Qualitative and quantitative estimation of comprehensive synaptic connectivity in short- and long-term cultured rat hippocampal neurons with new analytical methods inspired by Scatchard and Hill plots

Ryo Tanamoto¹, Yutaka Shindo¹, Mariko Niwano¹, Yoshinori Matsumoto², Norihisa Miki³, Kohji Hotta¹, Kotaro Oka¹

1) Department of Biosciences and Informatics, Faculty of Science and Technology, Keio University

2) Department of Applied Physics and Physico-Informatics, Faculty of Science and Technology, Keio University

3) Department of Mechanical Engineering, Faculty of Science and Technology, Keio University

3-14-1 Hiyoshi, Kohoku-ku, Yokohama, Kanagawa 223-8522, Japan

Corresponding author: Prof. Kotaro Oka

e-mail: oka@bio.keio.ac.jp

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Abstract

To investigate comprehensive synaptic connectivity, we examined Ca^{2+} responses with quantitative electric current stimulation by indium-tin-oxide (ITO) glass electrode with transparent and high electro-conductivity. The number of neurons with Ca^{2+} responses was low during the application of stepwise increase of electric current in short-term cultured neurons (less than 17 days *in-vitro* (DIV)). The neurons cultured over 17 DIV showed two-type responses: S-shaped (sigmoid) and monotonous saturated responses, and Scatchard plots well illustrated the difference of these two responses. Furthermore, sigmoid like neural network responses over 17 DIV were altered to the monotonous saturated ones by the application of the mixture of AP5 and CNQX, specific blockers of NMDA and AMPA receptors, respectively. This alternation was also characterized by the change of Hill coefficients. These findings indicate that the neural network with sigmoid-like responses has strong synergetic or cooperative synaptic connectivity *via* excitatory glutamate synapses.

Running title: Qualitative and quantitative estimation of comprehensive synaptic connectivity

Key words: indium-tin-oxide (ITO) glass, neural network maturation, synaptic connectivity, Ca^{2+} imaging

Abbreviations: ITO, indium-tin-oxide; DIV, days *in-vitro*; NMDA, *N*-methyl-D-aspartate; AMPA, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

Introduction

Complicated neural networks are formed according to the maturation of neurons and synaptic connections. To understand such neural network behavior, *in-vitro* dissociated monolayer cultures on micro-fabricated devices have been used [1–4]. These *in-vitro* neural networks retain many morphological, pharmacological, and electrophysiological properties of networks *in-vivo*, and allow us much more detailed observation and manipulation at the molecular, cellular, and also network levels [5–14]. Furthermore, *in-vitro* neuron culture enables us to investigate the synaptic connectivity during neural network maturation in detail [11, 15, 16].

The functional or effective synaptic connectivity among neurons has been evaluated with the synchronous responses of the neurons evoked by the stimulation of specific

neurons in the network [5, 17, 18]. To measure the neural activities of many neurons simultaneously, optical techniques have been used [14, 17–20]. Although it is a powerful technique to visualize the train of spontaneous activities of the neurons in the network, comprehensive network behavior in response to the stimulation of specific neurons has not been evaluated because the contribution of a single neuron for activating connecting neurons is negatively correlated with the density of neurons in the network [17]. To evaluate the connectivity among neurons quantitatively, simultaneous stimulation of many neurons in the network and optical measurements of their activities are required. To achieve it, we have developed an experimental system with transparent indium-tin-oxide (ITO) glass electrode for electric current stimulation, and evoked neural activities are visualized with Ca^{2+} fluorescent imaging [21], and also investigated the simultaneous Ca^{2+} and Mg^{2+} mobilization in hippocampal neurons [22] recently.

In this study, by using this experimental setup with ITO electrode, we investigated the maturation of synaptic connectivity in rat hippocampal neurons in detail. We analyzed how the number of responding neurons increases according to the quantitative electric current stimulation. We succeeded to evaluate the cooperativity of the neurons with new analytical methods inspired by Scatchard [23] and Hill [24], which were

originally developed for analysis of reaction dynamics of enzymes.

Materials and Methods

Fabrication of patterned ITO glass electrodes

The patterned ITO electrodes were fabricated as described before [21]. In short, designed pattern was reproduced on an emulsion mask (Konica, Tokyo, Japan), and composed a photomask with inverting black and transparent area. The pattern was reproduced on photoresist coated on ITO glass slides by UV illumination through the photomask. An acidic solution composed of 5% HNO_3 and 20% HCl was used to etch the portion of the ITO that was not protected by photoresist. Then, the remaining photoresist was removed, and the patterned ITO glass electrode was rinsed with acetone and dried.

