Dynamics of Learning in Cultured Neuronal Networks with Antagonists of Glutamate Receptors

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ABSTRACT Cognitive dysfunction may result from abnormality of ionotropic glutamate receptors. Although various forms of synaptic plasticity in learning that rely on altering of glutamate receptors have been considered, the evidence is insufficient from an informatics view. Dynamics could reflect neuroinformatics encoding, including temporal pattern encoding, spatial pattern encoding, and energy distribution. Discovering informatics encoding is fundamental and crucial to understanding the working principle of the neural system. In this article, we analyzed the dynamic characteristics of response activities during learning training in cultured hippocampal networks under normal and abnormal conditions of ionotropic glutamate receptors, respectively. The rate, which is one of the temporal configurations, was decreased markedly by inhibition of α-amino-3-hydroxy-5-methylisoxazole-4-proprionic acid (AMPA) receptors. Moreover, the energy distribution in different characteristic frequencies was changed markedly by inhibition of AMPA receptors. Spatial configurations, including regularization, correlation, and synchrony, were changed significantly by inhibition of *N*-methyl-D-aspartate receptors. These results suggest that temporal pattern encoding and energy distribution of response activities in cultured hippocampal neuronal networks during learning training are modulated by AMPA receptors, whereas spatial pattern encoding of response activities is modulated by *N*-methyl-D-aspartate receptors.

INTRODUCTION

Cognitive dysfunction concomitant with some cerebral diseases, such as schizophrenia (1,2) and Alzheimer's disease (3,4), may result from abnormality of ionotropic glutamate receptors (iGluRs), especially *N*-methyl-D-aspartate (NMDA) receptors (5,6). However, it is still unclear whether the mechanism mentioned above would occur in various forms of learning dysfunction. In an effort to understand mechanisms of learning dysfunction at a network level from the standpoint of informatics, we first constructed one selective learning model of cultured hippocampal neuronal networks, then studied the dynamic characteristics of response activities in neuronal networks during learning training under normal and depressed levels of iGluRs, respectively, based on the learning model.

The realization of higher functions, such as learning and memory, ultimately relies on information processing, storage, and transmission (7). In these circumstances, the brain may have one universal working principle. Discovering neuronal information encoding is crucial to understanding the basic working principle of the neural system. Although various forms of synaptic plasticity in learning that rely on altering of iGluRs have been considered in previous studies, the evidence is still insufficient from an informatics standpoint. Response activities of the neuronal network during learning training can be modulated by low-frequency electrical stimulation. This is a kind of activity-dependent neuronal plasticity. Thus, study of dynamic characteristics of response activities in the neuronal network during learning is helpful in understanding learning mechanisms at the network level, and could lead to an understanding of the working principle of the neural system. In addition, studying response activities during learning training under abnormal levels of iGluRs is useful for understanding mechanisms of learning dysfunction. In particular, studies carried out in cultured realistic neuronal networks may help us to discover one general mechanism of learning, since cultured neuronal networks could be a simplified model of the complex neural system (8,9).

The dynamics of electrophysiological activities in the neuronal network include primarily spatiotemporal configurations and energy distribution (10–15). Spatiotemporal configurations of electrophysiological activities in the brain are thought to contribute to neuronal information encoding and synaptic contacts (7,12), which may play a vital role in the formation of privileged pathways in neuronal population activities. Energy distribution in different characteristic frequencies reflects the functional status of the neuronal network (16). Therefore, determining spatiotemporal configurations and energy distribution of response activities is an important step in discovering the information encoding of neuronal networks during learning.

For cultured neuronal networks, learning is an exploration process that involves formation and modulation of associations between stimuli and responses (17–20). In fact, learning a new cognitive task is also the selective procedure of appropriate circuits in the neuronal network for information transmission. Repeated cycles of a stimulation procedure could

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lead to a desired response and learning at the network level. The learning model at the network level can be constructed by applying low-frequency electrical stimuli (17). We modified the learning model by altering stimulation patterns. Using this kind of learning model, dynamic characteristics including spatiotemporal pattern encoding and energy distribution of neuronal response activities in cultured hippocampal networks were studied during learning training under normal and abnormal conditions of iGluRs, respectively.

