A Two-Phase Growth Strategy in Cultured Neuronal Networks as Reflected by the Distribution of Neurite Branching Angles

Orit Shefi,1,2 Sharon Golebowicz,2 Eshel Ben-Jacob,2 Amir Ayali1
1 Department of Zoology, Tel-Aviv University, Tel-Aviv 69978, Israel
2 School of Physics and Astronomy, Tel-Aviv University, Tel-Aviv 69978, Israel

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ABSTRACT: Neurite outgrowth and branching patterns are instrumental in dictating the wiring diagram of developing neuronal networks. We study the self-organization of single cultured neurons into complex networks focusing on factors governing the branching of a neurite into its daughter branches. Neurite branching angles of insect ganglion neurons in vitro were comparatively measured in two neuronal categories: neurons in dense cultures that bifurcated under the presence of extrinsic (cellular environment) cues versus neurons in practical isolation that developed their neurites following predominantly intrinsic cues. Our experimental results were complemented by theoretical modeling and computer simulations. A preferred regime of branching angles was found in isolated neurons. A model based on biophysical constraints predicted a preferred bifurcation angle that was consistent with this range shown by our real neurons. In order to examine the origin of the preferred regime of angles we constructed simulations of neurite outgrowth in a developing network and compared the simulated developing neurons with our experimental results. We tested cost functions for neuronal growth that would be optimized at a specific regime of angles. Our results suggest two phases in the process of neuronal development. In the first, reflected by our isolated neurons, neurons are tuned to make first contact with a target cell as soon as possible, to minimize the time of growth. After contact is made, that is, after neuronal interconnections are formed, a second branching strategy is adopted, favoring higher efficiency in neurite length and volume. The two-phase development theory is discussed in relation to previous results. © 2004 Wiley Periodicals, Inc. J Neurobiol 62: 361–368, 2005

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INTRODUCTION

A fundamental process in the development of single neurons into elaborate, electrically active neuronal networks is neurite outgrowth and branching in order to connect target cells and tissues.

There are several factors or classes of mechanisms that influence the geometry of a neurite that bifurcates into its daughter branches. A key mechanism which attracted much attention and ample work is the growth cone’s ability to measure environmental cues (Dodd and Jessell, 1988; Whittington, 1993; Tessier-Lavigne and Goodman, 1996; Zinn and Schmid, 1999; Bagnard et al., 2000, Huber et al., 2003) — the motile growth cones at the tips of growing neurites are guided to their targets by searching and measuring concentration gradients of a large array of chem-
repelling and chemo-attractive molecules, many of which have been identified. Most research, however, has focused on the general issue of target recognition during neuronal development and less on the effect of the environment on the specific morphology of the branching point.

A second basic factor is the effect of physical constraints on the geometry of neuronal arborization. Rall (1959) suggested a power relation between the diameters of a parent neurite and the diameters of its daughter branches based on theoretical calculations of impedance matching. This relation was also investigated by Bray (1979), who found in cultured chick dorsal root neurons a different power relation. Recent theoretical work by Chklovskii and Stepanyants (2003) distinguished between myelinated and non-myelinated neurites and suggested different powers for the two cases.

Physical forces such as tension and friction also affect the angle between the two newly formed daughter branches (Bray, 1979; Mitchison, 1991; Cherniak, 1992; Condron and Zinn, 1997; Cherniak et al., 1999; Zheng, 2000; Chklovskii and Stepanyants, 2003). We recently tested the relationship between neurite diameters and neuronal branching angles (Shefi et al., 2004), and showed that this relationship is based on minimizing neuronal volume as a cost function, or equivalently on an equilibrium of tension forces with an additional force exerted by the attachment of the junction to the substrate.

In the current work we employed an invertebrate in vitro model system (Ayali et al., 2002; Shefi, Ben-Jacob, et al., 2002; Shefi, Golding, et al., 2002), which allowed investigating the presence of innate mechanisms that may dictate the arrangement of neuronal bifurcation during network development. We comparatively investigated branching angles in two neuronal categories: neurons in dense cultures versus neurons in practical isolation. The neurons in the dense cultures bifurcated under the presence of extrinsic cues released from the rich cellular environment while the isolated neurons developed their neurites following mostly their intrinsic, predetermined mechanisms. We found a preferable regime of bifurcating angles in the absence of extrinsic cues.

