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Neuronal differentiation and synapse formation of PC12 and embryonic stem cells on interdigitated microelectrode arrays: Contact structures for neuron-to-electrode signal transmission (NEST)

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Abstract

The development of neuron–microelectrode interfaces (neurochips) is highly desirable for the non-invasive recording of the cellular response to neuroactive drugs as well as the electrical stimulation of nervous tissue by implantable electrodes. A prerequisite for neuron-to-electrode signal transmission (NEST) is the formation of synapse-like contacts between the neuronal cell and the conductive surface of a microelectrode array. We attempted synapse formation by neuronal differentiation of rat pheochromocytoma cells (PC12) and blastocyst-derived murine embryonic stem cells (ES-J1) on interdigitated microelectrode arrays that were made of gold (Au), platinum (Pt), or indium tin oxide (ITO). PC12 or ES cells were in vitro differentiated by incubation with nerve growth factor (NGF) and forskolin, or by serum deprivation and treatment with basic fibroblast growth factor (FGF-2), respectively. On top of ITO electrodes, the neuronal cells extended extremely long processes that terminated in pili-like contact structures, which is typical for growth cone formation. ES cells differentiated into neurons as verified by immunofluorescence staining of MAP-2 and developed synapse-like junctions with the ITO electrode surface as indicated by synaptophysin staining. Differentiated PC12 and ES cells showed bona fide morphological characteristics of synaptic growth cones that were unprecedented in tissue culture. Cones formed by PC12 cells could be stimulated with KCl and carbachol as shown by uptake of FM1-43, a fluorescent marker for synaptic vesicle formation. In contrast to Electrical Cell Impedance Spectroscopy (ECIS) recordings, AC impedance spectrometry with differentiated PC12 cells settled on interdigitated microelectrode arrays revealed lower AC impedance than that with undifferentiated cells, indicating that the complex impedance is dependent on ion fluxes at the neuron-to-electrode contact surface.

Keywords: ECIS; Interdigitated electrode; Microarray; Neurochip; ES cells; PC 12 cells

1. Introduction

Non-invasive methods to monitor the electrical behavior of neuronal cells allow for the interfacing of microelectrode arrays and neurons without the cells being compromised by potential-induced cell damage. Such methods are highly desirable for the use of neuronal cells in biosensors or in neuron-to-electrode signal transmission (NEST) technology. Although recent studies have demonstrated the feasibility of non-invasive activity monitoring with single neurons that were grown on microelectrodes, there has not yet been a thorough analysis of the neuron-to-electrode junction or demonstration of active synapse formation (Fromherz and Stett, 1995; Gross, 1979; Gross et al., 1985; Maher et al., 1999; Mistry et al., 2002). This analysis, however, is a prerequisite for the identification of electrode designs and materials that promote a tight contact between the electrode and neuronal cell surface. Neuron-to-electrode signal transmission and thus, synapse formation on electrode surfaces, is a critical parameter in biosensor technology of so

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called neurochips, for the measurement and monitoring of pharmacologically affected neuronal activity. Materials and designs with tight neuron-to-electrode contact structures are also desired for efficient electrical stimulation of neurons by non-invasive current injection. As an example, direct electrical stimulation by implantable electrodes has been used for treatment of neurodegenerative diseases, such as Parkinson (Olanow et al., 2000).

In order to design optimized microelectrodes for tight junctions between cellular and electrode surfaces, we have analyzed the growth and differentiation characteristics of rat pheochromocytoma PC12 and murine embryonic stem ES-J1 cells that were in vitro differentiated with nerve growth factor (NGF) or basic fibroblast growth factor (FGF-2), respectively. PC12, as well as undifferentiated ES cells, are recyclable systems that can be conveniently propagated before seeding on the microelectrode surface. As shown in Fig. 1, the electrode surface area has been optimized by the design of comb-like interdigitations. The cells either settled onto the insulating surface between the interdigitations or onto the conductive electrode surface. The neuron networking and formation of neuron-to-electrode junctions has been modulated by the use of three different electrode materials: gold, platinum, and indium tin oxide (ITO). ITO electrodes are translucent and thus, allow for high resolution imaging of neuron-to-electrode synapses by confocal fluorescence microscopy. The functionality of these synapses has been verified by immunoflurescence microscopy using antibodies against the synaptic vesicle marker synaptophysin and by FM1-43 uptake studies upon neurotransmitter stimulation.

We have monitored the electrical stimulation of differentiated PC12 cells by AC impedance measurement using interdigitated platinum microelectrodes. The use of impedance stimulation with interdigitated microarrays

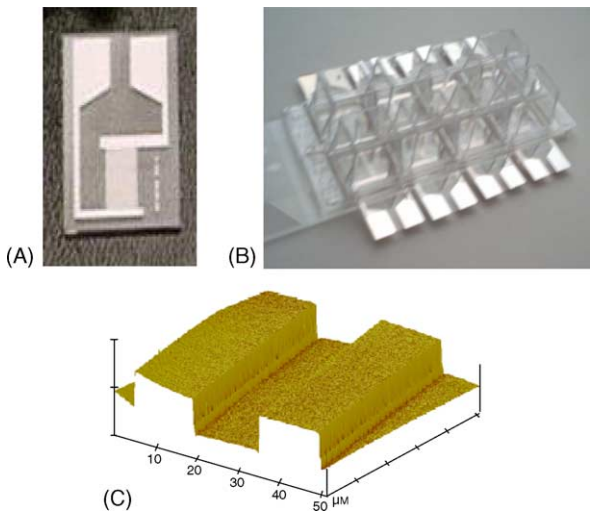

Fig. 1. Interdigitated microelectrode array design and multi-well chamber assembly of neurochips. (A) A single neurochip showing the region of electrode interdigitation. (B) An array of eight-cell culture wells. (C) Scanning probe micrograph of the interdigitated microelectrode array.