MATERIALS AND METHODS

Cell culture

Hippocampal cells were dissociated from embryonic rats of 18 days and plated on a multielectrode array (MEA). Animal use was in accordance with guidelines approved by Chinese local authorities. Cells were placed in a medium including Dulbecco's modified Eagle's medium (DMEM, Gibco, Carlsbad, CA), with 0.5 mM Glutamax (Invitrogen, Carlsbad, CA), 10% equine serum (HyClone, Logan, UT), and 10% fetal bovine serum (Gibco). One hundred thousand cells were planted in a 50-μL drop of modified DMEM on an MEA dish that was precoated with polyethyleneimine and laminin. This led to a planting density of 2000 cells/mm^2 in a monolayer. After half an hour of incubation, 1 mL of modified DMEM was added into each dish. After 24 h, the planting medium was replaced by a medium including DMEM with 0.5 mM Glutamax and 10% equine serum, but with no antibiotics or antimycotics. Cultures were maintained in an incubator at 37°C with 5% CO_2. One-half of the medium was changed every 3 days. Experiments were done when neuronal networks were 2–6 weeks in vitro.

Electrophysiological analysis

Electric activities were recorded with a square array of 60 substrate-embedded titanium nitride electrodes, with a diameter of 30 μm and 200-μm spacing (Multi Channel Systems, Reutlingen, Germany). Stimuli were generated by using a four-channel stimulator (Multi Channel Systems). After 1200× amplification, signals were sampled at 25 kHz. Thresholds (5 × root mean-square noise) were separately defined for each of the recording channels.

A learning model at the network level on the MEA system was constructed by applying 350 mV, 200 μs, 1 Hz pair stimulation, and the neuronal network responded to the stimulation by generating electric activities. The training protocol was similar for Shahaf et al. (17), except that the voltage stimulation mode was used, which consisted of biphasic rectangular voltage pulses and positive phase firsts. Further details can be found in our previous work (21,22). Four response modes were induced in cultured hippocampal neuronal networks during learning within the safe stimulation intensity range. Individual response mode was induced by 350–450 mV, 200 μs, and 1 Hz pair stimulation, mixed response mode was induced by 500–800 mV, 200 μs, and 1 Hz pair stimulation, periodic response mode was induced by 900–1500 mV, 200 μs, and 1 Hz pair stimulation, whereas quasiperiodic response mode was induced by 30–50 μA, 200 μs, and 1 Hz pair stimulation. In this article, the learning model we used was constructed by applying 350 mV, 200 μs, and 1 Hz pair stimulation. Individual response mode was induced by the training mode mentioned above (see Fig. 3 *A*).

Once the required response was attained, the stimulus was removed. If the response time (i.e., the time required for the selected electrode to fulfill the response/stimulus ratio (R/S) $\geq$ 2:10 criterion) decreased gradually in eight trials in the stimulation cycle, the simple learning phenomenon had been induced in the neuronal network. To ensure the stability of response activities in the network during training, we designed another series of experiments. After the first successful training trial, the neuronal network was trained every 0.5 h for several hours, and response activities were detected. We found that the R/S did not change much in 4 h, which suggested that the response activities were stable. The selective learning phenomenon has been induced if $R/S \geq$ 2:10 in the selected electrode but not in the monitored electrode.

To compare dynamic characteristics of response activities in cultured hippocampal networks during learning training under normal and abnormal conditions of iGluRs, specific antagonists were applied to the networks. First, the networks were trained to learn successfully; then, 50 μM *d,l*-2-amino-5-phosphonovaleric acid (APV), 50 μM 6-cyano-7-nitroquinoxaline-2,3-dione disodium (CNQX), 50 μM APV + 50 μM CNQX, or 2 mM Mg^{2+} was added into the bath solution, the networks were trained again, and response activities of the networks were detected. After that, the medicine was washed out, the networks were trained and the electric activities were recorded again.

