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Effect of viscosity on neurite outgrowth and fractal dimension

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The growth mechanism by which neurons achieve their characteristic ramified morphology has long been of interest, but determining whether physical parameters, such as viscosity, are important has been difficult due to a lack of useful hypotheses and standard reproducible techniques. We have recently shown that neurons exhibit fractal behavior and that their fractal dimension (d_f) is consistent with a physical process called diffusion-limited aggregation (DLA). We suggested that this DLA behavior might stem from viscosity differences, chemical gradients or electrical fields (Caserta et al., Phys. Rev. Lett., 64 (1990) 95–98). DLA is a model for a large family of growth processes. In order for a process to fit the DLA model, the growth rate must be proportional to the gradient of a field at a point on the growing structure (Feder, Plenum, New York, 1988, Ch. 4). Chemical, electrical, or fluid pressure fields can fit the model depending on the particular physical system under study. Here, we studied growth of retinal neurons from chick embryos in culture media of various fluid viscosities. Thus, we test whether DLA in this system was based on a fluid pressure field. As viscosity was increased from 1 to 4.3 cps, the number of neurite branches decreased 98%. However, there was no effect on d_f. Over this range of viscosities, total cellular protein synthesis decreased only 17%. The results indicate that, while differences in viscosity between the interior and exterior of the cell affect neurite outgrowth, they do not affect the fractal behavior of neurons. Thus, viscosity differences are not the basis for the DLA pattern of neuronal arborization.

CNS neurons generally and retinal neurons specifically, have a characteristic morphology — a cell body from which project axons and dendrites that constitute the neuronal arborization [1]. A neuron in vivo develops its arborization in response to genetic as well as environmental clues [13]. While this conclusion is well-accepted, it is not well understood. Major questions remain about the nature of the environmental clues and means by which they are sensed. The nature and complexity of the algorithm (the genetic component) necessary to generate a complex branched pattern in response to such clues is also not known. While there are several attractive hypotheses [11, 16], a difficulty in choosing among them is knowing how much of the final arborization pattern is a consequence of physical processes acting on the growing cell. Examples of these would be constraints imposed by the neuronal cytoskeleton and membrane structure at the growth cone. These questions are not independent since the amount of final shape that is determined by the basic biophysical constraints of the system will affect the complexity required of the algorithm.

We have recently suggested that one physical process that contributes to final shape of the neuronal arborization is diffusion-limited aggregation (DLA). DLA will produce a fractal shape in response to, at least, three environmental variables possibly relevant to growing neurons: electrical fields, chemical gradients and differences in viscosity [2]. The question was which, if any, of these factors was, in part, responsible for the pattern of neuronal arborization. Here, we asked if differences in viscosity between cytosol and tissue culture medium would alter neuronal arborization in a manner predicted by DLA. This type of analysis is possible because we and others have recently shown that the fractal pattern of a neuron can be reproducibly analyzed [3, 20].

We modified viscosity of the culture medium of embryonic chick retinal neurons with methyl cellulose and measured effects on neurite outgrowth. We found that increases in viscosity yielded fewer branched neurites. This is the opposite of what is predicted by DLA. Thus, we conclude that viscosity of the microenvironment does not determine the fractal dimension of the neuronal arborization.

To analyze the contribution of viscosity to final neuronal branching pattern we studied neurons that developed in a modified environment: retinal neurons grown under known conditions in vitro with various amounts of

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methyl cellulose, rated at 15 centipoise (cps) at 2% concentration by weight. We used concentrations of 1%, 0.5% and 0.25% of methyl cellulose to vary viscosity of the medium. Cells were prepared, plated at 2.3×10^5 per cm^2 in Dulbecco's-modified Eagle's medium (DME) with 5% fetal bovine serum and 1% glutamine and 1%, 0.5% and 0.25% methyl cellulose and maintained in culture on polyornithine according to Hausman et al. [10].

Cultures were maintained for two days before counting neuronal branches or analyzing the images for fractal dimension. For each viscosity the number of cells with arborizations were counted, the main neurite counted as one branch and any process coming off it was counted as an additional branch. Most cells had no processes initiating from any point on a main process, so the branch count was indicative of the number of cells found having processes in each area of the culture. However, there were few highly ramified cells, so the branch count was not indicative of the degree of ramification of the neuronal arborization. Cells were counted under a phase contrast microscope at a magnification of 20× at 15 different stage positions to sample different regions of the culture well. Cultures were then stained with 0.01% toluidine blue (1 h) and photographed. Neurons from the photographs were digitized and their fractal dimensions calculated [2, 3] as described below.

