● RESEARCH ARTICLE

# Quantitative evaluation of extrinsic factors influencing electrical excitability in neuronal networks: Voltage Threshold Measurement Method (VTMM)

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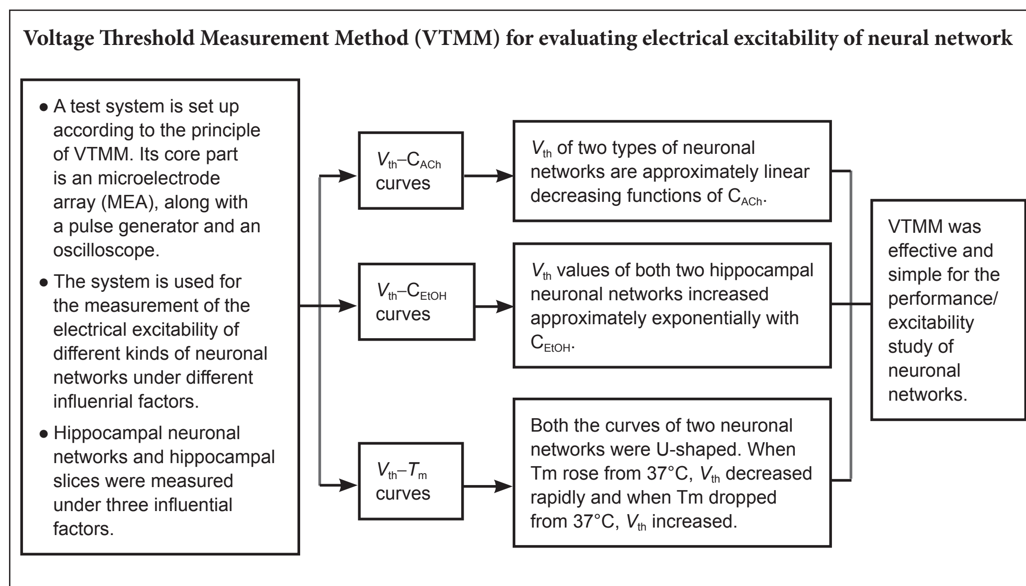
## Graphical Abstract

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## Abstract

The electrical excitability of neural networks is influenced by different environmental factors. Effective and simple methods are required to objectively and quantitatively evaluate the influence of such factors, including variations in temperature and pharmaceutical dosage. The aim of this paper was to introduce 'the voltage threshold measurement method', which is a new method using microelectrode arrays that can quantitatively evaluate the influence of different factors on the electrical excitability of neural networks. We sought to verify the feasibility and efficacy of the method by studying the effects of acetylcholine, ethanol, and temperature on hippocampal neuronal networks and hippocampal brain slices. First, we determined the voltage of the stimulation pulse signal that elicited action potentials in the two types of neural networks under normal conditions. Second, we obtained the voltage thresholds for the two types of neural networks under different concentrations of acetylcholine, ethanol, and different temperatures. Finally, we obtained the relationship between voltage threshold and the three influential factors. Our results indicated that the normal voltage thresholds of the hippocampal neuronal network and hippocampal slice preparation were 56 and 31 mV, respectively. The voltage thresholds of the two types of neural networks were inversely proportional to acetylcholine concentration, and had an exponential dependency on ethanol concentration. The curves of the voltage threshold and the temperature of the medium for the two types of neural networks were U-shaped. The hippocampal neuronal network and hippocampal slice preparations lost their excitability when the temperature of the medium decreased below 34 and 33°C or increased above 42 and 43°C, respectively. These results demonstrate that the voltage threshold measurement method is effective and simple for examining the performance/excitability of neuronal networks.

***Key Words:*** *nerve regeneration; threshold voltage; microelectrode array; electrical excitability of neural networks; acetylcholine; alcohol; temperature; hippocampal neuronal network; hippocampal slice; electrical stimulation; action potentials; neural regeneration*

## Introduction

Examining how information is coded at the level of neuronal networks is important for understanding nervous system function. Since the 1980s, microelectrode array (MEA) technology has been used to study neuronal networks. With this technology, neuronal signals can be transferred to peripheral circuits, enabling assessment of the generation, processing, and transmission of neural signals in neuronal networks (Steve, 2001; Wu and Wu, 2006; Liu et al., 2014). In the present study, we cultivated neural networks *in vitro*. Aside from the culture pattern, the growth of neural networks *in vitro* is mostly random. A high-throughput MEA can be used to assess the electrophysiological characteristics of a neural network with random and natural growth. Researchers can choose to use electrodes for stimulation or probing according to the specific characteristics of the neural network, which is grown on the MEA.

Currently, MEA applications mainly focus on electrical stimulation, toxicology, and drug screening (Otto et al., 2003; Danny et al., 2004; Micholt et al., 2006; Johnstone et al., 2010; Hill et al., 2010; Shein et al., 2010; Chong et al., 2011; Gonzalez-Sulser et al., 2011; Hogberg et al., 2011; Quintero et al., 2011; Chang et al., 2012; Monica et al., 2012; Frega et al., 2014; Shmoel et al., 2016). The way in which various factors affect the electrical activity of neuronal networks is an important topic. However, electrical stimulation is regarded mostly as a routine experimental condition used to induce action potentials or as an experimental way to modulate the electrical activity of neuronal networks. Such experiments primarily examine the degree of development of neuronal networks and the generation of spontaneous action potentials or other intrinsic properties. To date, no studies that used electrical stimulation as a reference parameter have evaluated the influence of extrinsic factors on neuronal networks.

Electrical signals in neural networks can be divided into two categories: spontaneous and evoked signals. Spontaneously generated signals have the following characteristics:

1) The signals are random in terms of shape and bursting site due to variability in connectivity, synaptic development, and the number and distribution of ion channels (Bean, 2007).

2) The temporal release pattern of a signal is related to the developmental period of the neural network. A neural network completes the basic link and begins to generate a low-frequency random-occurring signal *in vitro* after one week of cultivation, but no synchronous activity is present until the culture is at least two weeks old. The number and the strength of the synaptic connections in a cultured neuronal network will increase with the development. In the correlation studies of neural signal, it is indicated that a basic functional synaptic connection between neurons has been formed already, when a random spontaneously-generated or an impulse-stimulated neural signal can be observed in the neuronal network. Neural networks begin to produce synchronous activities *in vitro* at 3 weeks. During weeks 4 to 7, the spontaneous signal increases in frequency and activity in neural networks becomes synchronized. After 7 weeks, electrical activity in neural networks becomes complicated and random, and synchronization gradually diminishes (Van et al., 2004a, b; Ito et al., 2013; Bikbaev et al., 2015).

In contrast, evoked signals have the following characteristics:

1) Evoked signals are relatively stable in terms of synchronism, occurrence rate, amplitude, and duration due to similarities between the neural networks and the range of options for controlling the stimulation.

2) Thresholds above which a stimulus intensity can begin to elicit neural networks to generate one or more synchronous action potentials (Radivojevic et al., 2016) can be defined.

3) Evoked action potentials are mainly composed of postsynaptic potentials. Structural complexity in normally-cultured neural networks can counteract variability in individual neurons. Therefore, relatively stable stimulus intensity thresholds exist for neural networks with good coupling to the MEA. This phenomenon has been used to format logic gates (Feinerman et al., 2008; Soriano et al., 2008; Cohen et al., 2010).

In previous studies by our group, we found that the threshold stays constant in normal culture conditions (typical culture medium, 37°C temperature, and defined developmental cycle), but is influenced by individual or associated factors, such as drug applications and temperature. Therefore, the threshold can be used as a scale to measure the degree of influence of individual or associated factors.

