Biol. Cybern. 82, L1–L5 (2000)

Biological Cybernetics

Letter to the Editor

Electrical synapses by guided growth of cultured neurons from the snail *Lymnaea stagnalis*

Astrid A. Prinz, Peter Fromherz

Department of Membrane and Neurophysics, Max Planck Institute for Biochemistry, 82152 Martinsried/Munich, Germany

Received: 18 November 1999 / Accepted in revised form: 26 January 2000

Abstract. The ability to assemble neuronal networks with designed topology would allow uniquely defined experiments on neurocomputing. We describe a fundamental step, the controlled formation of synapses by guided outgrowth, *in vitro* for the first time combining simple neuritic geometry with predefined connectivity. We used neurons from the A-clusters in the pedal ganglia of the snail *Lymnaea stagnalis*. They were cultured on a substrate with linear patterns made by adsorption of brain-derived conditioning factors and photolithography. We induced and observed the frontal collision of two growth cones on narrow lanes. Following such encounters, individual electrical synapses formed that were sometimes strong enough for prolonged presynaptic stimulation to reach the threshold of postsynaptic firing.

1 Introduction

The structural complexity, unknown connectivity and limited accessibility of nerve cells and their neurites in brain tissue complicate the study of the dynamics of neural nets. A way to overcome this would be to assemble small networks of neurons with defined topology and connectivity in cell culture. Such an approach would allow us to ask elementary questions about neural signal processing in model systems of limited and controlled complexity. The dream system is an array of neurons with dendrites and axons growing in defined directions and with synapses formed at defined positions, as illustrated in Fig. 1.

We report here on a fundamental step towards such future-designed networks: the formation of a synapse by guided growth on a chemical trace. Although guided, outgrowth is well known in numerous variations for neurons of vertebrates (Gundersen 1985; Hammarback et al. 1985; Kleinfeld et al. 1988; Corey et al. 1991; Clark et al. 1993) and invertebrates (Fromherz et al. 1991; Fromherz and Schaden 1994), the formation of a synapse under control of a chemical pattern has not been achieved.

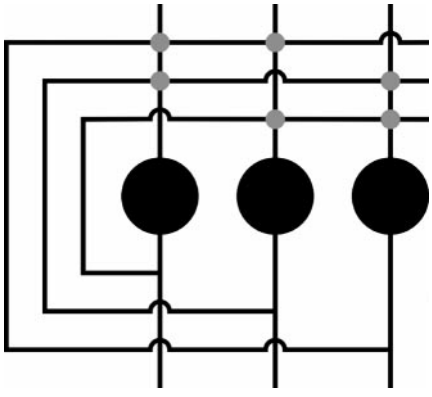

Fig. 1. Sketch of a synthetic neuronal network with defined positions of the cell bodies, with guided growth of dendrites and axons, and controlled position of synaptic contacts (indicated by *grey dots*)

Neurons from invertebrates are most adequate for the assembly of defined nets for two reasons: first, in invertebrates, networks of even just a few neurons can give rise to biological functions such as, for example, rythmic pattern generation (Kandel 1976; Selverston 1985). Second, small circuits from the molluscs *Aplysia californica* and *Lymnaea stagnalis* have been reconstructed by random outgrowth in culture, proving that these cells are able to reestablish specific and functional connections in vitro (Rayport and Schacher 1986; Kleinfeld et al. 1990; Syed et al. 1990). In our experiments, we used neurons from the A-clusters in the pedal ganglia of *L. stagnalis* (Slade et al. 1981; Syed and Winlow 1989) because they provide a source for many identified neurons with similar properties and because these neurons are joined in vivo by electrical synapses that can be expected to re-form in culture with sufficient strength to allow electrophysiological detection (Hadley et al. 1983, 1985; Bodmer et al. 1984, 1988; Guthrie et al. 1994).

