PHYSICAL REVIEW E **75**, 050901(R) (2007)

Towards neuro-memory-chip: Imprinting multiple memories in cultured neural networks

Itay Baruchi and Eshel Ben-Jacob
School of Physics and Astronomy, Raymond & Beverly Sackler Faculty of Exact Sciences, Tel-Aviv University, Tel-Aviv 69978, Israel
(Received 3 August 2006; published 16 May 2007)

We show that using local chemical stimulations it is possible to imprint persisting (days) multiple memories (collective modes of neuron firing) in the activity of cultured neural networks. Microdroplets of inhibitory antagonist are injected at a location selected based on real-time analysis of the recorded activity. The neurons at the stimulated locations turn into a focus for initiating synchronized bursting events (the collective modes) each with its own specific spatiotemporal pattern of neuron firing.

DOI: 10.1103/PhysRevE.75.050901 PACS number(s): 87.19.La, 84.35.+i

Understanding learning and memory in real neural networks is one of the central challenges in neuroscience. Adopting the cognitive perspective, Squire proposed that "Learning is the process of acquiring new information, while memory refers to the persistence of learning in a state that can be revealed at a later time" [1,2]. Hopfield and Tank used a statistical physics approach in relating memory and learning with global modifications in the network's connectivity [3]. Later studies extended these ideas with an attempt to understand the effects of synapse plasticity on the network activity, assuming that memory and learning are associated with the collective dynamics of the network as a whole [4–11]. The experimental efforts have traditionally been largely based on electrical stimulations [12,13], and more recently also on chemical stimulations [11,14–16]. Generally speaking, the approach is to monitor the response of the network activity to sessions of stimulations, looking for induced (imprinted) persistent alterations in the patterns of neuron firings. To allow systematic and parallel recording of several neurons, many investigators use cultured networks (dissociated neurons grown on an artificial substrate) as a model

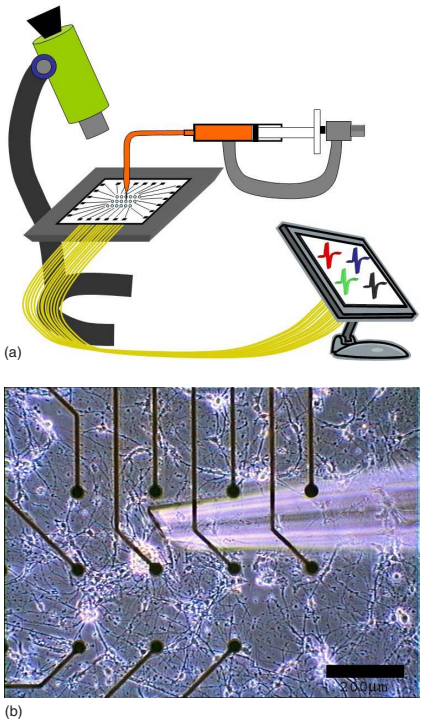

FIG. 1. (Color online) System used for local chemical stimulations. A micropipet mounted on a micrometer stage (a) is used for local chemical stimulation at a specific selected location. In (b) we show the tip of the micropipet placed above one of the recording electrodes 30 μm in diameter (the bar is 200 μm). To control the volume of the stimulating droplets, the micropipet is connected to a syringe that is mounted on a second micrometer stage.

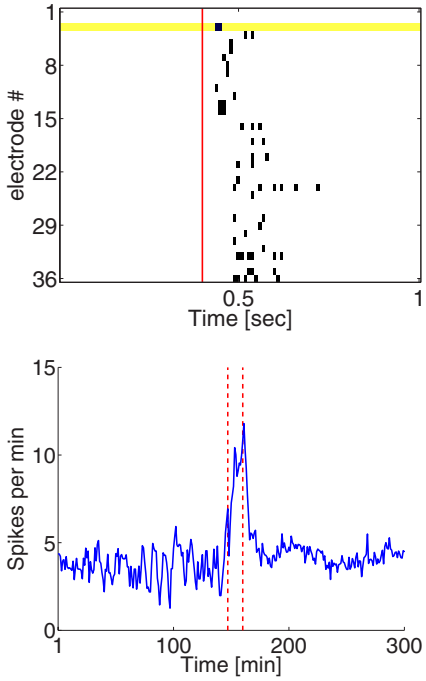

FIG. 2. (Color online) Network response to local chemical stimulations. (a) Raster plot representation of the network response to a stimulation of a single droplet. The time axis is divided into 10 ms bins. Each row is a binary bar-code representation of the activity of an individual neuron—a bar or 1 is plotted each time the neuron fires an action potential. The red (vertical) line indicates the time of injection. The yellow (horizontal) rmarker represents the recorded neuron closest to the location of the stimulation. As seen, one injection is sufficient to initiate a single synchronized bursting event with a distinct pattern of neuronal firing. The activity starts at the vicinity of the stimulated location and continues to propagate along a fixed path—a specific neuron fires second, another third, and so on [29,30]. (b) Network activity rate before, during, and after a stimulation session. As seen, the activity rate of the network is elevated while the network is stimulated. Once the stimulation session is over the network returns to its previous activity rate.

