Dual-compartment microfluidic device for neuronal co-cultures: Design, Implementation and validation
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Citation for published version (APA):

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Chapter 5

Functional Connectivity and Dynamics of Cortical-Thalamic Networks

Published as: Kanagasabapathi TT, Massobrio P, Barone RA, Tedesco M, Martinoia S, Wadman WJ, Decré MMJ. “Functional connectivity and dynamics of cortical-thalamic networks co-cultured in a dual compartment device” (Equal contribution)

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Functional connectivity and dynamics of cortical–thalamic networks co-cultured in a dual compartment device

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Spatial and temporal variability in response to hybrid electro-optical stimulation

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**ABSTRACT**

Co-cultures containing dissociated cortical and thalamic cells may provide a unique model for understanding the pathophysiology in the respective neuronal sub-circuitry. In addition, developing an in vitro dissociated co-culture model offers the possibility to study the system without influence from other neuronal sub-populations. Here we demonstrate a dual compartment system coupled to microelectrode arrays (MEAs) for co-culturing and recording spontaneous activities from neuronal sub-populations. Propagation of electrical activities between cortical and thalamic regions and their inter-dependency in connectivity is verified by means of a cross-correlation algorithm. We found that burst events originate in the cortical region and drive the entire cortical-thalamic network. Bursting behavior while mutually weak thalamic connections play a relevant role in sustaining longer burst events in cortical cells. To support these experimental findings, a neuronal network model was developed and used to investigate the interplay between network dynamics and connectivity in the cortical-thalamic system.
5.1 Introduction

The interactions between thalamus and cortex has been extensively studied following both in vivo (Nicolesis, 2005; Crunelli et al., 2011) and in vitro (Adams et al., 2011). Albeit prevailing opinion recognizes thalamus as a mere set of nuclei relaying signals to the cerebral cortex, recent advancement in the last decades have set forth new hypotheses regarding the thalamic functions and its interactions with the cortex. Accordingly, thalamus is recognized more importantly as a site for two-way interaction with the cortex than simply as a relay station to the same (Miller, 1996). The significance of the thalamus in the brain circuitry can be well understood considering that almost all neuronal signals from the sensory and motor periphery reach the cortex via the thalamus (Jones, 1985). Briggs and Usrey proposed that thalamus has more than a simple “gate function” for information flow (Briggs and Usrey, 2008) and that the thalamic neurons receive substantially strong input from cortico-thalamic feedback neurons thereby allowing the context to communicate continuously through the thalamus during sensory processing. This implies that cortical neurons can dynamically modulate the thalamic processing function, and ultimately shape the nature of its own input (Briggs and Usrey, 2008). The influence of cortical cues in the development of thalamo-cortical connectivity or in the remodelling of networks following environmental modifications has been demonstrated by Coronas et al. (Coronas et al., 2000).

In vitro studies have demonstrated that cortico-thalamic synapses have a low probability to discharge (Granseth and Lindstrom, 2003), that transition towards facilitation and augmentation when a high frequency stimulation is delivered (Granseth, 2004; Granseth and Lindstrom, 2004). In terms of thalamo-cortical connections, such synapses are relatively sparse and hence, the direct excitation provided by the thalamus is weak and requires a recurrent excitatory intra-cortical amplification to activate the cortex (Lubke et al., 2000).

These reciprocal connectivity pathways between cortex and thalamus are responsible for generating rhythmic neuronal network oscillations which, from a physiological point of view, play a significant role in fundamental functions such as sleep modulation, and sensor-motor information integration (Andolina et al., 2007; Iyengar et al., 2007; Crunelli et al., 2011). To better understand the interplay between thalamus and cortex, the development of in vitro systems utilizing organotypic slices or dissociated cells can offer a complementary approach to in vivo studies. In a recent work, Adams and co-workers (Adams et al., 2011) have pioneered protocols for developing organotypic co-cultures of cortex and thalamus slices extracted from P0-P3 rats to study and characterize the initiation and spreading of such oscillations within
thalamus and cortex. Although the organotypic slices provide better spatial resolution for electrophysiological recordings, selective manipulation (electrical and chemical) of a specific region in an organotypic slice is often not possible. Hence, developing such co-culture system with dissociated cells in compartmented devices may provide better manipulation capabilities that are not available from organotypic slice studies. Notwithstanding such potential advantages, preparation and survival of dissociated thalamic cells in vitro are often challenging. Earlier works have shown that thalamic cells in the early stages of development (~ E15) survive for up to 5 days in vitro (DIV) when isolated and grown in simple serum-free medium due to neurotrophic factor mediated interactions between themselves (Lotto and Price, 1995; Asavaritikrai et al., 2003). However, Survival of thalamic cells from older prenatal (> E15) and early postnatal brains in isolation was not successful (Magowan and Price, 1996; Asavaritikrai et al., 2003) and it was attributed to the absence of external influences such as the absence of cortical signals with specific developmental properties. The influence of cortical cues in the development of thalamo-cortical connectivity or in the re-modeling of networks following environmental modifications has been demonstrated (Cunningham et al., 1987; Coronas et al., 2000; Asavaritikrai et al., 2003).

