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ORIGINAL PAPER

The Effects of Acute GABA Treatment on the Functional Connectivity and Network Topology of Cortical Cultures

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Abstract γ-Aminobutyric acid (GABA) is an inhibitory transmitter, acting on receptor channels to reduce neuronal excitability in matured neural systems. However, electrophysiological responses of whole neuronal ensembles to the exposure to GABA are still unclear. We used micro-electrode arrays (MEAs) to study the effects of the increasing amount of GABA on functional network of cortical neural cultures. Then the recorded data were analyzed by the cross-covariance analysis and graph theory. Results showed that after the GABA treatment, the activity parameters of firing rate, bursting rate, bursting duration and network burst frequency in neural cultures decreased as expected. In addition, the functional connectivity also decreased in similarity, network density, and the size of the largest component. However, small-worldness was not found to be influenced by the acute GABA treatment. Our results support the position that using graph theory to evaluate the functional connectivity of neural cultures may enhance understanding of the pharmacological impact of neurotransmitters on neuronal networks.

Keywords Neural culture · Graph theory · Connectivity · Microelectrode arrays

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Introduction

Neurons from different brain areas of rodents can be cultured on MEAs and remain spontaneously active and stable for several months [1–3]. Moreover, the responses of neural cultures to neurotransmitters and their blockers are similar to those found *in vivo* [4–6]. Thus, the study of neural networks on MEAs would enable us to study how neuroactive substances influence the electrophysiological behaviors of neurons [7–11].

One of the major modes of activity in neural cultures is globally synchronized bursting, which is also called network bursting. This activity can be observed using electrophysiological recordings at about 10–14 days in vitro (DIV) [12–14]. In a typical network burst, most of neurons fire a cluster of spikes within hundreds of milliseconds [15–17]. Many studies have been carried out to study the characterization or pattern of spontaneous network bursts activity [18–24]. It has been reported that network bursts can be divided into subgroups with distant spatiotemporal correlation patterns [22], and that inhibitory antagonists [23] or the architecture of the culture [24] can produce new network burst patterns. However, interactions between the individual spiking units during the network burst are still unclear.

Recently, functional connectivity (statistical dependence between nodes activities) of the neural networks or brains gained further attention by modeling them as graph, whose nodes represent the spiking units and the edges represent the interactions between nodes [25]. Graph theory provides a valuable tool to study functional connectivity of neural cultures using MEAs [26–29] and to evaluate the global properties of the neuronal network. Researchers have found some non-random features in neural cultures. One of these features is small-worldness, which indicates a network

architecture that most of the nodes were not connected directly but can communicate with few intermediate relay steps. Functional networks of neural cultures display small-world structure after weeks of culture [27, 30]. In addition, a rich-club topology emerges during the development of neural cultures [29], which implies a network architecture in which nodes rich in connections tend to form strongly interconnected clubs. With graph theory, the above studies propose ways in network view to describe how the integrative nature of neural network function can be illuminated from a complex network perspective, rather than from individual neuron perspective.

To our knowledge, the neural pharmacological studies that used electrophysiological techniques to evaluate the impact of drug on neural cultures were mainly used patch clamp or multi-electrodes array to measure the changes of membrane potentials, firing rates, bursting rates, bursting duration etc. [31–34] of neuron or neural network induced by drugs. However, the drug effects on functional interactions within neural networks have yet to be examined. Functional connectivity of neural cultures has recently become valuable tools to assess the effects of drug son neurons. There is currently evidence indicating that drugs can be evaluated using graph theory by analyzing factors such as small-worldness in neuronal networks in vitro [26, 28].

In this study, the inhibitory neurotransmitter GABA was used to acutely treat cortical neural cultures in this study. During a network burst, functional connectivity was assessed by using cross-covariance (determine functional connections) and graph theory (statistics of network property). The study is one of the first to investigate functional connectivity to evaluate the pharmacological effect of GABA, supporting the perspective that graph theory can provide useful information about pharmacological impacts on neuronal networks.

Materials and Methods

Animals

All animal procedures complied with the guidelines of the Recommendations from the Declaration of Helsinki, and were approved by the Institutional Animal Care and Use Committee of the Chinese Academy of Military Medical Science. We made all possible efforts to reduce the number of animals used.

