | 0-133 | 37.7 | 37.8 ± 6.6 * |
| LN | " | 0-482 | 75.0 | 131.6 ±19.5 |
| ASF | all | 0-120 | 43.0 | 29.8 ± 3.4 * |
| ASF + gal-1 | " | 0-133 | 48.0 | 47.4 ± 4.6 * |
| LN | " | 0-482 | 81.0 | 83.8 ± 8.7 |

**Table 2: Effect of soma size on rat DRG neuron response to galectin-1** Dissociated rat dorsal root ganglion neurons were prepared and applied to laminin (LN), asialofetuin (ASF) or asialofetuin and treated with 100 μg/ml galectin-1 (ASF + gal-1) as described in figure 6. Neurons were defined as small (cell body diameter approximately <15 μm) or large (>15 μm). An asterix (*) indicates that the number is significantly different ($p<.005$) from the number below it. See also figure 6.

## Discussion

In this study, the effects of galectin-1 on the behavior of dorsal root ganglion neurons were examined. This lectin was first tested in an *in vitro* assay used to screen for compounds that affect neurite guidance, specifically, the ability of neurites to follow discontinuous paths of laminin. The presence of galectin-1 altered dramatically the pattern of neurite outgrowth on the pattern, causing growth of long neurites on stripes with gaps, on which neurite extension is usually foreshortened compared to outgrowth on unbroken laminin stripes. Additionally, neurites were not confined to the laminin paths (Figure 1). One explanation for these effects was that galectin-1 alters neurites' interactions with the material in the gaps, i.e. UV-inactivated laminin. Consistent with this hypothesis, galectin-1 potentiates neurite outgrowth on UV-inactivated laminin in dissociated DRG neuronal cultures (Figure 2A). The alterations in neurite behavior on the discontinuous laminin pattern are likely due to this effect. Interestingly, galectin-1 had no significant effect on neurites extending on active laminin applied as for the guidance assay (20 μg/ml) .

Several experiments suggest that galectin-1 induces neurite outgrowth by interacting with both substratum-bound and cellular carbohydrates. First, galectin-1's neurite-outgrowth promoting activity correlates with substrata that bear its carbohydrates ligands. Whether galectin-1 interaction with the substratum is necessary and/or carbohydrate-dependent was not assayed directly. However, galectin-1 has been shown to interact with laminin-1 (Cooper, et al., 1991; Zhou and Cummings, 1990) and indeed, neurite outgrowth on suboptimal laminin was enhanced by galectin-1. Laminin's carbohydrates residues are likely to be intact in UV-inactivated laminin, as another lectin-like molecule, pertussis toxin, binds UV-inactivated laminin at least as strongly as active laminin (see chapter 3 table 2). It was possible that binding of galectin-1 to irradiated laminin somehow restored the neurite-outgrowth promoting activity of the native molecule. There is precedent for antibody binding to active laminin modulating its biological activities (Calof et al., 1994). Also,

there is evidence that the oligosaccharides of laminin have biological activity (Dean et al., 1990), which could potentially be modulated by galectin-1 binding. However, it appears that the association of galectin-1 and laminin is not so complex, as another carbohydrate-rich molecule is a competent substratum for galectin-1 neurite-promoting activity. Asialofetuin-coated plastic supports robust neurite outgrowth when (and only when) galectin-1 is present (Figure 3A). Second, the neurite-stimulating activity of galectin-1 was blocked under all conditions in which it was observed by the addition of thiodigalactoside, a potent competitive inhibitor of galectin-1's interaction with B-galactoside sugars (Leffler and Barondes, 1986).

Third, the observation that DMN treated neurons do not respond to galectin-1 demonstrates that cellular N-linked complex type carbohydrates are required (Figure 4A). Formally, the requisite glycoconjugates could be on secreted glycoproteins as well as cell surface glycoproteins. In this discussion I will focus on the latter possibility. Galectin-1, a homodimer, is proposed to alter cellular behavior by crosslinking matrix and cell surface carbohydrates, or, if present in solution, to crosslink cells to other cells and cause aggregation. In these experiments an equilibrium of bound and soluble galectin-1 is expected. This is based on the finding in another study that galectin-1, even if preapplied to a laminin substratum, will dissociate from it during a culture period equivalent to that used in this study (Mahanthappa, et al., 1994). Yet, there was no noticeable cellular aggregation in the presence of galectin-1 for either chick or rat DRG neurons (not shown). This result is in contrast to the results of Outenreach and Jones (1992) who observed neuronal aggregation on a galectin-1 substratum. However, they applied galectin-1 directly to coverslips by a different plating method. In my hands galectin-1 applied alone to glass or plastic did not have any detectable effect on neurons (data not shown).

Interestingly, galectin-1-mediated neurite outgrowth appears to be integrin dependent. Not only could galectin-1 not induce neurites from CSAT-treated neurons cultured on active laminin (figure 5A), but the neurite outgrowth potentiated by galectin-1 on asialofetuin was sensitive to the anti-β1 antibody (figure 5B). One trivial explanation

for these results is that the CSAT antibody disrupts neuronal cell body attachment such that no neurite outgrowth is possible. However, the density of attached cells on asialofetuin-coated wells in the presence of galectin-1 and CSAT was not noticeably lower than in cultures without the CSAT antibody (data not shown).

How could galectin-1 act via integrin receptors? First, galectin-1 could be a new ligand for β1 integrins. An assortment of β1 integrins are found on DRG neurons including α1β1, α3β1, α5β1, and α8β1 (Tomaselli et al., 1993; Varnum-Finney et al., 1995). Both alpha and beta subunits bear N-linked complex-type carbohydrates so it is possible that the DMN-sensitivity of the effect is a result of galectin-1's interaction with these carbohydrates on the integrin receptor. In fact, the β1 chain (as isolated from melanoma cells) has N-acetyllactosamine structures (Chammas et al., 1993) which galectin-1 could specifically bind. Second, galectin-1 could bind integrin receptors, but at a site distinct from the ligand binding site, possibly inducing a conformational change in the integrin that results in the potentiation of neurite outgrowth in the absence of an integrin ligand (i.e. on an asialofetuin substrate). There are many precedents for extracellular modulation of integrin activity (Chan and Hemler, 1993; Loftus et al., 1994). For example, the monoclonal antibody TASC binds to the β1 integrin subunit and in chick retinal neurons can induce neurite outgrowth on laminin from neurons at a developmental stage where they otherwise do not respond to laminin. TASC's activity, like that of galectin-1 is CSAT-sensitive and it binds the integrin at a site distinct from the ligand binding site (Neugebauer and Reichardt, 1991). It is also possible that galectin-1 acts by binding to and crosslinking integrins on the cells surface to each other or to other glycoproteins. Crosslinking of integrins by antibodies can activate them. In this scenario, the CSAT antibody could interfere with the lectin's effect by sterically hindering the access of galectin-1 to binding sites on the ligand. Experiments are in progress to test if integrins from DRG neurons interact directly with galectin-1 .

