



Developmental Electrophysiology of Cultured Neuronal Networks at Early Stage

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Citation: Kuang SY, Wang Z, Yang X, Huang T, Xi T, et al. (2018) Developmental Electrophysiology of Cultured Neuronal Networks at Early Stage. Biosens Bioelectron Open Acc: BBOA-122. DOI: 10.29011/2577-2260.100022

Received Date: 20 February, 2018; **Accepted Date:** 31 March, 2018; **Published Date:** 06 April, 2018

Abstract

This paper is a report of an investigation of how Spontaneous Spiking Activity (SSA) emerging from cultured neuronal networks develops/evolves within the first three weeks of culture. Chick Forebrain Neurons (C-FBNs) were dissociated and plated on Microelectrode Array (MEA) chips. After C-FBNs were plated, MEA recording of SSA was performed every other day from Day 4 to Day 22. SAS software was used to process the exported data. A Wilcoxon one-tailed test was used to examine whether the enhancing effect of 2% Fetal Bovine Serum (FBS) on SSA signaling previously reported by this group is statistically significant based on a series of descriptors (variables) of SSA. Results showed that 1) the distributions of Spike Rate (SR), Burst Rate (BR), and Inter Burst Interval (IBI) were left-skewed; spike frequency within bursts (Spf) and inter spike interval within bursts (ISI) had normal distribution; and Burst Duration (BD) and the percentage of spikes within bursts (Sp%) showed mixed features of distribution. 2) The patterns of development of the above variables were plotted; the intrinsic coherence of the burst inner structure-related variables (Sp%, BD, Spf, and ISI) were studied. 3) 2% FBS was found to statistically significantly enhance the signals of all 7 variables. 4) While all 7 variables described above evolved during the first three weeks, the BD mode remained as a stabilized parameter at either 120 or 125 ms on most days of MEA recording in both cultures, with or without FBS. The significance and the potential application of the above findings in the fields of *in vitro* electrophysiology and neurotoxicology are discussed.

Keywords: Biosensor; Chick Forebrain Neurons; Early Development; Electrophysiology; Microelectrode Array; Spontaneous Spiking Activity

The Need for Criteria to Assess the Developmental Stages of the Electrophysiology of Cultured Neuronal Networks

Introduction

To introduce why early stage (the first three weeks) developmental electrophysiology was studied in this paper, this section will address the need for criteria to assess the developmental stages of the Spontaneous Spiking Activity (SSA) emerging from cultured neuronal networks and outline previous findings by this research group.

Potential neurotoxicity of chemicals can be assessed from different aspects or levels. When a live cell for instance a neuron is insulted by adverse stimuli, such as being exposed to toxic chemicals, functional deviation of the neuron from its normal range (functional neurotoxicity manifested by changes in the electrophysiology of the neuron that are often temporal and reversible) often occurs before the occurrence of both cytotoxicity and structural abnormality. Conventional methods of neurotoxicity

assessment (such as using MTT assay, LDH assay, etc.) usually detect cytotoxicity and structural abnormality, but lack an approach for early functional neurotoxicity assessment. Four decades after Gross et al. [1], Microelectrode Array (MEA) technology coupled with animal neuron cultures are now filling the gap to offer rapid, sensitive, and functional neurotoxicity assessment.

Cultured animal neurons are able to form an *in vitro* neuronal network that fires action potentials spontaneously. The extracellular traces of these action potentials are referred to as “Spikes,” and spikes tend to fire in high frequency spike trains, or bursts. SSA is the physiological behavior, or the functional output, of the neuronal network. SSA is subject to both physical and chemical changes in the environment, and an MEA can record SSA and its changes in response to such changes. Hence, a neuronal culture on an MEA chip is a neuron-based biosensor that can detect and trace early functional deviations (changes in SSA) from the culture’s normal functional state prior to the occurrence of cytotoxicity or structural changes [2-4]. Moreover, the MEA’s great power is manifested by temporal and spatial tracing of SSA online and offline via computerized signal analysis and high throughput of endpoint data, thus making possible rapid screening for potential neurotoxic chemicals in the environment [5].

