Segmental Independence and Age Dependence of Neurite Outgrowth from Embryonic Chick Sensory Neurons

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SUMMARY

Targets in limb regions of the chick embryo are further removed from the dorsal root ganglia that innervate them compared with thoracic ganglion-to-target distances. It has been inferred that axons grow into the limb regions two to three times faster than into non-limb regions. We tested whether the differences were due to intrinsic properties of the neurons located at different segmental levels. Dorsal root ganglia (DRG) were isolated from the forelimb, trunk, and hind limb regions of stage 25-30 embryos. Neurite outgrowth was measured in dissociated cell culture and in cultures of DRG explants. Although there was considerable variability in the amount of neurite outgrowth, there were no substantive differences in the amount or the rate of outgrowth comparing brachial, thoracic, or lumbosacral neurons. The amount of neurite outgrowth in dissociated cell cultures increased with the stage of development. Overall, our data suggest that DRG neurons express a basal amount of outgrowth, which is initially independent of target-derived neurotrophic influences; the magnitude of this intrinsic growth potential increases with stage of development; and the neurons of the DRG are not intrinsically specified to grow neurites at rates that are matched to the distance they are required to grow to make contact with their peripheral targets in vivo. We present a speculative model based on Poisson statistics, which attempts to account for the variability in the amount of neurite outgrowth from dissociated neurons.

Keywords: dorsal root ganglion, neurite growth, chick embryo.

INTRODUCTION

Neurons grow cellular processes (neurites) quite readily when they are dissociated and cultured in vitro. The form and pattern of these neurites are often characteristic of the type of neuron. For instance, cultured dorsal root ganglion (DRG) neurons are bipolar or pseudo-unipolar (Mudge, 1983); other types of neurons, such as autonomic ganglion neurons or spinal motoneurons, adopt a multipolar shape in culture (Bray, 1973; Bloch-Gallego et al., 1991). Although these qualitative characteristics emerge largely independent of the composition of the culture environment, substrate-bound and soluble factors influence the amount of neurite outgrowth (Levi-Montalcini, 1987; Rathjen, 1988; Lander, 1989). Such observations are partly responsible for the notion that the amount of growth and the number and disposition of nerve branches in vivo are determined by trophic interactions between neurons and their targets (Purves, 1988). In at least one instance however, namely the neurons of the cranial sensory ganglia, the amount and rate of outgrowth are apparently determined by intrinsic properties of the neurons. Davies (1989) has shown that dissociated neurons from the petrosal, geniculate, nodose, and vestibular ganglia grow neurites at different rates in a uniform culture environment, but for each neuron the rate and amount of growth are directly related to the in vivo neuron to target distance.

In the chick embryo, sensory neurons in DRG positioned at different segmental levels appear to differ in the rate and extent of their initial outgrowth (Swanson and Lewis, 1982). The greatest
Figure 1  Innervation of the limbs and trunk of stage 28 chick embryos, compiled from camera lucida drawings of three separate embryos stained with silver and protargol. Rostral is to the right. Note that the axons innervating the limbs are about two to three times as long as those innervating the trunk at this stage. Scale bar = 1 mm.

difference is between neurons in the thoracic ganglia, which innervate the skin and musculature of the trunk, and those of the brachial or lumbosacral ganglia, which innervate the forelimbs and hindlimbs. Axons begin to enter the bases of the limb buds at stage 25 (approximately 4.5 days of incubation), after the formation of the brachial and lumbosacral plexuses (Roncali, 1970). By stage 28, the longest axons innervating the limb bud are two to three times longer than those innervating the trunk (Fig. 1; see also Fig. 3 in Swanson and Lewis, 1982); By stage 30 the basic adult pattern of innervation is established in the hindlimb and distal axon tips have reached the wrist of the developing forelimb (Honig, 1982; Scott, 1982, 1987; Swanson and Lewis, 1982; Tosney and Landmesser, 1985a,b,c).

The simplest interpretation of these observations is that neurites grow more rapidly into the embryonic limb bud than into the thoracic region between the limb buds. The object of the present study was to determine whether neurons projecting to these regions grow or regenerate at intrinsically different rates, or whether the differences are more likely to be explained by differences in local cues in the environment of the growing axons. We attempted to distinguish between these two possibilities by measuring, first, the amount of neurite outgrowth from dissociated DRG neurons after 24 hours in culture, and, second, by measuring the rate of growth cone advancement from substrate-bound explants of whole DRG. The data from both approaches suggest that DRG neurons at any given stage of embryonic development are not pre-specified on the basis of their segmental position to grow axons at the rates proportional to ganglion to target distances in vivo. However, we did find evidence of intrinsic, stage-dependent differences in the rate of neurite outgrowth from isolated cells and DRG explants.