In the culture dish, neurite outgrowth from *Lymnaea* neurons is promoted by proteinaceous conditioning

Correspondence to: P. Fromherz
(e-mail: fromherz@biochem.mpg.de,
Tel.: +49-89-85782820, Fax: +49-89-85782822)

factors that are secreted by the brain and adsorbed to a substrate coated with polylysine (Wong et al. 1981, 1983, 1984; Barker et al. 1982; Ridgway et al. 1991). We succeeded in creating patterns of these conditioning factors by locally inactivating them with UV-illumination through a metal mask. The outgrowth of neurons was guided by such patterns, and electrical synapses were formed between pairs of neurons by collision of their growth cones on linear lanes.

2 Materials and methods

2.1 Substrate

To prepare patterned substrates, we modified a procedure for the preparation of conditioned medium previously reported by Wong et al. (1983) and Ridgway et al. (1991). Glass coverslips with attached silicone chambers (flexiPERM, In Vitro Systems & Services, Osterode, Germany) were incubated overnight with 1 mg/ml poly-L-lysine (Sigma, P6516) in Tris buffer (0.15 M, pH 8.4) (Wong et al., 1983). After several washes with water, the chambers were filled with a custom-made, defined medium (PAN Systems, Aidenbach, Germany) containing (in mM) NaCl 40.0, KCl 1.7, $CaCl_2$ 4.1, $MgCl_2$ 1.5, glutamine 1.0, Hepes 10.0 (pH 7.9), all other ingredients of Leibovitz L-15 medium at half of the standard concentration, and 20 μg/ml gentamycin (Sigma, G3632) (Ridgway et al., 1991). Central ganglionic rings were isolated from *L. stagnalis* that were raised in tap water and fed on lettuce. Prior to dissection, the snails were deshelled and soaked in 25% listerine in normal saline consisting of (in mM) NaCl 51.3, KCl 1.7, $CaCl_2$ 4.1, $MgCl_2$ 1.5, Hepes 5.0 at pH 7.9 (all from Sigma), then pinned to Sylgard in antibiotic saline, which is normal saline with 150 μg/ml gentamycin (Ridgway et al., 1991). The dissected brains were soaked in antibiotic saline ten times for 10 min and added to the defined medium in the chambers (3 brains/ml). After 3–5 days at 20 °C, the conditioned supernatant medium and the brains were removed. The cover slips were dried and illuminated for 20 min with the full spectrum of a mercury lamp (Osram HBO200, intensity of the 366 nm line about 100 mW/cm^2) through a mask consisting of aluminium strips (14 μm wide and 33 μm apart) on a silica plate. We used masks that were fabricated by photolithography and etching of a thin film of aluminium (Fromherz and Schaden 1994). Then the chamber was filled with defined medium or with defined medium mixed with the conditioned medium in a ratio of 1:1.

2.2 Cell culture

After removing the outer connective tissue, the dissected brains of snails were incubated in antibiotic saline for 15 min and in defined medium with 1.33 mg/ml collagenase/dispase (Boehringer Mannheim) and 0.67 mg/ml trypsin (Sigma, T8253) for 30–35 min, washed several times with defined medium, treated with 0.67 mg/ml trypsin inhibitor (Sigma, T9003) in defined medium for 15 min, and again washed several times (Ridgway et al., 1991). Then a high-osmolarity medium was applied, consisting of defined medium with 30 mM glucose added. The pedal ganglia were opened using a microneedle. Neurons from the A-clusters were removed by suction through a fire-polished and siliconized (Sigmacote, Sigma) micropipette, immediately placed on the patterned substrate, and incubated for 1 day at 20 °C (Ridgway et al., 1991).

2.3 Electrophysiology

Microelectrodes were made from glass capillaries, filled with a saturated solution of K_2SO_4 and contacted with a chlorinated silver wire. The electrode resistance was 10–25 MΩ. An electrode amplifier operating in discontinuous mode (Ba-1S, npi Advanced Electronic Systems, Tamm, Germany) was used to inject current and monitor voltage. The electrode capacitance was carefully compensated to avoid artifacts in the voltage measurements due to the electrode resistance. We applied depolarizing or hyperpolarizing currents up to 0.5 nA with 900 ms duration.