To date, neurons dissociated from the rodent central nervous system (such as cortex and spinal cord) are the most common cell types to be cultured on the MEA platform to form biosensors [5]. Using these biosensors, the acute functional neurotoxicity of a chemical or mixed chemicals is often assessed by conducting concentration-response experiments. However, the time window during which experiments were conducted varied greatly among researchers: before the third week *in vitro* [2,6,7]; after the third week *in vitro* [8-12]; or across the third week (from 9 DIV to 74 DIV [13]). According to Novellino and Zaldivar [10], the third week *in vitro* seems to be a transition period during which bursting activity became synchronized network-wide across all active channels, and this synchronized bursting activity became dominant. This seems reasonable since the development and maturation of the synaptic connections take some time in neuronal culture, and SSA develops/evolves over time. However, there is no unified criterion to assess the developmental stage of SSA. In previous research studies of the authors’ group, the third week also appeared to be a transition period for the maturity of SSA in Chick Forebrain Neuron (C-FBN) cultures.

The Third Week of SSA in C-FBN Cultures in Previous Studies by this Research Group

A novel type of neuron biosensor on an MEA using dissociated C-FBNs was developed and characterized morphologically,

functionally, and developmentally throughout the entire life span of several months by the authors’ group [14,15]. Similar to rodent counterparts, the synchronized net-wide bursting activity is the dominant feature of the SSA of the Chick FBN Biosensors (C-FBN-bioS), and it became obviously dominant during the third week *in vitro*. Also during the third week *in vitro*, the percentage of spikes that were packed into bursts (which appeared to be another dominant feature of SSA) was approaching its maximum as reported in detail in this paper. Because of the observations above, the third week *in vitro* appeared to be a transition period for the development or maturity of SSA: Before the third week, the two dominant features (i.e., a high percentage of maximal packing of spikes into bursts and the net wide synchronicity of bursting activity) were forming, and after the third week, the dominance of above two features lasted for several months if the cultures were well maintained. Given these findings, acute functional neurotoxicity tests were conducted on the C-FBN-bioS using concentration-response experiments after the third week *in vitro* [16,17]. A further detailed investigation of the development or evolution of SSA within the first three weeks has not been conducted.

Hence, one of the purposes of this paper is to investigate how SSA develops/evolves during the first 3 weeks. The investigation of SSA in this paper includes the investigation of 7 variables in two groups that characterize SSA from multiple aspects:

- 1) Burst-related variables including burst rate (BR), burst duration (BD), inter burst interval (IBI); and
- 2) spike-related variables including spike rate (SR), percentage of spikes in bursts (Sp%), spike frequency in bursts (Spf), and inter spike interval in bursts (ISI).

Since this group’s previous observation and report that the firing rate (both SR and BR) of a C-FBN-bioS increased significantly in the presence of 2% Fetal Bovine Serum (FBS) in the culture medium [14], the study reported here also examined the effect of 2% FBS on above 7 variables.

In brief, this paper

- 1) reports how above 7 variables of SSA develop/evolve during the first three weeks in culture, respectively, which may answer “Are there parameters that can be used to indicate the stage or maturity of the SSA from a developing *in vitro* neuronal network?”
- 2) discusses the significance and potential applications of the above findings in the area of electrophysiology and the current paradigm of neurotoxicity assessment.
- 3) examines whether 2% FBS enhances the signals of these 7 variables.

Materials and Methods

Chick FBN culture on an MEA

Embryonic Day 9 (E9) White Leghorn chick forebrains were dissected according to Heidemann et al. [18]. MEA preparation, forebrain tissue dissociation and dissociated forebrain cells plating, preparation of the first day's culture medium and medium after the first day were the same as described previously [14]. Medium after the first day included Neural Basal (NB) and NB⁺ (NB supplemented with 2% FBS). A total of 18 cultures from 3 batches of dissections were grown on MEA chips. Each dissection produced 6 cultures with 3 cultures in NB and 3 in NB⁺. A plating density of 2000 cells/mm² was used for all cultures. No mitotic inhibitor was used to suppress glial growth.