In this paper, we take a complementary and integrated approach in which experiments and modelling investigate specific interaction mechanisms between thalamic and cortical populations. The developed experimental system with microfluidic separated dual compartments coupled to Micro-Electrode Arrays (MEAs) might open new perspectives in the studies of the cortical-thalamic system. We first demonstrate co-culturing dissociated cortical and thalamic cells in vitro in the dual compartment system compatible with MEA. The system integrates two closed interconnected micro-chambers on planar MEA that allow the compartmentalization of neuronal cells and the control of fluidic micro-environments (Kanagasabapathi et al., 2011). The micro-chambers (100 µm high, 1.5 mm long) made of polydimethylsiloxane (PDMS) is able to segregate the two subpopulations, while an array of thin microchannels (10 µm width, 3 µm high, and 150 µm length) connecting the two micro-chambers allow neurite outgrowth between the two subpopulations. By exploiting such features, we realized co-cultures of dissociated neurons of cortex and thalamus. We characterized the dynamics of such co-cultures by means of first-order statistics to investigate the influence of cortical cell dynamics in thalamic network activity and vice versa. Then, by applying cross-correlation based algorithms (Garofalo et al., 2009), we inferred the functional connectivity maps between the two populations, and the directionality in signal propagation. A relevant reciprocal connectivity between the cortical and thalamic region was observed. Burst events originate in the cortical region and the
5.2 Materials and Methods

The presence of strong cortico-thalamic connections drives the thalamic network to discharge bursts while reciprocal weak thalamo-cortical connections play a salient role in the cortical network behaviour by modulating the duration and shape of the burst event. The analysis presented in this work confirms the recent findings that cortical region is the site of initiation of burst firing events while reciprocal thalamo-cortical connections are required to maintain a prolonged synchronised bursting pattern in the cortical culture (Adams et al., 2011).

Finally, to further uphold such connectivity schemes, we introduced a simple model of two large-scale neuronal populations mimicking the cortical and thalamic dynamics. Each neuron was described following the Izhikevich equations (Izhikevich, 2003, 2004) and different connectivity schemes between the two populations were tested to better demonstrate the necessity of such reciprocal connectivity for the actual dynamics observed in the experimental conditions.

Figure 5.1: Dual compartment device used in co-culture preparation. (A) Schematic layout of the dual-compartment device; (B) Planar MEA substrate with dual-compartment PDMS device; (C) Cortical-thalamic co-culture in a dual compartment device (Cortical cells and thalamic cells are highlighted with red and green fluorescence staining respectively).
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Neurofluidic Device and Electrophysiological Recordings

Dual compartment devices made of PDMS as described in our earlier work was used for the co-culture experiments (Kanagasabapathi et al., 2009; Kanagasabapathi et al., 2011). The microfluidic compartments (figure 5.1A) of 1.5 mm width, 8 mm length and 100 µm in height are interconnected with microchannels of 10 µm height, 3 µm width and 150 µm length that are spaced at regular intervals of 50 µm (in total ~ 120 microchannels connect the two compartments). The small cross-section of the microchannels prevents the movement of cells between compartments while allowing neurites to cross-over to the adjacent compartment and form a functional network (Kanagasabapathi et al., 2011). The compartmented PDMS device (figure 5.1B) was selectively oxygen-plasma treated to render the compartments and microchannels hydrophilic while preserving hydrophobic contact surface. The oxygen-plasma treated device was reversibly bonded to microelectrode arrays (MEA) substrate (Multi Channel Systems, Reutlingen, Germany) as described in our earlier work (Kanagasabapathi et al., 2009). MEA substrates with 60 planar TiN/SiN micro-electrodes (30 µm diameter, 200 µm spaced) arranged in an 8 x 8 layout (without four corner electrodes) were used in this work and PDMS devices were aligned to include 30 electrodes per compartment. Prior to bonding, MEA substrates were coated overnight with polyethylenimine (PEI) solution at a concentration of 40 µg/ml. The spontaneous electrophysiological activities from the plated cells was recorded using MEA1060 system (Multi Channel Systems) and the raw data were band pass filtered at 10 Hz to 3 kHz (Fee et al., 1996; Ide et al., 2010) and sampled at 25 kHz per channel (Rolston et al., 2007).

Co-culture Preparation

As per the approved protocols for the care and use of lab animals in the Netherlands, primary cultures of Wistar rat embryonic cortical and thalamic neurons were prepared by Trypsine (GIBCO, Invitrogen, USA) digestion of day-18 embryonic rat whole cortices and ventral basal thalamus. The dissociated cortical cells were cultured in neurobasal medium (Lonza lifesiences, USA) and the dissociated thalamic cells were cultured in similar medium supplemented with an additional 3% Fetal bovine serum (FBS) and 1% Horse serum (HS). On the day of the experiment, PEI coated MEA substrates were rinsed 3x times in sterile water prior to bonding with PDMS device. After bonding PDMS and MEA substrate, dissociated cells were plated at a concentration of ~ 2 x 10^5 cells / cm^2 in both the compartments. The plating occurred by injecting the cell suspension from one reservoir of each compartment.

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Figure 5.1C shows the neurite arborization within cortical and thalamic compartment and crossing-over of neurites to the adjacent compartment: red stain highlights the neurites in cortical compartment, and green highlights those in compartment with thalamic cells. The devices were then incubated in a humidified incubator at 37º C supplied with 5% CO₂. Neurite growth in the co-culture was checked at equal intervals and the neurobasal medium was replaced by freshly prepared medium on DIV 4, 7, 9, 11, etc., The presence of serum in the culture medium for thalamic cells (3% FBS + 1% HS) was maintained for at least (DIV) 5 days in culture. The serum dosage was reduced during the following days to 2% FBS + 0.5% HS and finally completely eliminated after DIV 9 to avoid the glia overgrowth during long term culture (more than DIV 15).

