Growth morphology of two-dimensional insect neural networks

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Abstract

How a collection of single neurons self-organize to form a complex functional system, the neural network, is a fundamental question. Two-dimensional in vitro invertebrate preparations offer an attractive model system to tackle this question due to the large size of the neurons, and their ability to grow in relative isolation as well as to develop elaborate networks. We culture locust neurons, monitor and analyze their morphology and growth process under various density conditions. The neurons actively target neighbor cells, and their structure is affected by neuronal vicinity. As the network forms there is a tendency for simplification of neuronal morphology.

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1. Introduction

How a functioning brain emerges from a collection of individual neurons is one of the most profound open questions in science. The study of this question translates to investigation of how a collection of elements, namely neurons, self-organize to form a new and extremely complex system; the neural network [3,7,8,17–19,21,22,24].

Even the simplest neural networks in the nervous system show surprisingly complex wiring diagram [1]. Furthermore, the single entities, the neurons themselves, come in a bewildering variety of shapes and forms. In order to gain some insight into the rules governing neural network configuration, one needs both to choose a system that is simple (relative to any in vivo network), and to be able to control for as many of its variables as possible.

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We are studying the morphological development and organization of two-dimensional neural networks in cultures of neurons dissociated from locust ganglia. Our overall objective is to understand the adaptive self-organizing rules that govern the formation of neural networks and to translate these rules to simple model assumptions. Invertebrate neurons offer an attractive model because of their large size and the facility with which they can be cultured in various conditions (e.g. extremely low densities) [2,5,6,9, 10,12–16,23]. Most importantly, the in vitro two-dimensional culture enables easy access for non-invasive optical observations, allowing us to follow the dynamics of neural growth and network organization.

In our work we focus on two aspects of network formation. First, we characterize single neuron morphology, and study the effects of neuronal vicinity on the neuron’s growth pattern. Secondly, we investigate the dynamics of neuronal growth. We follow the timing and sequence of events leading from single cultured neurons to interconnected networks.

2. Methods

Our culturing methods follow Smith and Howes [20] with slight modifications. The neurons were dissociated from the frontal ganglion (FG) of adult locusts. The locust FG is a small ganglion located in the insect head just in front of the brain consisting of approximately 100 neurons.

After dissection, enzymatic treatment and mechanical dissociation, the neurons were plated on Petri dishes pre-coated with Concanavalin A (Sigma). The number of ganglia per dish varied determining the final density of the culture and thus the average distance between cells. Cultures were maintained in 70% relative humidity, at a temperature of 29°C, and complete darkness.

We monitored the cultured neurons for their entire growth period during which neurites regenerated, outgrew and connected to form a network. We first examined the cells 24 h after dissociation and then once a day. Single images were acquired using a CCD camera mounted on a phase contrast microscope. In order to observe the growing process continuously, some culture dishes were maintained in a special controlled chamber mounted on the phase contrast microscope. Time lapse observations were performed with the CCD camera connected and controlled by a PC computer. Due to the sensitivity of the cultured cells to light, an optical shutter was momentarily opened by an external trigger, simultaneously with the computer controlled frame grabber.

In order to gain a close examination of the neurons’ structure, we used a scanning electron microscope (SEM) that allowed us magnification of up to ×3000.

Descriptive morphometric parameters of single neurons, such as the number of processes originating from the neuronal soma, the number of segments per cell, the length of the segments, general cell orientation etc. [4,5,11] were measured. We concentrated on parameters, which relate to the arborization of a neuron’s neurites, and to the distribution of the neurites in the 2D-growth plane. We divided the developing neurons into different categories according to their cellular vicinity (e.g. isolated neurons vs. neurons with close neighbors), as well as the cell’s stage of development (days in
culture and connectivity to other neurons). Mean values of the morphometric parameters were calculated and compared for the different categories.

3. Results

Our cultured neurons were fully differentiated adult neurons that have lost their original neurites, leaving only the soma. They varied in size between 10 to 50 µm. After 24 h in culture about 50% of the cells survived, out of which 25% had already started developing neurites. Most neurons demonstrated multi-polar growth (Fig. 1).

We maintained the culture for up to a month. The most intense stage of development and process growing was between days 1 and 4. After this rapid growth stage there was a pronounced decrease in growth rate. We followed single neurons by time lapse observations, during the various stages of development, from single neurons to interconnected neurons (Fig. 2). Close examination of the neurite outgrowth process suggested that neurons actively targeted neighbor cells. To have a quantitative estimation of the effect of neighbor cells on neurons’ growth we measured an elongation index (EI), which reflects whether the arborization of the neurites is symmetrical or directed. This index measures the ratio between the smallest circle that encompasses the neuron and the area of the polygon that connects the neurites’ tips. The neurites of isolated cells, which were not affected by neighbor cells, showed more symmetrical growth pattern. Their average EI was closer to 1 compared to neurons with close neighbors (1.7 ± 0.1 vs. 2.9 ± 0.7, respectively).

Fig. 1. A single cultured neuron, two days after plating, demonstrating a multi-polar growth pattern.
Fig. 2. Time lapse observations of a developing network in culture during 40 h. (A) Cultured neurons 48 h after plating. Neurites had already started developing. (B) The same neurons 8 h later, neurites of a neighbor cell out of the frame outgrew towards the cell in the middle. (C) 2 h later. (D) After additional 4 h all the cells in the frame were connected and the processes continued to branch and elongate. (E) 6 h later polygonal shapes were formed. (F) 24 h later the growth rate was much reduced and nerve-like bundles started to appear. Scale bar = 50 μm.

