Biosensors and Bioelectronics 66 (2015) 572–578

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Detection of 5-hydroxytryptamine (5-HT) *in vitro* using a hippocampal neuronal network-based biosensor with extracellular potential analysis of neurons

Liang Hu [a,b,c], Qin Wang [a], Zhen Qin [a], Kaiqi Su [a], Liquan Huang [b,c], Ning Hu [a,*], Ping Wang [a,*]

[a] Biosensor National Special Laboratory, Key Laboratory of Biomedical Engineering of Ministry of Education, Department of Biomedical Engineering, Zhejiang University, Hangzhou 310027, China
[b] Monell Chemical Senses Center, Philadelphia, PA 19104, USA
[c] School of Veterinary Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA

ARTICLE INFO

Article history:
Received 17 November 2014
Received in revised form
5 December 2014
Accepted 6 December 2014
Available online 8 December 2014

Keywords:
5-hydroxytryptamine (5-HT)
Neuronal network-based biosensor
Hippocampal neuronal networks (HNNs)
in vitro multi-site recording
Neuron and network electrophysiology

ABSTRACT

5-hydroxytryptamine (5-HT) is an important neurotransmitter in regulating emotions and related behaviors in mammals. To detect and monitor the 5-HT, effective and convenient methods are demanded in investigation of neuronal network. In this study, hippocampal neuronal networks (HNNs) endogenously expressing 5-HT receptors were employed as sensing elements to build an *in vitro* neuronal network-based biosensor. The electrophysiological characteristics were analyzed in both neuron and network levels. The firing rates and amplitudes were derived from signal to determine the biosensor response characteristics. The experimental results demonstrate a dose-dependent inhibitory effect of 5-HT on hippocampal neuron activities, indicating the effectiveness of this hybrid biosensor in detecting 5-HT with a response range from 0.01 μmol/L to 10 μmol/L. In addition, the cross-correlation analysis of HNNs activities suggests 5-HT could weaken HNN connectivity reversibly, providing more specificity of this biosensor in detecting 5-HT. Moreover, 5-HT induced spatiotemporal firing pattern alterations could be monitored in neuron and network levels simultaneously by this hybrid biosensor in a convenient and direct way. With those merits, this neuronal network-based biosensor will be promising to be a valuable and utility platform for the study of neurotransmitter *in vitro*.

1. Introduction

5-hydroxytryptamine (5-HT) is an important neurotransmitter involved in numerous neural functions in the brain. It regulates neural activity, which is reported to be involved in emotion, sleep and some neural dysfunction diseases such as Alzheimer's disease (Canli and Lesch, 2007; Outhred et al., 2013; Palop and Mucke, 2010; Pringle et al., 2013). In order to study the 5-HT in the neural network activities, fast detecting and monitoring techniques are required for 5-HT *in vitro*. Electrophysiological technology has been a desirable method for detecting 5-HT with efficient, sensitive and specific merits. Compared with other detecting methods such as electrochemical sensors, electrophysiological changes of neuron activities are fast in responding to 5-HT, which is more convenient for monitoring (Ahammad et al., 2011; Li et al., 2009; Rand et al., 2013). Moreover, electrophysiological detection is sensitive and specific due to the specific interaction between neurons and neurotransmitters, which also provides more biological information for 5-HT pharmacological properties. Traditional electrophysiological approach patch clamp is gold standard recording method with high sensitivity and specificity. However, the throughput is low and cannot reveal the network characteristics. Besides, these study approaches are complicate and time-consuming in manipulations (Cai et al., 2013; Johnston et al., 2014; Lu and Gean, 1998). To address these problems, microelectrode array (MEA) is employed with advantages of high throughput, easy manipulation, and non-invasion in long term recording.