METHODS

Dissection and Culture

Dissociated Cells. DRG were dissected from the mid-brachial, mid-thoracic, and mid-lumbosacral levels of
4.5–6.5-day-old chick embryos, corresponding to stages 25-30 of Hamburger and Hamilton (1951). Between four and eight ganglia from each level were washed in cold (4°C) Tris-buffered saline (TD) transferred to 1 ml of 0.05% trypsin in cold TD, then placed in a gassed (5% carbon dioxide [CO₂]) incubator at 37°C. After 45 min, the tissue was crudely disaggregated, spun down, and resuspended in TD containing 0.025% soybean trypsin inhibitor and 0.02% DNase to prevent cell clumping. The crude disaggregates were triturated by 10 passages with a fire-polished Pasteur pipette to produce a dissociated cell suspension. This was briefly centrifuged and the cells were resuspended in 1.2 ml of Ham’s F12 medium (Flow) supplemented with 10% heat-inactivated fetal calf serum (Flow), 2 mM L-glutamine, 40 μg/ml conalbumin, 50 μg/ml gentamicin, 100 μg/ml kanamycin. Purified growth factors were not added to the culture medium. Aliquots of 50 μl of the cell suspension were plated onto 9 mm glass coverslips previously coated with poly-D-L-ornithine (PORN) and laminin (see later) in a 24-well plastic culture plate (Linbro space saver, Flow). The plates were transferred to the 37°C incubator for 1 h. Each well was flooded with a further 450 μl of complete medium. After 24 h of culture, coverslips were fixed in 4% paraformaldehyde for 10 min, rinsed in phosphate buffered saline (PBS), and mounted in Aquapolymount for viewing with phase-contrast microscopy.

Explants. Three to five ganglia were removed from brachial, thoracic, and lumbar sacral regions of embryos at stage 25-30. The ganglia were transferred to a previously prepared PORN/laminin-coated 22 mm coverslip in a heated chamber (Intracell). A grid was marked on the underside of the coverslip for reference. A layer of mineral oil was placed over the medium to reduce evaporation during culture, and the whole assembly was left for 45 min to allow the explants to adhere before transfer to the microscope stage of a Nikon TMS-F inverted phase-contrast microscope. The chamber was maintained at 37°C and a 95% air/5% CO₂ gas mixture was continuously blown over the surface.

Precoating of Coverslips. A 50 μl or 250 μl droplet of PORN at 250 μg/ml in sodium borate buffer (pH 8.4) was placed on each coverslip (9 and 22 mm, respectively) and left to stand for 30 min at room temperature. Excess PORN was removed and the coverslips were washed three times with sterile, double-distilled water and allowed to dry for 1 h under an ultraviolet lamp at room temperature. Coverslips were subsequently coated with laminin (Bethesda Research Laboratories). For the dissociated cell cultures, a 50 μl droplet containing 100 μg/ml laminin in TD was added to each coverslip and incubated for 30 min at 37°C. Excess laminin was drawn off and the coverslip was rinsed several times with sterile water. Fibronectin was sometimes used instead of laminin to coat the PORN-covered coverslips. There was no difference in outgrowth on fibronectin compared with laminin in the present experiments (data not shown). The laminin-coating procedure was altered slightly for the explant cultures, to increase their adherence and viability. A 200 μl droplet of 100 μg/ml laminin was added to a 22 mm coverslip and incubated at 37°C for 60 min. The laminin solution was drawn off and the coverslip washed only once with equilibrated, complete media. The coverslip was immediately sealed into an autoclaved, stainless steel chamber with vacuum grease, and 1.2 ml of equilibrated, complete media was added. In both cases, the amount of laminin used was in excess of that which promotes maximum outgrowth from sensory neurons in culture (Buettner and Pittman, 1991).

Histological Staining

Stage 28 chick embryos were stained with silver and pro-targol exactly as described by Lewis (1978). Figure 1 is a simplified, composite drawing from three embryos, showing the difference in the extent of nerve outgrowth into the trunk and limbs at this stage.

