



Original Contribution

NEURONAL AGGREGATES IN CULTURE

E. Marani^{1*}, W.L.C. Rutten² and M. Deenen¹

¹Neuroregulation group, Dept. Neurosurgery, LUMC, Leiden University, ²Biomedical Signals & Systems Faculty of Electrical Engineering, Mathematics and Computer Science, University of Twente, Enschede, The Netherlands

ABSTRACT:

Cultures of neurons can be applied in neural probes and be implanted for the restoration of human neuronal function. To reach that goal preliminary research is needed on electrode-neuron contact, placement of neurons on the electrodes and the adhesion of neurons to the underground. Neurons tend to cluster in so called aggregates. This paper describes the normal behaviour of neurons on an underground of natural coatings. This description is a prerequisite for the comparison to neuronal cultures on artificial substrates.

Key words: Neurons, Electrode-neuron contact, Neuron's adhesion, Morphology of the aggregates.

INTRODUCTION

Concentration of neuronal cells occurs when dissociated neuronal cells are brought in culture at a concentration higher than 10^4 cells/ml and is named aggregation. Aggregation seemingly is a *natural* socio-behaviour of neurons. The positioning of dissociated neurons using negative dielectrophoretic forces (NDEP) leads to the artificial concentration of neuronal cells (1). The same holds for additional chemical treatment of substrates to create enhanced neurophilic and neurophobic backgrounds (2). By these backgrounds neurons are forced to group.

Grouping of neurons is used to induce neuronal two-dimensional networks to produce cultured probes, which may be used in future prosthetic interfaces between the nervous system and the artificial steering module. However, the two dimensionality of the probes is easily lost, since neurons tend to aggregate in a three dimensional cluster.

This paper describes the characteristics of neuronal aggregates of several brain parts in order to compare their "Bauplan" *in the future* to those produced by NDEP but also by neurophobic backgrounds. The natural aggregates are cultured on a background of laminin, fibronectin and poly-D-lysine, while NDEP or neurophobic backgrounds are artificial manipulations, which can influence

the neuron's adhesion capability and thus the morphology of the aggregates.

MATERIALS AND METHODS

Several brain parts (cortex, hippocampus, SCN, Arcuate region and dorsal root ganglion cells) were dissected free, mechanically dissociated or trypsin treated and cultured in a serum-free medium (R12), (3) with added NGF for at least up to 14 days in vitro (DIV). Immunocytochemistry for phosphorylated neurofilament (L-M-H mol. w.) was carried out. Aggregates and their bundles were studied by confocal and scanning/transmission electron microscopy after appropriate fixation and (fluorescent) staining.

RESULTS

Morphology

Natural aggregates of dissociated *cortical* neuronal cells can arise from 2-7 DIV depending on the concentration of cells. At high concentrations ($> 10^6$ cells/ml) large aggregates will develop, that easily show necrosis of their cells. Aggregates will make interconnections of grouped cellular protrusions, which constitute bundles. The maturation characteristics of the neurons can be studied using antibodies against phosphorylated neurofilament comprising the detection of L, M and H molecular weights. In *hippocampus* aggregates (n=20) with a diameter of 100 μ m, the first positivity of phosphorylated neurofilaments can be noted at 10 DIV, while the number of positive fibres

*Correspondence to: E. Marani, Dept. Physiology, Wassenaarseweg 62, 2300 RC Leiden, The Netherlands
E-mail: E.Marani@LUMC.nl

in the bundles ($n=100$) starts to increase after 10 DIV. The number of interconnecting bundles among the aggregates ($n=60$) is seven, except for those at the outside of the culture (4) *Dorsal root ganglion cells* also regroup and reconstitute a dorsal root ganglion provided the glial cells are still present (5).

Using dissociated *suprachiasmatic* neuronal cells small aggregates can be obtained (Figure 1 and 2), that are easy to study with confocal microscopy using fluorescent antibodies and dyes. The neuronal cells are mainly placed at the outside of the aggregate, while cellular protrusions form the inside of the aggregate. Neurofilament positive fibres can be followed travelling through the bundles producing collaterals in the aggregates and making "boutons en passage" with cells. Several aggregates can be visited before the growth cone is reached (6). The same holds for the preinfundibular cells of the arcuate region (7).

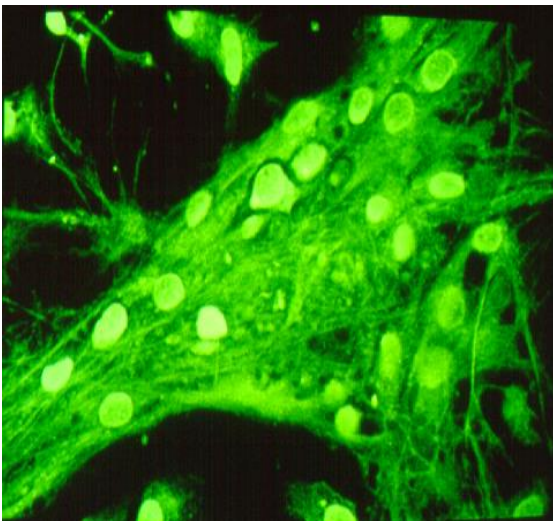


Figure 1. One out of a series of confocal microscopical images of an aggregate of the SCN fixed after 14 DIV stained with antibodies against phosphorylated neurofilament. Note the fibre concentration in the centre of the aggregate and its neurons at the outside

Physiology

Membrane currents can be elicited by step depolarisations and channel or action potential activity can be registered in these cultured neurons (5, 6, 7, 8), indicating that all these neuronal aggregates are functional. Moreover grouped neurons in culture start to be spontaneously active (6).

