Morphological characterization of *in vitro* neuronal networks

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We use *in vitro* neuronal networks as a model system for studying self-organization processes in the nervous system. We follow the neuronal growth process, from isolated neurons to fully connected two-dimensional networks. The mature networks are mapped into connected graphs and their morphological characteristics are measured. The distributions of segment lengths, node connectivity, and path length between nodes, and the clustering coefficient of the networks are used to characterize network morphology and to demonstrate that our networks fall into the category of small-world networks.

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I. INTRODUCTION

One of the most profound questions in science is how a collection of elements self-organize to form new and extremely complex systems ([1] and references therein, [2,3]). This question becomes far more challenging when talking about biological systems, where the building blocks themselves are living entities [4]. In the case of the nervous system this issue translates to the open question of how a functioning neuronal network (a small circuit as well as a complex brain) emerges from a collection of single entities, the individual neurons [5–9].

As in networks in general, there is a strong relation between the neuronal network structure, or ''wiring diagram,'' and its function, i.e. the form-function relation [10]. This enables determination of the dynamics and activity of a network by analyzing its morphology and topology of connectivity.

An attempt in this direction has been recently made by Watts and Strogatz in introducing their ''small-world networks'' concept [10–13]. A small world network is one that interpolates between the two extreme cases of a regular lattice, on the one hand, and a random graph, on the other. It is characterized by a local neighborhood, which is highly clustered (as in regular lattices), and by a short path length between vertices (as in random networks).

Watts and Strogatz state that small-world characteristics are a prevalent feature of real life biological networks. Yet, so far, only a few such systems have been examined experimentally. These include metabolic networks in various organisms [14], as well as the large-scale organization of metabolic networks [15], and the nervous system of the worm *Caenorhabditis elegans* [11].

We are presently studying two-dimensional *in vitro* neuronal networks. While these cultured networks lack some features of *in vivo* neuronal networks, they retain many others ([16] and references therein). They develop organotopic synaptic connections and exhibit a rich variety of electrical properties similar to those observed *in vivo*. The two-dimensional system enables easy access for noninvasive optical observations, allowing us to follow the dynamics of neuronal growth and network organization. In addition, our use of invertebrate (locust) cells is advantageous due to the large size of the neurons and the ease with which they can be cultured under various conditions [2,8,10,17–21]. All the above, together with recent progress in multielectrode array technology, optical imaging, and fluorescence microscopy, make invertebrate cultured neuronal networks a favorable model system for studies of neuronal networks and the nervous system.

In our culture preparations, fully differentiated adult neurons, which lose their dendrites and axon during dissociation, regenerate neurites that interconnect to form an elaborate network. During the growth process, growth cones connect to nonself, as well as self previously extended neurites, with no clear evidence for self-avoidance (see Fig. 1). It appears that the cultured neurons cannot be considered as simple elements; even the single isolated cell shows spontaneous electrical activity and forms a complex morphological structure.

Neuronal systems can be modeled as networks or graphs of coupled systems, where the vertices represent the elements of the system, and the edges represent the interactions between them. Once in the framework of a wired graph, one

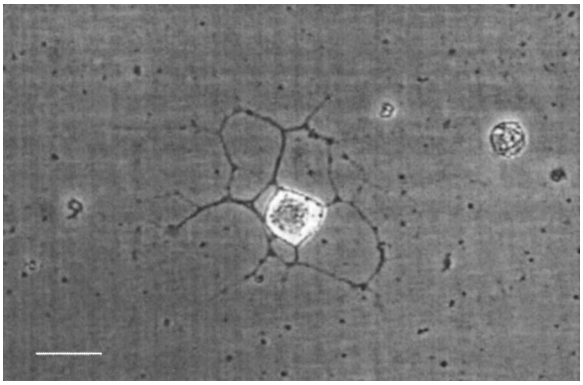

FIG. 1. A single cultured neuron, two days after plating. The neurites outgrow from the round soma, branch and connect to other neurites extending from the same cell. Scale bar = 50μ.