Anti-neurofilament staining of one culture was carried out by washing the culture three times in PBS, incubating for 30 min at 20°C with 10% fetal calf serum in PBS, followed by three further washes in PBS. The culture was then fixed in acetone at −20°C for 4 min, washed in PBS and incubated for 1 hour at 37°C in a 1:50 dilution of 68 kD neurofilament mouse antibody (Sigma) and washed in PBS. It was then incubated for another hour in fluorescein isothiocyanate conjugated goat anti-mouse immunoglobulin G (Sigma) at 1:5 dilution in PBS at 37°C, washed in PBS and mounted in Aquapolymount for viewing with fluorescence microscopy, using standard fluorescein filters.

Measurement and Analysis of Neurite Length

Dissociated Cell Cultures. The distribution of neurite lengths in 300–1500 neurons from each region was measured after 24 hours of culture. Each culture was viewed using an inverted phase-contrast microscope. Although we did not attempt to measure or control cell plating density, other than by using equal numbers of ganglia in each experiment, we studied microscope fields (×40 phase objective) containing not more than four cell bodies. Most fields contained no cell bodies. Neurons were selected for analysis if they satisfied two main criteria: that their cell bodies were rounded and discreet, not clustered nor associated with other cells; and that their neurites, when present, did not terminate on other neurites, cell bodies, or nonneuronal cells. Because of the relative sparsity of the cultures, very few neurons or neurites were disqualified from assay by these criteria.

Images of the neurons were captured using a JVC NK10 video camera and a Watford Video Digitizer (512 × 256 pixels; 64 gray levels) connected to an Acorn Archimedes computer. A laplacian digital filter was some-
times applied to enhance the contrast of the video image. Measurements of neurite length and cell body diameter were then made from the digitized images. Calibration of the measurements took account of the aspect ratio of the video image. The measurements of neurite length were estimated to have a maximum error of approximately 7%.

**Explant Cultures.** Time-lapse recordings of neurite growth from 80-90 growth cones from each segmental level were made using a Sony CCD monochrome video camera and monitor linked to a Mitsubishi HS-S5600, S-VHS time-lapse video recorder. The same digitizer and computerized system was used to process and measure the rate of movement of growth cones as for dissociated cells. The position of the growth cone was marked on the monitor at the beginning and at the end of a 30 min recording period. The shortest distance between the two points was then measured, from which the growth rate was calculated. After each period, the field of view was changed to a different explant, taking brachial, thoracic, and lumbosacral explants in rotation. The positions of each field were noted and it was thus possible to return to the same group of neurites after several hours. This technique allowed larger amounts of data to be acquired than if a small population of neurites had been followed exclusively over shorter intervals. In a few cases, small groups of neurons were monitored continuously; the rates of outgrowth measured from these were similar to those made by sampling every 30 min. With time, outgrowth from the explants became too complex for accurate measurements from individual growth cones to be made. In practice, this limit was reached after about 15 h.

**Statistics**

Most of the data were analyzed using nonparametric statistical methods, since the distributions of neurite length were skewed. These distributions were expressed in terms of their mode, median, and interquartile ranges and differences were tested using the Mann-Whitney U test. Student's t test were used to compare the mean rate of neurite outgrowth from DRG explants.

**RESULTS**

**Qualitative Appearance of Cultures**

**Dissociated Cells.** A number of different types of cells were present in the cultures (Fig. 2). First, there were neurons with small, phase-dark cell bodies and bipolar, unbranched neurites (SDB cells). The second type were neurons with large, phase-bright cell bodies from which single or multiple branched neurites extended (LBM cells). A third category of cells had large, phase-bright cell bodies, but no neurites were present after 24 hours in culture. We recorded these cells as “failures.” The cultures also contained cells that were clearly not neurons. These included flattened, granular cells with no neurites (possibly fibroblasts) and small phase-dark, spindle-shaped cells (probably Schwann cells). Cells with these characteristics were excluded from the analysis.

Most of the neurons were either unipolar or bipolar (Fig. 2): tripolar neurons were relatively uncommon and neurons with more than four neurites were never seen. We stained one culture with antibodies against the 68 kD fragment of neurofilament protein. Neurite-bearing cells stained intensely with the fluorescent antibody. Some of the failures showed a wispy staining in the cell body.

