ELSEVIER

Journal of Neuroscience Methods 104 (2001) 133–141

JOURNAL OF NEUROSCIENCE METHODS

www.elsevier.com/locate/jneumeth

# Adhesion proteins for a tight neuron–electrode contact

Helga Sorribas [a], Dieter Braun [b], Lukas Leder [c], Peter Sonderegger [c], Louis Tiefenauer [a,*]

[a] *Laboratory for Micro- and Nanotechnology, Paul Scherrer Institute, CH-5232 Villigen PSI, Switzerland*
[b] *Department of Membrane- and Neurophysics, Max-Planck-Institute for Biochemistry, D-82152 Martinsried, Germany*
[c] *Department of Biochemistry, University of Zürich, Winterthurerstr. 157, CH-8057 Zürich, Switzerland*

Received 5 May 2000; received in revised form 12 September 2000; accepted 13 September 2000

**Abstract**

The neural cell adhesion molecules axonin-1 and NgCAM have been genetically engineered and covalently immobilized on glass and silicon oxide surfaces in their correct orientation. Surfaces treated with these adhesion molecules were used as substrates for culturing dorsal root ganglion neurons. The cleft between the neuron cell membrane and the surface was determined using fluorescence interference contrast (FLIC) microscopy. For comparison, cell–material distances on laminin, RGDC, polylysine and amino-terminated surfaces were measured. When the neurons grow on axonin-1 the cell–surface distance is at a minimum (37 nm) probably because the glycocalyx hinders a closer contact. A selective treatment of extracellular electrodes with axonin-1 could be used to improve the cell–material contact and thus increase extracellularly recorded signals. © 2001 Elsevier Science B.V. All rights reserved.

*Keywords:* Neuron; Adhesion molecules; Cell–substratum distance; Extracellular electrodes

## 1. Introduction

In the last decade considerable progress has been made in culturing neurons on artificial surfaces as reviewed by Stenger and McKenna (1994). Since neurons need a biocompatible surface for their survival in vitro, a series of substances that enable adhesion has been investigated. Positively charged amino groups facilitate the adhesion of negatively charged cells as demonstrated when using short-chain amino silanes (Kleinfeld et al., 1988). It is now well known that the short peptide sequence Arg-Gly-Asp (RGD) found in the extracellular matrix protein laminin (Pierschbacher and Ruoslahti, 1984) promotes cell attachment (Hammarback et al., 1985). Many groups have investigated peptides containing the RGD-sequence for the biofunctionalization of surfaces (Tashiro et al., 1991; Heiduschka et al., 1996; Sorribas et al., 1999) and even a low surface coverage was shown to be sufficient to promote cell attachment and neurite outgrowth (Massia and Hubbell, 1990; Xiao et al., 1997). Differences in shape and motility of cells attached to polylysine and RGD-peptides (Glass et al., 1994) indicate that different structural elements of the cell membrane are involved in the interaction with the immobilized molecules.

In the last few years, several novel neural guidance proteins have been discovered (Tessier-Lavigne and Goodman, 1996). The neural cell adhesion molecules (CAM) of the immunoglobulin-superfamily are expressed in developing neurons and are responsible not only for adhesion and neurite outgrowth, but also for neurite pathfinding and fasciculation in vivo (Sonderegger, 1998). These adhesion proteins usually consist of several N-terminal Ig-like domains linked to fibronectin-like or other domains. The two-adhesion molecules axonin-1 and NgCAM have been isolated from chicken and investigated in detail (for a review see Sonderegger et al., 1998). Based on their amino acid sequence the domain structure could be postulated (Burgoon et al., 1991; Züllig et al., 1992). Membrane-anchored axonin-1 molecules become engaged in a homophilic trans-interaction (Rader et al., 1993) involving

* Corresponding author.
*E-mail address:* louis.tiefenauer@psi.ch (L. Tiefenauer).


PII: S0165-0270(00)00333-2

the first four Ig-domains of each axonin-1 molecule. When two cells interact, a heterodimer, consisting of an axonin-1 and an NgCAM molecule, is formed in the cell membrane (Buchstaller et al., 1996) leading to a tetrameric (axonin-1/NgCAM)$_2$ complex. The specific functions of adhesion proteins may also be useful for in vitro applications.

Adhesion of neurons on surfaces is the pivotal step for establishing neural networks from dissociated neurons (Letourneau, 1975). However, spontaneous or induced burst activity arise only after several days in culture and the formation of a network requires time (Jimbo et al., 1993). Patch clamping is a very sensitive technique to measure voltage and current changes but it is invasive and thus recording time is limited to a few hours. Therefore, devices aimed at a noninvasive extracellular stimulation and recording of dissociated neurons and slices by using microelectrode arrays have been developed (Gross et al., 1977, 1993; Thiébaud et al., 1999). Indium–tin-oxide (Gross et al., 1985; Jimbo et al., 1993) or gold (Breckenridge et al., 1995; Maher et al., 1999) serve as materials for electrodes. Alternatively, the electrophysiological activity of neuron cells was monitored extracellularly by field effect transistor (FET) devices (Fromherz et al., 1991). A difficulty in some experiments is that extracellularly recorded signals are about two orders of magnitude lower than signals measured with patch clamp techniques and thus a relatively low signal-to-noise ratio is achieved. In FETs the intensity of the signal is proportional to the cell–electrode sealing (Bove et al., 1995). Sealing impedance has been experimentally determined and equivalent circuits of the cell membrane–electrode junction have been presented (Fromherz et al., 1991; Bove et al., 1995; Maher et al., 1999; Sprössler et al., 1999). It can be suggested that a tight contact between the membrane and the electrode material will improve signals from extracellular recordings. The distance is an important but not the only determining parameter for the seal resistance of the cleft; induced ionic currents play also an important role in neuron junctions. Fluorescence interference contrast microscopy (FLIC) allows us to measure the cell membrane–material distance precisely and accurately (Braun and Fromherz, 1997). The method is based on an integrated intensity calibration and does not depend on assumptions for uncertain values for parameters required for calculations.

