PPy/SWCNTs-Modified Microelectrode Array for Learning and Memory Model Construction through Electrical Stimulation and Detection of In Vitro Hippocampal Neuronal Network

Yan Yang [a, b, ‡], Yu Deng [c, ‡], Shihong Xu [a, b], Yaoyao Liu [a, b], Wei Liang [a], Kui Zhang [a, b], Shiya Lv [a, b], Longze Sha [c], Huabing Yin [d], Yirong Wu [a, b], Jinping Luo [a, b], Qi Xu [c]*, Xinxia Cai [a, b]*

[a] State Key Laboratory of Transducer Technology, Aerospace Information Research Institute, Chinese Academy of Sciences, Beijing 100190, China.

[b] School of Electronic, Electrical and Communication Engineering, University of Chinese Academy of Sciences, Beijing 100049, PR China.

[c] State Key Laboratory of Medical Molecular Biology, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100005, PR China.

[d] Division of Biomedical Engineering, James Watt School of Engineering, University of Glasgow, Oakfield Avenue, Glasgow G12 8LT, United Kingdom

ABSTRACT: The learning and memory function of the brain remain unclear, which is in urgent need of the detection of both a single cell signal with high spatiotemporal resolution and network activities with high

throughput. Here, an in vitro microelectrode array (MEA) was fabricated and further modified with polypyrrole /carboxylated single-walled carbon nanotubes (PPy/SWCNTs) nanocomposites as the interface between biological and electronic system. The deposition of the nanocomposites significantly improved performance of microelectrodes, including low impedance (60.3 ± 28.8 k Ω), small phase delay (- 32.8 ± 4.4°) and good biocompatibility. Then the modified MEA was used to apply learning training and test on hippocampal neuronal network cultured for 21 days through electrical stimulation, and multichannel electrophysiological signals were recorded simultaneously. During the process of learning training, the stimulus-response ratio of the hippocampal learning population gradually increased and the response time gradually decreased. After training, the mean spikes in burst, number of bursts and mean burst duration increased by 53%, 191% and 52% respectively, and the correlation of neurons in the network was significantly enhanced from 0.45 ± 0.002 to 0.78 ± 0.002 as well. In addition, the neuronal network basically retained these characteristics for at least 5 hours. These results indicated that we have successfully constructed a learning and memory model of hippocampal neurons on the in vitro MEA, contributing to understanding learning and memory based on synaptic plasticity. The proposed PPy/SWCNTs-modified in vitro MEA will provide a promising platform for the exploration of learning and memory mechanism and their applications in vitro.

KEYWORDS: microelectrode array, learning and memory, cultured hippocampal network, PPy/SWCNTs, electrical stimulation

1. INTRODUCTION

Learning and memory are the basic and essential advanced neural functions of the brain, and the underlying mechanisms have always been mysteries that researchers continue to explore[1, 2]. Some researchers proposed that learning is the formation of a specific group of neurons, and memory is their maintenance and activation[3]. The physiological basis is generally believed to be that under the action of external stimuli, neurons produce long-term potentiation (LTP) and long-term depression (LTD), which bring changes in the connections between neurons through synaptic plasticity[4]. In addition, learning and memory are closely related to the hippocampus[5, 6]. However, to date, the mechanism of learning and memory at the micro-cell level remains inadequately clarified, which is significant to be explored continuously via adopting effective tools with high spatiotemporal resolution and simultaneous regulation and detection.

Action potential is the fundamental embodiment of neural activity[7], and electrical stimulation (ES) is an approach to induce neuronal network learning and memory[8-10]. A precedent for microelectrode arrays (MEA) in neural information detection was created, after Thomas and his coworkers[11] assembled multiple metal microelectrodes in a specific area for the first time and successfully detected isolated-cultured neural electrical signals. Currently, MEA has become an experimental platform widely used in the study of electrophysiology of neural network models in vitro because of its high-throughput and non-invasive characteristics[12, 13]. However, MEA capable of resolving single neuronal firing are limited by the small electrode geometry, hence the recording and stimulation performance is adversely affected.

Nanomaterials have important application significance in detecting weak signals and improving signal-to-noise ratio due to their special advantages caused by extremely tiny size, such as high specific surface area, low impedance, chemical catalytic ability, etc. Among them, Carbon nanotubes (CNTs) have become one of the ideal nerve electrode materials since their discovery because of their unique tubular molecular structure, good biocompatibility, and excellent mechanical, electrical, and chemical

properties[14, 15]. Similar to CNTs, conductive polymers have good electrochemical properties and biocompatibility[16]. Specifically, polypyrrole (PPy) is a commonly used biosensor modification material, and has a wide range of applications in the field of neural electrodes. Richardson et al. [17] used PPy-modified neural electrodes to successfully perform electrical stimulation and release of neurotrophic substances in rats. George et al. [18] confirmed the biocompatibility of PPy through implantation experiments in the rat brain.

Here, we combined Microelectro Mechanical System (MEMS) technology, nanomaterial modification technology and cell culture technology to develop a microelectrode array for investigating the learning and memory functions of primary hippocampal neurons. Electrochemical deposition of PPy/CNTs nanocomposites on the surface of microelectrodes was performed to improve the array performance. As a result, the MEA demonstrated its efficacy in the study of hippocampal neurons induced to produce learning and memory behaviors under specific electrical stimulation paradigm. Overall, the proposed MEA provides a reliable and sensitive bidirectional brain-computer interface for in vitro cultured neuronal networks, making it a useful tool for research in neuroscience, cell biology, and drug discovery.

2. MATERIALS AND METHODS

2.1 MEA design and fabrication. We designed a 60-channel MEA on 4.9 cm×4.9 cm glass substrate, which contains 59 working electrodes of 30 μm diameter and 1 inverted “Y” shaped reference electrode extending into the interior of the working electrodes. Figure 1a shows that the MEA consisted of four layers: glass substrate, conductive layer, insulating layer and PPy/SWCNTs nanocomposite-modified layer. The fabrication of the first three layers was accomplished by MEMS technology, which was briefly described as shown in Figure 1c. First, spin the photoresist AZ1500 on the substrate after concentrated sulfuric acid treatment, expose with the first photomask and develop. Second, sputter the conductive metal layer (30 nm/250 nm Ti/Pt) and lift off to form patterns such as microelectrode sites, wires, pads,

etc. An insulating layer (800 nm Si_3N_4) was used through plasma-enhanced chemical vapor deposition, followed by the second photolithography. Then, C_4F_8 reactive ion etch the insulating layer on microelectrode sites, reference electrode and pads, but keep other parts insulating. Finally, to form a container for subsequent electrochemical deposition and cell culture, a glass ring was sticked to the MEA surface (Figure 1b).