**Explants.** Explanted ganglia adhered to the substrate and began to extend long neurites terminating in large, flattened growth cones within 4 hours (Fig. 3). In addition, flattened nonneuronal cells migrated out of the explants. Fasciculation of neurites was relatively uncommon. Some of the longer neurites were attached only at the growth cone, and their neurites appeared to float free in the medium, (compare Letourneau, 1975). Other neurites were relatively taut. The traction exerted by some growth cones was sufficient to pluck their cell bodies forcibly out of the explant. There were no consistent attractive or repulsive interactions between neurites on contact (see Honig and Burden, 1993). Neurites would apparently randomly either cross or grow together before parting again. In all cultures, a few neurites grew considerably longer neurites than the majority. In the measurements described later neurites were selected at random; in other words, we did not deliberately select either the longest or the shortest neurites.

**Quantitative Analysis of Neurite Outgrowth in Culture**

**Dissociated Cells.** Distributions of neurite lengths are shown in Figure 4. These histograms show the total neurite length per neuron, that is, for each neuron the summed lengths of each neurite and any branches, for the three different segmental levels of stage 28 embryos. The numbers of failure neurons (as already defined) are also indicated on Figure 4.

Descriptive statistics for the histograms of neurite length are shown in Table 1. Non-Gaussian statistical values are given, because the distributions of neurite length were skewed. There was no evidence that neurons from the thoracic ganglia
grew neurites shorter or longer than neurons from the brachial or lumbosacral ganglia ($p > 0.05$; Mann-Whitney test).

**Stage-Dependent Outgrowth of Neurites.** Since the development of the nervous system follows a rostrocaudal progression, local differences in the maturity of neurons between brachial and lumbosacral ganglia could conceivably have obscured any intrinsic differences in the rate of neurite outgrowth in our cultures. We examined this possibility in three ways. First, we compared neurite outgrowth from dissociated neurons obtained from a more restricted region of the developing neural axis, that is, we examined outgrowth from rostral lumbosacral ganglia and compared this with outgrowth from more caudal lumbosacral ganglia. Second, we studied the outgrowth in cultures prepared from younger (stage 25) and older (stage 30) embryos. Finally, we selectively measured the outgrowth from developmentally immature SDB neurons.

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**Figure 2** Phase-contrast micrographs of dissociated DRG cells from a stage 25 embryo after 24 hours in culture. (A) Bipolar neuron and two phase-bright “failures”. (B) Unipolar neuron. (C) Phase-dark bipolar neuron. (D) Phase-bright bipolar neuron with long neurites. (E) Bipolar neuron with branched neurites. (F) Multipolar neuron with three neurites emerging from the cell soma. (G) Nonneuronal cell, probably a Schwann cell. (H) Nonneuronal cells, probably fibroblasts. Scale bar = 100 μm.
Outgrowth from Lumbosacral Ganglia. Axons enter the crural plexus from lumbosacral (LS) segments 1-3, and then project to proximal targets in the thigh and knee. The sciatic plexus receives axons from LS 4-8, which then project mainly to distal targets in the knee and shank (Landmesser, 1978, Scott, 1982). Thus, it appears from silver-stained preparations that ganglia positioned only slightly more rostral than others in the lumbosacral region normally project shorter neurites than those ganglia positioned only slightly more caudal. Of course, many neurons in the lumbosacral DRG also have proximal targets in the thigh (and even in the body wall). We attempted to accommodate this by testing for differences in the distributions (not means) of neurite lengths in large samples using nonparametric statistical methods.

Histograms of total neurite length from cultured
Figure 4  Histograms of total neurite length of dissociated DRG neurons from stage 28 embryos. The data were accumulated from four repeat cultures made from different embryos. Numbers of failures, i.e., neuronlike cells without neurites, are indicated in the top right corner. (A) Brachial DRG, $n = 393$. (B) Thoracic DRG, $n = 262$. (C) Lumbosacral DRG, $n = 195$.

DRG neurons supplying the sciatic or crural plexuses of stage 28 embryos are shown in Figure 5. Both histograms were positively skewed. There was no evidence that the neurons from DRG LS 4-8 grew longer neurites than those from LS 1-3 in vitro. Modal values (125 $\mu$m and 100 $\mu$m, respectively) and medians (450 $\mu$m and 375 $\mu$m) were not statistically significantly different ($p > 0.05$, Mann-Whitney test).