Data analysis

Electric activities of neuronal networks were recorded by Mc_Rack, and spike and burst analysis were done with Neuroexplore. Data are expressed as mean ± SE, and were normalized by MATLAB (The MathWorks, Natick, MA) programs; *t*-tests were used to detect differences between the two groups. $P < 0.05$ was considered statistically significant.

RESULTS

Cultured hippocampal neuronal networks and spontaneous burst activities

The hippocampal neurons cultured on the multielectrode array form numerous synaptic connections (Fig. 1). This is apparent from the observation of various independent activity patterns, especially the synchronized burst activities in the neuronal network (Fig. 2 *A*). The results imply that single neurons seldom fire spontaneously without being activated by other neurons in cultured hippocampal networks. In fact, many of the connections observed under the microscope are actually parts of larger groups of connected units in the neuronal network.

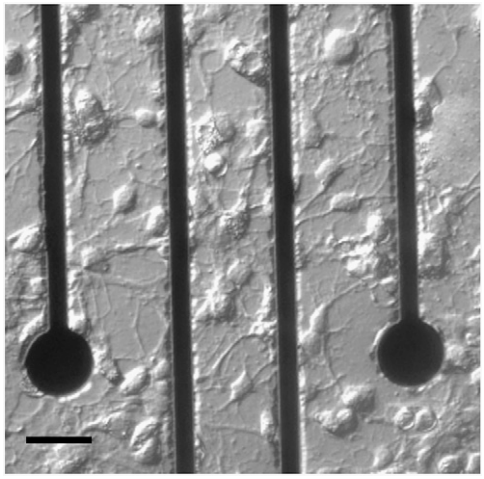

FIGURE 1 Representative cultured hippocampal neuronal network (25 div). Scale bar, 30 μm.

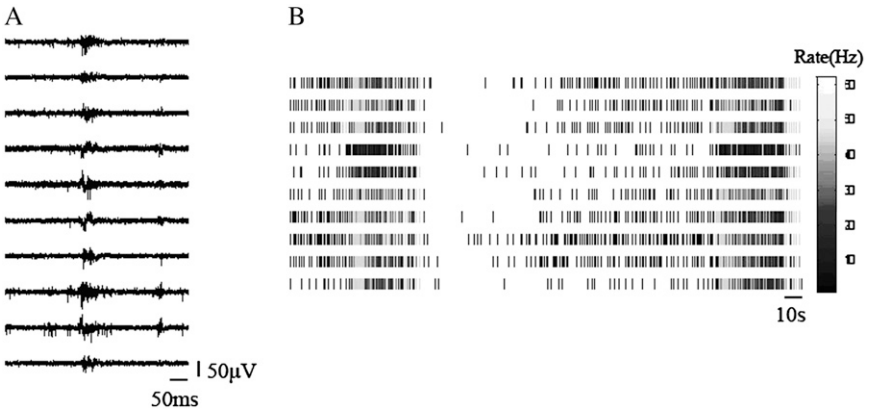

FIGURE 2 (*A*) Examples of spontaneous activity in cultured hippocampal neuronal networks (21 div). Each trace shows original recordings of electric activities on an electrode. (*B*) Raster examples of spontaneous activities in cultured hippocampal neuronal networks (21 div).

In our observation, most cultures showed initial spiking activities at ~1 week after cell seeding. With few exceptions, complex burst configurations were generated at 2–3 weeks after cell seeding. A raster of spontaneous activities in the neuronal network (21 days in vitro (div)) is shown in Fig. 2 *B*. If one spike event occurred, one vertical line is recorded. We observed that spontaneous synchronized oscillatory activities in the neuronal network occurred twice in 300 s (Fig. 2 *B*). Synchronized oscillatory activity is a major activity mode of mature and high-density dissociated neuronal cultures. Generally, spontaneous activities found in cultured hippocampal networks range from apparently stochastic spiking to organized bursting and even stable, long-term synchronized oscillatory activities.

