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## Guided outgrowth of leech neurons in culture

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Sensory neurons of *Hirudo medicinalis* were cultivated on patterns of extracellular matrix (ECM) protein which were prepared by UV irradiation using copper grid masks. The relation of the patterns and of the outgrowth of neurites was observed by scanning electronmicroscopy after gold decoration. The neurites were guided by narrow (10  $\mu\text{M}$ ) lanes of ECM protein. Branching of neurites was induced by branched lanes. Bent neurites were pulled off bent lanes at some distance behind the growth cone such that their length was reduced. Regularly spaced sites of adhesion became visible which remained connected to the neurites by extended filaments.

Simple circuits may be formed by invertebrate neurons in culture [8, 10]. To characterize and to control signal propagation in such networks we require (i) methods of multisite recording of electrical activity [4] and (ii) methods to prepare connections of defined geometry. With respect to the second aim we describe in the present paper guided outgrowth of leech neurons. We follow two well-known approaches: (i) outgrowth of arborizations of leech neurons on leech-specific laminin [1, 2]; (ii) guided outgrowth of neurons from chick dorsal root ganglia on tracks made by illumination of vertebrate laminin through copper grid masks [6, 7].

A laminin-like protein was obtained from the extracellular matrix (ECM) of the ganglia of *Hirudo medicinalis* by urea extraction [1]. The ECM protein was mixed with bovine serum albumin (BSA) labelled with fluorescein-isothiocyanate (Sigma, Heidelberg) (weight ratio about 1:1). Drops of 10  $\mu\text{l}$  of the ECM/BSA solution (about 1 mg protein per ml) were spread on a glass coverslip which had been cleaned extensively [5]. The thickness of the protein layer formed by air-drying was 500 nm as determined by angle-dependent light reflection. We placed quadratic or hexagonal copper grids (Plano, Marburg) on the ECM/BSA-layer and irradiated it by the total spectrum of a mercury lamp (Osram HBO 200) (6.5 mW/cm<sup>2</sup> of the 366 nm line) for 3–60 min. (Using the mixture of ECM protein and labelled BSA allowed us to see the pattern of shielded protein by fluorescence microscopy and by scanning electronmicroscopy after irradiation for 60 min.) The glass coverslip was attached

to the bottom of open silicone chambers (diameter 6 mm, Flexiperm-Mikro 12, Heraeus, Hanau). We removed individual cells from the group of large sensory neurons (N, T and P) at the ventral side of the segmental ganglia of the leech [3] and plated them on the coated coverslip in L-15 medium (Gibco) with 50  $\mu\text{g}/\text{ml}$  gentamycin sulfate (Sigma), 6 mg/ml glucose and 2% foetal calf serum (Gibco) at 20°C [1]. Growth was observed by phase contrast microscopy. After 2–5 days, the samples were fixed in 3% glutardialdehyde and dehydrated through a series of water/propanol mixtures. Gold was sputtered moderately to allow for selective deposition on the irradiated areas such that protein patterns and neurites were visible in the scanning electronmicroscope.

On a homogeneous ECM/BSA substrate the neurons formed irregular arborizations within 2–5 days as shown in Fig. 1a. The thin (secondary) neurites sprouted from the stump of the thick (primary) neurite as removed from the ganglion. Apparently the branching neurites avoided each other such that the tree covered the surface of the coverslip rather evenly. The distances between bifurcations were about 15–50  $\mu\text{m}$ . Occasionally junctions were formed. Homogeneous irradiation of the ECM/BSA layer before plating the cells lead to an inhibition of outgrowth of neurites within a few minutes as shown in Fig. 1b,c. The effect was observed without labelled BSA, too. We could assign the destruction of the growth promoting activity of laminin to UV-light (wavelength < 300 nm) by inserting a series of optical edge-filters (Schott, Mainz) into the light beam during irradiation.

On a patterned ECM/BSA substrate – made by irradiation of the ECM/BSA layer through a metal grid for



Fig. 1. Outgrowth of sensory neuron of leech. Phase contrast. Bar =  $50\ \mu\text{m}$ . a: arborization on extracellular matrix (ECM) protein mixed with bovine serum albumin (BSA). b: limited outgrowth on ECM/BSA irradiated for 3 min. c: suppression of outgrowth on ECM/BSA irradiated for 6 min. All pictures were taken in a state where no growth was observed for about 12 h.

