Single pulse responses in cultured neuronal networks to describe connectivity

Joost le Feber, and Michael Corner

***Abstract*—Synaptic connections between neurons play a crucial role in cognitive processes like learning and memory. In recent work we developed a method, using conditional firing probability (CFP analysis), to estimate functional connectivity in terms of strength and latency, and here we further explored on this method. CFP analysis estimates functional connectivity between pairs of neurons by calculating the probability that neuron *j* will fire at t=τ, given that neuron *i* fired at t=0. Because neuron *i* often fires more than once in the analysis interval of 500 ms CFPs don't indicate the average response to a single action potential in neuron *i*. Rather, CFPs are biased by the probability that neuron *i* will fire again in the analysis interval ($CFP_{i,i}$, or autocorrelation). We developed a method to estimate single pulse responses (SPRs), by deconvolving $CFP_{i,i}$ from the probability curve. We investigated the performance of this deconvolved measure in experiments with cholinergic network activation of cultured cortical networks. Ideally, acetylcholine should affect only the dynamic behavior of the system, but not the described (glutamatergic) connections in cortical networks. We found that changes in SPRs under different dynamic behavior were much smaller than those in CFPs. However, changes were still considerable, most likely reflecting the non-linear nature of synaptic transmission.**

I. Introduction

IT is generally believed that cognitive processes like learning and memory highly depend on the connections in neuronal networks. Therefore, it is an important issue in neuroscience to be able to estimate network connectivity, and several techniques have been developed. Most methods are based on theoretical considerations, and practical validation appears difficult. Very often these methods are based on, or related to, cross correlation. In our lab we developed a technique based on conditional firing probabilities ($CFP_{i,j}[\tau]$) to estimate functional connectivity [1]. This technique provides intuitive measures for strengths and latencies of functional connections, and has been shown to, at least to a certain extent, describe synaptic connections in cultured cortical networks [2].

Manuscript received December 17, 2010. Joost le Feber is with the Biomedical Signals and Systems Group, MIRA, Institute for Biomedical Engineering and Technical Medicine, University of Twente, PO Box 217, 7500AE, Enschede, the Netherlands (corresponding author e-mail: j.lefeber@ utwente.nl).

Michael Corner was with the Netherlands Institute for Brain Research, and performed part of the experiments after his retirement at Technion, Israel Institute of Technology , Haifa, Israel.

CFP analysis evaluates all spiking activity during 500 ms after any recorded action potential to calculate the probability that neuron j will fire at t=τ, given that neuron i fired at t=0. The number of action potentials at electrode i that is followed by a spike at j with a delay τ ($Nfollow_{i,j}[\tau]$) is calculated as

$$N_{follow_{i,j}}[\tau] = \quad X_i \;\; t \;\; \cdot X_j \;\; t + \tau \qquad (1)$$

Equation (1) holds because it is applied to binary arrays X_i and X_j, with $X_{i,j}[n] \in \{0, 1\}$ for all n, X_i was set to 1 whenever an action potential was detected and to 0 otherwise. CFP[τ] can be calculated by dividing $N_{follow}[\tau]$ by the total number of action potentials at electrode i (N_i):

$$CFP_{i,j} \;\; \tau \;\; = \frac{N_{follow_{i,j}}}{N_i} = \frac{{}_t X_i \;\; t \;\; \cdot X_j \;\; t+\tau}{{}_t X_i \;\; t} \;\; \forall \; 0 < \tau < 500\text{ms} \qquad (2)$$

This analysis yields a probability curve, to which a standard function is fitted to obtain measures for strength and latency. However, this measure does not reveal responses to single input spikes, because usually the initiating neuron fires more than once in the analysis interval. On average, this repeated firing may be captured by $CFP_{i,i}[\tau]$, which is closely related to the auto correlation function of the initiating neuron.