Dynamic characteristics of response activities during learning training with antagonists of *i*GluRs

Spatiotemporal configurations and energy distribution can reflect dynamics of neuronal activities in the network. Temporal configurations of neuronal activities include rate, amplitude, firing probability, and interval of spike. Spatial configurations of neuronal activities include regularity, correlation, and synchrony. In this study, change of temporal configurations, spatial configurations, and energy distribution were used to reflect dynamics characteristic of neuronal response activities in the network during learning training.

Temporal configurations of early postsynaptic responses were changed by special antagonists of iGluRs in the neuronal network during learning training. As shown in Fig. 3, application of 50 μM APV decreased the rate and amplitude of early postsynaptic responses by 32% and 37%, respectively. Application of 50 μM CNQX decreased these configurations by 76% and 31%, respectively. All synaptic events were abolished by subsequent application of 50 μM APV and 50 μM CNQX. Application of 2 mM Mg^{2+} reduced the rate and amplitude of early postsynaptic responses by 53% and 24%, respectively. In a word, APV, CNQX, and high-concentration Mg^{2+} simultaneously inhibited the mean firing rate and amplitude of early postsynaptic responses during learning training. At the same time, the distribution of firing probability of response activities in networks was changed markedly by applying specific antagonists of ionotropic glutamate receptors during learning training (Fig. 4). Briefly, the rate, one of the temporal configurations, was modulated primarily by α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptors.

Interspike interval (ISI) is defined as the time interval between two consecutive spikes in the spike trains:

$$ISI_{\mathrm{n}} = t_{\mathrm{n}} - t_{\mathrm{n}-1}$$

According to ISI_{n}, the mean of ISI is calculated, which is represented as

$$\mu_{\mathrm{Rj}} = \frac{1}{N_{\mathrm{j}}} \sum_{\mathrm{i}=1}^{N_{\mathrm{j}}} I_{\mathrm{ij}}.$$

The standard deviation of ISI is represented as

$$\sigma_{\mathrm{Rj}}^2 = \frac{1}{(N_{\mathrm{j}} - 1)} \sum_{\mathrm{i}=1}^{N_{\mathrm{j}}} (I_{\mathrm{ij}} - \mu_{\mathrm{Rj}})^2.$$

Here, we use the standard deviation of ISI and the mean of ISI to express precisely the characteristics of response activities in different spatiotemporal firing configurations. The coefficient of variation (CV) is represented as

$$\mathrm{CV} = \frac{\sigma_{\mathrm{R}}}{\mu_{\mathrm{R}}}.$$

Ranges of CV are set according to Young et al. (23). When $\mathrm{CV} \leq 0.35$, firing of neurons is highly regular; when $0.35 < \mathrm{CV} < 0.7$, firing of neurons is likely irregular.

The CV of early postsynaptic responses with 50 μM APV was >0.35, which indicated that response activities of the neuronal network during learning training were caused to become irregular by treatment with 50 μM APV. However, 50 μM CNQX or 2 mM Mg^{2+} seemed to have no effect on the variability of response activities in the neuronal network (Table 1).