There is evidence for a direct interaction of galectin-1 with an integrins from muscle. The integrin α7β1 from cultured skeletal muscle cells binds immobilized galectin-1 in a carbohydrate sensitive

manner (Gu, et al., 1994). This interaction could be responsible for the observed decrease of adhesion to laminin in the presence of galectin-1 (Cooper, et al., 1991). Instead, in the current study, galectin-1 does not appear to interfere with, but more to mimic, laminin-cell interactions. β1 integrin is also reported to be the predominant galectin-1-binding protein in smooth muscle cell extracts (D.N.W. Cooper, personal communication). Interestingly, galectin-1-mediated increase in olfactory neuronal adhesion to laminin was not found to be sensitive to an anti-β1 integrin antibody. However, the cellular receptor for galectin-1 in those neurons is believed to be a carbohydrate containing glycolipid, which is not consistent with results in DRG neurons, because DMN treatment does not affect glycosylation of lipids (Elbein, 1987). Undoubtedly, galectin-1 is capable of interacting with the cell surface in a variety of ways.

Two members of the galectin family have been identified in the chick (Hirabayashi and Kasai, 1993). Their expression pattern in the nervous system has not been determined. In the rat, galectin-1 mRNA is detected in all DRG neurons, starting after neuronal differentiation (E13) and continues throughout the time of axonal extension up to adulthood. Expression is much higher in small and medium diameter neurons (Hynes, et al., 1990). Immunostaining shows galectin-1 on fibers in the spinal cord in some (i.e. lamina I and II) but not all areas of the dorsal horn to which DRG neurons project (Regan, et al., 1986). Strikingly, high galectin-1 expression as detected by immunostaining correlates with subsets of DRG neurons that express B-galactoside-containing carbohydrate antigens (Regan, et al., 1986).

In all, these data suggest that small diameter cutaneous afferents both express galectin-1 and bear its carbohydrate ligands. Importantly, these correspond to the largest subset of neurons in the chick that are NGF responsive (LoPresti and Scott, 1994). Thus, it is not surprising that the response to galectin-1 in chick DRG culture is so vigorous. Preliminary experiments with dissociated rat dorsal root ganglion neurons suggest that they too can respond to galectin-1 when it is administered in the context of a carbohydrate-rich substratum (Figure 6 and Table 2). The strongest response was found with small diameter neurons, some of which may correspond to NGF dependent-

cutaneous afferents that project to layers I and II of the dorsal horn (Ruit et al., 1992).

Thus, the neurons that respond to galectin-1 in this study's culture system could be the *in vitro* correlates of the neurons that can secrete galectin-1. Since they would also be expected to bear the carbohydrate epitopes that may render them able to respond to the lectin, galectin-1 could have an autocrine role: If the lectin were secreted from the extending axon terminal, a locally high concentration of the molecule would be available to stimulate axonal extension, perhaps via a transient association with matrix molecules such as laminin. Such a model would be consistent with the relatively high concentrations of galectin-1 required for its effects on neurite behavior in this study. Galectin-1 could also act as a target derived, matrix-immobilized attractant for DRG axons. This is less plausible for DRG projections to the dorsal horn as the latter neurons do not express galectin-1 (Hynes, et al., 1990). Yet DRG neurons also project to the periphery, and galectin-1 is expressed in both muscle and skin. Or, galectin-1 could mediate axonal fasciculation, perhaps by crosslinking integrin receptors. Galectin-1 null mutant mice are viable and appear to develop normally (Poirier and Robertson, 1993); a careful examination of sensory neuronal projections in these animals, combined with *in vitro* experiments comparing the behavior of DRG neurons from mice with and without endogenous galectin-1 could provide more information about the role of galectin-1 in the developing nervous system.

## References


Barondes, S. H., Cooper, D. N. W., Gitt, M. A., and Leffler, H. (1994). Galectins: structure and function of a large family of animal lectins. J. Biol. Chem. *269*, 20807-20810.

Bottenstein, J. E., and Sato, G. H. (1979). Growth of a rat neuroblastoma cell line in serum-free supplemented medium. Proc. Natl. Acad. Sci. *76*, 514-517.

Brule, F. A. v. d., Buicu, C., Baldet, M., Sobel, M. E., Cooper, D. N. W., Marschal, P., and Castronovo, V. (1995). Galectin-1 modulates human melanoma cell adhesion to laminin. Biochem. Biophys. Res. Comm. *209*, 760-767.

Calof, A. L., Campanero, M. R., O'Rear, J. J., Yurchenco, P. D., and Lander, A. D. (1994). Domain-specific activation of neuronal migration and neurite outgrowth-promoting activities of laminin. Neuron *13*, 117-130.

Chammas, R., Veiga, S. V., Travassos, L. R., and Brentani, R. R. (1993). Functionally distinct roles for glycosylation of α and β integrin chains in cell-matrix interactions. Proc. Natl. Acad. Sci. USA *90*, 1795-1799.

Chan, B. M. C., and Hemler, M. E. (1993). Multiple function forms of the integrin VLA-2 can be derived from a single α2 cDNA clone: interconversion of forms induced by an anti-β1 antibody. J. Cell Biol. *120*, 537-543.

Cooper, D. N. W., Massa, S. M., and Barondes, S. H. (1991). Endogenous muscle lectin inhibits myoblast adhesion to laminin. J. Cell Biol. *115*, 1337-1448.

Dean, J. W., Chandrasekaran, S., and Tanzer, M. L. (1990). A biological role for the carbohydrate moieties of laminin. J. Biol. Chem. *265*, 12553-12562.

Dodd, J., and Jessell, T. M. (1986). Cell surface glycoconjugates and carbohydrate-binding proteins: possible recognition signals in sensory neurone development. J. Exp. Biol. *124*, 225-238.

Drickamer, K., and Taylor, M. E. (1993). Biology of animal lectins. Annu. Rev. Cell Biol. *9*, 237-264.

Elbein, A. D. (1987). Inhibitors of the biosynthesis and processing of N-linked oligosaccharide chains. Ann. Rev. Biochem. *56*, 497-534.

Gu, M., Wang, W., Song, W. K., Cooper, D. N. W., and Kaufman, S. J. (1994). Selective modulation of the interaction of α7β1 integrin with fibronectin and laminin by L-14 lectin during skeletal muscle differentiation. J. Cell Sci. *107*, 175-181.

Gupta, D., and Brewer, C. F. (1994). Homogeneous aggregation of the 14-kDa B-galactoside specific vertebrate lectin complex with asialofetuin in mixed systems. Biochem. *33*, 5526-5530.

Hadar, Y. R., Paz, K., Dekel, R., Mestrovic, T., Accili, D., and Zick, Y. (1995). Galectin 8. A new rat lectin, related to galectin-4. J. Biol. Chem. *270*, 3447-3453.