Pregnant Sprague–Dawley (SD) female rats were bought from the Experimental Animal Center, Academy of Military Medical Science (Beijing, PRC). The rats were placed in a room with constant temperature (24 ± 2 °C) and were individually housed in cages under a normal day/night cycle.

Neuronal Cell Culture on MEAs

Cortical tissues were dissected out from 18-day-old embryonic rats and were dissociated by enzymatic digestion in a 0.25% trypsin solution (30 min at 37 °C). The resulting tissue was resuspended in Dulbecco's modified Eagle's medium (DMEM, Sigma) containing 10% equine serum (Hyclone) and 10% fetal calf serum (Hyclone) at a final concentration of 1×10^6 cells/ml. Cells were plated onto MEAs previously coated with poly-L-lysine (Sigma, 0.1 mg/ml) and matrigel (Sigma, 0.2 mg/ml), resulting in a cell density of about 3000 cells/mm^2.After the cells were adhered onto the MEAs, the liquid was replaced by DMEM containing10% equine serum (Hyclone), and half of the medium was changed every 2 days. All cells were placed in a humidified incubator (5% CO_2, 95% air, 37 °C).

Acute GABA Treatment

The acute GABA treatment protocol was similar to that describe previously [35]. Briefly, at 21 DIV, the culture medium in each MEA was kept at 1 ml before treatment. The GABA mother solutions were prepared using DMEM to achieve the correct target concentrations (GABA concentrations: 1.25, 2.5, 5, and 10 μM). Before the cortical culture was treated with GABA, 200 μl of the cultured medium was pipetted out, mixed with a small amount (1 μl) of the GABA mother solution, and then carefully returned to the original medium to avoid osmotic or hydrodynamic stress.

Electrophysiology Recordings

The recording system and the MEAs were custom-made in our laboratory as reported previously [36, 37]. The recording system had 32 channels, including an amplifier, a NI USB-6259 data acquisition card, and software developed by LabVIEW (National Instruments, USA). The MEAs were made using indium tin oxide (ITO) glass, and the electrodes were electroplated using platinum. Each MEA had a large ground electrode and 59 microelectrodes (30 μm diameter, 200 μm inter-electrode distance).

Before recording, each MEA was placed in the recording system for 10 min to avoid shifts of neural activity from the MEA movement. Neural activity was then recorded for 10 min. All of the recordings were performed in a humidified incubator (37 °C with 5% CO2 and 95% air).

Spike Detection

Spike detection was performed using an Offline Sorter (Plexon, Inc.). Baseline shifts and high-frequency noise were removed using a band-pass filter (200–5000 Hz). The

spike detection threshold was set at five standard deviations of the background signal. Further analysis was carried out using Neuroexplorer(NexTechnologies, Inc.) and MATLAB (The Mathworks).

Burst Detection

Burst detection was performed using Neuroexplorer, as described in the literature [38]. Briefly, the spike train detection parameters were set as follows: minimum burst interval of 0.1 s, minimum burst duration of 0.1 s, and a minimum of five spikes per burst.

Network Burst Detection

A network burst consists of a fast sequence of spikes and can usually be observed in many channels simultaneously. A network burst represents the synchronous activity of a neural culture and provides information regarding interactions between neurons. The algorithm used to detect network bursts was the same as that reported previously [39]. Briefly, a network burst must be detected in at least four active channels within 250 ms, and each active channel must record at least three spikes within 100 ms. The onset and the end of a network burst were defined as the first and the last spike timestamp of the network burst respectively. "Tiny" network bursts were excluded from analysis. Thus, only network bursts with more than eight active channels (>3 spikes in 100 ms) were selected for further connection analysis.

Cross-covariance Method of Connection Determination

Functional connectivity was used to assess functional interactions between neurons in the neural network [40]. The evaluation of functional connectivity in neural cultures has been attempted using many different methods [26, 27, 30, 39–42]. However, there is no gold standard method for the optimal estimation of neural functional connectivity.