MEA recording

Typically, around 3 to 4 days *in vitro* (DIV) after cell plating, a morphological neuronal network was forming and could be seen under a microscope (Figure 1). We observed previously that the earliest emergence of SSA in the forms of spikes or bursts in our experimental setting was always at 4 DIV [14,15]. So MEA recording started at 4 DIV and then was performed every other day for each culture until 22 DIV. Each MEA recording lasted 10 min. SSA signals from the MEA were amplified using an MCS 1060-INV amplifier (MCS GmbH, Reutlingen, Germany) and collected using MC_Rack software (Version 4.3.0, MCS GmbH, Reutlingen, Germany) at a 25KHz sampling rate. All MEA recordings were performed inside a dry incubator set at 37° C with 5% CO₂ and 65% ambient relative humidity [19].

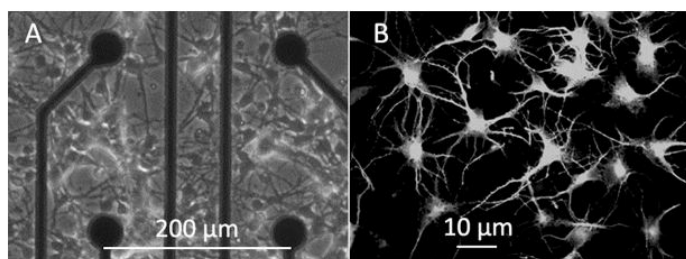


Figure 1: Phase-contrast images of two forming neuronal networks at 4 days *in vitro*. **A)** A culture in NB⁺ and **B)** a culture with positive staining of MAP2, the marker protein of neuronal soma and dendrites.

Spike and Burst Detection and Extraction of 7 Targeted Variables

Spikes and bursts were detected using MC Rack. The threshold for spike detection was set at -7 Standard Deviations (SD) lower than the mean noise amplitude for each active channel. The bursts were detected using the Burst Analyzer of MC Rack according to the following criteria:

1) The minimum number of spikes within a burst was equal to or greater than 3;

2) The maximum interspike interval (ISImax) within a burst was equal to or less than 100 ms; and

3) The minimum burst duration was equal to or greater than 10 ms.

MC Rack automatically extracts all 7 variables introduced previously and exports them to a .dat file. Microsoft Excel was used to open the .dat file and examine its contents. In an opened .dat file in Excel, these variables were automatically formatted as column names; the corresponding values from various channels were presented as rows that included all 60 channels of a standard 60-electrode MEA (with row 15 used as the reference channel). The value in each cell in the Excel spreadsheet was an average from a 10 min recording for a particular variable (column) in a particular active channel (row).

Statistical Analysis of 7 Variables

SAS software version 9.3 was used to process the .dat file opened with Excel. A filter of “6 spikes/min” was applied to determine the active channels to be used for data analysis: If a channel showed greater than 6 spikes/min, it was considered an active channel; channels that showed less than 6 spikes/min were considered silent and were excluded. Spike rate was statistically analyzed for active channels only. To process other selected parameters, active channels that showed a “0” value were also excluded.

