



## Biophysical constraints on neuronal branching

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### Abstract

We investigate rules that govern neuronal arborization, specifically the local geometry of the bifurcation of a neurite into its sub-branches. In the present study we set out to determine the relationship between branch diameter and angle. Existing theories are based on minimizing a neuronal volume cost function, or, alternatively, on the equilibrium of mechanical tension forces, which depend on branch diameters. Our experimental results utilizing two-dimensional cultured neural networks partly corroborate both the volume optimization principles and the tension theory. Deviation from pure tension forces equilibrium is explained by an additional force exerted by the anchoring of the junction to the substrate.

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### 1. Introduction

When examining the structure of the brain or even the structure of a simple neural network in the nervous system, one is faced with surprisingly complex wiring diagrams [1,15]. Even the neurons themselves, the individual entities that together construct neural networks, come in a wide variety of shapes and forms. As in networks in general, there is a strong relation between the neural networks' structure or wiring diagram, and function (see [15] and references within). Neurite outgrowth, the branching pattern of single neurons leading to the formation of specific neuronal and network morphology, as well as distinct synaptic connections, is a dominant factor in determining the future output of neural circuits-behavior.

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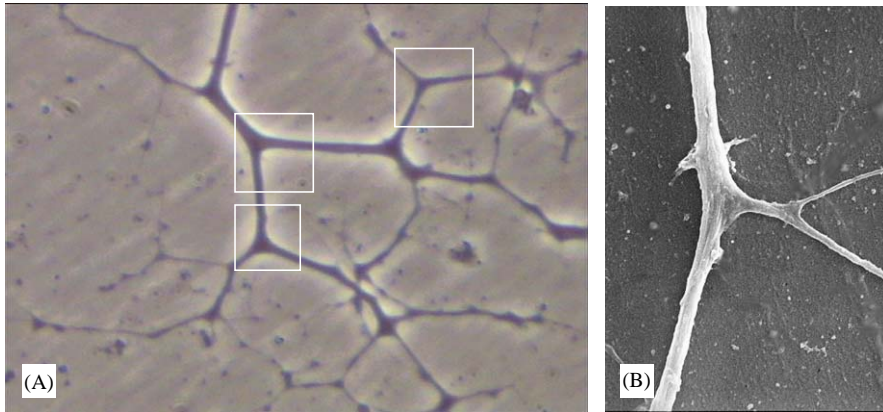


Fig. 1. (A) Cultured neurons arborize into multiple branching neurites. Examples of measured branch points (bifurcations) are marked. (B) A scanning electron microscope image of a neurite branching area.

In our search for rules that govern the complex structures of neurons and neural networks, we concentrate here on neuronal arborization, specifically on the local geometry of the bifurcation of a neurite into its sub-branches (Fig. 1).

The relationship between neuronal branching angles and neurites' diameters has been previously addressed by two major theoretical approaches. The first approach is derived from the concept of optimization, and has been previously applied to blood vessels, living trees and more [4,10,11]. This theoretical model is based on postulating a cost function and subsequently minimizing it under certain constraints. By suggesting the total volume of branches to be the cost function, Murray [10,11] calculated a relationship between branch diameters and angles at a single bifurcation in living trees and blood arteries. A similar model can be applied to neurons and neurite branching [4]. Indirect comparison of experimentally measured neurite diameters and branch angles with various model derived parameters (based on minimizing volume, length, signal propagation speed, or surface area) suggested that a volume minimization model provides the best fit to the data [4–7,9,14].

The second theoretical approach is based on postulating the existence of mechanical tension along the branches constructing neuronal arbors [3]. One can compare these branches to ropes being pulled by the growth cones at the tip of the growing neurites with forces proportional to the neurites' diameters. According to this approach, the neuronal arbor is attached to the substrate only by its growth cone. Hence, the junction geometry (i.e. the arrangement of the arbor segments) is determined by the equilibrium of tension forces.

Although the mechanical tension approach appears to differ from the volume optimization model, the two are mathematically equivalent. This is because the optimization problem can be formulated in a differential form, where derivatives of the cost function in respect to branch length are virtual forces. The requirement for the minimum cost function is then equivalent to the tension force equilibrium.

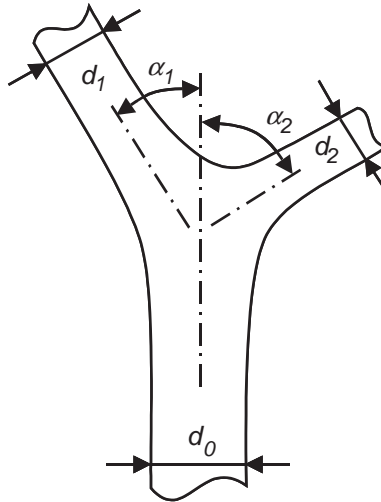


Fig. 2. Geometry of a neurite branch point is characterized by the diameters of the parent neurite ( $d_0$ ), and daughter neurites ( $d_1, d_2$ ) and by the branch angles ( $\alpha_1, \alpha_2$ ). We measured these parameters in cultured neural networks and compared them with theoretical predictions.