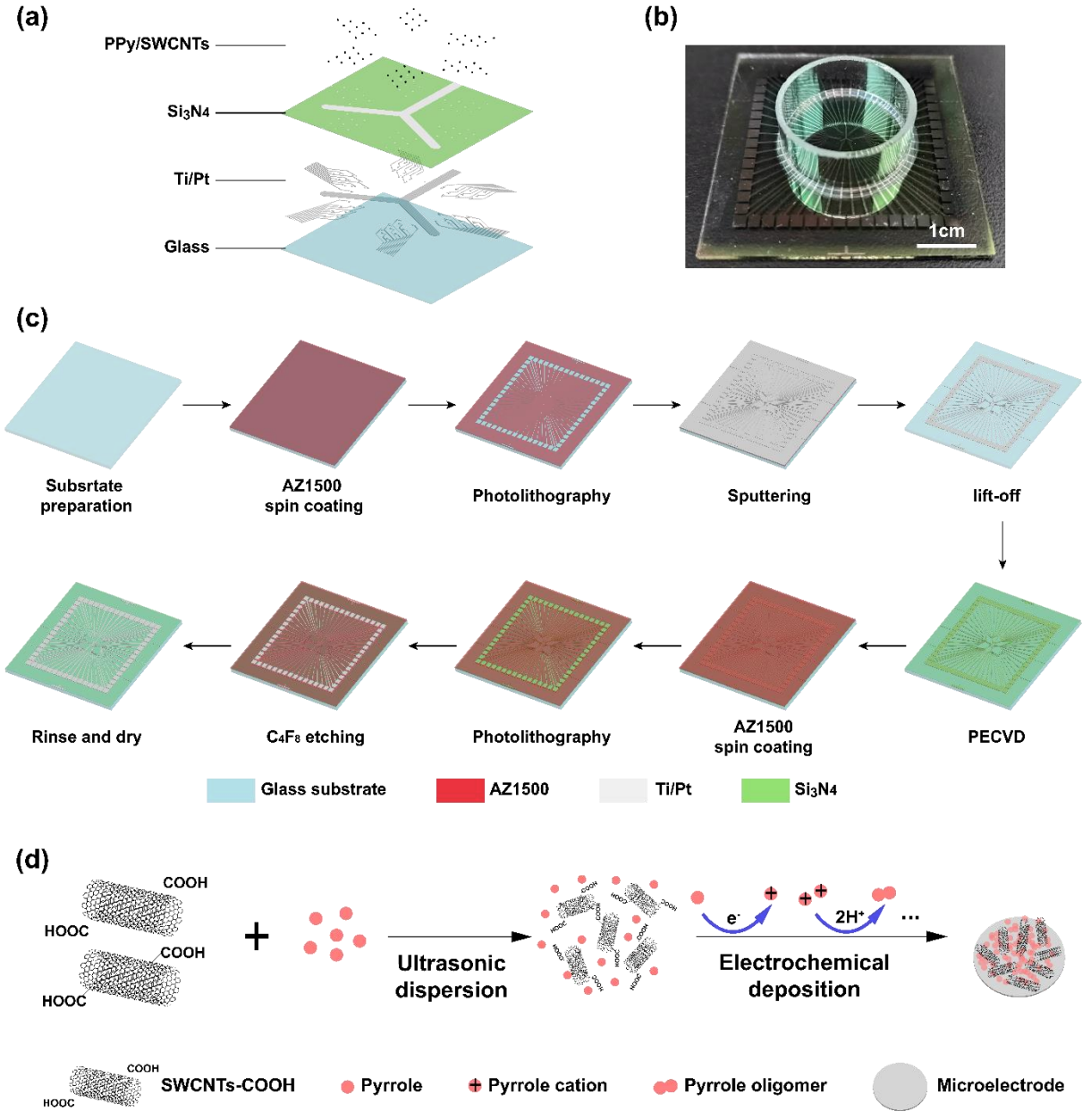

Figure 1. Schematic diagram of the MEA. (a) Exploded diagram of the MEA, consisting of glass substrate, Ti/Pt conductive layer, Si_3N_4 insulating layer and PPy/SWCNTs nanocomposite layer. (b) Photograph of the completed MEA. (c) MEMS fabrication process of the three layers: glass substrate, Ti/Pt conducting layer and Si_3N_4 insulating layer. (d) Process of electrochemical deposited PPy/SWCNTs nanocomposite coating on the Ti/Pt conducting microelectrodes.

2.2 Nanomodification for MEA-neuron interface. The 2 mg/mL carboxylated single-walled carbon nanotubes (SWCNTs, XFNANO, Nanjing, China) were diluted to 0.5 mg/mL by adding ultrapure water (Michem, Chengdu, China). Pyrrole (TCI Chemicals, Tokyo, Japan) was then added to this dispersion and kept ultrasonic vibration for 30 minutes to obtain a homogeneous dispersion. Before the electrodeposition, the MEA was oxygen plasma cleaned (50 w, 30 s) to attenuate the effects of the tiny impurities on electrode surface. The electrodeposition was carried out using a three-electrode system, in which the microelectrodes serves as working electrode, Pt as the counter electrode, and Ag/AgCl as reference electrode. Cyclic voltammetry (CV) was conducted on an Autolab PGSTAT302N electrochemical workstation (Autolab, Herisau, Switzerland) for the nanocomposites deposition. The scanning range of CV was 0-1.7 V, the scanning speed was 100 mV/s, and 11 cycles were scanned.

In the electrodeposition process, pyrrole monomer released an electron at the anodic electrode and became cationic pyrrole free radical, two of which would gather and produce an electroneutral dimer through disproportionation. Then dimer turned into pyrrole dimer cation by the loss of an electron, and then could be combined with monomer cation to form a trimer. Similar processes continued to synthetise PPy eventually[19]. Meanwhile, because carbon nanotubes were negatively charged, pyrrole cations with positive charges would also appeal to the surface of carbon nanotubes and form π-π conjugation[20]. As a consequence, PPy/SWCNTs nanocomposites was obtained. The process above is as shown in Figure 1d.

2.3 Mice embryos hippocampal tissue isolation and Primary hippocampal neuron culture. Primary neuron culture methods refer to the literature[21, 22]. Briefly, pregnant mice from Institute of Cancer Research (Vital River, Beijing, China) were sacrificed by cervical dislocation at gestational day 15.5, the abdominal skin was disinfected with alcohol, taken out the embryos carefully, and the hippocampal tissue was isolated under a dissecting microscope. Hippocampal tissue was digested with 0.25% EDTA-trypsin (Macgene, Beijing, China) in a 37℃-water bath for 15 minutes, then carefully separating the cells from the tissue blown by pipette. Cells centrifugation at 200 g for 5 min and resuspended by neurobasal plus medium (Gibco, Waltham, USA). After counting, cells were inoculated on Poly-L-Lysine (PLL, Sigma, St. Louis, USA) pre-coated MEA or cell dishes at a density of 1×10^5 cell/cm^2. Half-change the cells medium every three days. All animal experiments have passed the review of the Chinese Academy of Medical Sciences-Peking Union Medical College Ethics Committee, and all relevant personnel in animal experiments have the Beijing Municipal Laboratory Animal Practitioner Post Certificate.

