Highly active neurons emerging in vitro

Mami Okada,1 Rena Kono,1 © Yu Sato,1 Chiaki Kobayashi,1 Ryuta Koyama,1 and Yuji Ikegaya1,2,3
1Graduate School of Pharmaceutical Sciences, The University of Tokyo, Tokyo, Japan; 2Center for Information and Neural Networks, National Institute of Information and Communications Technology, Suita, Japan; and 3Institute for AI and Beyond, The University of Tokyo, Tokyo, Japan

Abstract
Mean firing rates vary across neurons in a neuronal network. Although most neurons infrequently emit spikes, a small fraction of neurons exhibit extremely high frequencies of spikes; this fraction of neurons plays a pivotal role in information processing, however, little is known about how these outliers emerge and whether they are maintained over time. In primary cultures of mouse hippocampal neurons, we traced highly active neurons every 24 h for 7 wk by optically observing the fluorescent protein dVenus; the expression of dVenus was controlled by the promoter of Arc, an immediate early gene that is induced by neuronal activity. Under default-mode conditions, 0.3%–0.4% of neurons were spontaneously Arc-dVenus positive, exhibiting high firing rates. These neurons were spatially clustered, exhibited intermittently repeated dVenus expression, and often continued to express Arc-dVenus for approximately 2 wk. Thus, highly active neurons constitute a few select functional subpopulations in the neuronal network.

NEW & NOTEWORTHY The overdispersion of neuronal activity levels can often be attributed to very few neurons exhibiting extremely high firing rates, but due to technical difficulty, no studies have examined how these outliers are selected during development and whether they are maintained over time. We optically monitored highly active neurons for as long as 7 wk in vitro and found that they constituted a unique population that was different from other “mediocre” neurons with normal firing rates.

INTRODUCTION
The nervous system is highly unequal in terms of neuronal activity levels. Only a few neurons exhibit extremely high firing rates, whereas many other neurons are most often inactive (1–4). Although highly active neurons are numerically less dominant than those with low activity, their spikes contribute to the majority of the total spikes in the brain and have a greater impact on neural computation than those emitted by many other “mediocre” neurons (3–5). Highly active neurons express the immediate early gene c-Fos (6) and are spatially clustered in the cerebral cortex in vivo (7). Given that the activity levels of individual neurons are invariant regardless of the behavioral states of an animal or the surrounding environment (3), they appear to be inherently determined, that is, highly active neurons are preconfigured, constituting a unique subpopulation in the neuronal network. However, there is no experimental evidence supporting this idea, and the mechanisms for the selection of highly active neurons and their long-term stability have not been clarified.
To address this gap, we employed a primary culture system that allows neurons to be maintained on a single layer on glass coverslips. Even without external stimuli, cultured neurons form synaptic connections and fire spontaneous spikes. This feature allows us to focus on the circuit properties that arise from the intrinsic properties of neurons. In addition, it is easy to observe identical neurons for a long time during development in primary culture and to manipulate them pharmacologically. In such “free-run” conditions in primary cultures, the firing rates of neurons are highly heterogeneous and exhibit a skewed distribution (8, 9). The skewed distribution is stable for at least a few days and provides a unique opportunity to elucidate the fate and dynamics of highly active neurons. However, no studies have addressed the long-term stability of the distribution with single-cell resolution, mainly because it is technically difficult to electrophysiologically trace each neuron for a long time.
Calcium imaging is available for long-term observation of neuronal activity, but calcium fluorescence may be saturated when neurons are highly active and the number of neurons observed at the same time is limited. To overcome this problem, we used the expression of Arc as a marker of the neuronal activity level. Arc is an immediate early gene that is rapidly induced by neuronal activity (10–12), and Arc-expressing neurons exhibit high firing rates (6). To ensure the long-term traceability of Arc-expressing neurons, we prepared cultured neurons from Arc-dVenus transgenic mice, in which an Arc promoter induced expression of dVenus, a rapidly degrading version of yellow fluorescent protein (13). By fluorescently monitoring dVenus-positive neurons, we discovered that a population of highly active neurons is likely fixed for at least weeks.

