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Research report

Investigating brain functional evolution and plasticity using microelectrode array technology

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ABSTRACT

The aim of this work was to investigate long and short-term plasticity responsible for memory formation in dissociated neuronal networks. In order to address this issue, a set of experiments was designed and implemented in which the microelectrode array electrode grid was divided into four quadrants, two of which were chronically stimulated, every two days for one hour with a stimulation paradigm that varied over time. Overall network and quadrant responses were then analyzed to quantify what level of plasticity took place in the network and how this was due to the stimulation interruption.

The results demonstrate that there were no spatial differences in the stimulus-evoked activity within quadrants. Furthermore, the implemented stimulation protocol induced depression effects in the neuronal networks as demonstrated by the consistently lower network activity following stimulation sessions. Finally, the analysis demonstrated that the inhibitory effects of the stimulation decreased over time, thus suggesting a habituation phenomenon.

These findings are sufficient to conclude that electrical stimulation is an important tool to interact with dissociated neuronal cultures, but localized stimuli are not enough to drive spatial synaptic potentiation or depression. On the contrary, the ability to modulate synaptic temporal plasticity was a feasible task to achieve by chronic network stimulation.

1. Introduction

External electrical stimuli are well-known to affect the functional activity of dissociated neuron networks (Marom and Shahaf, 2002). Jimbo et al. demonstrated that local tetanic stimulation induces long-lasting (longer than 30 min) changes in network responses, proving that potentiation and depression are pathway-specific and not neuron-specific, despite the large number of neurons and synapses in the network (Jimbo et al., 2000, 1999). In the literature, several studies have shown that these networks exhibit a variety of recurring activity patterns that can be modified by electrical stimulation. For instance, in Azouz and Gray (2000) the authors showed that cortical neurons are sensitive to the timing of their synaptic inputs and that can synchronize their firing with high temporal precision in Bologna et al. (2010), Chiappalone et al. (2006, 2008), Berdondini et al. (2009), Baljon et al. (2009), Wagenaar et al. (2006a,b) and Ide et al. (2010) the authors cultured neuronal networks on microelectrode array dishes to investigate neuronal development and used external electrical stimulus pulses to control neuronal activity pattern evolution and test synaptic plasticity. In Demarse et al. (2001) and Chao et al. (2008), MEA technology was used to integrate networks of cultured cortical neuronal networks with artificial intelligence. In Rolston et al. (2010), Potter et al. (2006) and Wagenaar et al. (2005) different types of closed loop feedback systems were implemented to modulate or change the network responses to external stimulation. The goal was to learn how to interpret the stimulus-evoked responses of dissociated cortical neurons to use these systems as in-vitro models of brain properties such as learning, memory and decision-making.

In Neves et al. (2008) and Wagenaar et al. (2006a), it was shown that low-frequency stimulation reduced synaptic transmission via long-term depression (LTD). LTD might serve as a learning mechanism in its own right or might be a means of ensuring homeostatic stability by preventing an increase in overall activity in potentiated networks.

The aforementioned experimental studies share a common objective, that is using external stimulation to modify the behavior of dissociated neuronal preparations and interact with them. The most common issues with such experimental setups are that the

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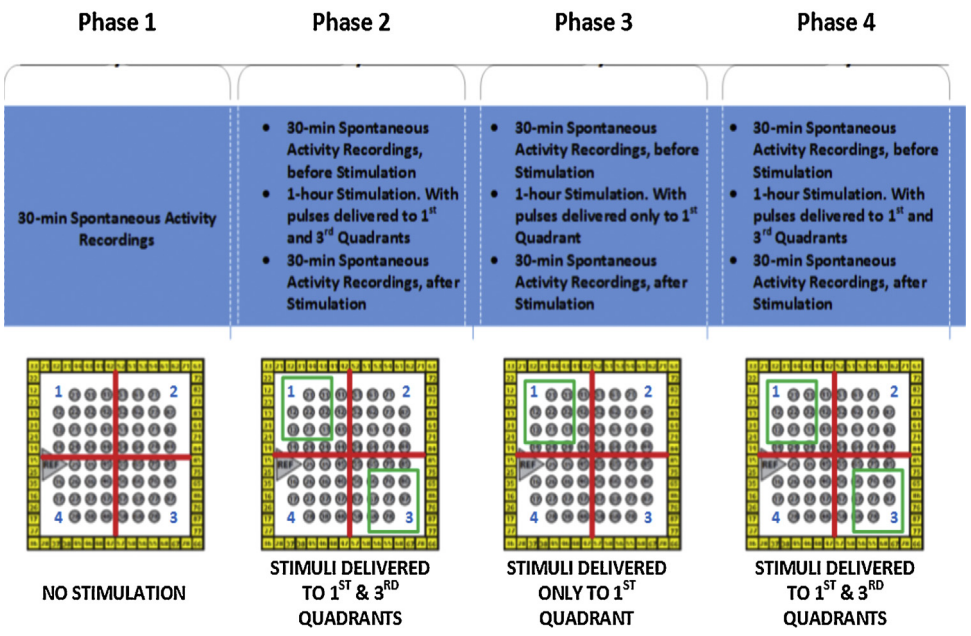

Fig. 1. Different experimental phases.

high variability in the network responses and the randomness in background activity make the neuronal activity difficult to interpret. Although the observed variability has often been attributed to the natural plasticity of the nervous system, there are no studies that have specifically investigated the extent to which this plasticity can be associated to in-vitro memory formation.

The aim of this work is to investigate whether dissociated neuron networks can exhibit memory phenomena, defined as the ability to learn and remember an applied stimulus paradigm. Specifically, it is sought to test whether preparations of dissociated neurons adapt to stimuli and respond to external inputs through plasticity and synaptic modification in a stable manner over both short and long time frames. The experimental protocol consisted of dividing the microelectrode array (MEA) electrode grid into four quadrants. After plating (Phase 1), two quadrants were chronically stimulated during Phase 2 (Q1 and Q3, see Fig. 3) while the remaining two were not stimulated at all (Q2 and Q4) and thus served an on-dish control. During Phase 3, the stimulus delivery was suspended in Q3 for ten days. Ten days later, in Phase 4, stimulation delivery was resumed into both quadrants (Q1 and Q3) and their responses were compared to quantify what level of plasticity took place in the network and how this related to the stimulation interruption.