In order to examine the origin of this favored regime we compared the experimental results to theoretical predictions. We simulated outgrowing single neurons and tested whether we could find a cost function that optimizes within this preferred regime of branching angles. We also compared the experimental results to theoretical bifurcation angles that were calculated based on physical constraints.

This approach proved to be very useful in predicting a two-phase branching strategy. In the first early phase, neurons are minimizing the time to first contact with other potential network members, while utilizing a preferred range of branching angles. In the second later developmental phase, a different strategy is adopted, one that favors higher efficiency in neurite length and volume. This phase is characterized by a broader range of branching angles than the previous one.

**MATERIALS AND METHODS**

**Cell Culture and Data Analysis**

We employed two-dimensional neuronal networks growing in cultures of neurons dissociated from insect ganglia. The experimental system has been described in detail elsewhere (Ayali et al., 2002; Shefi, Ben-Jacob, et al., 2002) and is only briefly outlined here. Neurons were dissociated from the frontal ganglion of anesthetized (CO2) adult locusts. After dissection, enzymatic treatment and mechanical dissociation, the neurons were plated on petri dishes, precoated with Concanavaline A, and maintained under controlled conditions. A CCD camera mounted onto a phase contrast microscope was used to acquire images of 2- to 6-day old cultured neurons into a PC for image processing analysis. We analyzed neuronal branch bifurcations in which a parent neurite splits into two daughter branches. Neurons and bifurcations of two categories were analyzed: (I) 60 bifurcated neurites of practically isolated neurons; neurons with no neighbor neurons in a radius of at least 500 μm [Fig. 1(A)]; and (II) 60 bifurcated neurites of neurons that were grown in dense cultures (above 20 cells/mm2) and were already part of a neuronal network [Fig. 1(B)]. After a branching area was chosen and its image acquired to the computer [Fig. 1(C)], the geometry of the bifurcation was traced to allow measurements of the angle formed between the orientations of the daughter neurites, angles were measured with accuracy of up to 2°. Using a special routine written in Matlab application, the average bifurcation angles, the corresponding errors and angle distributions were calculated.

**Theoretical Predictions: Biophysical Constraints**

We used the relation between the diameter of a parent neurite and the diameters of its daughter branches (Rall, 1959; Bray, 1979; Chklovskii and Stepanyants, 2003) and the relations between diameters and branching angles (Bray, 1979) to predict a regime of angles, which is a consequence of biophysical constraints in the absence of extrinsic cues.

The power law relating diameters is

\[ d_0 = d_1^n + d_2^n \]

where \( d_0 \) is the diameter of the parent neurite, \( d_1 \) and \( d_2 \) are the diameters of its daughter branches. With no extrinsic
cues, we assumed symmetrical bifurcations \((d_1 = d_2 = d)\). Thus the power law becomes \(d_0^2 = 2d^n\), or equivalently \(d_0/d = 2^{1/n}\), and the ratio between the diameters depends only on \(\eta\).

To relate the ratio between the diameters to branching angles we assumed that the bifurcations are in equilibrium of forces. If tension is proportional to branch diameter, following the Law of Sines, the ratio between the diameters is:

\[
\frac{d_0}{d_1} = \frac{\sin (\alpha_1 + \alpha_2)}{\sin (\alpha_1)}
\]

(assuming linear relation between tension and diameter; Shefi et al., 2004). According to the symmetry assumption presented previously, this ratio becomes:

\[
\frac{d_0}{d} = \frac{\sin (2\alpha)}{\sin (\alpha)} \text{ or } \frac{\sin (2\alpha)}{\sin (\alpha)} = 2^{1/n}.
\]

Thus we can calculate the branching angle \(\alpha\) based only on the power \(\eta\).

We tested our results against previous theoretically suggested and experimentally measured power \(\epsilon\) (Rall, 1959; Bray, 1979; Chklovskii and and Stepanyants, 2003; Scorcioni et al., 2004).

**Simulation**

To further test whether there is a preferable regime of angles in the process of neuronal branching, we constructed a computer simulation in which a single neuron outgrows and branch until contact is made between one of its neurites and one of the target cells that surround it. Simulation assumptions (based on Segev and Ben-Jacob, 1998) were: (a) The growing cell was surrounded by randomly positioned target cells. Neuronal density mimicked the experimental conditions. (b) Growth cones moved in biased random walk. (c) The probability of a neurite to branch increased exponentially with time. (d) A neurite branched into two daughter branches. (e) In each run all neurites branched at a distinct chosen angle.