We have prepared glass and silicon oxide chips endowed with recombinant axonin-1 and NgCAM molecules. A covalent and oriented immobilization is possible via a C-terminal cysteine. These surfaces induce adhesion and neurite outgrowth of dissociated dorsal root ganglia neurons (Sorribas et al., 1999). We have determined the minimal cell–surface distances by FLIC microscopy. Chips treated with aminosilane, polylysine, laminin and a covalently bound RGD-analog were investigated for a direct comparison. The results indicate that the cell membrane–surface distance is reduced to a minimum, if adhesion proteins are present.

## 2. Materials and methods

### 2.1. Fabrication of silicon oxide chips

Silicon oxide chips were produced as described by (Braun and Fromherz, 1997). Briefly, silicon (100) wafers were oxidized at 1000°C in wet oxygen up to 155 nm. In a first photolithography step 100 nm of the oxide were etched with hydrofluoric acid in stripes of 5 μm width. In the second photolithography step the same mask was turned by 90° in relation to the first step and now 45 nm of the oxide layer were removed. The resulting surface exhibited a pattern of squares of $5 \times 5$ μm. The heights of the resulting oxide squares were about 10, 55, 110 and 155 nm. The wafer was cut into chips of $34 \times 10$ mm. The thickness of the oxides was measured by ellipsometry after every experiment.

### 2.2. Production of recombinant axonin-1 and NgCAM

In order to achieve covalent and oriented immobilization of the neural cell adhesion proteins axonin-1 and NgCAM on the oxide surface, the transmembrane and intracellular domains of the proteins were deleted and a cysteine was inserted by genetic engineering at the C-term of the last extracellular domain near the membrane. This modification allowed the secretion of the recombinant proteins and also a covalent immobilization via the thiol group of the cysteine on silicon oxide and glass using a maleinimide crosslinker.

By genetical engineering the sequence Gly-Gly-Ser-Gly-Cys was introduced C-terminally to the position of the amino acid Glu-1012 at the end of the fourth fibronectin domain of wild-type axonin-1. A 950 bp fragment of axonin-1 cDNA was amplified using a backward primer that codes for the linker peptide sequence and introduces a KpnI restriction site and a forward primer located upstream of the HindIII restriction site at position 2070 of the wildtype axonin-1 gene (Züllig et al., 1992). The KpnI/HindIII digested 950 bp PCR-fragment was ligated into the expression vector pcDNA3.1 MycHis version A (InVitro Gen), previously cut with XbaI/KpnI, together with the HindIII/XbaI digested cDNA fragment of axonin-1 (2100 bp). HEK 293T cells were cultured in flasks in DMEM (Gibco-BRL) supplemented with 10% fetal calf serum (FCS) until a confluence of 20–30% was reached. Cells were transfected with 4 μg plasmid DNA/ml cell culture medium using the calcium phosphate precipitation tech-

nique. After 8 h incubation at 37°C the medium was changed to DMEM supplemented with 5% FCS. The supernatant with the secreted Cys-axonin-1 was harvested after 3–4 days of incubation. Purification of Cys-axonin-1 was done as described by Ruegg et al. (1989) for the purification of axonin-1 from the vitreous fluid of the eye of chicken embryos. Briefly, the filtered supernatant was loaded on a concanavalin A column (Pharmacia) after dialysis against loading buffer. Bound fractions were eluted with a gradient of α-mannopyranoside. The fractions containing Cys-axonin-1, as determined by immunoblotting were dialyzed against 20 mM Tris–HCl pH 8.0, loaded on an anion exchange column (MonoQ, Pharmacia) and eluted with a gradient of NaCl. Minor contaminants were separated from Cys-axonin-1 by gel filtration (Superose 12, Pharmacia). The purity of the Cys-axonin-1 fractions was confirmed with SDS polyacrylamide gel electrophoresis (PAGE) and immunoblotting.

For the production of recombinant Cys-NgCAM a similar strategy was pursued. A sequence coding for the peptide Gly-Gly-Ser-Gly-Cys was inserted at position Lys-1143 of the wild-type NgCAM cDNA. This amino acid is located at the end of the fifth fibronectin-domain of NgCAM (Buchstaller et al., 1996). The PCR was done using a backward primer containing the sequence of the linker peptide and a HindIII restriction site and a forward primer that was located upstream of the SacI site in the NgCAM cDNA. The HindIII/SacI digested 600 bp fragment introducing the peptide linker was ligated into the expression vector pcDNA3.1 MycHis version A (in vitro Gen) together with the XbaI/SacI digested NgCAM cDNA fragment. Transfection and expression were performed as described above for the recombinant Cys-axonin-1. The secreted protein was immunoaffinity purified from the supernatant with monocl onal anti-NgCAM antibodies as described by Stoeckli et al. (1991). The eluted fractions were tested with SDS-PAGE and immunoblotting.