3 Results and discussion

3.1 Growth cone collision

A few hours after positioning on the patterned substrate, *Lymnaea* neurons started to grow neurites that then elongated at a rate of a few tens of micrometers per hour. After 1 day in vitro, about 80% of all growing neurons perfectly followed the lanes of intact substrate, meaning that they did not deviate from the pattern with any of their newly extended processes. For longer culture periods, this high degree of compliance decreased, reaching about 30% after 3 days in vitro – a development that could be due to conditioning factors newly secreted by the neurons themselves during cell culture.

To induce the formation of a synapse, we placed individual *Lymnaea* neurons on the patterned substrate at distances of several hundred micrometers along the invisible lanes of conditioning factors. We observed pairs of neurons with their neurites growing collinearly on the same lane, with the two growth cones approaching each other. Figure 2 shows an encounter of two growth cones in three stages.

3.2 Strength of synapses

We checked the strength of electrical coupling between two cells about 1 h after an encounter of their growth cones. Both neurons were impaled with microelectrodes, a hyperpolarizing current step was injected into one cell, and the presynaptic and postsynaptic intracellular voltage transients were recorded. We did not electro-

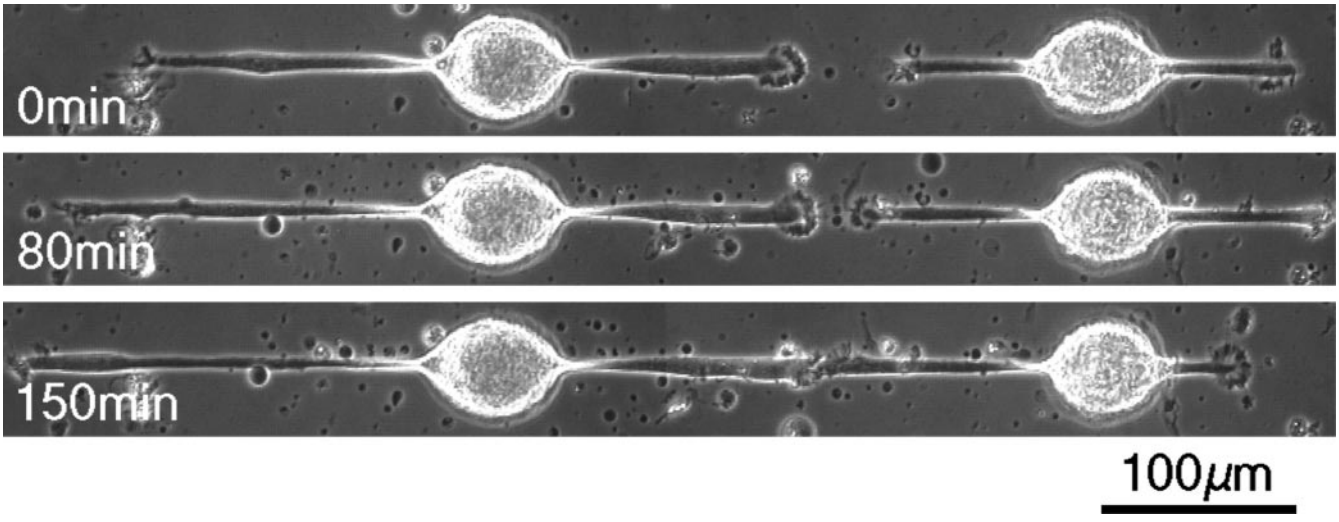

Fig. 2. Guided an encounter of the growth cones of two neurons from *Lymnaea stagnalis*. The width of the linear trace of proteinaceous conditioning factors is 14 μm. The cells were cultured for 1 day

