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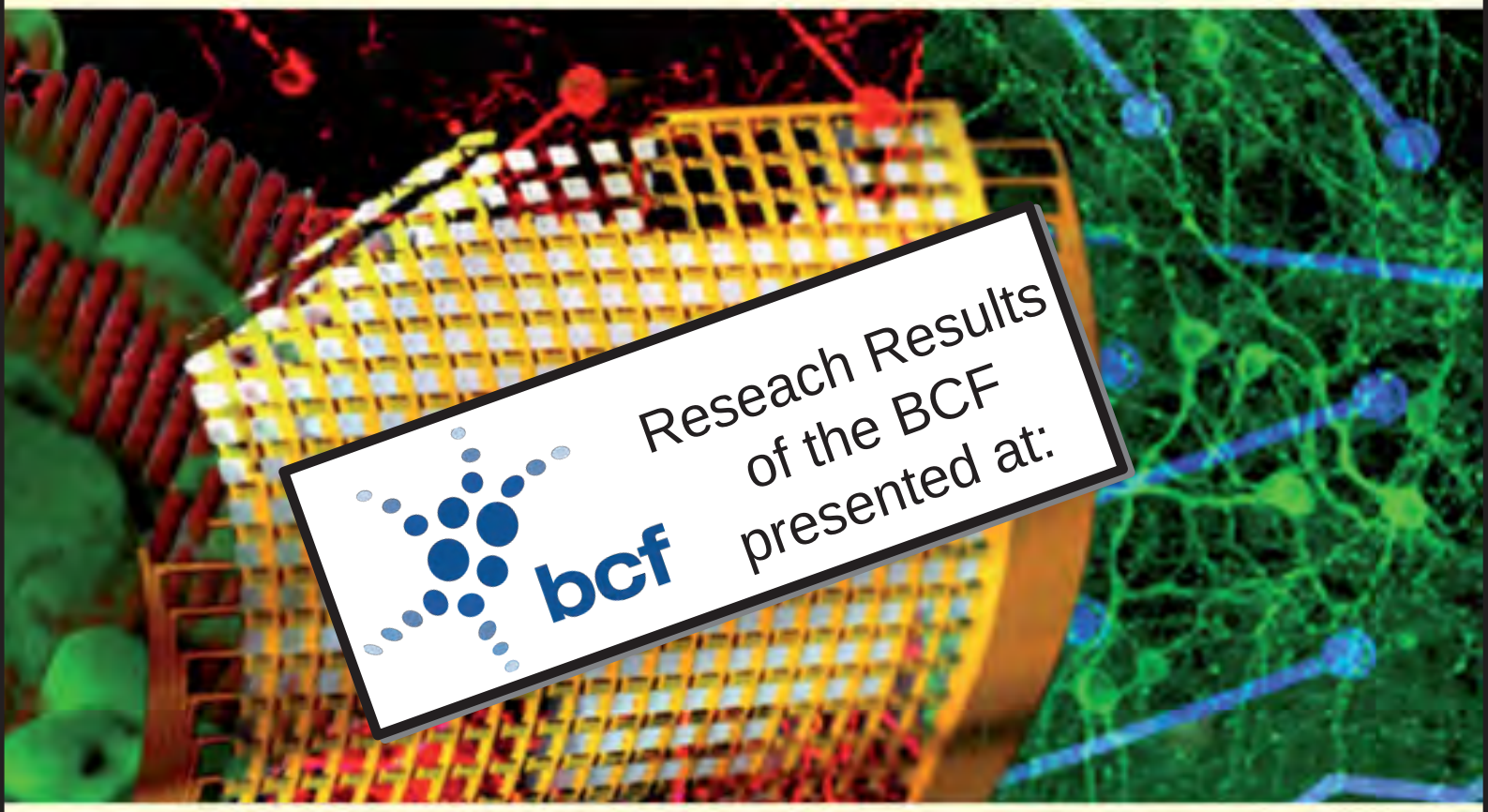

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Astrocytes drive neural network synchrony

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Abstract
While astrocytes have been repeatedly shown to play a functional and developmental role at the synapse, it remains unclear whether these cells contribute to neuronal network state and processing. Calcium imaging in conjunction with the extracellular recording of hundreds of neurons revealed complex relationships between the network activity of neurons and astrocytes. Using GFAP-Melanopsin, a light activated calcium channel, we induced transient calcium concentration increases in astrocytes, which led to prolonged periods (several seconds) of sustained, enhanced synchronized firing (spontaneous periods of bursts of bursts) in neurons throughout the network. The spatiotemporal properties of this behaviour indicate that astrocytes can modulate the overall state of synchronicity in neuronal networks.

1 Introduction

The neuroscience community is largely divided over the role of astrocytes in the brain's ability to process and store information. Several studies *(1–3)* cast doubt on the assertion that astrocytes serve a meaningful role in encoding by neural ensembles. While *(4–7)* among others argue that there is clear evidence that astrocytes are crucial players in computation, encoding and storage. We assert that the brain's functionality is a manifestation of the activity of networks of neurons (rather than the action of individual cells). Microelectrode arrays (MEAs) allowed us to measure the spiking activity of distal neurons while simultaneously observing calcium-related activity in astrocytes through live Ca^{2+} imaging. Since the network role of astrocytes is yet unclear and the functioning of the brain is achieved through the complex activity of neural ensembles, we sought to elucidate the contribution, if any, of astrocytes to the activity of groups of neurons.

