A Cytomechanical Investigation of Neurite Growth on Different Culture Surfaces

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Abstract. We have examined the relationship between tension, an intrinsic stimulator of axonal elongation, and the culture substrate, an extrinsic regulator of axonal elongation. Chick sensory neurons were cultured on three substrata: (a) plain tissue culture plastic; (b) plastic treated with collagen type IV; and (c) plastic treated with laminin.

Calibrated glass needles were used to increase the tension loads on growing neurites. We found that growth cones on all substrata failed to detach when subjected to two to threefold and in some cases 5-10-fold greater tensions than their self-imposed rest tension. We conclude that adhesion to the substrate does not limit the tension exerted by growth cones. These data argue against a "tug-of-war" model for substrate-mediated guidance of growth cones.

Neurite elongation was experimentally induced by towing neurites with a force-calibrated glass needle. On all substrata, towed elongation rate was proportional to applied tension above a threshold tension. The proportionality between elongation rate and tension can be regarded as the growth sensitivity of the neurite to tension, i.e., its growth rate per unit tension. On this basis, towed growth on all substrata can be described by the simple linear equation:

\[ \text{elongation rate} = \text{sensitivity} \times (\text{applied tension} - \text{tension threshold}) \]

The numerical values of tension thresholds and neurite sensitivities varied widely among different neurites. On all substrata, thresholds varied from near zero to >200 μdynes, with some tendency for thresholds to cluster between 100 and 150 μdynes. Similarly, the tension sensitivity of neurites varied between 0.5 and 5.0 μm/h/μdyne. The lack of significant differences among sensitivity or threshold values on the various substrata suggest to us that the substratum does not affect the internal "set points" of the neurite for its response to tension.

The growth cone of chick sensory neurons is known to pull on its neurite. The simplest cytomechanical model would assume that both growth cone-mediated elongation and towed growth are identical as far as tension input and elongation rate are concerned. We used the equation above and mean values for thresholds and sensitivity from towing experiments to predict the mean growth cone-mediated elongation rate based on mean rest tensions. These predictions are consistent with the observed mean values.

The environmental surface is one of the principal extrinsic regulators of axonal elongation (Letourneau, 1983; Reichardt et al., 1989; Sanes, 1989). The substratum surface plays a role in regulating axonal initiation (Ludueña, 1973; Collins, 1978; Smallheiser et al., 1984; Buettner and Pittman, 1991), axonal elongation rate (Ludueña, 1973; Bray et al., 1987; Kleitman and Johnson, 1989; Thomas et al., 1990; Buettner and Pittman, 1991), direction of growth (Letourneau, 1975; Gundersen, 1987; Burmeister and Goldberg, 1988), and branching (Bray et al., 1987; Burmeister and Goldberg, 1988; Buettner and Pittman, 1991).

Some of these effects seem to arise from cytomechanical interaction(s) between the neuronal growth cone and the different substrates. For example, growth cones pull (Lamoureux et al., 1989). A surface on which growth cones produced more traction force (pulled harder) could cause the growth cone to detach from a less adhesive surface. The growth cone would, thereby, steer the neurite in the direction of greater adhesion (Letourneau, 1983; Bray, 1987). This differential adhesion model may underlie growth cone guidance to particular pathways both in vitro and in vivo (Letourneau, 1983; Bray, 1987; Taghert et al., 1982; Nardi, 1983; Bastiani and Goodman, 1984; Caudy and Bentley, 1986).

A similar differential adhesion mechanism could underlie surface effects on axonal elongation rate. Recent evidence indicates that the rate of axonal elongation can be regulated by the tension exerted on the neurite (Zheng et al., 1991). Here again, if the pulling force exerted by a growth cone is limited by its adhesion, then elongation rates should correlate with surface adhesion. Similarly, tension experimentally applied to the margin of chick sensory neurons is known to initiate neurite outgrowth de novo (Bray, 1984; Zheng et al., 1991).
If this tension mechanism is part of normal growth cone–mediated neurite initiation and adhesion limits tension production, greater adhesion to a growth surface will enable incipient growth cones to exert greater pulling forces, thus increasing the rate of neurite initiation.