*Theoretical predictions:* According to Murray, the volume optimization approach leads to the three following equations (see Fig. 2 for definitions of parameters):

$$\cos(\alpha_1) = \frac{(d_0^4 + d_1^4 - d_2^4)}{(2d_1^2 d_0^2)}, \tag{1}$$

$$\cos(\alpha_2) = \frac{(d_0^4 + d_2^4 - d_1^4)}{(2d_2^2 d_0^2)}, \tag{2}$$

$$\cos(\alpha_1 + \alpha_2) = \frac{(d_0^4 - d_1^4 - d_2^4)}{(2d_1^2 d_2^2)}. \tag{3}$$

Assuming that the bifurcations are in mechanical equilibrium (or, equivalently, the cost function is minimized), there is a relationship between the tension forces and the branch angles. This relationship follows the Law of Sines for the force triangle and is given by the following three equations:

$$\frac{T_1}{T_0} = \frac{\sin(\alpha_2)}{\sin(\alpha_1 + \alpha_2)}, \quad \frac{T_2}{T_0} = \frac{\sin(\alpha_1)}{\sin(\alpha_1 + \alpha_2)}, \quad \frac{T_2}{T_1} = \frac{\sin(\alpha_1)}{\sin(\alpha_2)},$$

where  $T_0$  is the tension along the parent neurite and  $T_1$  and  $T_2$  are the tensions along the daughter neurites. The third equation follows the first two. If tension is proportional to branch diameter to some power  $v$ , then the above equations can be presented as

$$\frac{d_1^v}{d_0^v} = \frac{\sin(\alpha_2)}{\sin(\alpha_1 + \alpha_2)}, \tag{4}$$

$$\frac{d_2^v}{d_0^v} = \frac{\sin(\alpha_1)}{\sin(\alpha_1 + \alpha_2)}, \quad (5)$$

$$\frac{d_2^v}{d_1^v} = \frac{\sin(\alpha_1)}{\sin(\alpha_2)}. \quad (6)$$

Presented this way, Eqs. (1)–(6) predict the relations between diameters and angles.

Using cultured insect neurons, we set out to investigate whether neuronal arbors are adequately described by the above models and to determine the relationship between branch diameter and the cost function, or, alternatively, the dependence of the tension force on diameter. Additionally, in order to have a better understanding of tension force dynamics, we investigated the attachment of neuronal arbors to the culture substrate using mechanical manipulation.

## 2. Methods

### 2.1. Cell culture

The experimental system we employed, two-dimensional neuronal networks growing in cultures of neurons dissociated from insect ganglia, has been described in detail elsewhere [2,12] and is only briefly outlined here. This system is simple (relative to any in-vivo network), and allows control over many of its variables. It also enables easy access for optical observation and mechanical manipulation of the cells. Neurons were dissociated from the frontal ganglion of adult locusts. After dissection, enzymatic treatment and mechanical dissociation, the neurons were plated on Petri dishes, pre-coated with Concanavaline A, and maintained under controlled conditions. A CCD camera mounted onto a phase contrast microscope was used to acquire images of three to 6-day-old cultured neurons into a PC for image processing and analysis. Mechanical manipulation of single neuronal arbors was achieved using a glass rod mounted on a micromanipulator and constantly monitored by the phase contrast microscope.

### 2.2. Measurements and data analysis

We analyzed neuronal branch bifurcations in which a parent neurite splits into two daughter neurites (see Fig. 1). Bifurcations with obvious abnormalities such as abrupt thickness or angle changes and other irregular disruptions to growth were not measured. After a branching area was chosen and its image was acquired to the computer, the exact geometry of the bifurcation was traced to allow measurement of the diameters of the parent and daughter neurites and the angles formed between the orientation of the parent neurite and that of each of the daughter neurites (Fig. 2). In order to ensure systematic measurements and to overcome any deformation in neurite surface that might prevent a clear definition of the borders of the bifurcation area, we used a special routine written in Matlab application. Dots were marked along each of the three segments creating the bifurcation (parent and daughter neurites see Fig. 1), from

the junction point and up to 5 segment diameters away. A linear fit was calculated for each set of dots (at least 8 dots, marked on each of the two neurite faces, for all three segments, totaling 6 sets of dots altogether). When calculating the fit coefficients, the application discarded several points adjacent to the junction until the  $R$ -square was no longer compromised. The average diameters, average bifurcation angles and corresponding errors were calculated using the fitted parallel straight lines, accurately tracing the neurites. These measured experimental parameters were then tested against the theoretical predictions of the optimization and tension models by substituting them in the diameter and angles correlation functions presented in Eqs. (1)–(6).

### 3. Results

#### 3.1. Bifurcation measurements

Neuronal images were acquired from two to 6-day-old cultures. Diameters and related angles were measured from 22 neuronal branch bifurcations that complied with the criteria described above. All neurites were less than 5  $\mu\text{m}$  in diameter. Bifurcations were generally not symmetrical—i.e. the diameters and angles of the left and right daughter neurites were different. However, when averaging the measured angles this difference showed no statistical significance ( $58 \pm 16^\circ$  and  $61 \pm 20^\circ$  for left and right angles, respectively).