As a neurotransmitter, 5-HT could influence neural electrophysiological activities by specifically activating 5-HT receptors in the neurons. Hence hippocampal neurons endogenously expressing 5-HT receptors have been promising sensing elements of biosensors in 5-HT detection. In previous studies, cultured neurons on MEAs have been used as powerful sensing materials to assess the effects of neuroactive chemicals and toxics (Charkhkar et al., 2012; Pancrazio et al., 2014; Weiss, 2011). In these studies, the firing characteristics of single neurons such as firing rate and amplitude were analyzed to reveal the chemical function in neural physiology and pharmacology. However, information from single

* Corresponding authors.
E-mail addresses: huning@zju.edu.cn (N. Hu), cnpwang@zju.edu.cn (P. Wang).

http://dx.doi.org/10.1016/j.bios.2014.12.019
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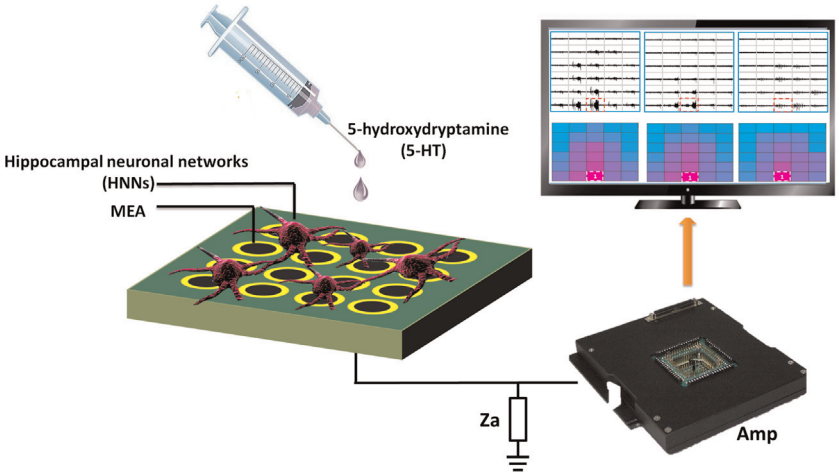

Fig. 1. Schematic of multi-site. recordings of HNNs with MEAs. Hippocampal neuron networks (HNNs) cultured on MEAs surface. Signal detected by microelectrodes were amplified by the amplifier (Amp) and then displayed on computer screen.

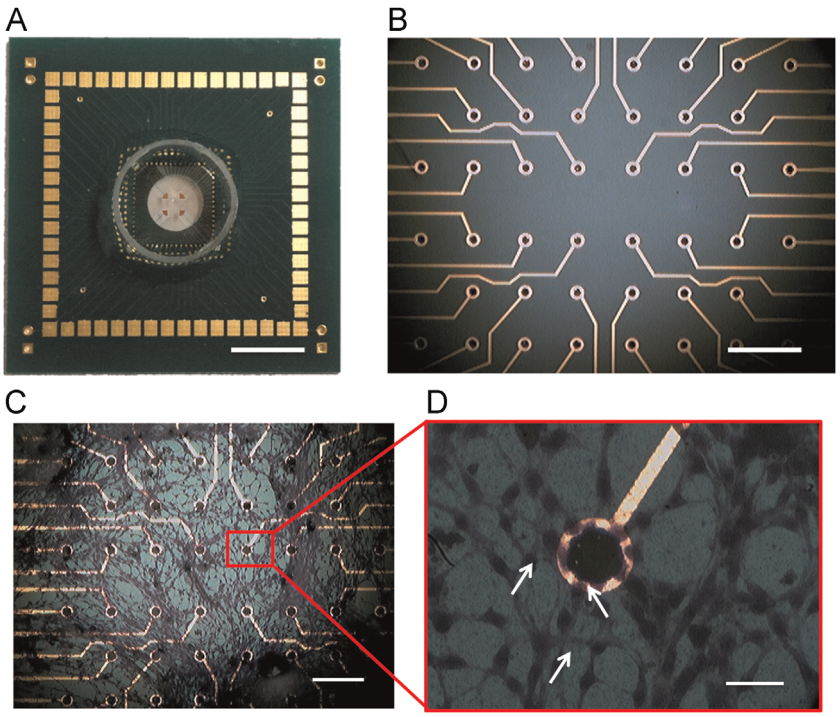

Fig. 2. (a) The MEA chip in package. Scale bar is 10 mm. (b) Microelectrodes array after electroplated with platinum black under microscope. The scale bar is 150 μm. (c) Stained HNNs on MEA chip under microscope. The detail in red squares will be magnified in (d). The scale bar is 150 μm. (d) Magnified stained hippocampal neurons from red squares in (c). White arrows indicated three neurons. The scale bar is 50 μm.

neurons was limited to specifically detect and determine the chemicals. In the present study, primary hippocampal neurons were cultured to hippocampal neuronal networks (HNNs) on MEA surface. Apart from the electrophysiological characterization from single neurons, neural network activities were also analyzed for 5-HT detection and determination. From neural network activities, signal transmission and network connection properties could also be applied for 5-HT detection, which contributes more specific information to 5-HT's pharmacology. Thus electrophysiological information from neuronal networks provides specificity to 5-HT detection.