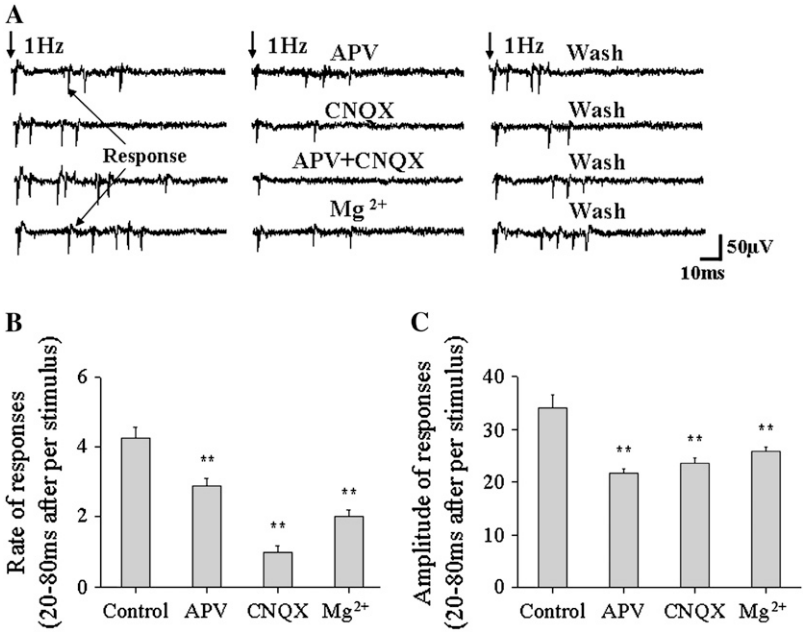

FIGURE 3 Effects of APV, CNQX, and Mg^{2+} on response activities in cultured hippocampal neuronal networks during learning training ($n = 24$ experiments in six cultures). (*A*) Examples of original traces of response activities with 50 μM APV, 50 μM CNQX, 50 μM APV + 50 μM CNQX, and 2 mM Mg^{2+}. (*B*) Effects of 50 μM APV, 50 μM CNQX, and 2 mM Mg^{2+} on the rate of early postsynaptic responses. **, $p < 0.01$. (*C*) Effects of 50 μM APV, 50 μM CNQX, and 2 mM Mg^{2+} on the amplitude of early postsynaptic responses. **, $p < 0.01$.

Joint peristimulus time histography (PSTH) is used to estimate correlation and synchrony between two neurons (24–26). Here, we used joint PSTH to estimate correlation and synchrony among neuronal units in the neuronal network. Fig 5 shows examples of correlation and synchrony of response activities between one recording channel and another in physiological solution and during drug treatment. The main joint PSTH matrix shows the correlations between electric activities of two channels. The middle histogram shows the near-coincident correlations. Where the diagonal alignment is clearer, the synchrony is better. The far-right histogram shows the correlations of electric activities of two channels around reference events.

In the case of addition of APV, the disordered status occurred in the neuronal network, as evidenced by an immediate decrease in correlations of response activities (correlation coefficient = 0.111) (Fig. 5). Application of CNQX decreased the correlations (correlation coefficient = 0.251) of neuronal response activities to a certain extent, along with the synchrony. Since all postsynaptic responses were abolished by subsequent application of 50 μM APV and 50 μM CNQX, we didn't evaluate the correlations under such circumstances. In the case of 2 mM Mg^{2+} treatment, the correlations (correlation coefficient = 0.312) remained quite similar with respect to the basal value (Fig. 5). In fact, we found a very high variability for Mg^{2+} experiments in terms of correlation analysis and synchrony analysis, including prosperity and decadence. But the general trend seems to be an immediate depressed response. This high variability of results should be further investigated with respect to the initial activity of the neuronal network.

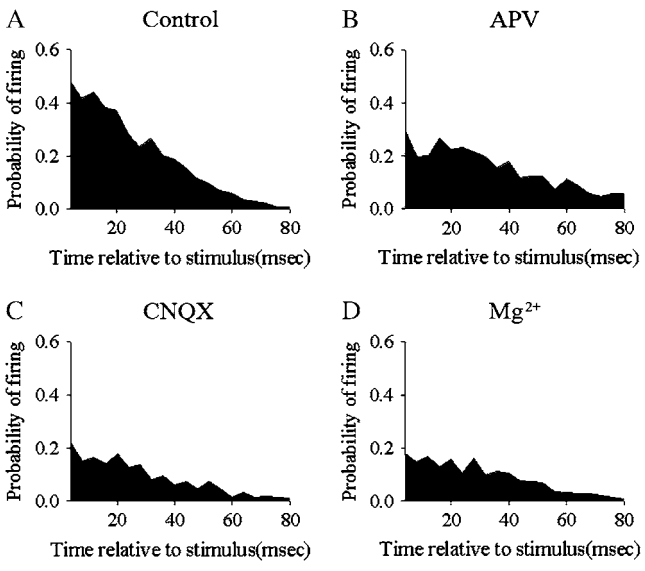

FIGURE 4 PSTH ($n = 36$ experiments in six cultures) within 80 ms after stimulus during learning training in cultured hippocampal neuronal networks without treatment (*A*), and after treatment with 50 μM APV (*B*), 50 μM CNQX (*C*), and 2 mM Mg^{2+} (*D*).

