Pertussis toxin specifically inhibits growth cone guidance by a mechanism independent of direct G protein inactivation.
Pertussis Toxin Specifically Inhibits Growth Cone Guidance by a Mechanism Independent of Direct G Protein Inactivation

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Summary

An assay employing patterned laminin substrata was used to screen for compounds that disrupt neurite guidance. One molecule, pertussis toxin, caused neurites to wander from patterns that normally guided them, yet had no significant effect on rates of neurite outgrowth. Wandering was greatest on patterns requiring frequent guidance (e.g., laminin stripes with periodic gaps). Surprisingly, the B oligomer of pertussis toxin, which lacks the subunit that inactivates G proteins, was equipotent at disrupting neurite guidance. Pertussis toxin probably acts by binding cell surface carbohydrates, since neurites lacking complex-type N-linked oligosaccharides were insensitive to the effects of the toxin. The B oligomer also blocked growth cone collapse induced by a brain membrane-derived factor; such factors are thought to act as repulsive guidance cues in vivo. That a single reagent can inhibit neuronal responses to both attractive and repulsive guidance cues suggests that such cues may share signaling pathways.

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 these molecules stimulate neurite growth; others mediate or regulate fasciculation of neurites; others act as soluble chemoattractants; others influence rates of neurite elongation; others render substrata nonpermissive 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; Letourneau et al., 1994; also see Pittman et al., 1989; O'Leary et al., 1991; Ochira 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, multidomain 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 (also called EHS laminin and, hereafter, simply "laminin") both promotes neurite outgrowth (i.e., it increases neurite initiation and rates of neurite elongation) and, if presented in a pattern such as stripes, guides growth cones (Hammaback 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.

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. In brief, a laminin-coated glass coverslip is exposed to ultraviolet (UV) 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.
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 (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 (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 Experimental Procedures. 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, which 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 nonneuronal cells surrounding each ganglion. Bar, 100 μm.

(C) 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, and 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. Note that the dimensions of regions of immunoreactive laminin are somewhat smaller than the dimensions of the chrome-coated areas of the photomask (see [A]). This was presumably due to refraction and reflection of ultraviolet light during irradiation. Bar, 50 μm.

Figure 2. Effect of Pertussis Toxin on Neurite Outgrowth on Patterned Laminin

(A and 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) shows representative neurites growing in the absence of drug. (B) shows representative neurites treated with pertussis toxin (0.5 μg/ml). The arrow marks the beginning of the outer zone. As shown by (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 Experimental Procedures). Each datapoint represents the mean ± SEM of values obtained from at least three groups of four 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 0 (open squares), 50 ng/ml (closed triangles), 250 ng/ml (closed squares), or 500 ng/ml (closed circles).
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 Experimental Procedures). 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 (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 20 μm gaps (Figure 1C). Measuring neurite bundle lengths from the beginning of the outer zone and pooling the data from each kind of stripe confirmed that mean neurite progression in the outer zone fell steadily as a function of gap size (data not shown).

These behaviors of neurites are consistent with previous observations that 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, whereas 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. In fact, cytochalasin B, an inhibitor of actin polymerization, had such an effect. At 50 ng/ml, it decreased the distance traveled by neurite bundles on uninterrupted laminin stripes by only 28%, whereas it reduced mean outgrowth on interrupted laminin stripes by 68% (5 μm gaps) and 93% (20 μm gaps; data not shown). Since cytochalasin B interferes with the formation of filopodia (see Maroh and Letourneau, 1984), and filopodia have been implicated in the crossing of laminin gaps (Hammarback and Letourneau, 1986), this result was not unexpected.

Several other reagents suspected to affect signaling processes within growth cones were also tested. Most had no detectable effect on neurite outgrowth on either interrupted or continuous stripes; these included cholera toxin (1–500 ng/ml), 8-bromo-cyclic GMP (1 mM), phosphoinositol-specific phospholipase C (0.05 U/ml), chondroitinase ABC (0.1 U/ml), and heparin (0.5–1.0 μg/ml; data not shown). Others strongly inhibited neurite outgrowth on all stripes; these included phorbol 12-myristate 10-acetate (1 μM) and staurosporine (10 μM).