Harlow, E., and Lane, D. (1988). Antibodies: a laboratory manual. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).
Hirabayashi, J., and Kasai, K. (1993). The family of metazoan metal-independent beta-galectoside-binding lectins: structure, function and molecular evolution. Glycobiology *3*, 297-304.
Hynes, M. A., Gitt, M., Barondes, S. H., Jessell, T. M., and Buck, L. B. (1990). Selective expression of an endogenous lactose-binding lectin gene in subsets of central and peripheral neurons. J. Neurosci. *10*, 1004-1013.
Kindt, R. M., and Lander, A. D. (1995). Pertussis toxin specifically inhibits growth cone guidance by a mechanism independent of direct G protein inactivation. Neuron *15*, 79-88.
Kleinman, H. K., McGarvey, M. L., Liotta, L. A., Robey, P. G., Tryggvason, K., and Martin, G. R. (1982). Isolation and characterization of type IV procollagen, laminin, and heparan sulfate proteoglycan from the EHS sarcoma. Biochemistry *21*, 6188-6193.
Leffler, H., and Barondes, S. H. (1986). Specificity of binding of three soluble rat lung lectins to substituted and unsubstituted mammalian B-galactosides. J. Biol. Chem. *261*, 10119-10126.
Letourneau, P. C., Pech, I. V., Rogers, S. L., Palm, S. L., McCarthy, J. B., and Furcht, L. T. (1988). Growth cone migration across extracellular matrix components depends on integrin, but migration across glioma cells does not. J. Neurosci. Res. *21*, 286-297.
Loftus, J. C., Smith, J. W., and Ginsberg, M. H. (1994). Integrin-mediated cell adhesion: the extracellular face. J. Biol. Chem. *269*, 25235-25238.
LoPresti, P., and Scott, S. A. (1994). Target specificity and size of avian sensory neurons supported *in vitro* by nerve growth factor, brain-serived neurotropic factors, and neurotrophin-3. J. Neurobiol. *12*, 1613-1624.
Mahanthappa, N. K., Cooper, D. N. W., Barondes, S. H., and Schwarting, G. A. (1994). Rat olfactory neurons can utilize the endogenous lectin, L-14 in a novel adhesion mechanism. Development *120*, 1373-1384.
Neugebauer, K. M., and Reichardt, L. F. (1991). Cell-surface regulation of β1-integrin activity on developing retinal neurons. Nature *250*, 68-71.
Outenreach, R. L., and Jones, A. L. (1992). Influence of an endogenous lectin substrate on cultured dorsal root ganglion cells. J. Neurocytol. *21*, 788-795.
Poirer, F., Timmons, P. M., C.-T.Chan, Geunet, J.-L., and Rigby, P. W. J. (1992). Expression of the L-14 lectin during mouse embryogenesis suggests multiple roles during pre- and post-implantation development. Development *115*, 143-155.
Poirier, F., and Robertson, E. J. (1993). Normal development of mice carrying a null mutation in the gene encoding the L14 S-type lectin. Development *119*, 1229-1236.

Regan, L. J., Dodd, J., Barondes, S. H., and Jessell, T. M. (1986). Selective expression of endogenous lactose-binding lectins and lactoseries glycoconjugates in subsets of rat sensory neurons. Proc. Natl. Acad. Sci U.S.A. *83*, 2248-2252.

Ruit, K. G., Elliott, J. L., Osborne, P. A., Yan, Q., and Snider, W. D. (1992). Selective dependence of mammalian dorsal root ganglion neurons on nerve growth factor during embryonic development. Neuron *8*, 573-587.

Timpl, R., Rohde, H., Risteli, L., Ott, U., Robey, P. G., and Martin, G. R. (1982). Laminin. Meth. Enzymol. *82*, 831-338.

Tomaselli, K. J., Doherty, P., Emmett, C. J., Damsky, C. H., Walsh, F. S., and Reichardt, L. F. (1993). Expression of β1 integrins in sensory neurons of the dorsal root ganglion and their functions in neurite outgrowth on two laminin isoforms. J. Neurosci. *13*, 4880-4888.

Varnum-Finney, B., Venstrom, K., Muller, U., Kypta, R., Backus, C., Chiquet, M., and Reichardt, L. F. (1995). The integrin receptor α8β1 mediates interactions of embryonic chick motor and sensory neurons with tenascin-C. Neuron *14*, 1213-1222.

Whitney, P. L., Powell, J. T., and Sanford, G. L. (1986). Oxidation and chemical modification of lung beta-galactoside-specific lectin. Biochem J. *238*, 683-689.

Zhou, Q., and Cummings, R. D. (1990). The S-type lectin from calf heart tissue binds selectively to the carbohydrate chains of laminin. Arch. Biochem. Biophys. *281*, 22-35.

Zhou, Q., and Cummings, R. D. (1993). L-14 lectin recognition of laminin and its promotion of *in vitro* cell adhesion. Arch. Biochem. Biophys. *300*, 6-17.

# Chapter 6

# Conclusions

*Overview*

In my thesis, I have used two distinct approaches to study how cells interact with their extracellular environment. First, I explored the mechanism of laminin-mediated anti-adhesion. This work was based on the observation that laminin reduces the adhesion of olfactory neuronal cells to fibronectin when laminin and fibronectin are present together on a substratum (Calof and Lander, 1991). The results of this investigation suggested two models, which were discussed. The second half of my thesis took advantage of a different property of laminin: the ability to guide neurites when presented in a substratum-bound pattern. As little is known about the cellular mechanisms underlying the neuronal growth cone's response to extracellular guidance cues, I designed an *in vitro* assay to screen for treatments that affect laminin-mediated growth cone guidance. I identified two treatments that affect neurite behavior, both by binding cell surface carbohydrates. Pertussis toxin (and the plant lectin MAL) alters the ability of laminin to guide neurites but not laminin-mediated neurite elongation. In contrast, galectin-1 potentiates neurite growth on carbohydrate-rich substrata.

*An investigation of the mechanism of laminin-mediated anti-adhesion*

In this study, non-neuronal cell lines susceptible to laminin-mediated anti-adhesion were used to determine the mechanism of this effect. Laminin could block adhesion by interacting with the cell--possibly interfering with adhesive receptors or sending "anti-adhesive" signals--or it could block the cell's access to the adhesive molecule. The observation that laminin interfered with adhesion even in the absence of extended time, physiological temperature, and an intact cytoskeleton suggested that no intracellular signaling events are involved in anti-adhesion (Ch. 2, Fig. 7). Furthermore, laminin blocked interaction with fibronectin in a cell-free system: integrin-containing liposomes did not bind to fibronectin in the presence of laminin (Ch. 2, Fig. 8). Interestingly, laminin does not appear to significantly decrease antibody binding to specific sites on fibronectin (Ch. 2, Fig. 9).