Cultures during the second week of development (DIV 14) were histologically stained with a single pair of monoclonal NeuN (Sigma-Aldrich, USA) and polyclonal Neurofilament 200 (NF200) Kda (Sigma-Aldrich, USA) antibodies. Immunofluorescence staining was carried-out with the PDMS mask attached to the substrates and by passing various solutions through the reservoirs connected to the compartments. Secondary antibody with red fluorochrome and with green fluorochrome was added in cortical and thalamic compartment respectively, to visualize neurite specificity from both cell types simultaneously.

Dataset

Spontaneous electrophysiological activity was recorded in a development period starting from DIV 21 up to DIV 35. We choose such period since dissociated cortical and thalamic neurons reach a stable state of maturation after the 3rd week in vitro. The results presented in this work come from a dataset made up of n = 15 cortical-thalamic co-cultures. In addition, we also used as controls n = 12 cortical and, n = 15 thalamic cultures in isolation, as well as n = 8 cortical-cortical and n = 6 thalamic-thalamic co-cultures.

To perform the cross-correlation analysis and the estimation of the functional connectivity maps, we considered n = 5 Cx-Th, and n = 8 Cx-Cx co-cultures. This reduction is mainly due to the level of activity of the considered networks. With low firing activity, the cross-correlation algorithm may include false functional connections and hence, it may not represent the connectivity accurately (Garofalo et al., 2009).
Figure 5.2: Raw data of spontaneous activity of cortical and thalamic cells in isolation and in co-cultures. (A) Activity recorded from a cortical culture in isolation - 10s of spontaneous activity recorded from a sample electrode showing burst events in the cortical cells cultured in isolation. Bottom: A closer look at burst shape; (B) Activity recorded from a thalamic culture in isolation - 10s of spontaneous activity recorded from a sample electrode showing tonic firing in the thalamic cells cultured in isolation. Bottom: A closer look at the tonic firing; (C) Cx-Th co-culture - 10s of spontaneous activity recorded from a sample electrode placed in the compartment of cortical neurons in a co-culture system; (D) 10s of spontaneous activity recorded from a sample electrode placed in the compartment of thalamic neurons in a Cx-Th co-culture system.
5.2 Materials and Methods

SPIKE AND BURST DETECTION

Spontaneous spiking within the culture is detected using threshold based ‘Precise Timing Spike Detection’ (PTSD) algorithm (Maccione et al., 2009) using an independent threshold for each channel computed according to the standard deviation (i.e. 7 x SD) of the biological and thermal noise of the signal (Chiappalone et al., 2003).

Bursts and network bursts are detected by using the algorithm devised by Pasquale et al. (Pasquale et al., 2010). The algorithm is based on the computation of the logarithmic inter-spike interval histogram in order to detect automatically the best threshold between inter-burst (i.e., between bursts and/or outside bursts) and intra-burst (i.e., within burst) activity for each recording channel of the array. Once the burst detection is performed, we used the extracted burst event trains (i.e. trains containing only the first spike of each burst) to detect the network bursts, following a procedure similar to that used for the detection of single-channel bursts (Pasquale et al., 2010).

INSTANTANEOUS FIRING RATE (IFR)

The level of activity of a cell or a network of cells is characterized by its firing rate (FR). The firing rate is defined as the number of spikes in a rather large time window and it can be measured from the neural activity as follows (Adrian, 1928):

\[
FR = \frac{1}{T} \left( \sum_{n=1}^{N} \delta(t - t_n) \right) dt = \frac{N}{T}
\]  

With T representing the duration of the recording and N the number of spikes occurring at time ts. The instantaneous firing rate (IFR) is computed by dividing the spikes in a small window of size Δt by the bin width (Rieke et al., 1997). Such small window is realized by means of a Gaussian kernel of width equals to Δt. In the results presented in this paper, we set Δt = 100 ms. The IFR can be evaluated for each single channel and/or averaged among all the active electrodes of the MEA, obtaining the IFR of the whole network.
Cross-Correlation and Functional Connectivity Maps

Cross-Correlation (CC) function was built by considering the spike trains of two recording site (Garofalo et al., 2009). The frequency at which a spike firing was recorded in one recording site (‘target site’) relative to the spike firing in another recording site (‘reference site’) as a function of time was measured and a CC function was evaluated considering all the pairs of spike trains. Mathematically, CC reduces to a simple probability $C_{xy}(\tau)$ of observing a spike in a train Y at time $(t + \tau)$, because of a spike in another train X at time $t$; $\tau$ is called time shift or time lag, and in the analysis presented in this work was set to 0.1 ms. Connection strength between the recording sites was evaluated on the basis of the peak value of the CC function, named $C_{\text{peak}}$. Among them, the highest value corresponds to the strongest connections while the directionality was accounted from the sign of the corresponding peak latency. From the statistically relevant $C_{\text{peak}}$, functional connectivity maps were estimated.

Functional connectivity captures patterns of deviations from statistical independence between distributed and often spatially remote neuronal units (Friston, 1994), measuring their correlation/covariance, spectral coherence or phase-locking. Functional connectivity is time-dependent and “model-free”, that is, it measures statistical interdependence without explicit reference to causal effects. Different methodologies for measuring brain activity could generally result in different statistical estimates of functional connectivity (Horwitz, 2003).