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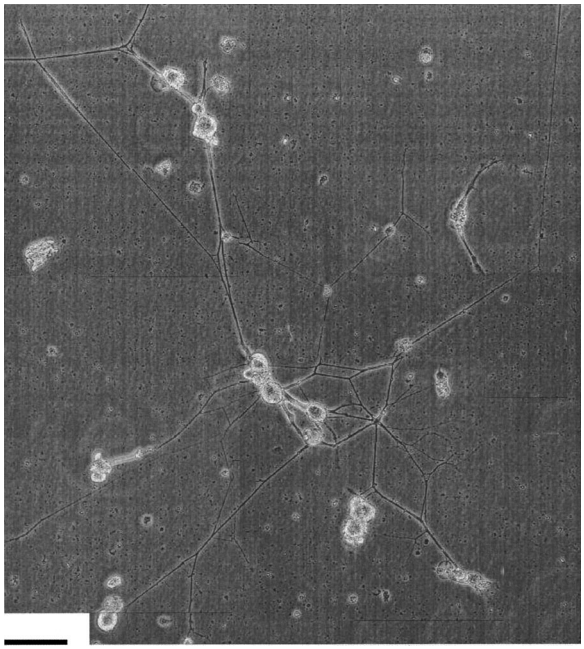

FIG. 2. A part of a mature 6-day-old network. At this stage all the neurons in the network are connected to each other. The neurites are straight segments that show high tension along the processes between junctions. The junction points appear to be more firmly attached to the substrate than are the neurites that connect them. Scale bar $= 50\mu$.

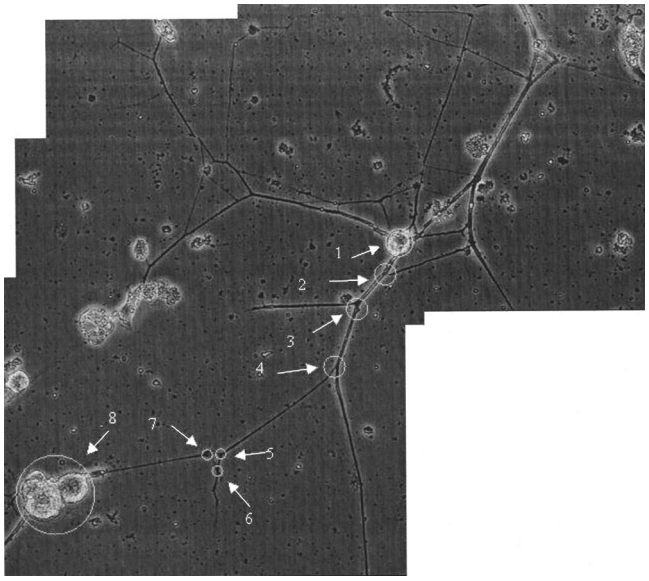

FIG. 3. Illustration of the points considered as vertices in the networks: numbers 1 and 8 are somata of neurons and numbers 2–7 are connection points between neurites. In the adjacency matrix pairs of vertices that are connected as 1-2, 2-3, 3-4, 4-5, 5-6, 5-7, etc. obtain the value 1 while the nonconnected pairs obtain the value 0.

can apply the mathematical tools of graph theory to analyze the system under study and to look for universal, generic features that are common to different kinds of networks within as well as outside the nervous system.

As a first step we needed to define vertices and edges in our system. According to the ''neuron paradigm'' the building blocks of the nervous system are the neurons (vertices) and synapses (edges). However, based on the branching and growth process of the cultured networks, we chose the neurons, the synapses, and synapselike connections between the neurites of the same neuron, to be the vertices. Our main working assumption was that these structures are essential for information processing in the network.

In this work we describe the results for three different neuronal networks grown in culture under controlled conditions. These networks are characterized by a high clustering coefficient compared to random graphs, and a path length that is closer to random networks than to regular ones. We thus classify the studied neuronal networks as small-world networks.