I presented two models that could explain these results. First, laminin could interact directly with the fibronectin-binding integrin in a manner that blocks the adhesive interaction but does not in itself promote adhesion. Second, laminin, with its large size and rigid structure, could alter the topography of the substratum such that cells' access to fibronectin is limited. The first model is less likely, as studies by others have shown that integrin-independent adhesion is also susceptible to laminin-mediated anti-adhesion (A.L. Calof and A.D. Lander, pers. commun.). To address the second model, electron micrographic analysis of surfaces coated with laminin, fibronectin, or a mixture of the two, could be used to provide some information about the arrangement of these molecules on the substratum. According to the model, fibronectin-coated substratum would be expected to be relatively flat, and laminin-coated substratum raised. In fact, laminin molecules should protrude at least 20 nm on average (the height of integrin molecules above the cell surface; Nermut et al., 1988) above the fibronectin molecules to block the binding of integrins to fibronectin. Atomic force microscopy could also confirm whether this condition is met by providing measurement of the molecular height of substratum-bound laminin and fibronectin (for review see Lal and John, 1994).

While these studies do not definitively identify the mechanism of anti-adhesion, they focus further investigation on how laminin might act outside the cell to alter cellular behaviors. Such behaviors would be likely to include ones in which adhesion may play some role, such as neurite outgrowth. For example, some known examples of inhibition of neurite outgrowth might be explained by an anti-adhesion-like mechanism. According to the second model described above, one molecule could physically shield neurons and their neurites from other, neurite outgrowth-promoting molecules. Molecules such as tenascin and chondroitin sulfate proteoglycan that are anti-adhesive for cell bodies under some conditions sometimes also inhibit neurite outgrowth *in vitro* (Lochter et al., 1991; Oohira et al., 1991). In fact, it might be possible to test if the E1' fragment of laminin could inhibit

neurite outgrowth on, for example, fibronectin, by neurons that do not extend neurites on that fragment.

The ordered arrangement of extracellular matrix in structures such as basement membranes supports the hypothesis that the topology of laminin itself could alter cell-matrix interactions. For example, many basement membranes are quite thin (~100 nm), barely thick enough to be spanned by two laminin molecules. Depending on the orientation of those laminin molecules, cellular access to other molecules in the basement membrane--or even to domains of laminin itself--might be blocked. In one ~100 nm basement membrane, that of the corneal epithelium, immunoelectron microscopic studies have suggested that E1' domains of laminin are confined to the middle of the structure, and long-arm domains protrude outward (Schittny et al., 1988). This configuration should maximize cell access to cell binding sites found in the long arm (e.g., sites for integrin $\alpha 6\beta 1$) and minimize cell binding to sites found in the short arms (e.g., sites for integrin $\alpha 1\beta 1$) (Colognato-Pyke et al., 1995). On the other hand, if such a basement membrane is injured, its cut surface would expose short arm binding sites, which could be used by cells (e.g., inflammatory cells) as one way of detecting a site of injury. Since basement membranes of widely varying thicknesses exist *in vivo*, it is not known to what degree laminin's arrangement varies from one type of basement membrane to another. Nevertheless, it is possible that topological effects of laminin--which are observed as anti-adhesion *in vitro*--play an important role in regulating cell-ECM interactions *in vivo*.

*Identification of a specific inhibitor of laminin-mediated guidance*

In the second part of this thesis, I designed a novel *in vitro* assay based on the *in vitro* guidance activity of laminin. I used the assay to test for pharmacological treatments that interfere with growth cone guidance. In the assay, neurites from immobilized neuronal tissue (embryonic chick dorsal root ganglia (DRG)) elongate onto continuous and discontinuous laminin stripes created by selectively inactivating regions of substratum-bound laminin with ultraviolet light (Ch. 3, Fig.

1). A treatment that specifically disrupted guidance was predicted to have a greater effect on neurite elongation on broken, discontinuous paths of laminin than on unbroken paths, as the growth cones would need to be "guided" each time they encountered a discontinuity. The pharmacological treatments of interest were expected to interfere with the ability of neurites to cross gaps of UV-inactivated laminin and thus cause a decrease in the length of the neurites observed on the discontinuous stripes.

One treatment, cytochalasin B, did inhibit neurite extension on discontinuous stripes significantly more than on continuous stripes (Ch. 3, Fig. 2). Cytochalasin B is believed to act here by disrupting filopodia and other actin-based structures in the growth cone (e.g., Marsh and Letourneau, 1984). Of a relatively small number of treatments tested, no others had such an effect on neurite behavior (Ch. 3, Table 1). However, more extensive screening of pharmacological agents might reveal other compounds with the same effect as cytochalasin in the assay. These would be candidates for treatments that affect filopodial length and dynamics.

Instead, the effect of pertussis toxin on neurite behavior was strikingly different from that originally expected. Pertussis toxin caused neurites to grow off laminin stripes that normally confined them. The effect was more pronounced for stripes with larger gap width (Ch. 3, Fig. 3). While this result suggested that pertussis toxin disrupted the ability of neurites to follow paths of laminin, there could have been other explanations for this neurite behavior. For example, if pertussis toxin disrupted neurite-neurite interactions, a neurite might be freer to encounter the laminin borders and thus might leave the laminin stripes more frequently. When the behavior of single neurites from dissociated DRG neurons was tested on a different laminin pattern, they too responded to pertussis toxin by crossing boundaries of laminin that normally stop them (Ch. 3, Fig. 4). Together, the results strengthen the conclusion that pertussis toxin interferes with the ability of laminin to guide neurites.

For both the whole ganglion stripe assay and the dissociated cell guidance assays, the possibility was considered that, in the presence of pertussis toxin, UV-inactivated laminin becomes a better substratum for neurite outgrowth, or unirradiated laminin a poorer substratum. If the growth cone chooses its substratum based on relative growth rates, either change could conceivably facilitate neurite crossing from laminin to UV-inactivated laminin. However, when the effect of pertussis toxin on neurite outgrowth on uniform active laminin or uniform UV-inactivated laminin was tested over the same time course as the dissociated cell guidance assay, there was no evidence for any change in neurite behavior on either substratum (Ch. 3, Fig. 5). Thus, pertussis toxin alters the ability of neurites to choose between two substrata without altering the relative rate of neurite extension on the two substrata. This suggests that the ability of laminin to guide is more than the ability to promote rapid outgrowth, and that growth cone choicemaking is more than preferential extension where growth will be the fastest.