Previous studies have shown that the efficacy of electrical stimulation depends on the amplitude, length, and waveform of stimulus signals. In this study, we chose a biphasic asymmetrical voltage pulse as the electrical stimulation signal. The duration of the positive phase was 10 times that of the negative phase, but its amplitude was one tenth that of the negative phase, providing near balance of charge for the two phases. Asymmetric biphasic voltage pulses with the positive phase first followed by a negative phase have higher stimulation efficiency and can effectively avoid "electrochemical damage" and "anodic break excitation", thus reducing nerve injuries (Brummer et al., 1977; Shen et al., 2001; Wagenaar et al., 2004; Egert et al., 2008; Weihberger et al., 2011, 2013). A unidirectional pulse causes an imbalance of anions and cations in the vicinity of the electrodes, which in turn causes electrochemical damage to the nerve. A bidirectional symmetric pulse will form a strong depolarization region near the anode electrode which can also produce excitatory stimuli to the nerve, when the amplitude is increased to a certain degree. Therefore, bidirectional asymmetric pulses have been used in our study.

Here, we report the results of experimentation with our Voltage Threshold Measurement Method (VTMM), which is introduced for the first time in this paper. Our system enables quantitative evaluation of the influence of individual or associated factors on the electrical excitability of a target/candidate neuronal network. We sough to validate the quantification, reliability, and simplicity of this method. We chose acetylcholine (ACh), ethyl alcohol (EtOH), and temperature as influencing factors due to the large body of previous work on these variables. We measured the influence of these factors on electrical excitability in a hippocampal neuronal network and hippocampal slice preparation using the VTMM.

## Materials and Methods

### Measurement setup

The VTMM system is shown in **Figure 1**.

A voltage pulse generator (Agilent 33220A, Palo alto City, CA, USA) is used as the electrical stimulator. The core part of the system is an MEA module, which includes a glass-based 60-electrode MEA (Multi Channel Systems, Reutlingen City, Baden-Württemberg, Germany) with electrode diameters of 10 μm and spacing of 100 μm, an MEA holder (MEA1060, Multi Channel Systems, Baden-Württemberg, Germany) with a temperature controller (TC01, Multi Channel Systems, Baden-Württemberg, Germany), and a multi-channel neural signal amplifier (Cerebus Front-End Amplifier, Salt Lake City, UT, USA). The MEA module holds the cultured neuronal networks or dissected brain slices. The impulse sequence from the generator is applied to one electrode in the MEA and the other electrodes are used to detect the evoked action potentials. The MEA outputs are connected to the multi-channel neural signal amplifier. The amplified signals are accessed *via* an oscilloscope (Agilent 2024A, Palo alto City, CA, USA) for waveform display and also by a multi-channel neural signal analysis system (with a 128-channel neuro-signal processor and Cerebus analysis software). We used two parallel monitoring systems for the MEA output signals to enable cross-validation of the results of the signal analysis. The cross-validation indicated that multi-channel neural signal analysis system is not necessary in future VTMM systems.

### Cells and reagents

We used the following two types of models to obtain neuronal networks in the present study:

1) Primary hippocampal neuronal cells were isolated from fetuses taken from 18-day pregnant Sprague-Dawley rats (QingLongShan Animal Breeding Center). The mother rats were specific-pathogen-free and had a body mass of approximately 350.00 g. The total number of pregnant rats was 60. Approximately eight embryos were isolated from each rat. The number of male and female embryos was equal.

2) Hippocampal slices from 14-day neonatal Sprague-Dawley rats (QingLongShan Animal Breeding Center) were dissected and prepared. The rats were specific-pathogen-free and had a body mass of approximately 35.00 g. The total number of rats was 100, with an equal number of males and females. Approximately three brain slices containing the bilateral hippocampus area were collected from each rat.

We used the following reagents: Dulbecco's modified Eagle's medium and neurobasal medium (Gibco, Grand Island, NY, USA), fetal calf serum (HyClone, Logan, UT, USA), acetylcholine and agar A (Generay, Shanghai, China), double resistant, B27, glutamine, glutamic acid, D-glucose, and poly-L-lysine (Sigma, Milwaukee, WI, USA), alcohol, NaCl, KCl, $KH_2PO_4$, $MgSO_4$, $NaHCO_3$, and $CaCl_2$ (Nanjing Reagent, Nanjing, China).

All animal procedures were approved by the Institutional Animal Care and Use Committee of Southeast University of China for the use of laboratory animals (approval No. SYXK (Su) 2010-0004, Southeast University, Nanjing, China).

All animal experiments were performed in strict accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals (1988.11.1), and all efforts were made to minimize suffering (approval No. 20100831001; Southeast University, Nanjing, China).

### Preparation of neuro-chips with hippocampal neurons

At the beginning of each experiment, we prepared hippocampal neuronal chips for use with the MEA according to the following steps:

1) The MEA chamber was immersed in 75% ethanol for 30 minutes, dried, and sterilized using ultraviolet light for 8 hours.

2) Poly-L-lysine solution (0.10 mg/mL) was added to the culture chamber, completely immersing all electrodes.

3) The MEA was incubated for 24 hours at 37°C with 5% $CO_2$ and saturated humidity.

4) The poly-L-lysine solution was removed, and the MEA was rinsed with sterilized ultra-pure water and dried on a medical purification worktable.

For the hippocampal neuronal network, whole brain tissue was first dissected at a low temperature (4°C) from a fetal rat taken from an 18-day pregnant Sprague-Dawley rat euthanized *via* cervical dislocation. The brain tissue was dissected and subjected to digestion with pancreatic enzymes to obtain isolated/scattered hippocampal neurons. Afterwards, the primary hippocampal neuronal cells were seeded on the surface of the MEA with a cell density of $4 \times 10^5/cm^2$ in inoculation medium, which comprised neurobasal medium supplemented with 1% B27, 1 mM glutamine, and 25 μM glutamine acid. After 4 hours, the inoculation medium was replaced with growth medium (neurobasal medium supplemented with 1% B27 and 0.50 mM glutamine). The neurons were cultured in an incubator at 37°C with 5% $CO_2$ and saturated humidity for 13 days for successive experiments.

### Preparation of brain chips with hippocampal slices

We obtained rat hippocampal slices according to the following steps:

1) Whole brain tissue was quickly separated from 14-day-old neonatal Sprague-Dawley rats euthanized *via* cervical dislocation.

2) The brain tissue was washed with cool (≤ 4°C) oxygenated (95% $O_2$, 5% $CO_2$) artificial cerebrospinal fluid (132.0 mM NaCl, 2.0 mM KCl, 1.2 mM $KH_2PO_4$, 1.1 mM $MgSO_4$, 19.0 mM $NaHCO_3$, 2.5 mM $CaCl_2$, 10.0 mM D-glucose, pH 7.2–7.4).

3) The brain tissue was embedded in 5% agar. After the agar solidified, the brain tissue was transversely dissected (coronal slice) along the longitudinal axis of the brain into 400-μm-thick slices using a tissue slicing machine (McIlwain tissue chopper, Redding City, CA, USA). The slices were soaked in oxygenated artificial cerebrospinal fluid (25°C, 95% $O_2$, 5% $CO_2$).

4) The hippocampal area was dissected from each brain tissue slice and placed onto the electrode area of the MEA (no directional restrictions other than aim to cover the entire

electrode area). Growth medium (the same as that used for primary hippocampal neurons) was added and replaced for successive experiments.

**Measurement of normal voltage thresholds and voltage thresholds under different influential factors**

After preparing the two kinds of neural chips, we divided them into four experimental groups. The purpose of the first group was to confirm the normal voltage threshold without interference factors ($n$ = 6). We subjected the neural chips in the other three groups to acetylcholine, alcohol, and temperature ($n$ = 5), respectively.

After allowing 13 days for the hippocampal neuronal network cultures to grow, we examined the neuronal networks or hippocampal slices prepared on the MEA using a microscope (BX51; Olympus, Tokyo, Japan). One electrode located under the neuronal network was selected as the stimulation site, and the remaining electrodes were used as detection sites.