Cell Culture

All animal procedures were approved by the ethical committee of Keio University (permit number is 09106-(1)).

Primary culture of hippocampal neurons was prepared from day 18-19 embryonic wistar rats (Charles River Laboratories Japan, Tokyo, Japan). The hippocampal neurons were isolated and dissociated with a dissociation solution (Sumitomo Bakelite, Tokyo, Japan) for 30 min. To culture neurons on the patterned ITO electrode, six Press-to-Seal silicone isolators (Invitrogen, Carlsbad, CA, USA) were reversibly sealed to the patterned ITO electrode slide in piles. The dissociated neurons were plated onto poly-D-lysine (Sigma-Aldrich, St. Louis, MO)-coated patterned ITO glass electrode chamber with 1 mL cell suspension of 400,000 cells/mL, and cultured in neurobasal medium supplemented with B-27, 2 mM L-glutamine, 50 U/mL penicillin, 50 µg/mL streptomycin (all from Invitrogen). Cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂. The neurons were cultured for 7-40 days before experiments.

Fluorescent Measurements

For optical imaging, neurons on the patterned ITO glass were incubated with 5 µM fluo-4 AM and 0.02% pluronic F-127 (Invitrogen) in the culture medium for 30 min at 37°C. Then, the neurons were washed twice with Krebs-Ringer Henseleit solution (KRH) containing (in mM): NaCl, 125; KCl, 5; MgSO₄, 1.2; CaCl₂, 2; KH₂PO₄, 1.2;

glucose, 6; HEPES, 25; pH adjusted to 7.4 with NaOH, and incubated in fresh KRH for 15 min at 37°C for complete hydrolysis of the acetoxymethyl ester form of fluo-4 AM in the neurons. Fluorescent imaging experiments were performed using a fluorescent microscope (ECLIPSE TE300, Nikon, Tokyo, Japan) equipped with a 10× objective (S Fluor, Nikon), a 505 dichroic mirror and a 535/55-nm barrier filter. Fluo-4 was excited at 488 nm selected from Xe lamp (150 W) with a monochromator unit, and fluorescence was measured with a CCD camera (HiSCA, Hamamatsu Photonics, Shizuoka, Japan). The fluorescence was calculated as the mean intensity over a defined region of interest (ROI) on cell body of each neuron by using software package, Aquacosmos (Hamamatsu Photonics).

Electric Current Stimulation

Stimulation current was applied with Electronic Stimulator (SEN-8203, NIHON KOHDEN) through Isolator (SS-403J, NIHON KOHDEN). The duration was varied from 40 μ s to 1500 μ s. The voltage was set at 10 V, interval at 16 ms, and train at 625 times. These settings yielded square wave voltage with frequency of 62.5 Hz, voltage of 10 V, and periods of 10 s. Neurons were stimulated every 30 s by increasing the duration from 40 μ s to larger value, gradually. A Digital Multimeter (CDM-2000D,

CUSTOM, Tokyo, Japan) was connected to the circuit comprising cell-stimulating ITO electrode and the stimulator in series in order to measure the current flowing through the ITO electrode current. The Digital Multimeter was set for ACA mode.

Inhibition of Glutamate Receptors

The glutamate receptor was blocked with *N*-methyl-D-aspartate (NMDA) receptor antagonist D,L-2-amino-5-phosphonovalerate (AP5, TOCRIS, Bristol, UK) at final concentration of 200 μ M, and α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, Sigma-Aldrich) at final concentration of 10 μ M. These inhibitors were applied to cells more than 10 min prior to fluorescent measurements.

Detection of Ca^{2+} responses

In order to determine the response of Ca^{2+} to the electrical stimuli, the time differentials of the relative fluorescent intensities ($d(F/F_0)/dt$, F_0 is the initial value of fluorescent intensity) were calculated for each neurons. The onset of the response timing was detected as the supra-threshold period ($2.58 \times \text{SD}$ of noise) using the value

$d(F/F_0)/dt$, with the noise value being the calcium signal associated with the non-stimulated period (0–1 min) [25].

Result & Discussion

In our previous study, we have developed patterned ITO glass electrode (Fig. S1c) to stimulate cells without the use of a counter electrode, and observed the excitabilities in PC12 cells and hippocampal neurons as Ca^{2+} responses [21]. It has been revealed that ITO glass electrode is available for quantitative and local electrical stimulation with fluorescent imaging because of its high electro-conductivity and transparency.