II. GROWTH OF THE NEURONAL NETWORK

Cell cultures. Neurons were dissociated from the frontal ganglion of adult locusts and maintained under controlled conditions. Culturing method followed Shefi *et al.* [2]. Plated neurons varied in size from 10 μm to 50 μm. The number of ganglia per dish determined the density of the culture, and thus the average distance between cells.

A charge coupled device camera mounted onto a phase contrast microscope was used to acquire images of the cultured neurons and networks into a PC for image processing analysis.

Growth process. Time-lapse observations on the growth process of cultured neurons revealed that the most intense stage of development was between day 1 and day 5. After this rapid growth stage there was a pronounced decrease in growth rate [2,3]. By day 6 in culture most of the neurons had developed interconnections and were already a part of an elaborate network (Fig. 2). Hence, we analyzed the network at that point.

During the growth process, growth cones connected not just to neighbor cells but also to neurites previously extended from their own cell body, with no evidence for self-avoidance. They thereby formed close loops. The junctions or interconnection points acted as anchors that seemed to be more firmly attached to the substrate than the neurites themselves. Tension was generated along the neurites as they stretched between these anchors to form straight segments, giving the close loops polygonal shapes (Figs. 1 and 2).

Connectivity statistics was found to change significantly with the age of the single neuronal cell in culture and the developmental stage of the network as a whole [2]. After the initial stage of intense neurite formation, the neuronal cell bodies started to aggregate into packed clusters. The clustering of cells was accompanied by absorption of branches and even whole neurites, together with rearrangements of neurites and what appeared to be fusion of parallel ones. The somata were observed to migrate along newly formed bundles toward one another. Thus, relatively homogenous cultures, in which single neurons were scattered, evolved into cultures organized into a few centers comprised of clusters of neurons connected by thick nervelike bundles [2,22].

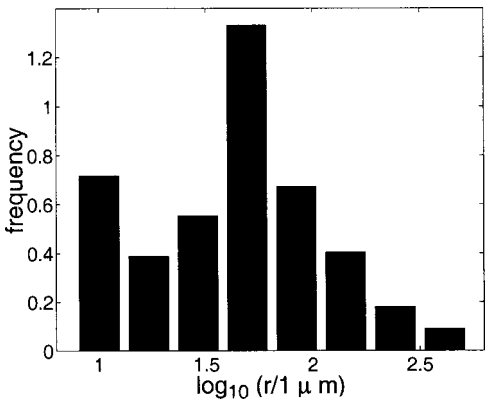

FIG. 4. Distribution of the physical lengths of connecting segments in network number 3 (240 nodes, 290 segments). Horizontal axis is $\log_{10}$ of segment length (measured in micrometers). Vertical axis is the normalized frequency of occurrence. Note the bimodality of the distribution in log space, and the apparent symmetry of the long-segments distribution.

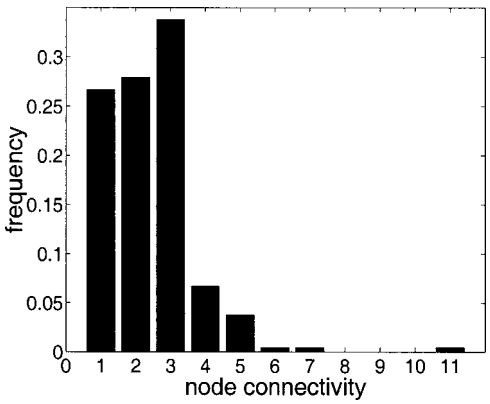

FIG. 5. Distribution of the node connectivity in network number 3 (240 nodes, 290 connections). Horizontal axis is *k*, the number of nodes to which each node is connected. Vertical axis is the normalized frequency of occurrence. $\overline{k}=2.38$.

III. MORPHOLOGY ANALYSIS

Abstraction process. We took a static snapshot of the evolving networks structure at a particular time point, day 6 in culture, on which the networks demonstrate maximum interconnections between neurites. We mapped the neuronal network into a simple graph using the following assumptions (see Fig. 3).

