33rd Annual International Conference of the IEEE EMBS
Boston, Massachusetts USA, August 30 - September 3, 2011

Autonomic Nervous System Driven Cardiomyocytes *in vitro*

Akimasa Takeuchi, *Student Member, IEEE,* Masahide Mori, Kana Kitagawa, Kenta Shimba, Yuzo Takayama, *Member, IEEE,* Hiroyuki Moriguchi, Keiko Miwa, Kiyoshi Kotani, *Member, IEEE,* Jong-Kook Lee, Makoto Noshiro, *Member, IEEE,* and Yasuhiko Jimbo, *Member, IEEE*

***Abstract*—Rat superior cervical ganglia (SCG), which are sympathetic ganglia, neurons and ventricular myocytes (VMs) were co-cultured separately in a minichamber placed on a microelectrode-array (MEA) substrate. The minichamber was fabricated photolithographically and had 2 compartments, 16 microcompartments and 8 microconduits. The SCG neurons were seeded into one of the compartments and all of the microcompartments using a glass pipette controlled by a micromanipulator and a microinjector. The VMs were seeded into the other compartment. Three days after seeding of the VMs, the neurites of the SCG neurons had connected with the VMs via the microconduits. Electrical stimulations, trains of biphasic square pulses, were applied to the SCG neurons in the microcompartments using 16 electrodes. Evoked responses were observed in several electrodes while electrical stimulation was applied to the SCG neurons. According to the two-way analysis of variance (ANOVA), the beat rate after electrical stimulation was affected by the frequency and the number of the stimulation pulses. These results suggest that pulse number and the frequency of the electrical stimulation contribute to modulation of the beat rate of the cardiomyocytes.**

I. Introduction

To examine the relationships between the activities of the sympathetic nervous system and cardiac functions, it is necessary to evaluate the activities of sympathetic neurons and cardiomyocytes in co-cultures. The development of sympathetic neurons co-cultured with cardiomyocytes has been electrophysiologically and morphologically investigated. [1], [2] The release properties of noradrenaline from sympathetic neurons were assessed by applying the electrical stimulation to the sympathetic neurons co-cultured with the cardiomyocytes. [3] The effects of the nerve growth factor (NGF), neurotrophin, produced by the cardiomyocytes, were estimated in sympathetic transmission between the sympathetic neurons and the cardiomyocytes. [4] These earlier co-cultures had difficulty in evaluating separately the activities of the sympathetic neurons and the cardiomyocytes, because the sympathetic neurons were randomly mingled with the cardiomyocytes. In the body, the sympathetic neurons are morphologically separated from and functionally connected with the cardiac tissue. Separated co-culture of a small number of the sympathetic neurons and the cardiomyocytes can be used for investigating the generation of cardiac fibrillation related with the activity of the sympathetic nervous system, because it would be possible to control the activity of the neurons artificially by treatment with nicotine [2] or electrical stimulation. [3]

Microfabrication techniques are one of the suitable approaches to study the dynamics of a small number of electrical excitable cells and have actually been utilized to investigate the functional characterization of cultured neurons or cardiomyocytes. [5]-[8] However, separate co-culture of small numbers of sympathetic neurons and cardiomyocytes has not yet been achieved using microfabrication techniques.

The purpose of the present study was two-fold. First, to develop a new technique for co-culture of sympathetic ganglion neurons and cardiomyocytes using microfabrication, and second, to evaluate the functional relationship of the two components in terms of changes in the beat rate of the cardiomyocytes after applying electrical stimulation to the sympathetic neurons.

II. Materials and Methods

A. Culture Device Fabrication

The co-culture device consisted of a microelectrode-array (MEA) substrate [9] and a minichamber. The latter contained 2 compartments, 16 microcompartments and 8 microconduits (Fig. 1A). The compartments were 360 μm in width, 2.5 mm in length and 70 μm in depth. The microcompartments were about 50-70 μm in diameter and 70 μm in depth. The microconduits were approximately 50 μm in width and 5 μm in height. The minichamber was formed on a soft-lithography master (Fig. 1B) using the photolithography technique. The soft-lithography master was produced through a two-stage process. At the first stage, negative photoresist (SU-8 3005, Microchem Corp.) as thin as 5 μm was spin-coated onto a 0.7-mm-thick glass substrate (Matsunami Glass Ind., ltd), and eight strips (50

Manuscript received April 15, 2011. We gratefully acknowledge the partial financial support provided by the Japan Society for the Promotion of Science (JSPS) via a Grant-in-Aid for JSPS Fellows, 22·6118, 2010.