One of three possible outcomes was expected:

1. The experimental quadrant responds to the resumed stimulation with a lower activity compared to the test quadrant to which chronic stimulation was regularly applied. Meaning that the applied stimulation potentiates the evoked responses (Potentiation) and that the quadrant in which the stimulation was first suspended and then resumed displayed no memory effects (Potentiation duration).
2. The experimental quadrant responds with higher activity compared to the test quadrant. Meaning that the applied stimulation attenuates the evoked responses (Depression) and that the quadrant in which the stimulation was first suspended and then resumed displayed no memory effects (Depression duration).
3. The experimental quadrant shows no significant difference from the always-stimulated quadrant. Meaning that potentiation or depression effects are either not present in dissociated cultures neurons under these experimental conditions.

2. Materials and methods

2.1. Cell culture preparation

Cortical tissue from embryonic Sprague-Dawley rats at day 18 of gestation was obtained from BrainBits, LLC (Springfield, IL). MEA dish preparation and neuron dissociation was informed by methodology described in Wagenaar et al. (2006b). Namely, cortical tissue was dissociated by enzymatic digestion in papain solution (20 min at 37 °C) followed by mechanical dissociation with a fine tipped Pasteur pipette. The resulting tissue was re-suspended in Neurobasal medium supplemented with 2% B-27 and 1% Glutamax-I (NbActiv4, BrainBits) at 4000 cells/μl. The dissociated neurons were then plated onto MEAs that had been coated with poly-D-

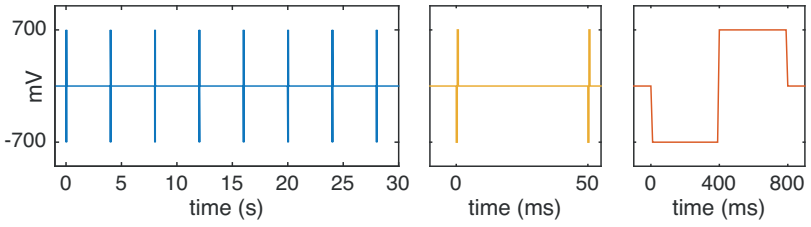

Fig. 2. Delivered stimulus pulses. Left-hand panel shows the eight paired-pulse stimulus sequence, with one pulse delivered to each of the selected stimulating electrodes every four seconds. Center: inter-pulse time interval = 50 ms. Right: characteristics of a single bipolar pulse.

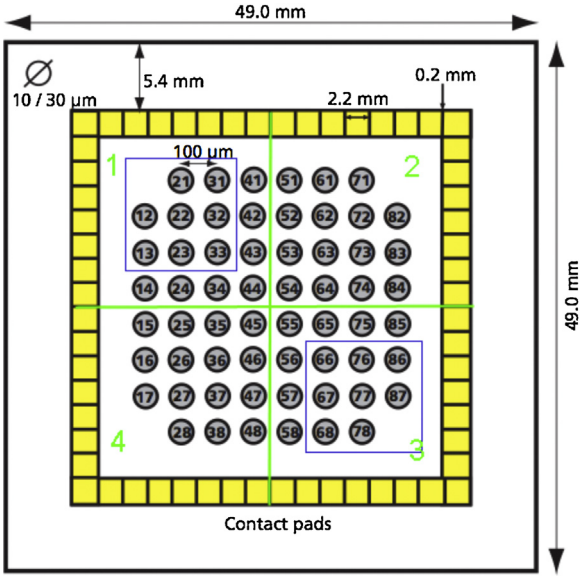

Fig. 3. Electrode grid. The blue rectangles show the eight electrodes per quadrant used to deliver stimulus pulses. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

lysine and laminin to promote cell adhesion. Cultures were kept in an incubator at 5% CO_2 at 37 °C and transferred to a dedicated acquisition incubator with the same environmental conditions during the experimental sessions. To reduce thermal stress of the cells, MEAs were kept at 37 °C by means of a controlled thermostat (Multi Channel Systems [MCS], Reutlingen, Germany). Half of the culture medium was changed twice per week, immediately after the recording sessions.

2.2. Data collection

Microelectrode arrays 60MEA20030iR-Ti (MCS) consist of 60 TiN/SiN planar round electrodes (30 μm diameter; 200 μm center-to-center inter-electrode intervals) arranged in a square grid (without corners; see Fig. 3), with the only exception of a larger ground electrode replacing one of the recording electrodes. All dish chambers are sealed with a gas permeable Teflon membrane to prevent contamination and evaporation (Potter and DeMarse, 2001). The activity of all cultures is recorded using a MEA1060-INV System (MCS). Voltage signals are amplified 1200×, sampled at 20 kHz and acquired through the data acquisition card and MC_Rack software (MCS). During stimulation sessions, electrical stimuli were delivered through a two-channel stimulator MCS STG1004. Data processing is performed off-line in a two-step process: first spike detection and parameter extraction are carried out using MC_Rack software, (MCS); then data analysis and statistical testing are performed with MATLAB© (Mathworks, Natick, MA).

3. Experimental procedure

The stimulation protocol implemented in this work was designed to investigate two phenomena. The first was whether electrode stimulation created localized (near Q1 and Q3) or general (dish-wide) stimulus-evoked responses. The second was whether the external stimulation protocol generated short-term or long-term potentiation and/or depression in dissociated cultured neurons. The experiments were thus designed to elucidate both temporal and spatial aspects of network activity and to reveal variations in synaptic plasticity.

The MEA electrode grid was divided into four quadrants: two test quadrants and two control quadrants. Voltage stimulus pulses were delivered using eight preselected electrodes in each of the two test quadrants. The experiments were divided into four ten-day long phases in which different stimulation protocols were implemented. No stimulation was delivered in the first experimental phase, while during the last three experimental phases, chronic external stimulation was delivered for one hour every other day. Every recording session started 20 min after placing the dish onto the amplifier, thereby giving the neurons time to recover from mechanical stress due to movement. Every complete experiment consisted of the following experimental phases:

Phase 1: Lasting from plating until the network displayed clear spontaneous activity, defined as a steady firing activity exhibited from most electrodes. During this period, the acquisitions consisted of spontaneous activity recordings with no stimulation delivery. This phase usually lasted between six to eight days in-vitro.