2.4 Immunocytochemistry. Immunocytochemistry methods refer to the literature[23, 24]. After 21 days of culture, cells were carefully washed twice with 37℃ 1× phosphate-buffered saline (PBS, Gibco, Waltham, USA), fixed by adding 4% formaldehyde solution (Gibco, Waltham, USA) for 10 min, then discarded PBS cautiously, and carefully washed 3 times with 1× PBS, 10 minutes each time. 5% bovine serum albumin (BSA, Gibco, Waltham, USA) solution containing 2‰ Triton-X-100 (Gibco, Waltham, USA) was added and incubated for 1 h, then discard the supernatant, wash carefully 3 times with 1× PBS, 10 minutes each time. Add MAP-2 primary antibody (Abcam, Cambridge, UK) diluted in 1% BSA solution by 1:200 and incubate overnight at 4°C. Subsequently, the fluorescent secondary antibody (Invitrogen, Carlsbad, USA) diluted in 1% BSA solution was incubated at 37°C for 1 h, discarded the supernatant, and carefully washed 3 times with 1× PBS, 10 min each time. Counterstained with DAPI (Abcam, Cambridge, UK), mounted, and microscopically examined.

2.5 Experimental workflow for learning and memory model construction. Negative phase first biphasic voltage pulses with ±300 mV amplitude, 200 μs pulse-width and 1 Hz frequency were employed as stimulus pulses. Biphase is to ensure the charge balance and avoid excessive accumulation of charges on the electrode. Specific frequency, amplitude, and pulse-width were chosen because pulses at these parameters have been reported to impact on synaptic plasticity as well as learning and memory function of neuron[25].

Spontaneous discharge was recorded at the beginning (control). Then we conduct electrical stimulation on cultured neuronal networks as learning training (learning phase), which contained 8 cycles with 4 trials in each cycle and 10 stimulus pulses in each trial. 10 consecutive stimulus pulses were followed by a 20-second break (i.e. the interval between trials in a cycle was 20 s), and when 4 trials were done, an 1-minute break was taken (i.e. the interval between cycles 1 min). There were 320 stimulus pulses overall in the learning phase, lasting for 20 minutes and 20 seconds. After learning, the neuronal network rest for 20 minutes, then stimuli-evoked response was tested (test phase). The test method is to apply 10 stimulus pulses (i.e. one trial) to the neuronal network at the same position once an hour, and detect its response for further analysis (Figure 2). All these stimulus pulses were conducted through the STG4002 dual-channel stimulus generator (Multi Channel Systems, Baden-Württemberg, Reutlingen, Germany).

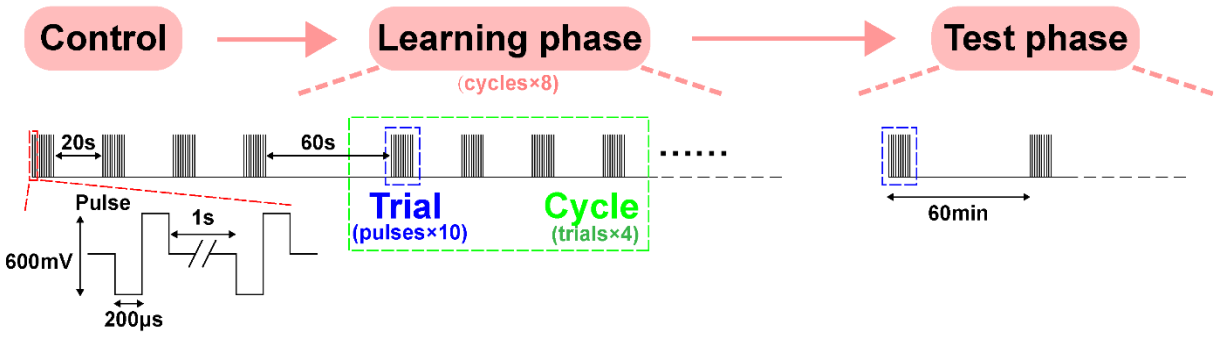

Figure 2. Schematic illustration of experimental workflow to explore learning and memory function of cultured hippocampal neuronal networks, including the electrical stimulation protocol applied in learning phase and test phase and the basic unit of stimulus pulses.

2.6 Electrophysiology recordings and data preprocessing. MEA was connected to the 128-channel Cerebus neuro data recording system (Blackrock Microsystems, Salt Lake City, USA) through a customed Printed Circuit Board (PCB). The signal was displayed and stored using Central Software Suite (Blackrock Microsystems, Salt Lake City, USA). Spikes was sampled at 30 kHz and extracted by 250 Hz-5 kHz band-pass filter. In addition, only spikes that reached the threshold (three times the amplitude of baseline) would be displayed and recorded.

To extract single unit activity, valley-seeking principal component analysis (PCA) algorithm was applied for spike sorting, then remove units with an average firing rate below 0.1 Hz and waveforms with interspike intervals (ISIs) below the refractory period (1000 μs) from spike sorting output.

3. RESULTS AND DISCUSSION

3.1 Characterization of PPy/SWCNTs-modified MEA. Compared with the bare microelectrode, the PPy/SWCNTs-modified microelectrode shows a dark surface (Figure 3a), From the scanning electron microscopy (SEM), there are reticulated interwoven nanostructures formed on the modified MEA (Figure 3b), significantly increasing the surface roughness and expanding the specific surface area of microelectrodes. The electrochemical impedance spectroscopy (EIS) was carried out in a PBS solution from 10 Hz to 100 kHz, and the results showed that both the impedance and phase of the modified electrode were significantly reduced (Figure 3c-e). Compared with the bare electrode, the impedance at 1 kHz (characteristic frequency of neuron spike) decreased from 251.7 ± 28.8 kΩ to 60.3 ± 28.8 kΩ, and the phase from -73.6 ± 0.3° to -32.8 ± 4.4°. The significant decrease in impedance after modifying the

microelectrodes with PPy/SWCNTs can be attributed to several potential reasons: Firstly, the PPy/SWCNTs may increase the effective surface area of the electrode, allowing for more efficient charge transfer between the electrode and the surrounding cells, which can contribute to a reduction in impedance. Secondly, the presence of SWCNTs in the PPy/SWCNTs may promote the formation of a more uniform and compact coating on the electrode surface, and this can reduce the contact resistance between the electrode and the surrounding cells, leading to a decrease in impedance. Finally, both PPy and SWCNTs have been reported to possess excellent electrical conductivity, so that PPy/SWCNTs may improve the conductivity of the electrode, further contributing to the reduction in impedance.

The coarsening of the surface is conducive to the close adhesion of cells on MEA. The reduced impedance and the decreased phase delay are of great significance for the detection of weak signals such as neural discharge. In conclusion, the modification of PPy/SWCNTs nanocomposites improved performance of MEA.

