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Understanding Spatial and Temporal Patterning of Astrocyte Calcium Transients via Interactions Between Network Transport and Extracellular Diffusion

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Abstract

Astrocytes form interconnected networks in the brain and communicate via calcium signaling. We investigate how modes of coupling between astrocytes influence the spatio-temporal patterns of calcium signaling within astrocyte networks and specifically how these network interactions promote coordination within this group of cells. To investigate these complex phenomena, we study reduced cultured networks of astrocytes and neurons. We image the spatial temporal patterns of astrocyte calcium activity and quantify how perturbing the coupling between astrocytes influences astrocyte activity patterns. To gain insight into the pattern formation observed in these cultured networks, we compare the experimentally observed calcium activity patterns to the patterns produced by a reduced computational model, where we represent astrocytes as simple units that integrate input through two mechanisms: gap junction coupling (network transport) and chemical release (extracellular diffusion). We examine the activity patterns in the simulated astrocyte network and their dependence upon these two coupling mechanisms. We find that gap junctions and extracellular chemical release interact in astrocyte networks to modulate the spatiotemporal patterns of their calcium dynamics. We show agreement between the computational and experimental findings, which suggests that the complex global patterns can be understood as a result of simple local coupling mechanisms.

Introduction

Networks of interacting components are known to self-organize and form complex macroscopic processes, which emerge from local coupling between individual network components[1,2]. The brain is an example of a system with self-organizing dynamics; it contains an astronomical number of neurons and glia that interact over multiple spatial and temporal scales. Patterns of brain activity within these interacting networks underlie our

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thoughts and memories, and drive behavior. These interactions are mediated through different cell populations and through various mechanisms, such as synaptic coupling, gap junctions, and neuromodulation. The most researched types of interactions in the brain are those mediated by neurons. Traditionally, glial cells were thought to provide primarily structural and metabolic support for neurons[3]. However, recent work suggests that glia may be active participants in brain information processing[4]. Within the last decade it has become clear that astrocytes, a sub-population of glial cells, play an important role as modulators of neural activity and brain function.

Astrocytes do not signal directly through electric depolarization of their membranes as neurons do, however they have been shown to display well-defined dynamic calcium transients in both *in vitro* and *in vivo* preparations[5–9]. This calcium-based excitability is generated through calcium release from endoplasmic reticulum stores that in turn is being mediated by activation of IP3 receptors by the IP3[10]. Furthermore Ip3 plays the role of the intracellular transport vehicle of the calcium excitation through the astrocytes, as well as intercellular signaling as it diffuses through cellular processes and gap junctional couplings connecting adjacent cells or their processes[10]. The IP3 subsequently activates the calcium release from the ER stores, which in turn activates additional IP3 production. The calcium itself is relatively rapidly buffered by the cytosol[11]. The astrocytes can be thus modeled in the first order approximation as excitable cells with well-defined propagation of calcium signaling.

Further downstream, astrocytic calcium signaling is involved in many processes including biochemical pathways that activate the extracellular release of a variety of transmitter molecules, which can interact with other astrocytes and also with neurons. For example, the release of ATP or glutamate from a single astrocyte has been shown to activate calcium transients in other astrocytes[12–14]. Moreover, it has been shown that a single astrocyte can envelope up to 140,000 neuronal synapses, putting them in a unique position to control neuronal domains[10]. These astrocytic processes are endowed with various G-protein coupled receptors that, among others, evoke the cells' calcium responses[15]. Thus it became clear that there is a bidirectional communication between the neurons and astrocytes, and this notion led to the formulation of a “tripartite” synapse concept, in which astrocytes play a prominent role in regulating neuronal signaling at a synaptic level[16]. Therefore, patterns of calcium signaling in astrocytes can be important for shaping neuronal network formation and development. Even though the functional effect of astrocytes on neurons is complex and poorly understood, it is clear that astrocytes have a role in brain function that needs further study.

The GJ coupling between astrocytes and Ip3 diffusion provides for complex network interaction between the cells[17] [4]. At the same time astrocytes secrete and uptake signaling molecules from the extracellular space. Here, ATP is implicated as one of major signaling molecule allowing for extracellular communication between the astrocytes[15]. The interaction of these two coupling mechanisms, diffusion of extracellular chemical signaling and direct gap junction coupling, can lead to complex spatiotemporal patterning of the network activity, which has been demonstrated in recent work[18].