After each MEA was connected to the test system, we assessed the state of the connected neural network for 10 minutes. If we observed no synchronous spontaneous activity on the oscilloscope, the cultured neural network was considered to meet the experimental requirements. We then applied an asymmetric charge-balanced biphasic pulse to the stimulating electrode. The durations of the positive and negative phases were 2.00 ms and 0.20 ms, respectively (**Figure 2**). At body temperature (37°C), the amplitude of the negative phase began at 0 mV and increased in 1-mV steps, while the amplitude of the positive phase was adjusted proportionally. The test period was 5 minutes for each amplitude, with a 3-minute interval between stimuli.

During testing, the electrical signals from the detection electrodes were observed in real time via the oscilloscope. When one or more typical neural action potentials were observed in the waveforms at different sites, the amplitude of the negative phase was reported as the voltage threshold $V_{Th}$. The neural responses monitored using the oscilloscope were compared with the synchronous recordings made by the 128-channel neuro-signal processer (the signal waveforms recorded by the 128-channel neuro-signal processer were read and plotted by using Matlab software). In this paper, we defined the $V_{Th}$ value measured under normal conditions (hippocampal neuronal networks at 37 °C with a cell density of $4 \times 10^5/cm^2$ after 13 days of culture; 400-μm-thick hippocampal slices from 14-day-old neonatal Sprague-Dawley rats) as the normal $V_{Th}$. This was used as the reference value for defining excitatory and inhibitory states.

We conducted six independent threshold evaluations with six different batches of mice. Given the cumulative effect of external factors during continuous testing, each chip (hippocampal neuronal network or hippocampal slice) could only be used for one threshold detection experiment (we conducted 5 independent parallel experiments to measure $V_{Th}$ under different influential factors).

The VTMM determines the influence of an extrinsic factor on a quiescent neuronal network by measuring a series of $V_{Th}$ values for different quantities of that factor. We demonstrated the efficacy of the method using the following three influential factors—ACh, EtOH, and temperature.

The first influential factor was ACh. After preparing a neuronal network or hippocampal slice on the MEA, the culture medium in the MEA chamber was replaced with test medium, *i.e.*, fresh growth medium containing a given concentration of ACh ($C_{ACh}$). $C_{ACh}$ was increased from 0 μM in 5.5 μM steps. After 3 minutes of incubation, we measured the $V_{Th}$ values under the influence of $C_{ACh}$, and obtained $V_{Th}$– $C_{ACh}$ data.

EtOH was chosen as the second influential factor. The growth medium in the neuronal network or hippocampal slice prepared in the MEA chamber was replaced with test medium containing a defined concentration of EtOH ($C_{EtOH}$). $C_{EtOH}$ was increased from 0 mM in 10.00 mM steps. After 3 minutes of incubation, we measured $V_{Th}$ values under the influence of $C_{EtOH}$, to assess the $V_{Th}$–$C_{EtOH}$ relationship.

The third influential factor was the temperature of the medium ($T_m$). $T_m$ was adjusted from 30–45°C (the range of human body temperature) in 1°C steps. The $V_{Th}$ value under a given $T_m$ was measured 3 minutes after the temperature adjustment to obtain a set of $V_{Th}$–$T_m$ data.

**Data processing and analysis**

We used SPSS 20.0 software (IBM, Armonk, NY, USA) to test the normal distribution of the results from parallel groups. When the data were normally distributed ($P$ > 0.05), we calculated the mean and standard deviations (mean ± SD) of test results from the parallel groups ($n$ = 6 for the normal $V_{Th}$ and $n$ = 5 for the acetylcholine, alcohol, and temperature groups) under the same conditions. We then plotted the relationship curves of the threshold voltages for the two kinds of neural networks for the four conditions. The threshold variation results for the different $C_{ACh}$ levels had a linear fit, the threshold variation results for the different $C_{EtOH}$ levels had an exponential fit, and the threshold variation results for the different temperatures had a U-shape.

## Results

**Neuronal networks and the locations of the stimulating electrodes on the MEA**

**Figure 3A** shows photos of hippocampal neurons cultured under normal growing conditions for 13 days, forming the desired neuronal networks. **Figure 3B** shows that the hippocampal slice effectively covered the electrode area on the MEA. **Figure 3** also shows the electrode chosen as the stimulating site in each case (the position of the stimulating electrode was not constant among samples. In practice, stimulating electrodes were chosen among any of the electrodes that were covered by the cultured neural network, and the remaining electrodes were used for detection).

**Normal voltage thresholds of neuronal networks**

Because the waveform and the frequency of the stimulus artifacts clearly indicated that they were artificially generated, and the interval between stimulus artifacts was constant, they were easily distinguished from the evoked action potential signals. Therefore, we focused on the waveform details of the captured

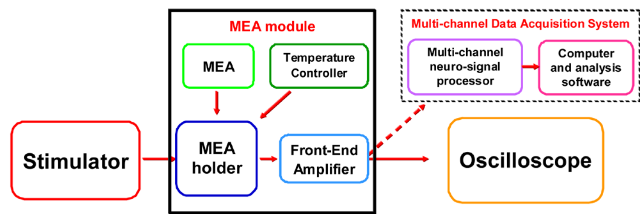


**Figure 1 Block diagram showing our Voltage Threshold Measurement Method (VTMM) system.**
The arrow and block with a dashed line denote a more sophisticated recording set-up that was for cross-validation and not necessary to the operation of the VTMM system.

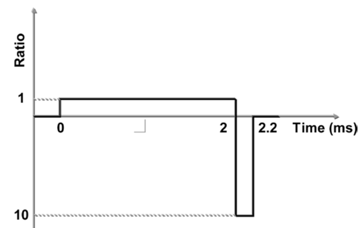


**Figure 2 Waveform of the stimulation pulse.**
Threshold was defined as the amplitude of the negative phase. The ordinate is the ratio of the voltage amplitude between positive and negative pulses (no unit), indicating that the amplitude of the negative wave is 10 times that of the positive wave. The abscissa denotes time in ms, indicating that the duration of the forward wave was fixed at 2 seconds and the duration of the negative wave was fixed at 0.2 seconds.

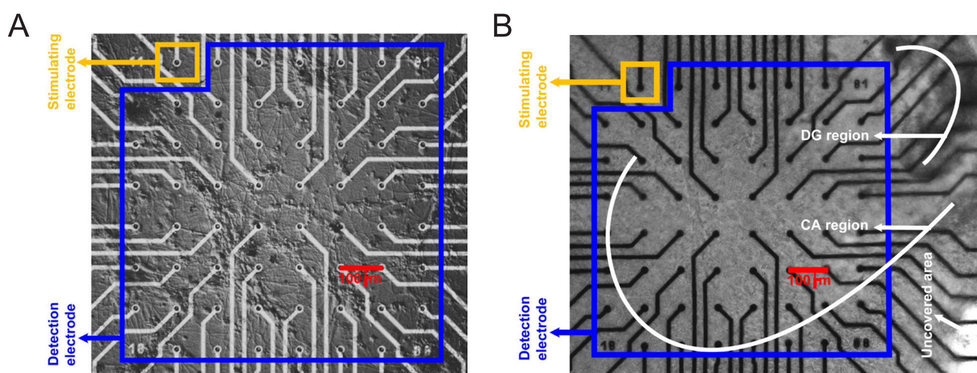


**Figure 3 Microscope images of the neuronal networks and the locations of the stimulating electrodes on the microelectrode array.**
(A) Cultured hippocampal neuronal network after 13 days; (B) hippocampal slice. The electrical potential in the region denoted by the yellow box has a highly excitatory effect, and the electrodes in the region denoted by the blue frame are extremely sensitive.