Image acquisition was performed with an Apple-scanner (637 × 825 pixel resolution) and fractal dimension (d_f) of the digitized patterns was determined as follows. We chose a neuronal point at random as a 'local origin', and constructed a sequence of concentric disks around this, each with a different radius r. For each disk, we counted mass $M(r)$, the number of pixels constituting the neuronal shape in that disk. We averaged over all possible local origins within the radius of gyration of the object (square root of the sum of the squares of the radii from the center of mass to all points on the object, divided by square root of the number of points). Fractal mathematics predicts $M(r)$ to scale as

$$M(r) \sim r^{df}. \quad (1)$$

[21]. The slope of log $M(r)$ plotted against log r gives a quantitative value of d_f. Images of digitized neurons were input into a sand box method program written in FORTRAN, as previously [2, 3]. This yielded a plot of natural log of mass versus natural log of radius. From this plot we determined slope (d_f) as follows. For each region of the curve to be analyzed, we fit a least squares line to the data and determined the slope of the line. Although the plot had a relatively constant slope, there were variations from region to region. For the best slope, we first chose the region with the highest slope and calculated its slope, then chose the region with the lowest slope and calculated its slope. We then combined the high-slope and low-slope regions into a single region from which we calculated a best slope. The best slope resulted in the average d_f.

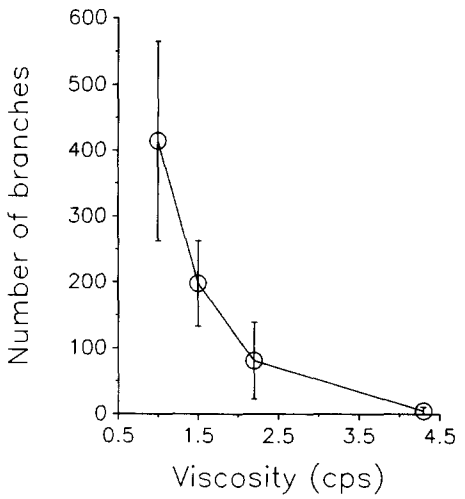

Fig. 1. Number of neurite branches in cultures of chick embryo retinal neurons grown at increasing viscosity of the culture medium. Branches were counted per microscope field as described in the text. Most cells had no processes initiating from any point on a main process, so the branch count was indicative of the number of cells found having processes in the areas of the culture counted. There were few highly ramified cells, so the branch count was not indicative of the degree of ramification of the neuronal arborization. Each point represents the mean and SD of 15 different fields. This is one of three experiments.

The effect of culture in methyl cellulose on cellular biosynthesis was determined by measuring protein synthesis as described previously [9]. Briefly, cultures were exposed to 15 μCi/ml ^{14}C-mixed amino acids (New England Nuclear) for 60 min, synthesis stopped by treatment with cold 15% trichloroacetic acid (TCA) and the incorporation into TCA-precipitable counts determined by scintillation counting.

We chose retinal neurons in vitro as a system to study the effect of viscosity changes on neurite growth because of extensive experience growing the cells and because they are nearly two dimensional in vivo. Thus, measurements on cells in vitro can be directly related to cells in vivo. Preliminary experiments with methyl cellulose polymers over the viscosity range (at 2%) from 4000 to 15 cps established that viscosities at the lower end of this range allowed neurite outgrowth. Such experiments also established that medium viscosity had no measurable effect on cell viability (determined by dye exclusion) or on initial cell attachment to polyornithine. For the experiments described here, we diluted 15 cps methyl cellulose in culture medium at the following viscosities and strengths: 4.34 cps (1%), 2.15 cps (0.5%), and 1.48 cps