Phase 2: Training Phase, duration: ten days. The network underwent one hour of stimulation every two days; eight electrodes in Q1 and eight electrodes in Q3 were sequentially used to deliver the stimuli. Data acquisitions were composed of:

1. 30 min spontaneous activity before stimulus delivery.
2. 1 h evoked activity recording during stimulus delivery.
3. 30 min spontaneous activity.

Phase 3: Test Phase, duration: ten days. Only the eight electrodes in Q1 were used to deliver the stimuli, in a one-hour session every two days. No stimuli were delivered to Q3.

Phase 4: Identical to Phase 2, duration: ten days. This phase was designed to expose differences between Q1 and Q3 (test) quadrants with respect to temporal changes in the stimulation delivery and to assess the memory (plasticity) mechanisms in the neural network.

Dividing the experiments into four distinct phases made it possible to emphasize differences in functional responses between the test quadrant and the experimental quadrant due to stimulus delivery interruption. The temporal organization of the implemented experimental approach is shown in Fig. 1.

3.1. Stimulation protocol

The stimulation paradigm consisted of a simultaneous paired-pulse stimulus delivered to two electrodes in the two test quadrants. The paired-pulse stimulation approach was chosen to maximize the probability of excitation without over-stimulating the neuronal cultures. This is based on previous studies in which it has been shown that the use of paired pulses allows one to decrease the amount of current/voltage necessary to evoke meaningful network responses (Bakkum et al., 2008; Bi and Poo, 1998). Paired-pulses evoke larger responses with lower voltages because the first pulse activates presynaptic voltage-gated calcium channels, allowing for calcium influx into the neurons. When the second pulse follows the first by a few milliseconds, it raises the calcium concentration before the calcium released by the first pulse has been reabsorbed (Ide et al., 2010). Every stimulus pulse consisted of two bipolar voltage square waves (negative phase first) with amplitude respectively of −700 mV and 700 mV and duration 400 μs per phase. Bipolar stimulation between one electrode and a distant large ground electrode was used to minimize the effects of electrolysis since this can damage both the MEA electrodes and the cultured neurons. It has been shown that utilizing charge-balanced stimuli, especially for long-term applications, drastically reduces the risk of generating electrolysis (Wagenaar et al., 2004). The pulse widths were sized at 400 μs to reduce the probability, of large artifacts masking neuronal signals during stimulus presentations (Wagenaar et al., 2004). To increase the probability of neuron

Table 1
Stimulation electrode sequence.

Pulse	Channels
1	21 & 66
2	31 & 76
3	12 & 86
4	22 & 67
5	32 & 77
6	13 & 87
7	23 & 68
8	33 & 78

excitation without over-stimulating the network (Brewer et al., 2009), the stimulus pulses were always delivered in pairs with an inter-pulse interval of 50 ms and with an inter-pair interval of 4 s. The temporal characteristics of the implemented stimuli are shown in Fig. 2.

3.2. Stimulation: electrode pattern

The eight electrodes in each of the test quadrants were stimulated sequentially for one hour in each experimental session. During stimulus delivery, two electrodes, one per test quadrant, were simultaneously stimulated. The stimulus pulses were always delivered between a stimulating electrode and the ground electrode. Eight pulses per cycle were delivered to the two quadrants as shown in Table 1. The electrodes that we used to deliver the stimulus pulses, their spatial location and the quadrant division are shown in Fig. 3.

4. Signal processing

4.1. Spike detection

Continuous voltage traces were band-pass filtered to enhance the spiking components of the signals. In order to implement a band-pass filter we fed our signals into the cascade of a high-pass 2nd order Butterworth filter with cut off frequency set at 180 Hz and a low-pass 2nd order Butterworth, whose cutoff frequency was 3 kHz.

Spike detection was carried out using a simple threshold mechanism. The threshold was individually set for each channel and chosen as 6 times each band-pass filtered signal's standard deviation, as measured by MC_Rack software, within a 500 ms time window. To reduce the possibility of detecting duplicated spikes, a detection refractory period of 1 ms was used, during which no multiple detection events were accepted.

4.2. Feature extraction

Raw voltage signals were sampled at 20 kHz and amplified 1200× before being digitized and stored onto the acquisition computer. In order to perform off-line analysis, significant parameters such as neural spikes and LFPs were extracted from the raw acquired signals, and the overall spike rate and burst parameters were also recorded.

Given their importance in characterizing the behavior of cultured neuronal networks, bursting activity patterns were characterized according to the number of bursts occurring in each recording session, the number of spikes per burst, and the frequency of burst occurrence. Bursts were detected using MC_Rack software by searching for instances where the interval between spike trains was less than 10 ms. Events were then included in a detected burst until the interspike interval became larger than 100 ms. A single bursting event included all the detected events whose interspike interval was less than 15 ms. A burst was not counted if its duration was less than 50 ms or if it contained four or fewer spikes.

4.3. Data analysis

Given the high variability and randomness associated with the MEA recording features, a custom statistical analysis procedure was implemented to account for the temporal and spatial spontaneous variability that MEA activity displays. This was especially critical considering that the temporal evolution of these neuronal cultures could play an important role in modulating their firing characteristics over the duration of the 30-day experiment. Temporal variability was assessed by dividing acquisitions into 5-min time windows during which network performance was presumed to be stationary. Spatial variability was quantified by computing the mean firing activity and bursting parameters with respect to every quadrant in each experimental condition, both pre-stimulus and post-stimulus.

4.4. Temporal statistical analysis

Network spontaneous firing before stimulation sessions was considered as a natural characteristic of the network during a specific experimental session and it was used as a baseline to measure stimulation-induced changes. Specifically, to test whether stimulation had an effect on spontaneous activity, a series of firing parameters, such as the number of bursts and the Array-Wide Spike Detection Rate (ASDR), defined as the number of spikes detected per unit time, summed over all electrodes in the array (Wagenaar et al., 2006b), were measured. Such parameters were computed with respect to recordings acquired immediately before the stimulation sequence (N_{base}), as well as in the recordings after stimulation (N_{post}). Next, the 30-min long spontaneous recording sessions were divided into six 5-min bins. Bursts and ASDR were then measured within these time windows, for both pre and post-stimulation recording sessions. Stimulation-induced changes were quantified as

$$\Delta N_{ind} = \frac{N_{post} - N_{base}}{N_{base}}$$

This normalization controlled for detected changes that were not statistically significantly larger than spontaneous changes.