TABLE 1 CV of early postsynaptic responses during learning training in cultured hippocampal neuronal networks in the absence and presence of pharmacological inhibitors

	Control	APV	CNQX	Mg^{2+}
σ_R (ms)	4.64 ± 0.19	13.40 ± 0.64	31.70 ± 1.74	14.96 ± 0.73
μ_R (ms)	29.08 ± 1.22	36.23 ± 1.63	125.09 ± 6.88	75.09 ± 3.68
CV	0.16 ± 0.01	0.37 ± 0.02	0.25 ± 0.01	0.20 ± 0.01

Values given are for untreated cells (Control) and cells treated with 50 μM APV, 50 μM CNQX, or 2 mM Mg^{2+} ($n = 28$ experiments in six cultures). CV, coefficient of variation.

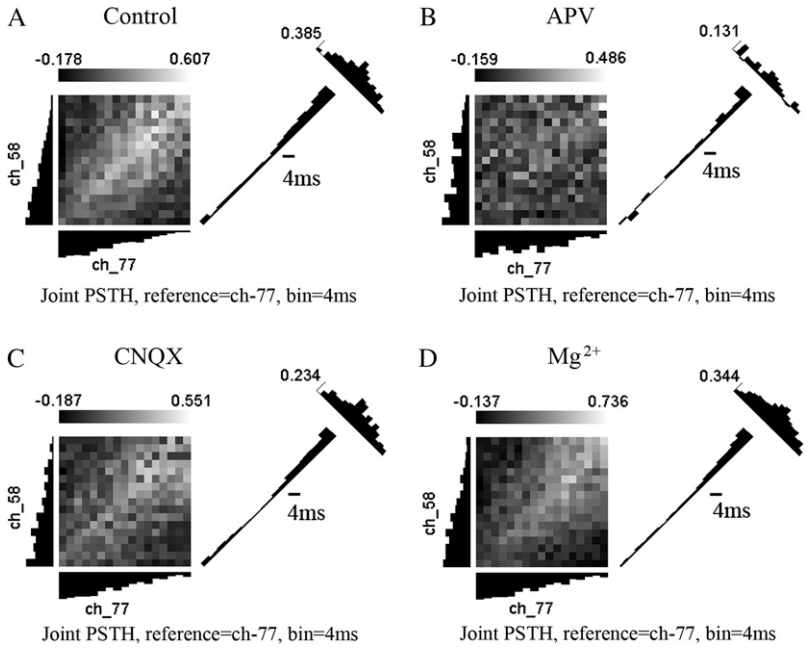

FIGURE 5 Correlations and synchrony of response activities within 80 ms after stimulus in cultured hippocampal neuronal networks during learning training without treatment (*A*), and after treatment with 50 μM APV (*B*), 50 μM CNQX (*C*), and 2 mM Mg^{2+} (*D*).

Statistically (Fig. 6), the correlation and synchrony of response activities within 80 ms after stimulus in the neuronal network were both decreased by 72% with 50 μM APV and by 48% with 50 μM CNQX. However, interestingly, the correlation and synchrony of response activities were increased by 6% and 1%, respectively, with 2 mM Mg^{2+}. In brief, spatial configurations of neuronal response activities in the networks, including regularity, correlation, and synchrony, are modulated primarily by NMDA receptors.