In the present study, a neuron network-based biosensor was established for detection of 5-HT employing HNNs as sensing elements which expressed high density of 5-HT receptors. With multi-site simultaneous recording, single neuron and neural network firing characteristics were derived for the electrophysiological signals. All the details were discussed in following sections.

2. Method and experiments

2.1. Fabrication of MEA

The MEA chip was designed and prepared by the standard microfabrication technology. First, a layer of Ti (30 nm) was deposited onto the glass substrate as the adhesion layer, followed by the Au layer (300 nm) as working electrode. Then the metallic layer was coated by the photoresist and exposed to ultraviolet light under the mask with defined electrode layout. The exposed

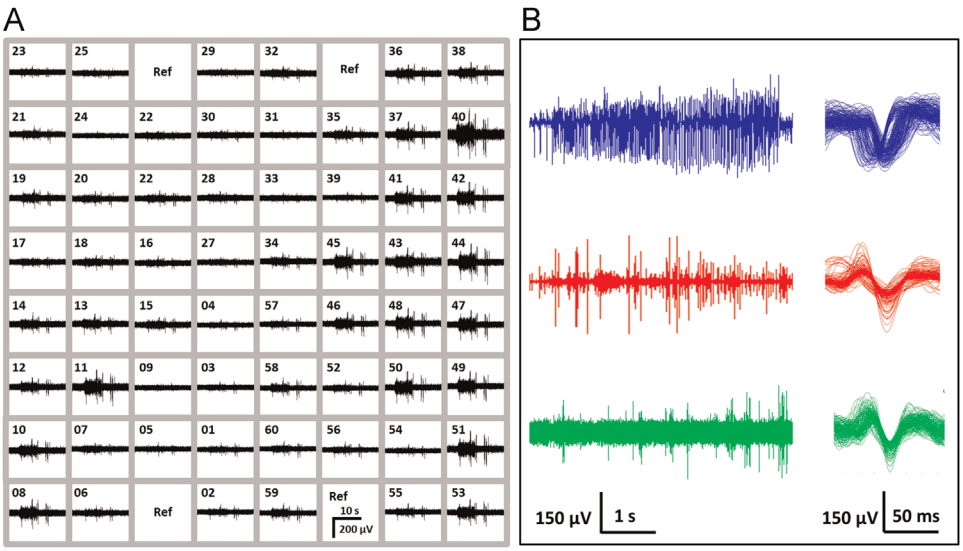

Fig. 3. (a) Multi-site. recording signals of HNNs in spontaneous state. The signals shown in each channel were filtered by bandpass filter from 10–40 Hz. multiple channels were valid in recording simultaneously. (b) Magnified signals from three different MEA recordings and their waveforms respectively. (For interpretation of the references to color in this figure,the reader is referred to the web version of this article).

metallic area was etched. As a result the electrodes and interconnects were formed. Subsequently, the photoresist was removed and a layer of Si_3N_4 1 μm was deposited by PECVD techniques. Finally the electrodes and contact pads were exposed after the Si_3N_4 etched in the KOH solution. The contact pads were connected to the ports on the printed circuit board (PCB) by gold thread. A detection chamber with diameter of 15 mm was fixed on the chip by epoxy. The MEA chip was designed as 8×8 array pattern (Fig. 2b). The diameter of single electrode was 20 μm and the distances between each electrode were 150 μm. Prior to experiments the MEA was electroplated with platinum black by chronoamperometry to reduce the impedance of the electrodes and to improve the signal to noise ratio (SNR). The deposition potential was 0.01 V and the duration was 30 s. After electroplating, the impedance of electrodes was decreased to 20 ± 10 kΩ at 1 kHz. The noise was reduced to 20 ± 5 μV.