combines the principle of Electrical Cell Impedance Spectroscopy (ECIS) with the recently emerging neurochip technology (Lacy et al., 1996; Lo et al., 1999; Sharma et al., 2001; Wegener et al., 2000). ECIS uses high frequency AC impedance measurement of cells grown on a small area, single electrode surface. In this arrangement, the capacitance depends upon the quality of the cell-to-cell rather than on the cell-to-electrode contact. In contrast, neurochips with cells grown on microelectrode arrays have been used for single cell recordings with comparably low capacitance due to a rather small surface of cell-to-electrode contact points (Fromherz and Stett, 1995; Gross, 1979; Gross et al., 1985; Maher et al., 1999; Mistry et al., 2002). The novel, interdigitated microelectrode arrays designed for this study have a total surface area that is 10^4 times larger than that of neurochip electrodes and ten times larger than that of ECIS electrodes (Fig. 1). The overall dimensions, however, are comparably small (5 mm × 5 mm) and can be integrated by chip technology in isolated microfluidic chambers. The high capacitance allows for sensitive impedance measurement that is affected by small resistance variations resulting from the cell-to-electrode contact characteristics.

2. Materials and methods

2.1. Materials

Monoclonal mouse antibody against MAP-2a/b, forskolin, laminin, and polyornithin were from Sigma (St. Louis, MO). Polyclonal rabbit anti-synaptophysin antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Carbachol was from Calbiochem (San Diego, CA). FM1-43, FITC-maleimide, goat anti-mouse IgG Alexa 488-conjugate and goat anti-rabbit IgG Alexa 546-conjugate were from Molecular Probes (Eugene, OR). Cell culture media, FCS, NGF, and FGF-2 were from Life Technologies Inc. (Gaithersburg, MD). All other chemicals were of analytical grade or higher.

2.2. Fabrication and preparation of microelectrode arrays

Microlithographically fabricated chips are interdigitated microsensor electrodes of sputter-deposited platinum or gold, or indium tin oxide on oxide-grown silicon or borosilicate glass. Chips were fabricated from wafers that began as 6 in. × 6 in. glass plates or 6 in. diameter silicon wafers carrying 100 Å of magnetron sputtered titanium/tungsten (Ti/W) and 1000 Å sputter deposited platinum or gold. Electrodes were defined by ion-bean milling through hard-baked photoresist. The photoresist was subsequently stripped and finally, a silicon nitride overlayer was deposited and subsequently plasma etched to create windows that exposed the original region of electrode interdigitation and the bonding pads. Each chip comprised platinum, gold, or ITO lines or digits (10 μm width) separated by similarly dimensioned spaces (5, 10, 15 and 20 μm). Digits were 5 mm long and

50 per bus giving rise to a working area for cell culture that can vary from 2.50 to 9.86 mm^2. The metallic digits were electrochemically platinized from an aqueous solution of chloroplatinic acid to minimize the faradaic component of the overall device interfacial impedance. Each chip had a 20 mm^2 surface area and each well was 3 mm high for a 60 mm^3 volume. Eight chips were assembled into a multi-element array microfluidic chamber and each microfluidic well treated with 3-aminopropyltrimethoxy silane (1% (v/v) in anhydrous ethanol) followed by scrupulous washing with ethanol. The metallic digits of each chip were then cathodically cleaned to remove adsorbed siloxane polymer by repeated cyclic voltametric scanning over the range −1.5 to +0.0 V versus Ag/AgCl, 3 M Cl^-. Wells were further sterilized with 70% (v/v) ethanol for 2 h before coating with laminin or poly-L-ornithin/laminin for differentiation of PC12 or ES-J1 cells, respectively.

2.3. Cell culture and in vitro neuronal differentiation

Rat pheochromocytoma PC12 cells were from the American Type Culture Collection (ATCC CRL-1721) and were grown in medium that was recommended by the supplier. Murine pre-implantation blastocyst embryonic stem cells (ES-J1) were purchased from the Embryonic Stem Cell Core Facility, Medical College of Georgia, Augusta, GA, and cultivated as previously described (Bieberich et al., 2001, 2003). All cells were propagated in a humidified incubator at 37 °C in a humidified atmosphere with 5% CO_2. For in vitro differentiation of PC12 cells, platinum, gold, or ITO—multielectrode arrays (5 mm × 5 mm) were coated with laminin at a concentration of 100 μg/cm^2 (Weeks et al., 1991). Undifferentiated PC12-cells (1×10^4 to 5×10^4 cells) were seeded onto the laminin-coated microelectrode arrays and incubated for 24 h without any medium supplements. Neuronal differentiation was initiated by the addition of 100 ng/ml NGF and incubation for 48 h (Kaplan and Stephens, 1994). Neurite outgrowth and branching was enhanced by a 24 h bolus of 5 μM forskolin, followed by incubation with NGF alone for another 48 h. Pre-implantation blastocyst-derived embryonic stem cells (ES-J1) were grown on γ-irradiated feeder fibroblasts and neuronal differentiation was induced by serum deprivation of embryoid bodies (EBs) as previously described (Bieberich et al., 2001, 2003; Hancock et al., 2000). In brief, serum-starved EBs were trypsinized and plated on poly-L-orinithin/laminin coated ITO-neurochips followed by incubation with 100 ng/ml FGF-2 in N2-supplemented serum-free medium for 4 days. Neuroprogenitors were then fully differentiated by the addition of serum and incubated for another 4 days before fixation with 4% (w/v) paraformaldehyde in phosphate-buffered saline.