A crucial step for estimating reliable connectivity map is the thresholding procedure. Once that a general connectivity map is obtained by the application of the CC algorithm, high and low values in the connectivity map are expected to correspond to strong and weak connections. A procedure to select the strongest functional links is necessary because a CC value (i.e., a $C_{\text{peak}}$) is computed for each electrode pair independently of the existence of a direct or indirect (causal) link, a simply random co-activation or a noisy link. Since the connection strength is proportional to the peak amplitudes (Garofalo et al., 2009), a threshold value for creating statistically significant and reliable connectivity maps preserving relevant links and discarding noisy and weakest ones is necessary. In this analysis, we estimate the functional connectivity maps by considering only the strongest links to avoid any possible false positive connection and to focus on the most reliable connections (i.e., by considering a small number of links with respect to the total connections identified by the statistical method). In particular, we considered the 20 strongest connections intra-cluster (i.e., cortico-cortical, or thalamo-thalamic connections), and the 10 inter-cluster (i.e., cortico-thalamic, or thalamo-cortical connections).
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Neuronal Network Models

A neuron model based on the Izhikevich equations was used (Izhikevich, 2003). The Izhikevich model depends on four parameters, which allow reproducing the spiking and bursting behavior of specific types of neurons. From a mathematical point of view, the model is described by a two-dimensional system of ordinary differential equations (Izhikevich, 2003):

\[
\frac{dv}{dt} = 0.04v^2 + 5v + 140 - u + I_{\text{tot}}
\]

(2)

\[
\frac{du}{dt} = a(bv - u)
\]

(3)

With the after-spike resetting conditions:

\[
\text{if } v \geq 40mV \Rightarrow \begin{cases} 
  v &\leftarrow c \\
  u &\leftarrow u + d
\end{cases}
\]

(4)

In equations (2-4), \(v\) is the membrane potential of the neuron, \(u\) is a membrane recovery variable which takes into account the activation of \(K^+\) and inactivation of \(Na^+\) channels. \(I_{\text{tot}}\) takes into account all the inputs which each neuron receives, and can be split in two components according to Equation (5).

\[
I_{\text{tot}} = I_{\text{syn}} + I_{\text{noise}}
\]

(5)

\(I_{\text{syn}}\) describes the synaptic input from other neurons; \(I_{\text{noise}}\) is a current source generator introduced to model the spontaneous sub-threshold electrophysiological activity of the neurons. It is a stochastic source of noise, which was modeled according to an Ornstein-Uhlenbeck process (Cox and Miller, 1965):

\[
dI_{\text{noise}} = \frac{I_{\text{noise}}}{\tau_I} dt + \frac{m_I}{\tau_I} dt + s_I \sqrt{\frac{2dt}{\tau_I}} \xi_I
\]

(6)

In Equation (6) the quantity \(\Delta t\) is a white noise with zero mean and unitary variance. In this way, \(I_{\text{noise}}\) is Gauss-distributed at any time \(t\) and, after a transient of magnitude \(\xi_I\) (correlation length), converges to a process with a mean equal to \(m_I\) and standard deviation \(s_I\). For the simulation, we set \(\tau_I = 1\) ms, \(m_I = 25\) pA, and \(s_I = 9\) pA.
Figure 5.3: Instantaneous Firing Rate (IFR) in isolated cultures. (A) IFR profiles evaluated over 300 s of spontaneous activity of a cortical culture (Binsize = 100 ms); (B) IFR distribution of the activity shows a linear relationship with a slope of -1.06; (C) IFR profiles evaluated over 300 s of spontaneous activity of a thalamic culture (Binsize = 100 ms); (D) IFR distribution of the thalamic firings with tri-exponential fit shows an exponential relationship.
Figure 5.4: Instantaneous Firing Rate (IFR) in Cx-Th co-cultures. (A) IFR profiles evaluated over 300 s of spontaneous activity of a cortical-thalamic co-culture (Binsize = 100 ms, red and green lines represent cortical and thalamic firings respectively); (B) IFR distribution of the activity depicted in (A); (C) Average values of the slope of the linear fitting of cortical neurons when are cultured alone (1.03 ± 0.04) and with thalamic neurons (1.66 ± 0.25) (p < 0.01, Kruskal-Wallis nonparametric test); (D) Time of initiation of network bursts in Cx-Th co-culture system showing higher probability of cortical origin of synchronized bursts.
The possibility to obtain several different patterns of activity depends on the choice of the $a$, $b$, $c$, $d$ parameters (Izhikevich, 2004). Cortical cells have been modeled with two different families of neurons: the family of regular spiking neurons (RS), and the family of fast spiking neurons (FS), respectively. Such dynamics were achieved by defining the following arrays (Equation 7), where the first row relates to the excitatory, while the second row relates to the inhibitory neurons.

\[
\begin{align*}
a_{\text{CX}} &= \begin{bmatrix} 0.02 \\ 0.02 + 0.08 r_i \end{bmatrix} \\
b_{\text{CX}} &= \begin{bmatrix} 0.2 \\ 0.25 - 0.05 r_i \end{bmatrix} \\
c_{\text{CX}} &= \begin{bmatrix} -65 + 15 r_i^2 \\ -65 \end{bmatrix} \\
d_{\text{CX}} &= \begin{bmatrix} 8 - 6 r_i^2 \\ 2 \end{bmatrix}
\end{align*}
\]