In this study, we applied this setup for investigating the neuronal activities of hippocampal neuron network during the application of stepwise increase of electric current stimulation to many neurons in the network (Fig. S1a, upper panel). Several neurons showed large Ca^{2+} responses by the application of relatively weak current. After the first and large Ca^{2+} responses, excited neurons showed fluctuated Ca^{2+} responses synchronizing to the ON/OFF of electric current stimulation. In several other neurons, the first Ca^{2+} responses require stronger electric current stimulation (Fig. S1b, upper panel). From these results, we confirmed that the ITO device enables to

simultaneously stimulate many neurons with non-invasively, and observe their Ca^{2+} responses through transparent ITO glass.

We investigated the neural responsibility to current stimulation as the function of neural network maturation. It has been known that the neural responsibility to the stimulation increases with culturing days *in-vitro* (DIV) [16], and formation of matured neural networks and appropriate distribution of excitatory ion channels on the cell membrane require from two to three weeks [15]. We compared the ratio of the neurons responded to the stimuli to all neurons observed. In our experiments, long-term cultured neurons (cultured for longer than 17 DIV) were highly sensitive to stimuli and short-term cultured ones (cultured for less than 17 DIV) showed low sensitivity ($P < 0.05$, Fig. 1a). The cumulative number of responding neurons in long-term culture drastically increased in response to weak range of stimuli while it increased gradually with the saturation at the low ratio value in short-term cultured neurons (Fig. 1b). Furthermore, over 17 DIV, neurons show two typical response patterns: monotonous saturation (orange) and S-shaped (sigmoid) like saturation (red). In the sigmoid like saturation, there are some sharp increases of responding neurons. We suppose that it is based on cooperative activity of neurons in the network, that is, one responding

neuron enhances to excite the connecting neurons. To examine this assumption in more detail, Scatchard plots were applied to representative example showing sigmoid like and monotonous saturation, respectively (Fig. 1c and d). The plots show the relationship between the ratio of responding neurons and the quotient obtained by dividing the ratio value by current value. It is now widely recognized that downward-curved Scatchard plots are obtained when there is positive cooperativity in substrate binding to an enzyme [26]. Our results showed the typical form of plot for sigmoid like and monotonous saturation, respectively, and the sigmoid like shape indicates positive and high cooperativity in general. To investigate whether some responding neurons excite the connecting neurons to be activated, the delay time to the onset of stimulation was obtained from concerning samples showing sigmoid and monotonous curves, respectively (Fig. 1e and f). The delay time of the Ca^{2+} response from the onset of stimuli varied widely in the network with sigmoid-like responses. Because Ca^{2+} response observed here probably induced by burst-like activities of neurons, not by single spike, the propagation across synaptic connection was slower than action potential. The Scatchard plot and histogram of delay were also obtained for short cultured samples (<17DIV). The Scatchard plot was linear and the ratio of

responses with delay time of several seconds was smaller than the samples showing monotonous curve (Fig. S2). These results indicate that short-term cultured network has low cooperativity.

We consider that the high cooperativity of long-term cultured network results from the dense synaptic connection. To directly demonstrate it, NMDA and AMPA type glutamate receptors were pharmacologically blocked by selective glutamate receptor antagonists, AP5 (200 μ M) and CNQX (10 μ M). Although the mixture of the antagonists suppressed the activity of some neurons, Ca^{2+} responses were observed again in response to higher current stimulation in several neurons (Fig. S3, raster diagram of Ca^{2+} responses). In these neurons, minimum current intensities that evoked Ca^{2+} responses before and after application of antagonists were compared (Fig. 2a). In those neurons, the delay time to the onset of electrical stimuli was shortened by the inhibition of glutamate receptors (Fig. 2b). It is probably caused by the change in the cooperativity among neurons. While some neurons showed delayed Ca^{2+} response enhanced by connecting neurons before the inhibition of glutamate synapses, after the inhibition, these neurons were directly activated by higher electric current stimulus.

To analyze the change in the cooperativity, the Scatchard plot was obtained for one

representative sample in control and antagonist condition (Fig. 2c). The downward-curves in Scatchard plot were shown in control condition, whereas linear plots in inhibited condition. It indicates that the cooperativity is lowered by the inhibition of glutamate synapses. Moreover, we applied the Hill equation to analyze them further:

$$y = \frac{n (K[S])^n}{1 + n (K[S])^n}$$

where n is the Hill coefficient, K is the coupling constant, $[S]$ is the substrate molecule in the field of biochemistry [24]. In this study, to compare the cooperativity between control and inhibited condition, Hill coefficient n was calculated from the data of those conditions, respectively. The Hill coefficient n is larger in the control conditions than ones in the inhibited conditions for every sample, and $n < 1$ for several samples in inhibited conditions (Fig. 2d). According to the present study, $n < 1$ means that the system is anti-cooperative [26]. It is supposed that the GABAergic synapses dominantly remained in the antagonist condition, and response of one neuron would negatively facilitate responses of neighbor neurons.