In addition, power spectral density (PSD) of early postsynaptic responses at different characteristic frequencies during learning training was changed distinctly with 50 μM CNQX. However, it was not changed much by treatment with 50 μM APV or 2 mM Mg^{2+} (Fig. 7). The result showed that energy distribution of neuronal response activities in the network was modulated primarily by AMPA receptors. Moreover, the power of low-frequency elements ($<$10 Hz) clearly decreased with 50 μM CNQX, which indicated that the fast response component of postsynaptic responses during learning training was controlled primarily by AMPA receptors.

From an informatics point of view, we showed that rate, one of the temporal configurations, was modulated primarily by AMPA receptors; spatial configurations, including regularity, correlation, and synchrony, were modulated primarily by NMDA receptors. Furthermore, we identified that the fast-response component of response activities was produced primarily by AMPA receptors during learning training.

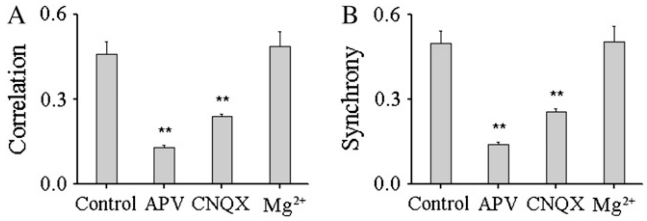

FIGURE 6 (*A*) Correlations of response activities within 80 ms after stimulus in cultured hippocampal neuronal networks during learning training, with or without antagonists of iGluRs (n = 36 experiments in six cultures). (*B*) The synchrony of response activities within 80 ms after stimulus in cultured hippocampal neuronal networks during learning training with or without antagonists of iGluRs (n = 36 experiments in six cultures).

DISCUSSION

Based on the selective learning model of cultured hippocampal neuronal networks, we analyzed dynamics adopted in spatiotemporal encoding of early postsynaptic response activities in cultured hippocampal neuronal networks during learning training under normal and abnormal levels of iGluRs, respectively. From an informatics standpoint, we determined that rate, one of the temporal pattern encoders, was modulated primarily by AMPA receptors; spatial pattern encoding, including regularity, correlation, and synchrony, was modulated primarily by NMDA receptors. Moreover, we observed that the fast-response component of neuronal activities in the network was produced primarily by AMPA receptors during learning training. Our results are consistent with simulation results, which will help the study of information encoding of neuronal response activities in the networks during learning (27,28).

Understanding learning in real neural networks is one of the central challenges in neuroscience. In an attempt to understand learning dynamics at the network level, we constructed