**MATERIALS AND METHODS**

**Animals**

All experiments were performed with the approval of the Animal Experiment Ethics Committee at the University of Tokyo (Approval No.: P29-9) and according to the University of Tokyo guidelines for the care and use of laboratory animals.

**Cultures**

Primary hippocampal cultures were prepared from neonatal wild-type (WT) or Arc-dVenus mice of either sex. Arc-dVenus mice were provided by Prof. Yamaguchi at Gifu University. Mouse pups were anesthetized by isoflurane and the brains were removed. The hippocampi were dissected out in Hank’s balanced salt solution at 37°C, minced, and incubated at 37°C for 15 min with trypsin and ethylenediaminetetraacetic acid, followed by incubation with DNase at room temperature for 5 min. Tissues were washed in Hank’s balanced salt solution; triturated using a fire-polished Pasteur pipette in neurobasal medium containing 2% B27 supplement, 0.5 mM glutamine, 25 μM glutamate, 1 mM HEPES, and 10% horse serum; and filtered through 40-μm pore cell strainers. Cells were dispersed on 12-mm-diameter glass coverslips coated with poly-D-lysine. They were placed in which an Arc promoter induced expression of dVenus, rapidly degrading version of yellow fluorescent protein (13). By fluorescently monitoring dVenus-positive neurons, we discovered that a population of highly active neurons is likely fixed for at least weeks.

**Immunohistochemistry**

Cultures were fixed with 4% paraformaldehyde and 4% sucrose in phosphate-buffered saline for 10 min, permeabilized with 0.1% Triton-X solution for 10 min, and blocked with 10% goat serum for 1 h at room temperature. Cells were incubated with primary antibodies overnight at 4°C and then with secondary antibodies for 1 h at room temperature. The primary antibodies used were as follows: mouse anti-NeuN (1:1,000, Millipore, Bedford, MA), chicken anti-MAP2 (1:1,000, Synaptic Systems, Göttingen, Germany), rabbit anti-Arc (1:1,000, Millipore, Bedford, MA), guinea pig anti-Iba1 (1:1,000, Synaptic Systems, Göttingen, Germany), mouse anti-S100β (1:1,000, Sigma-Aldrich, St. Louis, MO), and mouse anti-RFP (1:1,000, MBL International Corporation, Nagoya, Japan). The specificity of this anti-Arc antibody has been shown by experiments using Arc-knockout mice (14). The secondary antibodies used were Alexa 405/-488/-594/-647-labeled anti-mouse/rabbit guinea pig/chicken IgG (1:500, Thermo Fisher Scientific, Waltham, MA). The antibodies were diluted in 1% goat serum solution. Nuclei were labeled with DAPI (1:1,000, Thermo Fisher Scientific, Waltham, MA).

**Electrophysiology**

Recording was performed at 35°C in a submerged chamber perfused at 6–8 ml/min with oxygenated artificial cerebrospinal fluid, which consisted of 127 mM NaCl, 26 mM NaHCO₃, 1.6 mM KCl, 1.24 mM KH₂PO₄, 1.3 mM MgSO₄, 2.4 mM CaCl₂, and 10 mM glucose. For cell-attached recordings, glass pipettes (3–6 MΩ) were filled with artificial cerebrospinal fluid. Signals were amplified and digitized at a sampling rate of 2 kHz using a MultiClamp 700B amplifier and a Digidata 1440 A digitizer, respectively, that were controlled by pCLAMP 10.4 software (Molecular Devices).
Statistics

Data are reported as the means ± standard errors (SEs). The Kolmogorov–Smirnov test, jackknife resampling test, Z-test, Student’s t-test, and two-way repeated-measures ANOVA were used to assess the significance of the differences. \( P < 0.05 \) was considered statistically significant. Post hoc power analysis was conducted to evaluate the statistical power. The geometric energy \( E \) was calculated by the following equation (15):

\[
E = \sum \frac{1}{r_{ij}} Z_i Z_j,
\]

where \( r_{ij} \) is the Euclidean distance between neurons \( i \) and \( j \). The \( Z_i \) value was designated as 1 or 0 depending on whether neuron \( i \) was dVenus positive or not, respectively. The geometric energy was compared with the chance level estimated by 10,000 surrogates in which \( Z \) was randomly swapped across neurons.