Neurite Outgrowth at Earlier and Later Stages. Measurements on 24-hour cultures were obtained...
from embryos at stage 25, just prior to the invasion of the limb buds by the growing axons, and at stage 30 when the adult pattern of limb innervation is essentially established. Figure 6 shows the distribution of neurite lengths from the brachial DRG neurons at stages 25, 28, and 30. Similar results were obtained from thoracic and lumbosacral regions (Table 1). In no case were there any significant differences in neurite outgrowth from the different segmental levels. There were clear differences in

Table 1  Summary of Statistical Data on Neurite Growth in Cultures Obtained from Brachial (B), Thoracic (T), and Lumbosacral (L) Levels of Embryos at Stages 25 to 30

<table>
<thead>
<tr>
<th>Stage</th>
<th>Segmental Level</th>
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<th>Failures (%)</th>
<th>Mode</th>
<th>Median</th>
<th>Quartile Point</th>
<th>Interquartile Range</th>
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<td>250</td>
<td>150 350</td>
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Figure 5  Histograms of total neurite length in DRG neurons from stage 28 embryos dissociated from lumbosacral ganglia 1-3 (A; n = 140); lumbosacral ganglia 4-7 (B; n = 141). The number of failures are again indicated.
Segmental Independence of Neurite Growth

Figure 6 Median neurite lengths and interquartile ranges for repeat cultures from the brachial region of stage 25 to 30 embryos.

the overall amount of outgrowth at each stage, however. The median neurite length increased significantly, the variance also increased but the proportion of failures decreased (Table 1). Differences in the morphology of the neurons were also apparent. The proportion of multipolar neurons increased from 0.02% to 2.1% and 3.2% of the total number of neurons at stages 25, 28, and 30, respectively. Similarly, the percentage of cells with branched neurites increased from 7.6% to 9.8% and 18.4% at the same stages.

Outgrowth from Immature (SDB) Neurons. Neurons located in the DRG are generated over an extended period (Carr and Simpson, 1978). At all the stages studied here, ganglia contain indifferent cells (undifferentiated, neural progenitors), primitive neuroblasts, and intermediate neuroblasts (Pannese, 1974). It has been shown that neurons that have only recently developed from precursors are identifiable as SDB cells (Rohrer et al., 1985; Ernsberger and Rohrer, 1988; Wright et al., 1992). Like these authors, we, therefore, assumed that SDB cells were not axotomized by the isolation procedure and that they therefore grew their first neurites in culture. We only analyzed data from stage 25 embryos, as these contained the greatest percentage of SDB neurons (Table 2).

Neurite outgrowth from SDB neurons was about 25% less from thoracic than from limb DRG regions; from a median of 225 to 250 μm to one of 175 μm (p < 0.0001, Mann Whitney test). However, this difference in outgrowth is much less than that inferred from fixed preparations over a 24-hour period comparing thoracic and limb regions in vivo.

Time-Lapse Microscopy of Neurite Outgrowth from Explant Cultures. Our measurements of the amount of neurite outgrowth from dissociated cells permitted a relatively large quantity of data to be collected and analyzed. However, it is appropriate to ask whether the amount of growth recorded after 24 hours reflected a uniform underlying rate of neurite growth. Initially, we attempted to address this question using time-lapse microscopy of dissociated neurons, but we found that the cell bodies often moved small amounts during the recording period, and this made accurate measurement of changes in neurite length and, therefore, rates of outgrowth difficult to obtain. Rates of neurite growth were more readily monitored using explants of DRG cultured under similar conditions to the dissociated cells. Rates of growth cone advance were calculated from measurements over 30-minute intervals.

Explants of ganglia from different segmental levels at stage 28 showed no overt or statistically significant differences in rate of growth cone advance (Fig. 7). Values of between 0.4 and 3.75 μm/min were recorded. The form and extent of the neuritic halos around the explants were similar in all cases. Measurements were also obtained from stage 25 and stage 30 embryos. Least square fits for the mean growth cone advance rates at stage 28 showed they were fairly constant throughout the culture period (Fig. 7). Data for the other developmental stages are given in Figure 8. At stage 25, no difference was found between segmental levels, and the rates recorded were comparable with those at stage 28. At stage 30, however, growth rates were reduced at all segmental levels compared with the earlier stages. Interestingly, this was most pronounced in the thoracic region, where there was a further small but significant decrease in growth rate compared with brachial and lumbosacral levels (1.0 μm/min compared with 1.3 μm/min; p < 0.01, t test).