### 2.3. Immobilization of adhesion molecules

The silicon oxide chips were cleaned with Caro solution (96% sulfuric acid, 30% hydrogen peroxide, 3:1) and extensively rinsed in ultra high pure water before each use. Laminin and polylysine were adsorbed, whereas the other adhesion molecules investigated were covalently immobilized on the silicon chips (Fig. 1). Briefly, 230 μl of a 20 μg/ml mouse laminin (GibcoBRL) solution in phosphate buffered saline (PBS) or of a 100 μg/ml poly-L-lysine (Sigma) solution were applied on the chips. After 2 h the chips were rinsed with PBS. For the covalent immobilization procedure the chips were incubated for 1 h in a 2% aminopropyltriethoxy silane (APTES, Sigma) solution in toluene and washed with toluene afterwards. Some of these chips were subsequently used as amino-terminated substrate for cell–surface distance measurements. For the immobilization of the cysteine terminated recombinant proteins (Cys-axonin-1 and Cys-NgCAM), and the adhesion peptide Arg-Gly-Asp-Cys (RGDC) the following protocol was used: a 5 mM solution of the heterobifunctional crosslinker *N*-[γ-maleimidobutyryloxy]sulfosuccinimide ester (sGMBS, Pierce) was prepared solving it in 30 μl dimethyl sulfoxide and diluting it in 50 mM phosphate buffer pH 7.0. The solution was passed through a 0.2μm sterile filter and 230 μl were applied on each chip. The samples were rinsed with phosphate buffer after 2 h. The recombinant adhesion proteins Cys-axonin-1 and Cys-NgCAM were diluted to a final concentration of 150 μg/ml in 50 mM phosphate buffer pH 7.4. A 2 mM solution of RGDC (Bachem, Switzerland) was prepared in 50 mM phosphate buffer pH 7.0. The protein or peptide solutions (230 μl) previously filtered sterile were applied on the chips for 2 h and rinsed with phosphate buffer.

### 2.4. Cell preparation

Dorsal root ganglia (DRG) were dissected from 10-day-old chicken embryos as described by Sonderegger et al. (1985). The isolated ganglia were dissociated during a 15-min incubation in 0.25% trypsin in PBS (GibcoBRL) at 37°C, followed by mechanical dissocia-

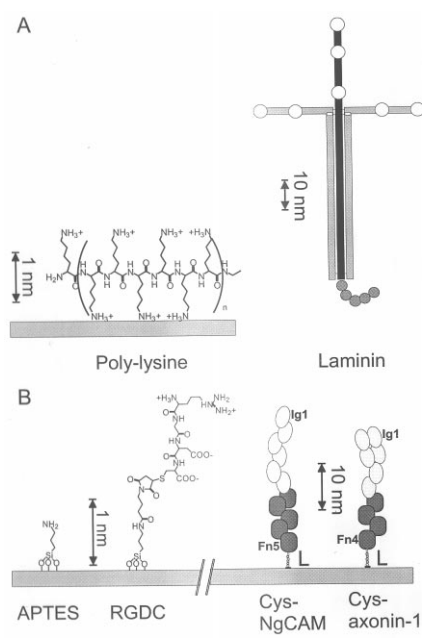


Fig. 1. Dimensions of adhesion molecules immobilized on silicon oxide. (A) Polylysine and laminin were immobilized by physisorption. (B) APTES, RGDC, Cys-axonin-1 and Cys-NgCAM were covalently immobilized on the oxide via silane chemistry with a crosslinker molecule for an oriented immobilization. (L, peptide linker with C-terminal Cys, crosslinker and amino silane; Ig, immunoglobulin-like domain; Fn, fibronectin-like domain).

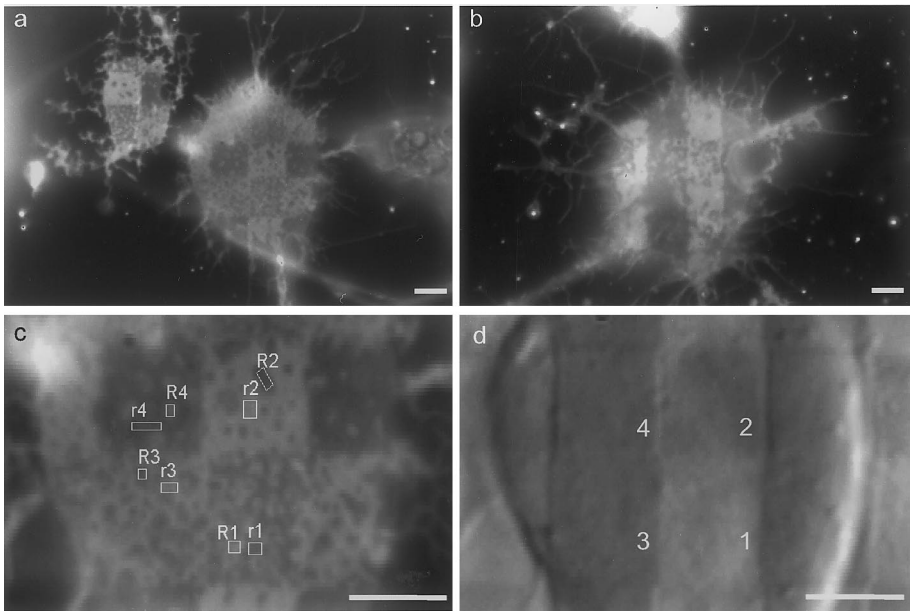


Fig. 2. Fluorescence micrographs of DRG neurons cultured 20 h on APTES (a) and axonin-1 (b). (c) shows a detailed view of the cell in the center of (a). Fluorescence intensities were measured over the regions delimited by white boxes (r1–r4 and R1–R4 in (c)) and the correspondent oxide thickness assigned comparing with the picture of the cell at the same position made with closed Abbe condensor in white light (d). Oxide thickness 1–4 in (d) correspond to 10, 55, 110 and 155 nm respectively. Scale bars 5 μm.

tion with a pipette tip. Dissociated cells were suspended in serum free defined MEM (Stoeckli et al., 1996) and plated on the chips at a density of 5000 cells/cm$^2$. For the assessment of cell adhesion and neurite outgrowth the cells were seeded on glass treated as described for the silicon chips and cultivated during 4 days. Optical inspection using phase contrast microscopy was carried out on day 1, when neurites had formed. DRG neurons could be identified by a typical halo around the cell body whereas glia cells appeared flat. Cell debris and dead cells were observed frequently as a consequence of the preparation, but could clearly be distinguished from living cells by the dotted appearance of the soma. For an assessment of the influence when using different substrates, the percentage of cells with neurites was counted and the neurite lengths determined using the NIH image software. Only neurites that emerged from an isolated neuron (not from a clump of cells), and that did not contact other neurites or cells, were considered. The neurite length of the longest branch was measured from the cell soma to the most remote tip. Data in Table 1 are from three culture preparations using three chips of each substrate and are means of $n > 30$ measured neurites.

