Electrophysiological and Morphological Characterization of Rat Embryonic Motoneurons in a Defined System

Mainak Das, Peter Molnar, Halagowder Devaraj, Matthew Poeta, and James J. Hickman*

Department of Bioengineering, Clemson University, 420 Rhodes Hall, Clemson, South Carolina 29634-0905

In an attempt to integrate biological components with silicon-based devices and systems, artificial silane surfaces have been successfully used to grow motoneurons in a defined environment. In this study we characterized the morphology and electrophysiology of purified rat embryonic (E14) motoneurons grown on a self-assembled monolayer (SAM) of N-1[3-(trimethoxysilyl)propyl]diethylenetriamine (DETA) versus that on ornithine/laminin surfaces in serum-free media. On DETA motoneurons were flat and grew more processes, whereas on ornithine/laminin they tended to aggregate. The membrane time constant, a characteristic associated with electrotonic compactness, was significantly longer for motoneurons grown on DETA. Other electrophysiological parameters were similar for the motoneurons on the different surfaces. This is the first study where purified ventral horn motoneurons were cultured in a completely defined (nonbiological surface, serum-free) environment.

Introduction

The integration of living cells with silicon-based systems is essential for the frontier areas of bioengineering, biosensor development, prosthetics, high throughput drugtoxin screening, neurocomputing, and biorobotics (1–3). An enabling factor for research into these topics is to create in vitro systems where a majority of the variables are known or defined. A representative system of the type of in vitro model that would have a tremendous impact if developed is the reflex arc, one of the most fundamental circuits for motor control. The recreation of this system in a defined in vitro environment will allow researchers to address a number of the above topics and also to gain a more fundamental understanding of the basic neuroscience of this complex system.

The first step to create an in vitro system composed of all of the elements of the reflex arc integrated with silicon-based systems (i.e., microelectromechanical systems, or MEMS, devices) involves the modification of the surface of the silicon to enable the development and the long-term survival of cells in a defined hybrid environment. In addition, for many cases biological variability in the components of the culture media are not desirable for the above applications and the neurons need to be cultured in a completely defined media that approximates cerebral spinal fluid (CSF) (4). We have experimentally established a system where all of the major neuronal culture parameters are known and quantifiable (5), which consists of a reproducible dissociated culture methodology, serum-free media, and a surface composed of self-assembled monolayers (SAMs) (4).

Motoneurons are one of the most extensively characterized neuronal cell types (6–8). Recently, much attention has been focused on motoneuron physiology driven by the partial success in the repair of spinal cord injuries (9). With the development of tissue engineered nerve constructs (3), the study of the interaction of motoneurons with engineered surfaces has become crucial for their successful application. However, there is little reported effort to create a defined in vitro system for studying isolated motoneurons or motoneurons in conjunction with other components of the reflex arc, except for rudimentary systems such as Campenot chambers (10). It is important to coculture the motoneurons with their targets because, without the appropriate intracellular and extracellular signals, motoneurons do not survive for significant periods of time (9). Motoneurons can be maintained in serum-free culture media with the addition of several growth factors (8, 11), but in most of the published methods motoneurons are initially plated in serum-containing media, where serum is an undefined component and not reproducible. Thus, this research is important because, although the electrophysiological characteristics of developing motoneurons have been studied extensively (6, 7, 12), no reports of their morphological or electrophysiological properties in a defined, reproducible environment have been reported.

The use of SAMs for chemical surface modification enables that surface to be equipped with a variety of functional groups that possess required specific electrical, optical, or chemical properties (13). SAMs have already been used for studying cell-surface interactions (4), the control of protein adsorption (14), prosthetic device biocompatibility (15), and most importantly for recreating a Campenot-like system, cell patterning (16). In addition to the above reasons, serum-containing culture media should not be used in chemical patterning of neuronal networks because proteins deposited from serum can cover the original chemical pattern on the substrate (17). Previously, a self-assembled monolayer of N-1[3-(trimethoxysilyl)propyl]diethylenetriamine (DETA) was suc-
cessfully used to pattern (5, 18) and determine polarity (16) of hippocampal pyramidal cells.

