GROWTH MORPHOLOGY OF TWO-DIMENSIONAL INSECT NEURAL NETWORKS

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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.

Submitted for oral presentation

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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 relatively simple elements, namely neurons, self-organize to form a new and extremely complex system; the neural network [2,6,7,16,17,18,19,22].

Precise neurite outgrowth and synaptic interconnections characterize the lying down of neuronal networks, the basic hardware framework of the brain. Single neurons' branching pattern and the formation of distinct synapses, are dominant factors, instrumental in the future output of neural circuits.

The overall objective of our research is to study the adaptive self-organizing rules that govern the formation of neural networks. *In vivo* nervous systems are inaccessible for such a task. We therefore investigate two-dimensional *in vitro* preparations, in which invertebrate neurons are grown in a controlled environment. These cultures offer an attractive model system due to the large size of the neurons, which enables manipulation, and the ability of the cultured neurons to grow in relative isolation as well as to develop elaborate networks [1,5,8,9,11,12,13,15,21].

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 the neuron growth. We follow the timing and sequence of events leading from single cultured neurons to interconnected network.

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Methods

Our culturing methods follow Smith & Howes [19] with slight modifications. The neurons are dissociated from the frontal ganglion of adult locusts. This is a small ganglion consisting of approximately 100 neurons. In the locust, neurons within the ganglion form neural circuits that generate rhythmic motor output controlling gut movements. After enzymatic treatment and mechanical dissociation the neurons lose their original neurites, leaving only the soma. The number of ganglia per dish determines the density of the culture and thus the average distance between cells.

We monitor the cultured neurons for their entire growth period during which neurites regenerate, outgrow and connect to form a network. We first examine the cells 24 h after dissociation and then once a day. Single images are acquired by a camera mounted on a phase contrast microscope. We measure morphometric parameters such as number of processes originating from the soma, number of segments, length of segments, soma area, cell orientation etc. [3, 4,10, see Fig. 1]. Mean values are calculated, and we compare sets of neurons in different stages of development, as well as neurons connected versus not connected to neighboring cells.

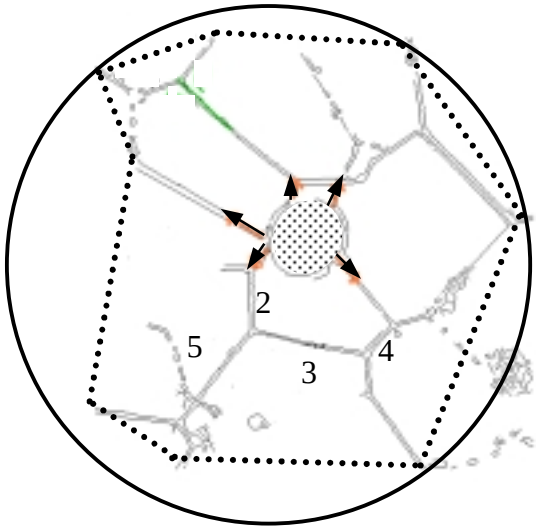

Figure 1: A processed image of a single neuron and some of the morphometric parameters that are analyzed. Arrows mark processes that originate from the soma. We count the total segments of the neurite tree. The dashed line makes a polygon that represents the area of the cell. The solid line is the smallest circle that encompasses all the cell neurites. The shaded area is the soma area.

In order to observe the growing process continuously, we grow the cells in a special controlled chamber mounted on the phase contrast microscope, and perform time lapse observations with a CCD camera connected to a VCR.

Results

We cultured the neurons at very low densities (practically isolated neurons) as well as in dense conditions (Fig. 2). Cell size was between 10 μm to 50 μm, and the average distance between cells varied between 20 μm, in a high-density dish, to 350

μm, in a low-density dish. After 24 h about 50% of the cells survived, and around 25% of these had already developed neurites.

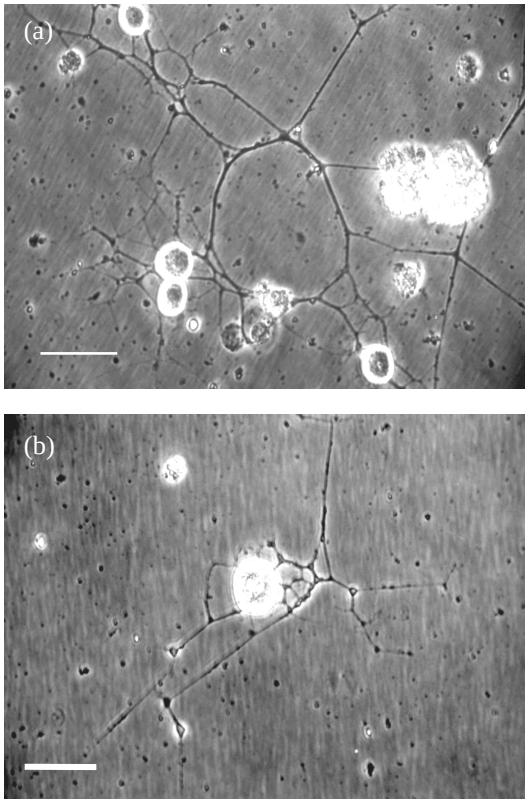

Figure 2: Locust neurons cultured in high density (a), or in very low density (b), 2 days after plating. In the phase contrast microscope cell bodies show as bright areas, and the processes as dark lines. A complex net was formed in the high-density culture. Scale bar = 50 μm.