60 min – outgrowth of thin secondary neurites was observed only if the primary neurite was in contact with protein shielded during irradiation. The neurites grew exclusively along the narrow lanes ( $10\ \mu\text{m}$  wide) which were previously protected by the grids. An electronmicrograph – taken at a stage where no further outgrowth was observed – is shown in Fig. 2a. The neurites were located in the middle of the lanes. Short filaments (filopodia) connected them with the borders. Two bifurcations were induced at almost every crossing as shown in Fig. 2a. The irregular spontaneous arborization reappeared as soon as the neurites reached the field of homogeneous ECM-protein as shown in Fig. 2b. The neurites remained on the lanes behind the growth cone. However, neurites were pulled off bent lanes such that their length was shortened as illustrated in Fig. 3. These neurites remained connected to the lanes through thin filaments and foot-like adhesion sites spaced at intervals of about  $10\ \mu\text{m}$  (Fig. 4). Growth cones collided on the hexagonal net. They passed each other. Antiparallel and parallel neurites fasciculated as may be inferred from Figs. 3 and 4.

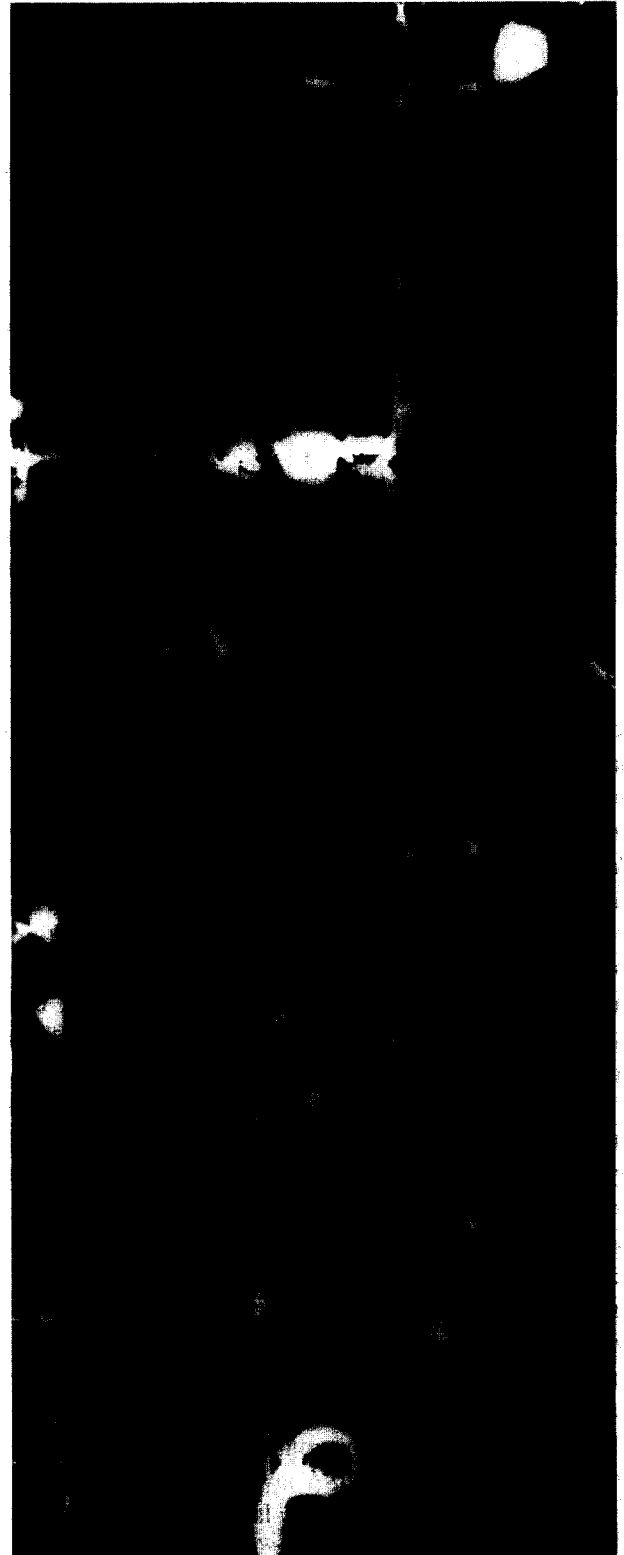


Fig. 2. Guided outgrowth on quadratic nets of ECM/BSA. Scanning electronmicrographs. Bar =  $50\ \mu\text{m}$ . The area marked by dark lanes was shielded by a copper grid during irradiation. The greyish tone is due to a preferred deposition of gold on irradiated substrate. a: narrow lanes induce straight growth. Short filaments (filopodia) connect the neurites and the borders of the lanes. Crossing of lanes induces a sequence of two bifurcations. b: irregular branching is induced as the neurites reach homogeneous substrate.

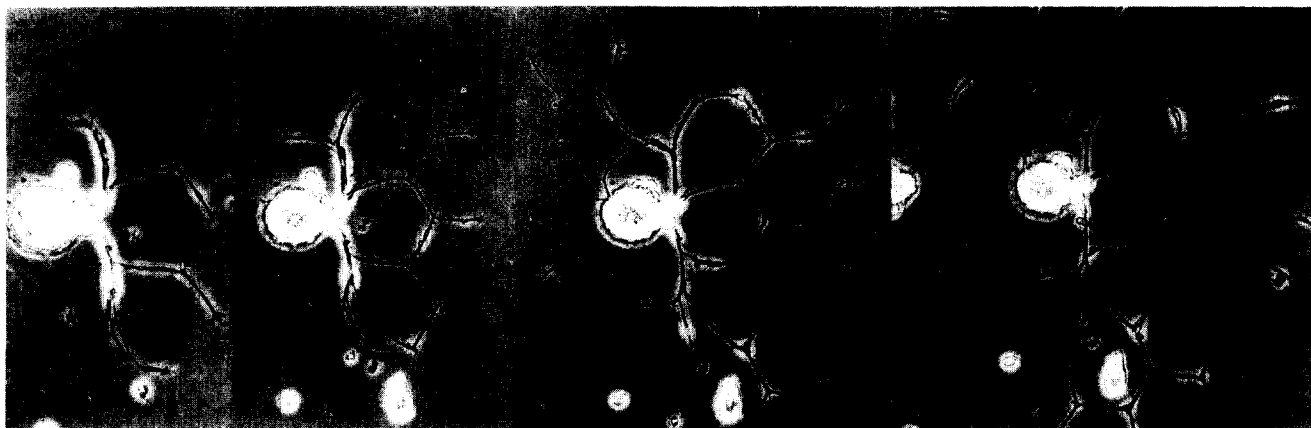


Fig. 3. Guided outgrowth on hexagonal net of ECM/BSA. Phase contrast. Bar = 50  $\mu\text{m}$ . a: 1 day after plating. b: 8 h later. c: 14 h later. d: 19 h later. The lanes guide the leading tip. Colliding neurites fasciculate. At later stages bent neurites are pulled off bent lanes such that their length is shortened. Fasciculated neurites separate from each other.