(1) All vertices are identical.

(2) All edges are identical. That is, we ignored edge length, the possibility that different edges have different synaptic efficacies and/or edges directionality.

(3) We ignored edge multiplicity, i.e., we considered only whether vertices were adjacent or not.

Physical properties of the networks. Our main findings obtained for three 6-day-old networks cultured and grown under the same conditions are summarized in Table I. Before taking the graph theory approach and describing the networks as simple labeled graphs (which are devoid of any physical properties), we examine in further detail the statistics obtained for one network, the largest of the three (network number 3 in Table I, with 240 nodes and 290 edges). Distribution of the measured lengths between all pairs of nodes on a semilogarithmic scale is shown in Fig. 4. The bimodal distribution, with the majority of the samples at the lower end of the distribution (short segments, $r\sim 10\ \mu$m), and the remaining samples distributed in a symmetrical manner in the log space between $>10^1$ and $10^{2.5}\ \mu$m (10–300 μm), is typical for networks with spatially clustered structures [22].

TABLE I. Descriptive parameters measured for three 6-day-old neuronal networks, cultured and grown under controlled conditions: *n*, number of nodes; $\overline{k}$, average node connectivity, $\overline{l}$, characteristic path length. Values for a regular ring graph ($l_{reg}=n/2k$) and a random graph are also given for comparison. c_{rand} and l_{rand} are calculated for ten numerically generated random graphs with the same parameters as the corresponding studied network.

Net	n	$\overline{k}$	$l_{reg}/l/l_{rand}$	c/c_{rand}
1	104	2.33	22.35/11.03/4.88±.28	0.092/.017±.010
2	140	2.62	26.70/9.66/4.82±.12	0.129/.016±.010
3	240	2.38	50.53/17.58/5.90±.13	0.113/.009±.007

Mapping of the network into a graph. The neuronal network is described as labeled graph *G*. Such a graph can be described solely by its adjacency matrix $A(G)$ [23]. This symmetric matrix is defined as follows: Let the nodes of *G* be labeled $v_1, v_2, \ldots, v_n$. The Adjacency Matrix $A=a_{i,j}$ of *G* is a binary matrix of order *n*, with

$$a_{ij}=\begin{cases}1 & \text{if } v_i \text{ and } v_j \text{ are joined by an edge}\\ & \text{(i.e., adjacent, or neighbors)}\\ 0 & \text{otherwise.}\end{cases} \quad (1)$$

For each of the networks under study, $A(G)$ is constructed by manually labeling all nodes and marking all connected pairs (see Fig. 3). The properties of the adjacency matrix facilitate calculation of all of the required characteristics of the graph (see below), through simple algebraic manipulations. In particular, we stress the following important property [23]: If *G* is a labeled graph with adjacency matrix *A*, then the (i,j) element of A^l is the number of walks of length l from v_i to v_j.

Node Connectivity. The degree *k* of a vertex is the number of other vertices to which it is directly connected (=adjacent). For each vertex v_i, k is obtained by adding up the elements in row (or column) i in $A(G)$. The distribution of *k* values for network 3 is depicted in Fig. 5. The average connectivity is $\overline{k}=2.38$. It can be seen that the network has a

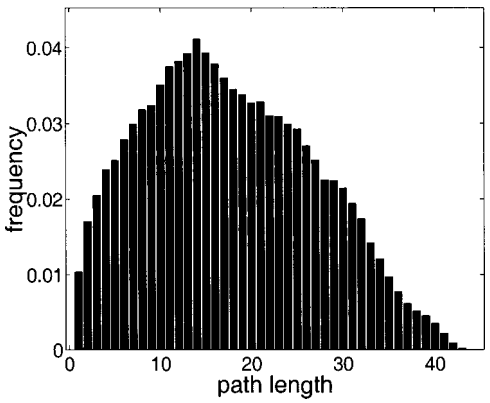

FIG. 6. Distribution of the path length values in network number 3 (240 nodes, 290 connections). Horizontal axis is the path length, i.e., the number of edges in the shortest path between two nodes. Vertical axis is the normalized frequency of occurrence. $\overline{l}=17.58$.

pronounced scale of connectivity. That is, it is far from being ''scale free,'' a feature claimed to be common among ''real-world'' networks [24]. However, as Amaral *et al.* have shown [25], the node connectivity of various networks (real as well as manmade) can exhibit either scale-free, broad scale or single scale statistics.