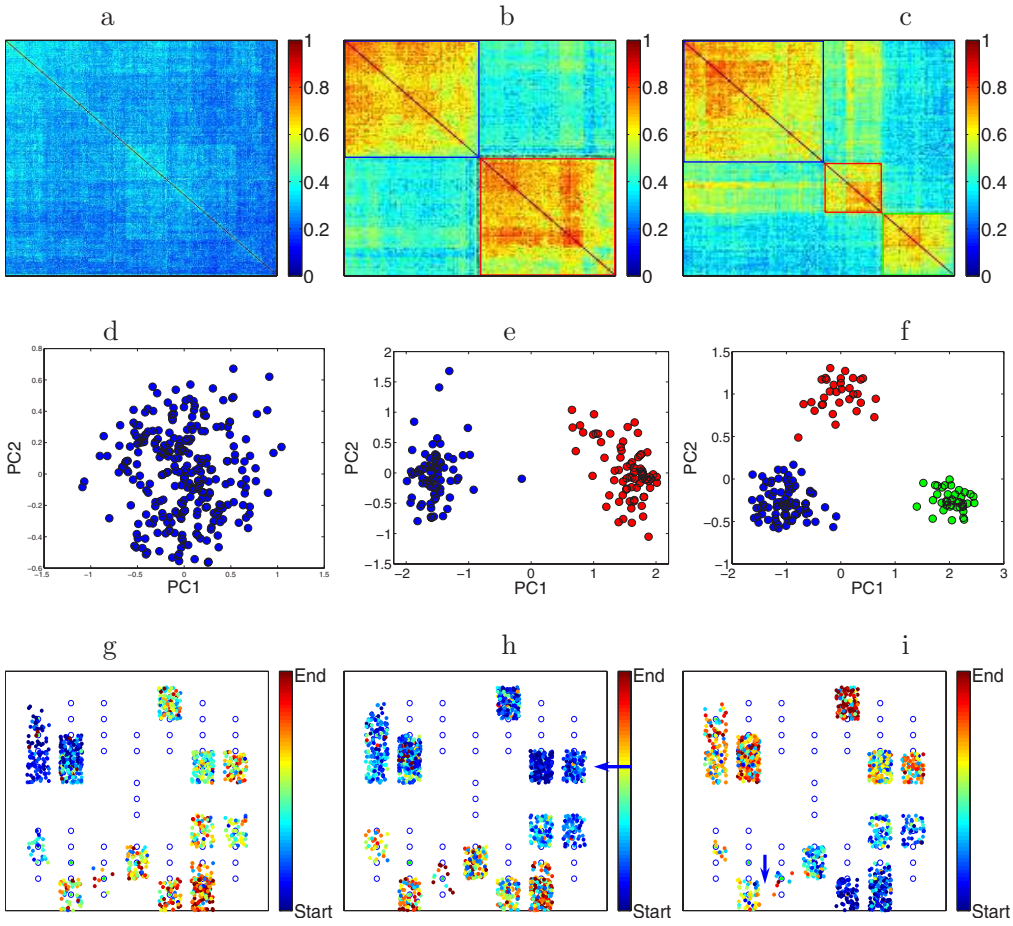

FIG. 3. (Color online) Imprinting new classes of SBEs. (a) The inter-SBE correlation matrix before stimulations. (b) The effect of the first session of stimulations and (c) after the second stimulation session. The matrices in the three cases are reordered using the dendrogram clustering algorithm. (d)–(f) Result of projecting the correlation matrices on the two leading principal vectors of the PCA clustering algorithm. As seen on the left, (a) and (d), before stimulation, only one type of SBE is exhibited by the network. In the middle, (b) and (e), a new class of SBEs appears (red/right) in addition to the previously existing one (blue/left) indicating that it has been created by the stimulations. On the right, (c) and (f), we see that a third class of SBEs (the green cluster/right) is created by the second set of stimulations without erasing the two existing ones. The second session was applied 24 h after the first one. To present the propagation patterns, we plot the propagation diagram for the three classes of SBEs (g)–(i). We define for each neuron i the time it fires its first spike SBE no. n as T_n^i. We then draw for each SBE a colored dot for each neuron to indicate its relative time of firing T_n^i using the jet color code: blue (dark) for early firing and red (light) for later firing. The blue (dark) arrows in the middle and right panels indicate the locations where the stimulations were applied.

system to study these aspects [8,9] as is done here.