Given that changes in relative growth rate are apparently not necessary for disruption of neurite guidance, it is interesting to consider whether such changes are always sufficient. In this regard, some of the data obtained in the studies of galectin-1 (Ch. 5) are relevant. Galectin-1 greatly increases the extent of neurite outgrowth on UV-inactivated laminin (Ch. 5, Fig. 2) but not on active laminin (Ch. 5, Table 1). For the neurons that respond to galectin-1, the treatment apparently increases the rate of neurite outgrowth on UV-inactivated laminin to levels similar to that seen on active laminin (though rates were not measured directly, this conclusion is suggested by neurite length measurements). So, does galectin-1 affect growth cone guidance? Galectin-1 treatment does increase the amount of neurite mass found off the laminin stripes (Ch. 5, Fig. 1E) and the calculated percentage of neurites off the stripes is in the same range as pertussis toxin (about 20-30%). Yet, the measured wandering is not nearly as great as would be expected if the neurites crossed freely from active to UV-inactivated laminin (i.e., if there were a complete loss of guidance). Given that, in the presence of galectin-1, neurite lengths

on uniform (unpatterned) laminin and uniform inactivated laminin are essentially equal (mean neurite length of 114 μm vs. 118 μm after 14 hours; cf. Ch. 5), in the absence of any guidance the percent found "off-stripe" should approach the percentage of the substratum that lies between stripes. Since the stripes are 40 μm wide and the spaces between them 60 μm, that number should be ~60%, or 2-3 times the value observed.[1] Although some of this difference could reflect the fact that, prior to the addition of galectin-1 22 hours before fixation, neurites were initially confined to stripes, and fiber-fiber interactions or directional persistence may have favored them remaining so, this effect seems unlikely to be responsible for such a large difference between observed and predicted outcomes. Precisely how much of the difference is due to bundling or persistence could be resolved by repeating the galectin-1 experiments on substrata consisting of laminin squares, rather than laminin stripes (cf. Ch. 3, Fig. 4). The observation that galectin-1 can essentially equalize the neurite outgrowth-promoting abilities of laminin and UV-inactivated laminin substrata, but apparently causes only a partial disruption of guidance, supports the view that relative differences in the growth-promoting ability of substrata may play a role in axon guidance, but cannot be the whole story.

Note that the effects of both pertussis toxin and galectin-1 are distinct from the effect observed with cytochalasin B, which does not cause neurites to leave the laminin stripes. This comparison is interesting in light of other studies showing that *in vivo* application of cytochalasins in some cases causes axonal pathfinding errors and that filopodial contact with guidance cues can alter a growth cone's direction (e.g., Chien et al., 1993; O'Connor et al., 1990). The comparison also shows that, however pertussis toxin acts on neurites,

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[1]It is interesting to try this calculation for neurites treated with pertussis toxin, which also causes 20-30% of neurite mass to leave laminin stripes. In this case, it is also true that 60% of the substratum lies between the laminin stripes, but here, measurements of neurite length on uniform active and inactive laminin substrata indicate that neurites traveling on the inactive laminin are expected to progress only 1/10th as far, during any given period, as those traveling on active laminin. Taking this into account, it is apparent that pertussis toxin causes substantially more neurite misguidance than galectin-1.

it is apparently not acting only by globally disrupting actin polymerization. It would be interesting to treat neurites extending on patterned laminin with both cytochalasin B and pertussis toxin. If the effect of the two treatments combined resembled one the treatments alone, this would suggest that the targets of pertussis toxin and cytochalasin are in the same pathway.

*The growth cone has a common mechanism to integrate disparate types of guidance cues*

Several molecules that induce growth cone collapse have been shown to alter the direction of neurite growth *in vitro* (Baier and Bonhoeffer, 1992; Fan and Raper, 1995) and are candidate *in vivo* inhibitory guidance molecules. In fact, the B oligomer of pertussis toxin (see below) blocks growth cone collapse induced by two different factors: a crude collapsing factor from embryonic chick brain (crude collapsin-1) and thrombin (Ch. 3 Fig. 8; Ch. 4, Fig. 5). It would be useful to confirm that the effect of pertussis toxin on brain-membrane induced growth cone collapse is not limited to blocking collapse (a somewhat artificial assay) but is actually related to the guidance-inhibiting effects of the molecule inducing collapse. For example, one could test if pertussis toxin blocks the observed turning of growth cones from collapsin-coated beads (Fan and Raper, 1995). Or, one could test if pertussis toxin treatment causes neurites that normally avoid collapsin-1-bearing substrata to grow on this substratum.

While collapsin-1 is believed to affect growth cones through an as yet unidentified cellular receptor, thrombin is known to act by both receptor-independent and receptor-mediated mechanisms. First, thrombin is a serine protease, and so could act by cleaving cell surface molecules or by altering the extracellular environment of the growth cone--for example, by proteolytically cleaving the substratum-bound laminin (Liotta et al., 1981). Second, thrombin could act by a receptor-mediated mechanism. The thrombin receptor is widespread in the nervous system, and is known to be expressed in (rat) dorsal root ganglia (Niclou et al., 1994). Thrombin activates this G protein-

coupled receptor by cleaving a 41 amino acid (for the human thrombin receptor) peptide off the N-terminal of the receptor. The new N-terminal is believed to serve as an activating peptide, as exogenously applied peptides with the new N-terminal sequence can activate the receptor (Vu et al., 1991). Such a peptide can mimic thrombin-induced neurite retraction by mammalian neurons, indicating that this response is receptor-mediated (Jalink and Moolenaar, 1992; Suidan et al., 1992).

Using a thrombin receptor peptide (TRP) to induce growth cone collapse would be advantageous for this study because, if pertussis toxin (or its B oligomer) could block TRP-induced collapse, it would strongly demonstrate that pertussis toxin could interfere with thrombin receptor-mediated events (see below). However, the sequence of the thrombin receptor in chicken (or other avian species) is not known, and the effects of the agonist peptide appear to be species selective (Connolly et al., 1994; Gerszten et al., 1994). While murine or human TRP's could certainly be tested, they might not induce growth cone collapse in the chick DRG neurons due to the species difference. Alternatively, the bioactive phospholipid lysophosphatidic acid (LPA) could be used in these assays as the agent inducing neurite retraction. LPA, the primary collapsing factor in serum, is believed to induce neurite retraction and growth cone collapse via a receptor distinct from the thrombin receptor. Yet, it mimics the effects of thrombin with regard to the morphological changes induced in cells and neurites, and is believed to share downstream targets with thrombin (Bend et al., 1992; Jalink et al., 1993, 1994). If LPA induces growth cone collapse in DRG neurons, and B oligomer blocks this collapse activity, it would suggest that the B oligomer is interfering with a receptor-mediated event with some known downstream signaling events.