For the FLIC experiments cell membranes were stained 20 h after seeding with the amphiphilic dye 1,1′ - didodecyl - 3,3,3′,3′ - tetramethylindocarbocyanine perchlorate ($DiIC_{12}$, Molecular Probes). A 5 mM $DiIC_{12}$ stock solution in ethanol was diluted 1:1000 in PBS resulting in a suspension of dye aggregates. The medium was removed and substituted with 3 ml of this solution. The dye was added, removed and re-added carefully to the cells in order to enhance staining. After 5 min at 37°C this procedure was repeated once. The living cells were kept in PBS during observation with the fluorescence microscope. For the FLIC measurements two independent cell culture preparation and two chips per coating were used.

### 2.5. Determination of cell–surface distances

Theory and set-up used for the calculation of the cell–surface distance with fluorescence interference contrast microscopy (FLIC) have been described by Lambacher and Fromherz (1996), Braun and Fromherz (1997). Briefly, fluorescence pictures (Fig. 2) were taken through a water immersion objective (100 ×, numerical aperture 1.0, Zeiss Axioskop) with opened Abbe condensor at 546 nm excitation wavelength. Emitted light was filtered and detected between 580 and 640 nm with a CCD camera (Sony chip ICX039AL, HRX, Theta Systems, Germany) that had an effective pixel size of 90 × 90 nm. Exposure times varied between 40 and 320 ms.

In order to identify the oxide squares below the cell a reference picture was made through the same objective filtering light from a halogen lamp at 630 nm and with closed Abbe condensor (Fig. 2d). Pictures taken with open Abbe condensor showed no intensity contrast coming from the cells. On each oxide square regions of equal fluorescence intensity were defined and fitted by the mentioned optical theory of interference.

Table 1
Neurite outgrowth of DRG neurons cultured on different substrates measured as described in Section 2[a]

| Substrate | Outgrowth (% of total cells) | Mean neurite length (μm) |
|---|---|---|
| Aminosilane | 20 | $40 \pm 10$ |
| RGDC | 60 | $124 \pm 60$ |
| Laminin | 90 | – |
| Axonin-1 | 50 | $80 \pm 30$ |
| NgCAM | 80 | $231 \pm 86$ |

[a] The lengths are means of at least 30 inspected cells.

FLIC-microscopy takes advantage of the interference of incident and reflected light above a mirror (Wiener effect). The standing modes of the electromagnetic field above the surface of silicon modulate the excitation and the emission of a fluorescent dye, which is inserted in a membrane. The observed photons per unit time $J_{fl}$ depend on the probabilities of excitation under stationary illumination ($P_{ex}$) and of emission into the detector ($P_{em}$) according to Eq. (1) with a scaling factor $a$ and a background $b$.

$$J_{fl}(d_{ox}) = aP_{ex}(d_{ox}, d_{cleft})P_{em}(d_{ox}, d_{cleft}) + b \quad (1)$$

For the calculation of the cell–substrate distance $d_{cleft}$ an optical model with five layers (bulk silicon, oxide layer, extracellular cleft, cell membrane and cytoplasma) was assumed described by thickness and refractive index (Braun and Fromherz, 1997).

## 3. Results

### 3.1. Neurite outgrowth on surfaces treated with different cell adhesion molecules

The outgrowth of neurites on the different substrates was examined as described in Section 2 and the findings are summarized in Table 1. Neurite outgrowth was found in 90% of the neurons when they were cultured on laminin, but only in 20% of the cells grown on the amino-terminated silane APTES. Neurite outgrowth on Cys-NgCAM was comparable to outgrowth on laminin whereas on RGDC and on Cys-axonin-1 about half of the neurons had neurites. The mean neurite lengths for each substrate were calculated for comparison. They ranged from 40 to 230 μm. On APTES neurites were thick and only rarely longer than two cell diameters (Fig. 3a). On laminin and Cys-NgCAM the neurites were the thinnest and longest (Fig. 3b), whereas on Cys-axonin-1, RGDC and polylysine the neurites where slightly shorter and broader. These findings indicate that neurite outgrowth is strongly influenced by the molecule present on the surface.

Generally, when neurons had begun to interconnect and fasciculate the surface–contact area was smaller as the soma was often sitting on top of neurites. This seems to be a property of these sensory neurons as such behavior was rarely observed with hippocampal neurons (D. Braun, personal communication).

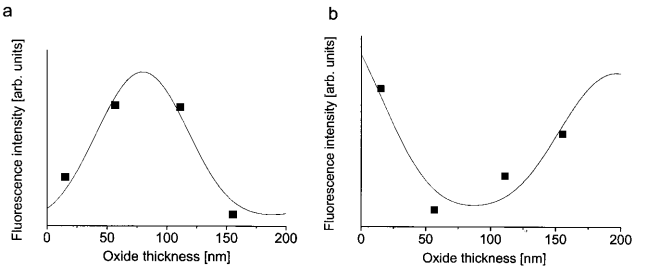


Fig. 4. Determination of minimum and maximum cell–surface distances. The average fluorescence intensities of boxes r1–r4 (a) and R1–R4 (b) of Fig. 2c were plotted vs. the four different oxide thickness (■). The curve was fitted with the distance calculation program (see text). The calculated distances were $34 \pm 5$ nm for (a) and $154 \pm 7$ nm for (b).