The algorithm used to estimate functional connectivity was based on one reported in the literature [27], with small modifications. Briefly, we calculated the cross-covariance sequence $\Phi_{xy}(\mathrm{m})$ between spike trains of two channels during a network burst using the equation below:

$$\Phi_{\mathrm{xy}}(\mathrm{m}) = \mathrm{E}\{(\mathrm{x}_{\mathrm{n+m}} - \mu_{\mathrm{x}})(y_{\mathrm{n}} - \mu_{\mathrm{y}})\}$$

where $\Phi_{xy}(\mathrm{m})$ represents a similarity evaluation between vector x and vector y after vector x shifts m time bins, E {} is an expected value operator, and $\mu_x$and μ_y are the mean values of vector x and vector y, respectively. In this study, vector x and vector y represents vectors that collect the numbers of spikes within each time bin (1 ms) during a network burst.

The cross-covariance values of the spike trains may increase with firing rate [43]. The cross-covariance values cannot simply be regarded as functional interaction or functional connectivity weights. This is to avoid the confounding caused by random spiking. Thus, a shuffling procedure was applied to compare the differences in cross-covariance between neural activity and surrogate neural activity. In this manner, real neural functional connections could be distinguished from functional connections due to chance. Briefly, recording spike trains were randomly shuffled and were used as surrogates to real neural activity in calculations of the cross-covariance sequence. This step was repeated 100 times. A z-score was then used to normalize the difference between the cross-covariance of the real neural activity and that of the surrogate data. The z-score equation is as follows:

$$Z(\mathrm{m}) = \frac{\Phi_{\mathrm{xy}}(\mathrm{m}) - \Phi^{S}_{xy}(\mathrm{m})}{\delta^{\mathrm{S}}_{\mathrm{xy}}(\mathrm{m})}$$

where $\Phi^{\mathrm{S}}_{\mathrm{xy}}(\mathrm{m})$ is cross-covariance value of surrogate pairs and δ^{S}_{xy} (m) is the standard deviation of the cross-covariance surrogate values set at $\Phi^{\mathrm{S}}_{\mathrm{xy}}(\mathrm{m})$. $Z_{\max} = \max(Z(\mathrm{m}))(\mathrm{m} \neq 0)$ was used as the connectivity weight.

In this study, we produced a 32×32 connectivity weight matrix for each selected network burst. Determination of the presence of a connection was based on two threshold schemes. The networks were computed across a range of absolute thresholds (0.05–1.0, in steps of 0.05) for basic functional connectivity metrics (degree and network density). A range of proportional thresholds (2–40% maximum network density, in steps of 2%) was also used to construct a network. The largest connected components were then evaluated and used to compute complex topological metrics, such as small-worldness.

Graph Metrics

Graph metrics were selected to assess the functional networks of the neural cultures. To evaluate the similarities in functional networks during a recording episode, pairwise Spearman correlation was used to compute the two upper triangles of the 32×32 connectivity weight matrices. The connection strength of the functional network represents the mean value of all of the connection weights in all selected connection weight matrices. Network density was defined as the percentage of all possible connections that were realized. The degree was the number of connections linked with other nodes. Nodes to which no other nodes were linked were defined as having a degree of 0.Small-worldness [44, 45] was used to measure the presence of

small-world organization. If $C^*L_{random}/C_{random}^*L > 1$, then the network has a small-world organization. C and C_{random} represent the average clustering coefficients, and L and L_{random} represent average path lengths of the target network and the surrogate network, respectively. The surrogate network was constructed using the Brain Connectivity Toolbox [25] using the same size and degree distributions as those in the original network (100 iterations).

Statistical Analysis

All data are expressed as means ± standard errors of the mean. Four neural cultures on MEAs were used in this paper. We used statistics software SAS (version 9.0) to perform the statistical analyses. A one-way analysis of variance (ANOVA) with Tukey's studentized range (HSD) test and a two-way ANOVA with a Bonferroni post-hoc test were used to evaluate the data. A p-value < 0.05 was considered significant.