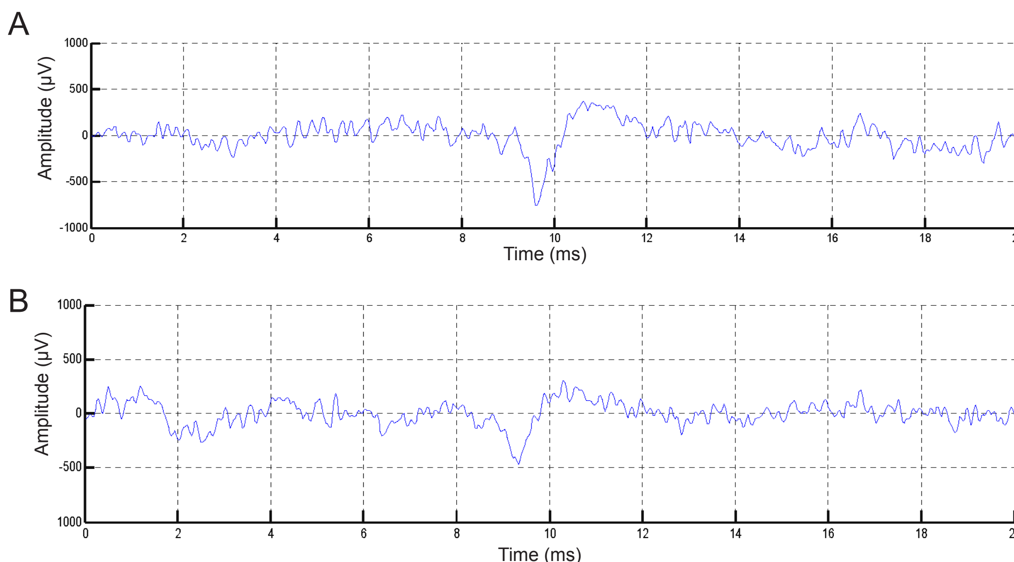


**Figure 4 Typical waveforms of the evoked neural action potentials for two types of neuronal networks recorded by a 128-channel neuro-signal processer.**
(A) Hippocampal neuronal network at 60-mV stimulation amplitude; (B) hippocampal slice at 30-mV stimulation amplitude.

evoked action potential signals. Upon applying electrical stimulation under normal conditions at 37°C, **Figure 4** shows the typical signal waveforms produced by the two types of neuronal networks recorded by the 128-channel neuro-signal processer. **Figure 5** shows screenshots of the oscilloscope with the signals detected from four channels. Both scales are 2 ms/grid.

As shown in **Figures 4** and **5**, all action potentials exhibited a consistent pattern. Their waveforms contained two parts: the first was a negative peak with a high amplitude but short duration, and the second was a positive peak with a low amplitude and long duration. The action potentials were approximately 1.50 ms long.

As listed in **Table 1**, the normal $V_{Th}$ of the hippocampal neuronal networks (56.00 mV) was higher than that of the hippocampal slices (31.17 mV). The threshold is expressed as a positive potential because the recordings were extracellular field potentials.

### $V_{Th}$ under the influence of ACh

The measured $V_{Th}$ values from the two types of hippocampal neuronal networks under the influence of different $C_{ACh}$ levels

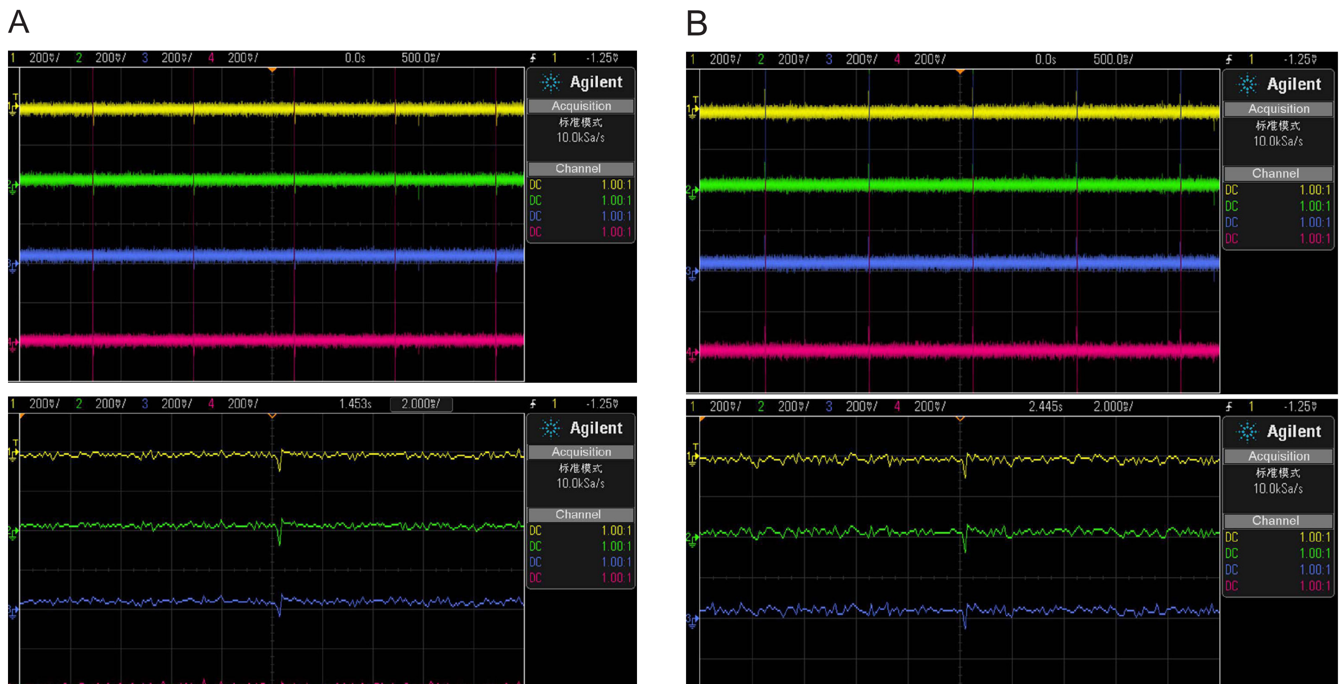

**Figure 5 Oscilloscope images of typical waveforms of the evoked neural action potentials for two types of neuronal networks**
(A) Hippocampal neuronal network at 60-mV stimulation amplitude; (B) hippocampal slice at 30-mV stimulation amplitude.

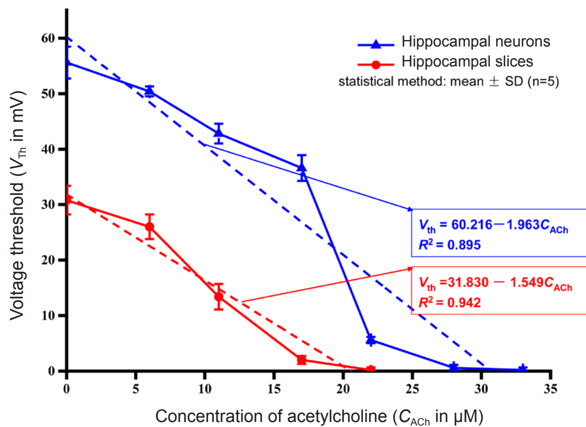


**Figure 6 The measured and fitted voltage threshold ($V_{Th}$) *versus* the concentration of acetylcholine ($C_{ACh}$) in two types of hippocampal neuronal networks.**
Each $V_{Th}$ curve encompasses the mean ± SD ($n$ = 5) of five independent experiments (five different cultures from five different animals).