### 3.2. Calculation of minimal cell–surface distances

Areas of homogeneous fluorescence intensity were defined (Fig. 2c) and the averaged intensities were plotted versus the oxide square thickness (Fig. 4). For the regions r1–r4 defined in Fig. 3c the lowest intensity was on the thinnest oxide indicating a narrow cleft (Fig. 4a), whereas in the regions R1–R4 the intensity was

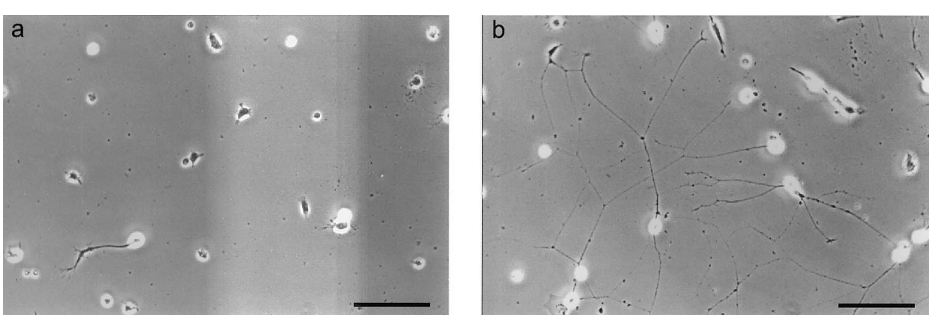


Fig. 3. DRG neurons cultured for 24 h on amino-silane (a) and NgCAM (b), respectively. Note the difference in the frequency and the length of neurites between the two substrates. Scale bar 100 μm.

highest on the thinnest oxide indicative of a large cell–surface distance (Fig. 4b), as the standing modes have a node near silicon. The distance values were fitted into the distance curve calculation of the program developed by D. Braun (Fig. 4, solid lines). For an interactive FLIC simulation and a distance fitting program see http://mnphys.biochem.mpg.de/projects/flic/flic_root.html.

As it can be seen in Fig. 2 the fluorescence intensity is not homogeneously distributed over the whole oxide square area, rather it shows an irregular, dotted pattern. Analyzing the cell–surface distance these membrane patches revealed differences from 30 to 100 nm (Fig. 4). This phenomenon was observed on all substrates and has been mentioned by Braun and Fromherz (1998) for rat neuron cultures on chips with adsorbed polylysine. For the calculation of the minimal cell–surface distance for each substrate type only the smallest distance value of every cell was taken.

The results are summarized in Fig. 5. The closest cell–surface contact was found on the Cys-axonin-1 substrate ($37 \pm 10$ nm, $n = 16$) and on the APTES surface ($39 \pm 3$ nm, $n = 7$). On Cys-NgCAM substrate a longer distance was determined ($47 \pm 8$ nm, $n = 14$), whereas on the polylysine substrate the minimal distance was found to be $54 \pm 9$ nm ($n = 7$). The cleft separating the cell membrane grown on laminin from the surface ($91 \pm 4$ nm, $n = 7$) was much wider and comparable to the previously reported value for hippocampal cells of $104 \pm 1$ nm (Braun and Fromherz, 1998). On chips treated with the short adhesion peptide RGDC we found a heterogeneity in terms of the cell–surface distances. A cluster for cells with a mean distance of $39 \pm 4$ nm ($n = 5$) can be defined (Fig. 6, dashed line), whereas the distance of the further cells ranged between 80 and 160 nm.

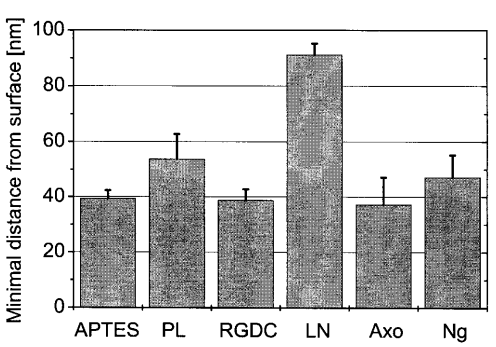


Fig. 5. Average minimal distances and S.D. of DRG neurons cultured on amino-terminated surfaces (APTES), on poly-L-lysine (PL), on RGDC, on laminin (LN), on Cys-axonin-1 (Axo) and on Cys-Ng-CAM (Ng). The distance values are given in the text and are based on seven to 16 measurements.

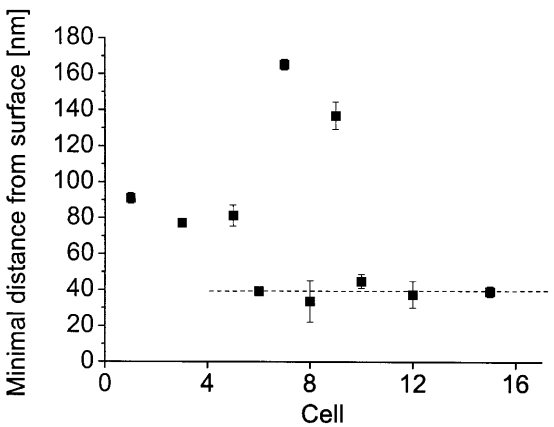


Fig. 6. Calculated minimal distances from all cells measured on RGDC-treated chips. Low values are clustered in a mean cell–substrate distance of 39 nm (dashed line).