The above differential adhesion model postulates a permissive role for the substratum. That is, growth cone adhesion to surfaces limits their ability to exert tension. Surfaces allowing greater adhesion permits the growth cone to exert more tension. Alternatively, or in addition, the substrate may play an instructive role in some aspects of neurite growth stimulation. For example, the interaction of extracellular adhesive molecules with their receptor involves modulation of chemical second messengers (Bixby, 1989; Danilov and Juliano, 1989; Schuch et al., 1989). Such instructive interactions might cause the growth cone to exert more tension, increasing elongation rate or initiation.

Yet another alternative for substratum regulation is that the substratum might affect the response of a neuron to a fixed tension level. For example, "towed" axonal elongation requires forces above some tension threshold, which varies from cell to cell (Dennerli et al., 1989; Zheng et al., 1991). A substratum interaction that increased or decreased this threshold would slow or speed neurite outgrowth without necessarily affecting the neurite tension. Also, individual neurites vary in their sensitivity to applied tension, i.e., their elongation rate per unit of applied tension (Zheng et al., 1991). The substratum might affect growth by altering this sensitivity.

We report here a cytomechanical investigation of cultured chick sensory neurite outgrowth on three growth substrata: (a) plain tissue culture plastic; (b) plastic treated with collagen type IV; and (c) plastic treated with laminin. We wished to determine whether growth cones function near their adhesive limit on these surfaces, so that small increases in tension would prove significant for the attachment and detachment of growth cones. We investigated whether cytomechanical parameters previously shown to regulate neurite elongation (tension, thresholds, and sensitivities) differ among neurites grown on the different substrata. We also compared the relationship between tension and elongation rate for growth cone–mediated elongation and elongation induced by experimental towing with a glass needle.

Materials and Methods

Neuronal Culture

Chick sensory neurons were isolated as described by Sinclair et al. (1988) from lumbosacral dorsal root ganglia of 11-12-d-old chicken embryos. Cells were grown at 37°C in L-15 medium (Sigma Chemical Co., St. Louis, MO) supplemented with 0.6% glucose, 2 mM L-glutamine, 100 U/ml penicillin, 136 μg/ml streptomycin sulphate, 10% FCS, and 100 μg/ml NE serum growth factor isolated from mouse salivary glands (Varon et al., 1972). Cells were grown on three different culture surfaces, (a) 60-mm Corning tissue culture dishes as supplied, (b) same dishes treated with collagen type IV, and (c) same dishes treated with laminin.

For cells grown on collagen, a few drops of collagen Type IV (Sigma Chemical Co.) at 1 mg/ml in PBS were placed onto the bottom of a dish, aggregated to wet the entire culture surface, and allowed to incubate for 2 h at room temperature before the addition of cells suspended in complete medium. Culture surfaces were treated with laminin in a similar manner except that laminin (Collaborative Research Inc., Lexington, MA and a kind gift from the laboratory of Dr. M. Wiche, University of Michigan) was used at a concentration of 10 μg/ml in L-15 medium (without supplements).

Cytomechanical Measurements

Cytomechanical measurements used glass needles calibrated for their bending modulus. Flint glass (R-6) tubing 0.8 mm o.d. × 0.6 mm i.d. (Drummond Scientific Co., Broomall, PA) was fabricated into needles with an effective lever arm of 3.5-mm in length and ~6 μm in diameter by a BB-CH programmable needle puller (Mechanex S.A., Geneva, Switzerland). The bending modulus of this region was determined by the calibration procedure described by Dennerli et al. (1988). In these experiments, eight different needles were used varying in bending modulus from 4.4 to 14.5 dynes/μm, four of the needles were between 4.4 and 6.0 μdyne/μm. The departure of these needles from Hookean behavior was estimated to be <4% based on their average working deflection and the fit of calibration data to first and second order equations.