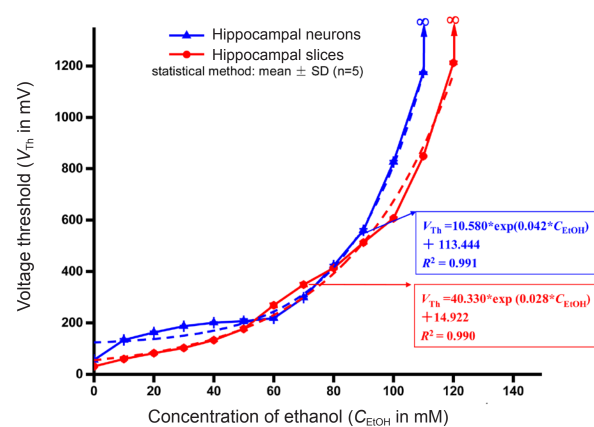


**Figure 7 The measured and fitted voltage threshold ($V_{Th}$) versus the concentration of ethanol ($C_{EtOH}$) in two types of hippocampal neuronal networks.**
Each $V_{Th}$ curve encompasses the mean ± SD ($n$ = 5) of five independent experiments (five different cultures from five different animals).

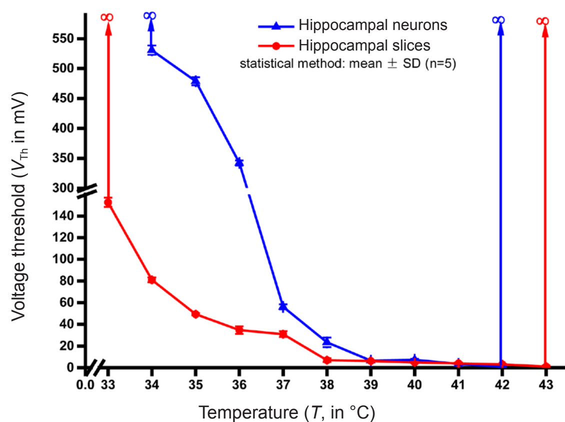


**Figure 8 The measured voltage threshold ($V_{Th}$) *versus* the temperature of the medium ($T_m$) in two types of hippocampal neuronal networks.**
Each $V_{Th}$ curves encompasses the mean ± SD ($n$ = 5) of five independent experiments (five different cultures from five different animals).

**Table 1 Normal $V_{Th}$ of the neuronal networks**

| Neuronal networks | Normal $V_{Th}^*$ (mV) |
|---|---|
| Hippocampal neuron | 56.00±2.53 |
| Hippocampal slice | 31.17±2.48 |

*Voltage threshold ($V_{Th}$) is the mean ± SD of six independent experiments (six different cultures from six different animals).

are listed in **Additional Table 1**, and the fitted $V_{Th}$-$C_{ACh}$ curves are plotted in **Figure 6**. The mean $V_{Th}$ was taken from five independent experiments (five different cultures from five different animals).

The curves plotted in **Figure 6** indicate that the $V_{Th}$ values for the two types of neuronal networks form an approximately linear decreasing function of $C_{ACh}$. Thus, the electrical excitability of the neuronal networks decreased with increasing $C_{ACh}$. When 33 μM and 22 μM of $C_{ACh}$ were applied to the hippocampal neuronal networks and hippocampal slices, respectively, action potentials could be elicited from both hippocampal neuronal networks without any electrical stimulation. The linear fits of the $V_{Th}$ versus $C_{ACh}$ values shown in **Figure 6** had negative slopes of −1.96 and −1.55 mV·μM$^{-1}$ for the hippocampal neuronal networks and hippocampal slices, respectively. The negative slope for the hippocampal neuronal networks was steeper than that for the hippocampal slices.

### $V_{Th}$ under the influence of EtOH

The measured $V_{Th}$ values from the two types of hippocampal neuronal networks under the influence of different $C_{EtOH}$ levels are listed in **Additional Table 2**, and the fitted $V_{Th}$- $C_{EtOH}$ curves are plotted in **Figure 7**. The mean $V_{Th}$ was taken from five independent experiments (five different cultures from five different animals).

The curves plotted in **Figure 7** indicate that the $V_{Th}$ values for the two types of hippocampal neuronal networks increased approximately exponentially with $C_{EtOH}$, indicating that the electrical excitability of the neuronal networks decreased with increasing $C_{EtOH}$. When $C_{EtOH}$ exceeded 110 mM and 120 mM for the hippocampal neuronal networks and hippocampal slices, respectively, no action potentials could be elicited even with extremely high stimulating voltages (> 2000 mV). This indicates that both hippocampal neuronal networks lost their electrical excitability completely under the influence of high $C_{EtOH}$. Within the range of exponential dependence, the influence of $C_{EtOH}$ was similar for the two hippocampal neuronal networks. However, the exponential growth index of the hippocampal neuronal networks (0.04) was larger than that of the hippocampal slices (0.03).

### $V_{Th}$ under the influence of moderate temperature

The measured $V_{Th}$ values of the two types of hippocampal neuronal networks under the influence of moderate temperature $T_m$ are listed in **Additional Table 3**, and the fitted $V_{Th}$-$T_m$ curves are plotted in **Figure 8**. The mean $V_{Th}$ was also taken from five independent experiments (five different cultures from five different animals).

**Figure 8** shows that both curves have a U-shape and the following features:

1) When $T_m$ rose above 37°C, $V_{Th}$ decreased rapidly, indicating that the electrical excitability of the neuronal networks increased with increasing medium $T_m$.

2) When $T_m$ exceeded 42°C and 43°C, no action potentials could be elicited from the hippocampal neuronal networks and the hippocampal slices, respectively, and the neuronal networks abruptly lost their electrical excitability. The highest temperature at which the hippocampal neuronal networks lost electrical excitability was lower than that for the hippocampal slices.

3) When $T_m$ dropped below 37°C, $V_{Th}$ increased, indicating that the electrical excitability of the neuronal networks decreased with decreasing moderate $T_m$.

4) When $T_m$ dropped below 34°C and 33°C, no action potentials could be elicited from the hippocampal neuronal networks and the hippocampal slices, respectively, and the neuronal networks abruptly lost their electrical excitability. The lowest temperature at which the hippocampal neuronal networks lost electrical excitability was higher than that for the hippocampal slices.

## Discussion

Neural networks cultured *in vitro* grow randomly, such that a basic network forms after a week and an obvious increase in action potentials appears after two weeks. In this study, we chose hippocampal neuronal networks cultured *in vitro* for 13 days as the candidate network for testing the VTMM. We chose this model because it was a quasi-mature network without spontaneous action potentials. In our study, we did not observe any synchronous spontaneous action potentials during the 10 minutes before each test.

If we did observe spontaneous action potentials from either the 13-day *in vitro* cultured hippocampal neuronal networks or the hippocampal slices, we would expect them to be highly random and non-synchronized. In this paper, we used electrical stimulation to induce action potentials. There was an obvious causal relationship between evoked action potentials and stimulus pulses, indicating the evoked action potentials had a strict "full" or "no" effect, which is similar to the threshold effect seen in individual neurons. Furthermore, the evoked action potentials had a high and stable degree of network synchronization, and all of the detection electrodes could record signals synchronously.

In current MEA-based studies, captured action potentials are mainly judged by the parameters of intracellular and external action potential waveforms. The waveform of such evoked action potentials is relatively simple, fixed, and similar to a typical action potential in a behaving organism (Lewicki, 1998; Scott et al., 2013; Xu et al., 2014). Thus, the evoked action potentials are easily identified using an oscilloscope. Oscilloscopes are capable of real-time, complete, and objective displays of detected signals. Thus, they are frequently used for real-time recording, observation, and screening of detected signals.

Our results show that 1) the neural signal acquisition system and the oscilloscope could record the evoked signals, and 2)

all of the evoked signals recorded were consistent with the relevant parameters for extracellular stimulation and thus could be classified as typical evoked action potentials. However, given that the signals recorded by the neural signal acquisition system require redrawing by other built-in signal processing software or custom software for analysis, use of the oscilloscope is a simpler method. Our results also show that the normal voltage thresholds of hippocampal neuronal networks are higher than those of hippocampal slices, indicating that hippocampal neuronal networks have lower electrical excitability and require a higher stimulus current to induce action potentials.