In this study, we construct a highly reduced model with aim to understand how these two modes of coupling within astrocyte networks influence the astrocyte network activity patterns. We take the approach of studying the pattern formation in reduced astrocyte networks in dissociated rat hippocampal cultures. To probe the network dynamics in this system, we perform calcium imaging to record the spontaneous calcium signaling in developing cultures. We grow cultured networks on microelectrode arrays in order to monitor neuronal activity, while simultaneously imaging astrocyte calcium signals. During the early development of cultures, astrocytes form networks and display spontaneous calcium signaling while there is no electrical neuronal activity. However, this is an active period for synapto-genesis and it is known that neurons release various signaling molecules at that time[19]. We observe robust patterns of calcium activity during the first week *in vitro* before spontaneous action potentials in neurons were observed on the microelectrode arrays, while the neurons subsequently became electrically active after 7 DIV (days *in vitro*). To investigate the interaction of extracellular diffusion and network transport mechanisms on spatio-temporal pattern formation within astrocyte networks, we study the network dynamics of calcium signaling during the first week of development in dissociated cultures,.

Methods

Dissociated cell cultures

We prepared hippocampal cell cultures of astrocytes and neurons from Wistar rats on postnatal day 1 using a protocol modified from[20]. Tissue was digested with %0.5 Trypsin-EDTA (Invitrogen) diluted in Hanks Balanced Salt Solution (Invitrogen) and then dissociated by titration with a pasteur pipette. Cells were centrifuged and re-suspended in Neurobasal-A medium (Gibco) supplemented with 2% B-27 (Gibco), 5% heat-inactivated horse serum (Gibco), 0.5 mM L-glutamine (Sigma-Aldrich), 0.5 mM pen-strep (Sigma-Aldrich) and 10 mM HEPES (Sigma-Aldrich). The cell density was adjusted to a final plating density of 1500 cells/mm². Bi-weekly half the culture medium was replaced with fresh medium.

Calcium imaging

Astrocytes were imaged at 5 days *in vitro* using calcium indicator Fluo 4-AM (Invitrogen) and astrocyte marker[21] sulforhodamine 101 (Invitrogen). Fluo 4-AM and sulforhodamine 101 were diluted to final concentrations in HEPES-buffered saline containing the following: 119 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂, 30 mM Glucose, 10 mM HEPES, and pH adjusted to 7.4. For imaging, the culture medium was replaced by 5µg/mL fluo4-am and incubated for 40 minutes at room temperature, following a second incubation with 5 µM sulforhodamine 101 for 20 minutes at room temperature. Cultures were gently washed with HEPES-buffered saline 3 times following incubations. Cultures were maintained at 35 degrees Celsius during imaging. Olympus microscope IX71 was used for wide-field fluorescence imaging, and recorded with Hamamatsu Digital CCD camera C10600 at (6.4 Hz frame rate). MetaMorph software was used for data collection; all further analysis was done with Matlab.

Micro-electrode Arrays

Dissociated cells were plated on 60-electrode glass microelectrode arrays (Multi Channel Systems, Reutlingen, Germany), with 30 μm diameter electrodes and 200 μm spacing between electrodes. Contact pads and tracks were transparent to allow for optical imaging of astrocyte calcium signaling while simultaneously monitoring neuronal electrical activity with the microelectrode array. Microelectrode arrays were cleaned and treated with 0.05% poly-ethylene-imine (Sigma-Aldrich) in borate buffer followed by the 20 $\mu\text{g mL}^{-1}$ laminin (Roche Applied Science) solution in media following the protocol[20]. Cultures were recorded at 25 kHz using multi-channel systems acquisition card and MC-Rack software. Cultures were maintained at 35 degrees Celsius during recording, and each micro-electrode array recording was two minutes long.

Pharmacological Treatment

18- β glycyrrhetic acid (BGA) (Sigma-Aldrich) was dissolved in dimethyl sulfoxide (DMSO) into 1000x stocks and stored at -20°C . BGA stock was diluted in HEPES buffered saline to a final concentration of 25 μM . To disrupt gap junctions, cultures medium was replaced by 25 μM BGA solution and incubated for 15 minutes at 35 degrees[22]. An equivalent volume of vehicle replaced the medium in control cultures. Following incubation, cultures were gently washed with HEPES-buffered saline three times and imaged five minutes later.

Cell detection and event detection

Fluorescent imaging was acquired with commercially available MetaMorph Microscopy Software for Olympus Basic and imported into MATLAB for semi-automatic cell contour detection using custom software[23] modified from the Rosa Cossart lab. All subsequent analysis was performed with custom programs in MATLAB. Astrocyte somas were detected in the sulforhodamine 101 image, and the fluo4-am fluorescence signals were processed by wavelet filtering of the raw signal and event detection was determined by thresholding the signal above baseline. Each fluorescence signal was examined and any false positives and negatives in the event detection were corrected.