In this study, which is the first step in creating an in vitro reflex arc, we demonstrate that purified rat embryonic motoneurons could be cultured in a defined environment (serum-free) on a synthetic surface (DETA), and we report their electrophysiological characterization compared to a more biologically based system (ornithine/laminin). The passive membrane properties of the motoneurons were determined because they are the major determinants of the summation of synaptic inputs (6, 19). The development of the specific ionic conductances on the soma are also necessary for sustained repetitive firing of action potentials and ultimately muscle action (20).

Materials and Methods

Surface Modification. DETA. Glass coverslips were cleaned using HCl/methanol (1:1), soaked in concentrated H2SO4 for 30 min, and then rinsed in double distilled H2O. Coverslips were next boiled in deionized water, rinsed with acetone, and then oven dried. The DETA film was formed by reaction of cleaned surfaces with a 0.1% (v/v) mixture of the organosilane in toluene. The DETA cover glasses were heated to just below the boiling point of toluene, rinsed with toluene, reheated to just below the boiling temperature, and then oven dried.

Surface Characterization. Surfaces were characterized by static contact angle measurements using a Cam 200 contact angle goniometer (KSV) utilizing standard protocols and X-ray photoelectron spectroscopy (XPS). XPS is a technique for the characterization of the top 50–100 Å of a surface that identifies elements present in the surface layer, their oxidation states, and their relative amounts. For XPS analysis, the Kratos 165 XPS instrument was used according to a published protocol (21).

Ornithine/Laminin. Stock solution of polyornithine (Sigma, 1.5 mg/mL /1000 × /) was prepared in water, whereas the stock solution of laminin (natural mouse, Invitrogen, CA, 1.5 mg/mL /500 × /) was prepared in phosphate-buffered saline (PBS) (Invitrogen). Coverslips (Thomas Scientific, Swedesboro, N.J.) cleaned by acid treatment were incubated with polyornithine (1:1000 dilution) from stock in water overnight at room temperature. Coverslips were dried in the laminar flow hood. Laminin was dissolved in the culture medium at a dilution of 1:500. Ornithine-coated coverslips were placed in 35 mm cell culture dishes, and then 3 mL of the laminin solution was added and incubated in a CO2 incubator overnight at 37°C (8).

Rat Embryonic Motoneuron Culture. Rat spinal motoneurons were purified from ventral cords of embryonic day 14 (E14) embryos as described by Henderson et al. (8). Briefly, rats were anaesthetized and killed by inhalation of an excess of CO2. This procedure was in agreement with the Animal Research Council of Clemson University, which adheres to IACUC policies. Ventral spinal cells from the embryo were dissociated after trypsin (Invitrogen, 0.05%) treatment and centrifuged for 15 min at 500g over a 6.5% metrizamide cushion (22). The large cells remaining above the cushion were further selected using the immune interaction between motoneurons and the 192 antibody (1:2 dilution, ICN Biomedicals, Akron, OH) coated on the dishes (8). The antibody recognizes the low affinity NGF receptor expressed only by ventral motoneurons at this age (23).

Purified motoneurons were plated on 22 × 22 mm2 ornithine/laminin-coated coverslips and DETA-coated coverslips at a density of 1.5–2.0 × 103 cells/mm2. The culture medium was Neurobasal (Gibco-BRL) supplemented with B27 (2% v/v; Invitrogen), L-glutamine (0.5 mM), and 2-mercaptoethanol (25 μM). Three growth-promoting factors were added: glial cell line-derived neurotrophic factor (100 pg mL−1 GDNF; Invitrogen), brain-derived neurotrophic factor (100 pg mL−1 BDNF; Invitrogen), and ciliary neurotrophic factor (1 ng mL−1 CNTF; Cell Sciences) (24). The culture medium was changed every 5 days and L-glutamate (25 μM) was added to the culture medium during the first 5 days of growth. Motoneurons were maintained in vitro for 14 days.