2.4. FM1-43 assay and immunofluorescence microscopy

PC12 cells, in vitro differentiated on laminin-coated, ITO interdigitated microelectrode arrays, were incubated for 10 min in medium supplemented with 10 mM KCl and 5 μM FM1-43 (Ryan, 2001). Acetylcholine receptor-mediated depolarization and synaptic vesicle formation was induced by the addition of carbachol at a final concentration of 50 μM (Berkeley and Levey, 2000; Furukawa et al., 1994). Fluorescence was monitored at 480 nm excitation and 550 nm emission with an inverted Nikon TE300 fluorescence microscope. For immunofluorescence staining, differentiated and fixed ES-J1 cells were incubated with 1 μg/ml primary antibody (anti-synaptophysin, anti-MAP-2a/b) and 5 μg/ml Alexa 546 anti-rabbit or Alexa 488 anti-mouse secondary antibody (Bieberich et al., 2001; Becher et al., 1999).

2.5. AC impedance recording and analysis

AC electrical impedance was used as an interrogating technique to probe changes in electrical behavior of the cells pursuant to induced differentiation. AC impedance was monitored following a procedure previously used for ECIS (Wegener et al., 2000). An eight-element array of individual neurochips (device under test, DUT) was assembled and interfaced to the interrogating equipment via a custom interface. The array was maintained within an incubator chamber at 37 °C in a humidified atmosphere with 5% CO_2. Multiplexed data acquisition from each sensor element was performed using a Solartron 1260 Frequency Response Analyzer (FRA) (Slumberger, CT) connected to the eight-element array via a Hewlett-Packard HP 34970A Data Acquisition Interface unit. A precisely controlled interrogating sine wave of 100 mV peek-to-peek voltage (100 mV p-t-p), at a frequency of 4000 Hz, was sequentially applied to each sensory element (DUT). The transfer function yielded real and imaginary components of the impedance.

3. Results

3.1. Electrode shape and material are critical parameters for in vitro neuronal differentiation

Fig. 2 shows the NGF and forskolin-induced differentiation of PC12 cells on interdigitated gold (Fig. 2A), platinum (Fig. 2B and C), or ITO (Fig. 2D) electrodes. The cells first settled within the interdigit spaces at high density (>20 cells/100 μm electrode length), but then attached in between two electrode lines (alternating cathode and anode) when platinum was used (Fig. 2C), whereas in the case of gold and ITO, cells predominantly attached on top of the electrode surface (Fig. 2B and D). Accordingly, networking of differentiated cells on platinum microarrays appeared in the form of linear cables, whereas cells on gold and ITO electrodes formed a branched network. Most strikingly, neurite outgrowth on these electrode surfaces was significantly enhanced over that of PC12 cells grown on tissue culture dishes. As shown in Fig. 2D, the length of neurite processes (200–1000 μm) was up to 100-fold the diameter of the cell