Fractional Change Measures

Fractional change was calculated for various quantities as the value after condition (A) minus the value before condition (B), and normalized by their sum (A+B).

$$\Delta = \frac{A - B}{A + B} \quad (1)$$

Spatial clustering

We defined the spatial clustering distance, D_{exp} , as the average distance between all closest pairs of astrocytes with calcium events during an imaging experiment. To compare between experimental conditions with varying numbers of active astrocytes, we compared D_{exp} to the value of the spatial clustering distance for randomized networks (D_{rand}). Namely we

randomized locations of active astrocytes distributing them randomly among experimentally identified locations (as measured with sulforhodamine 101) of all astrocytes. The average randomized distance between active cells, D_{rand} , was computed for over 50 sets of randomizations and compared to that observed experimentally. To quantify the spatial clustering we define a spatial clustering measure given by,

$$C = \frac{D_{rand} - D_{exp}}{D_{rand} + D_{exp}} \quad (2)$$

where C is the relative change of the spatial clustering distance for experimental data (D_{exp}) with the spatial clustering distance for randomized networks (D_{rand}).

Autocorrelation Widths

The autocorrelation function was calculated of each astrocyte calcium fluorescence signal. To determine a measure of the autocorrelation signal width, we calculated the time lag at a half maximum of the autocorrelation, where the total height is determined by the peak of the autocorrelation and the first derivative of the autocorrelation crossing zero. Using the time lag at the autocorrelation half maximum as an indicator for the timescale of astrocyte calcium signal duration, we computed the average time lag for all signals in an imaging experiment and the ratio change for before and after pharmacological treatments.

Cross Correlations

The cross correlation function was calculated pairwise between all pairs of astrocytes in a network for time lag shifts between -90 seconds and 90 seconds. Pairs of astrocytes within a range of distances were binned and the average cross correlation functions was computed for pairs within distance ranges. The temporal ordering is not relevant for obtaining a measure of total average correlation at a distance. Therefore we utilized the following strategy to avoid averaging out correlations that were maximal for either positive or negative time lags. Any correlation functions whose global maximum occurred for a negative time lag value, we reflected the cross correlation function across the y-axis, so that only the magnitude of the time lag information was averaged over pairs.

Golomb Synchrony Measure

To quantify the level of synchrony within the neuronal network activity, the burst synchronization χ , a measure developed by Golomb et al. [24], was applied to the microelectrode array recordings. The Golomb synchrony measure is defined as:

$$\chi^2 = \frac{\sigma_v^2}{\frac{1}{N} \sum_{i=1}^N \sigma_{v_i}^2}, \quad (3)$$

where σ_v^2 is the time-averaged variance of the mean voltage signal from all N neurons, and $\sigma_{v_i}^2$ is the variance of the i th neuron's voltage signal. The synchrony measure χ depends upon

the increase in variance of the mean voltage signal as bursting synchronization increases. χ is bounded on the interval $[0,1]$, and reaches a value of 1 when all recorded neurons are perfectly synchronous and spike at exactly the same time. To apply this measure to microelectrode array recordings, the discrete spike time series data was convolved with a Gaussian signal to create a continuous voltage signal representative of the neural spiking. The Gaussian signal for a particular Gaussian standard deviation (σ_g), spanned from $-3\sigma_g$ to $3\sigma_g$, and had a maximal peak amplitude of 1. Taking advantage of this Gaussian convolution approach, we applied convolutions of Gaussians with varying standard deviations (σ_g) to the same spike time series data. Gaussians of larger width (standard deviation) will effectively smooth over the smaller timescale of individual spiking patterns, while smaller gaussian widths can preserve the individual or groups of spike timings, allowing us to investigate synchrony across multiple timescales.

Results

Imaging astrocytic network activity

We imaged the spontaneous astrocyte calcium transients of hundreds of astrocytes over several minutes during the first week *in vitro* (Fig. 1). To identify individual astrocyte somas, we used concurrent staining with sulforhodamine 101 which selectively labels astrocytes[21]. The astrocytes were identified as the cells that were co-labeled with both markers.

Figure 2a shows an example raster plot of the fluorescence intensity over time for an astrocyte network during four minutes of imaging. Each row displays the changes in calcium fluorescence over time for individual astrocytes rescaled between the fluorescence intensity signal's minimum (black) and maximum (white). To better understand the effects of calcium signaling on an astrocytic network, we perturbed these spontaneous Ca^{2+} patterns by pharmacological disruption of the gap junction coupling strength between astrocytes.