A. Takeuchi is with the Graduate School of Frontier Sciences, The University of Tokyo, Chiba, 277-8563 Japan (phone: +81-047-136-4635; fax: +047-136-4648; e-mail: takeuchi@ bmpe.k.u-tokyo.ac.jp).

M. Mori, K. Shimba, K. Kitagawa, H. Moriguchi, K. Kotani, and Y. Jimbo are with the Graduate School of Frontier Sciences, The University of Tokyo, Chiba, 277-8563 Japan. Y.

Takayama is with the Graduate School of Information Science and Technology, The University of Tokyo, Tokyo, 113-8656 Japan.

K. Miwa is with the Research Institute of Environmental Medicine, Nagoya University, Nagoya, Japan.

J. K. Lee is with the Graduate School of Medicine, Osaka University, Osaka Japan.

M. Noshiro is with the Graduate School of Medical Sciences, Kitasato University, Kanagawa, xxx-xxxx Japan.

μm in width, 650 μm in length, 5 μm in height) were developed. At the second stage, these strips were covered with 100-μm-thick photoresist (SU-8 3050, Microchem Corp.), and 16 cylinders (50 μm in diameter, 100 μm in height) and 2 blocks (0.36 mm in width, 2.5 mm in length and 100 μm in height) were developed on the SU-8 strips.

Next, 70-μm-thick polydimethylsiloxane (PDMS, Dow Corning Toray Co., Ltd.) was spin-coated onto the soft-lithography master. A 2-mm-thick PDMS ring was then placed on the coated PDMS. This ring acted as a reinforcement when the minichamber was carefully released from the master. The released minichamber was placed on the MEA substrate so that the center of a microcompartment was placed over that of an electrode.

B. Cell Culture

The superior cervical ganglia (SCG), which are sympathetic ganglia, were taken from 2- to 3-day-old Wistar rats and collected in ice-cold Leibovitz's L-15 medium (Invitrogen Corp.) containing 0.5 mg/ml collagenase (Wako Pure Chemical Ind., Ltd.). The SCGs collected were incubated for 60 minutes at 37 ºC. Cardiac ventricles obtained from the same rats were incubated for 12-16 hours at 4 ºC in 10 ml of Hank's balanced salt solution (Invitrogen Corp.) containing 50 μg/ml trypsin (Worthington Biochemical Corp.), followed by an additional 3-5 minutes of incubation at 37 ºC with 16 ml L-15 medium supplemented with 0.13 mg/ml collagenase (Worthington Biochemical Corp.). The incubated ventricular cells were preplated for 30 minutes at 37 ºC in a tissue culture flask (BD Corp.) for removal of most of the fibroblasts. As a result, the ventricular myocytes (VMs) were separated as a suspension.