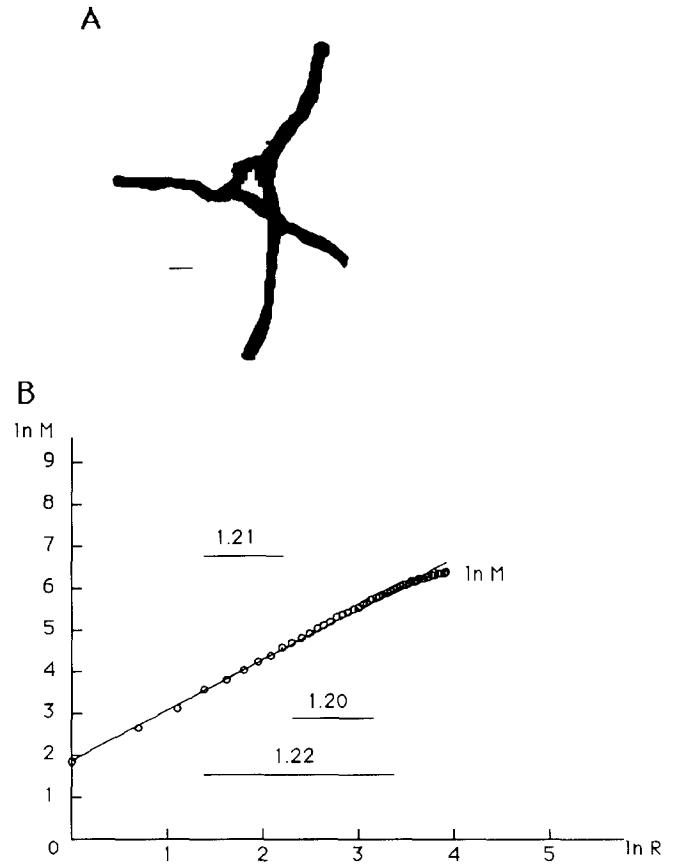

Fig. 2. Fractal analysis of a representative retinal neuron. A: digitalized image of the neuron in vitro. Bar = 10 μm. B: the fractal analysis for the neuron in A. From this plot of natural log of mass versus natural log of radius, we determined the slope (d_f). For the linear region of the curve, we fitted a least squares line and determined the slope. Although the plot had a relatively constant slope, there were variations from region to region. For the intermediate slope, we first chose the region with the highest slope and determined it, then chose the region with the lowest slope and determined it. We then determined a single region from which we determined the slope. This was defined as the intermediate slope and was used to calculate the average d_f. In this figure, regions of the plot used for straight line fits are shown as horizontal bars with the corresponding slopes written above them. The variability range was calculated as the difference between the high-slope measurement and the low-slope measurement [3].

(0.25%). Cells were maintained in methyl cellulose for four days of culture before analysis.

Outgrowth of neurites from retinal neurons was markedly affected by increases in viscosity of the culture medium. As viscosity increased the number of neurons with dendritic arborizations decreased (Fig. 1). Neuron branches decreased from 414 per microscope field in cultures without added methyl cellulose to 5 in the 4.3 cps medium.

Digitizing these photographs produced images of individual neurons (see Fig. 2A for a typical neuron). Analysis by the sand box method produced plots of natural log of mass versus natural log of radius as shown in Fig. 2B. In contrast to the effect on neurite branching, we found no significant change in fractal dimension of the neuronal arborization with increased viscosity of the culture medium (Fig. 3). The fractal dimension was mainly in the range from 1.0 to 1.2 (Fig. 3). This is lower than those obtained previously [2] for cultured cells ($d_f = 1.4$), due to a shorter culture period (4 days for the current study versus 14 days for the neurons with $d_f = 1.4$) used here to detect differences during the process of neurite outgrowth. The relevant point is that the fractal dimension of retinal neurons was not changed by variation in viscosity of the culture medium over a range that had striking effects on neurite branching.

The fact that viscosity had little effect on fractal dimension suggested that it was not as sensitive as the number of branches. To determine the extent of the medium viscosity effect on total cell metabolism, we investigated protein synthesis in the cultures. The results (Fig. 4) show that protein synthesis was little affected by the increase in methyl cellulose concentration. There was only a 17% decline at the lowest viscosity of methyl cellulose and no greater effect with further increases in viscosity.

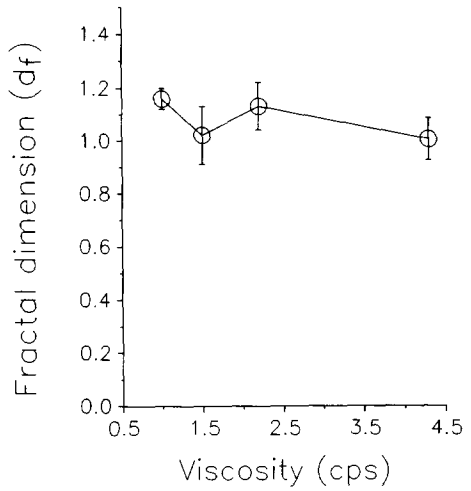

Fig. 3. Fractal dimension (d_f) of chick embryo retinal neurons as a function of viscosity of the medium (1.0, 1.48, 2.15 and 4.34 cps). The fractal dimension was calculated for each neuron as illustrated in Fig. 2. Each point represents the mean and SD of 5 different fields. This is one of two experiments.