We maintained the culture for one week. The most intense stage of the growing process occurred between day 1 and day 4. After this rapid growth stage there was a pronounced decrease in growth rate. Similar results were previously reported by Rossler *et al*. in a locust system [14].

Our morphometric measurements 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 (Fig. 1).

The cellular vicinity of the neuron affected the elongation index (the ratio between the smallest circle that encompasses the neuron and the area of the polygon that connects the neurites tips, Fig. 1). This parameter reflects whether the growth of a single neuron is homogenous or directed. The elongation index was higher in neurons with close neighbor cells, compared to neurons with no neighbors around. The latter demonstrated a more homogeneous growth pattern (index closer to 1).

Even more striking were the differences between neurons connected vs. not connected to neighbor neurons. As long as the neurons were isolated, the number of processes originating from their soma significantly increased with time; a 50% increase between day 2 and 3 after plating, $p<0.05$. Neurons that seemed connected to neighbor cells, demonstrated a 50% reduction in the number of processes originating from the soma between day 2 and 3 ($p<0.005$). Connected neurons also had a significantly lower average number of neurite segments per cell compared to not connected neurons ($p<0.005$).

We followed single neurons by time lapse observations for up to 90 hours after plating, during which they interconnected to generate small networks (Fig. 3). Close examination of the neurite outgrowth process suggested that neurons actively targeted neighbor cells. The neurites elongated toward specific neighbors, split and connected to the target neurons. Cell bodies that have not yet regenerated processes were awaken by the contact with the approaching neurite, and started growing their own neurites.

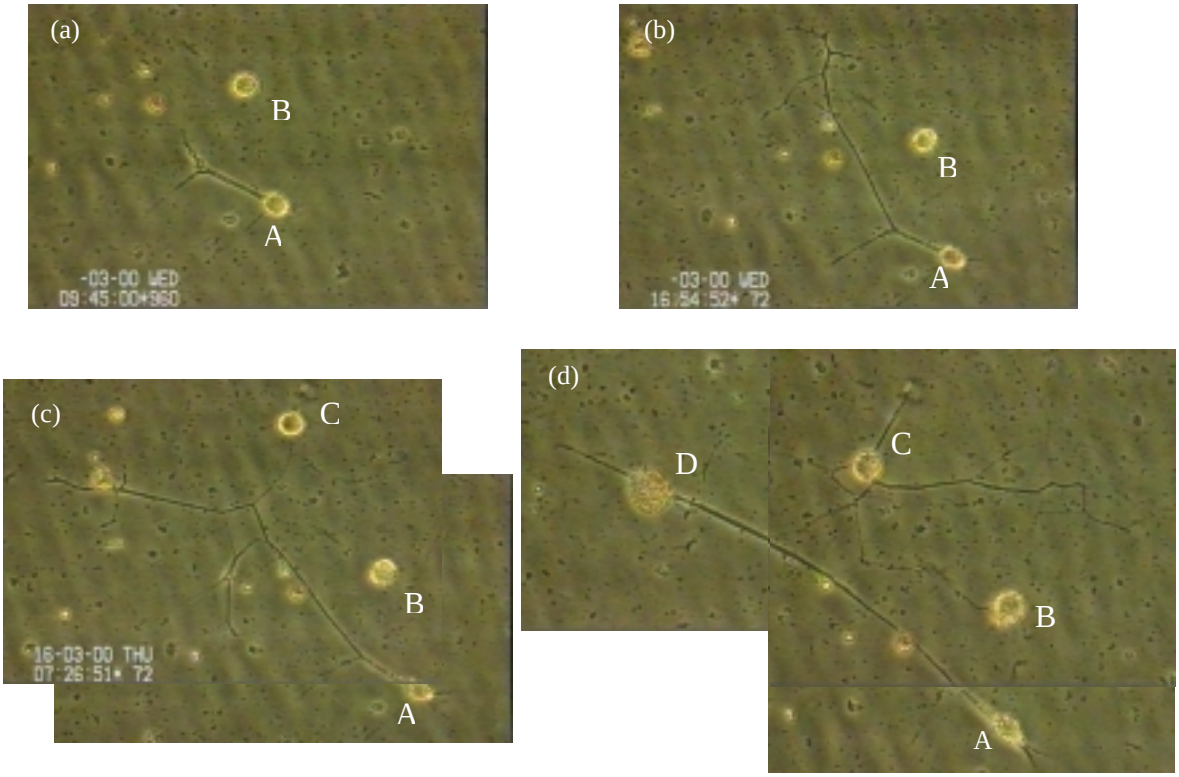

Figure 3: (a) Cultured neurons 48h after plating. (b) The same neurons 7h later. Neurites of cell A elongated and sprouted in a very directional manner. (c) 14h later, branches of cell A targeted and connected cell C. (d) After additional 22 h, Cell C now awaken, regenerated its own neurites. A not yet regenerated cell B is also targeted.

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. Analyzing the morphology of growing cultured neurons as they form networks is a valuable tool in studying this issue. Neurons are affected by their neuronal vicinity; they actively target neighbor cells. Connections formed between neurons lead to a simplification process of the morphology of the

network members. These results will serve as a basis for a study of the interrelations between morphology and activity of evolving networks.

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