The observation that pertussis toxin blocks both thrombin-induced neurite retraction and laminin-mediated guidance raises the possibility that these two events are mediated by related mechanisms. Some intracellular signaling events involved in TRP- and LPA-

mediated neurite retraction have been identified. For example, treatment of neuronal cells with the C3 exoenzyme from *Clostridium botulinum*, which selectively inhibits the small GTP-binding protein rho by ADP-ribosylation, blocks TRP- and LPA- induced neurite retraction (Jalink, et al., 1994). The members of the rho family have been shown to play a role in regulating the organization of actin in non-neuronal cells (for review see Hall, 1993). For example, microinjection of constitutively active RhoA protein into fibroblast cells induces the formation of stress fibers, and inhibition of rho activity with the C3 exoenzyme makes cells refractory to growth factors that normally induce focal contacts and stress fiber formation (Paterson et al., 1990; Ridley and Hall, 1992). It is conceivable that rho proteins could regulate the movement of actin in response to a growth cone encountering a stimulus, and thus rho activity could be required for neurite guidance as well as for thrombin-induced neurite retraction. In this case, application of the C3 exoenzyme might cause neurite wandering. This drug became commercially available while this thesis was written, and would be a good candidate to test in the patterned laminin guidance assay. Because this drug is not membrane permeable for all cell types, it would be important to test that the C3 treatment used for the guidance assay can ADP-ribosylate rho in these intact cells. This could be done by comparing the ability of cellular homogenates from C3-pretreated and -untreated intact DRG neurons to be further ADP-ribosylated by C3 exoenzyme, as detected by $^{32}$P-NAD incorporation (see Jalink et al., 1994). If the C3 exoenzyme were able to permeate the intact cells and ADP-ribosylate its substrate, then the amount of *de novo* ADP-ribosylation by C3 in the homogenates would be decreased. If C3 exoenzyme did disrupt the ability of neurites to respond to a laminin pattern, this would suggest that a rho protein may play a role in laminin-mediated neurite guidance.

*First steps toward the identification of a pertussis toxin "receptor"*

A number of experiments together strongly suggest that pertussis toxin affects neurites by interacting with cellular N-linked complex-type carbohydrates. First, the B oligomer of pertussis toxin, which lacks the subunit containing the ADP-ribosyltransferase that

inactivates G proteins, is equipotent at causing neurite wandering and blocking brain membrane-induced growth cone collapse (Ch. 3, Fig. 6). The B oligomer contains two carbohydrate binding sites. Second, treatment with deoxymannojirimycin (DMN), an inhibitor of N-linked complex-type glycosylation, blocks the effect of pertussis toxin on neurite guidance (ibid.). Third, N-linked complex-type carbohydrates on laminin are not necessary for pertussis toxin's effect on neurons (Ch. 3, Fig. 7). Finally, the plant lectin from *Maackia amurensis* (MAL), which binds to a subclass of sialylated carbohydrates, also causes neurites to wander from laminin stripes and squares (Ch. 4, Fig. 1-2).

Given that cellular N-linked complex-type carbohydrates are necessary, pertussis toxin could influence neurite behavior by binding to cell surface glycoproteins. In one model, this binding could interfere with growth cone guidance by directly blocking the interaction of growth cone receptors with guidance cues. This model would require a common "guidance receptor" for disparate molecules such as laminin and thrombin. Alternatively, pertussis toxin binding could elicit signals that interfere with the normal functioning of the growth cone when it encounters a discontinuous substratum. For example, pertussis toxin could mimic a natural ligand for a signaling receptor. There is precedent for pertussis toxin co-opting a cell surface receptor and inducing the same physiological effect as the normal ligand. For example, the B oligomer of pertussis toxin has been shown to activate platelets by binding to platelet glycoprotein Ib, a receptor for a natural ligand, von Willebrand factor, whose normal function is to activate platelets (Sindt et al., 1994). Note that this putative natural ligand would not need to be a lectin, though this is certainly an intriguing possibility.

Identification of the molecules on DRG neurons to which pertussis toxin binds is an important step in determining how the toxin alters neurite behavior. To this end, radioiodinated DRG neuronal extracts were passed over a pertussis toxin affinity column and proteins eluted with a trisaccharide, sialyllactose. This procedure

yielded many proteins as identified by gel electrophoresis of the eluate (Ch. 4, Fig. 3 and Table 2). Several experiments could confirm which of the observed bands on the gel actually represent cellular proteins that bind pertussis toxin via their N-linked complex-type carbohydrates. First, metabolic labeling of the DRG neurons with $^{35}S$ methionine (instead of directly radioiodinating cell extracts) would have the advantage of labeling only cellular proteins, not residual proteins from the culture medium or the substratum-bound laminin. Second, protein extracts from DMN-treated neuronal cultures could be prepared in parallel and subjected to pertussis toxin affinity chromatography. As DMN treatment of neurons blocks the biological effects of pertussis toxin, a protein important to the neurites' response to pertussis toxin would be expected to be absent from the eluate when extracts from neurons treated with DMN are passed over the column.

The eventual purification of the biologically relevant pertussis toxin-binding proteins could take advantage of the availability of other lectins that do and do not mimic the effects of pertussis toxin. As mentioned above, MAL mimicked the guidance effect of pertussis toxin while lectin from *Sambucus nigra* (SNA) did not. These results suggest the following scheme for purification: passing neuronal extracts over a SNA column and taking the flow through for passage over a pertussis toxin column. The eluted material (with the eluting sugar removed) could then be passed over a MAL column. Preliminary results suggest that some proteins from the SNA "flow through" do bind to a pertussis toxin column. However, it is possible that the biologically relevant pertussis toxin-binding proteins also bind to SNA, but via a different carbohydrate residue (containing sialic acid linked 2-6 and not 2-3) situated in the protein such that the binding of SNA does not have the same biological effect as the binding of MAL or pertussis toxin. Alternatively, SNA might bind the "pertussis toxin receptor" with a low affinity such that an interaction with immobilized SNA occurs, even though the lectin is not effective in the biological assay at the concentrations used.

Using purified neuronal preparations as the source for a biochemical purification has the advantage that any cellular proteins eluted from the column are more likely to be derived from neurons and not non-neuronal cells. However, the quantity of material that can be obtained by culturing these primary neurons is quite small. Approximately 6 million ganglia would be required to generate 10 μg of pertussis toxin column eluate, based on the yields of protein extract from the DRG neuronal cultures and the fraction of protein and counts eluted from the WGA and pertussis toxin columns, respectively (see Ch. 4 Methods). In future experiments, more abundant sources of material, such as whole dorsal root ganglia or embryonic chick brain, are more likely to be used to compile enough material to generate, for example, antibodies against pertussis toxin-binding proteins. In these cases, the extracts could be treated with sialidases to remove sialic acid residues, and the bands missing from the elution profile of the enzymatically treated material would represent candidates for carbohydrate-dependent pertussis toxin-binding proteins. One drawback of this control is that not all sialic acid residues are equally susceptible to sialidase treatment. However, if the same enzymatic treatment blocked the neuronal response to pertussis toxin in intact cells, this would indicate that the biological relevant pertussis toxin-binding proteins were susceptible to the treatment. Sialidase treatment has been shown to block B oligomer-mediated effects of pertussis toxin in other cell types and to inhibit the binding of pertussis toxin to proteins immobilized on nitrocellulose (Brennan et al., 1988; Witvliet et al., 1992).