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 (Figures 2A and 2B).

As shown in Figure 2C, 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 2C, 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 Inhibits Neurite 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 that multiple 40 μm squares of active laminin were separated by 25 μm gaps of inactivated laminin (Figure 3A). 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 Figures 3B–3E. Most neurites remained confined to the laminin square on which they originated, with only the occasional filopodium leaving the squares (e.g., Figure 3E). This is not surprising, given that the gaps between the squares exceed what these neurites seem to be able to cross (see Figure 1C; 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 3F–3J). This occurred regardless of whether pertussis toxin was added at the time of cell plating or 1 hr later, when cells had already attached (data 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 3K, 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 legend to Figure 3). In un-
Neuron

Figure 3. 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 (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 \( \mu \)m squares of active laminin, spaced 25 \( \mu \)m apart. (B-E) Laminin-coated coverslips were patterned using the photomask depicted in (A). Dissociated dorsal root ganglion neurons were plated at 5000 cells per 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; these have been marked on the photomicrographs with dotted white lines. Note that the actual dimensions of the laminin squares were somewhat smaller than the chrome squares on the photomask; as in Figure 1 and Figure 2, this was presumably due to refraction and refraction of ultraviolet light during irradiation. Bar, 10 \( \mu \)m.

(F-I) Phase contrast micrographs of representative neurons from cultures prepared as in (B-E), except that pertussis toxin (0.8 \( \mu \)g/ml) was included in the culture medium at the time of plating. Laminin squares are illustrated as above. (K) Neurons cultured as in (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 (closed bars) of pertussis toxin (0.8 \( \mu \)g/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, with 100 neurite-bearing neurons counted in each case. In category I, the neurite did not extend beyond the boundary of the laminin square (e.g., [B]); category II, the neurite left the laminin square but did not contact any adjacent laminin square (e.g., [I]); category III, the neurite reached one but not two adjacent squares (e.g., [G]); category IV, the neurite extended at least two squares beyond its square of origin (e.g., [J]). The normal confinement of neurites to laminin squares is markedly disrupted by pertussis toxin (p < .005 by \( \chi^2 \) analysis).

treated 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 the Ability of Laminin 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 (data not shown). This result, combined with the observation that pertussis toxin–treated neuritis sometimes appeared to travel preferentially from one laminin square to another (e.g., Figure 3J), 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 4, neither neurite initiation nor outgrowth on active laminin (Figure 4A) or inactivated laminin (Figure 4B; 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 \( \mu \)g/ml) or vehicle alone was added to cultures of dissociated neurons plated on uniform, active laminin and rates of neurite elongation were measured 3–5 hr later, the mean values obtained were not statistically different (111 ± 25 \( \mu \)m/hr [SD] vs. 100 ± 27 \( \mu \)m/hr, respectively; n = 15; p > .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 \( \mu \)g/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 S1 subunit of the toxin (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 Boligomer, that lacks the S1 subunit (Tamura et al., 1983; Gray et al., 1989; for review, see Kaslow and Burns, 1992). Apparently, the effect of pertussis toxin on neurite guidance falls into this latter category. In assays of neurite growth on continuous and interrupted
Penicillin Toxin Inhibits Neurite Guidance

Figure 4. Lack of Effect of Pertussis Toxin on Neurite Outgrowth on Unpatterned Substrata

Laminin coverslips were prepared as described in Experimental Procedures 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 (closed circles) or absence (open circles) of pertussis toxin (0.6 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 point of origin of the neurite 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 (see Neugebauer et al., 1966) and reveal no significant effect of pertussis toxin on either substratum (for mean neurite length, p > .5 in both cases by Student's t test).