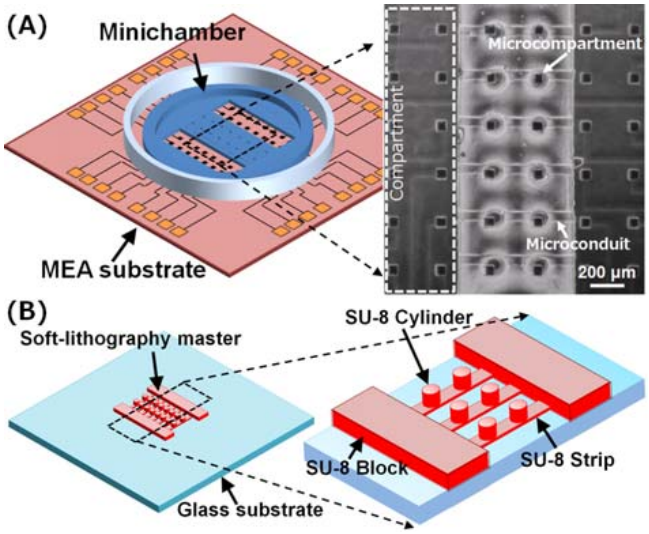

Fig. 1 Minichamber on an MEA substrate. (A) Sixty-four electrodes were embedded in the center of the MEA substrate. Each electrode covered an area of 50×50 μm square. One electrode was 250 μm apart from the other. Center-to-center distances of the microcompartments were 250 μm. (B) The soft-lithography master consisted of 16 cylinders and 2 blocks developed on the SU-8 strips.

The culture device was coated with type I-C collagen (Nitta Gelatin Inc.) and filled with culture medium before seeding of the SCG neurons and VMs. Dissociated SCG neurons were seeded into the left compartment and 16 microcompartments using a glass pipette under phase-contrast microscopic observation. Sixteen to twenty hours after seeding of the SCG neurons, dissociated VMs were seeded into the other compartment in the same way as the SCG neurons. In addition, separation of the cardiomyocytes from the SCG neurons was confirmed.

The culture medium in this study was based on Brum's medium [10], and contained 90% ($^{V}/_{V}$) Dulbecco's modified Eagle medium (DMEM, Invitrogen Corp.) and 10% ($^{V}/_{V}$) fetal bovine serum, supplemented with a set of 25 ng/ml 2.5S Nerve Growth Factor (Invitrogen Corp.), 10 ng/ml Glial Cell-line-derived Neurotrophic Factor (Sigma-Aldrich Inc.), 2 mM Glutamax (Invitrogen Corp.), and 1% Penicillin-Streptomycin (Invitrogen Corp.). The medium was replaced every 24 hours.

C. Extracellular Recording

The MEA substrate in a temperature- and CO_2-level-controlled box (37 °C, the CO_2 concentration at 5%) was used to measure spontaneous electrical activities of the cell cultures. A reference electrode was located separately from the minichamber in the culture medium.

The electrical signals obtained from the cultures were amplified (26 dB) with preamplifiers located at the electrodes. The preamplified signals were filtered with a band-pass filter (-3 dB at 10 Hz and 3 kHz), and then amplified (80 dB) with a main amplifier (NF Corp.). The amplified signals were sampled at intervals of 40 μs using an AD converter (National Instrument Corp.) with a resolution of 12 bits, and recorded on the hard disk drive of a personal computer.

D. Electrical Stimulation and Evaluation of the Effects

First, spontaneous electrical activities of the cardiomyocytes were recorded for 3 minutes. Then 9 different electrical stimulations, trains of biphasic square pulses (1 ms at +1 V, followed by 1 ms at -1 V), were applied to the cultured SCG neurons in all of the microcompartments. These electrical simulations were determined by a combination of 3 different frequencies (1, 5 and 10 Hz) and 3 different numbers of pulses (60, 300 and 600 pulses). The applied current flowed from each electrode under a microcompartment to the reference electrode. After each stimulation had been discontinued, spontaneous activities were recorded for 1 minute.

The effects of electrical stimulation were evaluated on the basis of changes in the cardiomyocyte beat rate. The beat rate of the VMs was obtained by counting the number of spontaneous electrical activities recorded form the electrode on the MEA substrate. The frequency change ratio was calculated by dividing the beat rate after the stimulation by that before the stimulation.

III. Results

A. Cell Culture

The SCG neurons adhered to the left compartment and all of the microcompartments, and the VMs adhered to the other compartment. The SCG neurons and the VMs were successfully compartmentalized in the minichamber. The SCG neurons 3 days after VM seeding (3 days *in vitro*, DIV) grew into the left compartment and all of the microcompartments in the co-culture (Fig. 2). On the other hand, the VMs proliferated only in the right compartment, and generated spontaneously and intermittently synchronized electrical activities. Separation of the SCG neurons from the cardiomyocytes was still maintained at 5 DIV, as confirmed by immunostaining with beta-3 tubulin, a specific marker of neurons (Fig. 3). Moreover, synaptic connections were formed between the SCG neurons and the cardiomyocytes (Fig. 4). From these fluorescence imagings, we concluded that the cell bodies of the neurons were fixed in the microcompartment, and the synaptic connections were formed between the cardiomyocytes.