4.5. Spatial statistical analysis

The average firing parameters measured from the electrodes within each quadrant were used as a metric of local activity. In addition to changes induced by the four experimental phases, it was also investigated whether the spatial distribution of the delivered stimuli could play a role in shaping the spatial patterns of network responses.

In summary, both ASDR and burst parameters were measured in the network responses in each quadrant before and after stimulation delivery, and significant changes in spatial response patterns were assessed. To have an activity baseline of the network electrical behavior, the ASDR and burst parameters were also computed in each quadrant within the spontaneous recording sessions. These were taken as representative of the spontaneous spatial variations or pattern drifts in the network.

5. Results

Experiments were performed on eight MEA dishes plated with E-18 dissociated rat neurons. All the neuronal cultures came from the same brain tissue and the dishes were plated simultaneously.

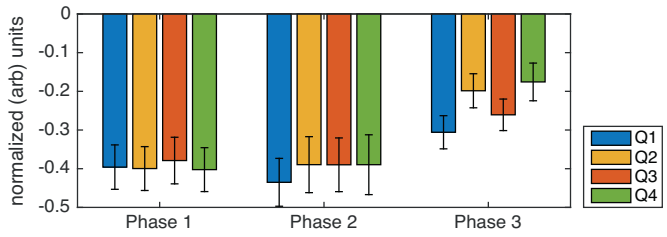

Fig. 4. Differences between average spike counts from post and pre-stimulation (normalized by pre-stimulus values) by experimental phase and quadrant. None of the in-Phase differences across quadrants are statistically significant (i.e. $p > 0.01$).

The results represent the mean values of the data collected from all the MEA experiments.

5.1. Spike count spatial analysis

Fig. 4 shows the average spike count, computed as normalized differences in network firing before and after stimulation. To quantify statistically significant changes in quadrant activity due to local stimulation, one-way ANOVA tests were carried out on spike counts averaged across all the experimental sessions within every experimental phase. Significance threshold was set to 5%. The *p*-values reported in the figures measure the statistical significance of changes that occurred across quadrants within each experimental phase. Non-significant *p*-values are reported in red boxes. It was found that, on average, the networks responded to electrical stimulation by reducing their firing activity. This can be seen in Fig. 4 in the negative values of the normalized average spike counts. As expected, no significant differences in network behavior were observed between the quadrants in Phase 2. On the contrary, such changes became statistically significant in Phase 3 post-stimulation sessions and in phase 4 (for both pre and post-stimulation sessions).

The first significant finding is that the local stimulation protocol was not able to evoke any localized changes in network activity, since all quadrants display similar activation activity independent of the stimulus location. Furthermore, the suspension of stimulation in Q3 during Phase 3, and then its resumption in Phase 4, did not induce any detectable activity changes, neither in that specific quadrant nor in the others.

Another approach that was used as a measure of spatial changes in network activity was to measure the changes that occur between experimental phases within each quadrant, as shown in Fig. 5. Fig. 5 shows that, in all four quadrants, the average spontaneous pre-stimulation network activity (blue bars) in Phase 3 was significantly larger than those in Phases 2 and 4. In contrast, the yellow bars suggest no significant post-stimulation differences between quadrants. Whereas Fig. 4 shows no significant difference between the four quadrants in each of the three experimental phases (i.e. no detectable spatial plasticity), Fig. 5 shows significant *temporal* plasticity within quadrants and across the experimental phases.

Fig. 5 (orange bars) shows normalized post-pre stimulation difference variability across the three experimental phases with average spike counts grouped by quadrant. Differences observed between the three experimental phases are not statistically significant for Quadrant 1. On the contrary, Quadrants 2–4 display significant variations during phase 4 compared to phases 2 and 3. The differences between the groups were assessed using *p*-values computed using a one-way ANOVA test followed by a multi comparison test based on the Tukey–Kramer difference criterion, also known as Tukey's Honest Significance Difference Test (HSD test).

5.2. Spike count temporal analysis

Array-Wide Detection Rate (ASDR), defined as the number of spikes detected per unit time, summed over all electrodes in the array, has been used extensively to evaluate total network activity (Wagenaar et al., 2006a,b). Fig. 6 shows the overall ASDR, averaged across 5-min time bins within experimental sessions, with every panel showing a different experimental phase. Within experimental sessions, changes due to spontaneous fluctuations of neuronal activity were not significant. This was especially apparent when comparing the magnitudes of the changes that occurred between pre and post-stimulation recordings. The effects due to external stimulation were therefore significantly larger than the

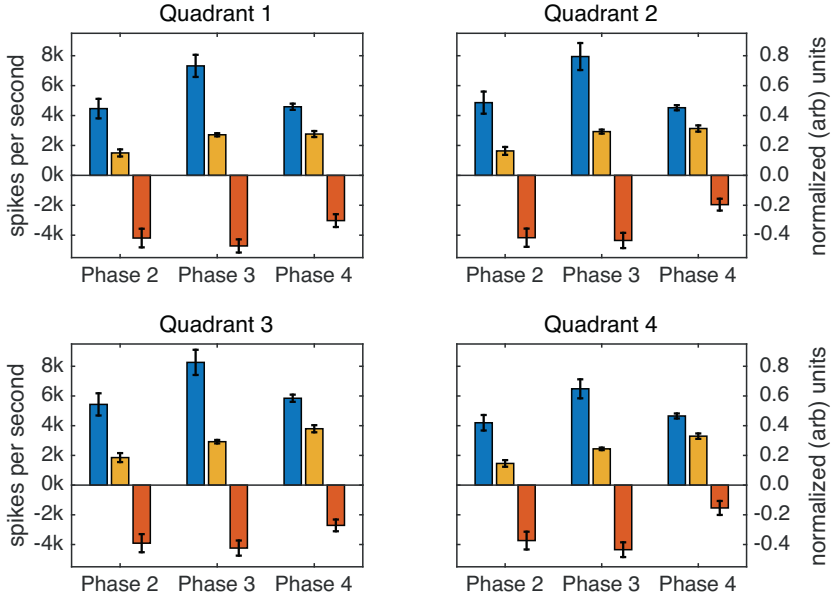

Fig. 5. Neuronal activations by dish quadrant and experimental phase. Pre-stimulation activations: blue; post-stimulation activations: yellow; normalized difference between post-stimulation and pre-stimulation: orange. Left-axes labels refer to activations [blue and yellow]; right-axes labels refer to normalized differences [orange]. All groups are significantly different ($p < 0.01$) excepting normalized differences in quadrant 1. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