We have previously shown that retinal neurons exhibit fractal dendritic arborization patterns after neurite outgrowth both in vivo and in vitro [2]. The fractal dimension (d_f), the mathematical description of that fractal behavior, falls within a narrow range across several types of CNS neurons and might reflect a basic property of neurite outgrowth [3]. We noted that a common physical process, diffusion-limited aggregation (DLA), acting on the neuronal growth cone would yield fractal dimensions within the observed range. We also suggested three environmental factors that might be responsible for this DLA fractal behavior. These are: a viscosity differential between cell and culture medium, a chemical gradient, or a non-uniform electrical field [2]. Because it could be more readily controlled, we chose here to investigate the role of viscosity. DLA theory predicts a transition from a branchless to a ramified morphology when the viscosity outside the cell goes from less than that inside to greater than that inside. If the DLA theory holds and fluid viscosity is the determining factor then we would expect fewer or no branches at low culture medium viscosities. We would expect no branches until the viscosity of the medium exceeds that inside the cell. For any viscosity level higher than that we would expect a branched structure with the number of branches again unchanged with increasing viscosity. We did not find such a transition to a ramified morphology but, instead, when we increased viscosity we found fewer neurons with branches. At the highest viscosity level (4.3 cps) the number of cells with branches was only 1% of those in the control medium. The remaining neurites were single processes without branches. Since branching was strongly inhibited, there is unlikely to be a transition above 4.3 cps. We conclude that the viscosity differential between the inside and the outside of a neuron during neurogenesis is not a factor in the development of the fractal dimension. Few studies have directly investigated the effects of viscosity on neurite outgrowth. Harris et al. [8] investigated neurite outgrowth in a collagen matrix. They found that neurite outgrowth was initiated sooner, proceeds at a faster speed and that the shape of the growth cones was more like that seen in vivo when the cells were grown in the matrix. Clearly, these results are quite different from those obtained here and it is likely that factors other than viscosity are responsible. Harris et al. [8] suggest that three-dimensional matrices can maintain a growth factor gradient. Since the growth rate would be expected to decline when the growth factors are depleted, this might lead to a higher average growth rate. Schwarz et al. [19] give a similar explanation: suggesting that the matrix provides a slow release mechanism for neurotrophic factors such as NGF. Alternatively, matrices may preserve neurotrophic factors that otherwise would be degraded by cellular proteases. Schwarz et al. [19] also note the importance of matrix adhesive components such as laminin in supporting neurite extension. It is likely that the extra substratum contact area available in matrices leads to more contact with extracellular matrix components and the faster growth observed. Thus, the fact that neurons grow well in matrices does not contradict our observations that they produced fewer branches in more viscous liquid media.

Our results demonstrate that viscosity differences are not a physical factor affecting the fractal dimension of

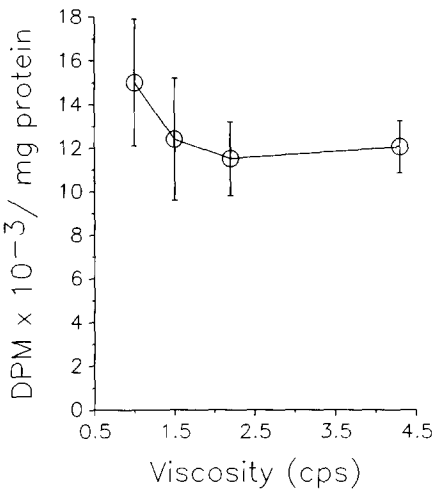

Fig. 4. Effect of viscosity of the culture medium on protein synthesis in retinal neurons in culture. Thirty minutes before each assay period the cultures were provided with [^{3}H] amino acids and TCA-precipitable counts determined as previously [9]. One of two experiments is shown with the mean and S.D. from three measurements.

neurite outgrowth. From the DLA model we predicted two other sources of fractal behavior (chemical gradients and electrical fields) for embryonic neurons. Chemical gradients [7] and electrical fields are both known to affect neurite outgrowth [18]. Since neurites growing in response to an electrical field are simultaneously influenced by a chemical gradient of growth factors, it is more complicated to relate this situation to DLA than in the case where there are only chemical gradients. We are currently investigating these possibilities.

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