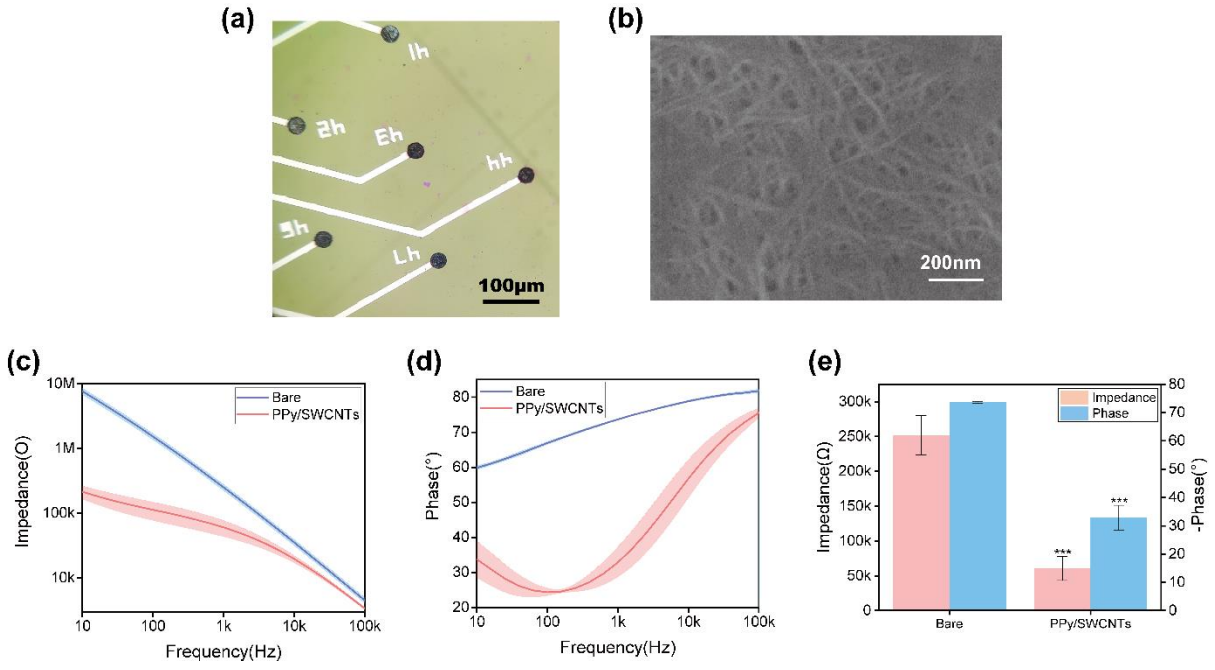

Figure 3. Topography and electrochemical characterization of PPy/SWCNTs-modified microelectrodes. (a) Optical microscope photograph of PPy/SWCNTs-modified microelectrodes. Scale bar = 100 μm. (b) SEM at 50kx magnification of PPy/SWCNTs modification. Scale bar = 200 nm. (c) Impedance characteristics of bare and PPy/SWCNTs-modified microelectrodes at different frequencies. (d) Phase characteristics of bare and PPy/SWCNTs-modified microelectrodes at different frequencies. (e) Average impedance and phase of bare and PPy/SWCNTs-modified microelectrodes at 1kHz. *** = $P < 0.001$, t-test, n = 5 for each group.

3.2 Monitoring of electrophysiological signals from primary hippocampal neurons on MEA. The cultured primary hippocampal neurons on the proposed MEA demonstrated a good condition. After cultured for 21 days in vitro, the neurons grew strong neurites and formed complicated connections (Figure 4a). MAP-2 and DAPI were used to identify the hippocampal neuronal network (Figure 4b). Fig.4c illustrated raw data recorded by our MEA, including high-frequency spike signals and low-frequency local field potential signals, thus demonstrated the electrophysiological signal detection capability of the MEA by showing active spontaneous discharges through multiple channels, with the waveforms being typical neuron discharges.

To study learning and memory function of the cultured neuronal network through our MEA, we used electrical stimulation as a learning paradigm with similar procedures to those previously reported to induce learning[26, 27] (see the Materials and Methods for more details). The electrophysiological recording of neuronal networks included three phases: spontaneous discharge, learning phase and test phase. The function of spontaneous discharge was to serve as a control group. The purpose of the learning stage was to let neurons gradually acquire the external input information and respond to electrical stimulation stably at fixed positions. The test stage tested the stimulus-induced response.

Spike signals after high-pass filter and classification in three different experimental phases were shown in Figure 4d, which demonstrated a significant difference. The spontaneous spikes in control phase behaved relatively calm and distributed relatively even. In the learning phase, neurons were activated by continuous and regular electrical stimulation and responded to it, therefore the discharge turned to be very intense. After the learning and memory process of fixed electrical stimulation pattern in the learning phase and a period of rest, the neurons tended to fire synchronously at the specific time in the test phase. These transformation in firing pattern, synchronization and other aspects indicated that the neuronal network became more mature after electrically stimulated[28]. Further in-depth analysis such as quantification of these transformation will be carried out as below.

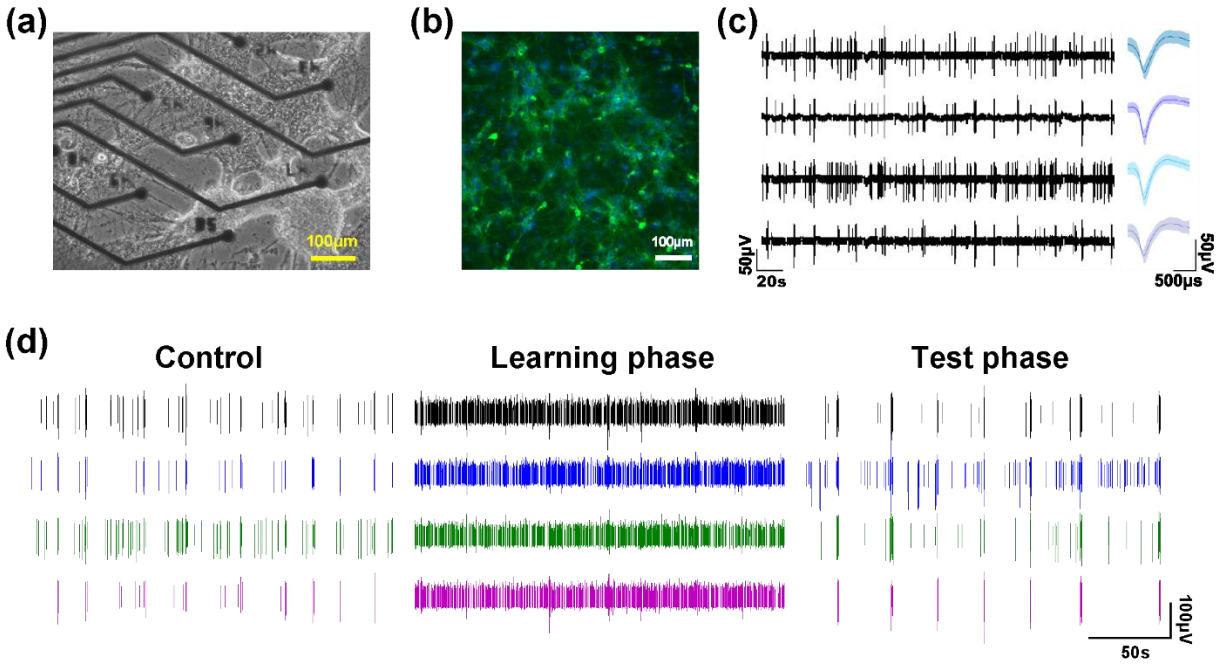

Figure 4. Interconnected networks and electrophysiological activity of the hippocampal cells. (a) Neurons maintained on MEA for 21 days. Scale bar:100 μm. (b) Immunocytochemistry (ICC) images of hippocampal neuronal networks. Neuronal structures stained with MAP-2 (green)， while nuclei were subjected to DAPI staining (blue). Scale bar: 100 μm. (c) Original recorded electrophysiological signals (Scale bars: 50 μV, 20 s) and their corresponding spike waveforms (Scale bars: 50 μV, 500 μs) of four

representative channels. (d) Neural spikes in spontaneous discharge, learning phase and test phase respectively. Scale bars: 100 μV, 50 s.