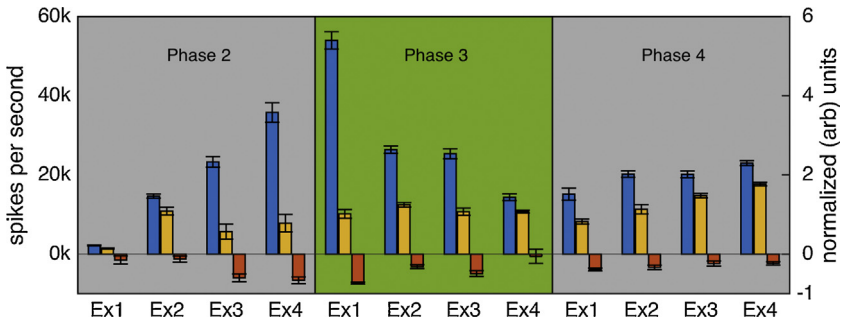

Fig. 6. Overall ASDR, averaged across 5-min time bins within experimental session (Ex 1–4) pre-stimulus activations: blue. Post-stimulus activations: yellow. Normalized difference between post-stimulus and pre-stimulus: red. All differences between experimental sessions were statistically significant ($p < 0.01$) excepting post-stimulus activations in Phase 3. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

natural variability in neuronal network activity. Furthermore, all observed differences were statistically significant, demonstrating a clear trend: throughout the pre-stimulation experimental sessions in Phase 2, the network's overall activity increased, then started to decrease in Phase 3 before becoming stable in Phase 4.

Pre and post-stimulation recordings differed substantially. Chronic stimulation inhibited activity, as evidenced by post-stimulation ASDR levels being consistently lower than pre-stimulation (see Fig. 6). More importantly, the trend that can be seen in pre-stimulation activity was not present in post-stimulation recordings. Fig. 6 shows that network activity after stimulation was more stable over experiments and across phases, with mean values across experiments that stayed more stable than corresponding mean values derived from pre-stimulation recordings.

Fig. 6 also shows the normalized differences in average overall ASDR between post-stimulation and stimulation (red bars). The evolution of the stimulation effect can be evaluated using the changes in the means of the activity differences. During Phase 2 the changes became more negative over time, until they began to be less negative in Phase 3 and were more consistently low during Phase 4. All observed variations are statistically significant based on a one-way ANOVA hypotheses test. Fig. 6 shows that differences between average post and pre-stimulation spike counts had a decreasing trend in Phase 2, reached a negative peak on the first experimental day of Phase 3, after which activity differences started to become less negative throughout Phases 3 and 4. During the last two experimental days of Phase 4, the differences between post and pre-stimulation spike counts steadily reached their closest values to zero (that is smallest changes). This suggests that over repeated experimental sessions neurons became less sensitive to the stimulation delivery and that the long-term depression effects linked to electrical stimulation faded. This finding confirms what was previously found in Wagenaar et al. (2005) and Napoli et al. (2013).

The final network evolution characteristic measured in this work is how the average overall ASDR evolved across different experimental phases (long-term induced plasticity). Fig. 7 shows the overall mean ASDR changes across experimental phases. All differences between phases scored significantly different (one-way ANOVA test) and the decreased effects of the applied stimulation due to the fact that neuronal networks are habituating to the presented stimulation can be seen.

5.3. Burst parameter spatial analysis

Burst parameter analysis is a well-known technique for investigating the characteristics of neuronal networks (Wagenaar et al., 2005). In this section, the average (across eight neuronal cultures) burst parameter spatial evolution is presented. A moving average boxcar filter with a 3-day sliding window has been implemented to average the results derived from different neuronal networks.

Fig. 8 shows the average number of bursts during pre and post-stimulation delivery sessions. Burst detection was performed using the algorithm introduced in Section 4.2. The burst analysis results confirm the finding obtained from analyzing the mean spike counts, in which it was found that the stimulation protocol did not generate any kind of spatial effects in the network responses. This is demonstrated here by the fact that the mean number of bursts across the four experimental phases did not display meaningful variations between quadrants. Fig. 8 also shows that the number of bursts displays similar behavior across quadrants both pre and post-stimulation. This demonstrates that the dish responded to electrical stimulation as a single neuronal ensemble across the four quadrants, independently of the stimulation phase.

Another burst parameter typically associated with changes in network activity is the average number of spikes detected during bursting events. This differs from the ASDR or overall spike counts because the mean number of spikes in a burst does not count spikes that occurred outside bursting events. Results with respect to pre and post-stimulation session recordings are shown in Fig. 9. Here, it can be seen that the differences of the mean numbers of spikes per burst reached a minimum at the beginning of Phase 3 and then became less negative (e.g. smaller changes between pre and post) towards the end of Phase 4.

Collectively, Figs. 8 & 9 demonstrate no spatial differences in activity between quadrants due to the experimental stimulation. In contrast, the stimulation protocol generated temporal changes in the network responses, as seen in Fig. 9, in which the post-pre stimulus response differences reached their peak at the beginning of Phase 3 and then became smaller. This demonstrates the neuronal desensitization to the stimulation protocol.

6. Discussion

It is common practice in MEA research studies to use activity-based criteria for two purposes: to select the stimulating electrodes that evoke larger network responses and to select neuron cultures whose behavior fits some predetermined conditions (Bologna et al., 2010). On the contrary, in this work, activity-based selection criteria were not used, neither to select stimulating electrodes nor to select experimental results to be included in the analysis.