RESULTS

Few Highly Active Neurons Are dVenus Positive

Arc was immunohistochemically detected in hippocampal neurons of primary cultures prepared from WT mice (Fig. 1A). The fluorescence of dVenus was also microscopically detected in hippocampal neurons of primary cultures prepared from Arc-dVenus transgenic mice (Fig. 1C). To confirm that Arc and dVenus expression was induced during increased neuronal activity, we treated DIV 28 cultures with 50 \( \mu \)M of picrotoxin, a GABA\(_A\) receptor antagonist, for 5 h and then immunostained the cultures with anti-NeuN, a neuronal marker; note that the effect of picrotoxin becomes saturated at 50 \( \mu \)M (16). In control cultures without picrotoxin treatment, only a few neurons exhibited strong Arc or dVenus fluorescence, producing a long-tailed distribution of fluorescence intensity as a whole (Arc: Fig. 1B, \( n = 3,560 \) neurons in 4 cultures, dVenus: Fig. 1D, \( n = 4,480 \) neurons in 4 cultures; also see Fig. 3B). Picrotoxin increased the proportions of...
neurons with strong Arc or dVenus fluorescence (Arc: Fig. 1B, n = 3,576 neurons in 4 cultures, P < 2 × 10^{-306}, D = 0.54, power = 1, Kolmogorov–Smirnov test; dVenus: Fig. 1D, n = 4,537 neurons in 4 cultures, P = 3.5 × 10^{-36}, D = 0.13, power = 1). The overall proportions of Arc-positive neurons were 1.5% ± 0.2% and 45.4% ± 5.6% for the control and picrotoxin conditions, respectively. The proportions of dVenus-positive neurons were 0.84% ± 0.21% and 6.8% ± 0.81% for the control and picrotoxin conditions, respectively. Thus, both endogenous Arc and artificially introduced dVenus were induced in an activity-dependent manner in our in vitro system.

To examine the dynamics of dVenus expression in individual cells, we labeled any given cell at each moment as "dVenus-positive" or "dVenus-negative." Specifically, a neuron with a dVenus fluorescence intensity above the means + 3 × SD of the background intensity was considered dVenus-positive. We recorded spontaneous spikes of DiV 28 neurons in the cell-attached configuration using patch-clamp pipettes (Fig. 1E). dVenus-positive neurons emitted spikes at higher frequencies than those of dVenus-negative neurons (Fig. 1, F and G, n = 8 cells each, P = 0.040, jackknife resampling test, power = 0.54). In addition, the interevent interval of dVenes-positive neurons was shorter than dVenus-negative neurons (Fig. 1H, n = 8 cells each, P = 3.7 × 10^{-28}, D = 0.40, Kolmogorov–Smirnov test, power = 1). This result suggests that spontaneous dVenus expression under control conditions was also induced in an activity-dependent manner.

**Arc-Positive Neurons Have Complex Morphology**

To examine the cell types in our cultures, cultures prepared from WT mice were immunostained with four cell type-specific markers: neuron marker MAP2, microglia marker Iba1, astrocyte marker S100β, and inhibitory neuron marker GAD67 (Fig. 2A). These cell-type-specific markers were not merged...
each other. MAP2-positive neurons accounted for 79.6% of all cells, whereas 13.6% were SI00β-positive astrocytes. There were no Iba1-positive microglia (Fig. 2B). GAD67-positive inhibitory neurons accounted for 11.0% of MAP2-positive neurons (Fig. 2C). We confirmed that the proportion of GAD67-positive inhibitory neurons to the total number of Arc-positive neurons was 16.7% (Fig. 2D). Thus, the majority of Arc-positive cells were excitatory neurons.