ACh is widely distributed in the central and peripheral nervous systems as an excitatory neurotransmitter and participates in the regulation of physiological functions and information transmission. Nevertheless, it has mostly been studied in terms of its physiological functions, and at a restricted concentration range of 10–40 μM (Gross et al., 1995).

To data, no studies have reported on the $V_{Th}$-$C_{ACh}$ relationship. Here, we found that for $C_{ACh}$ levels equal to or greater than 5.50 μM (1 μg/mL), ACh increases the electrical excitability of hippocampal neuronal networks and hippocampal slices by linearly decreasing $V_{Th}$. When $C_{ACh}$ was increased to 33 μM and 22 μM in the hippocampal neuronal networks and hippocampal slices, respectively, action potentials could be evoked in both types of neuronal networks without electrical stimulation. We found that the negative slope for the hippocampal neuronal networks was steeper than that for the hippocampal slices, indicating that the decrease in $V_{Th}$ had an earlier effect on the hippocampal neuronal networks, and that the effect of Ach on hippocampal neuronal networks is more prominent.

Previous studies have shown that ethanol can affect the synthesis and delivery of many types of neurotransmitters, interfere with the activity of receptor pathways and voltage-gated ion channels, induce hyperpolarization of the postsynaptic membrane, affect the generation, transmission, and processing of neural signals, thus altering neural activity in the brain, and cause extensive inhibition in the central nervous system (Ramezani et al., 2003; Moonat et al., 2010; Azam et al., 2012; Li et al., 2013). Benson et al. (1989) studied the effect of ethanol on cultured hippocampal neurons using the patch clamp method and found that ethanol concentrations ranging from 50 to 100 mM induced marked inhibition of neuronal discharge. Based on data collected from cortical neurons cultured on an MEA and statistical analyses of long-term neural signals detected at all of the electrodes (30–60 minute recordings for each ethanol concentration), Xia and Gross (2003) reported that ethanol ranging from 10 to 160 mM decreased electrical excitability in neuronal networks. The activity of neuronal networks, *i.e.*, the rate and duration of activity, decreased with increasing $C_{EtOH}$ until activity stopped upon $C_{EtOH}$ reaching 100 mM (Xia and Gross, 2003).

In the present study, $V_{Th}$ increased with increasing $C_{EtOH}$, and the electrical excitability of the neuronal networks was progressively inhibited. When $C_{EtOH}$ exceeded 110 mM, the electrical activities of the hippocampal neuronal networks were completely suppressed. These results are in accordance with the aforementioned studies. Similar results have been reported in hippocampal slices, specifically, that electrical activity was completely suppressed when $C_{EtOH}$ exceeded 120 mM. Thus, the upper limits of electrical excitability of the two types of neuronal networks can be defined for ethanol.

Furthermore, the exponential growth index for hippocampal neuronal networks is larger than that for hippocampal slices, meaning that hippocampal neuronal networks respond more quickly to increasing $V_{Th}$ levels, and that the influence of EtOH on hippocampal neuronal networks is more prominent.

Variations in medium temperature can cause changes in neuronal parameters. Higher temperatures can increase the ion-exchange rate, accelerate the transformation of channel proteins, influence the activity of different types of ion channels, and alter the resting potentials and depolarization rates of neurons (Schiff and Somjen, 1985; Griffin et al., 1996; Burgoon and Boulant, 2001). On the contrary, lower temperatures decrease metabolism, affect the release of neurotransmitters (Dietrich, 2009; Lenhardt, 2010), sharply reduce the $Ca^{2+}$ current in hippocampal neuronal networks, suppress the activity of ion pumps and glutamate-gated ion channels (Tymianski et al., 1998), alter the triggering, transmission, and relative refractory period of neural signals (Micheva and Smith, 2005; Sajikumar et al., 2005), and change the rhythm of electrical activity, the firing rate, and the amplitude of spike potentials (Schiff and Somjen, 1985; Karlsson and Blumberg, 2004; Li et al., 2004). In a study on the discharge frequency of neurons at temperatures ranging from 32–39°C, Griffin et al. (1996) found that raising $T_m$ increased the discharge frequency and excitation rate, and also shortened the interval of activation. In previous electrophysiological studies, raising $T_m$ within the range of 32–40°C accelerated the depolarization rate of neuronal membranes, shortened the time interval of the bursts, and increased the burst rate of the signal (Burgoon and Boulant, 2001).

In this study, $V_{Th}$ generally had a decreasing dependency on the $T_m$ in the range around the body temperature: when $T_m$ increased above 37°C, $V_{Th}$ quickly dropped to several millivolts with small fluctuations, indicating that the electrical excitability of the two types of neuronal networks had been enhanced. However, when $T_m$ decreased below 37°C, increased $V_{Th}$ indicated weakened electrical excitability. Our findings are consistent with the aforementioned studies, and we further demonstrated that no action potentials could be evoked from hippocampal neuronal networks and hippocampal slices when $T_m$ exceeded 42 and 43°C or dropped below 34 and 33°C, respectively. The observed abrupt and complete loss of electrical excitability delineates the temperature limits for electrophysiological activity of neuronal networks.

The high temperature at which hippocampal neuronal networks lost electrical excitability was lower than that for hippocampal slices, indicating that the influence of high temperatures on hippocampal neuronal networks was more prominent. The low temperature at which hippocampal neuronal networks lost electrical excitability was higher than that for hippocampal slices, indicating that the influence of low temperatures on hippocampal neuronal networks was also more prominent.

The basic concept of the VTMM was to develop a method by which the activity of neural networks could be quantitatively measured when they do not or do not yet have synchronous spontaneous activity. This is we used young (13-days) cultured hippocampal neural networks, which do not show synchronous spontaneous activity, in the present study. Cultured hippocampal neural networks and hippocampal slices were chosen as research platforms in this study because they are classic *in vitro* models that resemble the functions of normal neuronal tissue. Although their normal $V_{Th}$ values vary due to their different tissue structures and cellular compositions, **Figures 6–8** show the observed similarities in the trends of electrical excitability among the two types of neuronal networks under the influence of temperature, ethanol, and Ach. Our data indicate that the short culture time was appropriate.

VTMM uses an MEA, a low-cost pulse generator and an oscilloscope as measuring instruments, and takes the easily-observed amplitude of the stimulating pulse voltage as "scale" for quantitatively assessing the influence degree of external factors such as drugs and temperature on the electrical activity of neuronal networks. Using such a method, the test data defined as "voltage threshold" can be obtained from a neuronal network cultured for only 13 days. In addition, the voltage threshold can be determined by means of the observation of the waveforms on the oscilloscope, and no large-scale data processing and complicated statistical algorithms are required. The shortcoming is that there is not yet a program to automatically recognize the action potentials induced by the stimulating voltage pulses.

## Conclusions

We validated the efficacy and simplicity of the VTMM, introduced in this study, based on measurements from hippocampal neuronal networks and hippocampal slices under the influence of three different extrinsic factors. Our main electrophysiology results are as follows:

1) We determined the normal $V_{Th}$- and $C_{ACh}$-threshold values for the two types of neuronal networks, and observed approximately linearly decreasing relationships between $V_{Th}$ and $C_{ACh}$.

2) We determined the $V_{Th}$–$C_{EtOH}$ relationships, as well as the $C_{EtOH}$ limits beyond which electrical excitability is suppressed in the two types of neuronal networks.

3) The $V_{Th}$–$T_m$ curves of the neuronal networks were in the vicinity of normal body temperature. We found both the upper and lower limits of the $T_m$ beyond which electrical excitability in the two types of neuronal networks is fully suppressed.

VTMM represents an accurate and simple method for quantitatively evaluating the influences of various extracellular factors on the electrical excitability of different neuronal networks, including environmental factors such as temperature, dietary factors such as ethanol, and pharmacological factors such as ACh. Drug screening for treatment of neurological diseases is also a potential application.