B. Effects of Electrical Stimulation

Immediately after the electrical stimulation, evoked responses of the SCG neurons were observed from several electrodes directly under the microcompartments, irrespective of the number and frequency of the pulses. Figure 5 shows the evoked responses recorded from 3 electrodes located directly under the microcompartments.

Table 1 shows the mean values and standard deviations of the increase in the beat rate ratio of the VMs after 9 different stimulations had been applied to the SCG neurons (5 DIV). Two-way analysis of variance (ANOVA) revealed that the pulse frequency had a significant effect on the beat rate ratio, and that the interaction between the number of pulses and their frequency also did (Table 1). Bonferroni's multiple comparison *post hoc* test demonstrated a significant difference between 1 Hz and 10 Hz at 600 pulses (Fig. 6).

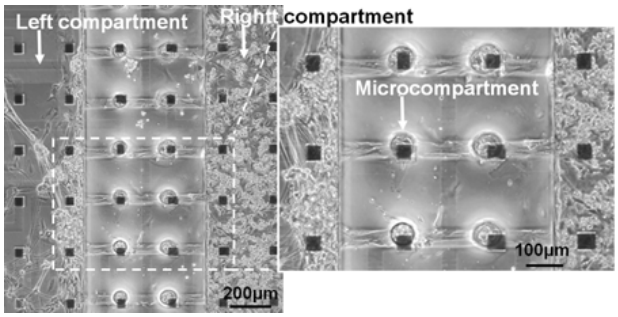

Fig. 2 Co-culture of the SCG neurons and the VMs. The SCG neurons were confined to the left compartment and all of the microcompartments, and the VMs were in the right compartment. Three days *in vitro* (3 DIV).

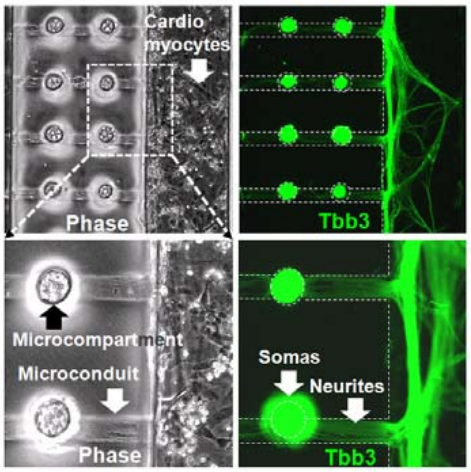

Fig. 3 The neurites of the SCG neurons extended along the microconduits only and reached the VMs. Cardiomyocytes were confined in the left compartment at 5 DIV. The SCG neurons were immunostained with the anti- tubulin beta-3 (Tbb3) antibody. Somata were not seen in the microconduits.

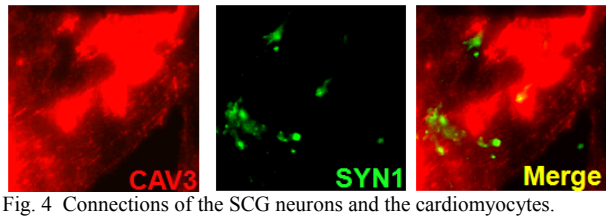

Fig. 4 Connections of the SCG neurons and the cardiomyocytes. The SCG neurons and the cardiomyocytes at 5 DIV immunostained with antibodies to caveolin-3 (red) and synapsin I (green), and the synapses were formed on the cardiomyocytes.