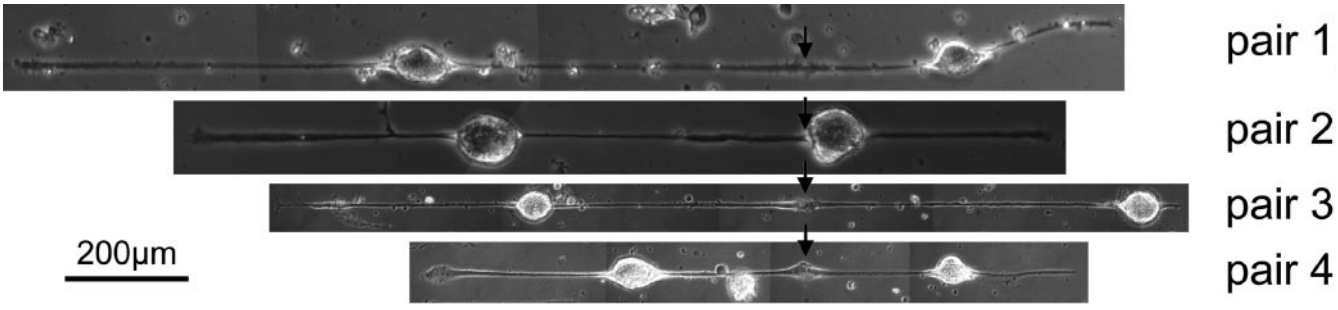

Fig. 3. Pairs of *Lymnaea* neurons after collision on a linear trace of conditioning factors. The position of collision is marked by an arrow. Both neurons of each pair were impaled with a microelectrode. The measurements are shown in Fig. 4

physiologically monitor the functional transformation of the two growth cones into a synaptic junction to avoid the interference of such an invasive long-term recording with the viability of the cells.

Figure 3 shows four representative pairs of neurons. Their presynaptic and postsynaptic recordings are depicted in Fig. 4 for current injection in the left and in the right neuron. The strength of electrical coupling is characterized by the ratio of the stationary postsynaptic and presynaptic response to a presynaptic current step, the coupling coefficient $k_{\mathrm{pre,post}} = \Delta V_{\mathrm{M}}^{(\mathrm{post})} / \Delta V_{\mathrm{M}}^{(\mathrm{pre})}$ (Bennett 1966; Kiss 1979; Bodmer et al. 1984). We found considerable variation in the coupling coefficient with $k_{\mathrm{LR}} = 0.02, k_{\mathrm{RL}} = 0.02$ in pair 1, $k_{\mathrm{LR}} = 0.10, k_{\mathrm{RL}} = 0.21$ in pair 2, $k_{\mathrm{LR}} = 0.22, k_{\mathrm{RL}} = 0.08$ in pair 3, and $k_{\mathrm{LR}} = 0.29, k_{\mathrm{RL}} = 0.11$ in pair 4. The histogram of $k_{\mathrm{pre,post}}$ for 15 pairs in shown in Fig. 5.

We then elicited trains of action potentials in one of the cells by injecting a depolarizing current. The pre- and postsynaptic voltage responses are shown in Fig. 4. Corresponding to the large variability in coupling coefficients, we found a wide range of postsynaptic responses to such presynaptic bursts: the postsynaptic depolarization was weak in the pair with weak coupling under hyperpolarization. For a pair with intermediate coupling, the depolarization in the postsynaptic cell was superimposed with small modulations correlated to the presynaptic action potentials. And for a strongly coupled pair, a few presynaptic action potentials could depolarize the postsynaptic cell enough to trigger a postsynaptic action potential through temporal summation.

3.3 Discussion

To guide the outgrowth of snail neurites in cell culture, we patterned a layer of unknown conditioning factors that were directly deposited onto the substrate by secretion from snail brains rather than relying on the differential adhesion of nerve cells to synthetic substrates (Kleinfeld et al. 1988; Corey et al. 1991) or lanes of purified natural substances (Gundersen 1985; Hammarback et al. 1985; Clark et al. 1993). While being chemically less well defined, this brain-derived substrate presumably more closely mimics the environment encountered by growing nerve cells in the snail brain itself.