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***Additional file 1***: *Open peer review report 1.*
***Additional Table 1***: *$V_{Th}$ values of the neuronal networks under the influence of different $C_{ACh}$.*
***Additional Table 2***: *$V_{Th}$ values of the neuronal networks under the influence of different $C_{EtOH}$.*
***Additional Table 3***: *$V_{Th}$ values of the neuronal networks under the influence of different $T_m$.*

## References

Bean BP (2007) The action potential in mammalian central neurons. Nature reviews: Neuroscience 6:451-465.
Benson DM, Blitzer RD, Landau EM (1989) Ethanol suppresses hippocampal cell firing through a calcium and cyclic AMP-sensitive mechanism. J Pharmacol 164:591-594.
Brummer SB, Turner MJ (1977) Electrochemical consid-erations for safe electrical stimulation of the nervous system with platinum electrodes. IEEE Trans Biomed Eng 24:59-63.
Burgoon PW, Boulant JA (2001) Temperature-sensitive properties of rat suprachiasmatic nucleus neurons. J Physiol Regul Integr Comp Physiol 281:706-715.
Chang WC, Lee CM, Shyu BC (2012) Temporal and spatial dynamics of thalamus-evoked activity in the anterior cingulate cortex. J Neurosci 222:302-315.
Chau TT, Izazola-Conde C, Dewey WL (1982) Evidence for the existence of peptide and nonpeptide morphine-like materials in mouse brain: effect of an analgesic intracerebroventricular dose of acetylcholine on their levels. J Pharmacol Exp Ther 222:612-616.
Chong SA, Benilova I, Shaban H, Strooper BD, Devijver H, Moechars D, Eberle W, Bartic C, Leuven FV, Callewaert G (2011) Synaptic dysfunction in hippocampus of transgenic mouse models of Alzheimer's disease: a multi-electrode array study. J Neurobiol Dis 44:284-291.
Cohen O, Keselman A, Moses E, Rodr´ıguez Mart´ınez M, Soriano J, Tlusty T (2010) Quorum percolation in living neural networks. Europhys Lett 89:18008.
Danny E, Amir M, Noam Z, Shimon M (2004) Dopamine-induced dispersion of correlations between action potentials in networks of cortical neurons. J Neurophysiol 92:1817-1824.
Dietrich WD (2009) Therapeutic hypothermia for spinal cord injury. J Crit Care Med 37:238-242.

Egert U, Okujeni S, Weihberger O, Mikkonen JE (2008) Improved response reproducibility in neuronal networks in vitro by means of phase-coupled electrical stimulation and stimulation at pre-selected sites. Front Comput Neurosci 2:120.

Feinerman O, Rotem A, Moses E (2008) Reliable neuronal logic devices from patterned hippocampal cultures. Nat Phys 4:967-973.

Frega M, Pasquale V, Tedesco M, Marcoli M, Contestabile A, Nanni M, Bonzano L, Maura G, Chiappalone M (2012) Cortical cultures coupled to micro-electrode arrays: a novel approach to perform in vitro excitotoxicity testing. J Neurotoxicol Teratol 34:116-127.

Frega M, Tedesco M, Massobrio P, Pesce M, Martinoia S (2014) Network dynamics of 3D engineered neuronal cultures: a new experimental model for in-vitro electrophysiology. Sci Rep 4:05489.

Gage FH (2000) Mammalian neural stem cells. Science 287:1433-1438.

Gonzalez-Sulser A, Wang J, Motamedi GK, Avoli M, Vicini S, Dzakpasu R (2011) The 4-aminopyridine in vitro epilepsy model analyzed with a perforated multi-electrode array. J Neuropharmacol 60:1142-1153.

Griffin JD, Kaple ML, Chow AR, Boulant JA (1996) Cellular mechanism for neuronal thermosensitivity in the rat hypothalamus. J Physiol 492:231-242.

Gross GW, Rhoades BK, Azzazy HME, Wu MC (1995) The use of neuronal networks on multielectrode arrays as biosensors. Biosens Bioelectron 10:553-567.

Guan XH (2002) The diagnosis and treatment of acute alcohol intoxication. Kongjun Yixue Zazhi 18:37-39.

Guan YJ, Gao YM, Xie ZD, Li FJ (2000) Effects of high temperature on the nerve cells of primary culture. Jiepouxue Zazhi 23:15-19.

Hamurtekin E, Gurun MS (2006) The antinociceptive effects of centrally administered CDP-choline on acute pain models in rats: the involvement of cholinergic system. J Brain Res 1117:92-100.

Hill AJ, Jones NA, Williams CM, Stephens GJ, Whalley BJ (2010) Development of multi-electrode array screening for anticonvulsants in acute rat brain slices. J Neurosci Meth 185:246-256.

Hogberg HT, Sobanski T, Novellino A, Whelan M, Weiss DG, Bal-Price AK (2011) Application of micro-electrode arrays (MEAs) as an emerging technology for developmental neurotoxicity: evaluation of domoic acid-induced effects in primary cultures of rat cortical neurons. J Neurotoxicol 32:158-168.

Ito D, Komatsu T, Gohara K (2013) Measurement of saturation processes in glutamatergic and GABAergic synapse densities during long-term development of cultured rat cortical networks. J Brain Res 1534:22-32.

Johnstone AF, Gross GW, Weiss DG, Schroeder OH, Gramowski A, Shafer TJ (2010) Microelectrode arrays: a physiologically based neurotoxicity testing platform for the 21st century. J Neurotoxicol 31:331-350.

Jones PG, Dunlop J (2007) Targeting the cholinergic system as a therapeutic strategy for the treatment of pain. J Neuropharmacol 53:197-206.

Jones RW (2003) Have cholinergic therapies reached their clinical boundary in Alzheimer's Disease. J Geriatr Psychiatr 18:S7-S13.

Karlsson KA, Blumberg MS (2004) Temperature-induced reciprocal activation of hippocampal field activity. J Neurophysiol 91:583-588.

Lenhardt R (2010) The effect of anesthesia on body temperature control. J Front Biosci 2:1145-1154.

Lewicki MS (1998) A review of methods for spike sorting: the detection and classification of neural action potentials. Network 9:R53-R78.

Li C, McCall NM, Lopez AJ, Kash TL (2013) Alcohol effects on synaptic transmission in periaqueductal gray dopamine neurons. J Alcohol 47:279-287.

Li XN, Zhou W, Yao S, Luo QM (2004) Effects of temperature on the activity of cultured hippocampal neuronal networks. J Acta Biophysica Sinica 20:477-482.

Liu Q, Wu C, Cai H, Hu N, Zhou J, Wang P (2014) Cell-based biosensors and their application in biomedicine. J Chem Rev 114:6423-6461.

Lossi L, Alasia S, Salio C, Merighi A (2009) Cell death and proliferation in acute slices and organotypic cultures of mammalian CNS. Prog Neurobiol 88:221-245.

Mauch DH, Nägler K, Schumacher S, Göritz C, Müller EC, Otto A, Pfrieger FW (2001) CNS synaptogenesis promoted by glia-derived cholesterol. Science 294:1354-1357.

Micheva KD, Smith SJ (2005) Strong effects of subphysiological temperature on the function and plasticity of mammalianpresynaptic terminals. J Neurosci 25:7481-7488.

Micholt E, Jans D, Callewaert G, Bartic C, Lammertyn J, Nicolaï B (2006) Extracellular recordings from rat olfactory epithelium slices using micro electrode arrays. J Sens Actuator B-Chem 184:40-47.

Moonat S, Starkman BG, Sakharkar AS, Subhash CP (2010) Neuro-science of alcoholism: molecular and cellular mechanisms. J Cell Mol Life Sci 67:73-88.

Newman EA (2003) New roles for astrocytes: regulation of synaptic transmission. J. Trends Neurosci 26:536-542.