Path length. The path length l between v_i and v_j is defined as the number of edges included in the shortest path between v_i and v_j. The characteristic path length $\overline{l}$ of the graph G is l averaged over all pairs of vertices. The distribution of path length values in network 3 is shown in Fig. 6. The characteristic path length of the corresponding graph is $\overline{l}=17.58$.

Clustering coefficient. Another important parameter in the context of small-world networks is the clustering coefficient of the graph. The clustering coefficient of vertex v, $C(v)$, is defined as the number of edges among the k_v neighbors of v (=adjacent vertices), divided by the maximal number of such edges, $k_v(k_v-1)/2$. Thus, $C(v)$ (which is in the range [0–1]) measures the ''cliquishness'' of the neighborhood of v, i.e., what fraction of the vertices adjacent to v are also adjacent to each other. By extension, the clustering coefficient of the graph G, $\overline{C}$, is the average of $C(v)$ over all vertices. For network 3 we obtain $\overline{C}=0.113$.

Small-world test. Using the results presented in Table I, we can now attempt to test whether our *in vitro* neuronal networks fall into the category of small-world networks. For this purpose, the table contains a comparison to two benchmark cases: a random graph and a regular graph [11], with the same number of nodes n and average connectivity $\overline{k}$ as the network under study. The formal definition of a small-world network requires that such a network satisfies (1) $\overline{C}\gg C_{random}$ ($C_{random}\sim k/n$), that is, a small-world network is much more highly clustered than the corresponding random graph and (2) $l_{regular}\gg\overline{l}\geq l_{random}$, i.e., the characteristic free path of a small-world network is close to that of a random graph, and much smaller than that of a regular graph. Specifically, $\overline{l}$ should scale as $\ln(n)/\ln(k)$, rather than as $n/2k$.[1]