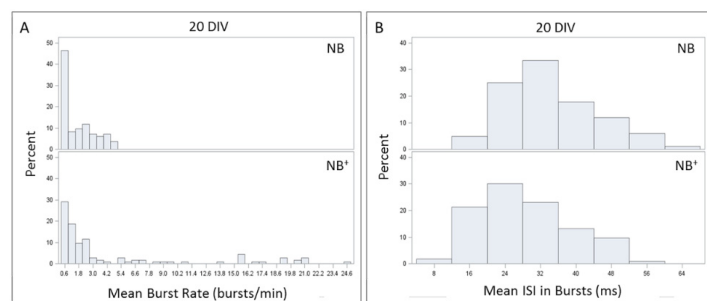
To explore how SSA evolved during the first three weeks and whether 2% FBS-treated cultures in NB⁺ medium had a significantly enhanced effect on the signals of the targeted 7 variables when compared to those signals from cultures in NB medium, SAS was first used to produce, for each recorded day (i.e., 4, 6, 8, 10, 12, 14, 16, 18, 20, and 22 DIV), a set of histograms with respect to the value of each selected parameter. The histograms indicated that a small fraction of variables (2 out of 7) showed typical normal distributions, and others did not. Consequently, a nonparametric method, Wilcoxon one-tailed test, was selected ($\alpha = 0.05$). Although MEA recording was started at 4 DIV for each culture, the sample sizes (total number of active channels of all cultures from all three dissections for both NB and NB⁺ groups) at 4 DIV were too small and were not applicable for further statistical tests, so all data from 4 DIV were excluded in this section.

Results and Discussion

Classification of 7 variables based on their distributions

According to the distributions of the 7 sets of histograms automatically generated by SAS, variables were classified into three groups. In Group I, SR (spike rate), BR (burst rate), and IBI (inter-burst interval) exhibited left-skewed distribution all the way from 6 to 22 DIV, so these data were non-parametric. In Group II, Spf (spike frequency in bursts) and ISI (inter-spike interval in

bursts) showed normal distribution in general all the way, so they were parametric data. And in Group III, BD (burst duration) and Sp% (spike percentage in bursts) showed mixed features: like-normal distribution, but skewed, with BD skewed left and Sp% skewed right. A representative Group I variable, left-skewed BR distribution, is shown in Figure 2A. The distribution of SR is very similar to the distribution of BR [14]. The distribution of IBI is also left-skewed (not shown) because IBI is inversely correlated to BR. A representative Group II variable, normally distributed ISI, is shown in Figure 2B.



Figures 2(A-B): Examples of Group I nonparametric and Group II parametric distributions of SSA variables. **A)** A typical left-skewed distribution of Burst Rate (BR) at 20 DIV for cultures in NB and NB⁺; Effect of 2% FBS on BD at higher rates seen in NB⁺ group and absent in NB group. **B)** A normal distribution of Inter Spike Interval (ISI) in bursts at 20 DIV; that the ISI mode in NB⁺ was smaller than the ISI mode in NB indicates an increase in the Spf in the presence of 2% FBS.

The distributions of the Group III variable, Sp%, are presented at 8 DIV and 20 DIV to show the tendency of their evolution with time (Figure 3).

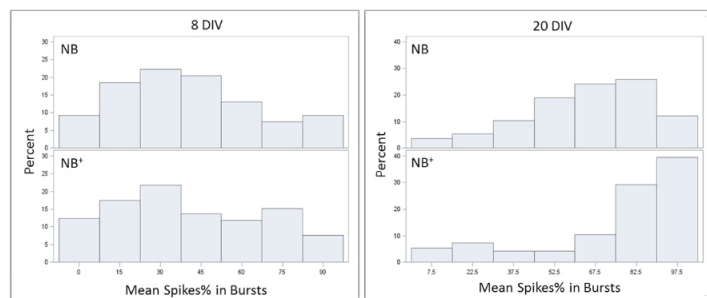


Figure 3: The distribution of percent of spikes in bursts (Sp%) at 8 DIV and 20 DIV. Over time, this distribution in both NB and NB⁺ groups tended to be right-skewed, meaning more and more spikes packed into bursts over time, and 2% FBS promoted this process.

The distributions of the Group III variable, BD, are also presented at two different DIVs, but with the purpose of showing a stable, unchanged BD mode (the value of the mean BD that appears most frequently) over time for both NB and NB⁺ groups (Figure 4). This discovery is unique to BD because similar stability was not seen in the modes of the other 6 targeted variables. The significance of this unique finding is discussed in section 3.5 after discussion of the effects of 2% FBS on the 7 variables.