3.3 Real-time learning and enduring memory of neurons to electrical stimulation. The response of neurons in the learning phase was shown in Figure 5a. The discharge of hippocampal neurons gradually became regular and coupled with electrical stimulation, indicating that neurons progressively adapted to external stimuli and learn their characteristics in the learning phase. This phenomenon could be quantified by stimulus response/stimulus ratio (R/S ratio) and response time (RT)[29, 30]. The R/S ratio referred to the proportion of stimulus pulses that caused the expected response in a trial (including 10 stimulus pulses), that is, the proportion of the number of stimulus pulses that successfully induced the action potential within 20-80 ms to the total number of stimulus pulses in a trial. Response time was defined as the time required to reach the expected response. To get rid of the impact of stimulus artifacts, we took the data from 10 ms to 200 ms after stimulus pulses for analysis, and considered it unresponsive if there was no spike within 10-200 ms. The difference between the average timestamp of the first three action potentials evoked by the stimulus pulse and the timestamp of the stimulus pulse was taken as the RT. After calculating the R/S ratio and RT of each neuron in the 32 trials in the learning phase, we performed linear fit on the 32 R/S ratios and 32 RTs of every 45 neurons respectively. Only when the condition that slope of R/S ratio was positive and the slope of RT was negative are both fulfilled, the neuron was categorized as learning population. Through calculation, 13 of the total 45 neurons were learning population.

Linear fit of R/S ratio and RT of the learning population at the learning phase were performed (Figure 5b), the former of which had a positive slope and the latter had a negative one. The results demonstrated an increase in R/S ratio and a decrease in RT, indicating that the hippocampal neurons were successfully induced to learning and memory behavior. In 5 h of the test phase, the R/S ratio and

RT of the learning population fluctuated slightly and kept at a better level than that of the learning phase (Figure 5c). We inferred that with some rest after the learning phase, the neuronal network had stabilized from an excited state, formed a learning population through specific connections and learned and memorized specific external inputs, thus became a more focused-functional neuronal network.

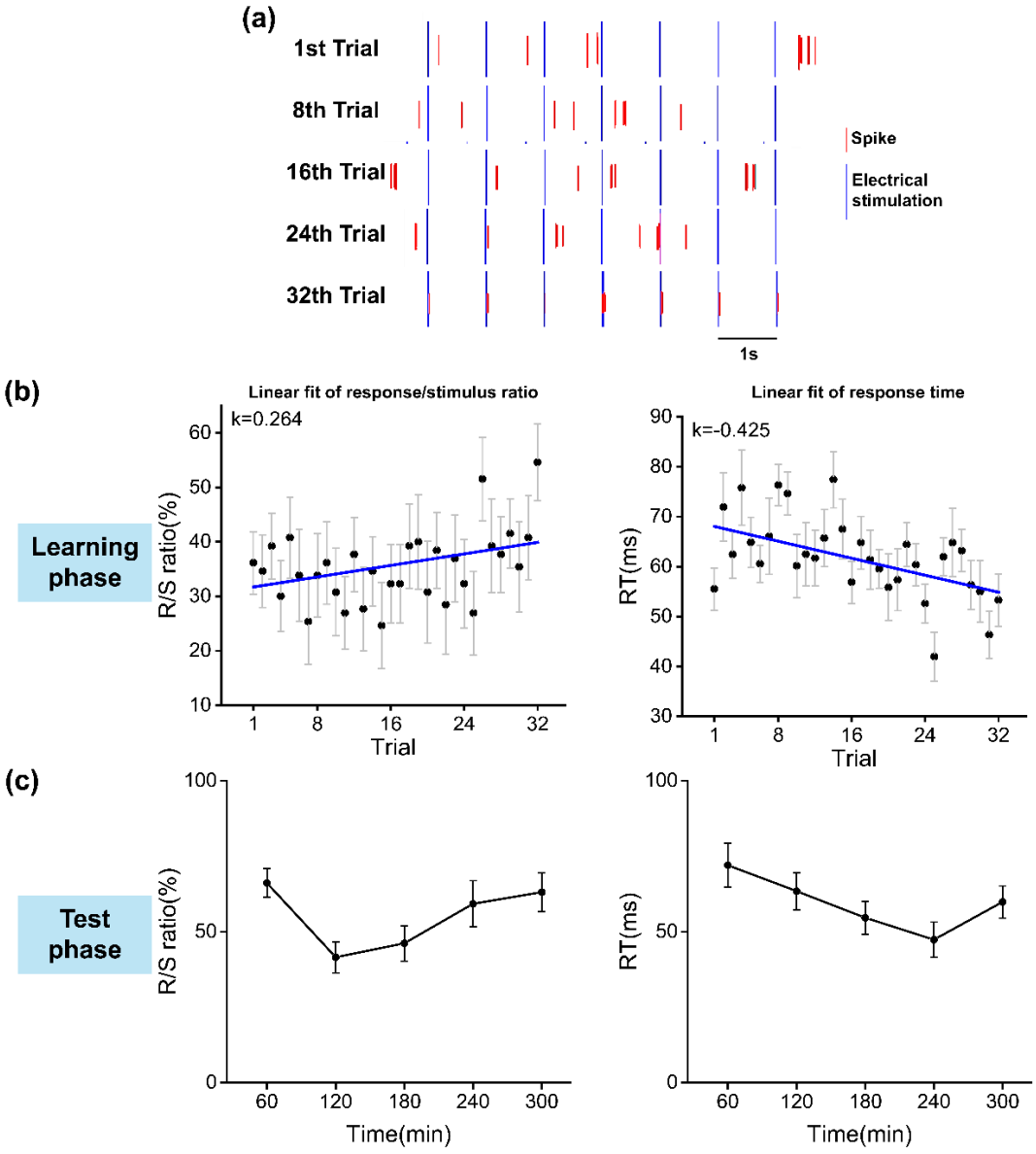

Figure 5. Learning response and memory retention phenomena through quantification of stimulus-evoked firing. (a) Diagram of spike and ES timestamps, illustrating learning response along with trials in

learning phase. (b) Linear fit of trial vs. the R/S ratio and RT of the learning population in learning phase. (c) The R/S ratio and RT in test phase.