Simplified Network Model of Astrocytic Interactions—In parallel we have constructed a highly simplified computational model of astrocyte-astrocyte interactions. Based on the known experimental results[10] we simulated two major means of signal transduction: network transport through astrocytic gap junctions, and chemical transport through diffusion in extracellular space[18]. The reduced cellular activity is modeled through integrate-and-release dynamics with an appropriate time constant resembling calcium release. In short, incoming excitation can cause Ca^{2+} to be released from the internal stores to propagate throughout the cell, and then continue on to spread to the associated network. While it is clear that astrocyte signaling is multimodal and extremely complex, we implicitly assumed that the extracellular agents act directly on calcium concentration within a cell and we modeled it as one species. The properties of this model were extensively studied in recent work[18]. We have shown that depending on connectivity properties of gap junctions the two processes (network transport and extracellular diffusion) can cooperate, strengthening the network activity, or they can compete, inhibiting each other and subsequently lowering the network response[18]. Based on existing experimental

literature here we assumed that astrocytic connectivity is primarily local as there are no long-distance connections between astrocytic processes[25].

Astrocytes are modeled as the nodes of the network and nodal activity, is driven by random noise, internal release, and extra-cellular excitation from other active astrocytes. The release of Ca^{2+} from the endoplasmic reticulum (ER) is represented by an internal flush of nodal agent upon exceeding a charging threshold. At all times the internal concentration is allowed to interact with other connected astrocytes through gap junctions via an equilibrating term (see equation below). In addition to the internal release, breaching the charging threshold is accompanied by a diffusive flush of signaling into the extracellular space, which allows astrocytes to passively influence neighboring astrocytes. While it is clear that these two pathways are mediated by (many) other signaling molecules, here we assumed for simplicity that the extracellular signaling will inadvertently affect the calcium levels in an astrocyte by activating G protein coupled receptors.

The full equation describing the dynamics is as follows:

$$\tau_E \frac{dX_i}{dt} = -\alpha X_i + I_{noise,i}(t) + I_{flush,i}(t) + \beta \sum_j A_{ij} (X_j - X_i) + \gamma \sum_j D_{ij} (r, t - \tau_j) \quad (4)$$

X_i represents the amount of active agent within the i th node and is related to the astrocytic calcium concentration. The element time constant is $\tau_E = 3$, while α is the leak constant controlling the auto-decay of calcium levels in the cell ($\alpha = 0.5$). The time units are arbitrary but overall activation is to follow ~ 100 ms calcium release timescale. The $I_{noise,i}$ is an instantaneous sub-threshold release, that could correspond to random neuronal firing. It is defined as

$$I_{noise,i}(t) = \sum_{t_{n,j}} I_n [H(t - t_{n,i}) - H(t - t_{n,i} - \Delta T)] \quad (5)$$

H denotes the Heaviside function, the noise amplitude $I_n = 2.1$; $t_{n,i}$ is a specific noise instance at which the noise is applied. We set the pulse duration to be $\Delta T = 1$. The noise is applied randomly at any given node with probability $p_n = 0.01$.

I_{flush} , represents the release of Ca^{2+} from the ER into the interior of the cell. The form of the release is modeled as an exponential decay in order to capture experimental timescales. The autorelease is activated when level of the agent reaches the preset threshold, $X_i = 1$:

$$I_{flush,i}(t) = I_{amp} \left(e^{\frac{-(t-\tau_i)}{c_1}} - e^{\frac{-(t-\tau_i)}{c_2}} \right) \quad (6)$$

where τ_i denotes the timing of the last threshold crossing in simulation steps. The amplitude of the internal release, $I_{amp} = 1.5$. The activation waveform is represented as the difference between a slow, $C_1 = 300$, and a fast, $C_2 = 30$, exponential. Thus, I_{flush} has a strong positive

phase shortly after its activation, which decays away over the duration of its refractory period. The refractory period is set to 1000 timesteps and prevents continuous activation of the system.

The next term on the RHS of Equation (4) describes the gap junction coupling term. Astrocytes are placed on 40×40 grid. The lattice constant, which is the minimal distance between the cells, was set to 1. Here the parameter β denotes the efficacy of coupling. The range of gap-junction strengths is limited by the overall amount of agent (Ca^{2+}) an astrocyte can send out. Higher values indicate a greater sharing of agent amongst astrocytes and less retention of their individual concentration level. Network connections are represented by the astrocyte adjacency matrix A_{ij} , where $A_{ij}=1$ if cells i and j are directly coupled and $A_{ij}=0$ otherwise. The gap junction coupling, $X_j - X_i$, is a standard term describing gap junctions that allows for rapid equilibration of Ca^{2+} concentrations at different nodes. Astrocytes are coupled via gap-junctions to all neighbors within a radius, $R=2$. Results are shown for local connectivity, and similar results were obtained for networks with a small portion of the connections (10-20%) randomly rewired.