In Equations (7), $r_i$ is a uniform random variable which spans from 0 to 1, and $i$ the neuron index. This variable was added in order to introduce more variability in the neuron dynamics: a RS neuron is obtained if $r_i = 0$, whereas if $r_i = 1$, a bursting neuron is obtained. Such distribution is biased towards RS neurons. Similarly, to model the dynamics of thalamic cells, we set the aforementioned parameters as follows:

\[
\begin{align*}
a_{\text{MT}} &= \begin{bmatrix} 0.02 \\ 0.03 + 0.02 r_i \end{bmatrix} \\
b_{\text{MT}} &= \begin{bmatrix} 0.2 \\ 0.25 - 0.03 r_i \end{bmatrix} \\
c_{\text{MT}} &= \begin{bmatrix} -65 + 15 r_i^2 \\ -52 \end{bmatrix} \\
d_{\text{MT}} &= \begin{bmatrix} 6 r_i^2 \\ 0 \end{bmatrix}
\end{align*}
\]

Thalamic neurons present two firing regimes: when they are depolarized, starting from a resting condition, they exhibit a tonic firing; on the other hand, if they receive an inhibitory input, the neurons fire rebound bursts of action potentials. To preserve some characteristics of the composition of in vitro networks, we introduced 70% of excitatory neurons, and 30% of inhibitory ones (Marom and Shahaf, 2002). From a morphological point of view, neurons are modeled as punctual processes and no axonal conduction delay was taken into account.

To model the co-culture connectivity, two independent networks made up of 512 nodes each, one for the cortical ($\text{CX}_{\text{net}}$), and one for the thalamic network ($\text{TH}_{\text{net}}$) were designed. Both $\text{CX}_{\text{net}}$ and $\text{TH}_{\text{net}}$ are fully connected, as found experimentally, and the average degree was set at $1500 \pm 97$. Autapses are avoided. The interconnections between the two sub-populations were modeled as follows: the presynaptic neurons in a compartment (e.g., $\text{CX}_{\text{net}}$) were chosen among the ones that establish the strongest connections within the same cluster; the targets were randomly chosen in the other compartments (e.g., $\text{TH}_{\text{net}}$). In the simulated model, 10% strongest connections was set from cortical to thalamic compartment, while 5% of strongest connection was set from thalamic to cortical compartment.
5.3 Results

Viable Co-culture and Spontaneous Activity Recordings

Viable cortical-thalamic co-culture was maintained for up to DIV 35 and the spontaneous activities were recorded twice per week between DIV 21 and DIV 35. Figure 5.2 shows a raw data sample from 1 of the 60 recording sites in a MEA. Electrophysiological activities recorded by a single electrode of a cortical and thalamic culture in isolation are shown in figure 5.2A and 5.2B respectively. Figure 5.2C and 2D shows an example of electrophysiological recordings of a cortical-thalamic co-culture in the dual compartment device. It should be noted that the spontaneous activity of an isolated cortical culture (figure 5.2A) is markedly different than those observed in a co-culture model (figure 5.2C). Thalamic cells are mainly characterized by a tonic firing both in isolation (figure 5.2B) and when co-cultured with cortex (figure 5.2D). However, the presence of cortical neurons forces the genesis of burst events also in the thalamic population with features (e.g., duration, frequency intra burst) that resemble the cortical ones.

Network Dynamics in Thalamic and Cortical Cultures

To quantify the dynamics in such a co-culture system and to better understand the electrophysiological signal interaction between the two cell types, firstly an Instantaneous Firing Rate (IFR) analysis of the spontaneous activities was performed. We evaluated the IFR of 12 cortical and 15 thalamic cultures in isolation and figures 3A-3D show a representative example. In the case of cortical cultures, IFR computed over a time window of 300 seconds with a bin width of 100 ms shows dynamics characterized by bumps of activity corresponding to the well-known network bursts appearing during the development and maintained in the mature phase of cortical cultures (figure 5.3A). Distribution of the IFR, plotted in a bi-logarithmic plot shows a linear trend (figure 5.3B). By interpolating the experimental data with a linear fit, we found a linear relationship for more than two decades with a slope equals to -1.06. On the average (figure 5.4C), for cortical cultures, we found a mean slope equals to 1.03 ± 0.04 (mean ± standard deviation, absolute value). This behavior can be explained by the presence of a rich and well-structured spiking activity that originates the bursting behavior in a cortical culture in isolation (Wagenaar et al.,2006).

IFR analysis performed over 15 devices with thalamic cell cultures (figure 5.3C) shows that the IFR is characterized by a sustained activity as a result of the high percentage of cells characterized by a tonic spiking. Such activity generates an IFR distribution with an exponential shape, as depicted in figure 5.3D, where a long linear segment
Figure 5.5: Burst analysis. Raster plots of 1-second of spontaneous activity of (A) cortical culture in isolation; (B) thalamic culture in isolation; (C) cortical-thalamic co-culture (red is cortical, green is thalamic); (D) Shape of the cortical bursts, as demonstrated by the burst duration bars, is modulated by the presence of thalamic cells (p < 0.01, Kruskal-Wallis nonparametric test).
5.3 Results