The spatial analysis of network activity shows that the local stimulation protocol implemented in this work was not able to evoke any localized changes in network activity. This is proven by the fact that when comparing activity pre and post-stimulus, all the quadrants display similar trends in firing activity independently of the delivered stimulus location (position), as seen in Fig. 5. This rejects the hypothesis that neurons that are closer to the stimulating electrodes respond with stronger activity. This also means that

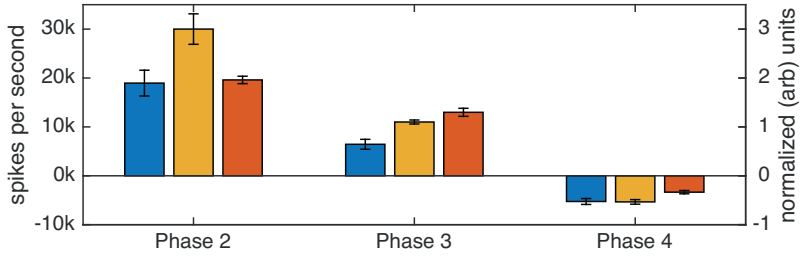

Fig. 7. Overall ASDR mean changes across experimental phases. Pre-stimulation data: blue. post-stimulation data: yellow. Normalized difference between post-stimulation and pre-stimulation: red, right axis. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

network connectivity is stronger than expected, that the neurons are so well-connected that even the application of localized stimulus pulses made the networks respond as a whole with activity that was widespread over all the electrodes. Another explanation could be that using the larger reference electrode as the negative one to deliver the stimulus voltage pulses might induce currents in the MEA dishes that are not localized as previously thought.

Even though the electrode grid was divided in different areas, such a division did not carry over to the underlying neuronal networks. In fact, the cultured neurons are capable of generating connections that spread out across any area of the MEA dish. This implies that stimulation, even if localized to a specific site, could evoke responses and modifications in different locations of the dish. As a result, the observed spatial changes in the neuron activity might not be exclusively contained within a given quadrant. Nonetheless, it was anticipated that the effects of the applied stimulation should be stronger or more lasting in proximity of the delivered stimulus pulses. In order to identify spatial changes in network activity evoked by local stimuli, both the total activity (entire electrode grid) and local activity (in each quadrant) were quantified. This allowed for detecting statistically significant local changes (across quadrants) with respect to the induced spatial activity changes in the networks.

Despite the fact that the stimulation parameters used here were either adapted from previous research studies (Wagenaar et al., 2004) or justified physiologically, they do not appear to be optimal for driving spatial changes in network activity patterns. Before concluding that local effects cannot be generated in an MEA preparation, further testing is required using different stimulation parameters.

Furthermore, the suspension of stimulation in Q3 during Phase 3 and its resumption in Phase 4, did not induce any detectable activity changes, neither in Q3 specifically nor the other quadrants. The results in Fig. 4 shows the average differences in firing activity before and after stimulus delivery measured in the four quadrants. No evident changes can be detected among network responses across different experimental phases, as demonstrated by the non-significant ANOVA test p-values for all of the three phases.

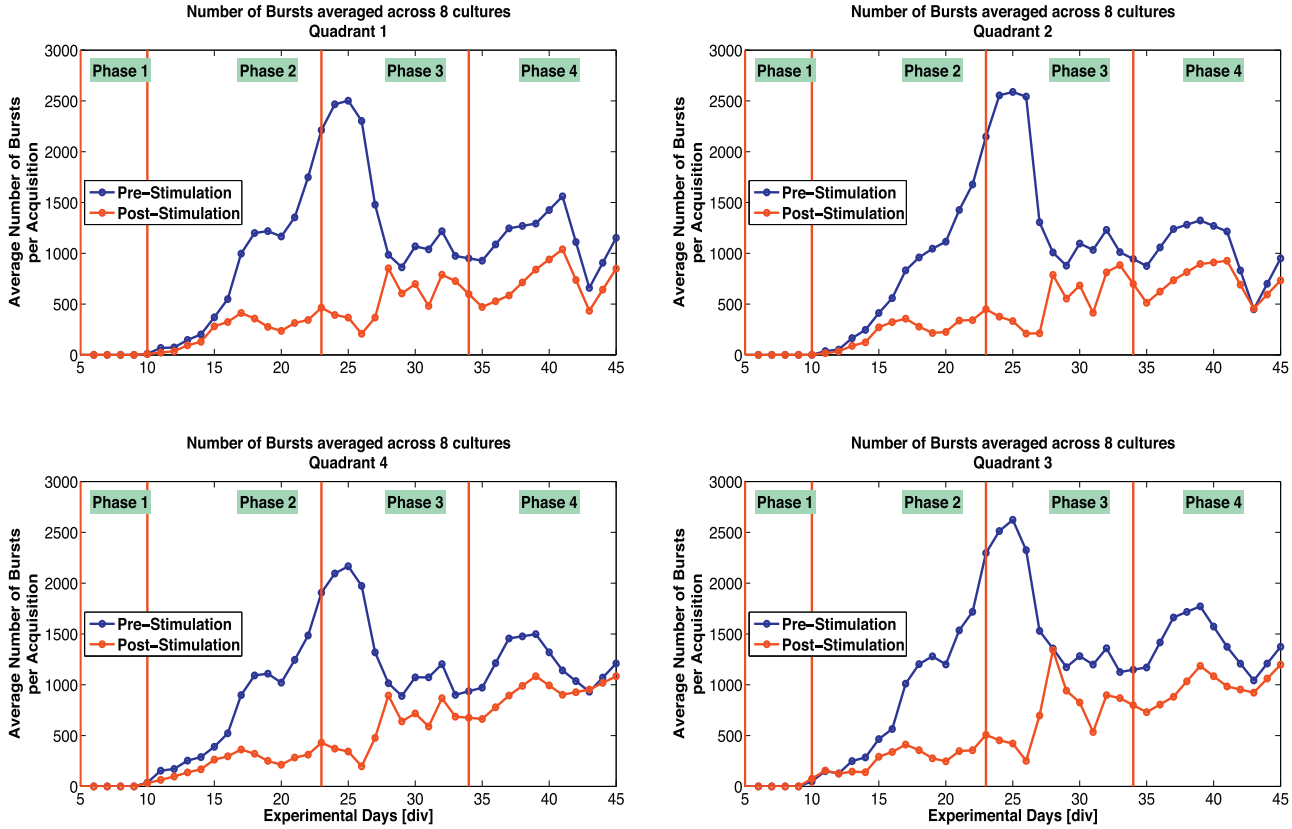

Fig. 8. Average number of bursts grouped by quadrant. Blue and red lines represent respectively pre-stimulation and post-stimulation curves. Four experimental phases are shown. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