Figure 5. 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

(A) Data from cultures treated with 0.5 μg/ml pertussis toxin (closed circles); an equimolar amount (0.66 μg/ml) of the B oligomer of pertussis toxin (open circles); or no drug (open squares). 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 from the laminin stripes, even though the same amount of the toxin caused extensive wandering of neurites from non-DMN-treated ganglia (Figure 5B). These data indicate that interaction of pertussis toxin with cellular N-linked carbohydrates is necessary for pertussis toxin to cause neurite wandering on laminin substrata. In addition, it was found that pretreatment of patterned laminin substrata with pertussis toxin failed to cause neurite wandering (substrata were pretreated with 2.5 μg/ml pertussis toxin, washed, and neurones cultured for 12 hr; under these conditions the amount of substratum-bound toxin at the end of the experiment was at least as great as when 0.5 μg/ml was added to the culture medium at the time of cell plating [data not shown]).

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
be 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 (see 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 somitely 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 6). 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 to two very different types of guidance cues.

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 (see Figure 1) or widely spaced laminin squares (see Figure 3). 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.

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 (see Figure 2). 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 neurones (see Figure 3). 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 (see Figure 3). The effect did not depend upon the addition of pertussis toxin at the time of cell or ganglion plating (see Experimental Procedures), 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 "recaptured" by the pattern (see Figure 3J).

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, though plausible, has been largely discredited, not only for laminin but for other guidance molecules (Gundersen, 1987; Lemmon et al., 1992; Gomez and Letourneau, 1994; Zheng et al., 1994).

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 (see Figure 4). Such a mechanism of neurite guidance can be seen as a special case of chemokinesis, the phenomenon in which motile cells are guided toward a chemoattractant simply because their rate of random motility is increased by the chemoattractant (see Devreotes and Zigmond, 1988).

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.

This conclusion raises the possibility that laminin guides by a means analogous to chemotaxis, the phenomenon...
in which cells migrate toward a chemoattractant because individual cells measure local differences in chemoattractant level and reorient to move in the direction of increased concentration (Devreotes and Zigmond, 1988). Fundamentally, chemotaxis requires cells to make and compare local measurements of chemoattractant concentration and direct their movement accordingly. When growth cones confront laminin borders, they do much the same thing. Filopodia and lamellipodia that explore the substratum may make individual determinations of the presence or absence of laminin on either side of a border, and the machinery of the growth cone may integrate those measurements so as to determine a direction for growth.

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 (see Figure 5A), 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 presence of laminin (see Figure 5B) and that prebinding of pertussis toxin to laminin substrata fails to perturb neurite guidance (data not shown) strongly suggest that the toxin inhibits neurite guidance by binding to neuronal glycoproteins via their N-linked complex-type carbohydrate chains. That such carbohydrate-mediated binding is not only necessary for the disruption of neurite guidance but probably is also sufficient is suggested by the fact that wheat germ agglutinin (a plant lectin with a broad binding specificity that includes sialic acids), at appropriate doses (~2.5 μg/ml), can partially mimic the effects of pertussis toxin on neurite guidance (unpublished data).

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). Preliminary results from pertussis toxin blotting of SDS-PAGE-fractionated dorsal root ganglion membrane proteins suggest that the same is likely to be true for neuronal cells (unpublished data). 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; Saukkonen et al., 1992; Stein et al., 1994; for review, see Kaslow and Burns, 1992). However, 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 (Tomasselli 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 (see Figure 4) suggests that integrins might not be among the targets of the toxin, 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 the receptors of pertussis toxin on neuronal cells may lead to the identification of novel growth cone signaling molecules.

The observation that the B oligomer inhibits growth cone collapse (see Figure 6) 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 (see Baier and Bonhoeffer, 1992), their effects are essentially opposite in direction to those of laminin; i.e., growth cones prefer to grow where collapsing factors are not. 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.