Identification of Motoneurons by Islet-1 Immunostaining and Live-Dead Assay. The purity of the motoneurons was verified using Islet-1 immunostaining. Briefly, purified motoneurons on coverslips were fixed using 4% paraformaldehyde in PBS plus 0.1% glutaraldehyde at 4°C for 15 min. Quenching of excess aldehyde groups and permeabilization were performed with 50 mM lysine plus 0.1% Triton X-100 for 15 min at room temperature. Nonspecific staining was blocked using 2% BSA and 2% goat serum in PBS. Anti-Islet antibody 4D5 (Developmental Studies Hybridoma Bank, University of Iowa) was used at a dilution of 1:200. The secondary antiserum antibody was coupled to Cy3 (Jackson labs). Molecular Probe’s L-3224 Live/Dead Assay kit was used for the live-dead assays.

Morphological Analysis. Phase-contrast pictures were taken with a commercial Nikon Coolpix 990 camera using the 40× objective of a Zeiss Axiovert S100 microscope. Pictures were enhanced in Photoshop (Adobe) and analyzed using Scion Image Software (Scion Corp., Maryland). The long and the short axes of the soma as well as the number of processes originating in the soma were measured.

Electrophysiology. Whole-cell patch clamp recordings were performed in a recording chamber on the stage of a Zeiss Axiovert 2 FS Plus upright microscope. The chamber was continuously perfused (2 mL/min) with the extracellular solution (Neurobasal culture medium; pH was adjusted to 7.3 with HEPES, 35°C). Patch pipettes were prepared from borosilicate glass (BF150-86-10; Sutter, Novato, CA) with a Sutter P97 pipet puller and filled with intracellular solution (in mM: K-glutamate 140, EGTA 1, MgCl2 2, NaATP 2, phosphokreatine 5, phosphokreatine kinase 2.4 mg, Hepes 10; pH = 7.2). The resistance of the electrodes was 6–8 MΩ. Voltage clamp and current clamp experiments were performed with a Multiclamp 700A amplifier (Axon, Union City, CA). Signals were filtered at 2 kHz and digitized at 20 kHz with an Axon Digidata 1322A interface. Data recording and analysis were performed with pClamp 8 software (Axon). Membrane potential was corrected by subtraction of a 15 mV tip potential, which was calculated using Axon’s pClamp 8 program. The membrane resistance and capacitance were calculated using a 50 ms voltage step from −85 to −95 mV without any whole-cell or series resistance compensation. Sodium and potassium currents were measured in voltage clamp mode using voltage steps from a −85 mV holding potential. Whole cell capacitance and series resistance was compensated using a p/6 protocol. Only cells with access resistance less than 22 MΩ were analyzed. The membrane time constant was determined using a 1 ms −0.8 nA current impulse in current clamp mode. Six traces were averaged and 3 exponentials were fitted to the averaged trace using the pClamp program. The electrotonic length of neurons was calculated using the ratio of the 0th and 1st order time
constants \(L = \pi ((r_1/r_2) - 1)^{1/2}\) (25). Repetitive firing, firing thresholds, and after-hyperpolarization were measured with 1 s depolarizing current injections from a \(-85\) mV holding potential.

**Statistical Analysis.** A two-sample t test was performed for the statistical analysis of morphological and electrophysiological data. Parameters obtained from DETA-plated neurons were compared with the ornithine/laminin controls.

**Results**

**Surface Modification.** The surface modification controls were tested by contact angle and X-ray photoelectron spectroscopy (XPS). The contact angle for DETA covered coverslips was \(40.62 \pm 2.92^\circ\) (mean ± SD), which had been shown previously to be acceptable (4). XPS indicated that the glass surface was modified by a complete layer of DETA (characterized by the 399 eV N peak) (4).