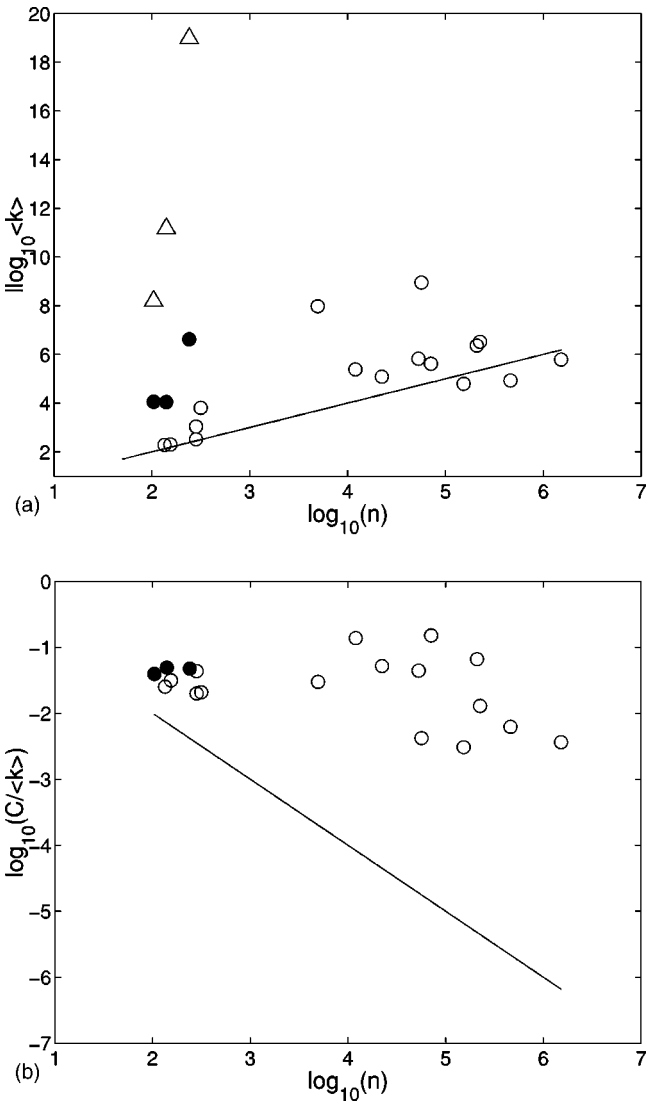

FIG. 7. (a) Average path length of the neuronal networks studied (filled dots), compared to the prediction of random graph theory [$l_{rand}=\ln(N)/\ln(\langle k\rangle$, solid line] and regular graphs [$l_{reg}=n/2k$, open triangles]. Data for our networks are also compared to other real networks (open dots, data taken from Albert and Barabasi [26], Table I). (b) The network's clustering coefficient compared to the prediction of random graph theory ($c_{rand}=\langle k\rangle/N$), and other real networks [26] [symbols as in (a)].

A comparison between the average path length and clustering coefficient of our three networks and various real networks (following Albert and Barabsi [26]), with the theoretical values for random and regular graphs, is presented in Fig. 7. It can be seen that our three networks fall within the

[1]One should note that a random graph with the same n and $\overline{k}$ as our networks will not, in general, be fully interconnected. The characteristic path length for such a graph corresponds only to connected subgraphs. The fact that our graphs are completely connected at these parameter values is, of course, a feature that distinguishes them from random graphs.

''cloud'' of real-world networks. As presented in Table I, the clustering coefficient is indeed much higher (5–13 times) than that of the corresponding random graphs. The data do not allow us to verify that $\overline{l}$ scales as $\ln(n)/\ln(k)$ but the characteristic path length is closer to l_{random} than to $l_{regular}$, as in small-world networks.

IV. DISCUSSION

During development of the nervous system, two opposing forces impose the morphology of the evolving neuronal networks. On the one hand, single neurons grow axons and highly branched dendritic trees in order to achieve maximum interconnected networks. This enables efficient information flow, and adds to the strength of the networks as computational units. On the other hand, developing extended and vastly branched neurites has a high energetic cost. Hence, the final structure of the neuronal network is a consequence of the interplay between these factors. One category of networks that could be the result of such competition is small-world networks, combining fast information transmission with maximal economy in wiring length (energetic cost).

We studied *in vitro* two-dimensional neuronal networks generated by culturing neurons dissociated from locust ganglia. The *in vitro* networks were mapped onto graphs where the vertices represent the elements of the system and the edges represent the interactions between them. We examined our networks at the stage where they were practically fully connected. In order to determine whether the networks fall within the small-world regime, we calculated the clustering coefficient and path length of each network and compared these parameters to random and regular graphs with the same n and $\overline{k}$.

For the three networks tested, the clustering coefficients were indeed much higher than those of the corresponding random graphs, and the characteristic path lengths were closer to l_{random} than to $l_{regular}$. According to this test, the networks can be classified as small-world networks.

Distribution of the lengths of segments connecting the nodes in our networks is typical to networks with a spatially clustered structure. This becomes very apparent as the networks mature. The culture goes through a dynamical process, starting with single entities, continues to a fully connected network, and finally develops to cultures organized into a few centers comprised of groups of neurons connected by thick nervelike bundles. The latter can be characterized by efficient information transmission together with tight and thrifty structure, the features of a small-world network.

The growing process that was observed in our two-dimensional cultures serves to demonstrate the self-organization process that leads to the characteristic structure of the nervous system *in vivo*: concentrations of neuronal cell bodies, namely, ganglia, interconnected by nerve tracts.

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