Rest tensions were measured by a refinement of the method of Dennerli et al. (1988), where unbranched chick sensory neurites were plucked to the side (like a guitar string) by a needle of known compliance at the midpoint between the cell body and the growth cone attachment. Each pluck required 4–6 s and no deflection was evident with similar movements of needles in water (i.e., there was no measurable deflection because of viscous drag). The plucking of neurites were recorded on videotape in real time. The change in neurite length was calculated geometrically from the easily measured lateral displacement imposed by the needle. The axial force on the neurite was calculated from lateral deflection of the needle (force perpendicular to the neurite) using vector algebra (See Dennerli et al., 1988, Fig. 1). The rest tension was obtained by extrapolation of the linear relation between force and length change to the zero distension intercept (y-intercept). The sole modification to the previous method (Dennerli et al., 1988) was that the force-measuring needle was mounted in the micromanipulator next to a reference needle that bore no load, as described in Lamoureux et al. (1989) and Dennerli et al. (1989). Thus, the bending of the force-measuring needle could be accurately measured from the distance between it and the reference needle throughout the process of neurite distension. During the course of each pluck, we stopped the videotape at ~0.1 s intervals to obtain needle deflection and neurite distension data. In the earlier method (Dennerli et al., 1988), the bending of the force-measuring needle could be measured at only a single time point, when the neurite was released from the needle. This enables us to make fuller use of the experimental observation and produces tension measurements more purely elastic and less "contaminated" by viscoelastic effects in the neurite.

To measure tension thresholds for elongation and tension sensitivities, neurites were towed from their growth cone as described by Zheng et al. (1991). Neurites were subjected to their growth cone as described by Zheng et al. (1991). Neurites were subjected to a step-function protocol of applied tensions: a constant force was applied for periods of 30–60 min, the tension was incremented 25–125 μdyne then applied constantly for another 30–60 min, and so on. A given neurite was subjected to 3–7 such steps of constant force. The elongation response of these neurites were interpreted in terms of a linear fit of tension and the average extension rates during that tension step. The data from the first 5 min of each step was excluded to correct for viscoelastic, nongrowth contributions to elongation (see Zheng et al., 1991). This plot is interpreted to yield cytomechanical parameters of growth: The x-intercept is the tension at zero growth rate and so is interpreted as a tension threshold for growth. The slope of the line (growth rate per tension unit) is the sensitivity of the neurite to tension.

Measurements of Growth Cone–mediated Elongation Rates and Extents of Elongation

The microscope used for observations of neuronal growth was housed in an insulated box, warmed to 37°C by air curtain incubators. No experimental manipulations were performed on cells observed for growth other than those required to place the cultures onto the stage of the inverted microscope. Cells were observed for varying periods between 1 h 2 min and 8 hr 10 min via phase optics, recorded in 24× time lapse on videotape and subsequently analyzed for growth cone advance.

Elongation rates for individual growth cones were measured by counting the number and position of individual growth cones every 10 min. This short time period allowed us to account for changes in the number of growth cones as neurites branched and retracted, i.e., we noted the "births" and "deaths" of neurites. The points were derived from plots of neurite advance. The net forward advance of the growth cone by controlling for lateral movements as described by Katz et al. (1984). The growth rate is defined by the forward movement during a single growth cone's lifetime (or during the period of observation) divided by the time period. Periods of retraction were therefore included in the rate calculation except in those cases in which uninterrupted retraction caused the complete elimination of a neurite.
branch. In these cases, data from times after the beginning of the uninterrupted retraction were not used. That is, our data included the normal retraction periods occurring during a growth phase, but did not include data from periods of complete neurite retraction.

Results

Neurite Rest Tensions on Different Growth Substra\a\a

We measured the static tension of neurites from 11-12-d-old embryonic chick sensory neurons growing on plain tissue culture plastic, culture plastic treated with collagen type IV, and plastic treated with laminin. The method was a refinement of the earlier method of Dennerll et al. (1988), as described in Materials and Methods. Fig. 1 shows typical results of this intervention. As in previous studies (Dennerll et al., 1988, 1989), neurites on all three substrata behave purely elastically during this intervention, i.e., the neurites obey Hooke's law for springs. Presumably, plucking on this time scale is too rapid to engage viscous or growth elements of the overall neurite response to tension (Dennerll et al., 1989). Average rest tensions on the differing substrata were: 141.9 μdyn ± 12.0 (n = 21) on untreated tissue culture plastic; 149.9 μdyn ± 24.9 (n = 12) on collagen type IV treated plastic; and 200.9 μdyn ± 19.5 (n = 19) on laminin-treated dishes. Two-tailed, pairwise comparison of these means via a group comparison t test showed that the rest tension for laminin differed significantly (P < 0.05) from both untreated and collagen-treated plastic. However, the rest tensions for untreated and collagen-treated plastic did not differ at this level of significance.