## 4. Discussion

From early experiments with dissociated neuron cultures it was recognized that a positively charged surface promotes adhesion of neurons (Yavin and Yavin, 1974; Letourneau, 1975). Presently, the positively charged polyamino acids polyornithine and polylysine are well established as coating molecules for culture dishes. The exact nature of the attachment mechanism to these surfaces is not yet clear. However, it seems widely accepted, that negatively charged proteoglycans or lipids of the cell membrane could interact with the surface-bound polycations (Yavin and Yavin, 1974; McKeehan and Ham, 1976; Glass et al., 1994). The assumption that a positive charge is sufficient for cell adhesion was refined by Kleinfeld et al. (1988). They postulate that the chemical structure may additionally be important as indicated by the finding that di-amino terminated molecules on surfaces were more potent adhesion promoters than surfaces treated with mono-amino-molecules. This is in agreement with our observation that cells are growing poorly on the mono-amino silane APTES, but the distance of the cell membrane to the substrate on APTES is at a minimum. Adhesion mechanism of cells on APTES and polylysine is probably comparable. The measured cell–surface distance, however, is larger on polylysine (54 nm). Polylysine, which is physisorbed onto the chip surface, is more extended than the short-chain APTES and the coating is not as stable as the covalently bound APTES, probably resulting in an increased mean distance value.

In contrast to APTES and polylysine, the other adhesion molecules studied bind cells specifically via receptor molecules. The extracellular matrix protein laminin is recognized by integrin molecules present in the cell membrane (McKerracher et al., 1996). The integrins are a family of receptor proteins consisting of heterodimers of distinct $\alpha$ and $\beta$ chains that protrude

20–22 nm into the extracellular space (Nermut et al., 1988). Neurons cultured on laminin coated chips are separated from the surface by a rather wide cleft of 91 nm as it can be expected considering the dimensions of laminin ($72 \times 113$ nm; Engel et al., 1981). Braun and Fromherz (1998) have reported a homogeneous 104-nm-thick cleft for rat neuron cultures on adsorbed laminin. A cleft of this width will result in a loose sealing to extracellular FETs. Some of the cell adhesion functions of laminin are mimicked by the short peptide sequence RGD that is recognized by different integrins (Glass et al., 1994; Burridge and Chrzanowska-Wodnicka, 1996). A theoretical distance of 25 nm can be assumed by adding the dimension of the RGDC covalently immobilized on the surface (2–3 nm) and the mentioned length of the integrin molecule protruding form the cell membrane (20–22 nm). The actual determined distance was slightly longer (39 nm), because as pointed out below, the glycocalyx of the cell may also contribute to the distance value. It is not clear, why in some cases we found longer distances for cells on RGDC chips. Further receptor molecules may be involved or we observed different neuron subtypes. Since this phenomenon was not observed with other adhesion molecules we can exclude a methodical fault.

Several studies have been made to unravel the interactions of axonin-1 and NgCAM with other cell adhesion proteins. Although a homophilic binding of axonin-1 between two cells could be shown with heterologous expression on myeloma cells (Rader et al., 1993), the biological function of this complex is not yet clear. Neurite outgrowth on axonin-1 substrates is probably mediated by NrCAM (Suter et al., 1995; Lustig et al., 1999) whereas neurite outgrowth on Ng-CAM substrates is thought to be mediated by the tetrameric $(\text{axonin-1/NgCAM})_2$ complex. Considering that an Ig-domain is about 4 nm long (Schiffer et al., 1973) and assuming similar dimensions for the fibronectin-like domains, the length of the immobilized Cys-axonin-1 would be 32 nm, and Cys-Ng-CAM is estimated to be 44 nm long. From the structural models of interactions we would expect that the cleft between cell and these substrates should be in the range of 50–70 nm. The cell–surface distances measured by FLIC confirm these expectations. On Cys-axonin-1 substrate the cells are at a minimum distance (37 nm) and slightly more distant on Cys-NgCAM-treated surfaces (47 nm).

The fact that no cell–surface distance below 35 nm was found for DRG neurons cultured on different substrates indicates that additional bulky molecules may be present. It is known, that cells are coated with a carbohydrate-rich layer, the so-called glycocalyx. Early electron microscopy works on chicken DRGs had revealed the existence of a cell coat on these neurons (James and Tresman, 1972). Depending on the staining method the thickness of the cell coats measured varied from 30 nm (ruthenium red staining) to 120 nm (lanthanum permanganate staining). The glycocalyx of red blood cells has been estimated to be only 6 nm thick (Linss et al., 1991). Indeed, red blood cell ghosts adhered to polylysine coated surfaces at a very low distance of 12 nm as determined using FLIC microscopy (Braun and Fromherz, 1997). We thus conclude that the glycocalyx may prevent a closer contact of the cell membrane to the surface by steric hindrance. Therefore, we postulate that 35–40 nm is the minimum cell–surface distance for DRG neurons. Whether this is sufficient to improve the signal-to-noise ratio in electrophysiological experiments with extracellular electrodes has to be shown.

Neurons have been reported to adhere to the surface in so called point contacts (Streeter and Rees, 1987; Arregui et al., 1994), i.e. small (90–200 nm) regions of the membrane closely apposed to the substrate, in which other types of integrins and cytoskeletal anchor proteins than those found in focal contacts are involved (Tawil et al., 1993; Arregui et al., 1994). Note the irregular dotted pattern of the membrane in the fluorescence images of the cells (Fig. 2). Patches of membrane appear darker than other areas indicating that some parts of the membrane are more distant from the surface than others (Fig. 2c and Fig. 4). The difference between the distances of these patches was in the range of 30–100 nm for most cells. The lateral dimensions of these dots ranged from 100 to 500 nm, but sometimes they were even larger than 1 μm. Thus, the observed dots may represent point contacts. Identification of the involved proteins by using immunocytochemical methods could help to confirm this assumption.