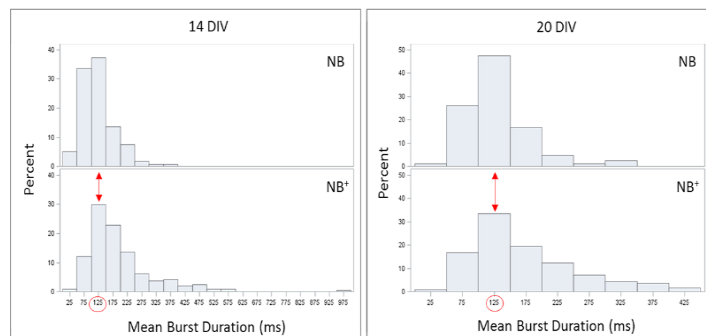


Figure 4: The distribution of BD for both NB and NB⁺ groups at 14 and 20 DIVs. 1) In both groups, the distribution of BD showed a mixed feature of normal and left-skewed distribution; 2) bidirectional red arrows point out the overlapped mode between two groups at each presented DIV; and 3) the circled numbers indicate these overlapped BD modes are 125 ms, and this value was not affected by 2% FBS.

Notably, the three groups of variables reflect SSA from three levels:

- 1) Group II variables that had normal distribution (Spf and ISI) constitute the Burst Inner Structure (BIS), so they were intrinsically related to BIS;
- 2) Group I variables that had left-skewed distribution (SR, BR, and IBI) are variables beyond the scope of BIS; and
- 3) Group III variables that had mixed features (BD and Sp%) were both BIS-related and beyond. Table 1 summarizes these findings and presents the appropriate statistical methods that should be used for a test of significance if a treatment is applied. In this paper, our treatment was 2% FBS in NB⁺; the control group was NB.

Variable Group	Variable Abbreviation	Variable Unit	Level	Variable Distribution	Recommended Statistical Method
Group I	SR	spikes/min	beyond BIS	left-skewed	non-parametric
	BR	bursts/min		left-skewed	non-parametric
	IBI	ms		left-skewed	non-parametric
Group II	Spf	Hz	intrinsic to BIS	normal	parametric and/or non-parametric
	ISI	ms		normal	parametric and/or non-parametric
Group III	BD	S	BIS related and beyond	mixed	non-parametric
	Sp%	%		mixed	non-parametric

Table 1: Classification of the 7 targeted variables based on their distribution.

The effect of 2% FBS on the 7 variables

The effect of 2% FBS can be seen in Figures 2, 3 and 4:

- 1) A higher BR can be seen for NB⁺ than for NB (Figure 2A);
- 2) The mode of ISI for NB⁺ is smaller than that in NB (Figure 2B), meaning Spf is higher in NB⁺ than in NB;
- 3) Spikes have a tendency to be organized into bursts along with DIVs (right-skewed at 20 DIV), and 2% FBS promotes this tendency-more spikes were organized into bursts in NB⁺ than in NB on the same DIV (Figure 3);
- 4) Larger BDs were seen in the NB⁺ group than in the NB group (Figure 4).

To examine whether the above fire rate-enhancing effect on each variable was statistically significant, a Wilcoxon one-tailed test was employed: Because it can be used for both nonparametric and parametric statistical tests, it applied to all 7 variables in this study. The Wilcoxon test compares the median value of a variable from all active channels in all cultures (Active Channel Count [ACC], the sample size) in control and experimental groups instead of means of these variables. The following sections (5.3 and 5.4) further report the development of these variables with the fire rate-enhancing effect of 2% FBS on each variable.