The extracellular activation arriving at each astrocyte is represented by the final term on the RHS of Equation 4. T is the release amplitude, and $D_{ij}(r, t - \tau_j)$ is the solution of the two dimensional diffusion equation at a distance r and time t in the space embedding the astrocytic network.

$$D_{ij}(r, t - \tau_j) = \frac{N}{4\pi D^* (t - \tau_j)} e^{\frac{-r^2}{4D^*(t - \tau_j)}} e^{-\xi(t - \tau_j)} \quad (7)$$

Here $N=1000$ is the amplitude of neurotransmitter release. The diffusion constant is $D^*=0.001$ [1/ms], and the time decay constant of the agent in the external environment is $\xi=0.01$. We assume that the element releases the agent into the physical space only when its value is supra-threshold. The gap junction and diffusive coupling parameters are both set to the range of values so that the network activity ranges from quiescence to bursting behavior.

The cells' dynamics was updated, via Euler's method, for 40,000 time-steps after randomizing the initial conditions by evolving the network for 10,000 time-steps. The figures displayed represent the averages over 4 simulations.

Effects of blockage of Astrocytic Gap Junctions—To disrupt gap junction coupling, we incubated cultures in 25 μM 18- β glycyrrhetic acid (BGA) for 15 minutes (see methods section). We found that the number of astrocytes with spontaneous calcium transients is dependent on gap junction coupling strength between cells (Fig. 2b). Following incubation with gap junction blocker BGA ($n=2$ cultures), the number of astrocytes with spontaneous calcium events during imaging decreases relative to the vehicle control ($n=3$ cultures). We quantified this effect by calculating the ratio change in number of astrocytes with spontaneous calcium transients between before and after BGA treatment. We measured a negative value in the fractional change in number of astrocytes with spontaneous calcium

transients for BGA treatment, and no significant ratio change for vehicle control (Fig. 2b). This indicates that astrocytic gap junctions play an important role in network signaling.

Parallely, to mimic partial BGA block of astrocytic gap junctions, we lowered the conductance of gap junction coupling, β , in our model. The simulated astrocyte network shows a decrease in activity for weaker gap junction coupling parameter values (Fig. 2c). Figure 2c depicts the activity of astrocytic calcium events as a function of both gap junction coupling and the interaction strength between the extracellular signaling in astrocytes. In the presence of local gap junction coupling the astrocytic activity scales monotonically with strength of β . Thus, in this case, weakening gap junctions always results in decrease of astrocytic activity independent of extracellular signaling strength, except for the $\gamma=0$ case. For more discussion on parameter regimes see [18]. Figure 2d shows an example of the number of active cells within 100 time-steps for parameters in regions (i) and (ii) of Figure 2c. These parameter regions (i) and (ii) will be used for all examples in the following figures.

After weakening gap junction coupling in the astrocyte network, in addition to fewer astrocytes with spontaneous calcium transients, we also observed significant changes in the distribution of active cells in cultures. The spatial distribution of the remaining active astrocytes stopped being uniform, and instead formed locally clustered domains (Figs. 3a and 3b). To quantify this effect we defined the clustering measure C (see methods) as a fractional change of Euclidian distance between locations of observed active astrocytes and the randomized ones. Higher values of spatial clustering, C , indicate that more clustered groups of astrocytes were active. We observed that for control conditions the spatial distribution of active astrocytes is only minimally different from randomized locations, indicating only a small propensity for clustering. However, the spatial clustering increased significantly when cultures were treated with BGA. This effect is again not present for the vehicle control (Fig. 3e). This indicates that blocking gap junction greatly diminishes one mode of coupling, leaving the other still intact. The higher clustering value in the absence of gap junctions indicates that communication is still occurring, and that the remaining coupling is effectively highly clustered (short range). We hypothesize that the remaining coupling mechanism is linked to extracellular signaling and diffusion transport.

To support this hypothesis we conducted similar *in silico* experiments using the developed model. Analogous to the experimental results, we observe that in networks with strong gap junction coupling large numbers of cells activate, forming randomly distributed activity domains (Fig. 3c). Spatial randomization of active sites yields no measurable effect in this case. However, when the network coupling is lowered, the large domains do not form and we observe formation of small clusters of active cells (Fig. 3d) with high spatial clustering. These observations are summarized on Fig. 3f.