In this paper we have presented neural adhesion proteins as potentially useful specific molecules for surface and electrode modification, in order to induce a tight cell–material contact. The specific functionality of such cell adhesion molecules could also be used to induce axon vs. dendrite growth or even synaptogenesis. Preliminary experiments in our group have shown, that the recombinant adhesion proteins still promote neurite outgrowth when they are directly immobilized on gold. Whether the cell-contacts with these treated gold electrodes result in improved electrophysiological signals for extracellular recordings has to be shown. Furthermore, the benefit of these specific proteins in combination with other adhesion molecules, their fate in long-term cultures and their influence on various cell functions have to be investigated. Our findings indicate that adhesion proteins are different in some respects to laminins and polycations and that they may be useful for neuron cultures.

## Acknowledgements

Kurt Ballmer and Sandra Ruch are acknowledged for their support in the preparation of the recombinant proteins. We thank Georg Dechant for the cell culture support at the MPI of Neurobiology. We thank Rodney Douglas, Institute for Neuroinformatics, Zürich for helpful discussions and Peter Fromherz, MPI-München, for making available the FLIC equipment and for discussions. This work was supported by the Swiss National Science Foundation, SPP Biotechnology.

## References

Arregui CO, Carbonetto S, McKerracher L. Characterization of neural cell adhesion sites: point contacts are the sites of interaction between integrins and the cytoskeleton in PC12 cells. J Neurosci 1994;14:6967–77.

Bove M, Grattarola M, Martinoia S, Verreschi G. Interfacing cultured neurons to planar substrate microelectrodes: characterization of the neuron-to-microelectrode junction. Bioelectrochem Bioenerg 1995;38:255–65.

Braun D, Fromherz P. Fluorescence interference-contrast microscopy of cell adhesion on oxidized silicon. Appl Phys A 1997;65:341–8.

Braun D, Fromherz P. Fluorescence interferometry of neuronal cell adhesion on microstructured silicon. Phys Rev Lett 1998;81:5241–4.

Breckenridge LJ, Wilson RJA, Connolly P, Curtis ASG, Dow JAT, Blackshaw SE, Wilkinson CDW. Advantages of using microfabricated extracellular electrodes for in vitro neuronal recordings. J Neurosci Res 1995;42:266–76.

Buchstaller A, Kunz S, Berger P, Kunz B, Ziegler U, Rader C, Sonderegger P. Cell adhesion molecules Ng-CAM and axonin-1 form heterodimers in the neuronal membrane and cooperate in neurite outgrowth promotion. J Cell Biol 1996;135:1593–607.

Burgoon MP, Grumet M, Mauro V, Edelmann GM, Cunningham BA. Structure of the chicken neuron-glia cell adhesion molecule NgCAM: origin of polypeptides and relation to Ig superfamily. J Cell Biol 1991;112:1017–29.

Burridge K, Chrzanowska-Wodnicka M. Focal adhesions, contractility, and signalling. Ann Rev Cell Biol 1996;12:463–519.

Engel J, Odermatt E, Engel A, Madri JA, Furthmayr H, Rohde H, Timpl R. Shapes, domain organizations and flexibility of laminin and fibronectin, two multifunctional proteins of the extracellular matrix. J Mol Biol 1981;150:97–120.

Fromherz P, Offenhäuser A, Vetter T, Weis J. A neuron–silicon junction: a retzius cell of the leech on an insulated-gate field-effect transistor. Science 1991;252:1290–3.

Glass J, Blevitt J, Dickerson K, Pierschbacher MD, Craig WS. Cell attachment and motility on materials modified by surface-active RGD-containing peptides. Ann New York Acad Sci Biochem Eng VIII 1994;745:177–86.

Gross GW, Rieske E, Kreutzberg GW, Meyer A. A new fixed-array multi-microelectrode system designed for long-term monitoring of extracellular single unit neuronal activity in vitro. Neurosci Lett 1977;6:101–5.

Gross GW, Wen W, Lin J. Transparent indium–tin oxide patterns for extra-cellular, multisite recording in neuronal culture. J Neurosci Methods 1985;15:243–52.

Gross GW, Rhoades BK, Reust DL, Schwalm FU. Stimulation of monolayer networks in culture through thin-film indium–tin oxide recording electrodes. J Neurosci Methods 1993;50:131–43.

Hammarback JA, Palm SL, Furcht LT, Letourneau PC. Guidance of neurite outgrowth by pathways of substratum-absorbed laminin. J Neurosci Res 1985;13:213–20.

Heiduschka P, Göpel W, Beck W, Kraas W, Kienle S, Jung G. Microstructured peptide-functionalised surfaces by electrochemical polymerisation. Chem Eur J 1996;2:667–72.

James DW, Tresman RL. The surface coats of chick dorsal root ganglion cells in vitro. J Neurocytol 1972;1:383–95.

Jimbo Y, Robinson HPC, Kawana A. Simultaneous measurement of intracellular calcium and electrical activity from patterned neural networks in culture. IEEE Trans Biomed Eng 1993;40:804–10.

Kleinfeld D, Kahler KH, Hockberger PE. Controlled outgrowth of dissociated neurons on patterned substrates. J Neurosci 1988;8:4098–120.

Lambacher A, Fromherz P. Fluorescence interference-contrast microscopy on oxidized silicon using a monomolecular dye layer. Appl Phys A 1996;63:207–16.

Letourneau PC. Cell-to-substratum adhesion and guidance of axonal elongation. Dev Biol 1975;44:92–101.

Linss W, Pilgrim C, Feuerstein H. How thick is the glycocalyx of human erythrocytes? Acta Histochem 1991;91:101–4.