The method for measuring rest tensions requires that neurites accommodate considerably greater tensions than their rest tensions without detaching, as shown in Fig. 1. A frequency distribution of the maximum experimental tensions applied to neurites as a function of their rest tension for all three substrata is shown in Fig. 2. The load applied to most experimental neurites was greater than twice the neurite's rest tension, but more than 3x the neurite's rest tension in 30% of the cases. These values are simply the greatest force applied experimentally, and are thus all smaller than the force at which the growth cone or cell body would lose adhesion. Although tension measurements require that neurites do not detach at low forces, this condition is met by virtually all neurites studied. Thus, neurites were not selected for their ability to remain attached.

Sensitivity and Threshold for Tension-induced Elongation

Chick sensory neurons can be elongated by "towing" with a needle (Bray 1984; Zheng et al., 1991). Elongation requires forces above some tension threshold, which varies somewhat from cell to cell (Zheng et al., 1991). Also, individual neurites vary in their sensitivity to applied tension. That is, neurites differ in their elongation rate per unit of applied tension (Zheng et al., 1991). Systematic differences in these cytomechanical parameters could underlie differences in axonal growth on different surfaces. For example, neurites would grow faster, tensions being equal, if a substratum caused cells to become more sensitive or lowered their threshold.

Chick sensory neurites were subjected to increasing tensions by applying steps of constant force with a glass needle of known compliance (Zheng et al., 1991). As shown in Fig. 3, neurites on both collagen- and laminin-treated plastic showed elongation rates proportional to tension above a threshold, as previously reported for plain tissue culture plastic (Zheng et al., 1991). As before, the correlation of elongation rates with applied tension was quite high. Of 33 total experiments (14 on plain, 6 on collagen, and 13 on laminin), 29 had r values > 0.9, and in only one case was r < 0.8. Thus, the elongation rate of neurites in this towing regime are described by an empirical linear equation:

\[
growth\ rate = sensitivity \times (\text{neurite tension} - \text{tension threshold})
\]

(Eq. 1)
Tension thresholds for elongation (zero-growth rate intercept) varied substantially from cell to cell, but the same variability was found on all substrata. Fig. 4 is a frequency distribution of thresholds (as a percentage of the total sample size) for neurites on each substratum. As found previously (Zheng et al., 1991), there is some tendency for thresholds to cluster between 100 and 150 μdynes. Mean values of the tension thresholds on these substrata were virtually identical: 111.2 ± 27.8 μdynes for neurites on plain plastic; 115.8 ± 18.9 μdynes on collagen-treated plastic; and 120.2 ± 16.8 for neurites on laminin. Two-tailed pairwise differences between these thresholds are not significant even at the 0.20 level.

Fig. 5 shows the frequency distribution histogram for the sensitivity of neurite elongation to applied tension. Tension sensitivity on laminin and collagen is similar to one another and similar to that previously found for plain tissue culture plastic (Zheng et al., 1991). Tension sensitivity of neurites varied between 0.5 and 5.0 μm/h per μdyne of applied tension. Average values for tension sensitivity were 1.39 ± 0.33 μm/h/μdyne on plain plastic; 1.32 ± 0.37 μm/h/μdyne on collagen; and 2.39 ± 0.53 μm/h/μdyne on laminin. Again, two-tailed, pairwise differences between these sensitivities are not significantly different at the 0.2 level of significance.

**Neurite Elongation Rates on Differing Substrata**

Neurite growth on a culture substratum involves a variety of different phenomena; growth cone advance rates, frequencies of growth cone branching, and lag times of neurite initiation from cell bodies. Previous work from this laboratory on tensile stimulation of neurite growth showed that elongation rates of unbranched, individual neurites were proportional to neurite tensions above some threshold (Denner et al., 1989; Zheng et al., 1991). Consequently, we were interested in measuring growth cone advance minimally influenced by factors for which we have no cytomechanical data, such as branching and initiation.