#### 3.2. Theoretical prediction validation

Statistically significant correlations between the functions of the measured diameters and angles were demonstrated when testing the predictions in Eqs. (2)–(4), but not for Eqs. (1),(5) and (6) .

As can be seen, the correlations are significant for only part of the predicted relations. Furthermore, the data show considerable variation (Fig. 3). The exponent  $\nu$ , which relates tension to diameter, was found to be 2.9.

#### 3.3. Mechanical manipulation

To test the assumption that the arbor is attached to the substrate only by its growth cones, we mechanically manipulated a daughter neurite by means of a fine glass rod until its growth cone was detached from the substrate and tension along the neurite was released. We monitored the position of the junction during the process of neurite detachment and absorption in several cultured neurons. Fig. 4 presents an example of a bifurcation before and after such manipulation. It is clear that although the neurite is entirely absent, the junction point itself has only slightly moved.

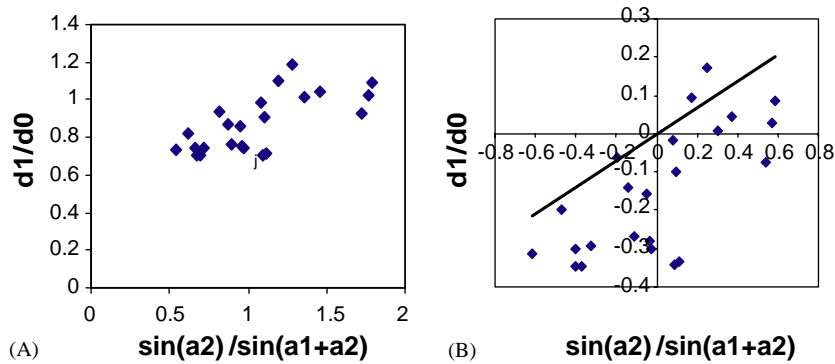


Fig. 3. Mechanical tension equations tested for 22 measured neuron bifurcations. (A) The graph shows the results for Eq. (1) (dots). (B) The same data plotted on the log–log graph. The data are fitted by a straight line passing through the origin. The slope of the line gives the exponent  $\nu$  in the dependence of tension force on diameter (or cost function on diameter).

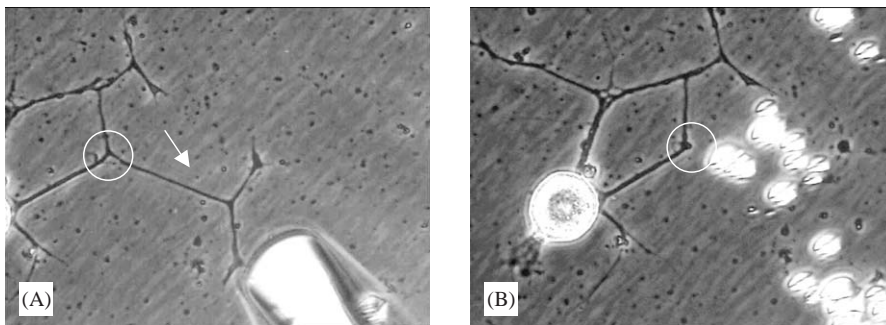


Fig. 4. An example of a bifurcation before (A) and after (B) mechanical manipulation. It is clear that although the neurite is entirely absent (marked with an arrow in A), the junction point itself has only slightly moved (marked with the circle). The junction point itself is attached to the substrate, where it acts as an anchor, thus adding an additional force to the local equilibrium of forces.

#### 4. Discussion

In order to examine the biophysical constraint on neuronal branching, we employed two-dimensional neuronal networks growing in cultures of neurons dissociated from insect ganglia. We tested two theoretical approaches—volume optimization and tension equilibrium, both of which relate the diameters of a neuronal arbor to its bifurcation angles. To do so, we measured the dimensions of branch points and tested the validity of the theoretical predictions.

Our results show that both the tension theory and the volume optimization principles provide reasonable approximations to the data. However, the predictions demonstrate some disparity and are not all significant.

These deviations from the expected values may be explained either by the fact that the relationship between the tension force and diameters is more complicated than was assumed, or by additional forces other than tension that take part in determining branch angles. We investigated the second possibility, assuming that the junction point itself may be attached to the substrate and thus act as an anchor to the branching point [2,12,13], adding an additional force to the local equilibrium of forces. Mechanical manipulation demonstrated that this is indeed the case.

In summary, we suggest that elements of both theories tested here participate in ensuring the construction of neurite bifurcations that will satisfy functional requirements. The variations from pure tension forces equilibrium is explained by an additional force exerted by the attachment of the junction to the substrate. This force confers plasticity upon the structure of the branch bifurcation, which enables optimization of bifurcation geometry without contradicting physical constraints. Plasticity is achieved by an additional degree of freedom to the relation between diameter and angle and is limited by the magnitude of the force produced by the junction as an anchor. We further believe that the principles demonstrated in our two-dimensional experimental system are also applicable *in vivo* [8], where the neuronal growth and branching process take place within a three-dimensional substrate.

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