We tested branching angles between 0° to 360° (0°, reflecting daughter branches maintaining the exact vector of the mother neurite; 360°, the reverse). Once a target cell was reached, the growth process stopped and one of several different cost functions, including total length of all neurites, volume of the dendritic tree, and time of growth, was calculated. We calculated the cost functions in 1000 non-dependant runs for each branching angle and used the averaged values to fit a polynomial curve. We tested for an optimized regime of angles that minimized the tested cost function.

**RESULTS**

**Bifurcation Measurements**

Neuronal images were acquired from 2- to 6-day-old neuronal cultures. Angles were measured from bifurcations of neurons that were practically isolated (category I) and from bifurcations of neurons growing in dense cultures (category II). All angles were measured at a rather early stage after bifurcation occurred, before the daughter branches outgrew and went through further bifurcation themselves [Fig. 1(C)]. The rich bifurcations and neurite arborization behavior we observed is a characteristic of neuronal dendritic trees (e.g., Uylings and Van Pelt, 2002). Yet, we did not positively discriminate between axons and dendrites in our cultured
neurons and thus refer to them only generally as neurites. We also did not attempt to distinguish between cell types and we control for this variable only by consistent culturing procedure and totally arbitrary choice of measured angles in the two neuronal categories tested.

Figure 2 demonstrates the distribution of measured angles for the two neuronal categories. The isolated neurons bifurcated at angles between 30° to 150°, where most of the angles (near 50%) were concentrated between 75° to 105°. Neurites of the second category of neurons, that are surrounded and connected to neighbor cells, split at a somewhat wider range of angles, between 30° to 165°. Moreover, the distribution histogram appears flatter and does not demonstrate a preferred regime of branching angles. Both categories showed a similar distribution of angles at the lower range; 20% of the neurites had a bifurcation angle below 60°. Based on our previous work and time lapse observations (Shefi et al., 2004), this narrow range of angles is a consequence of very thin daughter branches that can exert low tension forces, and we believe that these thin neurites reflect an initial premature stage of the bifurcation process. Disregarding this lower regime (below 60°), we could calculate a similar average angle for both categories (98° ± 20° and 98° ± 30° for categories I and II respectively. As mentioned, error in measurement was up to 2°).

Biophysical Predictions

As presented above, we used the relation between the diameter of a parent neurite to the diameters of its daughter branches and the relations between diameters and branching angles to predict an optimized regime of angles dictated by biophysical constraints (in the absence of extrinsic cues). Thus, we calculated the bifurcation angle $\alpha$ that is affected only by the power $\eta$. Substituting $\eta = 1.5$ according to Rall’s law (Rall, 1959) gives an angle of 74° between the two daughter neurites. Substituting $\eta = 1.73$ according to Bray (1979) gives an angle of 84°. The calculated angle between the two daughter neurites based on $\varepsilon = 2.52$, as predicted for non-myelinated fibers by Chklovskii and Stepanyants (2003) is 98°. The latter is similar to the average angle we found experimentally.

Simulation Results

An example of one run of a simulated neuron that bifurcates at one particular angle is presented in Figure 3. We tested the neurite outgrowth and branching process for each of 60 angles between 0° and 360° (see Method section). Figure 4(A) presents the dependence of total growth time of the simulated neuron on the bifurcation angle. An average of 1000 simulated neurons (simulation runs) is shown for each angle. As can be seen, the lower and upper ranges of branching
angles have a high cost (the cost function being the total time from growth initiation to contact with a target cell). In between these high cost regions, a lower cost or total time is obtained with a clear minimum around the branching angle of $\alpha = 112^\circ$ (angle between the two daughter branches). The graph is not distributed symmetrically around the minimum; the upper range shows the highest cost. Testing the total length of the neuritic tree as the cost function [Fig. 4(B)] resulted again in a parabolic-like curve. The higher and the lower range of angles yielded high cost and the medium range a lower cost. Again the upper range was higher than the lower range. This time however the area around the minimum value $106^\circ$ was wider in comparison to the total time model. Similar results were found when the total volume of the dendritic tree was calculated (not shown).