DISCUSSION

Inherent to central nervous system dissociated neuronal cells are their possibility to aggregate and to constitute connections,

forming a network: "a dispersed brainstem". Although cortical and hippocampus neurons are stored in vivo in layers, in vitro they form "nuclei". The aggregates are the cellular knots and the bundles of protrusions their interconnecting cables in the network. Some bundles are occupied with single cells. The studied small natural aggregates have a mushroom like appearance and their cells are placed at the exterior of the head in order to get maximum exchange with the culture fluid. Phosphorylated forms of neurofilament characterise the development of immature neurons and axons to mature ones and help growing axons to accommodate the demands for plasticity and stability by modulating the structure of the axonal cytoskeleton. Moreover phosphorylated neurofilament expression correlates with the electrophysiological maturation. These labelled axons visited several aggregates, which will be connected to the neuron the axon originated from.

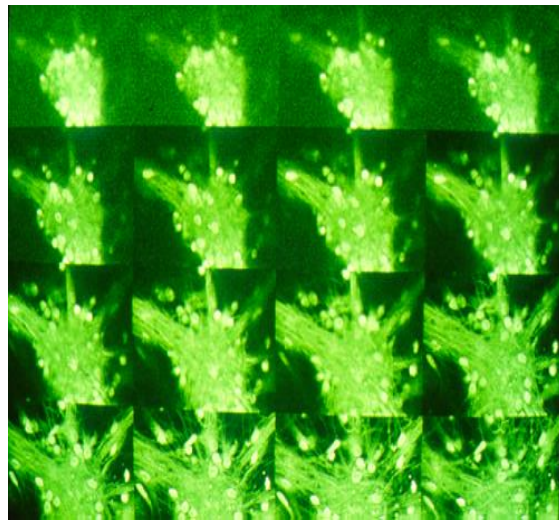


Figure 2. Whole series of confocal microscopical sections through an aggregate of the SCN after 14 DIV stained with antibodies against phosphorylated neurofilament. Large bundles of protrusions are leaving the aggregate towards a nearby aggregate already at the top of the aggregate

Circular island networks are produced by bio-friendly surroundings for the outgrowing sprouts in such a way that these sprouts are adhered to artificial undergrounds. In this way prepatterned surfaces produce the desired network organisation with the desired interconnections of the circular islands. Polyethyleneimine and fluorocarbon monolayers increase or decrease adhesion of neurons. Moreover polyethylenoxidecoated hydrophobic surfaces inhibit the absorption of proteins and are produced as neurophobic background surfaces (2).

Neurons can be cultured on multi-electrode arrays with a mapped pattern of the interconnections. Most neuronal cultures are spontaneous active after 5-7 DIV and can be kept in culture for a few months. This opens the possibility for long-term, longitudinal recording of activity levels in individual neurons and the influence of the desired pattern on this neuronal activity. A two dimensional layer of neurons enhances these studies.

Two-dimensional networks are mainly induced by using a strong hydrophilic background to let the neurons adhere to. The best-known hydrophilic chemical substance is polyethyleneimine (PEI), which is frequently used in the culture of two-dimensional networks (1, 8, 9), (1). However over time the substrate's hydrophilic capacity diminishes and the grouped neurons tend to form an aggregate. Even absorbed layers of block copolymers that are neurophobic over time cannot overcome this return to the three dimensionality of the grouped neurons.

Therefore, the molecular adhesion in and as a consequence the morphology of two and three-dimensional neuronal groups should be studied to understand the force that drives neurons to aggregate three dimensionally.

REFERENCES

1. Heida, T., Rutten, W.L.C., Marani, E., Viability of dielectrophoretic trapped neural cortical cells in culture. *J.Neurosci Meth.* 110: 37-44, 2002.
2. Ruardij, T.G., van den Boogaart, M.A.F., Rutten, W.L.C., Adhesion and growth of electrically active cortical neurons on polyethyleneimine patterns microprinted on

PEO-PPO-PEO triblockcopolymer-coated hydrophobic surfaces. *IEEE Transactions on Nanobioscience*, 1:1-8, 2002.

3. Romijn, H.J., Van Huizen, F., Wolters, P.S., Towards an improved serum free, chemically defined medium for long term culturing of cerebral cortex tissue. *Neurosci Biobehav Rev* 8: 301- 344, 1984.

4. Van Welsum, R.A., van der Voet, G.B., Marani, E. et al., Aluminum affects interconnections between aggregates of cultured hippocampal neurons. *J. Neurosci Meth*, 93: 157-166, 1989.

5. Van Dorp, R., Jalink, K., Oudega, M., Marani, E., et al. Morphological and functional properties of rat dorsal root ganglion cells cultured in a chemically defined medium. *Eur J.Morphol*, 28: 430-444, 1990.

6. Walsh, I.B., van den Berg, R.J., Marani, E., Spontaneous and stimulated firing in cultured rat suprachiasmatic neurons. *Brain Res*, 588: 120-131, 1992.

7. Marani, E., Corino, M., van den Berg, R. et al., Ionic conductances in cultured pre-infundibular cells from the hypothalamic arcuate region. *Neuroendocrin*, 48:445-452, 1988.

8. Buitenweg, J.R., Rutten, W.L.C., Marani, E. et al., Extracellular detection of active membrane currents in the neuron-electrode interface. *J.Neurosci Meth*, 115: 211-221, 2002.

9. Rutten, W.LC, van Pelt, J., (2001) Activity patterns of cultured neural networks on microelectrode arrays. Proc. 23rd Int. Conf. IEEE Engin. Med Biol Soc. Istanbul, Turkey 4 pp ISBN 0-7803-7213-1 CD-ROM