*Identification of two distinct carbohydrate-mediated effects on neurons*

Another lectin-like molecule, galectin-1, was tested in the guidance assay. Galectin-1, like pertussis toxin, caused neurites to leave laminin stripes, but, more strikingly, the lectin also caused a significant increase in neurite length on the discontinuous laminin stripes (Ch. 5, Fig. 1). Galectin-1 treatment also caused a marked increase in neurite extension on uniform UV-inactivated laminin (Ch. 5, Fig. 2). This result suggests that, unlike pertussis toxin, galectin-

1's effect on neurite behavior on the discontinuous substratum was caused by a change in the neurites' response to the UV-inactivated laminin.

It is interesting that, as lectins, galectin-1 and pertussis toxin have similar carbohydrate binding properties yet have different effects on neurons. Pertussis toxin is expected to bind to sialyllactosamine-containing structures. Galectin-1 binds preferentially to lactosamine-bearing structures and terminal sialic acid linked 2-3 (though not 2-6) is tolerated (Leffler and Barondes, 1986). They are both dimeric lectins that could potentially act on cells by crosslinking cell surface molecules. They both bind to laminin (Witvliet et al., 1989; Zhou and Cummings, 1990). However, laminin's N-linked complex-type carbohydrates are not necessary for the effects of pertussis toxin whereas galectin-1 seems to require an appropriate carbohydrate-bearing substratum, as it potentiates neurite outgrowth on asialofetuin but not on bovine serum albumin (Ch. 5 Fig. 3 and Table 1). For both lectins, their effects on DRG neurons (i.e., disruption of guidance for pertussis toxin and increased neurite outgrowth on asialofetuin for galectin-1) require cellular N-linked complex-type carbohydrates (Ch. 5, Fig. 4). Yet, their biological effects suggest that they are acting through distinct cellular receptors. While the cellular receptors mediating pertussis toxin's effects on neurons are as yet unknown, one candidate receptor for galectin-1 has been identified, as galectin-1-mediated neurite outgrowth is sensitive to anti-β1 integrin antibodies (Ch. 5, Fig. 5). Evidence for a direct interaction between galectin-1 and a β1 integrin will be sought by determining if β1 integrins from DRG neuronal extracts interact with immobilized galectin-1.

Galectin-1 is expressed at the time and place in the developing rat to participate in axonal elongation and/or guidance (Hynes et al., 1990). Preliminary studies in this thesis indicate that a subset of rat DRG neurons, like chick DRG neurons, respond to galectin-1 when plated on an asialofetuin substratum (Ch. 5, Fig. 6 and Table 2). There is some evidence from other studies that the same DRG neurons that secrete galectin-1 also bear the carbohydrate epitopes that would

enable them to respond to the lectin (Regan et al., 1986). Using antibodies to these epitopes (e.g., Dodd and Jessell, 1985), it would be possible to determine if the subset of DRG neurons that extend neurites in response to galectin-1 *in vitro* do indeed bear these carbohydrates. If so, this result would be consistent with a model in which galectin-1 stimulates axonal extension *in vivo* by an autocrine and/or paracrine mechanism.

*What have we learned about axon guidance?*

The molecular complexity of the environment in which an axon travels and the specificity with which it attains its targets suggests that the axon and its growth cone are capable of integrating a range of stimuli into fairly precise behaviors. Guidance was previously explained by some by reference to adhesive interactions, i.e., the substratum to which the growth cone adheres best is selected. However, a number of studies have demonstrated that neurites do not always chose to grow on the substratum to which they adhere best (e.g., Gundersen, 1987; Lemmon et al., 1992). Thus, growth cone behavior cannot be predicted by adhesive measurements alone. Nevertheless, the intracellular events that accompany an adhesive interaction, such as reorganization of the actin cytoskeleton and tyrosine phosphorylation, could certainly play a role in growth cone motility and/or guidance. Similarly, the data presented in this thesis do not imply that differences in rate of neurite outgrowth are irrelevant to guidance, but suggest that there are other events in guidance that are not immediately dependent on the rate and extent of neurite outgrowth. This is true for eukaryotic chemotaxis; increase in speed is only one of the changes a cell can undergo in response to a chemoattractant (Devreotes and Zigmond, 1988).

How could a treatment affect guidance but not growth? One attractive model is that a growth cone elongating on uniform laminin would theoretically receive a laminin "signal" everywhere, while a growth cone in contact with both laminin and non-laminin--a situation where guidance would be required--will only be receiving that signal from the part in contact with laminin. Just as a migrating cell in a

gradient is though to "measure" the levels of chemoattractant across its cell body, so might the growth cone need to measure the relative levels of laminin with which it is in contact in order to be guided. If the intracellular "machinery" for spatial measurement is sensitive to pertussis toxin, then a pertussis toxin treated growth cone, while still able to recognize laminin and grow on it, will not be able to respond to a change in laminin concentration.

In summary, pertussis toxin and other lectins may provide the basis for a novel approach to axon guidance.

## References


Baier, H., and Bonhoeffer, F. (1992). Axon guidance by gradients of a target-derived component. Science *255*, 472-475.

Bend, R. L. v. d., Brunner, J., Jalink, K., Corven, E. J. v., Moolenaar, W. H., and Blitterswijk, W. J. v. (1992). Identification of a putative membrane receptor for the bioactive phospholipid, lysophosphatidic acid. EMBO J. *11*, 2495-2501.

Brennan, M. J., David, J. L., Kenimer, J. G., and Manclark, C. R. (1988). Lectin-like binding of pertussis toxin to a 165-kilodalton chinese hamster ovary cell glycoprotein. J. Biol. Chem. *263*, 4895-4899.

Calof, A. L., and Lander, A. D. (1991). Relationship between neuronal migration and cell-substratum adhesion: laminin and merosin promote olfactory neuronal migration but are anti-adhesive. J. Cell Biol. *115*, 779-794.

Chien, C.-B., Rosenthal, D. I., Harris, W. A., and Holt, C. E. (1993). Navigational errors made by growth cones without filopodia in the embryonic *Xenopus* brain. Neuron *11*, 237-251.

Colognato-Pyke, H., O'Rear, J. J., Yamada, Y., Carbonetto, S., Cheng, Y. S., and Yurchenco, P. D. (1995). Mapping of network-forming, heparin-binding, and alpha 1 beta 1 integrin-recognition sites within the alpha-chain short arm of laminin-1. J. Biol. Chem. *270*, 9398-9406.

Connolly, T. M., Condra, C., Feng, D. M., Cook, J. J., Stranieri, M. T., Reilly, C. F., Nutt, R. F., and Gould, R. J. (1994). Species variability in platelet and other cellular responsiveness to thrombin receptor-derived peptides. Thromb. Haemost. *72*, 627-633.