DISCUSSION

During the early development of the chick embryo, DRG neurons project axons into the periphery and ultimately connect with a variety of presumptive target tissues (mostly skin and muscle). The basic
Table 2  Summary of Statistical Data for SDB Neurons from Stage 25 Embryos

<table>
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\[1\] These data represent a part of that which was originally presented in Figure 4.

body plan of the embryo determines that some axons have much further to grow than others in order to contact or maintain connection with their peripheral targets. This is especially apparent for those ganglia that innervate limb versus nonlimb regions. Indirect measurements indicate that DRG neurons grow processes toward more distant targets at greater rates than toward closer targets (Honig, 1982; Scott, 1982; Swanson and Lewis, 1982). The main objective of the present study was therefore to determine whether growth rate is determined more by an intrinsic, segmentally based program, as has been suggested for cranial sensory neurons by Davies (1989), or whether it is determined more by the environment of the limbs, which regulates neurite outgrowth, as suggested indirectly by earlier work (Hamburger, 1939; Bennett et al., 1980; Swanson and Lewis, 1982). We investigated this problem by culturing isolated cells and explants from DRG taken from chick embryos during the stages over which DRG neurons normally grow axons into the periphery. Our data suggest that at the stages when segmental differences are clearly apparent in vivo, differences in either the amount or the rate of neurite outgrowth are insufficient to account for the growth observed in vivo. However there were intrinsic, stage-dependent differences in both amount and rate of neurite outgrowth, whatever the segmental origin of the DRG neurons. We also found that the distributions of neurite lengths in culture were positively skewed. We are unable to account for this simply in terms of neuronal subtypes (SDB/LBM) in the ganglia. We propose a speculative model to account for the distributions.

Segmental Independence of Neurite Outgrowth

It is clear that neurons have an intrinsic capacity to grow neurites, and the form of these is often characteristic for the type of neuron, even in a foreign culture environment. Our results suggest that when DRG neurons are placed into a controlled, homogeneous culture environment, they exhibit similar neurite outgrowth characteristics, irrespective of the segmental level from which they were isolated. In only one case (stage 25) was the median neurite length from the thoracic region less than those from limb-innervating regions, but the difference was far less than the amount predicted over a comparable period of growth in vivo, about 400 to 500 μm. At stage 28, the median amount of neurite outgrowth from dissociated cells was 230 μm after 24 hours in culture. This is comparable to the mean length of neurites from the thoracic region at the same stage of development (approximately 24 hours after emerging from the developing DRG). Thus, the simplest explanation of our data is that the outgrowth seen in vitro represents a basal level that is intrinsic to the neurons, and this may be elevated to greater levels in the limb regions by the activity of (unidentified) limb-derived factors.

There are rostrocaudal differences in the maturity of neurons. We investigated the possibility that this could have biased the data by culturing neurons from the lumbosacral region of the embryo. Here, neurons that differ in age by only a few hours appear to show differential growth into target tissues in the hind limb (Scott, 1982, 1987). The more caudally located neurons innervate the more distal targets of the hind limb at the same time as proximal targets become innervated by more rostral neurons. However, when neurons from these two regions of the lumbosacral plexus were cultured, no differences in the amount of neurite outgrowth after 24 hours were observed (Fig. 5).

It is possible that our results reflected differences in the control of regeneration rather than de novo growth of axons. To investigate further the possibility that newly formed neurons may behave differently from others in culture, measurements of neurite length were made from a morphologically distinct population of immature (SDB) neurons. These neurons had not been axotomized; in other words, they extended their first neurites in culture (Rohrer et al., 1985; Ernsberger and Rohrer, 1988; Wright et al., 1992). When these new neurons...
were taken from different segmental levels and cultured, statistically significant differences in the amounts of neurite outgrowth after 24 hours were observed. However, the differences in outgrowth were insufficient for the magnitude of the differences observed in vivo. By contrast, in the study by Davies (1989), neurites from different cranial sensory ganglia grew neurites in culture that were com-

Figure 7  Neurite growth rates (μm/min) from explanted DRG from stage 28 embryos. Data from four repeat cultures from different embryos. (A) Brachial DRG. (B) Thoracic DRG. (C) Lumbosacral DRG. Open circles represent data points; filled circles are the means for each 30-minute time interval recorded. The lines are least square fits for the mean data points.
parable both in rate and magnitude to observations made in vivo.