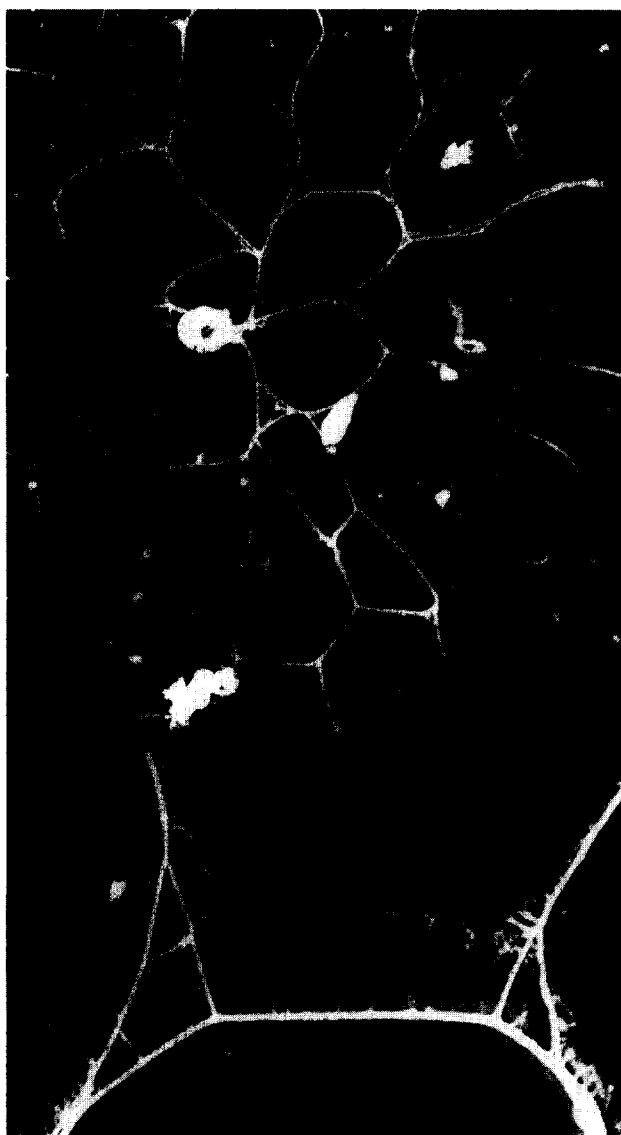


Fig. 4. Guided outgrowth on hexagonal net of ECM/BSA. Scanning electronmicrograph of the neuron shown in Fig. 3d. a: overview. Bar = 50  $\mu\text{m}$ . b: Detail. Bar = 20  $\mu\text{m}$ . Bent neurites are pulled off from bent lanes. Foot-like sites of adhesion are revealed at intervals of about 10  $\mu\text{m}$ . The neurites are connected to these sites by filaments.

Guided outgrowth of leech neurons by lanes of native ECM protein resembles guidance of DRG neurons [6, 7]. The length of guided neurites, however, is hundreds of micrometers, i.e. distinctly longer than reported for DRG neurons. Leech neurons do not grow on irradiated substrate in contrast to the DRG neurons [6]. The difference may be due to a higher light intensity used here to affect the substrate. The neurites of DRG neurons were guided as they came into contact with an unirradiated lane. Guidance was thought to result from an enhanced adhesion of neurites to the nonirradiated substrate [7]. In our case a distinction of guidance mechanisms such as 'differential adhesion' or 'substrate limitation' [7] is not possible. Meandering of neurites within the lanes was not observed in contrast to the outgrowth of retinal neurons from chicken and goldfish on patterned substrate [11, 12]. The perfect guidance is probably due to the width of the lanes (10  $\mu\text{m}$ ) which is much smaller than in refs. 11 and 12 (50–90  $\mu\text{m}$ ), and is within the range of the filopodia of the growth cone. Neurites avoid each other on homogeneous substrate much like the self-avoidance of P-type sensory neurons *in vivo* [9] whereas extensive fasciculation occurs in the case of restricted growth on patterned substrate.

A bifurcation originates from a genuine branching of a single neurite or from a separation of fasciculated neurites. Considering the dynamics of the leading tip during growth, the thickness of the neurites and the morphology of the branching points we assign most bifurcations on the patterned substrate to genuine branching, in particular those on the quadratic grid shown in Fig. 2. Preliminary results of time-lapse video studies confirm this interpretation (P. Fromherz et al., unpublished).

The separation of neurites from guiding lanes is at variance with observations of guidance of DRG neurons [6]. It was claimed that neurites follow strictly the path of their growth cone. The discrepancy may be due to the

shorter neurites evaluated in ref. 6. We conclude that guidance of the leading tip and adhesion of the guided neurite have to be distinguished. Laminin is effective with respect to stimulation of outgrowth and with respect to adhesion of the neurites. The final arborization is a result of guidance and of reorganization of the neurites according to a 'rule of shortest connection'.

Our results show that narrow lanes of leech-specific laminin promote guided outgrowth of leech neurons and that branched tracks induce branching of neurites. On this basis arbitrary arborizations may be prepared using metal masks of arbitrary shape. The approach will be useful not only with respect to the design of connections in neural networks but also with respect to the study of outgrowth and branching of neurites themselves.

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