This study revealed that the neural activities drastically change during the culture. In short-term cultured neurons, no neural responses to the higher electric current stimulation were observed, and this indicates that individual neuron has low sensitivity to the stimulation, and/or synaptic connections are sparse and weak. In long-term cultured neurons, the neural network alters its construction and components; high sensitivity and/or low threshold neurons with high synaptic connectivity and follower neurons. In previous studies on spontaneous firing, it has been found that depending on the size of network system [17, 27, 28] and DIV [29], the properties of firing drastically vary, such as the frequency of spontaneous spikes and bursts, inter-spike-interval and duration of burst. In addition, the phenomenon is thought to be due to the variation of the density of membrane-dependent sodium and potassium ion-channel, synapses [30, 31] and transporters of synaptic vesicle [31, 32]. On the other hand, after 3 weeks *in-vitro*, the firing rate gradually decrease while the feature of each burst becomes more general. The reason is thought to be that the reorganization of internal structure accommodates the number of excitatory and inhibitory synapse [15]. Our analysis applied here successfully reflected those changes in network properties (Fig. 1). Moreover, in long-term cultured networks, changes in the

cooperativity elucidated by the inhibition of glutamate synapses were evaluated. These analyses proposed here would be a powerful tool to evaluate the properties of neural networks.

In this study, we describe the cooperative responses among neurons connecting each other in the cultured hippocampal network. Applying the ITO electrode device developed previously, we evaluated the cooperativity of networks as the function of culturing days (Fig. 1) and the effect of glutamate synapse on it (Fig. 2). The combination of ITO glass electrode and Ca^{2+} imaging technique will be the plausible tool for understanding the maturation and cooperative phenomenon of the neural network in brain and spinal cord because of the advantage of this system that quantitative current stimulation and spatiotemporal neural activity analysis.

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Figure legends

Fig. 1 Responsibility of neurons to current stimuli as a function of DIV.

(a) The ratio of the number of responding neurons to that of total neurons for long-term (≥ 17 DIV, red and orange gradation, N=28) and short-term (<17 DIV, blue, N=10) cultured samples. Error bars indicate SEM. *P<0.05 in t-test.

(b) Changes in the ratio of cumulative number of responding neurons to the number of total neurons as a function of the intensity of electric current stimuli. The red and orange markers show results of long-term cultured neurons (≥ 17 DIV, circle plots indicate 17 DIV, square 24 DIV, diamond 23DIV and triangle 38 DIV, respectively), and blue markers indicate short-term cultured neurons (<17 DIV, circle plots indicate 10 DIV and diamond 15 DIV, respectively). In long-term cultured neurons, two typical response patterns were shown: sigmoid like (red) and monotonous (orange) saturation curve.

(c), (d) Scatchard plots for representative example showing sigmoid-like (c) and monotonous (d) saturation, respectively. The plots show the relationships between the quotient obtained by dividing the ratio of responding neurons by electric current stimulation value and the ratio of responding neurons.

(e), (f) Time delay of neuronal responses to the onset of electrical stimuli in samples showing sigmoid-like (e) and monotonous (f) saturation curves, respectively. The data were summarized of all the Ca^{2+} responses detected in entire measurements for the

neurons showed sigmoid-like (N=2131 events from 8 experiments) and monotonous (N=266 events from 3 experiments) responses.

Fig. 2 Inhibition of neural activities by antagonists of glutamate receptor.

(a) Comparison of minimum electric current intensity that induced Ca^{2+} responses without and with inhibition of glutamate receptors by AP5 (200 μM) and CNQX (10 μM).

These data are shown as box-and whisker plot, showing median, maximum, minimum and first, third quartile values, respectively. Circles indicate the values of each neuron.

(** indicates $P < 0.001$ in paired t-test, $n=24$).

(b) Time delay of neuronal responses to the onset of electric current stimulation without inhibition (red) and with inhibition (blue) of glutamate receptors, respectively. The data

were summarized of all the Ca^{2+} responses detected in entire measurements before

(N=139 events) and after (N=74 events) the inhibition (8 Experiments). Histograms

were normalized by the value of 0–2 sec.

(c) Scatchard plots for representative example for control condition and inhibited condition, respectively.

(d) The Hill coefficient n for control and inhibited conditions, respectively. These data are shown as box-and whisker plot, showing median, maximum, minimum and first, third quartile values, respectively. Circles indicate the values calculated from each experiment. The coefficient is larger in control conditions than in antagonist conditions (** indicates $P < 0.001$ in paired t-test, $N=6$).

Figure 1

Figure 2