2.2. Hippocampal neuron culture

Experiments were carried out in accordance with the guidelines of the local animal welfare committee. Sprague Dawley (SD) rats were obtained from Zhejiang Academy of Medical Sciences. All solvents/reagents were analytical grade. Hippocampal neurons were obtained from 1 or 2-day-old newborn SD rats (4 male, 3 female). Hippocampus was rapidly dissected under sterile conditions, kept in cold Hanks balanced salt solution (HBSS) (4 °C) with 3 g/L fetal bovine serum (FBS) (Sijiqing, Hangzhou, China). Then the hippocampal tissue were incubated in the enzyme solution of 0.25% trypsin (Boshide, Wuhan, China) at 37 °C for 15 min and mechanically dissociated by trituration with Pasteur pipet in Dulbecco's modified Eagle's medium (DMEM) (Gibco, USA) with 10% FBS. Subsequently the dissociated cells were filtered with sieve of 200 holes per inch to get cell suspension. Cells were collected by centrifugation and resuspended in DMEM containing 10% FBS supplemented with 1% L-glutamine (Puzhen, Shanghai, China), 1% Penicillin–Streptomycin (MP Biomedicals, Shanghai, China). Cells were plated at the final density of 10^5 cells/MEA into the MEA chamber (10 mm internal diameter) and maintained at a humidified atmosphere at 37 °C with 5% CO_2. The cell density was relatively high so as to acquire relatively dense networks which could maintain more stable than simple networks. Each MEA dish was placed in a petri dish to reduce medium evaporation and maintain sterility. After 24 h the medium was replaced by DMEM with 2% B27 (Gibco, USA), 1% N2 (Gibco, USA), 1% L-glutamine, 1% Penicillin–Streptomycin and 10% FBS. After another 24 h final concentration of 10 μmol/L cytosine arabinoside (Sigma, USA) was added into the medium to prevent the outgrowth of non-neuronal cells (Robertson et al., 1991). To improve the biocompatibility of the silicon device, poly-L-lysine and laminin mixture (100 μg/mL poly-L-lysine (Sciencell, USA) and 8 μg/mL laminin (Gibco, USA) mixed by the rate of 1:1) was coated on the surface of MEA chip prior to seeding cells. The culture medium was half replaced every 2–3 days. When the neurons formed networks after being cultured for 14 days *in vitro* (DIV), the measurement experiments began to perform. The cultured neuronal networks were stained by Wright's dye (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China).

2.3. HNN-based biosensor detection of 5-HT

After 14 DIV culture, the HNN-based biosensor was used to detect 5-HT by recording and analyzing the extracellular potentials of HNNs. (Fig. 1). The MEA-1060 system (Multi Channel Systems, Germany) was applied for simultaneously recording. It was placed in the shielding box to avoid external electromagnetic interference. The sampling rate was set at 10 kHz and system noise level was maintained at about 15 μV. Ag/AgCl electrode was employed as reference electrode. The software MC_Rack (Multi Channel Systems, Germany) was utilized for real-time display and data storage. The temperature of the MEA chip was maintained at 37 °C to keep the physiological activity of HNNs.

Protocols: In our experiment, the concentrations for 5-HT was chosen from 0.01 μmol/L to 10 μmol/L to evaluate the sensor properties. All the 5-HT solutions were prepared in culture medium. All the solutions (5-HT, medium) were maintained warm at 37 °C in water bath during experiments in order to eliminate the influence of temperature difference with extracellular environment. Before the delivery of 5-HT, the spontaneous network activities of HNNs were recorded in medium (pH 7.4) as control condition. Then the electrophysiological signals of the HNNs were recorded with the stimulation of 5-HT from low concentrations to higher ones. Each condition was recorded for 300 s. Finally the