3.4 Firing pattern transformation to bursts. Burst firing refers to the continuous and high-frequency discharge of neurons in a short time[31]. Synchronous burst activity plays an essential role in advanced brain functions such as learning and memory[32]. We could see from figure 4d intuitively that before the learning training, the spontaneous firing pattern of the cultured hippocampal neuronal network appeared random, consisting of single spike and burst firing. When the neuron network was successfully trained to learn, its electrical activity was transformed into synchronous bursts. The burst analysis showed that compared with the situation before ES, the mean spikes in burst went up from 2.98 (±0.41) to 4.57 (±0.47), the number of bursts of the neuronal network increased from 6.35 (±1.12) to 18.45 (±1.34) and the mean burst duration rose from 23.21 (±3.58) ms to 35.17 (±1.67) ms, displaying 53%, 191% and 52% uplift respectively (Figure 6a-c). These numbers illustrated that the burst firing of the neuronal network was improved, and we conducted successful learning training by means of electrical stimulation.

The spontaneous synchronous burst activity of the neuronal network induced by successful learning training lasted in test phase, which took 5 hours (Figure 6d). It was an expression of LTP, which is a cellular mechanism closely related to learning and memory[33]. The long-term changes in the electrophysiological activity of this neuronal network were manifestations of enhanced synaptic plasticity, indicating that the information transmission and processing functions of the neuronal network were strengthened.

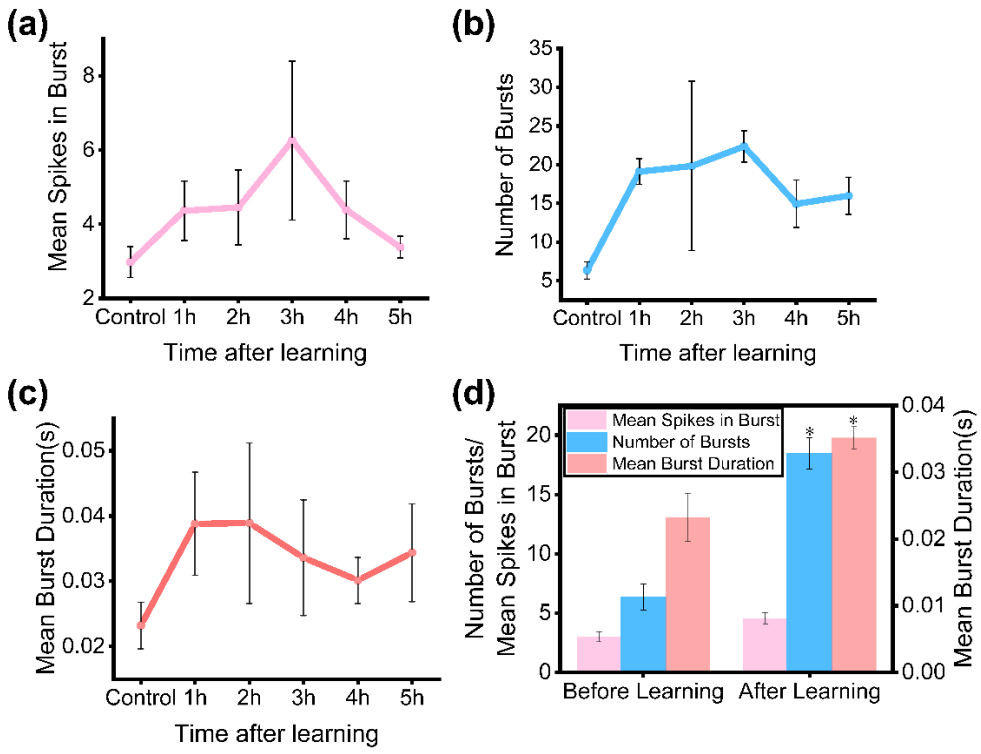

Figure 6. Burst analysis under control and after learning. (a) Mean spikes in burst, (b) Number of bursts and (c) Mean burst duration under the condition of control, 1 h, 2 h, 3 h, 4 h, 5 h after learning respectively. (d) Comparison of three parameters before and after stimulation. * = $P < 0.05$, t-test.

3.5 Enhancement of network correlation after learning. The response of the neuronal network to electrical stimulation is not only reflected at the cell level, but also at the network level. Quantifying the correlation between spike trains of neurons is a crucial part of neuron network analysis. When the firing rate is different, some indicators commonly used to measure the correlation cannot be objectively compared, such as Pearson correlation coefficient, correlation index, etc. In other words, they fail to distinguish whether the increase in probability of simultaneous neurons firing is due to the neurons become more active or the neuron connections become tighter. Therefore, an improved indicator, spike time tilling coefficient (STTC), was proposed in 2014[34]. The outstanding advantage of STTC is that it is insensitive to the firing rate, hence it is eligible to exclude the interference of the firing rate from

correlation quantification. Therefore, STTC has been widely used in measuring the network correlation of neural spike trains recorded from MEA[35, 36].

We calculated the STTC of every two cells through their spike trains, and it is demonstrated from the color change in STTC correlation matrix heatmap that after the electrical stimulation in the learning phase, the network correlation was significantly enhanced, and only slightly weakened despite the passage of time. This strong synchronization in this network was generally maintained during 5 hours in the test phase (Figure 7a). The average results also confirmed this point, and the difference between control and test phase was significant, even in the fifth hour (Figure 7b).

The above results showed that after the hippocampal neuronal network on the MEA underwent electrical-stimulation-form learning training, not only the overall burst firing of the neuronal network was enhanced, but also the correlation and synchronization of the electrophysiological activities in the neuronal network had been improved. These were manifestation of significant alterations in the electrophysiological characteristics of neuronal networks after effective learning training. The reason for these significant alterations might be that learning training changed the connection among neurons in the network. Through the growth of new synapses and the decay of old synapses, special neuronal clusters had been formed, and the formation and preservation of such neuronal clusters is learning and memory of neuronal network[37-39].

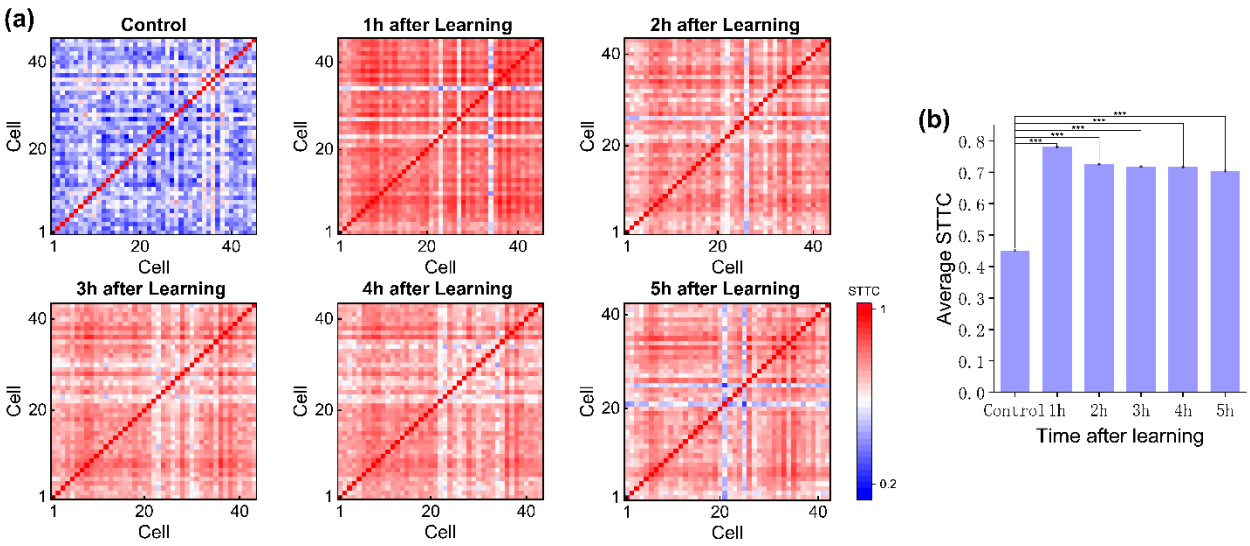

Figure 7. Changes in network correlation brought by learning. (a) Heatmap of STTC correlation between every two cells under the condition of control, 1 h, 2 h, 3 h, 4 h, 5 h after learning respectively. (b) Average STTC correlation under the condition of control, 1 h, 2 h, 3 h, 4 h, 5 h after learning respectively. *** = P < 0.001, t-test.