The frontal collision of two growth cones on a linear lane that we used to induce synapse formation is the geometrically most simple connection one can form between two unbranched neurites. In addition, it has other

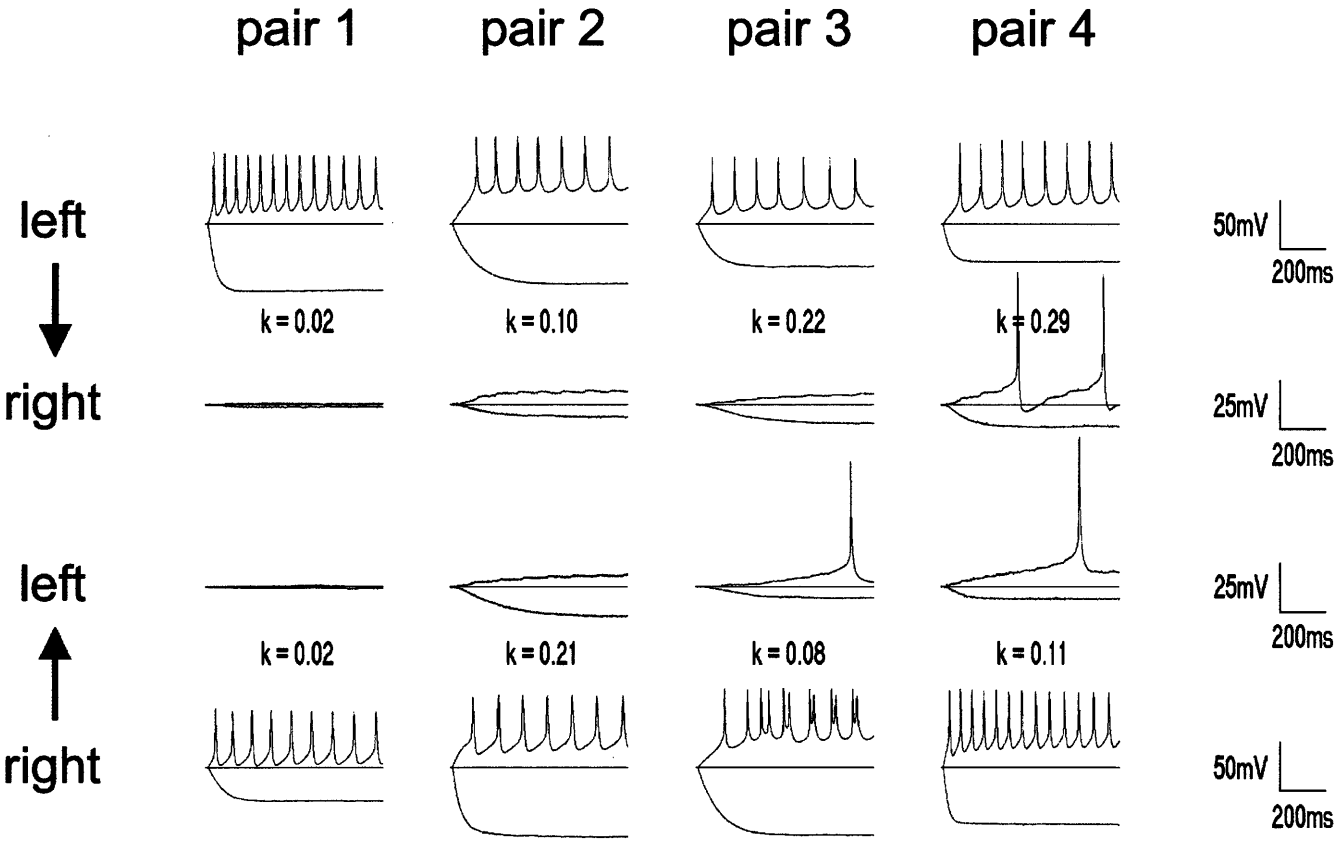

Fig. 4. Synaptic coupling of pairs of *Lymnaea* neurons from Fig. 3. The first and second rows refer to the signal transfer from left to right, the third and fourth rows to the signal transfer from right to left. The *lower trace* in each diagram shows the voltage change for hyperpolarizing current injection in the presynaptic cell; the *upper trace* refers to the depolarizing current injection. The values of the coupling coefficient $k_{pre,post}$ in both directions are indicated