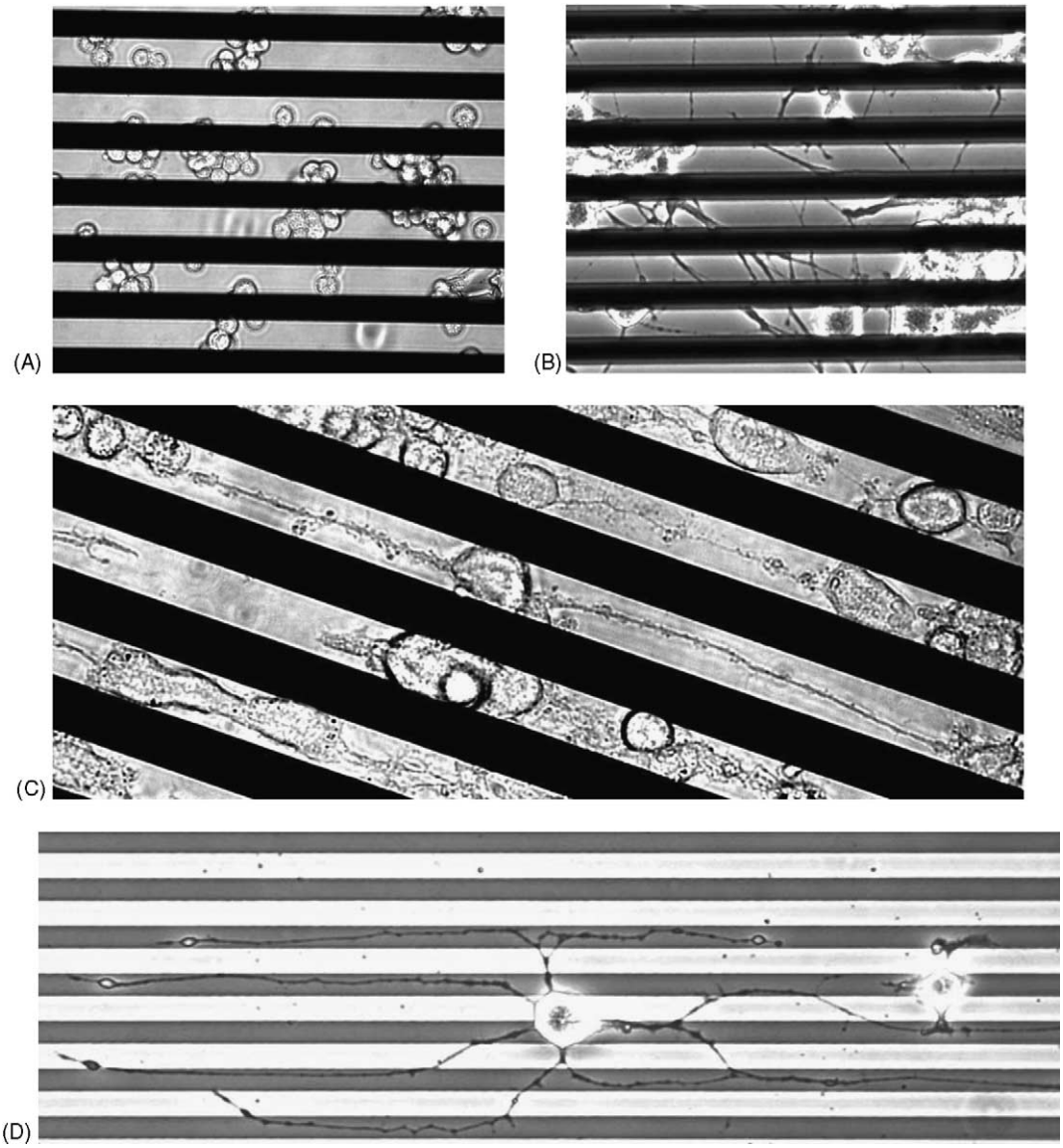

Fig. 2. In vitro differentiation of PC12 on gold, platinum, or ITO neurochips. (A and B) Gold electrodes; (A) undifferentiated; (B) differentiated PC12 cells; (C) platinum electrode; (D) ITO electrode.

body. On ITO electrodes, the growth path was strictly confined to the electrode surface.

3.2. In vitro differentiated PC12 and ES cells form neuron-to-electrode synapses

PC12 cells, when differentiated on ITO electrodes, developed neuron-to-electrode junctions with morphological characteristics typical of growth cones (Fig. 2D). As shown in Fig 3A, DIC microscopy demonstrated that the processes and growth cones extended numerous filipodia. Fluorescence labeling of membrane protein with FITC-maleimide indicated that growth cones were attached to the electrode surface (Fig. 3B and C). The synaptic function of these neuron-to-electrode contacts was analyzed by the ability for uptake of FM1-43, a fluorescent marker for synaptic vesicle formation upon receptor stimulation. Fig. 4 shows that carbachol, an agonist for cholinergic receptors, stimulated uptake of FM1-43 concurrent with swelling of growth cones immediately (5–10 min) after the addition of the drug to the medium. A potential neuron-to-electrode synapse formation was also analyzed with murine ES cells that were in vitro differentiated on interdigitated ITO microelectrode arrays using a serum deprivation/FGF-2 differentiation protocol. As shown in Fig. 5A and B, neuroprogenitor cells settled preferably on the electrode surface and extended processes 2 days after incubation in FGF-2-supplemented medium (Fig. 5C). Fig. 5D shows fully differentiated neurons at day 7 post seeding on the ITO microelectrode arrays. Neuronal differentiation was verified by immunostaining with an antibody