**Survival of Motoneurons in Defined Environment.** Purified rat embryonic motoneurons, plated either on DETA or ornithine/laminin surfaces (Figure 1), survived at least 12 days in serum-free Neurobasal culture media. The initial number of attached cells was significantly higher on DETA than on ornithine/laminin, but the number of surviving cells decreased more rapidly on DETA during the first 4 days (Figure 2). Islet-1 immunostaining was performed after day 6 in culture. More than 90% of the purified motoneurons were positive to Islet-1.

**Effect of DETA Surface on the Morphology of Motoneurons.** The morphology of motoneurons on DETA and on ornithine/laminin was different (Figures 1 and 2, Table 1). On DETA the motoneurons were flat, based on phase contrast microscopy observation, and sent out more processes, whereas on ornithine/laminin they tended to aggregate after day 10 in culture. The number of processes originating from the soma was significantly higher with neurons plated on DETA than with neurons plated on ornithine/laminin (Figure 2, Table 1). There was no significant difference in the size of the cells on the two surfaces. The diameter of the cells (long axis in Table 1) increased as the cells matured in the culture system (Table 1).

**Effect of Surface on Electrophysiological Characteristics of the Motoneurons.** The resting membrane potential of the motoneurons became more negative with time and stabilized by day 6 (Table 2). The amplitudes of the voltage-dependent sodium and potassium currents also increased with time, and the neurons became capable of sustained repetitive firing after ap-

**Table 1. Summary of Morphological Parameters of Motoneurons Grown on DETA and Ornithine/Laminin Surfaces**

<table>
<thead>
<tr>
<th>Day</th>
<th>Long Axis (μm)</th>
<th>SD</th>
<th>O/L</th>
<th>SD</th>
<th>Short Axis (μm)</th>
<th>SD</th>
<th>O/L</th>
<th>SD</th>
<th>Num. Proc.</th>
<th>SD</th>
<th>O/L</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>17.13</td>
<td>2.90</td>
<td>18.40</td>
<td>3.83</td>
<td>9.98</td>
<td>1.87</td>
<td>10.68</td>
<td>2.95</td>
<td>9.10</td>
<td>2.66</td>
<td>4.50</td>
<td>2.04</td>
</tr>
<tr>
<td>6</td>
<td>19.25</td>
<td>3.53</td>
<td>20.91</td>
<td>4.38</td>
<td>12.47</td>
<td>2.31</td>
<td>13.57</td>
<td>3.57</td>
<td>8.48</td>
<td>3.19</td>
<td>4.44</td>
<td>1.54</td>
</tr>
<tr>
<td>9</td>
<td>21.40</td>
<td>4.76</td>
<td>22.72</td>
<td>4.54</td>
<td>12.28</td>
<td>1.76</td>
<td>14.30</td>
<td>2.48</td>
<td>10.13</td>
<td>3.54</td>
<td>7.20</td>
<td>2.59</td>
</tr>
<tr>
<td>12</td>
<td>24.69</td>
<td>4.37</td>
<td>25.60</td>
<td>5.84</td>
<td>14.27</td>
<td>2.94</td>
<td>12.36</td>
<td>2.76</td>
<td>10.25</td>
<td>2.79</td>
<td>5.77</td>
<td>2.20</td>
</tr>
</tbody>
</table>

\(P < 0.005\), two-sample Student’s t test.
proximately 6 days (Figure 3). There was no significant difference between the passive or active membrane properties of motoneurons plated on DETA or ornithine/laminin surfaces except for the membrane time constant, which was longer for neurons grown on DETA (Figure 3). This difference was observable for each time period checked. The calculated electrotonic length of motoneurons was shorter for the DETA surface than for ornithine/laminin. On the basis of firing properties of the neurons they were classified into 4 "firing type" categories: category 0, no action potential; category 1, or 2 action potentials; category 3, repetitive firing but duration of the firing is less than 1 s; category 4, continuous repetitive firing. The maximum firing frequency of the matured motoneurons was 18 Hz, observed during the first 1 s measurement period.