4. CONCLUSION

In the present work, we designed and fabricated an in vitro MEA to simultaneously detect electrophysiological signals of neurons and apply electrical stimulation to them. The MEA was prepared by MEMS technology, and the electrode-neuron interface was modified by electrochemical deposition technology of nanomaterial. The modified PPy/SWCNTs nanocomposites had low impedance, small phase delay and good biocompatibility, which benefited the detection of high-quality signals in cultured neurons. Then we conducted learning training and test through electrical stimulation on the primary hippocampal neurons cultured 21 days in vitro. In the learning phase, the R/S ratio gradually increased while the RT progressively shortened. After stimulation, both bursts of neurons and the network correlation enhanced. These changes were maintained within five hours in the test phase, indicating the

generation of learning behavior induced by electrical stimulation and the retention of memory. Consequently, PPy/SWCNTs-modified in vitro MEA, with simultaneous record and stimulation functions, provides a promising tool for study of simplified cultured neuronal networks.

AUTHOR INFORMATION

Corresponding Author

Jinping Luo − State Key Laboratory of Transducer Technology, Aerospace Information Research Institute, Chinese Academy of Sciences, Beijing 100190, China; University of Chinese Academy of Sciences, Beijing 100049, China; Email: jpluo@mail.ie.ac.cn

Qi Xu − State Key Laboratory of Medical Molecular Biology, Institute of Basic Medical Sciences Chinese Academy of Medical Sciences, School of Basic Medicine Peking Union Medical College, Beijing 100005, China; Neuroscience Center, Chinese Academy of Medical Sciences, Beijing 100005, China; Email: xuqi@pumc.edu.cn

Xinxia Cai − State Key Laboratory of Transducer Technology, Aerospace Information Research Institute, Chinese Academy of Sciences, Beijing 100190, China; University of Chinese Academy of Sciences, Beijing 100049, China; orcid.org/0000-0001-5997-7252; Email: xxcai@mail.ie.ac.cn

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. ‡These authors contributed equally. Y. Yang. brought up conception and specific design of the experiments. Y. Deng cultured primary hippocampal neurons and performed fluorescence staining. J. Luo, W. Liang, H. Yin, Y. Wu, Q. Xu and X. Cai guide the whole process. Y. Yang, S. Xu and Y. Liu designed and fabricated the MEA. Y. Yang and K. Zhang complete electrode modification. Y. Yang, S. Xu, Y. Liu and K. Zhang carried out electrical

stimulation experiments. Y. Yang analyzed the data. All authors have given approval to the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS

MEA, microelectrode array; ES, electrical stimulation; R/S ratio, response/stimulus ratio; RT, response time; STTC, spike time tilling coefficient.

REFERENCES

1. Tonegawa, S.; Liu, X.; Ramirez, S.; Redondo, R., Memory Engram Cells Have Come of Age. *Neuron* **2015,** *87* (5), 918-31.

2. Josselyn, S. A.; Tonegawa, S., Memory engrams: Recalling the past and imagining the future. *Science* **2020,** *367* (6473), 39-+.

3. Marom, S.; Shahaf, G., Development, learning and memory in large random networks of cortical neurons: lessons beyond anatomy. *Q. Rev. Biophys.* **2002,** *35* (1), 63-87.

4. Bear, M. F.; Malenka, R. C., Synaptic plasticity: LTP and LTD. *Curr. Opin. Neurobiol.* **1994,** *4* (3), 389-399.

5. Scoville, W. B.; Milner, B., Loss of recent memory after bilateral hippocampal lesions. *J. Neurol. Neurosurg. Psychiatry.* **1957,** *20* (1), 11-21.

6. Ji, J.; Maren, S., Differential roles for hippocampal areas CA1 and CA3 in the contextual encoding and retrieval of extinguished fear. *Learn. Mem.* **2008,** *15* (4), 244-251.

7. Hodgkin, A. L.; Huxley, A. F., Action potentials recorded from inside a nerve fibre. *Nature* **1939,** *144*, 710-711.

8. Chiappalone, M.; Massobrio, P.; Martinoia, S., Network plasticity in cortical assemblies. *Eur. J. Neurosci.* **2008,** *28* (1), 221-37.

9. le Feber, J.; Witteveen, T.; van Veenendaal, T. M.; Dijkstra, J., Repeated stimulation of cultured networks of rat cortical neurons induces parallel memory traces. *Learn. Mem.* **2015,** *22* (12), 594-603.

10. Poli, D.; Massobrio, P., High-frequency electrical stimulation promotes reshaping of the functional connections and synaptic plasticity in in vitro cortical networks. *Phys. Biol.* **2018,** *15* (6), 06LT01.

11. Thomas, C. A.; Springer, P. A.; Loeb, G. E.; Loeb, Y.; Berwald, N.; Okun, M., A miniature microelectrode array to monitor the bioelectric activity of cultured cells. *Exp. Cell Res.* **1972,** *74*, 61-66.

12. Kim, R.; Joo, S.; Jung, H.; Hong, N.; Nam, Y., Recent trends in microelectrode array technology for in vitro neural interface platform. *Biomed. Eng. Lett.* **2014,** *4* (2), 129-141.

13. Tanwar, A.; Gandhi, H. A.; Kushwaha, D.; Bhattacharya, J., A review on microelectrode array fabrication techniques and their applications. *Mater. Today Chem.* **2022,** *26*, 101153.

14. He, E.; Xu, S.; Xiao, G.; Dai, Y.; Li, X.; Song, Y.; Gao, F.; Zhang, Y.; Xu, S.; Cai, X., MWCNTs/PEDOT:PSS nanocomposites-modified microelectrode array for spatial dynamics recording of epileptic discharges in multi-subregion of hippocampal slice. *Sensor. Actuat. B-Chem.* **2021,** *329*, 1-9.

15. Li, X.; Song, Y.; Xiao, G.; He, E.; Xie, J.; Dai, Y.; Xing, Y.; Wang, Y.; Wang, Y.; Xu, S.; Wang, M.; Tao, T. H.; Cai, X., PDMS-Parylene Hybrid, Flexible Micro-ECoG Electrode Array for Spatiotemporal Mapping of Epileptic Electrophysiological Activity from Multicortical Brain Regions. *ACS Appl. Bio Mater.* **2021,** *4* (11), 8013-8022.