The neurons’ neurites elongated toward specific neighbors, split and connected to target neurons. We measured the angle between two second-order processes immediately after new branches had formed to reveal that 36% of the branching angles measured in isolated cells (with no close neighbors) were $80^\circ \pm 5^\circ$, demonstrating an innate, preferred branching angle. In marked contrast, in the presence of nearby neurons and their growth cones, presumably generating some stimuli from the environment, this preferred angle was masked to give rise to a much wider, rather uniform, range of angles between $30^\circ$ and $120^\circ$ (Fig. 3).

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. Doing so, they formed close loops of neurites, a phenomenon that is clearly demonstrated in the SEM image presented in Fig. 4. The junctions or interconnections points acted as anchors that seemed to be more 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. 4 and 5).

As the neurons regeneration process proceeded and connections between neurons had been established, the first outburst of neurite outgrowth and rich branching gave rise to a second very different process of reduction and simplification of the pattern. As long as the neurons remained isolated, the number of processes originating from their soma significantly increased with time, culminating in a 50% increase between days 2 and 3 after plating ($p < 0.05$). In marked contrast, neurons that were already connected to
Fig. 3. Branching angles measured at newly created branching points just after splitting of the new neurites in practically isolated neurons (A, \( n = 11 \)) and in neurons with close neighbors that had already formed connections to other cells (B, \( n = 43 \)). Data suggest that the neurons have an innate preferred branching angle that is masked by the effects of the neuronal vicinity.

Fig. 4. (A) Scanning electron microscope image of a single neuron (magnification of \( \times 800 \)). (B) Close look at the polygonal shape that was formed by the straight neurites. The soma is on the left and two junctions on the right side of the image (magnification of \( \times 3000 \)). Scale bar = 10 \( \mu \text{m} \).

neighbor cells, demonstrated a 50% reduction in the number of processes originating from the soma between days 2 and 3 (\( p < 0.005 \)). Thus, by day 3 there was a highly significant difference between connected and not yet connected neurons in the number of processes originating from the soma (\( p < 0.005 \)) (Fig. 6A). Connected neurons also had a significantly lower average number of neurite segments per cell compared to not connected neurons (\( p < 0.005 \), (Fig. 6B). The previously very complicated web of neurites started forming much thicker bundles. In time-lapse observation this process was comprised of two events; absorption of “unneeded” branches and even whole neurites, together with re-arrangements of neurites and what appeared to be fusion of parallel ones. As the networks reached days 6 or 7 in culture, another phenomenon that involved re-arrangements and migration was observed. The neuronal cell bodies started to aggregate into packed clusters. Again in time-lapse observation, the somata were observed to migrate along the newly formed bundles toward one another. Thus relatively homogenous cultures in which single neurons were scattered, evolved into
Fig. 5. Straight segments show high tension along the processes between junctions. It looks as if the junction points are better attached to the substrate than the neurites connecting them. A few examples of junctions are marked by dotted circles.

Fig. 6. (A) The number of processes originating from the soma in not yet connected neurons (open bars) shows a significant increase with time, culminating in a 50% increase between days 2 and 3 after plating. Neurons that were already connected to neighbor cells (shaded bars), demonstrated a 50% reduction in the number of processes originating from the soma between days 2 and 3. (B) Connected neurons (shaded bar) had a significantly lower average number of neurite segments per cell compared to not connected neurons (open bar). * \( p < 0.05 \), ** \( p < 0.005 \).

cultures organized into a few centers comprising clusters of neurons and thick nerve-like bundles connecting them (Fig. 7). This simplification processes was very slow and continued steadily for few more weeks.
4. Summary

The adaptive self-organizing rules that govern the formation of neural networks are a fundamental topic in developmental neuroscience, computation science, and pattern formation [7,17–19,24]. As in networks in general, there is a strong relation between the network’s wiring diagram or structure, and its function. Individual neurons’ branching pattern, and the formation of distinct interconnection between neurons, are dominant factors, instrumental in the future output of the neural circuit, behavior.

In our work we concentrated on the changing morphology of single neurons as they regenerate and outgrow processes to become members of the evolving neural network. By controlling the culture density, we tested the effect of neurons on each other’s structure and growth pattern. By following the culture continuously throughout the generation of the neural network we could identify and characterize landmarks or milestones in neural network formation and to add them as assumptions to a previous model constructed in our lab [not presented here; 17–19]. This model treated growth cones as autonomous entities, which navigate in biased random walk toward neighbor target soma, with sensitivity to concentration gradients of chemo-repellents and chemo-attractants.

Our results demonstrate that neurons actively target neighbor cells, but also connect to their own previously extended neurites, following no rules of self-avoidance. After connections have been formed, tension is generated along the processes. The neurons’ morphology is affected by their neuronal vicinity, by the presence of close neighbors.
In the absence of neuronal stimuli the growth pattern reflects pre-determined factors. After connections have established the neurons structure go through a simplification process. This is part of a general simplification and reduction process that characterizes the mature neural network and culminates in the generation of cell clusters that are connected by nerve-like structures.

The present results will be extended to a more detailed account of network structure, and will serve as a basis for a study of the interrelations between morphology and activity of evolving networks.

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