Figure 5.6: Functional connectivity maps. (A-B) Two examples of functional connectivity map evaluated by considering the strongest 20 connections intra-cluster and the 10 strongest inter-cluster; (C) Distribution of the inter-cluster connections evaluated over 5 cortical-thalamic co-cultures. By considering the 10 strongest connections, we found ~77% of cortico-thalamic connections, and 23% of thalamo-cortical. By relaxing the number of the strongest inter-cluster connections, we can observe an increasing of the thalamo-cortical links. For example, taking into account the 30 strongest inter-cluster links, we counted ~60% of cortico-thalamic, and 40% of thalamo-cortical connections (+17%); (D) Distribution of the inter-cluster connections evaluated over 8 cortical-cortical co-cultures. By considering the 10 strongest connections, we found ~53% of comp A to comp B connections, and 47% of comp B to comp A. The percentage of connections originating from compartment A to compartment B, and vice versa, was observed to be completely random also by varying the number of links from 10 to 30.
cannot be detected. We fit the distribution with three exponential functions \( f(x) = a \cdot e^{bx} \) as shown by the three black and gray segments of figure 5.3D. The choice to divide the distribution in three tracts was made in order to generate a correspondent number of fitting functions that exhibit a lower possible root mean square error (RMSE) value. For the example of figure 5.3D we found: \( a = [0.07, 0.15, 13.2]; b = [-0.03, -0.05, -0.11]; \) \( \text{RMSE} = [2.0 \times 10^{-3}, 2.1 \times 10^{-4}, 1.9 \times 10^{-5}] \). This tonic spiking cells are practically absent in cortical cultures (figure 5.3A) while network bursting behavior, typical of cortical cells in isolation (figure 5.3A), is less frequent in the thalamic cultures (figure 5.3C) in isolation and very much different to the cortical ones in terms of duration (Cf. figure 5.5D).

To verify whether these particular IFR distributions are influenced by the presence of the same culture type (either cortex or thalamus) in a dual compartment device, we also analyzed the activity of \( n = 8 \) cortical-cortical (Cx-Cx) co-cultures and \( n = 6 \) thalamic-thalamic (Th-Th) co-cultures. No significant change in IFR distribution was observed both in Cx-Cx and in Th-Th co-cultures with respect to the control cultures on standard single-compartment devices (see supplementary figures S5.2 and S5.3 - where IFR profiles and distributions are shown). By computing the IFR distributions of cortical cultures both in two compartments as well as in single compartment devices, we obtained mean slope values equals to, 1.03 ± 0.08 (compartment A), 1.05 ± 0.09 (compartment B), and 1.03 ± 0.04 (single compartment device) (supplementary figure S5.1), which are indeed not statistically different (\( p < 0.01^1 \)). Regarding the thalamic IFR distributions, in both experimental conditions a clear exponential trend was observed.

**Network Dynamics in Co-culture**

Under similar experimental conditions, 15 dual compartment devices with cortical-thalamic cells co-cultured in adjacent compartments were used for the IFR analysis to study the network dynamics in such systems. IFR analysis of these dual compartment devices are shown in figure 5.4A. The IFR pattern follows the dynamics typically expressed by isolated cortical cultures (figure 5.3A). It should be noted that the IFR of thalamic neurons loses part of its random spiking, and become time-locked to the bumps of the activities generated by the cortical cells. The distribution of the IFR changes dramatically for both thalamic (green squares, figure 5.4B) and cortical neurons (red squares, figure 5.4B). Network burst patterns were observed

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1 Kruskal-Wallis nonparametric test was applied, since the normality assumption was not verified by our datasets (Kolmogorov- Smirnov normality test).

in the compartment with thalamic cells and they appeared to be time-locked to the cortical bursting phenomenon. However, the presence of the thalamic neurons can be recognized by the tail of the distribution which drops at a frequency of about 100 sp/s and by the increase in the slope of the distribution. The linearity of the cortical (red line) and thalamic (green line) IFR lasts less than two decades, while we found a linear behavior lasting more than two decades in isolated cortical cultures. By averaging the IFR distribution slope values, when cortex is co-cultured with thalamus (n=15), we found a value of 1.66 ± 0.25, statistically different (p < 0.01) from the one found when cortex is cultured alone (n=12) (figure 5.4C).

To further analyze the influence between the two cultures we investigate the time of initiation of network bursts in both compartments of Cx-Th co-cultures. Timestamp of the beginning of network burst and the distribution of bursts in both compartments were computed. For each network burst in compartment A, the burst in compartment B immediately before and after is located. Considering the time of initiation, network bursts that originate within 20 ms of each other were considered to be synchronous and those network bursts that originate at least 20 ms before or after the network bursts in the other compartment were considered to be leading and trailing bursts, respectively. Distribution of network burst initiation in Cx-Th co-culture devices is shown in figure 5.4D. The obtained results shown that in the Cx-Th co-cultures (n=15 devices) network bursts were observed to originate in the Cx compartment in majority of the cases closely followed by thalamic bursts. Finally we look at the burst duration by hypothesizing a possible feed-back influence of the thalamic population on the behavior of cortical cells. The occurrence of burst events in a sample cortical culture in isolation is shown in the raster plot of figure 5.5A while figure 5.5B shows the spiking pattern of a sample thalamic culture in isolation. In a co-culture system with cortical and thalamic cells (figure 5.5C), burst patterns in the cortical compartment appear to be highly influenced by the presence of the thalamic population. The duration of cortical bursts in the co-culture is elongated (figure 5.5D) with an average burst duration of 389 ± 24 ms (mean ± standard error), when compared to the cortical bursts in isolation - with an average duration of 246 ± 4 ms, (p < 0.01, Kruskal-Wallis nonparametric test). In addition, also the burst duration of the thalamic subpopulation is modulated by the presence of cortical neurons: when thalamic neurons are cultured alone, they display a burst duration equal to 397 ± 2 ms, which is reduced to 281 ± 4 when cultured with cortical neurons (figure 5.5D).
Figure 5.7: Simulation Results. (A) Profile and (B) distribution of the IFR evaluated over a simulated co-culture with unidirectional cortico-thalamic connections. Neither the IFR profile nor the distribution matched the experimental data depicted in figure 5.4; (C) Profile and (D) distribution evaluated over a simulated co-culture with bi-directional cortico-thalamic and thalamo-cortical connections resembles the experimental condition.
5.3 Results