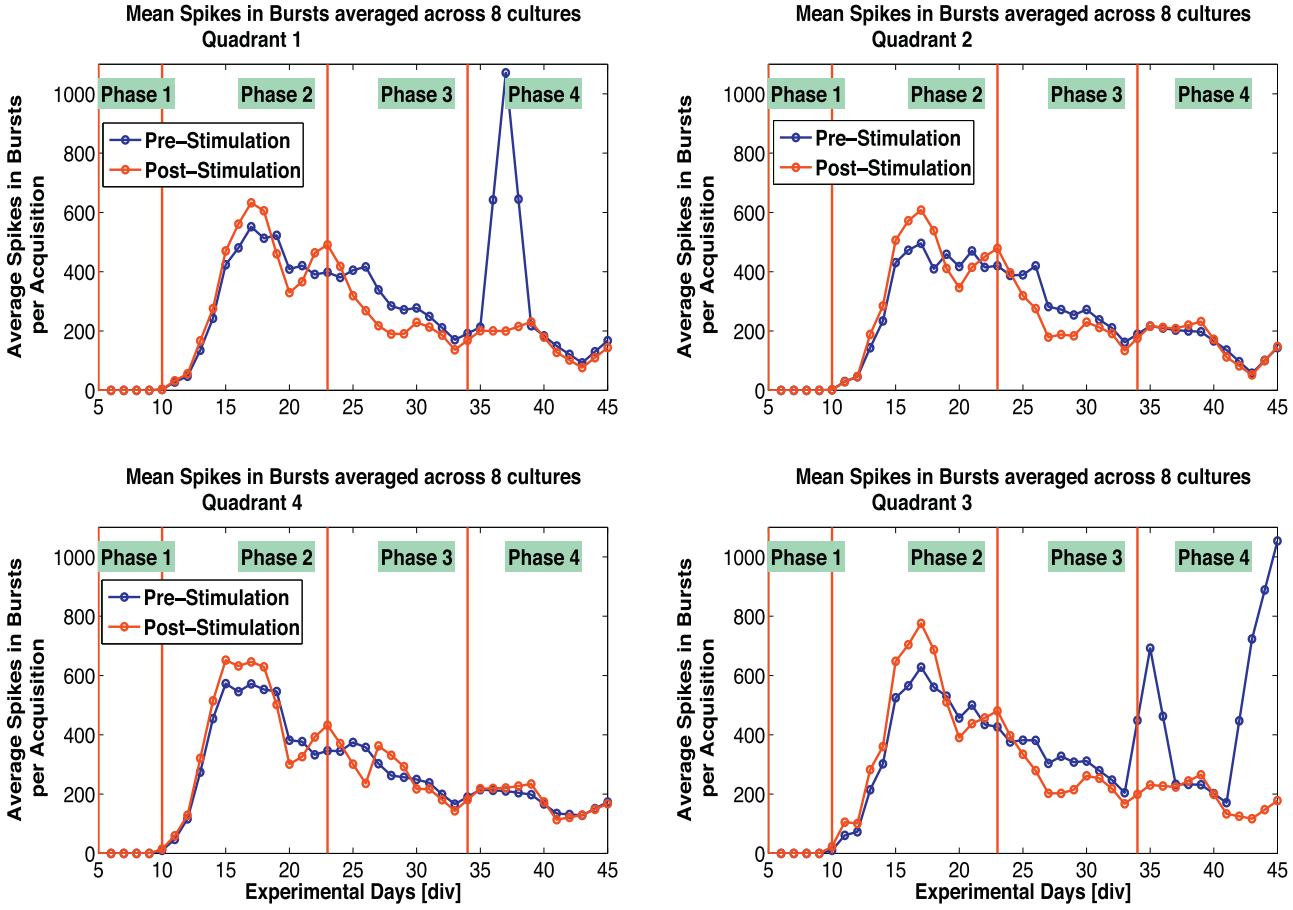

Fig. 9. Average number of spikes in bursts grouped by quadrant.

Fig. 5 again shows two important findings, (1) it is not possible to identify any stimulus-evoked spatial changes in the network; (2) over time, the neuronal networks tend to respond with smaller variations to the chronic external stimuli, as demonstrated by the smaller differences in average activity recorded in Phase 4. In other words, stimulation produced smaller inhibitory effects on the network behavior over time, meaning that the neurons habituated to the presence of the electrical stimuli. Inhibitory effects due to external stimulation are expected in these neuronal preparations. Most components of the nervous system modulate activity and propagate information by means of inhibition and the absence of inhibition is what causes the system to become more active (Wagenaar et al., 2005; Gonzalez et al., 2014). In this view, it is to be expected that the presence of external electrical input signals induced the network to be less active compared to the absence of stimulation. Furthermore, results derived from the analysis of network bursting activity patterns did confirm what was observed in spike count analysis. These results suggest that these dissociated neuronal network features are more interrelated than previously thought and that their combined use could lead to a better understanding of neuronal network connectivity evolution.

MEA experiments have typically proven difficult to control, owing to the multitude of parameters that must be considered. In Wagenaar et al. (2006b) the authors demonstrated that neuronal network temporal spontaneous activity evolved, changing its activity level. Specifically, neuronal networks exhibited a steady ASDR increase during the first three weeks in vitro, then their activity leveled off, while the degree to which culture-wide bursts dominated the activity kept increasing.

The temporal analysis results presented in this work show that daily network activity varied in a statistically significant manner (see Fig. 6), suggesting that short-term plasticity phenomena took place. In addition, the meaningful differences in activity between pre and post-stimulation sessions compared to changes within single sessions demonstrate that our stimulation protocol was successful in generating stimulus-evoked responses. Moreover, a stimulation habituation effect was highlighted in the neuronal networks over the course of 30 days, with network stimulus-evoked responses growing in Phase 2, reaching their maximum in Phase 3 before decreasing in Phase 4, when the network had become desensitized to external voltage pulses. Long-term plasticity phenomena are exposed in Figs. 6 and 7 that display how differently the neuronal networks reacted to the same stimulation protocol that was delivered in Phases 2 and 4. One last consideration regards differences across experimental phases. They were significant, but they did not reflect the amount of delivered stimulation within each phase. For instance, in Fig. 7 differences in average activity are not higher in Phases 2 and 3 during which the largest amount of electrical stimulation was delivered.