To examine the effect of the presence of glial cells on the localization of highly active neurons, the density of glial cells within a radius of 250 μm from a given Arc-positive neuron was compared with that within a randomly selected field of view (Fig. 2E). The mean glial density around Arc-positive neurons seemed to be slightly higher than that within the random field of view, but the difference was not statistically significant (Fig. 2F, n = 12 and 15 images from 3 cultures, P = 0.085, t25 = −1.793, Student’s t test, power = 0.60). This result suggests that glial cells are unlikely to affect neuronal Arc expression, at least under our experimental conditions.

We next investigated the morphology of Arc-positive neurons. To observe the morphology of Arc-positive or Arc-negative neurons, we infected cultured neurons prepared from WT mice with AAV2retro-Cre-EBFP and AAVdj-CBA-Flex-mCherry; neurons were sparsely labeled. Neurons were immunostained with Arc (Fig. 2G), and Sholl analysis was used to evaluate the morphological complexity of these neurons. The number of interactions of Arc-positive neurons across concentric circles was greater than that of Arc-negative neurons [Fig. 2H, n = 21 (Arc-negative) and 6 cells (Arc-positive) from 3 cultures, P = 0.035, F = 4.988, two-way repeated-measures ANOVA, power = 0.47]. Thus, Arc-positive neurons had a more complex morphology than Arc-negative neurons.

dVenus-Positive Neurons Are Spatially Clustered

To investigate whether the locations of dVenus-positive neurons were spatially uniform or biased, neurons were immunostained with NeuN on DiV 49 (Fig. 3A). Geometric energy, an index of spatial clustering that was calculated as the sum of the inverse intersomatic distances between all possible dVenus-positive neuron pairs (7, 15), was higher than the 95% confidence interval of the stochastic chance levels estimated from 10,000 surrogates in which the dVenus expression label was randomly shuffled across all neurons (Fig. 3, B and C). Therefore, dVenus-positive neurons were clustered in the vicinity of each other. This clustering might be affected by cell density. We also prepared low-density cultures by dispersing cells at an initial density of 900 cells/mm2 instead of 1,800 cells/mm2 and found that dVenus-positive neurons were still spatially clustered in the low-density cultures (Fig. 3, B and C). The proportions of dVenus-positive neurons did not differ between the 900 and 1,800 cells/mm² cultures (Fig. 3D, n = 4 cultures, P = 0.70, ts = 0.40, Student’s t test, power = 0.19). Thus, the emergence of highly active cells seemed to be homeostatically balanced against cell densities.

dVenus-Positive Neurons Are a Small, Select Population

To examine the persistence of dVenus expression, we assessed dVenus fluorescence in individual neurons every 6 h for 49 days (Fig. 4A). dVenus-positive neurons began to emerge around DiV 21 (Fig. 4B). The proportion of dVenus-positive neurons remained at less than 0.1% until DiV 35 and then jumped to an apparently plateaued state of ~0.3%–0.4% (Fig. 4B). During our observation period of 49 days, 153 (1.9%) of a total of 8,188 neurons were dVenus-positive for at least 1 day (Fig. 4D, n = 8 cultures).

In individual cells, the duration of continuous dVenus expression ranged from 1 to 15 days. The distribution of the durations of dVenus expression events was compared with the chance level estimated from 10,000 surrogates in which the dVenus expression label was randomly shuffled across all neurons and DiVs (Fig. 4E, Z-test for 8 cultures). Events...
with durations of 1 day were less frequent than that indicated by chance level, whereas longer events were more frequent than that indicated by chance level. Given that dVenus is degraded within several hours (13), this result indicates that the high-activity state of neurons often persists for days. To further confirm the persistence of dVenus expression, we computed 2-successive-day stability values as the mean probability that a cell that was dVenus positive on a given DiV was also dVenus positive on the previous day. Data are shown as the means ± SE of eight cultures.