The interplay between cortico-thalamic and thalamo-cortical populations can be further investigated, in this specific experimental model system, by estimating the functional connectivity between the two regions. Two examples of functional connectivity maps evaluated by considering the strongest 20 intra-cluster and 10 inter-cluster connections in two sample co-culture experiments are shown in figure 5.6A and 6B. Direction of the links is derived by the peak latency of the cross-correlogram and allows to estimate the weight of the reciprocal influence of the two co-cultured populations. By considering 5 co-cultures used in this analysis, it was observed that ~77% of the connections are cortico-thalamic, while ~23% was thalamo-cortical.

By varying the number of connections (i.e., from 10 to 30), an increase in the fraction of thalamo-cortical links was observed indicating that the strongest connections are from cortical to thalamic population (figure 5.6C). This finding further supports the experimental observation of burst initiation in co-cultures. The results confirm that the majority of burst events originate in the cortical region and spreads to the thalamic region.

Figure 5.8: Simulated Burst durations of cortical neurons, as a function of the percentage of the inter-compartment connections. With no thalamic interconnections, the burst duration of Cx resembles the burst duration of a Cx alone culture (bar 1 and 2). Increasing the percentage of thalamic-cortical interconnections, the mean burst duration of Cx also increases and resembles the experimental value when thalamo-cortical links are 5% of the intra-compartment connections.
counterpart driving the thalamic network to burst, while reciprocal thalamo-cortical connectivity plays a significant role in modulating the shape of the bursts in the cortical region. Finally, as a control, we estimated the functional connectivity maps in n = 8 Cx-Cx co-cultures (figure 5.6D). In this case, percentage of connections originating from compartment A to compartment B, and vice versa, was observed to be completely random also by varying the number of links from 10 to 30.

Simulated Models

To support the experimentally found interplay between the cortical and thalamic populations, an Izhikevich based neuronal network model was developed to specifically study network dynamics in the two sub-populations (cortical and thalamus), influenced by the presence or absence of reciprocal connections. In this model, three different configurations were simulated: (i) populations of cortical and thalamic cells in isolated conditions; (ii) interconnected cortical-thalamic populations with unidirectional cortical to thalamic inter-connections (10% of the strongest connections of the cortical population), and (iii) interconnected populations with bidirectional strong cortico-thalamic (10% of the strongest connections of the cortical population) and weak thalamo-cortical inter-connections (5% of the strongest connections of the thalamic population), resembling the actual experimental situation. We implemented a cortical-thalamic network made up of 512 cortical and 512 thalamic neurons. Each neuronal population is fully connected (average degree 1500 ± 97). The dynamic of each cortical and thalamic neuron was modeled according to the parameters presented in equations 7 and 8 (cf., Sec. 5.2 - p. 96). For each configuration, 300 seconds of spontaneous activity were simulated, and analyzed by using the same metrics adopted from the experimental data, namely the IFR profile and its distribution. The IFR of the simulated spontaneous activity of the model for isolated conditions resembles that of the experimental results (data not shown).

Figure 5.7 shows the simulated cortical-thalamic co-culture model with unidirectional cortico-thalamic (figure 5.7A and B) and bi-directional inter-connections (figure 5.7C and D). When strong cortico-thalamic and weak thalamo-cortical connections are considered, the network dynamics well reproduce the experimentally observed phenomenon. The distribution of the IFR of thalamic neurons (figure 5.7D, green curve) is shifted towards the lower IFR value resembling the experimentally observed shape. Tonic spiking behavior of thalamic neurons was partly disrupted showing burst patterns time-locked to the cortical population. Thus confirming that cortical neurons drive the entire network in a more bursting regime while the presence of thalamo-cortical inter-connections play a fundamental role in the modulation
of cortical burst duration and shape. These results can be further supported by considering the simulated configuration with only unidirectional cortico-thalamic inter-connections. In this case, the global behavior is far from the experimental results supporting the evidence that thalamo-cortical connection play a major role in shaping the overall dynamics. The peaks of the thalamic neurons (figure 5.7B, green curves) are higher than the cortical counterpart (figure 5.7B, red curves) in contrast to the experimental observation.

To quantify these considerations, we computed the burst duration of cortical neurons by sweeping the percentage of the inter-connections between the two populations (figure 5.8). It can be noticed that if we consider only a pool of cortico-thalamic connections (unidirectional inter-compartment connectivity) equal to 10% of the total connections within the cortical compartment (second bar of figure 5.8), burst durations are close to those obtained in an isolated cortical population (first bar). Increasing the percentage of thalamo-cortical projections, the mean burst duration of cortical population also increases (third bar of figure 5.8 with 10% cortico-thalamic and 2% thalamo-cortical connections), and resembles the experimental values when thalamo-cortical links are 5% of the intra-compartment connections (fourth bar). Finally, if the percentage of the thalamo-cortical inter-connections is greater than cortico-thalamic connections (last bar), the mean burst duration assumes implausible large values (more than 600 ms). These results are qualitatively in agreement with functional connectivity analysis (Cf., Sec. 5.2 - p. 91) in Cx-Th devices, in which the ratio of cortico-thalamic functional connections was experimentally observed to be much higher in comparison to the thalamo-cortical connections (figure 5.6C).