Results

Primary cortical tissues were prepared from embryonic day 17–18 SD rats. Mixtures of neurons and astroglia were isolated and seeded on MEA chips. Neurons can attach onto the MEAs and develop into a mature neuronal network [46, 47]. Cortical neurons were cultured on MEAs for about 3 weeks and then treated with GABA at 21 DIV, as showed in Fig. 1a. Neural activities on MEAs were recorded by MEA recording system and functional connectivity changes were analyzed by graph theory (Fig. 1b).

Spiking Activities of Cultured Neural Networks During Acute GABA Treatment

The neural network was mature at 21 DIV. At this point, the neural activity was characterized by synchronized bursts and random spikes. In order to explore the effects of acute GABA treatment on the neural cultures, cortical neural cultures were treated with GABA gradually in a cumulative manner (1.25, 2.5, 5, and 10 μM). The recorded neural activities at each concentration of GABA are shown as raster plots in Fig. 2.

Statistical analysis indicates that the activities of the neural cultures were significantly inhibited by GABA in firing rate ($F = 60.56$, $p < 0.0001$, Fig. 3a), bursting rate ($F = 31.13$, $p < 0.0001$, Fig. 3b), and bursting duration ($F = 26.57$, $p < 0.0001$, Fig. 3c). Spike frequencies of the bursts did not change after treatment with GABA when compared to the initial state (Fig. 3d).

Network Burst Dynamic of Neural Cultures During Acute GABA Treatment

Typical network burst activity obtained without GABA treatment is shown in Fig. 4a. Network burst activity during treatment with 5 μM GABA is shown in Fig. 4b. It is suggested that during exposure to GABA concentrations under 5 μM, neural cultures display fewer spikes in a network burst. The statistical analysis of network burst rates indicated that the decrease was significant with treatments of 1.25, 2.5, 5, and 10 μM GABA (Fig. 4c). Acute GABA treatment (10 μM) completely inhibited network burst activity.

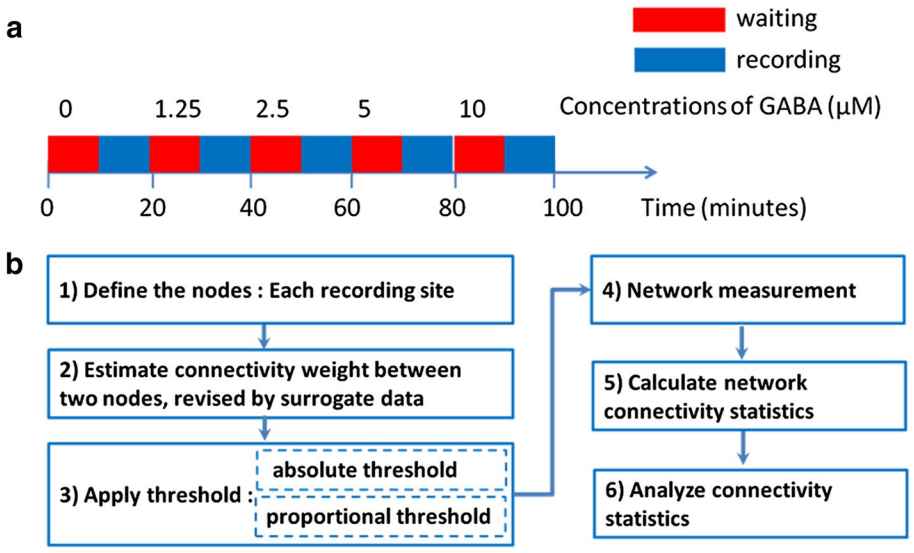

Fig. 1 The experimental procedure: **a** GABA was added to the culture medium in a concentration-accumulating manner. All cultures were stabilized for ten minutes before recording. **b** The protocol for the functional connectivity analysis