We further investigated what effect network coupling can have on the shape of calcium signaling in an individual astrocyte. Gap junction coupling being a bi-directional coupling rapidly equilibrates the Ca^{2+} levels among the coupled cells. Assuming that a relatively stereotypic amount of calcium is released from the ER into the cytosol during the calcium

event, the duration of event and the time evolution of the event may be strongly depend on the gap junction strength.

We measured the time course of calcium responses of individual astrocytes in the absence and in the presence of gap junctions coupling in both experiments and simulations (Fig. 4). Our simulations indicated that the duration of elevated calcium (the width of the calcium event) is strongly dependent on both the strength of gap junctions and the efficacy of extracellular signaling (Fig. 4F). For low β and low γ (strength of effect of extracellular signal on an individual astrocyte) the width of the calcium event initially decreases as gap junction coupling grows because of the equilibration effect discussed above. For strong β and even low γ , however, we observe subsequent broadening of the event width. This is due to the fact that the extracellular signaling lowers the calcium gradient, allowing the intracellular Ca^{2+} level to remain elevated. For high values of γ on the other hand the change of event width is largely independent of β . This is due to the fact that extracellular transport dominates network dynamics raising the resting calcium concentrations away from the original value.

Similarly, we investigated the time course of single astrocytic calcium events experimentally. Figure 4a and 4b shows an example of astrocyte calcium fluorescence transients before and after weakening gap junction coupling in the astrocyte network. We find that the duration of astrocyte calcium transients increases after BGA treatment. To quantify the change in timescale of calcium events, we computed the width of the autocorrelation of fluorescence signal of every astrocyte Ca^{2+} event (see Methods) as a measure for the time duration of calcium fluctuations in astrocytes (Fig. 4c,d). We find a significant increase in the width of the autocorrelation after decoupling gap junctions with BGA, seen as an increase in the fractional change compared to control (Figure 4e). We also plot the change of the calcium event time course for the vehicle, but here we do not observe significant change. These results are consistent with our simulations for relatively weak gap junction and extracellular coupling, possibly providing constraints on the biological values of the coupling parameters. Finally, we wanted to disambiguate the role of both coupling mechanisms in mediating formation of temporal correlations between the astrocytes. To study the network level spatio-temporal patterning of spontaneous calcium activity in cultured astrocyte networks, we measured how correlated calcium events were as a function of spatial distance between the cells, when gap junctions are left intact and when they are disrupted by BGA (Fig. 5). We plot an example of evolution of the cross correlation (CC) as a function of time lag for all astrocyte pairs falling into given distance ranges. Fig. 5a depicts an example control condition, while Fig. 5b shows an example BGA treated culture. We observe a significant change in the shape of the CC at all distances. For the controls there tends to be a peak in CC at relatively small lag. The lag increases significantly for cells that are located at a greater distance from each other. For the BGA treated astrocytes the peak of cross correlations is significantly shifted at all distances. However, notably there is not a big difference in the magnitude of cross correlation. These results are summarized over all cultures in Fig. 5e. We see that the lag values at which maximal CC is observed are significantly larger for the BGA treated cultures as compared to controls and vehicle treatment. It is interesting to note that the changes in the lag within the treatment group are only observed at shortest distances ($r < 50$ and $50 < r < 150$). The lags at which maximum

CC occurs are not significantly changing for longer distances between astrocytes. This is probably due to intrinsic timescales connected with calcium events of individual astrocytes.

We observe similar effects in our simulations. When gap junctions are present we observe much smaller lags at which maximal CC occurs (Fig. 5c). Lowering β , on the other hand, leads to much larger lags (Fig. 5d and Fig. 5f) that stabilize at larger cell distances (cell distances are calculated here in terms of lattice length). At the same time however we also observe significant decreases in the cross correlation amplitudes. This is a considerable departure from our experimental findings. This might indicate that the extracellular coupling is stronger than we assumed in the model.