In these experiments, chronic stimulation was applied for 30 days and although this represents a considerable period of time in comparison with the lifespan of MEA neuronal preparations (up to 3 months), this time range possibly could not have been optimal to elicit temporal changes in neuronal activity. In future work, other time scales should be tested to investigate if further network dynamics can be identified.

7. Conclusions

The results of this work demonstrate three main findings. First, there were no spatial differences in the stimulus-evoked activity within quadrants and that localized stimulation evoked responses whose effects extended well beyond the quadrants in which they were delivered. Secondly, the stimulation protocol induced depression effects in the neuronal networks as demonstrated by the consistently lower network activity following stimulation sessions. Interestingly, the post-stimulation and pre-stimulation recordings followed different behavioral trends. Finally, the inhibitory effects of the stimulation decreased over time, thus suggesting a habituation phenomenon.

Conflict of interest

The authors declare that there are no conflicts of interest.

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References

Azouz, R., Gray, C.M., 2000. Dynamic spike threshold reveals a mechanism for synaptic coincidence detection in cortical neurons in vivo. Proc. Natl. Acad. Sci. U. S. A. 97 (July (14)), 8110–8115.
Bakkum, D.J., Chao, Z.C., Potter, S.M., 2008. Spatio-temporal electrical stimuli shape behavior of an embodied cortical network in a goal-directed learning task. J. Neural Eng. 5 (September (3)), 310–323.
Baljon, P., Chiappalone, M., Martinoia, S., 2009. Interaction of electrically evoked responses in networks of dissociated cortical neurons. Phys. Rev. E 80 (September (3)), 031906.
Berdondini, L., Massobrio, P., Chiappalone, M., Tedesco, M., Imfeld, K., Maccione, A., Gandolfo, M., Koudelka-Hep, M., Martinoia, S., 2009. Extracellular recordings from locally dense microelectrode arrays coupled to dissociated cortical cultures. J. Neurosci. Methods 177 (March (2)), 386–396.
Bi, G.Q., Poo, M.M., 1998. Synaptic modifications in cultured hippocampal neurons: dependence on spike timing, synaptic strength, and postsynaptic cell type. J. Neurosci. 18 (24), 10464–10472.
Bologna, L.L., Nieus, T., Tedesco, M., Chiappalone, M., Benfenati, F., Martinoia, S., 2010. Low-frequency stimulation enhances burst activity in cortical cultures during development. Neuroscience 165 (February (3)), 692–704.
Brewer, G.J., Boehler, M.D., Ide, A.N., Wheeler, B.C., 2009. Chronic electrical stimulation of cultured hippocampal networks increases spontaneous spike rates. J. Neurosci. Methods 184 (October (1)), 104–109.
Chao, Z.C., Bakkum, D.J., Potter, S.M., 2008. Shaping embodied neural networks for adaptive goal-directed behavior. PLoS Comput. Biol. 4 (March (3)), e1000042.
Chiappalone, M., Bove, M., Vato, A., Tedesco, M., Martinoia, S., 2006. Dissociated cortical networks show spontaneously correlated activity patterns during in vitro development. Brain Res. 1093 (June (1)), 41–53.
Chiappalone, M., Massobrio, P., Martinoia, S., 2008. Network plasticity in cortical assemblies. Eur. J. Neurosci. 28 (April), 221–237.
Demarse, T.B., Wagenaar, D.A., Blau, A.W., Potter, S.M., 2001. The neurally controlled animat: biological brains acting with simulated bodies. Auton. Robots 11 (January (3)), 305–310.
Gonzalez, J., Morales, I.S., Villarreal, D.M., Derrick, B.E., 2014. Low-frequency stimulation induces long-term depression and slow onset long-term potentiation at perforant path-dentate gyrus synapses in vivo. J. Neurophysiol. 111 (March (6)), 1259–1273.
Ide, A.N., Andruska, A., Boehler, M., Wheeler, B.C., Brewer, G.J., 2010. Chronic network stimulation enhances evoked action potentials. J. Neural Eng. 7 (February (1)), 16008.
Jimbo, Y., Tateno, T., Robinson, H.P., 1999. Simultaneous induction of pathway-specific potentiation and depression in networks of cortical neurons. Biophys. J. 76 (February (2)), 670–678.
Jimbo, Y., Kawana, A., Parodi, P., Torre, V., 2000. The dynamics of a neuronal culture of dissociated cortical neurons of neonatal rats. Biol. Cybern. 83 (July (1)), 1–20.
Marom, S., Shahaf, G., 2002. Development, learning and memory in large random networks of cortical neurons: lessons beyond anatomy. Q. Rev. Biophys. 35 (February (1)), 63–87.
Napoli, A., Xie, J., Obeid, I., 2013. Investigating the role of chronic stimulation in dissociated cortical neuron development. Biomedical Engineering Society, Annual Meeting, 2013.
Neves, G., Cooke, S.F., Bliss, T.V.P., 2008. Synaptic plasticity, memory and the hippocampus: a neural network approach to causality. Nat. Rev. Neurosci. 9 (January (1)), 65–75.
Potter, S.M., DeMarse, T.B., 2001. A new approach to neural cell culture for long-term studies. J. Neurosci. Methods 110 (September (1–2)), 17–24.
Potter, S.M., Wagenaar, D.A., DeMarse, T.B., 2006. Closing the loop: stimulation feedback systems for embodied MEA cultures. In: Advances in Network Electrophysiology. Springer, pp. 215–242.
Rolston, J.D., Gross, R.E., Potter, S.M., 2010. Closed-loop, open-source electrophysiology. Front. Neurosci. 4 (September), 1–8.
Wagenaar, D.A., Pine, J., Potter, S.M., 2004. Effective parameters for stimulation of dissociated cultures using multi-electrode arrays. J. Neurosci. Methods 138 (September (1–2)), 27–37.
Wagenaar, D.A., Madhavan, R., Pine, J., Potter, S.M., 2005. Controlling bursting in cortical cultures with closed-loop multi-electrode stimulation. J. Neurosci. 25 (January (3)), 680–688.
Wagenaar, D.A., Pine, J., Potter, S.M., 2006a. Searching for plasticity in dissociated cortical cultures on multi-electrode arrays. J. Negat. Results Biomed. 5 (January), 16.
Wagenaar, D.A., Pine, J., Potter, S.M., 2006b. An extremely rich repertoire of bursting patterns during the development of cortical cultures. BMC Neurosci. 7 (January), 11.