Development of Group I variables and effect of 2% FBS

A Wilcoxon one-tailed test was used for both SR and BR to examine their significance between NB and NB⁺ groups from 6 DIV to 22 DIV (Figure 5). The same test was not applicable to 4 DIV because the sample size (ACC) was too small. From Figure 5, it can be seen that the development of SR and BR from 6 to 22 DIV in the NB group was relatively stable; in the presence of 2% FBS, the median SR and BR were significantly increased in general, and the increases of SR and BR were most rapid before 10 DIV.

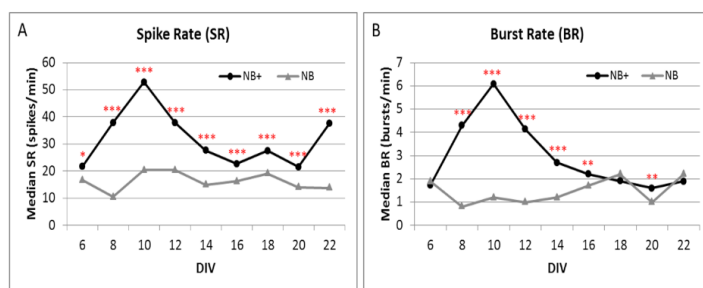


Figure 5: Graphic presentations of the development of SR (A) and BR (B) and the enhancing effect of 2% FBS on them. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

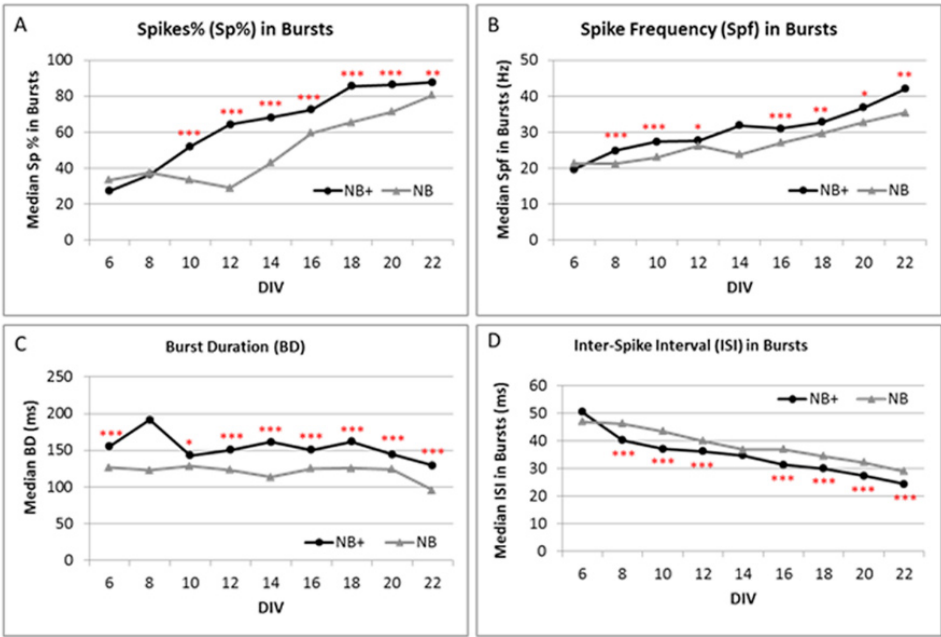
Development of BIS-related variables (Groups II and III) and effect of 2% FBS

Figure 6 shows the development of a group of BIS-related variables and the signal-enhancing effect of 2% FBS on these variables.

- 1) In Figure 6A, there is a gradual increase of median Sp% in bursts in NB group (grey line) with DIVs, indicating more spikes were organized into bursts with a maximum of 80% at 22 DIV; 2% FBS significantly increased median Sp% after 10 DIV (black line) with a maximum of nearly 90% at 22 DIV.
- 2) In Figure 6B, along with DIVs, median Spf increased gradually (grey line); 2% FBS significantly increased median Spf after 8 DIV (black line).
- 3) In Figure 6C, along with DIVs, the median BD tended to be stable except at 22 DIV in NB group (grey line). Addition of 2% FBS increased the median BD significantly at all DIVs (black line) and tended to stabilize the median at a higher value in general.
- 4) Considering Figs. 6A, 6B, and 6C together along with DIVs, bursts tended to comprise more spikes (6A) while median BD

tended to stay stable for both NB and NB⁺. This suggests BD was somehow resistant to increase over time, so an increased Spf within bursts should result from a combined effect of an increase in Sp% (Figure 6A) and a relatively stable median BD (Figure 6C). This phenomenon revealed that Sp%, Spf, and BD were 3 coherent variables that together determined BIS.