Time lapse video observations, as described in Materials and Methods, were made of 11-12-d-old embryonic chick sensory neurons growing on plain tissue culture plastic, culture plastic treated with collagen type IV, and plastic treated with laminin. Advance rates for each growth cone were calculated from the positions of growth cones measured every 10 min. This short time scale allowed new growth cone formation via branching to be noted and appropriately analyzed. Consequently, our measure of neurite elongation rate is a measure of the advance rate of individual growth cones. On this basis, the average rates of neurite elongation were 37.5 μm/h ± 2.1 (SEM) on plain tissue culture plastic (n = 47 growth cones); 45.0 ± 1.9 μm/h (n = 86 growth cones) on collagen type IV-treated substrata; and on laminin-treated substrates 51.0 ± 2.4 μm/h (n = 53 growth cones). Two-tailed, pairwise, t-test comparison of these mean elongation rates showed that the growth rate on untreated plastic differed from that on collagen and laminin at the 0.05 (95%) level of significance. A comparison of collagen and laminin showed that these were significantly different at the 0.05 (95%) level of significance for a one-tailed t test (laminin...
Relationship of Growth Cone-mediated Elongation to Experimentally Induced, "Towed" Elongation on Differing Substrata

As shown in Fig. 3 and described by Eq. 1, the rate of towed elongation in chick sensory neurites is linearly related to applied tension. Although the growth cone is known to pull on chick sensory neurites (Lamoureux et al., 1989; Heidemann et al., 1990), it is not clear that growth cone-mediated elongation is equally simple, i.e., neurite elongation rate can be predicted solely from the magnitude of tension exerted by the growth cone. A simple explanation is that the towed growth and growth cone-mediated growth are identical as far as tension input and elongation rate are concerned. We used Eq. 1 in an initial attempt to compare the tension–growth rate relationship of the two growth regimes. The tension input for rate > collagen rate), but not quite significant at the 0.5 level for a two-tailed test (t = 1.968, t0.05 = 1.977 for 137 degrees of freedom).

Discussion

As described in the introduction, one way in which different substrata have been postulated to affect axonal elongation and guidance is through permissive differential adhesion: adhesion to a surface permits or limits tension exertion by the growth cone. Growth cones and/or filopodia exerting more tension than the local adhesion limit pull free and are lost, leaving only the more firmly attached growth cones or filopodia (Letourneau, 1983; Bray, 1987). An untested assumption of this model is that the ability of growth cones to exert tension is limited by their adhesion. The data of Figs. 1 and 2 argue against this postulate on the three substrata used. The attachments of these growth cones withstood tension loads 2–3, and in some cases 5–10 times greater, than their rest tension, which we believe is a reasonable measure of their normal physiological tension loads (Dennerll et al., 1988, 1989).

Although technical limitations prevent us from measuring the adhesive force of individual filopodia, previous observations suggest that they too adhere to objects more strongly than required for the tension they exert (Heidemann et al., 1990). In one instance, for example, as a single filopodium retracted it became entangled with a number of additional neurites to which it was not attached. The filopodium continued to retract, pulling both the original neurite and the "new passengers" with no hesitation and no loss of adhesion to the neurite it initially contacted.

Our data are consistent with earlier studies indicating that growth stimulation by various substrata is not a simple matter of relative adhesion. Gundersen (1987, 1988) found that adhesion of chick sensory neurites to collagen type IV and to laminin is not correlated with guidance preferences. Also, McKenna and Raper (1988) found that despite laminin's growth stimulating activity, growth cones did not orient to follow a concentration gradient of substratum-bound laminin, as would be predicted by a guidance mechanism based on differences of adhesive force.

We believe we can reject another possible mechanism for substratum effects based on the towed growth data: The substratum does not seem to affect the internal "set points" of the neurite for its response to tension. When growth response to growth cone-mediated elongation was taken to be the average rest tensions measured on the three substrata. If the two growth regimes are similar, then tension sensitivities and tension thresholds should be similar in the two regimes. Consequently, we used the mean value of sensitivity and tension threshold from towing experiments on each substrate to generate a prediction from Eq. 1. That is, on each substratum,

predicted average advance rate = average towed sensitivity × (average rest tension – average towed threshold).