Devreotes, P. N., and Zigmond, S. H. (1988). Chemotaxis in eukaryotic cells. Ann. Rev. Cell Biol. *4*, 649-686.

Dodd, J., and Jessell, T. M. (1985). Lactoseries carbohydrates specify subsets of dorsal root ganglion neurons projecting to the superficial dorsal horn of rat spinal cord. J. Neurosci. *5*, 3278-3294.

Fan, J., and Raper, J. A. (1995). Localized collapsing cues can steer growth cones without inducing their full collapse. Neuron *14*, 263-274.

Gerszten, R. E., Chen, J., Ishii, M., Ishii, K., Wang, L., Nanevicz, T., Turck, C. W., Vu, T.-K. H., and Coughlin, S. R. (1994). Specificity of the thrombin receptor for agonist peptide is defined by its extracellular surface. Nature *368*, 648-651.

Gundersen, R. W. (1987). Response of sensory neurites and growth cones to patterned substrata of laminin and fibronectin *in vitro*. Dev. Biol. *121*, 423-431.

Hall, A. (1993). Ras-related proteins. Curr. Opin. Cell Biol. 5, 265-268.

Hynes, M. A., Gitt, M., Barondes, S. H., Jessell, T. M., and Buck, L. B. (1990). Selective expression of an endogenous lactose-binding lectin gene in subsets of central and peripheral neurons. J. Neurosci. *10*, 1004-1013.

Jalink, K., Corven, E. J. v., Hengeveld, T., Morii, N., Narumiya, S., and Moolenaar, W. H. (1994). Inhibition of lysophosphatidate- and thrombin-induced neurite retraction and neuronal cell rounding by ADP ribosylation of the small GTP-binding protein rho. J. Cell Biol. *126*, 801-810.

Jalink, K., Eichholtz, T., Stam, F. R. P., Croven, E. J. v., and Moolenaar, W. H. (1993). Lysophosphatidic acid induces neuronal shape changes via a novel, receptor-mediated pathway: similarity to thrombin action. Cell Growth Diff. 4, 247-355.

Jalink, K., and Moolenaar, W. H. (1992). Thrombin receptor activation causes rapid neural cell rounding and neurite retraction independent of classic second messengers. J. Cell Biol. *118*, 411-419.

Lal, R., and John, S. A. (1994). Biological applications of atomic force microscopy. Am. J. Physiol. *266*, C1-C21.

Leffler, H., and Barondes, S. H. (1986). Specificity of binding of three soluble rat lung lectins to substituted and unsubstituted mammalian B-galactosides. J. Biol. Chem. *261*, 10119-10126.

Lemmon, V., Burden, S. M., Payne, H. R., Elmslie, G. J., and Hlavin, M. L. (1992). Neurite growth on different substrates: permissive versus instructive influences and the role of adhesive strength. J. Neurosci. *12*, 818-826.

Liotta, L. A., Goldfarb, R. H., Brundage, R., Siegal, G. P., Terranova, V., and Garbisa, S. (1981). Effect of plasminogen activator, plasmin and thrombin on glycoprotein and collagenous components of basement membranes. Cancer Res. *41*, 4629-4636.

Lochter, A., Vaughan, L., Kaplony, A., Prochiantz, A., Schachner, M., and Faissner, A. (1991). J1/tenascin in substrate-bound and soluble form displays contrary effects on neurite outgrowth. J. Cell Biol. *113*, 1159-1171.

Marsh, L., and Letourneau, P. C. (1984). Growth of neurites without filopodial or lamellipodial activity in the presence of cytochalasin B. J. Cell Biol. *99*, 2041-2047.

Nermut, M. V., Green, N. M., Eason, P., Yamada, S. S., and Yamada, K. M. (1988). Electron microscopy and structural model of human fibronectin receptor. EMBO J. *7*, 4093-4099.

Niclou, S., Suidan, H. S., Brown-Luedi, M., and Monard, D. (1994). Expression of the thrombin receptor mRNA in rat brain. Cell. Mol. Biol. *40*, 421-428.

O'Connor, T. P., Duerr, J. S., and Bentley, D. (1990). Pioneer growth cone steering decisions mediated by single filopodial contacts *in situ*. J. Neurosci. *10*, 3935-3946.

Oohira, A., Matsui, F., and Katoh-Semba, R. (1991). Inhibitory effects of brain chondroitin sulfate proteoglycans on neurite outgrowth from PC12D cells. J. Neurosci. *11*, 822-827.

Paterson, H. F., Self, A. J., Garrett, M. D., Just, I., Aktories, K., and Hall, A. (1990). Microinjection of recombinant $p21^{rho}$ induces rapid changes in cell morphology. J. Cell Biol. *111*, 1001-1007.

Regan, L. J., Dodd, J., Barondes, S. H., and Jessell, T. M. (1986). Selective expression of endogenous lactose-binding lectins and lactoseries glycoconjugates in subsets of rat sensory neurons. Proc. Natl. Acad. Sci U.S.A. *83*, 2248-2252.

Ridley, A. J., and Hall, A. (1992). The small GTP-binding protein rho regulates the assembly of focal adhesion and actin stress fibers in response to growth factors. Cell *70*, 389-399.

Schittny, J. C., Timpl, R., and Engel, J. (1988). High resolution immunoelectron microscopic localization of functional domains of laminin, nidogen, and heparan sulfate proteoglycan in epithelial basement membrane of mouse cornea reveals different topological orientation. J. Cell Biol. *107*, 1599-1610.

Sindt, K. A., Hewlett, E. L., Redpath, G. T., Rappuoli, R., Gray, L. S., and Vandenberg, S. R. (1994). Pertussis toxin activates platelets through an interaction with platelet glycoprotein Ib. Infect. Immun. *62*, 3108-3114.

Suidan, H. S., Stone, S. R., Hemmings, B. A., and Monard, D. (1992). Thrombin causes neurite retraction in neuronal cells through activation of cell surface receptors. Neuron *8*, 363-375.

Vu, T.-K. H., Hung, D. T., Wheaton, V. I., and Coughlin, S. R. (1991). Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. Cell *64*, 1057-1068.

Witvliet. M. H., Burns, D. L., Brennan, M. J., Poolman, J. T., and Manclark, C. R. (1989). Binding of pertussis toxin to eukaryotic cells and glycoproteins. Infect. Immun. *57*, 3324-3330.

Witvliet, M. H., Vogel, M. L., Wiertz, E. J. H. J., and Poolman, J. T. (1992). Interaction of pertussis toxin with human T lymphocytes. Infect. Immun. *60*, 5085-5090.

Zhou, Q., and Cummings, R. D. (1990). The S-type lectin from calf heart tissue binds selectively to the carbohydrate chains of laminin. Arch. Biochem. Biophys. *281*, 22-35.