2 Methods

Melanopsin was mammalian codon-optimized, and was synthesized by Genscript (Genscript Corp., USA). All constructs were verified by sequencing. Human embryonic kidney cells (HEK-293T *(8)*) were cultured in advanced DMEM (Invitrogen, Germany) supplemented with 2 % fetal calf serum (FCS, PAN Biotech GmbH, Germany), 10 µM cholesterol (Sigma-Aldrich, USA), 10 µM egg lecithin (Serva Electrophoresis GmbH, Germany) and 1 % chemically defined lipid concentrate (Invitrogen, Germany). For the production of lentiviral particles, 500,000 HEK-293T cells were seeded in 2 ml medium 24 h before transfection. 100 µl of 0.25 M $CaCl_2$, 25 µM chloroquine (Sigma-Aldrich, Germany) containing 2 µg of the lentiviral expression vector fgfa2-melanopsin-mCherry and 1 µg of each helper plasmid (pCD/NL-BH**(9)* and pLTR-G *(10)* were mixed with 100 µl 2x-HBS solution (100 mM HEPES, 280 mM NaCl, 1.5 mM Na_2HPO_4, pH 7.1) and added to the cells. The medium was replaced after 5 h and viral particles were produced for 48 h. The cell culture supernatant containing the viral particles was collected, filtrated through a 0.45 µm filter (Schleicher & Schuell GmbH, Germany,) and stored at -80 °C. For transduction, 200 µl of thawed viral particles were added to a primary cortical culture in 2ml of MEM medium and incubated for 1 day followed by exchange to new MEM medium.

Rhodamine-3 (Invitrogen, Germany) was used according to the manufactures instructions. Calcium imaging was undertaken while the culture was maintained at 37°C (TC02, Multi Channel Systems, Germany) 5% CO_2 and pH 7.4. Ca-imaging was performed using a Zeiss Z1 Examiner microscope with an excitation wavelength of 550nm. To stimulate GFAP-Melanopsin, cultures were exposed to approximately 2 lm of 470 nm light at 100ms duty cycles.

Ca imaging movies were preprocessed with ImageJ, where regions-of-interest (ROI) were defined manually. Astrocyte traces were selected based on calcium-transient properties described in *(11, 12)*. The traces were then filtered to remove noise using a Savitzky-Golay filter *(13)* with a kernel width of 21 and a first degree polynomial. Slow trends were removed with an equiripple high-pass filter (sampling frequency:12.5 Hz, stop-band frequency: 0.0008 Hz, pass-band frequency: 0.008 Hz. amplitude attenuation: 20 db).

Spike data was sampled at 10 Khz, per channel (20 ms cut outs around threshold (at 5.5 standard deviations from average signal). The raw electrode data was imported into Matlab (Mathworks, USA) using the open-source software, Neuroshare *(14)* and spikes were detected using the wavelet packet discrimination algorithm described in *(15, 16)*. The spike times were then stored for each channel.

3 Results

All of the neural networks exhibited spontaneous activity with global network coordination manifested by the generation of bursting events – short time windows during which most of the neurons participate in more rapid neuronal firings. The time-averaged network firing rates in the baseline were the same as in the experiments (during Ca imaging or optogenetic stimulation). All the networks shared characteristic neuronal firing rates, inter-burst-intervals (IBI) distributions and burst width distributions in the baseline (data not shown). Calcium transients of astrocytes within a view field were comparable to astrocyte calcium dynamics observed in vivo and in slices. These calcium traces were observed to be coordinated with each other and with neurons throughout the network (even at great distances). The synchronized nature of the neuronal firing and the coincidence of astrocyte Ca increases with these bursts led us to investigate the functional relationship between these cell types as characterized by their observed activity. This analysis revealed, by the structure apparent in the dendrogram ordered correlation matrix *(17)*, the existence of correlated astrocytic “patches” (figure 1B). These patches have been reported in other works as being the result of either transmitted calcium through gap junctions *(4)* or through shared neuronal input *(5)*. The neural network activity showed variable coordination with astrocyte activity – that is, some astrocytic transients appeared to correspond to the network firing rate whereas other astrocytes appeared less well coordinated (figure 1A). The mix of similar and dissimilar calcium signals among different astrocytes within a view field and the fact that subsets of astrocyte traces appeared to be related to the neural network firing rate, suggests that astrocytes form functionally relevant ensembles. This complex structure of interactions between the two cell types led us to question whether astrocytes were simply responding to neural input or, as suggested by the synaptic studies *(4–7)*, capable of modulating the activity of neural networks.