In linear time invariant systems theory the output of a system in the frequency domain $Y(\omega)$ is calculated as the product of the input $X(\omega)$ and the transfer function $H(\omega)$ (Eq.3), or in the time domain as the convolution of the input $x(t)$ with the impulse response $h(t)$ (Eq.4).

$$Y(\omega \;=\; X(\omega \;\cdot H(\omega \qquad (3)$$

$$y(t) = x(t) \otimes h(t) = \int_{-\infty}^{\infty} x(t-\tau) \cdot h(\tau(\tau) \qquad (4)$$

Here, $\otimes$ denotes a convolution. On average, the input from neuron i may be described by the probability that it fires again at t=τ, given that it fired at t=0, calculated as $CFP_{i,i}[\tau]$. This probability is proportional to the autocorrelation of the point proces that describes the firing pattern of neuron i. This implies that, in a linear approach, we may deconvolve the autocorrelation (input) from the CFP (output) to obtain an 'impulse response'. However, because neuronal networks are non-linear, we will use the term 'single pulse response' (SPR) rather than impulse response.

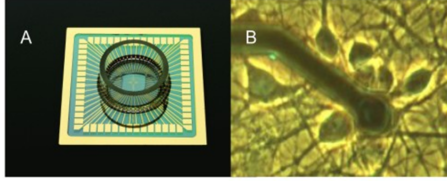

Figure 1. Multi electrode array (MEA) and close up of one of the electrodes. **A:** MEA, used to record neuronal activity in cultured cortical networks. It is based on a glass substrate with 60 embedded electrodes in the centre of the chamber, with 200 µm inter electrode distance. The glass ring glued on top was filled with glia conditioned growth medium and firmly sealed. **B**: close up of one of the electrodes and several neurons.

Two approaches may be followed to verify whether SPRs give a better description of actual (synaptic) connectivity than CFPs. First, realizing that CFPs mainly reflect excitatory connections [3-5], which are glutamatergic in cortical cultures, we may apply cholinergic stimulation to change the dynamic behavior of the system [6, 7] without directly imposing changes on the described glutamate driven connectivity. Whereas changing firing patterns (and thus $CFP_{i,i}[\tau]$) probably affect CFPs, ideally they should not alter SPRs. However, as cortical networks are not linear systems, reality will be that SPRs will also change, but possibly to a lesser extent. Alternatively, we may use a computer simulation [8] to generate firing patterns, and infer connectivity by CFP analysis and SPRs. Both measures may then be related to actual connectivity as present in the model.

Here we followed the first option and investigated the performance of CFPs and SPRs under cholinergic stimulation in cortical networks, cultured on multi electrode arrays (MEAs). Such networks have been extensively used to investigate network properties of cortical cultures [1]-[2], [6, 7],[9]-[10]. An example of a cortical culture on a MEA is shown in Figure 1.

II. Methods

A. Cell cultures

We obtained cortical cells from newborn Wistar rats at post natal day 1. After trypsin treatment cells were dissociated by trituration. About 400,000 dissociated neurons (400 µl suspension) were plated on a 60 electrode MEA (Multi Channel Systems, Reutlingen, Germany, see Figure 1), precoated with poly ethylene imine (PEI). This procedure resulted in an initial cell density of approximately 5000 cells per mm^2, which was in agreement with counted estimates in the first days after plating. With aging cell densities gradually decreased to ~2500 cells/mm^2. We used MEA's containing electrodes with 10 µm diameter (pitch 100 µm), or 30 µm diameter (pitch:200 µm)

Neurons were cultured in a circular chamber with inner diameter d = 20mm, glued on top of an MEA. The culture chamber was filled with ~700 µl R12 medium [11] MEAs were stored in an incubator, under standard conditions of 37°C, 100% humidity, and 5% CO_2 in air. For recording, we firmly sealed the culture chambers with watertight but CO_2 permeable foil (MCS; ALA scientific), and placed the cultures in a measurement setup outside the incubator. During recording we maintained the CO_2 level of the environment around 5% and we moisturized the air. For details about the recording setup see [9] Recordings were started after an accommodation period of at least 20 minutes.

After the measurements the cultures were returned to the incubator. We used four neuronal cultures obtained from different rats for our experiments, which were performed in the third week after plating of the dissociated cells.