We have shown here that gap junction coupling in astrocyte networks affects the spatial temporal patterning of astrocyte calcium signaling in young cultures and that a simple model can reproduce these patterning properties. Ultimately, we would like to understand how this astrocyte network pattern formation influences neuronal network dynamics. However, at this first week in cultured network development, neuronal networks are forming and do not yet exhibit spontaneous activity. After growing cultured networks for several weeks, neurons display complex spontaneous activity[26–28]. The role of astrocytic gap junctional networks in modulating neuronal dynamics is poorly understood. As a first step towards quantifying the effect of astrocyte network signaling on the modulation of neuronal network dynamics, we report changes in neuronal synchrony of three week old cultures after incubation with gap junction blocker BGA. Figure 6 shows the change in neuronal synchrony within cultured networks before and after acute gap junction blocker treatment. Relative to the vehicle controls, we find a small decrease in neuronal network synchrony after decoupling the gap junctional network. To quantify neural synchrony we applied the Golomb synchrony measure (χ) to spike time recordings convolved with gaussians of varying widths σ_g (see methods section) to reveal synchrony across varying timescales (Fig 6b). We find a small decrease in synchrony for BGA treated cultures over timescale of tens of milliseconds (p-value=0.16 for $\sigma_g=50\text{ms}$), corresponding to locking between individual action potentials, to longer timescales of seconds, corresponding to the scale of bursting of groups of action potentials. For even longer time scales we loose this effect and the synchrony measure is not significantly different between BGA and Vehicle control cultures (p-value=0.28 for $\sigma_g=5\text{sec}$). We found no change in synchrony for cultures incubated chronically with gap junction blocker (BGA supplemented in medium biweekly during culture development), which we attribute to possible homeostatic mechanisms of neuronal plasticity[29,30].

Discussion

It is a great challenge to reveal the effects of astrocytic gap junctions on neuronal activity because they arise from complex interactions among a large number of cells. Here we aimed at uncovering the network level astrocyte—neuron interactions in a highly reduced cultured system. Through a simple perturbation of decoupling gap junctions and quantifying changes in network activity, we investigated pattern formation in both young astrocytic networks and mature neuronal networks.

To gain insight into the mechanisms mediating pattern formation in astrocyte networks, we performed calcium imaging experiments to monitor spontaneous astrocytic Ca^{2+} activity in mixed dissociated cultures. We perturbed one mode of astrocyte coupling by pharmacologically blocking gap junctions in the network with application of BGA. We do not suggest that in this perturbation we are blocking all types of gap junctions in the cultured networks, as there may still be intact neuron-neuron and neuron-astrocyte gap junctions that BGA just not affect. For our purposes we are interested in disrupting the coupling between astrocytes, and its effect on the network calcium patterns. The extracellular pathway, on the other hand, is mediated by a host of signaling molecules, and therefore we were not able to successfully regulate it. We quantified the number of spontaneously active astrocyte cells, spatial clustering, temporal duration of calcium transients, and the dependence of temporal signal correlations on the spatial distance between cells for both spontaneous activity and decoupled networks.

To further understand these observed changes in spatio-temporal patterning, we utilized a computational model that takes into account these two major types of signaling pathways in astrocytes: transport through astrocytic gap junction coupling and diffusion in extracellular space. We show that the experimentally observed changes after the BGA treatment agree with our modeling results, lending credence to the hypothesis that both of these types of signaling pathways play an important role in network signaling. Furthermore, changes in the spatio-temporal patterning observed in our model when we were changing the relative coupling strength of both mechanisms provide general constraints on the biological values of these parameters.

Many experimental studies have addressed the correlations between calcium oscillations in astrocytes for both evoked and spontaneous activity. Calcium wave propagation has been observed in both cultured cell preparations[5,6,12,14] and in slices[7,8], where intercellular traveling waves propagate over astrocyte networks with speeds of 10-20 $\mu\text{m}/\text{sec}$ [31]. While localized synchronous calcium transients have been reported in slice[32,33] and in vivo[9,34]. To reconcile these conflicting observations, we suggest that the spatio-temporal propagation patterns could arise out of the network properties in different dynamic regimes. Therefore, we chose to study simplified networks and investigate the modes of astrocyte coupling and resulting pattern formation in network activity. Moreover, the interaction between astrocyte network communication and neuronal network activity is poorly understood.

Recent work has begun to investigate astrocyte network responses to neuronal activity[35], and how astrocyte calcium signaling can affect neuronal synchrony[36]. In a previous study, cultured neuronal-glial networks were manipulated through plating lower glial to neuronal cell densities and found changes in both neuronal functional clustering and synchrony[37]. In this article, we furthered this investigation by directly manipulating the coupling within the astrocytic network while preserving all other network properties, such as total number of astrocytes. Further studies need to test whether, and to what extent, changes in astrocytic spatio-temporal activity patterns affect activity patterns of neurons, and subsequently the involvement of astrocytes in cognitive functions of the brain. Additionally, there is experimental evidence linking astrocytic activity to several brain wide pathologies[38–41].

There is still much future work needed to disentangle the complex interactions underlying neuronal and astrocytic network activity pattern formation and its role in both cognition and diseased brain states.