5) It was predictable that while Spf increased along with DIVs, the ISI in bursts decreased correspondingly. Figure 6D confirmed this prediction: ISI and Spf showed a negative correlation in both NB and NB⁺ groups. So, the coherence described previously was among all 4 BIS-related variables.



Figures 6(A-D): Development of the 4 Burst Inner Structure (BIS)-related variables (group II and III) and their coherency. *p < 0.05; **p < 0.01; ***p < 0.001.

Knowing the patterns of these 4 BIS-related variables in the developmental window (the first three weeks, 6 to 22 DIV) of SSA is valuable. First, we can see how they evolved. Second, due to the nonparametric distribution of SR and BR, the mean SR and BR at each DIV varied greatly (not shown); the median SR and BR varied less than the variation in the mean SR and BR, but were still more variable (Figure 5A,5B) than these 4 BIS-related variables (Figure 6). Each of the 4 variables had its own relatively stable pattern during the early developmental window. These relatively stable patterns (in comparison to the unstable SR and BR development) and the coherence among them represent a degree of regularity. The discovery of the regularity leads us to propose that the 4 patterns of these 4 BIS-related variables and their coherency may be used as reliable endpoint criteria for assessing the maturity of the developing SSA as well as the potential neurodevelopmental toxicity of numerous chemicals.

The Stability of the BD Mode

BD is one of 7 variables studied in this paper, and as can be seen in Figure 4, the BD mode stabilized at 125 ms for cultures in both NB and NB⁺ and at both 14 DIV and 20 DIV. This stability was confirmed as can be seen in Table 2, from 6 DIV to 20 DIV.

DIV	6	8	10	12	14	16	18	20	22
NB	107	125	120	120	125	120	125	125	75
NB ⁺	107	125	120	120	125	120	125	125	105

Table 2: The BD mode in ms for NB and NB⁺ groups from 6 DIV to 22 DIV.

Table 2 shows that the BD mode is within a narrow time window from 120 ms to 125 ms from 8 DIV through 20 DIV. The reason for the BD mode drop at 22 DIV is unknown. One potential reason that cannot be excluded is the 22 DIV drop in the number of active channels in cultures, which was the sample size used for the Wilcoxon test. The development of the active channels usually peaked

between 10 DIV to 14 DIV, dropped gradually from 14 DIV to around 21 DIV, and plateaued thereafter. More research studies are needed to explore why. At 6 DIV, the BD mode for both the NB and NB⁺ group were 107 ms, smaller than 120 ms. Two factors may contribute to this result:

- 1) the sample size (ACC) for the statistical test was small at 6 DIV; and
- 2) the earliest time to observe the tissue-specific pattern of SSA (which implies the forming of stereotypical microcircuits in the culture plated at a density of 2000 cells/mm²) was 8 DIV.

In brief, the BD mode was unique because, first, it was quite stable with time and second, it was the only aspect of SSA examined in this paper that was not affected by 2% FBS. This finding may provide insight to further explore the tissue-specific functional feature of the underlying microcircuits connected by stereotypical synaptic connections for the following reasons.