Motivated by theoretical investigations regarding the important role of inhibitory neurons on the regulation of the internal dynamics of the synchronized bursting events (SBEs) [4,5,17,18] we decided to perform stimulations of inhibitory neurons. The chemical stimulations were performed by positioning a micropipet over a small group of neurons [Figs. 1(a) and 1(b)] [19] and injection of microdroplets (10 μl) at a rate of one every 20 s of 100 μM picrotoxin dissolved in neuron growth medium [an ionotropic gamma-aminobutyric acid (GABA) antagonist, which reduces the influence of inhibitory synapses [20]]. By using this method we guaranteed that the injection has a localized effect; during the short time of injection (a fraction of a second) the picrotoxin can diffuse to relatively short distances (the diffusion constant is about 25 $\mu m^2/s$ [21]). Using the latter and solving the diffusion equation we obtain that the concentration of the picrotoxin drops by factor of 10 at a distance of about 5 μm [22].

Many investigations showed that the activity of stand-alone cultured neural networks is marked by the spontaneous formation of synchronized bursting events—short time windows (~200 ms) of rapid neuronal firings separated by long time intervals (seconds) of sporadic firing [23]. From the dynamical systems perspective, the SBEs are collective modes of activity each with its own specific complex spatiotemporal patterns of neuronal firing—the activity starts at a specific location (the focus or initiating point) and propagates along a specific trajectory in space. It was suggested that the spontaneous formation of SBEs reflects the action of innate system level mechanisms that are associated with *in vivo* mechanisms of memory and learning [7,10,24,25]. The idea is that the spontaneous formation of SBEs in the activity of stand-alone cultured networks implies that they can be

regarded as memory templates or precursers of the *in vivo* memory templates.

Here we show that, by using focal chemical stimulations on cultured neuronal networks [26] at specific selected locations [27] and with a specific antagonist, picrotoxin (which satisfies the above requirements), we could create new SBEs in the network activity. To select a proper location for the stimulations, it is necessary to perform system level analysis of the network activity to reveal the activity propagation for the existing SBEs. Based on the analysis, a location that does not serve as an initiator (an activity starting point) for the existing SBEs is selected. Consequently the chemical stimulations are applied at this location.

Even an injection of a single microdroplet is sufficient to initiate a single synchronized bursting event with a distinct pattern of neuronal firing. The activity of the induced SBE starts at the vicinity of the stimulated location and continues to propagate along a fixed path as is seen in Fig. 2. Our method enables to store (imprint) the induced SBE in the network activity, i.e., to create a persistent new class of SBEs. The imprinting is performed by stimulating the network with a periodic (tonic) set of 20 injections at 20 s intervals [28]. To analyze the long-term effects of the stimulations and in particular the persistent and stability of the newly created SBE, we continue to record the spontaneous activity of the network for a long time (over 24 h) and apply the analysis methods describe next.

To identify and quantify the classification of the SBEs into distinct subgroups, each with its own characteristic internal patterns of neuronal activity, we first evaluate the inter-SBE correlation matrix $\rho(n,m)$ [7] defined as

$$\rho(n,m) = \max\left(\sum_{i=1}^{N} C_i^{n,m}(\tau)\right) \tag{1}$$

where $C_i^{n,m}(\tau)$ denotes the cross correlation between the activity of the ith neuron in the nth and mth SBEs. Next, the correlation matrix is clusterized using the standard dendrogram hierarchical clustering method [31]. To visualize the clusters, we use the principal component analysis (PCA) algorithm for dimension reduction [31]. We project the correlation matrix on the first two principal vectors obtained by PCA, and color each point according to its cluster obtained by the dendrogram hierarchical tree.