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Figure 1.

Fluorescence imaging of spontaneous astrocyte calcium transients in dissociated hippocampal cultures. (a) Example fluorescence image of calcium indicator fluo-4am. (b) Example fluorescence image of astrocyte marker sulforhodamine 101. (c) Overlay of fluo-4am and sulforhodamine 101 images, yellow marks astrocytes co-labeled with both dyes. (d) Schematic of model shows the gap junction and diffusion pathways of astrocyte coupling.

Figure 2.

Cultured astrocyte networks display spontaneous calcium transients mediated through gap junction (GJ) coupling. (a) Example raster plot of spontaneous astrocyte network activity. Fluorescence intensity is rescaled between 0 and 1, where maximum intensity (1) is white and minimum intensity (0) is black. (b) The number of astrocytes with spontaneous calcium transients is dependent upon gap junction coupling in the network. Gap junction blocker BGA decreases the number of astrocytes with spontaneous calcium transients relative to the vehicle control (p-value=0.027). Number is the ratio change in number after-before/after+before BGA (n=2) or vehicle treatments (n=3). (c) Simulated astrocyte network shows similar dependence on gap junction coupling strength. For decreasing gap junction coupling, activity decreases across various diffusion coupling strengths. Color bar defines activity as the average number of activations* refractory period /total simulation length. (refractory period=1000). (d) Example simulation for parameters in regions (i) and (ii) of (c) shows decreased activity for weaker gap junction coupling. The number of active cells is given for within 100 timesteps at point i) and ii) in (c).

Figure 3.

Decoupling gap junctions reveals underlying spatial clustering within astrocyte networks.

(a,b) Example bright-field image of a cultured network before (a) and after (b) BGA treatment. Spatial locations of astrocytes are outlined, where blue regions displayed calcium transients during the experiment and black regions had no spontaneous activity. (c,d) Simulated astrocyte networks have similar spatial clustering before (c) and after (d) gap junction coupling. Color indicates the number of timesteps from the most recent activation. (e) Disruption of gap junction coupling with BGA enhances spatially clustering of astrocyte network activity (p-value=0.05). Spatial Clustering is the ratio change in spatial clustering between experimental and random networks of the same spatial locations (see methods). (f) Simulated astrocyte network producing similar spatial clustering. Nearest neighbor distance ratio measured as (random-real)/(random+real).

Figure 4.

Weakening network wide gap junction coupling affects the timescale of calcium transients in individual astrocytes. (a,b) Example calcium transients of an astrocyte shown as the change in calcium fluorescence intensity over baseline fluorescence ($\Delta F/F$) before (a) and after (b) treatment with BGA. (c,d) Corresponding autocorrelation of the fluorescence trace. Insets are zoomed in to show timescale. Red dot marks the first local minimum of the slope of the autocorrelation (see methods) and the dashed red line marks the half maximum. (e) An increase in the width of the autocorrelation at half maximum shows an increased duration of calcium transient after BGA (p-value=0.02). Autocorrelation width is the ratio change in transient width after-before/after+before BGA or vehicle treatments. (f) Simulated astrocyte network shows similar timescale of calcium transients. Weaker gap junction coupling (ii) has increased transient width than stronger gap junction coupling (i). Color bar represents the width of the transient in number of timesteps.

Figure 5.

Spatio-temporal correlations between pairs of astrocyte calcium transients are mediated by gap junction communication. (a,b) Example cross correlations of calcium transients between all pairs of astrocytes within the network before (a) and after (b) BGA treatment across distances between all pairs of astrocytes. (c,d) Simulation results for cross correlations of calcium transients before(c) and after blocking gap junctions. Time lags are in number of timesteps. (e,f) Maximum cross correlation between pairs of astrocytes occurs at longer time lags as a function of distance between pairs of astrocytes for both experiments (e) and simulations (f). Weakening gap junction coupling with BGA increased the time lag for maximum cross correlation across distances ($r=50, 150, 300, 600$, and $800 \mu\text{m}$) between all pairs of astrocytes (p-values=0.03, 0.03, 0.04, 0.02, 0.01, respectively).

Figure 6.

Gap junction coupling influences neuronal synchrony across multiple timescales. (a.) Example of an acute BGA experiment. Neuronal network synchrony (χ) decreases after treatment with gap junction blocker, across multiple timescales ranging from tens of milliseconds to several seconds. (b.) Summary of the change in synchrony ($\Delta\chi$) for BGA cultures ($n=3$) and Vehicle control cultures ($n=2$). Error bars are SEM.