**DISCUSSION**

In this study, we constantly monitored the expression dynamics of Arc-dVenus, a fluorescent reporter of neuronal activity, in primary hippocampal cell cultures under spontaneous default-mode conditions. We discovered that a subclass of neurons preferentially expressed dVenus. The percentage of dVenus-positive neurons in cultures was almost identical to that under spontaneous conditions in vivo (17). These neurons were numerically sparse but spatially clustered. Our results were consistent with previous studies suggesting that, in primary hippocampal cultures, neuronal activity is heterogeneous, with only a few neurons showing high levels of activity (8). Given that in primary cultures, neurons form circuits depending on their intrinsic wiring programs in the absence of external stimuli, it was intriguing to find that dVenus-positive neurons were spatially clustered. Because the synaptic connection probability is not necessarily correlated with the
distance between cell bodies (18), we cannot directly discuss synaptic connectivity based on the intersomatic distance between highly active neurons, but we consider that there may be some spatially clustered function that is determined by the distance between cell bodies (19). Because a large proportion of neurons were dVenus negative, it is likely that “islands” of highly active neurons exist sparsely in a large network (“sea”) of neurons. In a computational neural network model, such patched structures of active neurons can be displayed through Mexican-hat patterned functional connectivity, with nearby excitation in a short spatial range flanked by surrounding inhibition in a longer range (20). Therefore, there may be some factor(s) that excite(s) nearby neurons and inhibit other neurons in primary neuron cultures.

dVenus-positive neurons started to appear around DiV 21. At this culture stage, the overall level of neuronal activity reaches a steady state (21). However, we found that the ratio of dVenus-positive neurons as well as the stability of dVenus expression in individual cells increased thereafter. After reaching the overall steady state, the network may further be reorganized to stabilize a few, select active neurons. The intrinsic properties and synaptic properties of these neurons may contribute to the emergence of heterogeneous networks. A previous report using cortical slices showed that highly active neurons receive more excitatory and fewer inhibitory inputs than less active neurons (6). Interestingly, the intrinsic excitability in highly active neurons is lower than that observed in less active neurons, probably due to homeostatic counterbalance against hyperexcitatory inputs. Therefore, highly active neurons may emerge mainly through the development of synapses. Although the overall activity level and connectivity of a neuronal network are affected by cell density (22), we found that the ratio of dVenus-positive neurons was invariant to neuron density. Thus, the proportion of highly active neurons seems to be regulated independently of the overall activity level. The activity levels of individual neurons are inherently determined and are kept constant across conditions (3).

Previous studies monitoring in vivo and in vitro neuronal activity revealed that the levels of neuronal activity are stable at certain time scales (9, 23, 24); however, the long-term stability of neuronal activity over weeks remains unknown. Our optical observation of dVenus expression revealed that as few as 0.3%–0.4% of neurons spontaneously expressed dVenus and that the dVenus-positive cells constitutively or repetitively expressed dVenus, forming a distinct subpopulation. Because a portion of the dVenus-positive cells seemed to drift over time during our observation period of 49 days, long-term monitoring is still necessary to verify whether all neurons become dVenus positive again after dVenus expression stops. It has been reported that highly active neurons encode more information than other neurons and play a central role in information processing (4). These neurons may act as a hub that has a significant impact on network activity (25). Experiments with specific ablation of highly active neurons will reveal the various functions of these neurons in organizing circuit activity. Our Arc-dVenus system may provide a unique experimental platform to investigate the function and behavior of highly active neurons. However, as the Arc protein is involved in long-term depression as well as long-term potentiation and is transsynaptically transferred to neighboring neurons, data derived using this protein have to be interpreted with care.

GRANTS
This work was supported by Japan Science and Technology Agency Exploratory Research for Advanced Technology (JST ERATO) Grant JPMJER1801 and Japan Society for the Promotion of Science (JSPS) Grants-in-Aid for Scientific Research Grant 18H05525. This research was partially supported by the Institute for AI and Beyond of The University of Tokyo.

DISCLAIMERS
Any opinions, findings, conclusions, or recommendations expressed in this article are those of the authors alone and do not necessarily reflect the views of United States Agency for International Development (USAID) or National Academy of Sciences (NAS).

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS
M.O., R.K., and Y.I. conceived and designed research; M.O., R.K., Y.S., and C.K. performed experiments; M.O. analyzed data; M.O. interpreted results of experiments; M.O. prepared figures; M.O. and Y.I. drafted manuscript; M.O., R.K., Y.S., C.K., and R.K. approved final version of manuscript.

REFERENCES