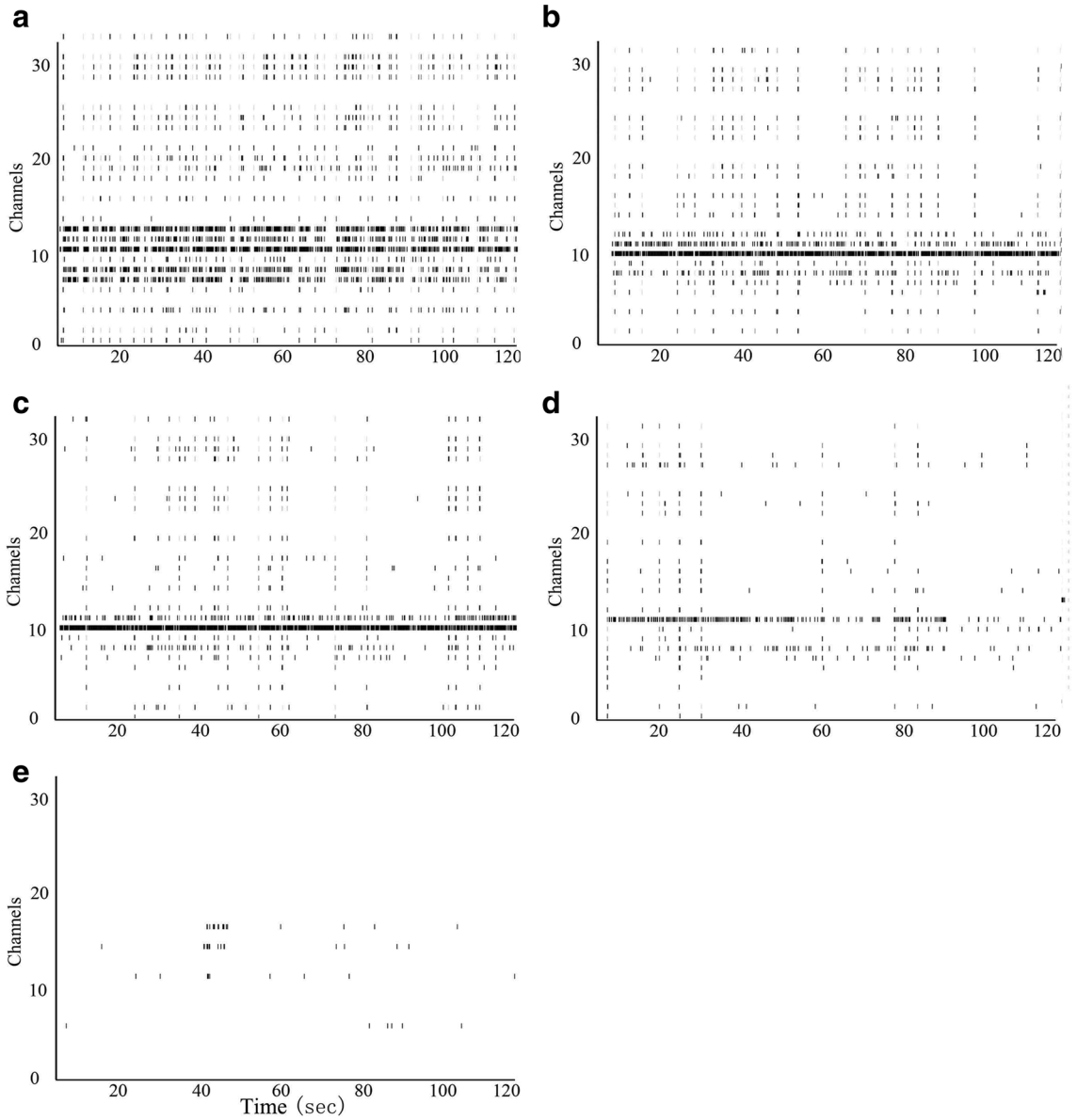

Fig. 2 The raster plotting of neural activity during treatment with different concentrations of GABA: **a** 0 μM, **b** 1.25 μM, **c** 2.5 μM, **d** 5 μM, and **e** 10 μM

Network Topology Following Acute GABA Treatment

To explore the influence of acute GABA treatment on functional connectivity of neural cultures, we focused on the synchronous activity known as the network burst. We used lagged inter-channel cross-covariance analysis for each GABA treatment condition over each network burst. A shuffling procedure [48, 49] was used to normalize the differences in connectivity weight between real neural data and surrogate data. The spatiotemporal structures of functional connectivity, evaluated by Spearman correlations between 50 randomly selected network bursts (for each recording), suggested a good similarity (0.5–0.7) between cultures treated with 0, 1.25, and 2.5 μM GABA. However, when the concentration of GABA reached 5 μM, the similarity was significantly decreased (Fig. 5a). Compared to the control (0 μM), application of GABA did not affect connectivity strength (Fig. 5b).