IV. Discussion

A. The Co-culture Device in Relation to Other Culture Devices

The cell bodies of SCG neurons are approximately 10-20 μm in diameter [11] and the microconduits connecting with the microcompartments were 5 μm in height. Consequently, the cell bodies of the SCG neurons were unable to enter the microconduits. Because the size of VMs is generally in the range of several tens to one hundred micrometers, [1] they were not able to enter the microconduits and were cultivated only in the right compartment.

Our co-culture device has mainly two major advantages over the devices already in use for studies of network neuronal activity. First, our device records the electrical activities generated by a small number of neurons cultured only in the micrometer-sized microcompartments, and stimulates a small number of neurons. A device fabricated photolithographically in previous report was previously used to record electrical activities and to stimulate the development of neuronal networks. [6] However, it recorded electrical activities generated by a large number of neurons, and not a small number, because many neurons were cultured in millimeter-sized compartments. Moreover, cortical neurons derived from rat embryos have been previously cultured on a grid-pattern developed by microcontact-printing. [7], [8] However, the neurites of some neurons deviated from the grid-pattern. Our present

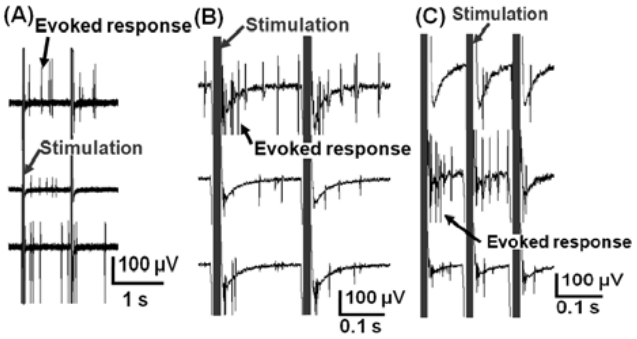

Fig. 5 Evoked responses of the SCG neurons were obtained in several electrodes directly under the microcompartments (5 DIV). (A), (B) and (C) indicate the evoked responses observed at frequencies of 1 Hz, 5 Hz and 10 Hz, respectively. In addition, no electrical activities associated with the contraction of the VMs were triggered by applying electrical stimulation to the SCG neurons (data not shown).

Table 1 ANOVA table of changes in beat rate of the VMs after the electrical stimulation applied to the SCG neurons was discontinued. According to the two-way repeated-measures ANOVA, significant differences in the beat rate after stimulation were caused by the frequency and the interaction effect of the frequency and the number of the pulses.

Source	Sum of Squares	Degrees of Freedom	Variance	Variance Ratio	P-Value
Number of pulses	3119.2	2	1559.6	1.53	$2.3*10^{-1}$
Frequency	36210	2	18104	17.7	$4.4*10^{-6}$
Interaction effect	11298	4	2824.4	2.76	$4.2*10^{-2}$
Repeatability error	36782	36	1021.7		
Total	87409	44			

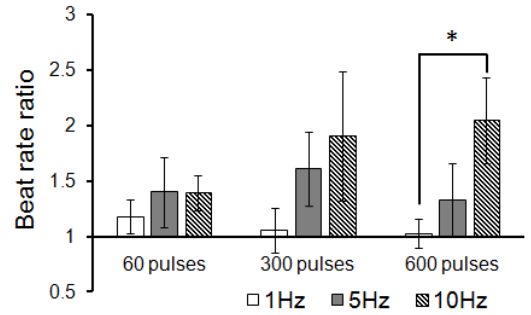

Fig. 6 Electrical stimulations to the SCG neurons induced the changes in the beat rate of the co-cultured cardiomyocytes. Bonferroni's multiple comparison *post hoc* test indicates that the change in beat rate after 600 pulses at 1 Hz were applied was significantly different from that after 600 pulses at 10 Hz (*P < 0.05).

method, on the other hand, ensures that SCG neurons and VMs are confined to the microcompartments and microconduits.

Second, we were able to explore the relationships between the activities of a small number of SCG neurons and those of VMs. An earlier study demonstrated the relationship between a SCG neuron and a VM. [2] However, it did not allow the evaluation of relationships between the activities of a small number of SCG neurons and VMs, because the two cell types were intermingled. Our device was able to detect the evoked responses generated by a small number of SCG neurons, because the neurons were confined to individual microcompartments.