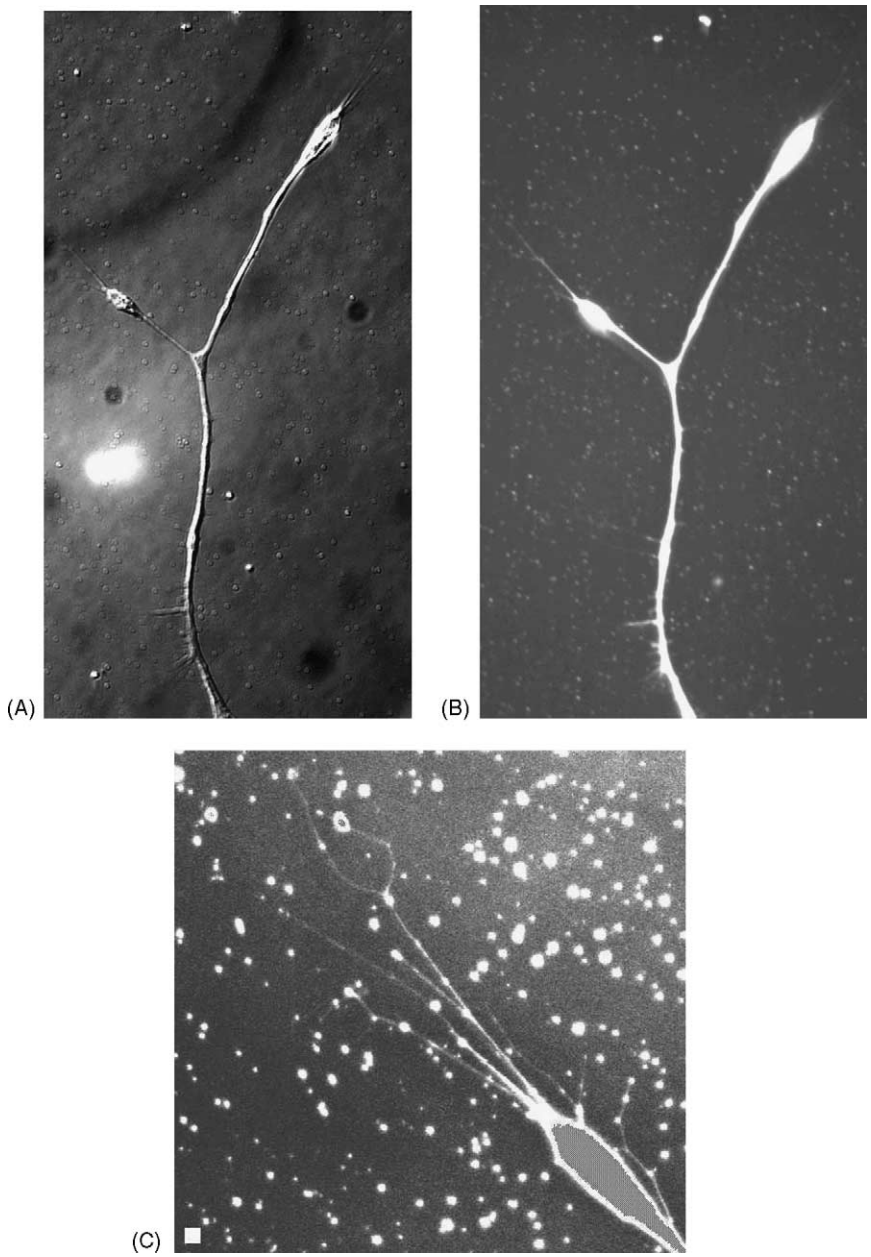

Fig. 3. PC12 growth cones on ITO neurochip. (A) Differential contrast (DIC) microscopy; (B) FITC-maleimide labeling of cell surface proteins; (C) confocal laser fluorescence microscopy of growth cone shown in B.

against MAP-2 (green fluorescence) and the synaptic vesicle protein synaptophysin (red fluorescence, Fig. 5E). Synaptophysin immunostaining was limited to the junctions of neuronal processes with the ITO electrode surface.

3.3. AC impedance of in vitro differentiated PC12 cells on interdigitated platinum microelectrode arrays

The electrical impedance of PC12 cells was monitored on interdigitated platinum microelectrode arrays. Changes in impedance of the array were due to the attachment of cells to the electrodes or within the interdigit spaces (10 μm width). About 2–5 cells/100 μm were in vitro differentiated by incubation with NGF and forskolin and AC impedance recorded for 12 h at 1 kHz and 50 mV. As shown in Fig. 6, peak impedance magnitude monitored with PC12 cells was lower (117.5 Ω) than with medium alone (121.0 Ω). The difference of about 4 Ω was concomitant with attachment and differentiation of about 5×10^4 PC12 cells that settled in the interdigit spaces between two electrode lines. The impedance increased sharply in the initial recording phase and dropped slightly during measurement due to temperature and gas equilibration processes within the CO_2-incubator after closing.