5.4 Discussion and Conclusions

The interaction between thalamus and cortex has attracted much attention in the recent past. Understanding the mechanism behind many pathological conditions such as Parkinson’s, epilepsy and schizophrenia involves a deeper understanding of the communication pathways involved in the cortical-thalamic brain circuitry. Although in vivo electrophysiological recordings provides capabilities to study such pathways, low spatial resolution of the recording electrodes and the influence of other regions of the brain in the cortical-thalamic communication pathways are often an hindrance in understanding the sole interaction between the cortex and thalamus. In vitro organotypic slice studies, on the other hand, offers possibilities to selectively culture individual regions on a MEA setup (Adams et al., 2011), however selective manipulation of network wide response of a particular region involved in the co-culture cannot be easily obtained. To circumvent these issues, a novel dual
compartment microfluidic system for co-culturing dissociated cortical-thalamic cells was demonstrated in our current study allowing investigation of specific interactions between the two populations. Protocols for successful long-term viability (for up to DIV 35) of dissociated thalamic cell types were developed. The long-term viability of thalamic neurons may be attributed to the neurotrophic support and signaling cues from the cortical cell types (Coronas et al., 2000; Asavaritikrai et al., 2003). We demonstrate that functional connectivity is re-established in dissociated cortical and thalamic cells indicating a natural inclination of the system to form reciprocal interconnections.

In a dual compartment device with cortical cells in both compartments (Kanagasabapathi et al., 2011; Pan et al., 2011), although functionally connected, the cells in individual compartments appear to exhibit network bursting behavior independent of the other compartment. With sequential plating of cortical cells in two compartments with a time delay of 10 days between plating, unidirectional propagation of cortical bursts and locally synchronized bursts in the time-delayed compartment has been reported (Pan et al., 2011). In our current work with cortical-thalamic co-cultures, reciprocal connections between the cortical and thalamic region were observed. Burst events for the vast majority originate in the cortical region and the presence of strong cortico-thalamic connections drives the network and in-turn, the thalamic cells discharge bursts. On the other hand, reciprocal weak thalamo-cortical connections were observed to play a relevant role in cortical behavior by modulating the duration of burst events. Thalamic cells are mainly characterized by a tonic firing both in isolation and when co-cultured with cortical cells. However, the presence of cortical projections produces burst events in the thalamic culture with features that resemble the cortical ones. This influence is reciprocal and, in the average found behavior in cortical-thalamic co-cultures, the burst duration in the cortical region is elongated by about 57%, while the burst duration in the thalamic region shortened by about 29%. Simulated neuronal network models, based on Izhikevich equations, further confirms the necessity of bi-directional cortico-thalamic connections to drive the network dynamics as observed experimentally.

The analysis presented in this work confirms the recent findings that cortical region is the site of initiation of burst firing events while reciprocal thalamo-cortical connections are required to maintain a prolonged synchronized bursting pattern in the cortical culture (Adams et al., 2011). In vivo, the results can be compared to multi-site recordings that support a cortical initiation of burst event discharge and subsequent recruitment of thalamic cells in the bursting behavior (Steriade and Amzica, 1994; Meeren et al., 2002). Further, the dual compartment system presented
in this work offers the unique possibility of probing specific neuronal circuitries independently from external influence of other neuronal populations. The system, in addition, offers capabilities to selectively manipulate the microenvironment of the individual cell types (i.e. influence of growth factors, chemical stimulant, etc.,) and this may prove instrumental in identifying the role played by individual cell types in a co-culture system.

**Acknowledgements**

This work is supported by Point-One grant (BrainMimic project) from the Dutch government, and by Philips Research Europe. The authors thank Frenk van Kan and Carlijn van Helvert (Life sciences facilities, MiPlaza Technologies) for their valuable assistance in cell culture work, and Matteo Garofalo for the cross-correlation algorithm.
CHAPTER 5

FUNCTIONAL CONNECTIVITY AND DYNAMICS OF CX-TH NETWORKS

REFERENCES


FUNCTIONAL CONNECTIVITY AND DYNAMICS OF CX-TH NETWORKS


5.5 **SUPPLEMENTARY INFORMATION**
Supplementary Figure S5.1: Slope of IFR distribution of Cx culture in isolation and in Cx-Cx co-culture. IFR distributions of cortical cultures in dual compartment device with mean slope values equals to 1.03 ± 0.08 in compartment A, 1.05 ± 0.09 in compartment B and in single compartment device with mean slope equal to 1.03 ± 0.04 has a linear relationship.
Supplementary Figure S5.2: Instantaneous Firing Rate (IFR) of Cx cultured in isolation and in Cx-Cx co-cultures. (A) IFR profiles evaluated over 300 s of spontaneous activity of a cortical culture in isolation (Binsize = 100 ms); (B) IFR distribution of the activity depicted in (A); (C) IFR profiles evaluated over 300 s of spontaneous activity of a cortical-cortical co-culture (Binsize = 100 ms); (D) IFR distribution of the activity depicted in (C).
Supplementary Figure S5.3: Instantaneous Firing Rate (IFR) of Th cultured in isolation and in Th-Th co-cultures. (A) IFR profiles evaluated over 300 s of spontaneous activity of a thalamic culture in isolation (Binsize = 100 ms); (B) IFR distribution of the activity depicted in (A); (C) IFR profiles evaluated over 300 s of spontaneous activity of a thalamic-thalamic co-culture (Binsize = 100 ms); (D) IFR distribution of the activity depicted in (C).