To compare the network topologies of the neural cultures during acute treatment with different concentrations of GABA, two threshold schemes were used to describe the changes in network topology. First, to compare network densities and the sizes of the largest components, functional networks of neural cultures were constructed across a range of absolute thresholds (0.5–10, in steps of 0.5; calculated for each network burst). Our data indicated

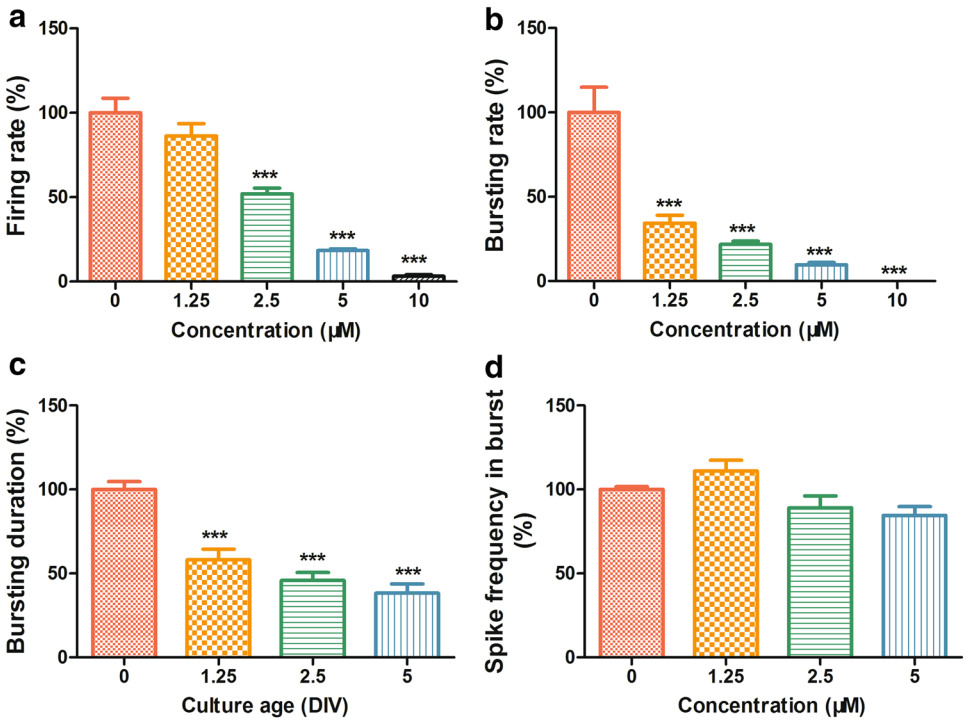

Fig. 3 Changesinactivity parameters of cortical neural cultures after acute treatment with increasing doses of GABA: **a** firing rate, **b** bursting rate, **c** burst duration, and **d** spike frequency of the bursts. ***p < 0.0001, comparison between acute GABA-treated cultures and cultures before GABA treatment

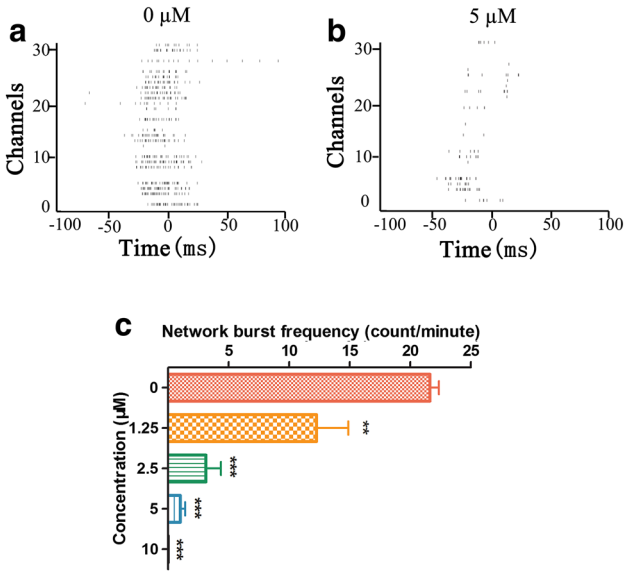

Fig. 4 Network burst dynamics during GABA treatment: raster plots of network bursts during **a** 0 μM and **b** 5 μM GABA treatment. **c** Network burst frequencies during treatment with different concentrations of GABA. **$p < 0.01$, comparison between activity of acute GABA-treated culture and activity of culture before GABA treatment