We assessed the rate of neurite outgrowth from DRG neurons, using explant cultures. Here, a lower rate of neurite outgrowth from thoracic DRG was found only at stage 30, after the differences are established in vivo. Furthermore, the difference reflected a decrease in rate of only about 25% (1.3 to 1.0 μm/min), which again is much less than the twofold to threefold difference inferred from fixed preparations of embryos between stages 28 and 30 (approximately 24 hours). Interestingly, the mean rates of neurite growth in explant cultures (1.0 and 1.75 μm/min), if maintained, should have produced 1440 to 2520 μm of growth in 24 hours, far greater than the single neurite lengths recorded in the isolated cell cultures. The reasons for this difference between dissociated cells and explant cultures remain unclear.

Thus, we are unable to account for the amount or rate of DRG neurite outgrowth in vivo from the behavior of the neurons in culture. As the neurons of the DRG represent a developmentally plastic population, both in terms of fate and final function (Le Dourain, 1983), it is perhaps not surprising that their initial growth characteristics are not pre-programmed. This contrasts with neurons derived from cranial sensory ganglia, which do appear to have intrinsic target-distance-related rates and amounts of neurite growth (Davies, 1989). Cranial sensory ganglia develop from ectodermal placodes rather than neural crest, so it is conceivable that the differences could be related to the different embryonic origins of these sensory neurons.

Age Dependence of Neurite Outgrowth

It is clear from the data that any segmental differences in neurite growth measured were insufficient to account for the differences in growth observed in vivo. There were, however, striking age-dependent differences in neurite growth. These were manifested in an increase in the growth potential of the neurons with increasing developmental stage. By growth potential, we mean the capacity to produce longer, more numerous and more highly branched neurites in culture. In other words, the older the embryo, the greater the likelihood that isolated neurons would grow one or more neurites and that these neurites would be branched. Bray et al. (1987) and Scott and Davies (1993) have demonstrated similar phenomena, of increasing complexity of neuronal arbors with stage of development in older chick DRG and trigeminal ganglion cultures. (In addition the numbers of neurons that failed to grow a process during the culture period, but otherwise appeared healthy, decreased with the stage of development). Buettner and Pittman (1991) have
demonstrated that the number of primary neurites extending from a neuron increases with increasing substrate laminin concentration, but the number of branch points decreases over the same concentration range. In our cultures laminin concentration was not varied. It could be that the increase in the length and complexity of neurites with embryonic age is a reflection of a stronger growth cone to laminin interaction, possibly mediated by an up-regulation of laminin receptors (Edgar, 1989) at the neuronal growth cone.

Stage-dependent differences were not observed in explant cultures. Here, growth rates were relatively constant between stages 25 and 28 but showed a decrease at stage 30. It is unclear why this should be so.

Distribution of Neurite Lengths

The distributions of neurite lengths from the dissociated cell cultures exhibited two consistent qualities; they were positively skewed and they contained a population of apparently healthy neurons that did not produce a neurite during the 24-hour culture period. Similar distributions were obtained from brachial, lumbar, and thoracic ganglia, which militates against the possibility that the skewness of the distributions is due to intrinsic, target-distance-dependent properties of the neurons. The shape of the distributions could be related to properties of neuronal subpopulations within the DRG, which might behave differently when placed into culture. However, although numerous cell surface and intracellular markers have been used to identify subpopulations of DRG cells (Rohrer et al., 1985, 1986; Marusich et al., 1986; New and Mudge, 1986; Sieber-Blum, 1989; Scott et al., 1990), it remains unclear how these are related to functionally distinct subsets within very immature DRG. For instance, mature ganglia contain histochemically distinct populations of small, dorsomedially located neurons, and larger, ventrolaterally located neurons (Hamburger and Levi-Montalcini, 1949; Hamburger et al., 1981). Dorsomedial but not ventrolateral cells express substance P and calcitonin gene-related peptide (New and Mudge, 1986). These cell types are not related by lineage, nor are they committed to innervate distinct targets in the periphery, and clonal analysis of neural crest cells shows that they are multipotential, only adopting the appropriate phenotype and forming central contacts after they have made their peripheral connections (Landis, 1990; Frank and Sanes, 1991). Furthermore, there is no evidence that dor- somedial and ventromedial cells are related to the SDB and LBM cells described in the present study. Hory-Lee et al. (1993) have demonstrated that a specific subpopulation of muscle sensory neurons exist within DRG that are maintained in culture by NT3. However, it is reasonable to expect that any neurotrophin-dependent subpopulation of neurons should have targets dispersed throughout the limb. That is, we would not expect such a group of neurons to show any distance-dependent outgrowth of neurites.