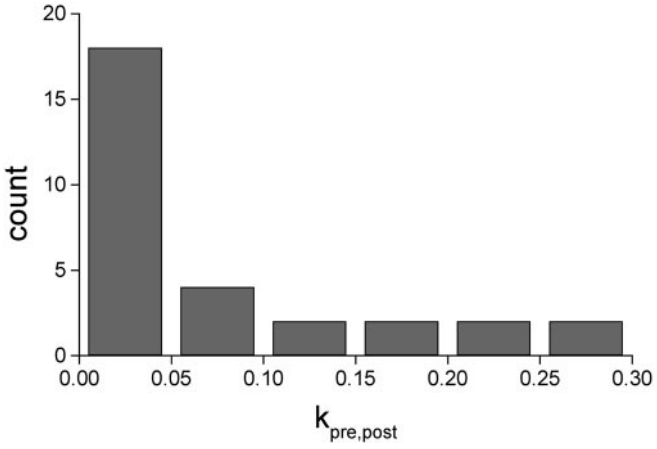

Fig. 5. Histogram of the coupling coefficients $k_{pre,post}$ for 15 cell pairs with a single contact formed by guided collision. For each pair, two coupling coefficients are included for the two directions of signal transfer

advantages: (1) it automatically satisfies the requirement that both cells have to be in an active state of growth to be able to form an electrical synapse (Hadley et al. 1983, 1985); (2) it makes cellular interactions more defined than in a random culture or in tissue by restricting the contact to a single and predictable location – a situation that would be a useful tool for the study of synapse formation itself.

Neurons from *Helisoma* and *Aplysia* grown on a homogeneous substrate can form electrical synapses with coupling coefficients of 0.39 ± 0.06 (Hadley et al. 1983) and 0.02–0.80, respectively (Bodmer et al. 1984), and we found similar values for *Lymnaea* neurons grown on a homogeneous substrate. In such random cultures, multiple contacts may exist between branched neurons (Guthrie et al. 1994), so it is striking that a single contact formed by guided collision of growing neurons can lead to a coupling coefficient of such similar strength. A rough comparison of morphology and electrophysiology suggests that a large collisional complex on the linear lanes leads to a high coupling coefficient.

For any given cell pair, k_{LR} and k_{RL} usually do not have the same value. This asymmetry of the coupling coefficient may be assigned to different input resistances of the two neurons, with $k_{pre,post} = R_{post}/(R_{syn} + R_{post})$ where R_{syn} is the synaptic resistance and R_{post} is the input resistance of the postsynaptic cell (Bennett 1966; Kiss 1979; Bodmer et al. 1984; Benjamin and Pilkington 1986). This relation is valid if the resistance of the neuritic cables leading from the cell bodies to the site of the synapse can be neglected. Indeed, there was no correlation of the coupling coefficient and the length of the neurites. Also, the electrotonic length constant of

the neurites was about 500 μm as determined by optical recording (Prinz and Fromherz, unpublished data), meaning that these neurons are electronically rather compact and most of the voltage drop between the two cell bodies actually occurs at the electrical synapse itself.

None of the electrical synapses we induced by growth cone collisions was so strong that it resulted in a one-to-one transmission of action potentials from the pre- to the postsynaptic neuron. This means that synapses produced in this manner have strengths in a range that will allow the study of signal processing mechanisms such as spatial and temporal summation (as seen in Fig. 4, pair 4) but do not couple the two cells so strongly that they always fire together and thus effectively behave like a single neuron.

4 Conclusions

Our experiments show that brain-derived conditioning factors can be used to control the outgrowth of snail neurons, and they demonstrate how to assemble and reconstitute simple neuronal networks with defined geometry and connectivity. Whether the approach is applicable to chemical synapses has yet to be investigated. Furthermore, the encounter of individual neurites by guided growth may become a novel tool to investigate the morphology and the electrophysiology of cellular and molecular mechanisms by which specific synapses are formed, since the growth patterns of the neurons are highly organized and the contact points between individual growth cones are limited much better than in a random cell culture.

Acknowledgements. We thank Dr. I. Pavenstädt, In Vitro Systems & Services, for help with the development of the culture chambers, Martin Jenkner for discussion and Xaq Pitkow for careful reading of the manuscript. The project is supported by the Bundesministerium für Bildung and Forschung.

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