that GABA treatment influences network density (two-way ANOVA, F = 0.31, $p = 0.0326$, Fig. 6a) and the size of the largest component (two-way ANOVA, F = 1.25, $p < 0.0001$, Fig. 6b) of functional networks of cortical neural cultures. Second, a range of proportional thresholds (2–40% maximum network density, in steps of 2%) were used to construct a functional network used to calculate small-worldness. Using no GABA exposure (0 μM) as a control, we found that small-worldness was unaffected by 1.25, 2.5, and 5 μM GABA exposure (two-way ANOVA, F = 0.69, $p = 0.1283$, Fig. 6c) and that small-world organization was robust in the face of GABA treatment.

Discussion

The effects of acute GABA exposure on cortical neural cultures were investigated by graph theory. Activity parameters, functional connectivity, and functional network topology were compared during the treatment with different concentrations of GABA. It was shown that while some activity parameters were influenced by GABA as expected, the connectivity and the network topology of cortical neural cultures were also found to be sensitive to GABA treatment.

Generally, small-world [27] and rich-club [29] organizations of neural cultures emerge during development without any external intervention. However, it has been reported that the topologies of neural cultures were influenced when the neural cultures were constructed as models of epilepsy by glutamate [26] and ischemic model by the combination of 4-aminopyridine and bicuculline [28]. Small world organization is a very important to neurosciences. In fact, "small-worldness" was found in multiple species and scales

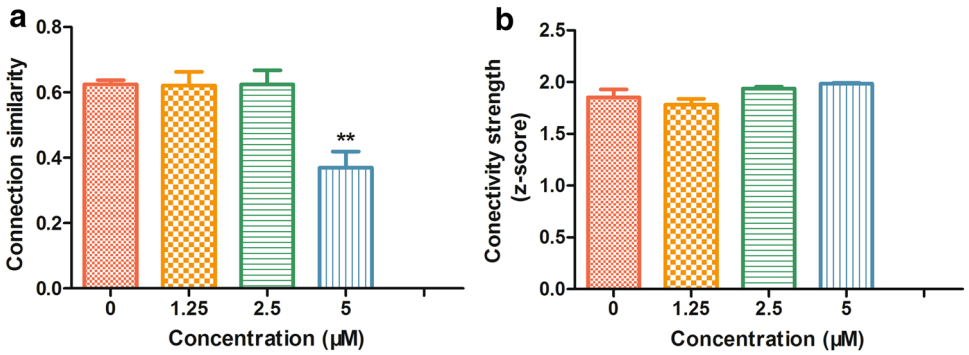

Fig. 5 Increasing concentrations of GABA induced changes in functional connectivity in cultured cortical networks. **a** Functional connectivity varied across network bursts within a recording episode. Five μM GABA may cause this similarity to decrease. **b** Functional connectivity strengths of neural cultures seemed to not be affected by 1.25, 2.5, or 5 μM GABA when compared to control. ** $p<0.01$, comparison between the activity of acute GABA-treated culture and that of the culture before GABA treatment