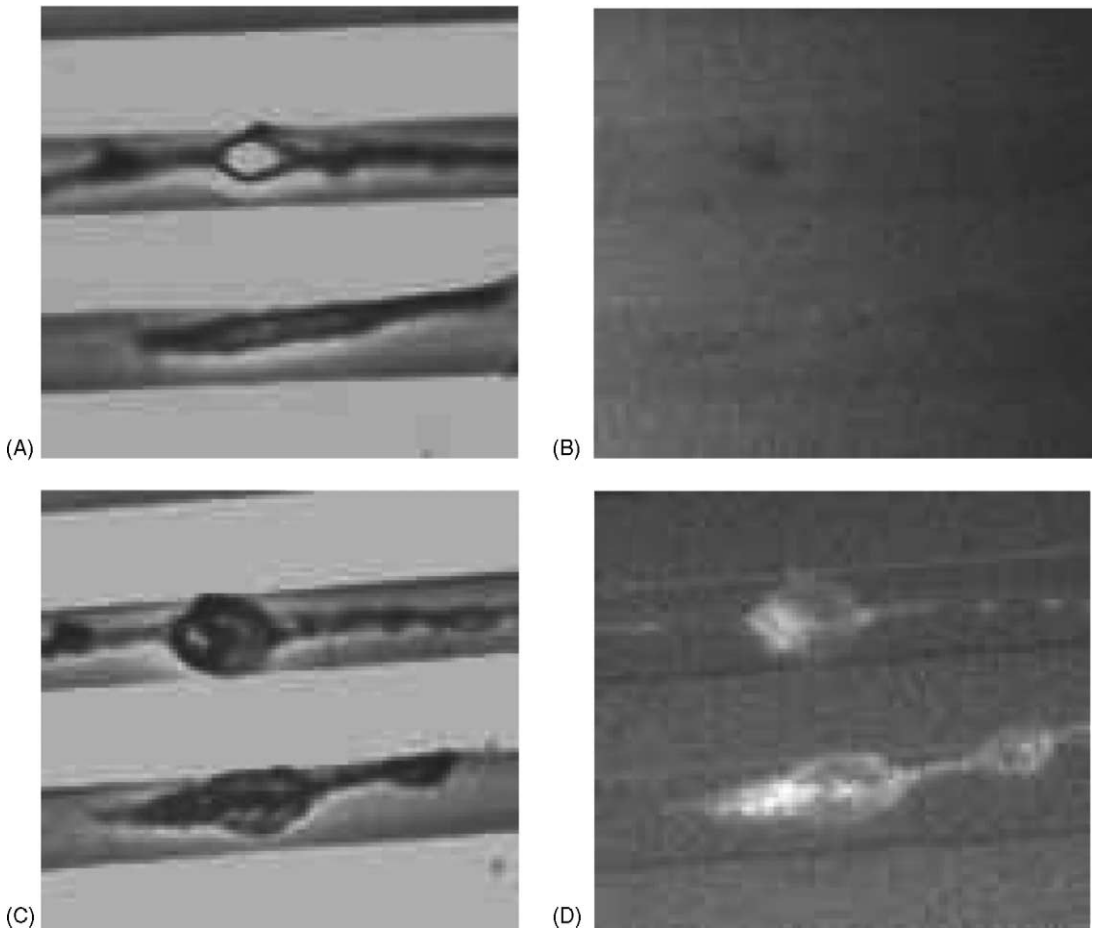

Fig. 4. Carbachol-stimulated FM1-43 uptake of PC12 growth cones on ITO neurochips. (A and B) Growth cones before the addition of carbachol; (A) phase contrast; (B) fluorescence; (C and D) growth cones 10 min after the addition of carbachol; (C) phase contrast; (D) fluorescence.

4. Discussion

The rapid establishment of protocols for in vitro neuronal differentiation of embryonic stem cells has opened exciting perspectives for engineering of neuron-to-electrode interfaces. The challenge, however, is given by the design of appropriate microelectrode arrays that accommodate the requirement of tight neuron-to-electrode contact structures and thus optimized surface characteristics with respect to electrode material and shape. In this study, we have analyzed the differentiation and growth behavior of two neuronal cell lines, rat pheochromocytoma PC12 and pre-implantation blastocyst-derived murine embryonic stem (ES-J1) cells, on interdigitated platinum, gold, or ITO microelectrode arrays. Interdigitated electrode arrays have a large surface area, which is the prerequisite for sensitive spectral impedance analysis and the formation of numerous, tight neuron-to-electrode contact structures. The co-planar configuration of the cathode and anode and the electric field (Guiseppi-Elie, unpublished data) offers the potential for impedance measurements to be highly sensitive to the neuronal material that is proximal to the device surface, i.e. on the electrode digits or within the interdigit space of the electrode.

Depending on the choice of electrode material or cell type these contact structures are established with the cell body and/or growth cones that are formed at the terminal extension of neuronal processes. PC12 cells extended neuronal processes whose lengths (200–1000 μm) were up to 100-fold of the diameter of the cell body. These processes were at least 10 times longer than those typically formed on a standard tissue dish (Parmar et al., 2002), indicating that the material and design of the microelectrode promotes the requisite properties of PC12 cell differentiation. Most likely, the extraordinary length of the processes results from intensive neurite outgrowth that is strictly limited to the surface of the electrode lines. To our knowledge, this is the first report on the exquisite neurite outgrowth characteristics of differentiating PC12 cells on microelectrodes. Previous studies have reported the in vitro differentiation of PC12 cells on ITO electrodes, but have not been able to improve neuronal process formation (Kimura et al., 1998). This improved process formation, however, is the prerequisite for extended, branched networking and the generation of ample