Thus, taking all our data together, we are left with the findings that the distributions of neurite length in cultures of dissociated cells are skewed; that neurons dissociated from older embryos grow longer neurites in culture than those from younger embryos; and that, from what is known, these differences cannot readily be accounted for by differentiation of subtypes of neurons within the DRG.

It is possible to account for and quantitatively to describe the distributions of neurite length using a simple, albeit speculative, model based on Poisson statistics. The assumption behind such a model is that some fundamental component or process controlling the amount or rate or both, of neurite growth may be expressed in multiples of a unitary amount, but each unit is expressed with a low statistical probability (see Appendix). The results of comparing the observed distributions and those predicted by the model for cultures of neurons from brachial DRG in stages 25, 28, and 30 embryos are shown in Figure 9. In each case, we have assumed that the expression of a single unitary component generates a range of neurite lengths distributed in accordance with a normal (Gaussian) distribution. The overall distribution of neurite lengths is thus represented by the sum of several gaussian distributions, each an integer multiple of the mean and variance of the unit Gaussian. Figure 9 shows that there was quite a good match between the distributions of neurite length predicted by the model and those observed from the data. What is particularly striking is that the mean “unit” neurite length is similar in each case: 100 to 125 μm.

It is, of course, premature to speculate at length about the significance of the correlation between the observed distributions of neurite lengths and those predicted by this model: but it is perhaps worth pursuing the notion that the amount or rate of neurite growth is regulated somehow in a discontinuous, quantized manner. The present experiments give no direct clue as to the nature of the unitary component or process(es) that may be responsible, however.
APPENDIX

A Poisson Statistical Model of Neurite Outgrowth

We were struck by the resemblance of the distributions of neurite length and the proportions of failures (neuron-like cells with no neurites) to the binomial or Poisson distributions that characterize a number of physiological properties of neural cells; for instance, the release of neurotransmitter at synapses (Del Castillo and Katz, 1954; Boyd and Martin, 1956; Korn and Faber, 1991). By analogy, we hypothesized that the distribution of neurite lengths in dissociated neuronal culture and the number of failures might be related phenomena. Specifically, we have assumed that the presence and length of a neurite are determined by the overall probability of expression of some unitary (quantal) component or process that is fundamental to neurite outgrowth. If it is supposed that the length of the neurite is determined by the average number of quantal components expressed, but the probability of expression of each is independent and small, then the mean number expressed, $m$, will be related to the probability of unitary expression, $p$, and the available total, $n$, by

$$m = np$$  \hspace{1cm} (1)$$

The distribution of numbers of quantal components in a population of cultured neurons will be given by a binomial distribution, which simplifies under the conditions of low individual probability of expression of the individual elements to a Poisson distribution:

$$P(x) = e^{-m^x} / x!$$  \hspace{1cm} (2)$$

where $P(x)$ is the fraction of the population expressing $(x)$ unitary elements underlying outgrowth.

Note that the number of failures in a sample of the population may be predicted by evaluating the case $P(x = 0)$. Conversely, the mean quantal content may be calculated from the fraction of the cells examined that fail to grow neurites:

$$m = \ln\left(\frac{\text{total number of neurons}}{\text{number of failures}}\right)$$  \hspace{1cm} (3)$$

If it is assumed that the expression of one unit of the quantized component or process produces an amount of neurite outgrowth that varies according to a Gaussian distribution, then the observed over-
all distribution will not be completely discontinuous, but rather described by the function:

\[
p(x) = N \cdot C_w \cdot \sum_{M_x=0}^{M_x=10} \frac{e^{-m} \cdot m^x}{x!} \cdot \left\{ \frac{1}{2\pi M_x \sigma^2} \cdot \exp \left[ -\frac{(x - M_x \bar{x})^2}{2M_x \sigma^2} \right] \right\}
\]

where \( N = \) total size of the population, \( C_w = \) class width of the histogram bins, \( M_x = \) mean quantal content for each iteration, \( P(x) = \) probability of any neurite length \( (x), \sigma^2 = \) standard deviation of the quantal unit neurite length.

We calculated the overall distribution of neurite lengths predicted by equation (4), assuming a unit mean and variance in the neurite length estimated from the left-hand tail and left-most peak (mode) of each observed distribution of neurite length, and a mean quantal content predicted from the number of failure neurons in each culture, using equation (3). The predicted unit Gaussian, and the sum of Gaussian distributions (computed for \( M_x = 1-10 \)) comprising integer multiples of the mean and variance of the unit Gaussian are superimposed on the observed distribution of neurite lengths in Figure 9.

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