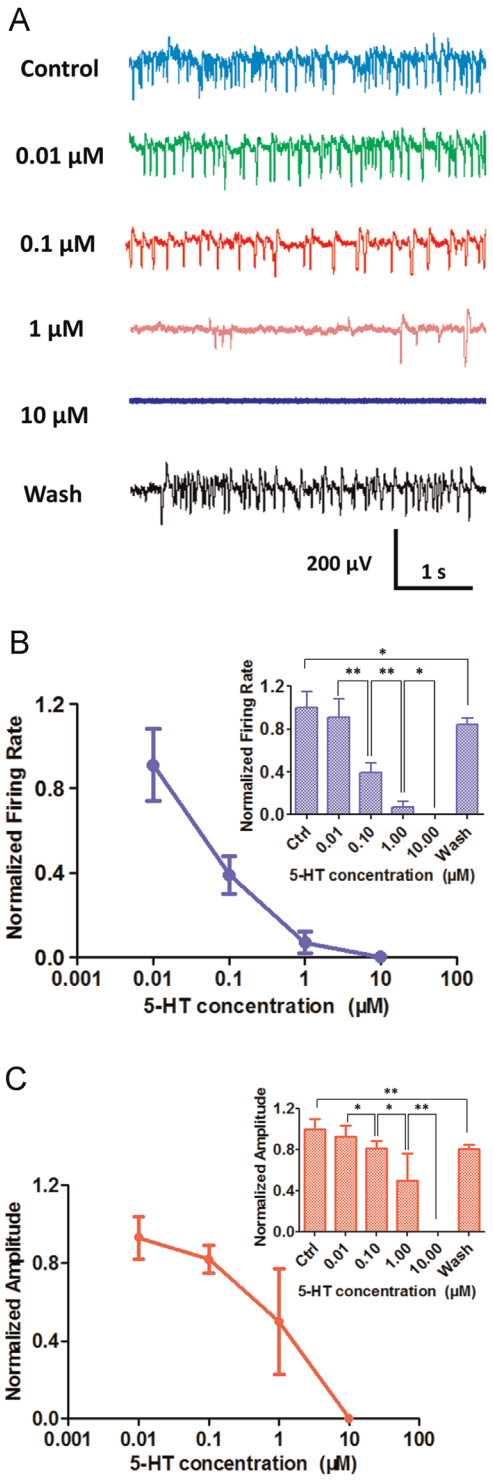

Fig. 4. (a) Typical original signals of hippocampal neurons with 5-HT treatment. Hippocampal neuronal activities are inhibited by increasing 5-HT concentrations. Individual trace shows electrophysiological recording of 5 s. (b) Standard curves of biosensor responses to 5-HT based on firing rate. Inset is the normalized firing rates with concentration gradient. (c) Standard curves of biosensor responses to 5-HT based on signal amplitude. Inset is the normalized signal amplitude with concentration gradient. All data in (b) and (c) are represented by the mean ± SD. Ctrl represents the control state and Wash indicates the state after 5-HT washed out. Student's *t* test (*, $P < 0.05$, **, $P < 0.01$) was used to evaluate difference significance between groups including 0.01 μmol/L versus Ctrl, 0.1 μmol/L versus 0.01 μmol/L, 1 μmol/L versus 0.1 μmol/L, 10 μmol/L versus 1 μmol/L and Wash versus Ctrl.

potential changes were recorded after 5-HT was washed twice by PBS and replaced by culture medium (pH 7.4). All the solutions were maintained at 37 °C during recording process.

2.4. Data processing and analysis

Data were recorded and replayed in MC_Rack (Multi Channel Systems, Germany). The waveforms of spontaneous action potentials were sorted out after the raw signals were filtered by a bandpass filter from 10 to 100 Hz in Matlab. The signals of single channels were analyzed by calculating firing rates and amplitudes of action potentials. Signals were displayed in MC_Rack, Clampfit or Matlab in different figures. The data, such as amplitudes, firing rates were represented as mean ± SD of n samples (*n* represent the number of valid electrodes). Each set of data were normalized to the mean value at the highest response. In this study the highest response of both firing rates and amplitudes was taken as control. Therefore normalization means values at each condition (control, three 5-HT treatments or after-wash) divide to the mean value of control group. Student's t test was used to evaluate the significant difference among different 5-HT concentration treatments. In the analysis of network activities, the number of active channels was calculated at different concentration treatments as mean ± SD of n sample (n represent the number of valid recording trials in which MEA could record the network activities). In this study, the active channel means those in which we can still detect the electrophysiological signals in each trial (300 s). Consequently the cross-correlation coefficients of relative channels were calculated and plotted in color-coded maps by Matlab. Strength was quantified by measuring the maximal cross-correlation coefficient.

3. Results and discussion

3.1. Results of HNN culture

Convincing spontaneously formed HNNs cultured on MEA chip for 14 DIV were shown in Fig. 2c. For cells grown in serum and/or glia containing culture, the development of networks is usually faster than those grown in serum free medium. Hippocampal neurons were able to produce action potentials after 3 DIV (Offenhäusser et al., 1997). And their activities could be detected extracellularly at 3-4 DIV (Jimbo et al., 1998; Liu et al., 1996, 1997). Therefore the developing HNNs at 14 DIV were capable to be used for electrophysiological recording. Fig. 2c showed the stained HNNs and Fig. 2d showed the magnified image from Fig. 2c. The white arrows indicate hippocampal neurons (Fig. 2d). The firing of neurons attached to the electrodes is the basis for MEA detection. As shown in Fig. 2d, more than one neuron were visibly attached to one single electrode, leading to assembly of signals from several neurons recorded by one electrode. Glial cells were functioning as support cells and could not generate action potentials.