**Discussion**

In this study purified rat embryonic motoneurons were cultured on a nonbiological surface, DETA, and on a standard biological surface, ornithine/laminin, in a completely serum-free environment. Immunostaining with Islet-1 was over 90% positive for motoneurons in both cases. The motoneurons survived and matured during a 12-day study period on both surfaces and were comparable to previous experiments with the control surface (11). The morphology and electrophysiology of motoneurons on DETA and ornithine/laminin were different; however, the only electrophysiological characteristic that differed significantly between the two surfaces was the membrane time constant.

The development of purified embryonic motoneurons on the biological surface (ornithine/laminin) was very similar to that described previously (7). Despite the general similarity of the results on both surfaces, however, there were some differences noted. The differences observed included the total number of neurons adhering to the surface after initial plating, the growth dynamics during the first 4 days following plating, the neuronal geometry, and the membrane time constant. Possible explanations for the higher initial cell number on the DETA surface is that cell attachment to DETA is much stronger than to ornithine/laminin or this combination of factors provides a more favorable initial environment for the neurons (4, 5, 16, 21, 26–28). On DETA, motoneurons were flat and grew more processes versus the biological surface, and in the older cultures (9–12 days) the motoneurons tended to aggregate on ornithine/laminin but not on DETA, as noted by qualitative visual observations. As noted above, a possible explanation for this is that the attachment of neurons to DETA is much stronger than to ornithine/laminin. Also, on ornithine/laminin, it is possible that the migration of the cells is facilitated. This is an area of continuing research. Another possible explanation might be that the laminin present in the substrate activated cell surface receptors (i.e., integrins), which then influenced the morphology of the motoneurons. This specific signaling could be missing or there could be different receptors activated in the case of DETA.

It is important to determine if embryonic motoneurons maintained in cell culture go through the proper developmental stages and become functional. During the maturation process for neurons, the membrane potential becomes more negative until it reaches a stable value of about −45 mV. In addition, during normal development, the amplitude of voltage-sensitive sodium and potassium currents increases, thus enabling the repetitive firing properties of the motoneurons. Our data are in good agreement with the published results for the emergence of the normal electrophysiology of the embryonic motoneurons (7, 25). The only observed difference in the membrane time constant, could be related to the altered morphology of the motoneurons on the DETA surface. The electrotonic length is a major parameter used in compartmental models of motoneurons, and it is highly dependent on the morphology of the neuron (29). The calculated electrotonic length was longer on ornithine/laminin than on DETA, indicating a possible difference in the physiology of the motoneurons, and this could be important because the electrotonic length is a parameter involved in the description of the summation of