Neurons *in vivo* do not form synaptic connections randomly, but have specific synaptic targets. The two major types of mammalian cortical neurons are excitatory glutamatergic pyramidal cells and inhibitory GABAergic interneurons. For example, chandelier interneurons target only the trigger zone of the pyramidal cells to form inhibitory synapses, Martinotti interneurons target only the dendrites of the pyramidal cells, and basket interneurons target the soma of the pyramidal cells [20]. Moreover, *in vivo* cortical columns in the mammalian cortex are thought to have canonical microcircuits [21, 22]. After dissociation of the cortical tissue, the cortical columns no longer existed; and after plating the dissociated neurons, the distribution of neurons was randomized in cultures. After these dissociated mammalian cortical neurons reformed a new network in culture, its SSA firing pattern exhibited its tissue specificity in comparison to the SSA pattern of a network formed from dissociated spinal cord neurons [5]. The tissue-specific pattern of mammalian SSA suggests that those *in vivo* stereotypical tissue-specific synaptic connections may be also formed *in vitro*; thus, one could hypothesize that many fundamental synaptic connections formed in a culture are the same as or similar to what forms *in vivo*. The same hypothesis can also be formulated for the chick forebrain neuronal culture for the following reasons.

First, chick forebrains, though architecturally quite different from the mammalian six-layered neocortex, have been confirmed to have the same types of input and output cell types as the mammalian cortex [23]. Second, remarkable anatomical similarity in the neuronal connectivity patterns has been found between the cortices of birds and mammals [24]. Third, the same *in vivo* information processing principles underlying the rodent canonical cortical circuits have also been found in avian cortices [25]. And fourth, through comparing the firing pattern of cortical neuronal

culture with that of the spinal cord neuronal cultures from both chicks and rats, this group's work has shown similarity in the tissue-specificity of the SSA firing pattern between C-FBN-bioS and rodent counterparts [16]. Based on the above findings, it appears that

- 1) chick forebrain neuronal tissue-specific stereotypical synaptic connections, like their rodent counterparts, are formed in C-FBN-bioSs and widely distributed in C-FBN-bioSs;
- 2) these stereotypical synaptic connections form many similar fundamental microcircuits with fixed patterns of wiring in the C-FBN-bioSs; and
- 3) when these fundamental microcircuits, which are conserved across species, are excited and fired, it appears likely that the excitability and refractory periods of the neurons in these microcircuits determine the maximum spike frequency. It is likely that the excitability and refractory periods also determine the BD mode; and 120 or 125 ms (in this research group's experimental setting) is the most "favored" BD mode of the fundamental microcircuits in the C-FBN-bioSs. Can the BD mode serve as a tissue-specific index that characterizes the fundamental microcircuits of a particular brain tissue (such as spinal cord, cerebellum, dorsal root ganglion) and, can the multiple aspects of BD provide more answers to biological questions about the fundamental microcircuit? Future research is expected to answer these questions.

Conclusion

The results reported in this paper indicate following:

- 1) The 7 variables that describe SSA characterize SSA at 3 levels: Beyond BIS, intrinsic to BIS, and BIS-related and beyond.
- 2) The early development (the first 3 weeks) of the 4 variables that are intrinsic to BIS (spf and ISI) or BIS-related and beyond (sp% and BD) showed predictable patterns that are coherently related. This result suggests that the patterns of their early development and their coherency may be used as the criteria to assess the maturity of the SSA from a developing *in vitro* neuronal network and to assess the developmental neurotoxicity of the chemicals in the environment.
- 3) 2% FBS significantly enhanced signals of the 7 variables, except one aspect of variable BD, the BD mode.
- 4) The BD mode shows a unique feature, i.e., its stability, which is not influenced by 2% FBS. This phenomenon is notable. If it can be confirmed by different labs, more research studies would be needed to reveal its implications, for example, whether it is a tissue-specific index that characterizes the electrophysiology of numerous stereotypical microcircuits formed in a neuronal culture with tissue-specific types of neurons?

Acknowledgements

The authors acknowledge funding from the National Institutes of Health through SC COBRE (P20RR021949) and an R01 grant (R01HL124782); the National Science Foundation (OIA-1655740); and the National Natural Science Foundation of China (31370956).

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