16. Aqrawe, Z.; Montgomery, J.; Travas-Sejdic, J.; Svirskis, D., Conducting polymers for neuronal microelectrode array recording and stimulation. *Sensor. Actuat. B-Chem.* **2018,** *257*, 753-765.

17. Richardson, R. T.; Thompson, B.; Moulton, S.; Newbold, C.; Lum, M. G.; Cameron, A.; Wallace, G.; Kapsa, R.; Clark, G.; O'Leary, S., The effect of polypyrrole with incorporated neurotrophin-3 on the promotion of neurite outgrowth from auditory neurons. *Biomaterials* **2007,** *28* (3), 513-23.

18. George, P. M.; LaVan, D. A.; Burdick, J. A.; Chen, C. Y.; Liang, E.; Langer, R., Electrically Controlled Drug Delivery from Biotin-Doped Conductive Polypyrrole. *Adv. Mater.* **2006,** *18* (5), 577-581.

19. Wang, L.; Xu, H.; Song, Y.; Luo, J.; Wei, W.; Xu, S.; Cai, X., Highly Sensitive Detection of Quantal Dopamine Secretion from Pheochromocytoma Cells Using Neural Microelectrode Array Electrodeposited with Polypyrrole Graphene. *ACS Appl. Mater. Interfaces* **2015,** *7* (14), 7619-7626.

20. Mosch, H. L.; Hoppener, S.; Paulus, R. M.; Schroter, B.; Schubert, U. S.; Ignaszak, A., The correlation of the binding mechanism of the polypyrrole-carbon capacitive interphase with electrochemical stability of the composite electrode. *Phys. Chem. Chem. Phys.* **2015,** *17* (20), 13323-32.

21. Seibenhener, M. L.; Wooten, M. W., Isolation and Culture of Hippocampal Neurons from Prenatal Mice. *Jove-J. Vis. Exp.* **2012,** (65), e3634.

22. Ray, J.; Peterson, D. A.; Schinstine, M.; Gage, F. H., Proliferation, Differentiation, and Long-term Culture of Primary Hippocampal Neurons. *P. Natl. Acad. Sci. USA* **1993,** *90* (8), 3602-3606.

23. Chamak, B.; Fellous, A.; Glowinski, J.; Prochiantz, A., MAP2 Expression and Neuritic Outgrowth and Branching are Coregulated through Region-specific Neuro-astroglial Inteactions. *J. Neurosci.* **1987,** *7* (10), 3163-3170.

24. Sciarretta, C.; Minichiello, L., The Preparation of Primary Cortical Neuron Cultures and a Practical Application Using Immunofluorescent Cytochemistry. In *Mouse Cell Culture: Methods and Protocols*, 2010; Vol. 633, pp 221-231.

25. Xu, S.; Deng, Y.; Luo, J.; Liu, Y.; He, E.; Yang, Y.; Zhang, K.; Sha, L.; Dai, Y.; Ming, T.; Song, Y.; Jing, L.; Zhuang, C.; Xu, Q.; Cai, X., A Neural Sensor with a Nanocomposite Interface for the Study of Spike Characteristics of Hippocampal Neurons under Learning Training. *Biosensors (Basel)* **2022,** *12* (7).

26. Jia, X.; Shao, W.; Hu, N.; Shi, J.; Fan, X.; Chen, C.; Wang, Y.; Chen, L.; Qiao, H.; Li, X., Learning populations with hubs govern the initiation and propagation of spontaneous bursts in neuronal networks after learning. *Front. Neurosci.* **2022,** *16*, 854199.

27. Hamilton, F.; Graham, R.; Luu, L.; Peixoto, N., Time-Dependent Increase in Network Response to Stimulation. *PLoS One* **2015,** *10* (11), e0142399.

28. Xu, S.; Deng, Y.; Luo, J.; He, E.; Liu, Y.; Zhang, K.; Yang, Y.; Xu, S.; Sha, L.; Song, Y.; Xu, Q.; Cai, X., High-Throughput PEDOT:PSS/PtNPs-Modified Microelectrode Array for Simultaneous Recording and Stimulation of Hippocampal Neuronal Networks in Gradual Learning Process. *ACS Appl. Mater. Interfaces* **2022,** *14* (13), 15736-15746.

29. Marom, S., Learning in Networks of Cortical Neurons. *J. Neurosci.* **2001,** *21*, 8782–8788.

30. Marom, S.; Eytan, D., Learning in ex-vivo developing networks of cortical neurons. *Prog. Brain Res.* **2005,** *147*, 189-99.

31. Krahe, R.; Gabbiani, F., Burst firing in sensory systems. *Nat. Rev. Neurosci.* **2004,** *5* (1), 13-23.

32. Li, Y.; Zhou, W.; Li, X.; Zeng, S.; Liu, M.; Luo, Q., Characterization of synchronized bursts in cultured hippocampal neuronal networks with learning training on microelectrode arrays. *Biosens. Bioelectron.* **2007,** *22* (12), 2976-82.

33. Dan, Y.; Poo, M. M., Spike timing-dependent plasticity: From synapse to perception. *Physiol. Rev.* **2006,** *86* (3), 1033-1048.

34. Cutts, C. S.; Eglen, S. J., Detecting pairwise correlations in spike trains: an objective comparison of methods and application to the study of retinal waves. *J. Neurosci.* **2014,** *34* (43), 14288-303.

35. Sharf, T.; van der Molen, T.; Glasauer, S. M. K.; Guzman, E.; Buccino, A. P.; Luna, G.; Cheng, Z.; Audouard, M.; Ranasinghe, K. G.; Kudo, K.; Nagarajan, S. S.; Tovar, K. R.; Petzold, L. R.; Hierlemann, A.; Hansma, P. K.; Kosik, K. S., Functional neuronal circuitry and oscillatory dynamics in human brain organoids. *Nat. Commun.* **2022,** *13* (1), 4403.

36. Giandomenico, S. L.; Mierau, S. B.; Gibbons, G. M.; Wenger, L. M. D.; Masullo, L.; Sit, T.; Sutcliffe, M.; Boulanger, J.; Tripodi, M.; Derivery, E.; Paulsen, O.; Lakatos, A.; Lancaster, M. A., Cerebral organoids at the air-liquid interface generate diverse nerve tracts with functional output. *Nat. Neurosci.* **2019,** *22* (4), 669-679.

37. Jimbo, Y.; Robinson, H. P. C.; Kawana, A., Simultaneous Measurement of Intracellular Calcium and Elenctrical Activity from Patterned Neural Networks in Culture. *IEEE Trans. Biomed. Eng.* **1993,** *40*.

38. Orenstein, O.; Keren, H., Development of Cortical Networks under Continuous Stimulation. *Front. Mol. Neurosci.* **2017,** *10*, 18.

39. Dias, I.; Levers, M. R.; Lamberti, M.; Hassink, G. C.; van Wezel, R.; le Feber, J., Consolidation of memory traces in cultured cortical networks requires low cholinergic tone, synchronized activity and high network excitability. *J. Neural Eng.* **2021,** *18* (4).