**Table 2. Passive and Active Membrane Properties of Embryonic Motoneurons on DETA and on Ornithine/Laminin Surfaces**

<table>
<thead>
<tr>
<th></th>
<th>day 3</th>
<th>day 6</th>
<th>day 9</th>
<th>day 12</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DETA STD OL</td>
<td>DETA STD OL</td>
<td>DETA STD OL</td>
<td>DETA STD OL</td>
</tr>
<tr>
<td>VM (mV)</td>
<td>−39.5 ± 5.2</td>
<td>−37.5 ± 7.0</td>
<td>−41.8 ± 6.8</td>
<td>−45.7 ± 6.2</td>
</tr>
<tr>
<td>RN (MΩ)</td>
<td>1233 ± 810</td>
<td>947 ± 414</td>
<td>583 ± 275</td>
<td>475 ± 108</td>
</tr>
<tr>
<td>CM (pF)</td>
<td>17.7 ± 10.7</td>
<td>17.4 ± 9.0</td>
<td>18.4 ± 11.7</td>
<td>12.8 ± 5.0</td>
</tr>
<tr>
<td>L ms⁻¹</td>
<td>1.97 ± 0.47</td>
<td>2.29 ± 0.37</td>
<td>1.43 ± 0.42</td>
<td>1.96 ± 0.39</td>
</tr>
<tr>
<td>tᵣ (ms)</td>
<td>1.96 ± 0.57</td>
<td>2.47 ± 0.32</td>
<td>2.37 ± 0.16</td>
<td>2.23 ± 0.18</td>
</tr>
<tr>
<td>tᵥᵣ (ms)</td>
<td>0.46 ± 0.22</td>
<td>0.54 ± 0.30</td>
<td>0.83 ± 0.30</td>
<td>1.18 ± 0.33</td>
</tr>
<tr>
<td>Na current (pA)</td>
<td>−1520 ± 930</td>
<td>−1243 ± 651</td>
<td>−2640 ± 1172</td>
<td>−2929 ± 1588</td>
</tr>
<tr>
<td>K current (pA)</td>
<td>1229 ± 714</td>
<td>1416 ± 749</td>
<td>2596 ± 1030</td>
<td>3833 ± 2340</td>
</tr>
<tr>
<td>t₀ (ms)</td>
<td>56.37 ± 15.2</td>
<td>31.0 ± 20.7</td>
<td>9.0 ± 54.0</td>
<td>2</td>
</tr>
<tr>
<td>AHP Ampl. (mV)</td>
<td>89.4 ± 53.7</td>
<td>53.8 ± 45.7</td>
<td>69.1 ± 16.9</td>
<td>34.3 ± 10.9</td>
</tr>
<tr>
<td>AP duration (ms)</td>
<td>7.99 ± 2.86</td>
<td>6.69 ± 2.87</td>
<td>3.67 ± 1.79</td>
<td>2.85 ± 1.51</td>
</tr>
<tr>
<td>AP duration (ms)</td>
<td>1520 ± 930</td>
<td>45.7 ± 6.2</td>
<td>57.3 ± 5.0</td>
<td>46.1 ± 5.1</td>
</tr>
<tr>
<td>n</td>
<td>10 ± 9</td>
<td>10 ± 9</td>
<td>10 ± 9</td>
<td>10 ± 9</td>
</tr>
</tbody>
</table>

- Electrophysiological parameters were measured using conventional voltage clamp and current clamp protocols during whole cell patch-clamp recordings from motoneurons growing on DETA and ornithine/laminin surfaces. VM: resting membrane potential. RN: input resistance. CM: membrane capacitance. L: electrotonic length. t₀: zero-order membrane time constant. Vᵥᵣ: longest membrane time constant in voltage clamp mode. Vₐᵥᵣ: action potential threshold voltage. Iₚᵥᵣ: action potential threshold current. Firing type: empirical classification of neurons (0-4) based on their repetitive firing properties. Significance difference (two-sample Student's t-test) compared to ornithine/laminin surface.
incoming synaptic inputs. This difference could be related to different receptors being activated on the different surfaces and will be one focus of the continuation of these investigations. However, these minor variations do not appear to be a hindrance in using this defined system to study the properties of developing motoneurons or the creation of a new in vitro system to study the segments of the reflex arc.

**Conclusion**

This is the first study where purified motoneurons were cultured and morphologically and electrophysiologically characterized on an artificial surface in a serum-free environment. In future experiments these artificial surfaces will be used to pattern immunopurified embryonic motoneurons, as seen with hippocampal neurons (5, 30), and to integrate them with silicon-based hybrid constructs to create the first segment of the reflex arc: motoneuron to muscle. Moreover, in this defined system the substrate composition can be systematically varied to study the contact signaling pathways influencing motoneuron survival, differentiation, and regeneration.

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**References and Notes**


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