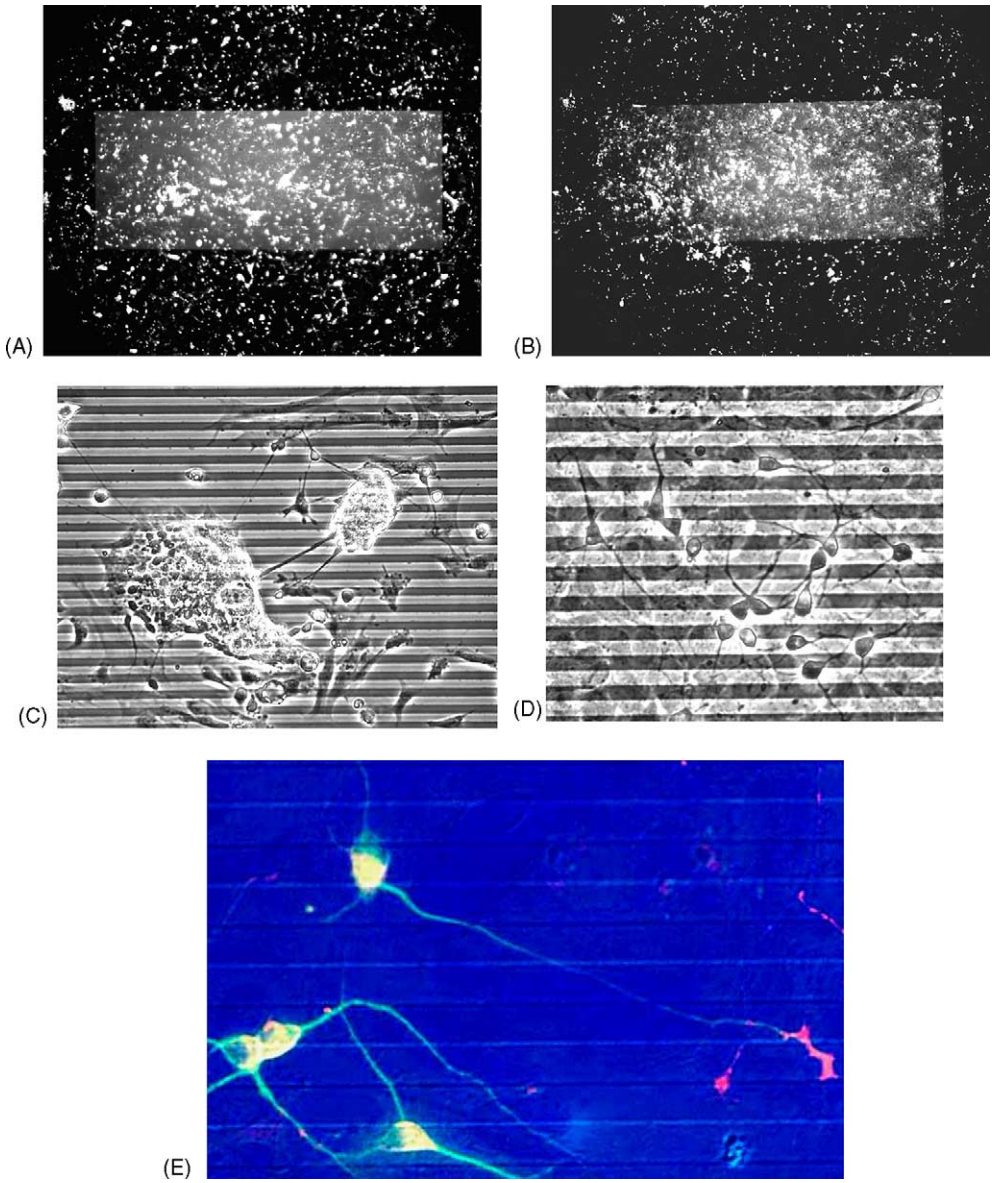

Fig. 5. Neuronal in vitro differentiation of embryonic stem cells on ITO neurochip. (A and B) Overview showing enrichment of differentiating ES cells on electrode surface; (A) day 2 post seeding; (B) day 7 post seeding; (C and D) higher maginification; (C) day 2 post seeding; (D) day 7 post seeding; (E) immunostaining of MAP-2 (green fluorescence) and synaptophysin (red fluorescence) at day 7 post seeding. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

neuron-to-electrode contact structures. It is not clear to us what specific characteristics of the conductor surfaces favor such extensive and directed neurite outgrowth or the favorable association of these outgrowths processes with ITO. However, it is apparent that while the other conductors are metallic, ITO is a semi-conductor that is rich in oxide surface chemistry and has a different work function than the metals (Pt = 5.6; Au = 5.1; ITO = 5.0) (Bruner et al., 2002).

We have analyzed the structure and function of neuron-to-electrode contact structures formed by differentiated PC12 and ES cells on ITO microelectrode arrays. The intensive pili formation indicates that the neurite processes and growth cones are anchored to the ITO surface and have established tight contact structures with the electrodes. The functionality of these contact structures has been demonstrated by carbachol-induced uptake of the fluorescent dye FM1-43 and translocation of synaptophysin, both are markers for synaptic vesicle formation (Becher et al., 1999; Ryan, 2001). Hence, the contact structures reveal characteristics of functional synapses, a prerequisite for neuron-to-electrode synaptic transmission.

We have analyzed the impedance characteristics of PC12 cells that were in vitro differentiated on interdigitated platinum microelectrode arrays. The formation of cable-like chains of interconnected cells within the interdigit spaces increases the contact surface area with the electrode lines