Lustig M, Sakurai T, Grumet M. Nr-CAM promotes neurite outgrowth from peripheral ganglia by a mechanism involving axonin-1 as a neuronal receptor. Dev Biol 1999;209:340–51.

Maher MP, Pine J, Wright J, Tai Y-C. The neurochip: a new multielectrode device for stimulating and recording from cultured neurons. J Neurosci Methods 1999;87:45–56.

Massia SP, Hubbell JA. Covalent surface immobilization of Arg-Gly-Asp- and Tyr-Ile-Gly-Ser-Arg-containing peptides to obtain well-defined cell-adhesive substrates. Anal Biochem 1990;187:292–301.

McKeehan WL, Ham RG. Stimulation of clonal growth of normal fibroblasts with substrata coated with basic polymers. J Cell Biol 1976;71:727–34.

McKerracher L, Chamoux M, Arregui CO. Role of laminin and integrin interactions in growth cone guidance. Mol Neurobiol 1996;12:95–116.

Nermut MV, Green NM, Eason P, Yamada SS, Yamada KM. Electron microscopy and structural model of human fibronectin receptor. EMBO J 1988;7:4093–9.

Pierschbacher MD, Ruoslahti E. Cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule. Nature 1984;309:30–3.

Rader C, Stoeckli ET, Ziegler U, Osterwalder T, Kunz B, Sonderegger P. Cell–cell adhesion by homophilic interaction of the neuronal recognition molecule axonin-1. Eur J Biochem 1993;215:133–41.

Ruegg MA, Stoeckli ET, Kuhn TB, Heller M, Züllig R, Sonderegger P. Purification of axonin-1, a protein that is secreted from axons during neurogenesis. EMBO J 1989;8:55–63.

Schiffer M, Girling RL, Ely KR, Edmundson AB. Structure of a lambda-type Bence–Jones protein at 3.5-Å resolution. Biochemistry 1973;12:4620–31.

Sonderegger P, Lemkin PF, Lipkin LE, Nelson PG. Differentiation modulation of the expression of axonal proteins by non-neuronal cells of the peripheral and central nervous system. EMBO J 1985;4:1395–401.

Sonderegger P, Kunz S, Rader C, Buchstaller A, Berger P, Vogt L, Kozlov SV, Ziegler C, Kunz B, Fitzli D, Stoeckli ET. Discrete clusters of axonin-1 and NgCAM at neuronal contact sites: facts and speculations on the regulation of axonal fasciculation. Prog Brain Res 1998;117:93–104.

Sonderegger P. Ig Superfamily Molecules in the Nervous System. Harwood: Amsterdam, 1998.

Sorribas H, Padeste C, Mezzacasa T, Tiefenauer L, Leder L, Fitzli D, Sonderegger P. Neurite outgrowth on microstructured surfaces functionalized by a neural adhesion protein. J Mater Sci Mater Med 1999;10:787–91.

Sprössler C, Denyer M, Britland S, Knoll W, Offenhäuser A. Electric recordings from rat cardiac muscle cells using field-effect transistors. Phys Rev E 1999;60:2271–6.

Stenger DA, McKenna TM. Enabling Technologies for Cultured Neural Networks. Academic Press: San Diego, CA, 1994.

Stoeckli ET, Kuhn TB, Duc CO, Ruegg MA, Sonderegger P. The axonally secreted protein axonin-1 is a potent substratum for neurite growth. J Cell Biol 1991;112:449–55.

Stoeckli ET, Ziegler U, Bleiker AJ, Groscurth P, Sonderegger P. Clustering and functional cooperation of Ng-CAM and axonin-1 in the substratum-contact area of growth cones. Dev Biol 1996;177:15–29.

Streeter HB, Rees DA. Fibroblast adhesion to RGDS shows novel features compared with fibronectin. J Cell Biol 1987;105:507–15.

Suter DM, Pollerberg EG, Buchstaller A, Giger RJ, Dreyer WJ, Sonderegger P. Binding between the neural cell adhesion molecules axonin-1 and Nr-CAM/Bravo is involved in neuron-glia interaction. J Cell Biol 1995;131:1067–81.

Tashiro KI, Sephel GY, Greatorex D, Sasaki M, Sirashi N, Martin GR, Kleinman HK, Yamada Y. The RGD containing site of the mouse laminin A chain is active for cell attachment, spreading, migration and neurite outgrowth. J Cell Physiol 1991;146:451–9.

Tawil N, Wilson P, Carbonetto S. Integrins in point contacts mediate cell spreading: factors that regulate integrin accumulation in point contacts vs. focal contacts. J Cell Biol 1993;120:261–71.

Tessier-Lavigne M, Goodman CS. The molecular biology of axon guidance. Science 1996;274:1123–33.

Thiébaud P, de Rooij N, Koudelka-Hep M, Stoppini L. Microelectrode arrays for electrophysiological monitoring of hippocampal organotypic slice cultures. IEEE Trans Biomed Eng 1999;44:1159–63.

Xiao SJ, Textor M, Spencer ND, Wieland M, Keller B, Sigrist H. Immobilization of the cell-adhesive peptide Arg-Gly-Asp-Cys (RGDC) on titanium surfaces by covalent chemical attachment. J Mater Sci Mater Med 1997;8:867–72.

Yavin E, Yavin Z. Attachment and culture of disociated cells from rat embryo cerebral hemispheres on polylysine-coated surface. J Cell Biol 1974;62:540–6.

Züllig R, Rader C, Schroeder A, Kalousek MB, von Bohlen und Halbach F, Fritz A, Hafen F, Sonderegger P. The axonally secreted cell adhesion molecule axonin-1: primary structure, immunoglobulin- and fibronectin-type-III-like domains, and glycosyl-phosphatidylinositol anchorage. Eur J Biochem 1992;204:453–63.