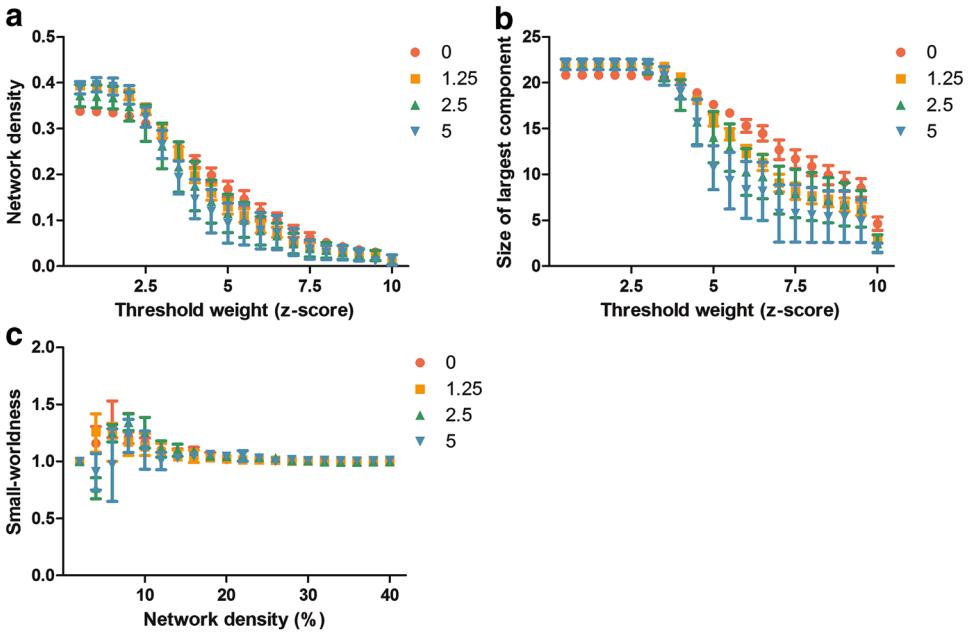

Fig. 6 Increasing concentrations of GABA induced changes in the network metrics of the network density (**a**), the size of the largest component (**b**), and small-worldness (**c**)

from structural and functional MRI studies of large-scale brain networks to MEA recordings of cellular networks [26–29] and intact animals [50, 51]. It was believed that a brain network with small-world structure had denser local clustering connection and could arrange some long-range connections to process information efficiently and economically. The rich-club topology refers to the tendency of nodes with high degree to form tightly-interconnected communities. They were found in the human brain, cultured neural and the other neural networks. Neural rich clubs have been hypothesized to act as a central high-capacity backbone for global communication [52] and integration [53] in the brain.

In our study, the "small-worldness" of neural culture was not influenced by 0~5 μM GABA treatments. In fact, there were inhibitory or excitory synapse and neurons in neural cultures. However, inhibitory synapse input would inhibit excitory neurons' activities. As a consequence, neurons, which are regulated by these excitory neurons, would be inhibited for lacking of input from excitory neurons. On the other hand, inhibitory synapse input would inhibit inhibitory neurons' activities. Therefore the neurons that regulated by these inhibitory neurons activity would be increased. There is an excitation-inhibition balance in neural cultures, which is critical for proper development and function of the central nervous system [54]. Hence, we speculate that this excitation-inhibition balance would be broken when neural cultures were added with GABA, but a new balance would emerge to fight against the extra amount of GABA. In this new balance, the firing rates of neural

cultures were significantly decreased, but the connectivity weight and the "small-worldness" may not be affected.

We also discovered that the similarities between functional networks during a recording episode decreased by the acute GABA treatment. Based on the data, GABA was verified to be involved in the regulation of the communications. However, the functional weight seemed to be unaffected by GABA treatment.

Network density, the size of the largest component, and the small-worldness of the cortical cultures were also found to be influenced by GABA exposure. Network density and the size of the largest component are reduced following GABA treatment. This means that GABA leads to a decrease in the connections within a neural network. However, small-worldness was not found to be influenced by an acute GABA treatment. Specifically, small-worldness remained above 1, which means that the small-world organization of the neural cultures was not interrupted by GABA.

It is known that GABA inhibits the excitability of individual neurons. However, it is unclear how GABA influences the global interactions of neural networks. Using graph theory, we can evaluate the connectivity of the whole neural network to acquire a better understanding of neural network functions. These functions include interactions between neurons and whole network dynamic properties. In addition, in vitro studies may help to improve our understanding of the functional network organization.

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Compliance with Ethical Standards

Conflict of interest The authors declare that there are no conflicts of interest.

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