Otto F, Görtz P, Fleischer W, Siebler M (2003) Cryopreserved rat cortical cells develop functional neuronal networks on microelectrode arrays. J Neurosci Meth 128:173-181.

Pan HX, Lü XY, Wang ZG, Ren TL, Fang T, Zhang J, Zhou CJ, Wang LG (2011) Silicon-based microelectrode arrays for stimulation and signal recording of in vitro cultured neurons. J Sci China-Inf Sci 54:2199-2208.

Quintero JE, Pomerleau F, Huettl P, Johnson KW, Offord J, Gerhardt GA (2011) Methodology for rapid measures of glutamate release in rat brain slices using ceramic-based microelectrode arrays: Basic characterization and drug pharmacology. J Brain Res 1401:1-9.

Radivojevic M, Jäckel D, Altermatt M, Müller J, Viswam V, Hierlemann A, Bakkum DJ (2016) Electrical identification and selective microstimulation of neuronal compartments basedeon features of extracellular action potentials. Sci Rep 10:31332.

Ramachandran V, Watts LT, Maffi SK, Chen J, Schenker S, Henderson G (2003) Ethanol-induced oxidative stress precedes mitochondrially mediated apoptotic death of cultured fetal cortical neurons. J Neurosci Res 74:577-588.

Ramezani A, Goudarzi I, Lashkarboluki T, Ghorbanian MT, Abrari K, Elahdadi Salmani M (2012) Role of oxidative stress in ethanol-induced neurotoxicity in the developing cerebellum. J Basic Med Sci 15:965-974.

Sajikumar S, Navakkode S, Frey JU (2005) Protein synthesis-dependent long-term functional plasticity: methods and techniques. J Curr Opin Neurobiol 15:607-613.

Schiff SJ, Somjen GG (1985) The effects of temperature on synaptic transmission in hippocampal tissue slices. J Brain Res 345:279-284.

Scott A, Weir K, Easton C, Huynh W, Moody WJ, Folch A (2013) A microfluidic microelectrode array for simultaneous electrophysiology, chemical stimulation, and imaging of brain slices. Lab Chip 13:527-535.

Shein IM, Ben-Jacob E, Hanein Y (2010) Innate synchronous oscillations in freely-organized small neuronal circuits. PLoS One 5:e14443-e14452.

Shen Q, Jiang D, Tai C (2001) Selective stimulation of smaller nerve fibers using biphasic rectangular pulses. Front Med Biol Eng 10:319-335.

Shmoel N, Rabieh Noha, Ojovan SM, Erez H, Maydan E, Spira ME (2016) Multisite electrophysiological recordings by self-assembled loose-patch-like junctions between cultured hippocampal neurons and mushroom-shaped microelectrodes. Sci Rep 6:27110.

Soriano J, Rodr´ıguez Mart´ınez M, Tlusty T, Moses E (2008) Development of input connections in neural cultures. Proc Natl Acad Sci U S A 105:13758-13763.

Steve MP (2001) Distributed progressing in cultured neuronal networks. J Prog Brain Res 130:49-62.

Tymianski M, Sattler R, Zabramski JM, Spetzler RF (1998) Characterization of neuroprotection from excitotoxicity by moderate and profound hypothermia in cultured cortical neurons unmasks a temperature-insensitive component of glutamate neurotoxicity. J Cereb Blood Flow Metab 18:848-867.

Ventura R, Harris KM (1999) Three-dimensional relationships between hippocampal synapses and astrocytes. J Neurosci 19:6897-6906.

Wagenaar DA, Pine SM, Potter J (2004) Effective parameters for stimulation of dissociated cultures using multi-electrode arrays. J Neurosci Methods 138:27-37.

Weihberger O, Lavi A, Okujeni S, Ashery U, Egert U (2011) State-dependent modulation of stimulus-response relations in cortical networks in vitro. BMC Neurosci 12:1-2.

Weihberger O, Okujeni S, Mikkonen JE, Egert U (2013) Quantitative examination of stimulus-response relations in cortical networks in vitro. J Neurophysiol. 109:1764-1774.

Whittingham TS, Lust WD, Christakis DA, Passonneau JV (1984) Metabolic stability of hippocampal slice preparations during prolonged incubation. J Neurochem 43:689-696.

Wu HY, Wu YY (2006) The research progress of neural chip technology. J Prog Biochem Biophys 34:89-93.

Xia Y, Gross GW (2003) Histiotypic electrophysiological responses of cultured neuronal networks to ethanol. J Alcohol 30:167-174.

Xu JM, Wang CQ, Lin LN (2014) Multi-channel in vivo recording techniques: signal processing of action potentials and local field potentials. Acta Physiologica Sinica 66:349-357.

Zhao DC, Xu T (1988) The effects of acetylcholine on the electric activities of pain reaction neurons in nucleus parafascicularis of thalamus and midbrain reticular formation in rats. J Acta Physiol Scand 40:326-334.

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Additional Table 1-3.

**Supplementary Table 1.** $V_{Th}$ values of the neuronal networks under the influence of different $C_{ACh}$.

| $V_{Th}$ values (mV)[a] / $C_{ACh}$ (μmol/L) \ Neuronal networks | Hippocampal Neuron | Hippocampal Slice |
|---|---|---|
| 0 | 56±2.881 | 31±2.588 |
| 5.5 | 50±0.894 | 26±2.236 |
| 11 | 43±1.789 | 13±2.302 |
| 16.5 | 37±2.302 | 2±0.707 |
| 22 | 6±0.548 | 0±0.447[b] |
| 27.5 | 1±0.548 | |
| 33 | 0±0.447[b] | |

[a]Data represent the mean $V_{Th}$ taken from five independent experiments ± the standard deviation of the mean.

[b]Data represent the activity evoked Ach.

**Supplementary Table 2.** $V_{Th}$ values of the neuronal networks under the influence of different $C_{EtOH}$.

| $V_{Th}$ values (mV)[a] / $C_{EtOH}$ (mmol/L) \ Neuronal networks | Hippocampal Neuron | Hippocampal Slice |
|---|---|---|
| 0 | 56±2.864 | 31±2.550 |
| 10 | 133±4.450 | 60±3.536 |
| 20 | 163±4.472 | 83±4.506 |
| 30 | 187±2.739 | 103±4.472 |
| 40 | 201±2.000 | 133±4.494 |
| 50 | 207±8.367 | 177±5.701 |
| 60 | 218±4.775 | 269±7.416 |
| 70 | 298±4.472 | 349±7.294 |
| 80 | 423±4.472 | 413±4.450 |
| 90 | 560±6.124 | 513±4.472 |
| 100 | 826±5.477 | 607±4.506 |
| 110 | 1175±1.789 | 849±2.236 |
| 120 | ∞ | 1212±5.701 |
| 130 | ∞ | ∞ |

[a]Data represent the mean $V_{Th}$ taken from five independent experiments ± the standard deviation of the mean.

**Supplementary Table 3.** $V_{Th}$ values of the neuronal networks under the influence of different $T_m$.

| $V_{Th}$ values (mV)[a] / Neuronal networks / $T_m$ (°C) | Hippocampal Neuron | Hippocampal Slice |
|---|---|---|
| 32 | ∞ | ∞ |
| 33 | ∞ | 153±4.336 |
| 34 | 531±7.823 | 81±2.236 |
| 35 | 479±6.633 | 49±0.894 |
| 36 | 342±4.147 | 35±3.493 |
| 37 | 56±2.280 | 31±2.646 |
| 38 | 23±4.336 | 7±1.732 |
| 39 | 7±0.418 | 6±0.837 |
| 40 | 7±0.894 | 5±0.707 |
| 41 | 3±1.140 | 4±0.707 |
| 42 | 1±0.164 | 3±0.447 |
| 43 | ∞ | 1±1.021 |
| 44 | ∞ | ∞ |

[a]Data represent the mean $V_{Th}$ taken from five independent experiments ± the standard deviation of the mean.