Next we studied the relationship between the cost functions, computed based on our simulations, and the experimentally measured branching angle distributions, shown in Figure 2. We tested the dependence of the probability measured for the different ranges of angles in both categories studied (isolated and connected neurons) on the two calculated cost functions: time and length. Interestingly, we found an exponential relationship: as the cost calculated for a specific branching angle increased, the probability to bifurcate at this angle reduced exponentially. This is clearly demonstrated at the upper range, where the costs were the highest and no experimental angles were measured for either category of neurons (Fig. 4). The different tested correlations could be described by a linear regression when plotted on a semi-logarithmic graph (log of measured probabilities vs. cost func-

![Figure 3](image_url)

Figure 3 An example of one run of a simulated neuron (marked in black) surrounded by randomly distributed target cells (grey). The neuron outgrows and bifurcates until one of its neurites makes contact with one of the target cells (arrow). In each run all neurites branched at a distinct chosen angle between $0^\circ$ to $360^\circ$ ($0^\circ$, reflecting daughter branches maintaining the exact vector of the mother neurite; $360^\circ$, the reverse). In the example shown the angle is $100^\circ$ between the two daughter branches. Once a target cell was reached, the growth process stopped and various cost functions were calculated.

![Figure 4](image_url)

Figure 4 The average cost function per branching angle ($0^\circ$ to $360^\circ$ between the daughter branches) was calculated for 1000 independent runs of simulated neurons. (A) Time of growth as the cost function showed a narrow regime of optimal branching angles around a minimum at $112^\circ$. (B) Calculating averaged total length of all neurites as the cost function resulted in a much wider range of low values around $106^\circ$. 

Neuronal Branching Angles
As shown in Figure 5, we found a similar slope of the regression line computed for the category I angles (isolated neurons) versus time as the cost function \((\lambda = -30 \pm 1)\) and for the category II angles (connected neurons) versus total length as the cost function \((\lambda = -32 \pm 1)\). These similar slopes suggest a single mechanism relating branching angles to cost in these two cases. Hence the experimental data obtained from the isolated neurons (category I) matches well the simulation results calculated for growth time as the cost function, and length or volume as the cost function describes best the data obtained from the neurons connected to close neighbors (category II). The two other category–cost combinations yielded different regression slopes (from each other and from the above).

**DISCUSSION**

Our search for the mechanisms that control neuronal branching angles was based on experimental observations, on generating theoretical prediction and simulations of developing neurons, and primarily on comparing the results of these complimentary research strategies. Our experimental data demonstrated a clear difference between neurons that outgrew their neurites in a relatively dense cellular neighborhood versus neurons that developed in isolation.

Biophysical constraints are major factors in determining the branching angle: both categories of neurons showed a close relation between neurite diameters and branching angles as a consequence of such constraints. It can be expected that at a very early stage of the bifurcation, when daughter neurites are just formed, they can only exert low tension forces. Thus the initial formed angles measured in both categories were narrower than angles of established bifurcations. This phenomenon was also confirmed by up to 90 h time lapse monitoring of developing neurons in culture (not presented here). As reported in Shefi et al. (2004), the junction itself is anchored to the substrate thus adding fraction to the equilibrium of forces. Fraction force is dependent on the area attached to the substrate (Zheng et al., 1994) thus it is negligible in the early stages of bifurcation.

Although both categories bifurcated with the same average branching angle, neurites of connected neurons surrounded by neighbor cells split in a wider range of angles, affected by the location of their target cells. In contrast, most of the branching angles of neurites that bifurcated in the absence of extrinsic cues were within a narrow range of angles. This may indicate on the presence of some intrinsic branching mechanisms and also further support the important

![Figure 5](image-url)

**Figure 5** The relationship between the computed cost functions and the experimentally measured branching angle distributions. Each point represents the log of the average probability to branch in a regime of angles versus the average cost calculated for that regime (normalized between 0 and 1). The selected ranges of angles follow the bin division in Figure 2. The first data point represents the regime of branching angles around the minimum cost function (see Fig. 4) and the rest are for wider angles. Average probability of zero is not presented. Errors in both axes (not shown for clarity) were taken into account in calculating the linear regression lines. (A) Probability measured for angles in category I neurons versus time as the cost function. (B) Angles in category II neurons versus length as the cost function. The slope of the two regression lines is very similar: \([\lambda = -30 \pm 1 \text{ and } -32 \pm 1] \text{ in (A) and (B) respectively}\).
role of the neuronal environment in neuronal morphology (Ayali et al., 2002; Shefi, Ben-Jacob, et al., 2002).