B. Electrical Stimulations to the SCG neurons Influence the Beat rate of the VMs

The key point of this study is the relationships between the parameters of electrical stimulations and the changes in beat rate of the VMs. Our results suggest that the change in the beat rate of the VMs after stimulation depended on two factors, the frequency and the effect of interaction between the frequency and the number of pulses. To our knowledge, no earlier researchers investigated the relationship between changes in the beat rate of VMs and the electrical stimulation of the SCG neurons. The relationship between the frequency and the pulse number of the stimulations will be investigated in our further study.

V. Conclusion

Rat SCG neurons and VMs have been successfully compartmentalized in a minichamber. Electrical stimulations were applied to the SCG neurons connected with the VMs. As a result, changes in the beat rate of the VMs after electrical stimulation of the SCG neurons were affected by both the frequency and the interaction between the frequency and the number of pulses.

References

[1] E. J. Furshpan, P. R. Macleish, P. H. O'laguet and D. D. Potter, "Chemical transmission between rat sympathetic neurons and cardiac myocytes developing in micro cultures: Evidence for cholinergic, adrenergic, and dual-function neurons", *Proc Natl Acad Sci U S A*, Vol. 73, pp. 4225-2429 (1976)

[2] O. G. Shcherbakova, C. M. Hurt, Y. Xiang, M. L. Dell'Acqua, Q. Zhang, R. W. Tsien and B. K. Kobilka, "Organization of β-adrenoceptor signaling compartments by sympathetic innervation of cardiac myocytes", *J Cell Biol*, Vol. 176, pp. 521-533 (2007)

[3] A. R. Wakade, D. A. Przywara, S. V. Bhave, V. Mashalkar. and T. D. Wakade, "Cardiac cells control transmitter release and calcium homeostasis in sympathetic neurons cultured from embryonic chick", *J Physiol*, Vol. 488, pp. 587-600 (1995)

[4] S. T. Lockhart, G. G. Turrigiano and S. J. Birren, "Nerve Growth Factor Modulates Synaptic Transmission between Sympathetic Neurons and Cardiac Myocytes", *J Neurosci*, Vol. 17, pp. 9573-9582 (1997)

[5] I. Suzuki and K. Yasuda, "Detection of tetanus-induced effects in linearly lined-up micropatterned neuronal networks: Application of a multi-electrode array chip combined with agarose microstructures", *Biochem Biophys Res Commun*, Vol. 356, pp. 470-475 (2007)

[6] R. Morales, M. Riss, L. Wang, L. Gavín, J. A. Del Río, R. Alcubilla and E. C. Tinturé, "Integrating multi-unit electrophysiology and plastic culture dishes for network neuroscience", *Lab Chip*, Vol. 8, pp. 1896–1905 (2008)

[7] J. C. Chang, G. J. Brewer and B. C. Wheeler, "Modulation of neural network activity by patterning.", *Biosens Bioelectron*, Vol. 16, pp. 527-533 (2001)

[8] A. K. Vogt, G. J. Brewer, T. Decker, S. Böcker-Meffert, V. Jacobsen, M. Kreiter, W. Knolla and A. Offenhäusser, "Independence of synaptic specificity from neuritic guidance", *Neurosci*, Vol. 134, pp. 783-790 (2005)

[9] Y. Jimbo, N. Kasai, K. Torimitsu, T. Tateno and H. P. C. Robinson, "A system for MEA-based multisite stimulation", *IEEE Trans Biomed Eng*, Vol. 50, pp. 241-248 (2003)

[10] P. C. Brum, C. M. Hurt, O. G. Shcherbakova, B. Kobilka and T. Angelotti, "Differential targeting and function of a2A and a2C adrenergic receptor subtypes in cultured sympathetic neurons", *Neuropharmacology*, Vol. 51, pp. 397-413 (2006)

[11] D. Bray, "Surface Movements during the Growth of Single Explanted Neurons", *J Cell Biol*, 1973, Vol. 56, pp. 702-712 (1973)