3.2. HNN-based biosensor can effectively detect neuronal extracellular potentials

After neurons were grown for 6 DIV, extracellular recordings of the HNNs were carried out. The recorded spontaneous signals of HNNs were shown in Fig. 3a. Among multi-recording sites, over 30 sites successfully recorded the signals. These signals were generally similar in firing duration and pattern, indicating the synchronized activities in the networks. Fig. 3b shows three segments of magnified filtered signals (blue, red, and green) and the corresponding waveforms. From the sorted waveforms, we could discriminate more than one waveforms, indicating assembly of the signals from more than one neuron recorded in that channel. The

duration of the waveform is much longer than one action potential (spike) of neurons (about 1 ms) (Stuart et al., 1997). Therefore the signal recorded in present study should be population spikes from small hippocampal neuron populations. This result was also consistent with the facts shown in Fig. 2a that more than one neuron were attached to single electrode. These results proved that this hybrid biosensor could detect and record the extracellular potentials of HNNs, giving feasibility for this biosensor in HNN electrophysiological recording.

3.3. Biosensor detection of 5-HT by single hippocampal neuron

On the basis of spontaneous electrophysiological signals, we recorded the response of neurons to 5-HT at various concentrations to determine the response characteristics of this hybrid biosensor. The original signals with 5-HT stimulation were shown in Fig. 4a. The concentrations of 5-HT ranged from 0.1 μmol/L to 100 μmol/L. The standard curves of biosensor based on normalized firing rate (NFR) and amplitude (NA) were shown in Fig. 4b and c. The insets of Fig. 4b and c are normalized firing rates (NFR) and amplitudes (NA) at different concentrations respectively (Fig. 4b and c). In Fig. 4b, the NFRs under treatments from left to right (control, 0.01 μmol/L, 0.1 μmol/L, 1 μmol/L, 10 μmol/L and after wash) were: 1 ± 0.15, $n=6$; 0.91 ± 0.17, $n=6$; 0.39 ± 0.09, $n=6$; 0.07 ± 0.05, $n=6$; 0 ± 0, $n=6$; 0.84 ± 0.06, $n=6$ (n represents the number of valid electrodes from 4 arrays). Respectively, the NA data are 1 ± 0.10, $n=8$; 0.95 ± 0.11, $n=6$; 0.82 ± 0.07, $n=6$; 0.51 ± 0.27, $n=6$; 0 ± 0, $n=6$; 0.81 ± 0.04, $n=6$ (n represents the same meaning as in NFR). From the original signals (Fig. 4a), the firing events decreased obviously with increasing concentrations. The analysis of firing rates also presented the significant difference (Fig. 4b). This result indicated that 5-HT presented a distinctive dose-dependent inhibitory effect on firing rate. When the concentration was 10 μmol/L, the firing activities were completely inhibited, which was also consistent with the pharmacological study of 5-HT in hippocampal neuron (Ashby Jr et al., 1994; Salgado and Alkadhi, 1995; Sprouse and Aghajanian, 1988). This inhibition should be due to the activation of 5-HT_{1A} receptor, which opens a G-protein-coupled K^+ channel leading to membrane hyperpolarization (Andrade and Nicoll, 1987). The hyperpolarizing response associated with a decrease in input resistance thereby inhibits cell firing and reduces population spikes. When 5-HT was washed out and replaced with medium, firing activities almost recovered from observation. However, there was still significant difference in firing rate as well as signal amplitude between these two groups. The significant difference between control and after washout groups could be due to the weaker status of neurons. The neurons usually became less responsive after long-term recording and multiple medium exchanges based on our experimental processes. The recovery of the inhibited