The results shown in Figs. 3(a)–3(f) illustrate that by local chemical stimulation it is possible to create several additional distinct classes of SBEs, without erasing the existing ones [32]. As seen in Figs. 3(g)–3(i), each class of SBEs has its own characteristic spatiotemporal pattern of activity propagation. The activity of each class starts at a specific location, close to the location of the chemical stimulations that created this class (accept for the spontaneous SBE), and then propagates along a specific trajectory [29,30]. These results indicate that our method enables us to imprint a collective mode with a predesigned spatiotemporal pattern. After the stimulation session, the network continues to spontaneously generate the newly created classes of SBEs (memories) for long periods of time (more than 40 h), as seen in Fig. 4. The imprinting of multiple collective modes

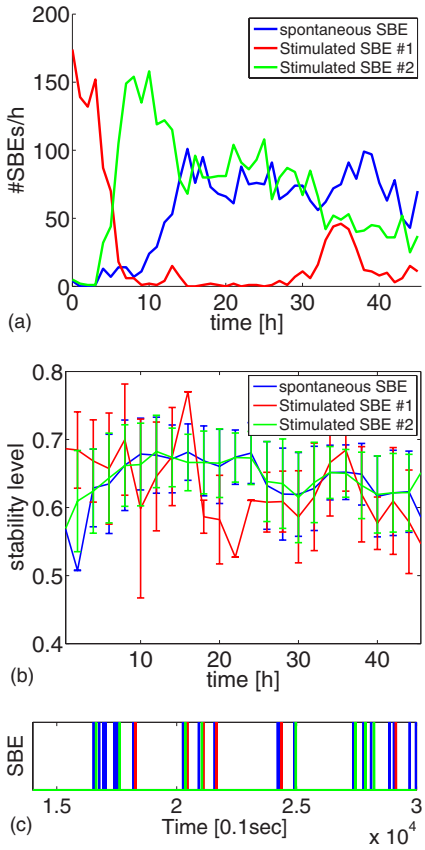

FIG. 4. (Color online) Persistence of the collective modes. (a) Rate of appearance of the three classes of SBEs after the second stimulation session (from a different network than the one whose results were shown earlier). The original existing class of SBEs is marked blue (dark), the first imprinted class of SBEs red (medium darkness), and the second imprinted class green (light). Although the marked red class was imprinted 24 h before the green one it still persists for more than 72 h. While the appearance rate of the green (light) class first goes down it goes up again, and so on. (b) Stability levels of each group of SBEs. Each line represents the level of correlations between each SBE (of a specific class) and the representative SBE of that class (an averaged one over all the SBEs that belong to the class). As seen, the correlation levels are significantly high (average correlation level of 0.65 ± 0.05) and preserved for more than 40 h. The results demonstrate the robustness of the specific spatiotemporal firing patterns of each of the imprinted memory templates. (c) A segment of the recorded sequence of SBEs colored according to their spatiotemporal patterns. As seen, the spontaneous and imprinted SBEs are exhibited by the network, mixed in their order of temporal appearance.

was performed on five different cultured networks and produced the same results. Stimulations with growth medium alone (no added chemicals) or with calcium as an excitatory promoting chemical initiate formation of new SBEs with each injection, but these do not persist after the stimulation session is terminated. That is, these stimulations did not lead to the imprinting of the induced SBEs.

In summary, we showed that by using a special protocol of local chemical stimulations it is possible to imprint predesigned new classes of SBEs in the network activity without erasing previously existing ones. It has been proposed before

that the SBEs in cultured networks can be viewed as memory templates or precursers of memory-related activity modes in task-performing *in vivo* networks; and that the SBEs indicate that the spatiotemporal patterns of neuronal firing represent information encoded in the correlations between the neuron activities during the SBEs [5–7,10,18,24]. In light of this perspective, we discovered a method for imprinting multiple predesigned and enduring memory templates in the activity of cultured networks. These findings hint that chemical signaling mechanisms might play a crucial role in memory and learning in task-performing *in vivo* networks.

In this regard we note that, since the activity of the created class of SBEs is initiated at the location of the stimulation, it brings to mind seizurelike events. The latter and other epileptiform events such as bursting and paroxysmal depolarizing shifts were suggested as likely candidates to create "memory" in a neuronal circuit [33].

To conclude, the method presented here using picrotoxin as a specific antagonist should be perceived as a proof of concept of the approach of learning by local chemical stimulations. It is likely that additional antagonists will be proven to work as well.

We are most thankful to D. Horn, A. Ayali, R. Segev, G. Bi, H. Levine, S. Schiff, and Y. Shapira for critical and constructive comments on the manuscript. We thank I. Brainis for technical assistance in preparation of the cultured networks. This research was partially supported by a grant from the Israeli Science Foundation and the Tauber Fund.