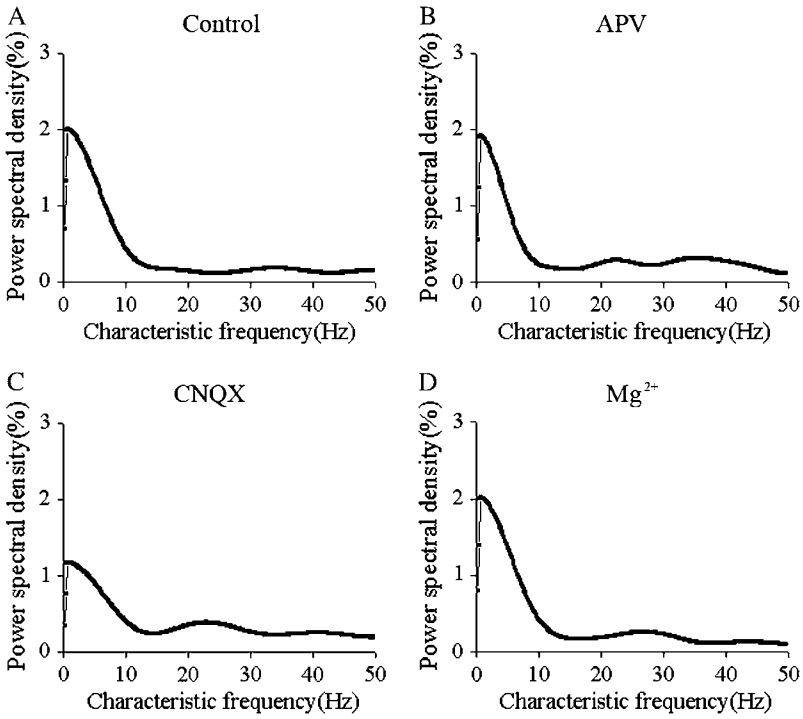

FIGURE 7 Effects of APV, CNQX, and Mg^{2+} on the PSD of early postsynaptic responses in cultured hippocampal neuronal networks during learning training ($n = 36$ experiments in six cultures). The figure shows PSD of early postsynaptic responses during learning training in networks without treatment (*A*), and after treatment with 50 μM APV (*B*), 50 μM CNQX (*C*), and 2 mM Mg^{2+} (*D*).

a learning model in cultured hippocampal neuronal networks (21,22), and based on this learning model, we studied dynamics characteristic of response activities during learning under normal and abnormal levels of ionotropic glutamate receptors. We know that activity varies among individual neurons and is not precise. The accurate activities of the neural system require integration of neuronal activities in the network. The integrated neuronal activities of the network are determined by neuronal intrinsic properties, including structural and functional properties, and extrinsic properties, including simultaneous electrical and chemical stimulation (8,29–31). In this study, we used low-frequency stimulation to induce stable system-level response activities, and used antagonists to inhibit the function of glutamate receptors in the whole network, thus changing the intrinsic properties and system-level activities of neurons in the network.

Although the learning phenomenon induced in cultured neuronal networks was not in agreement with the views of some researchers, one of our results, long-term potentiation of spontaneous activities in the neuronal network, could illuminate how learning occurs (21). As we know, long-term potentiation is one of the important mechanisms of learning (32,33). Based on the evidence, we considered that some kind of learning was induced in cultured neuronal networks, and that the low-frequency stimulation used here was similar to conditioned stimulation. Moreover, we found that synchronized oscillation in cultured realistic neuronal networks occurred after successful selective learning (21), which suggests that synchronized oscillation was associated closely with learning. Many current studies report that synchronized oscillation is vital to the survival of animals, and plays an especially important role in higher functions of the brain, such as learning, memory, and attention, as well (14,34–37). Many simulation studies of neuronal models support the above-mentioned results (38–41). Although the mechanisms of synchronized oscillation in the central neural system are still unclear, a molecular model has been presented that accounts for the main properties resulting from the coupling of a population of circadian oscillators (38).

In this study, one of the purposes was to indicate new possible parameters related to the associated strength and synchrony level among neuronal units, showing that joint PSTH can be utilized in conjunction with more standard parameters for evaluating electrophysiological activities of neuronal networks induced by pharmacological treatment. In fact, the issue of correlation has been deeply investigated to reveal the dynamics of cultured neuronal networks (42–44) and has been found to be related to external stimuli (45). These preliminary results suggest that parameters related to the associated strength and level of synchrony among neuronal units could reveal subtle changes in the network dynamics, thus indicating its promising application as a highly sensitive biosensing tool for learning study (46).

As widely demonstrated, mainly by Gross and co-workers (47,48), in vitro neuronal networks coupled to MEA-based devices constitute a suitable experimental model for pharmacological investigation. These systems show both a good sensitivity to neuroactive toxic compounds and reproducible results. Most of the works refer to spinal cord neurons that represent a more robust model in terms of network dynamics.

However, hippocampal neurons represent a more interesting and delicate model, likely to be the more advanced adaptive and sensitive system, which could be used for such applications.

In addition, as already stated, hippocampal neurons are less often utilized coupled to MEA-based devices, and therefore, detailed studies of the modulation of electrophysiological activity induced by chemical stimulation still need to be extensively and systematically performed at the network level. However, as foreseen by other investigators, application of MEA-based biosensors in the field of drug discovery seems to hold much future promise (49).

SUPPLEMENTARY MATERIAL

To view all of the supplemental files associated with this article, visit www.biophysj.org.

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