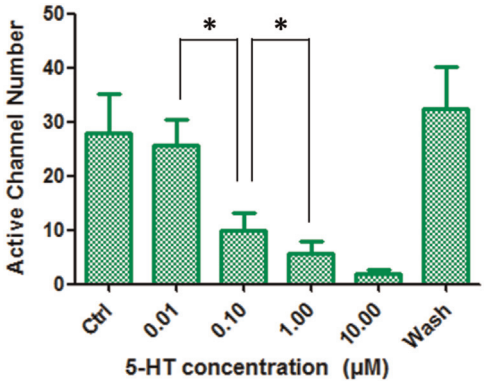

Fig. 5. Active channel number declined with 5-HT stimulations. The channels that managed to detect the signals during recording process (300 s for each condition) decreased when 5-HT concentration increased. Ctrl represents the control state and Wash indicates the state after 5-HT washed out. Data are represented by the mean ± SD. Student's t test was used to evaluate difference significance between groups including 0.01 μmol/L versus Ctrl, 0.1 μmol/L versus 0.01 μmol/L, 1 μmol/L versus 0.1 μmol/L, 10 μmol/L versus 1 μmol/L and Wash versus Ctrl (*, $P<0.05$; **, $P<0.01$).

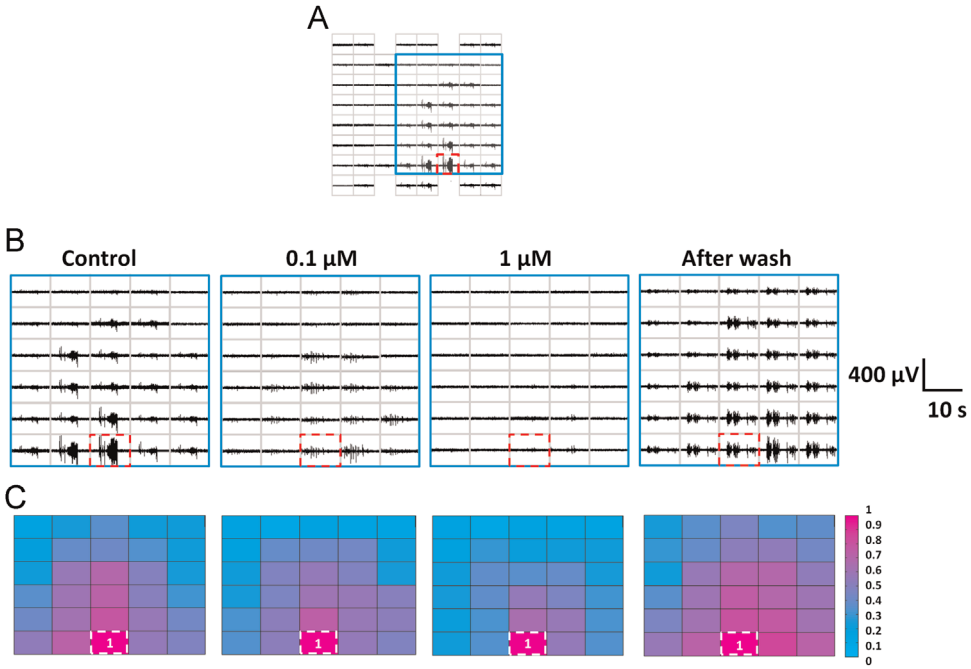

Fig. 6. (a) Consecutive recording sites (regions in blue square) were selected to illustrate the network responses. One channel was selected as reference (red dotted square) to make cross-correlation. analyzes with other channels. (b) Signals in corresponding recording sites in (a) at four conditions (blue squares). (c) Color-coded. maps of cross coefficients of corresponding recording regions in (b). The reference cross coefficient was 1 (white dotted squares). (For interpretation of the references to color in this figure legend,the reader is referred to the web version of this article).

activities proved the specificity of 5-HT's effect on hippocampal neurons. These results proved that this HNN-based biosensor could detect 5-HT in a dose dependent inhibitory way in the single neuron level. The detection limit of this biosensor is at least 0.01 μmol/L, which is lower than those by electrochemical sensors (Li et al., 2009; Rand et al., 2013). And the response range is from 0.01 μmol/L to 10 μmol/L. The response characteristics of this biosensor demonstrate its high sensitivity and specificity in 5-HT detection.