If the two regimes are similar, the predicted average advance rate should match the measured average growth cone advance rates, at least to within the (substantial) variation in the values of sensitivity and threshold. Table I shows this comparison. Two-tailed comparison of predicted and measured values shows that none differ significantly at the 0.05 (95%) level, although the laminin data is significantly different at the 0.1 (90%) level.

Figure 4. Frequency distribution histogram of tension thresholds (tension value of zero growth intercept from plots as in Fig. 3) for all towed neurites on the three different culture substrata. (o) Plain; (m) collagen IV; (s) laminin.

Figure 5. Frequency distribution histogram of tension sensitivities of elongation for all towed neurites on the three different substrata. The sensitivity is the growth rate (μm/h) per μdyne of applied tension, the slope from plots as in Fig. 3. (o) Plain; (m) collagen IV; (s) laminin.
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Table 1. Comparison of Mean Measured Growth Cone-mediated Elongation Rates with Predictions of Average Elongation Rates Based on Cytomechanical Parameters

<table>
<thead>
<tr>
<th>Substrata</th>
<th>Measured growth rates (μm/h) ± SEM</th>
<th>Expected growth rates* ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plain</td>
<td>37.5 ± 2.1</td>
<td>45.0 ± 1.9</td>
</tr>
<tr>
<td>Collagen</td>
<td>45.0 ± 41.8</td>
<td>51 ± 2.4</td>
</tr>
<tr>
<td>Laminin</td>
<td>192.9 ± 74.5</td>
<td>192.9 ± 74.5</td>
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</table>

*See text for description of calculation that produced expected values.

The neurite elongation we measured arises from two very different growth regimes; growth cone-mediated elongation and experimentally towed elongation. Our measurements of rest tension and of elongation rates were obtained from neurites elongating by growth cone advance. Our data on tension thresholds and sensitivities were obtained by experimentally imposing tension on the neurite. Neurite elongation in this towed regime, in contrast to growth cone-mediated growth, never pauses, the neurite cannot retract, and the growth cone serves, as far as we can determine, only as an attachment point.

The simplest cytomechanical model would assume that these two growth regimes are identical as far as tension and elongation rate output are concerned. That is, the relationship of Eq. 1 between cytomechanical parameters and growth rate obtained from towing experiments should predict the behavior of neurites elongating by growth cone advance. Table I shows that the measured values of growth cone–mediated elongation rate are consistent with the elongation rates predicted by Eq. 1. That is, on all three substrata, the measured growth cone advance rate is consistent with the idea that the growth cone exerts a tension that tows neurite extension, just as if the neurite had been towed with a glass needle. A stronger inference than "consistent" cannot be made because of the large variation in the measurements of sensitivities and thresholds in towed growth experiments. Because of this variation, a clear difference between observation and prediction would require, at the least, sensitivity/threshold measurements on many dozens of neurites.

We were surprised by the small differences in the growth cone elongation rates on the three substrata. By simple observation of our cultures, we see the well-documented growth stimulation on collagen and laminin (e.g., Bray et al., 1987, Fig. 1). However, the mean elongation rate on laminin was only 1.4× as great as the mean value on plain plastic, and the mean elongation rate on collagen was only 1.2× as great as that on plain plastic. Thomas et al. (1990) and Buettner and Pittman (1991) report very similar mean elongation rates for neurites growing on surfaces treated with the concentration of laminin used here. Other growth parameters appear to be more responsive to substratum differences. Bray et al. (1987) and Hanatz-Ambroise et al. (1987) reported a 2.5× increase in neurite branching on laminin relative to untreated plastic. Initiation of neurites also seems more strongly stimulated by laminin than elongation rate is. Davis et al. (1985) reported a 3× increase in ciliary neurons with neurites after 24 h on laminin compared with cells on untreated plastic. It seems that increases in elongation rate play a relatively small role in growth stimulation by collagen and laminin substrates.

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