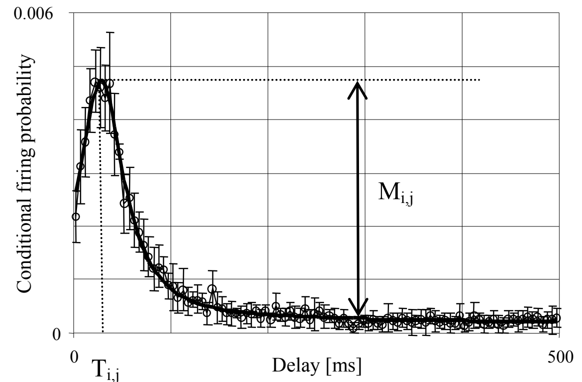

Figure 2. ○: Estmated probability that neuron *j* fires at t=τ, given that neuron *i* fired at t=0 (mean ± SD). Solid line: a standard function was fitted to this probability curve to obtain values for strength ($M_{i,j}$) and latency ($T_{i,j}$) of the functional connection between this pair of neurons.

B. Experiments

To evaluate the effect of firing patterns, in particular the effect of different auto correlations on the connectivity measures CFP and SPR, we first recorded spontaneous activity in standard medium (~2h; '*Control*' phase). Then we replaced 50% of the medium by medium containing 40µM carbachol, resulting in a final concentration of 20µM. Under these conditions we recorded spontaneous activity for ~20 hours ('*carbachol*' phase). Finally, we washed out carbachol by completely refreshing the medium and again we recorded spontaneous activity for ~20 hours ('*Washout*' phase).

C. Data analysis

In all cultures we first estimated functional connectivity in the control phase, using CFP analysis as explained in [1]. Then, we added carbachol (20 µM) and repeated the analysis to observed changes in functional connections. Finally, we repeated this analysis after carbachol washout. To assess the stability of functional connectivity we divided the *control*, *carbachol* and *washout* recordings of all experiments into data blocks of 2^{15} spiking events each. To evaluate the effect of carbachol administration on bursting, we applied a

measure introduced by Wagenaar et al.: burstiness index BI [10]. This is a normalized parameter with values between 0 (almost no bursts) and 1 (burst dominated).

In parallel we also estimated single pulse responses before, during and after carbachol application. This would in principle require deconvolution of the autocorrelation from the acquired probability curve, but unfortunately, deconvolution is a rather unstable process. To avoid deconvolution we convolved the standard curve from Figure 1 with the autocorrelation in each iteration step during the fit procedure. Thus we obtained parameters $M_{i,j}$ (strength) and $T_{i,j}$ (latency) that, convolved with the autocorrelation, yielded the CFP curve.

To evaluate the results, we investigated the temporal development of all functional connections. Because we were interested in the general effect of changing dynamic conditions on all connections, rather than differences between individual connections, each connection was normalized to its average value in the *carbachol* phase. Then all connections were averaged to assess the effect of carbachol on functional connectivity as estimated using CFPs or SPRs. All results are shown as mean ± SEM, unless indicated otherwise.

III. Results

We applied cholinergic activation in four cultures from

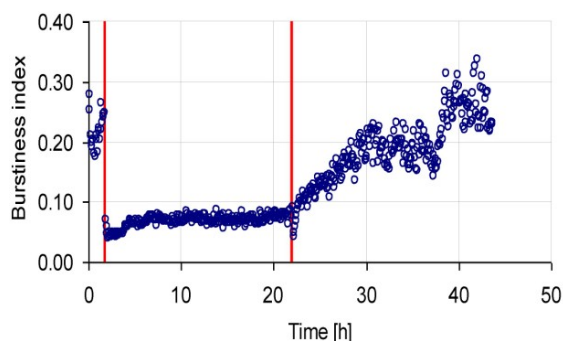

Figure 3. Example of the effect of carbachol on bursting. We calculated a burstiness index as described in Wagenaar 2005. Vertical red lines indicate the transitions from *Control* to *Carbachol* (t=2h) and from *Carbachol* to *Washout* (t=22h).

different rats. In all experiments the burstiness index (BI) decreased upon carbachol application, by 40% on average. Figure 3 shows an example of the effect of cholinergic activation on BI in one of the cultures.