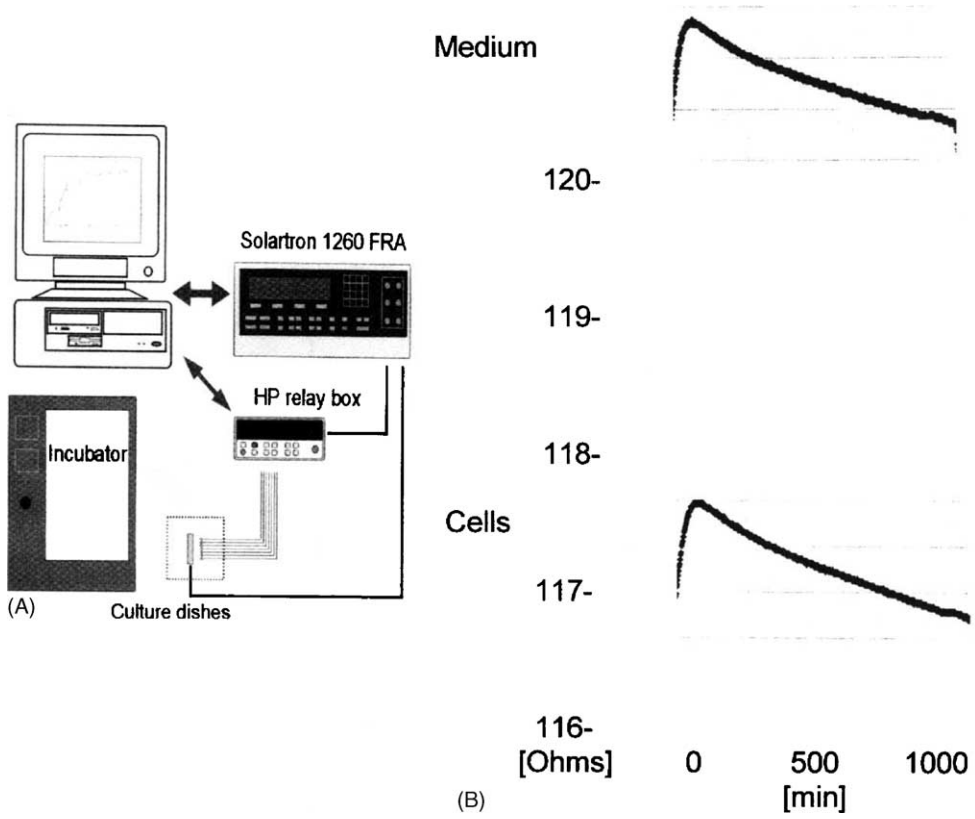

Fig. 6. AC impedance recording with PC12 cells on ITO neurochip. (A) Assembly for interrogation and recording; (B) AC impedance (1000 Hz, 50 mV) of in vitro differentiated PC12 cells. The initial peak is due to sample handling and re-equilibration of the medium to the CO_2-atmosphere in the incubator.

on both sides of each cell body. A large contact surface area between neurons and electrodes is significant because of the inverse correlation between impedance and electrode surface area. The electrical impedance between the culture medium and a noble metal electrode depends upon the electrified interface and the ion flux at the electrode surface (Maher et al., 1999). The attachment of cells to the electrodes reduces the effective electrode surface area that is accessible to the medium and thus increases the apparent impedance. This principle is well known from "Electric Cell-Substrate Impedance Sensing (ECIS)" that uses disc-shaped gold electrodes covered by a cell layer (Lacy et al., 1996; Lo et al., 1999; Sharma et al., 2001; Wegener et al., 2000). The impedance monitored with ECIS is thus inversely correlated to the extent of tight cell-to-cell contact structures. In contrast, the linear architecture of interdigitated microelectrode arrays does not result in tight cell-to-cell contact, but rather tight neuron-to-electrode contact structures. The impedance analysis of platinum arrays with PC12 cells differentiating within the interdigit spaces surprisingly resulted in a slightly lower impedance than monitored with cell-free medium as control. The reason for this observation that is inverse to the expectation as deduced from ECIS analyses, is still unknown. However, it should be noted that in contrast to conventional ECIS and previous microelectrode design, the dimension of the interdigit spaces of our microelectrodes allows most of the cells to establish a contact with both electrodes at the same time. Hence, it is very likely that the lower impedance may have been contributed by the tight neuron-to-electrode contact structures on both sides of the cell (Fig. 2C). This is now the subject of intense research within our group.

The integration of neuronal tissue and microelectrodes is a field of great medical promise. NEST can be helpful in transmitting electrical signals to prosthetic devices or for electrical stimulation of damaged or malfunctional brain areas. Electrical stimulation by implanted electrodes has been shown to significantly improve the condition of Parkinson patients (Olanow et al., 2000). The formation of synaptic contact structures with electrodes as part of neuronal differentiation takes this field to unforeseen possibilities. As we have demonstrated, differentiating embryonic stem cells actively contact the electrode surface with typical synaptic growth cones. This indicates a potential use of co-implants consisting of microelectrode arrays and embryonic stem cells for neuronal tissue repair. The electrodes can be hooked up to pacemakers that electrically stimulate re-growing neuronal tissue in severed nerves or even damaged brain areas. In contrast to the existing technology, the electrical transmission will be mediated by synaptic neuron-to-electrode contact structures that have been formed as part of a natural differentiation process. We will now further analyze the

effect of different electrode designs and neuronal differentiation protocols on the formation of hybrid synapses between neurons and microelectrodes. One such electrode format is the independently addressable, interdigitated electrode array. This format allows the measurement of cell impedances established between pairs of adjacent digits. We will also analyze the synaptic transmission process and the effect of membrane ion flux on the impedance characteristics of these hybrid structures. In particular, we will determine the biochemical mechanism that underlies the electrode-dependent enhancement of in vitro neuronal differentiation. A marriage of neurochips and in vitro differentiated embryonic stem cells is expected to result in novel and exciting medical applications in the field of neuronal tissue repair and biosensor technology.

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