[1] L. R. Squire, *Memory and Brain* (Oxford University, Oxford, 1987).
[2] L. R. Squire, Neurobiol. Learn Mem. **82**, 171 (2004).
[3] J. J. Hopfield and D. W. Tank, Science **233**, 625 (1986).
[4] E. M. Izhikevich *et al.*, Cereb. Cortex **14**, 933 (2004).
[5] V. Volman *et al.*, Phys. Biol. **2**, 98 (2005).
[6] I. Baruchi *et al.*, Chaos **16**, 015112 (2006).
[7] R. Segev *et al.*, Phys. Rev. Lett. **92**, 118102 (2004).
[8] E. Maeda *et al.*, J. Neurosci. **15**, 6834 (1995).
[9] D. A. Wagenaar *et al.*, J. Neurosci. **25**, 680 (2005).
[10] E. Schneidman *et al.*, Nature (London) **440**, 1007 (2006).
[11] D. Eytan *et al.*, J. Neurosci. **23**, 9349 (2003).
[12] B. Milner *et al.*, Neuron **20**, 445 (1998).
[13] Y. Mu and M. M. Poo, Neuron **51**, 115 (2006).
[14] D. Eytan *et al.*, J. Neurophysiol. **92**, 1817 (2004).
[15] J. M. Beggs and D. Plenz, J. Neurosci. **23**, 11167 (2003).
[16] T. I. Netoff and S. J. Schiff, J. Neurosci. **22**, 7297 (2002).
[17] T. J. Sejnowski and O. Paulsen, J. Neurosci. **25**, 1673 (2006).
[18] V. Volman *et al.*, Physica A **335**, 249 (2004).
[19] For local chemical stimulation, the local stimulation setup includes a micropipet connected by a short tube to a syringe. The syringe is mounted on an accurate micrometer, which allows precise injection of the chemical substance. The pipet is mounted on a microstage connected to a microscope. On the microscope plate we place the multielectrode array recording system. By using the microstage and looking through the microscope, we achieve high accuracy in placing the pipet tip over one of the recorded neurons.
[20] J. D. Goutman and D. J. Calvo, Br. J. Pharmacol. **141**, 717 (2004).
[21] V. N. Murthy *et al.*, Proc. Natl. Acad. Sci. U.S.A. **97**, 901 (2000).
[22] Picrotoxin has been used to generate foci of epileptic seizers in *in vivo* models using high concentrations that cause cell damage. G. Anlezark and B. Meldrum, Psychopharmacology (Berlin) **57**, 57 (1977). Here we followed Refs. [15,16], which used lower concentrations to stimulate the activity of slices without causing cell damage. In the local stimulations used here the the final concentration after the diffusion process is completed decreases to 6 μM. Using optical microscope observations we found that even concentrations of 250 μM did not cause cell death.
[23] R. Segev *et al.*, Phys. Rev. Lett. **88**, 118102 (2002).
[24] I. Baruchi and E. Ben-Jacob, Neuroinformatics **2**, 333 (2004).
[25] Y. Ikegaya *et al.*, Science **304**, 559 (2004).
[26] The cultured network preparation protocol is as described in Ref [23]. We use glia and neuron cells dissociated from the cortex of one-day-old Sprague-Dawley rats. The networks have relatively high neuron density—about 10 000 cells per mm^2. We start recording the network activity between the 10th and 25th day *in vitro*. We used a commercial multielectrode array (MEA) consisting of 60 substrate-integrated thin film microelectrodes (MEA chip, Multi Channel Systems) of $(10-20)\mu$. The electrodes are arranged according to our design of four subgroups of electrodes. The spacing between electrodes is 200 μm within the subgroups and 1000 μm between subgroups.
[27] O. Feinerman *et al.*, J. Neurophysiol. **94**, 3406 (2005).
[28] Each stimulation session consists of 20 stimulations, one every 20 s, of $\sim$10 μl growth medium to which we add picrotoxin to reach a concentration of 100 μM (raising the overall concentration of picrotoxin in the growth medium in which the cultured network is immersed by $\sim$6 μM).
[29] M. Abeles, *Corticonics: Neural Circuits of the Cerebral Cortex* (Cambridge University Press, New York, 1991).
[30] Y. Aviel *et al.*, Neurocomputing **44**, 285 (2002).
[31] W. J. Krzanowski, *Principles of Multivariate Analysis: A User's Perspective* (Oxford University Press, Oxford, 2000).
[32] To test the significance of the obtained partitioning we used the Kolmogorov-Smirnov statistical test, W. H. Press, S. A. Teukolsky, W. T. Vetterling, and B. P. Flannery, *Numerical Recipes in C* (Cambridge University Press, Cambridge, U.K., 1992) for comparing the distributions of two samples. We determined that the two clusters are indeed significantly different (P value 2.8×10^{-6}). Comparisons of the standard deviations of the two distributions yields that the mean correlations within a block and between blocks are 0.65 ± 0.09 (N=5625) and 0.4 ± 0.07 (N=5625), respectively.
[33] S. Z. Morgan *et al.*, Hippocampus **1**, 207 (2004).