In all four experiments both the SPRs as well as CFP's were larger in plain medium than during cholinergic stimulation. SPRs showed smaller changes than CFPs upon carbachol administration and wash out in all experiments. Figure 4 shows an example of the average development of CFPs and SPRs before (*Control* phase), during (*Carbachol* phase) and after cholinergic stimulation (*Washout* phase). On average CFPs were 3.97±0.82 times larger before and after carbachol than during cholinergic stimulation. SPRs were 2.72±0.38 times larger before and after carbachol application. In three out of four experiments the induced changes by carbachol administration were slightly larger than those observed after washout (on average a factor 4.5 vs. 3.4 for CFPs and 3.1 vs. 2.3 for SPRs). In the period before carbachol application, mean normalized CFP's were 40±21% larger than SPR's, after washout this difference was 33±18%. These differences were larger if we focused on functional connections with non-zero latency only: 46±18% and 44±17%, respectively.

IV. Discussion

In our study, cholinergic stimulation changed firing patterns from bursting into more dispersed activity (see Figure 3), as demonstrated in earlier studies [6, 7]. Because acetylcholine is not produced in cortical cultures, baseline functional connectivity is not cholinergic. This provides an opportunity to affect firing patterns without directly changing the described (glutamatergic) connections. Thus, we may bring the system into another dynamic state without explicitly changing the system itself, which enabled us to examine the effect of dynamic behavior on estimated functional connectivity.

In this study we avoided inverse convolution by convolving the standard curve from Figure 2 with the autocorrelation in each iteration step during the fit procedure. This approach assumes that SPRs share the same general shape of CFP's, as depicted in Figure 2. This assumption was supported by the observation that the ~20% of the neurons that had a flat autocorrelation under control conditions, showed probability curves with the same shape as the 80% with non-flat autocorrelations [1]. This indicates that we may use this basic shape to convolve with $CFP_{i,i}$ before curve fitting.

Ideally, estimated connectivity should not change with changing dynamic behavior. However, Figure 4 illustrates that estimated CFP's were highly affected, by a factor 4 on average. To obtain a better estimate, we estimated the single pulse response, analog to the impulse response in linear systems theory. However, synaptic strengths are highly non-linear and depend on activation frequency of pre- and postsynaptic neurons. It was therefore to be expect that also SPRs would change with altered activation patterns. This probably explains why SPRs did not yield identical values in the *Control*, *Carbachol* and *Washout* phases.

Our results showed that on average CFPs did indeed change with altered bursting behavior. SPRs were also affected by cholinergic activation, but to a much lesser extend (~40% less than CFPs). The remaining changes

probably reflected non-linearities in synaptic transmission. Thus, the changes in CFPs may result from a combined effect of autocorrelation pollution and non-linear synaptic transmission. The influence of the autocorrelation was removed, but still estimated connectivity depended on the dynamic state.

Figure 4 shows that, although mean values were rather stable, standard deviations were quite high. This might reflect different effects of altered dynamic behavior on individual SPRs. Alternatively, the high standard deviations might result from the spontaneous development of functional connections in a period of ~45 hours. Recent studies suggested that these spontaneously occurring changes are usually both up and down, hardly yielding hardly any effect on average values [12, 13]. This finding supports the view that the large standard deviations with a relatively stable average of Figure 4 may result from spontaneous development in individual connections.

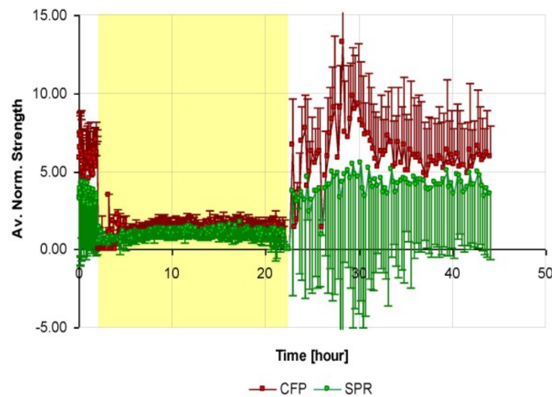

Figure 4 Example of the effect of cholinergic activation on CFPs (red) and SPRs (green). All individual functional connections were normalized to their mean values during cholinergic activation. Markers indicate mean values, error bars represent standard deviations. For clarity, only positive (red) or negative (green) error bars are shown. Yellow background indicates period of cholinergic activation; white background: before application and after washout.