3.4. Network response to 5-HT by HNN-based biosensor

From the firing patterns of single hippocampal neurons, the biosensor detected 5-HT with high sensitivity and specificity. In addition, information from network responses could assign more specificity to this biosensor for 5-HT detection. In order to evaluate the network response of HNNs to 5-HT, firstly the number of the active channels was calculated under each dose treatment (Fig. 5). Active channels represented those channels in which we can still detect the electrophysiological signals in each trial (300 s). The data in Fig. 5 were (from Ctrl to Wash): 28 ± 7.2; 25.7 ± 4.7; 10 ± 3.2; 5.7 ± 2.2; 2 ± 0.6; 32.3 ± 7.8, $n=5$ for all the groups (n represents the number of valid recording trials in which MEA could record the network activities from 7 trials). This result showed that the number of active channels declined with the increasing 5-HT concentrations, consistent with the alteration tendency of NFR and NA in Fig. 4b and c. The result of active channel analysis offered another aspect that determines the biosensor response characteristics.

Consequently, proper concentrations (0.1 μmol/L, 1 μmol/L) were selected to reveal the specific effect of 5-HT on network connections shown in Fig. 6. The selection of these two concentrations is due to the significant difference in active channels. Same recording sites (blue square) from one trial were picked out to illustrate the network characteristics (Fig. 6a). Fig. 6b shows the network responses of HNNs at four conditions (control, 0.1 μmol/L 5-HT, and 1 μmol/L 5-HT, 5-HT washed out). It is visible that the network activities generally decreased after application of 5-HT. It suggests that 5-HT could inhibit the neuronal activities in HNNs. When 5-HT was washed out, the electrophysiological network activities almost recovered. In order to determine biosensor's network responses to 5-HT, correlations of signals were also evaluated with the stimulation of 5-HT. Cross-correlation analysis for network activity between channels was carried out based on the multi-site signals. One channel was selected as reference (red dotted squares) and cross-correlation coefficients were calculated with other channels. And strengths were quantified by measuring the maximal cross-correlation coefficients. Fig. 6c shows the color-coded plots of the cross correlation coefficients for the same channels in Fig. 6a at four conditions: spontaneous, 0.1 μmol/L and 1 μmol/L 5-HT, and 5-HT after wash (Fig. 6a). The network activities were synchronized at these conditions. From observation, the spontaneous network activity synchronized in a neuronal network of about 600 μm in diameter (distance between 5 electrodes). As the concentration of 5-HT increased to 0.1 μmol/L and 1 μmol/L, the synchronous active areas became smaller (about 450 μm and 150 μm). After removal of 5-HT, active areas enlarged to be about 750 μm in diameter. At the same time, the cross-correlation coefficients maps also indicated an obvious regional weakening correlation with the 5-HT treatment. The connective area (area within cross-coefficients > 0.6) decreased from 450 μm to 150 μm in diameter. And the removal of 5-HT also led to an expansion of connective area. These results were consistent with the recorded signals in Fig. 6b, indicating that 5-HT could inhibit the neuronal connections of the network. Both the observation of network activities and the cross correlation analysis suggest that 5-HT could regulate network responses in an inhibitory way and that this inhibition could be reversed by removal of 5-HT. The inhibitory effects of 5-HT could be monitored effectively and conveniently by this hybrid biosensor, in particular with the color-coded cross-correlation maps. Above all, the network response analyzes provide network information to better determine and detect 5-HT, adding more specificity to this biosensor.

4. Conclusion

In the present study, we established a hybrid HNN-based biosensor with MEA chip by using HNNs as sensing elements. This biosensor is applied in detecting 5-HT from both single neuron and neuronal network levels. In general, this hybrid biosensor could detect 5-HT in a dose-dependent inhibitory way. This electrophysiological detection is superior in sensitivity and specificity due to the underlying biological interaction between 5-HT and hippocampal neurons. Moreover, electrophysiological recording by MEA is fast and easy to manipulate, making the biosensor convenient for monitoring for long term. With these advantages, it can be applied in detecting and monitoring neurotransmitters' effect on neural electrophysiology *in vitro*. However, neuron-electrode attachment need to be further studied to enhance the performance of neuron-based biosensor. In future work, surface modification would be made to increase the biocompatibility of MEA surface and to guide neurons adhere target positions. Overall, this study may provide a valuable sensing approach to know more about the 5-HT function in the neural system and find better ways to regulate and improve the neural function.

Acknowledgment

This work was supported by Major International Cooperation Project of Natural Science Foundation of China (No. 61320106002), Marine Public Welfare Project of China (No. 201305010), and Natural Science Foundation of China (No. 31228008).

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