When no extrinsic cues affect the bifurcation, one can select the appropriate theoretical models that are based on optimization principles and biophysical constraints, and under an appropriate set of assumptions generate a preferred angle that falls very nicely within the range shown by our real neurons. An angle of 98° between the two daughter branches was the mean value of our measured experimental angles and was also calculated using a power law that relates the diameters of a neurite and its daughter branches ($\eta = 2.5$). Thus our data favors the model suggested by Chklovskii and Stepanyants (2003) for non-myelinated fibers over other previously suggested power laws. Chklovskii and Stepanyants (2003) confirmed their other predicted power law for myelinated fibers by testing it against experimental measurements of cat motor fibers. They do not, however, refer to the deviation from the early studies (Rall, 1959; Bray, 1979) which is probably due to variation in neuronal types and model assumptions.

Recently, Scorcioni et al. (2004) reviewed morphometric parameters of reconstructed hippocampal pyramidal cells, both in vitro and in vivo. They show great variance in the measurements reported for different cell types as well as from different laboratories (including measures of neurites’ diameters and the angles between two-immediate daughters of bifurcating neurites). The two in vitro studies reviewed therein (Pyapali et al., 1998; Turner et al., 1995) report on a mean bifurcation angle of 86° and 88°. Much in accordance with our data, Pyapali et al. (1998) found the distribution of angles to have a major peak around 90° with an additional very low peak below 70°.

By comparing the simulated developing neurons with our experimental results we further looked for the basis of the mechanism that results in a preferred regime of branching angles. We tested a cost function for neuronal growth that would be optimized at a certain regime of angles, as was suggested in our data from real neurons. The fact that, independent of the cost function tested, our simulations generated a preferred range of branching angles (a minimum to the graph), is a first indication of the validity of this approach. We correlated the cost functions with the experimental results and found a consistent exponential mechanism that translates the cost function at a certain branching angle to the probability of a neurite to bifurcate at this angle.

Our model proved to be very useful in predicting two phases in the branching process of neurons during network development. In the first, early phase, neurons are tuned to find and make first contact with other potential network members as soon as possible. This phase is mirrored in our category I neurons that, while utilizing a preferred range of branching angles, developed in a way to minimize the time to first contact. In the second, later developmental phase, where neuron-to-neuron interactions are more rapid and abundant, a different strategy in branching is adopted, one that favors higher efficiency in neurite length and volume. This phase is characterized by a broader range of branching angles than the previous one as seen in our category II neurons. Minimizing wiring length and volume is an optimization approach that was previously suggested as a leading mechanism in the construction of the nervous system (Cherniak, 1995; Chklovskii et al., 2002; Laughlin and Sejnowski, 2003).

The adaptive branching strategy is also strongly supported by a previous study in which we reported that as long as neurons remained isolated, the number of processes originating from their soma significantly increased with time (Shefi et al., 2002). In contrast, neurons that were already connected to neighbor cells demonstrated a reduction in the number of neurites originating from the soma resulting in a highly significant morphological difference between connected and not yet connected neurons (Shefi et al., 2002). Van Pelt and Uylings (2002) studied branching rates of growth cones and their temporal patterns. By analyzing dendritic trees of Wistar rat multipolar non-pyramidal neurons, these authors came to a conclusion that a high baseline branching rate is rapidly decreasing as a function of time, indicating the largest baseline drive for branching in the early days of outgrowth. This strongly supports our proposed early phase strategy. Recently Baker et al. (2003) also defined clear phases in the development of sensory leech neurons in vivo, distinguishing between younger afferents and mature ones. These authors also observed a preferred arborization angle of 90° at the early stage.

In summary, our results shed light on key phenomena related to neuronal branching and suggest fundamental rules in the development of neuronal circuits. Moreover, our suggested model encompasses the reciprocal relationship between function and form of developing neuronal networks (see Connors and Regehr, 1996; Mainen and Sejnowski, 1996; Sporns et al., 2000, 2002). At the early phase of neuronal development functional requirements are dominant: minimizing time to the formation of synaptic connections. The establishment of functional synapses may be the trigger to switch to the second phase in which the major considerations are structural — optimization of the length and volume. This in turn reflects
back to function as it affects conductance velocity and information transmission in the network.

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REFERENCES