In a recent study we showed that functional connections do contain information about synaptic connections [2]. However, part of the functional connections may arise from pairs of neurons that receive input from a common source. The analysis proposed in this paper may not apply to functional connections resulting from a common input, thus obscuring the average result. This idea is supported by the fact that excluding all functional connections with zero latency, which are more likely to arise from a common input than from causal activation, yielded even larger differences between the changes in CFPs and those in SPRs before, during, and after carbachol application. Nonetheless, even when blindly applied to all functional connections, SPRs seem to better describe functional connectivity than CFPs.

We are currently running computer simulations with fully controlled synaptic non-linearities to further clarify the individual contributions of repeated activation and synaptic non-linearity.

References

[1] J. le Feber, W. L. C. Rutten, J. Stegenga, P. S. Wolters, G. J. Ramakers, and J. Van Pelt, "Conditional firing probabilities in cultured neuronal networks: a stable underlying structure in widely varying spontaneous activity patterns," *J. Neural Eng.*, vol. 4, pp. 54-67, 2007.

[2] J. le Feber, J. Van Pelt, and W. Rutten, "Latency related development of functional connections in cultured cortical networks.," *Biophys. J.*, vol. 96, pp. 3443-3450, 2009.

[3] A. M. H. J. Aertsen and G. L. Gerstein, "Evaluation of neural connectivity: sensitivity of cross-correlation," *Brain Res*, vol. 340, pp. 341-354, 1985.

[4] W. J. Melssen and W. J. M. Epping, "Detection and estimation of neural connectivity based on Crosscorrelation analysis," *Biol. Cybern.*, vol. 57, pp. 403-414, 1987.

[5] G. Palm, A. M. H. J. Aertsen, and G. L. Gerstein, "On the significance of correlation among neural spike trains," *Biol. Cybern.*, vol. 59, pp. 1-11, 1988.

[6] M. Chiappalone, M. Corner, and J. le Feber, "Persistent effects of cholinergic activation in developing cerebral cortex cultures: a model for the role of sleep activity patterns in early development?," 7th Int Meeting Substr-Integr MEAs, Reutlingen, Germany, 2010.

[7] M. Chiappalone, A. Vato, L. Berdondini, M. Koudelka-hep, and S. Martinoia, "Network dynamics and synchronous activity in cultured cortical neurons," *Int J Neural Syst*, vol. 17, pp. 87-103, 2007.

[8] T. Gritsun, J. le Feber, J. Stegenga, and W. Rutten, "Network bursts in cortical cultures are best simulated using pacemaker neurons and adaptive synapses.," *Biol. Cybern.*, vol. 102, pp. 293-310, 2010.

[9] H. J. Romijn, F. van Huizen, and P. S. Wolters, "Towards an improved serum-free, chemically defined medium for long-term culturing of cerebral cortex tissue," *Neurosc Biobehav Rev*, vol. 8, pp. 301-334, 1984.

[10] J. Stegenga, J. le Feber, E. Marani, and W. L. C. Rutten, "Analysis of cultured neuronal networks using intra-burst firing characteristics," *IEEE Trans Biomed Eng.*, vol. 55, pp. 1382-1390, 2008.

[11] D. A. Wagenaar, R. Madhavan, J. Pine, and S. M. Potter, "Controlling bursting in cortical cultures with closed-loop multi-electrode stimulation.," *J Neurosci*, vol. 25, pp. 680-8, 2005.

[12] M. Corner, "Reciprocal homeostatic responses to excitatory synaptic receptor inactivation in developing organotypic cortical networks in vitro," *Neurosci. Lett.*, vol. 438, pp. 300-302, 2008.

[13] J. le Feber, J. Stegenga, and W.L.C. Rutten, "The effect of slow electrical stimuli to achieve learning in cultured networks of rat cortical." *PLoS ONE* , vol. 5(1), e8871, 2010