**Experimental Procedures**

**Materials**

Chrome-on-quartz photomasks (thickness, 0.09 inch) were fabricated by Advance Reproductions (North Andover, MA). Laminin was purified from the mouse Engelbreth Holm Swarm sarcoma (Kleinman et al., 1982; Timpl et al., 1992). Rabbit anti-mouse laminin serum was pur-
chased from Polysciences (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-PAGE. Tissue culture media were purchased from Gibco-BRL or Caligro-Mediatech. Unless noted, all other reagents were from Sigma.

Preparation of Substrate

Laminin (20 μg/ml) was applied to sterile, acid-washed 12 mm round coverslips (number 1 thickness, Propper, Long Island City, NY) in calcium- and magnesium-free Dulbecco’s PBS and cleaned in holding medium (Leibovitz L15 medium with 0.5% glucose, 100 U/ml penicillin, and 100 μg/ml streptomycin) for 4 hr at 37°C, then washed five times with CMF-HBSS. To generate patterned substrata, laminin coverslips were washed five times with sterile distilled water, air dried, placed protein side down on a quartz photomask resting 0.5 cm above a short wave UV light source (UVP, model UVG-54, VWR Scientific), and irradiated for 10 min. In some cases, marks were made on the back of coverslips with an indelible lab marker to record pattern orientation.

Cell Isolation and Preparation

Fertile chicken eggs were obtained from Spafas (Storr’s, CT). Dorsal root ganglia from embryonic day 7–8 embryos were removed in sterile calcium- and magnesium-free Dulbecco’s 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 on –13 mm pieces of 8-0 nylron 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 1 (1 mg/ml) and soybean trypsin inhibitor (0.25 mg/ml) for 2 min, washed with complete medium (see below), and dissociated by trituration (10–20 strokes using a flame-polished Pasteur pipet). Nonneuronal 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 × 10⁶ cells/ml. Cell suspension (100 μl) was applied to prepared coverslips in a paraffin-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 2% additives (Bottenstein and Sato, 1979). 1 mg/ml crystalline bovine serum albumin (ICN Biochemicals), 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin, and 20 ng/ml 2.5S NGF (Collaborative Research). Ganglia were cultured in an 8% CO₂ atmosphere, dissociated neurons in a 5% CO₂ atmosphere. After 24 hr, ganglion explant cultures were supplemented with 10 μM 5-fluoro-2-deoxyuridine to inhibit nonneuronal cell growth. Compounds (e.g., pertussis toxin) were added to cultures of intact ganglia or dissociated neurons at the time of plating, unless otherwise indicated. Compounds 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 1A. Compounds were added to dissociated neurons at the time of plating, unless otherwise indicated. Cultures were fixed in 4% formaldehyde, 0.5% 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 11:000 in PBS-BSA (PBS with 1% BSA) for >30 min at 37°C. 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 antirabbit 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 three 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 TitreTek slide (Fisher) in PBS and viewed using a Bio-Rad MRC-800 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 Axiopt 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 traveled 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 quantitate neurite wandering, a variation on the pattern in Figure 1A was used so that the type of stripe from which a neurite had wandered could be determined unambiguously. In brief, 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 4 similar stripes by using the confocal microscope to measure total, background-subtracted fluorescence in the fluorescein channel (which detects the cytoskeletal dye CMFDA) in the four rectangular areas (starting from the beginning of the outer zone of the pattern) representing the stripes themselves ("on"), as well as the five rectangular areas constituting the lanes between the stripes ("off"). These areas were identified by reference to images of laminin immunostaining.

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 0.6% glucose. The dish was overlayed with light mineral oil (Sigma) to prevent evaporation and maintained at 37°C ± 0.2°C using a PDM-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 illumination using VisioN software (version 2.80, 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 traveled during 4 min intervals. Plotting the running total of distance against the total time gave the rate of outgrowth as the slope.

Assay of Growth Cone Collapse

A crude fraction from embryonic day 10 chick brain membranes was prepared as described (Raper and Kapfhammer, 1990), dialyzed against PBS overnight, and, for an additional 2–4 hr, against basal F12 medium. The material was added directly to cultures at a 10-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.
Pertussis Toxin Inhibits Neurite Guidance

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