Cortical cultures of neurons and glia, where exclusively astrocytes expressed Melanopsin, demonstrated a dramatic neuronal response upon stimulation. During stimulation, prolonged periods (several seconds long) of intense synchronized firing occurred across the neural network (Fig. 1D-F). That is, though the overall firing rate remained constant, the maximum burst duration during astrocyte stimulation increased considerably. This neuronal response to astrocyte stimulation was not observed to be sharply correlated in time with the 470 nm pulse nor did it show strong dependence on light intensity, exposure time or pulse rate. However, the network-wide super-bursting began shortly after stimulation and persisted only a short time following the end of stimulation.

To exclude neuronal expression of Melanopsin we performed immunohistochemistry (IHC). IHC revealed that astrocytoma cells that do not express GFAP and neurons labeled with anti-MAP2 antibodies both fail to immunoreact with Anti-OPN4 (anti-melanopsin) antibodies (data not shown). Cultures infected with GFAP-Mcherry only or not infected did not superburst in response to 470 nm light pulses (data not shown).

4 Discussion

Whether or not astrocytes have the potential to change a neural network’s state is crucial in determining their relevance to the aspects of brain function that, to date, have been solely considered neuronal. Using optogenetic tools, we prompted calcium transients in astrocytes (and only astrocytes). These cells, activated through Ca-influx, induced a state change in the neural network leading to repeated, highly synchronized super-bursts. Astrocytes are thus capable of not only modulating neuronal activity but changing a network’s overall working mode. Since astrocyte proliferation is a hallmark of various brain diseases, for example, gliosis is typical of epileptic foci *(18)*, our results suggest that this astrocytic potential to modulate network states may contribute to highly-active super-synchronized epileptic episodes.

Acknowledgments

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Clustered network structure promotes spontaneous burst initiation in vitro

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Abstract
To assess dependencies between evolving connectivity and emerging network dynamics we modified connectivity in developing networks of cortical neurons in vitro by pharmacological manipulation of morphological differentiation processes. Our data suggests that clustering of neurons and fasciculation of neurites increases the ability of cultured networks to spontaneously initiate synchronous network bursts.

1 Introduction

Synchronous bursting events (SBE) are widely observed in developing neuronal systems, suggesting that the ability to spontaneously initiate these dynamics reflects a crucial, though transient feature of forming networks. Similarly, SBE dynamics robustly emerge as the predominant type of activity in networks of cultured neurons in vitro. We suggest that general neuronal mechanisms might guide network self-organization in a way that establishes these dynamics. Interestingly, theoretical models have shown that hierarchical network structures embedding clusters of strongly inter-connected neurons are optimal for initiating and sustaining spontaneous activity [1] and clustered network structure typically emerges in networks forming in vitro [2] and in vivo [3]. We speculate that activity-dependent structural plasticity, being a principal driving force of network self-organization, establishes clustered network structures and thereby promotes spontaneous activity levels. Previous studies have shown that protein kinase C (PKC) inhibition promotes dendritic outgrowth and arborization [4], and impairs pruning [5], linking this protein closely to structural plasticity. To test our hypothesis, we thus inhibited PKC in developing networks of cortical neurons in vitro to modify network structure.

2 Materials and Methods

Primary cortical cell cultures were prepared as described previously [6]. Cells were extracted from cortices of newborn rats by mechanical and enzymatic dissociation and plated onto polyethyleneimine-coated micro-electrode arrays (6x10 with 0.5mm and 32x32 electrodes with 0.3mm spacing; Multichannel Systems). Cultures developed in growth medium (MEM) supplemented with heat-inactivated horse serum (5%), L-glutamine (0.5mM), glucose (20mM) and gentamycin (10µg/ml) under 5% CO2 and 37°C incubator conditions. PKC inhibitor Gö6976 1µM was applied starting from the 1st day in vitro (DIV). Staining against MAP2 protein was performed for morphological analysis of dendrites. Recordings were performed under culture conditions (MEA1600-BC and MEA30-1024, Multichannel Systems).

3 Results

We show that developmental inhibition of PKC in cortical cell cultures increased dendritic outgrowth, impaired neurite fasciculation and clustering, and abolished network pruning. This resulted in more homogeneous and potentially better connected networks. In consequence, SBEs propagated faster and in more regular wave fronts. Yet, in agreement with our hypothesis, SBEs were triggered from fewer sites and at lower rates suggesting that these homogeneous networks embedded fewer SBE initiation zones. We tested if the homogeneous networks were able to support higher SBE rates by providing additional input by electrical stimulation. Interestingly, homogeneous networks achieved higher SBE rates when electrically stimulated compared to the more clustered control networks. Our data suggests that activity-dependent structural plasticity promotes network clustering and thereby spontaneous SBE initiation during development. Based on recent evidence for a reciprocal scaling between synaptic strength and number of neuronal partners in vitro [7], we propose that locally more confined synaptic targeting within neuronal clusters promotes stronger and more recurrent coupling of neurons. The resulting connectivity structure could thereby more easily amplify spontaneous excitation locally beyond a critical threshold necessary for SBE initiation.

4 Conclusion

Our results indicate that activity-dependent structural plasticity promotes neuronal clustering and thereby the ability of in vitro networks to spontaneously initiate SBEs. We propose that this might be part of a general strategy pursued by neuronal networks to establish this crucial activity pattern during development.

Acknowledgements

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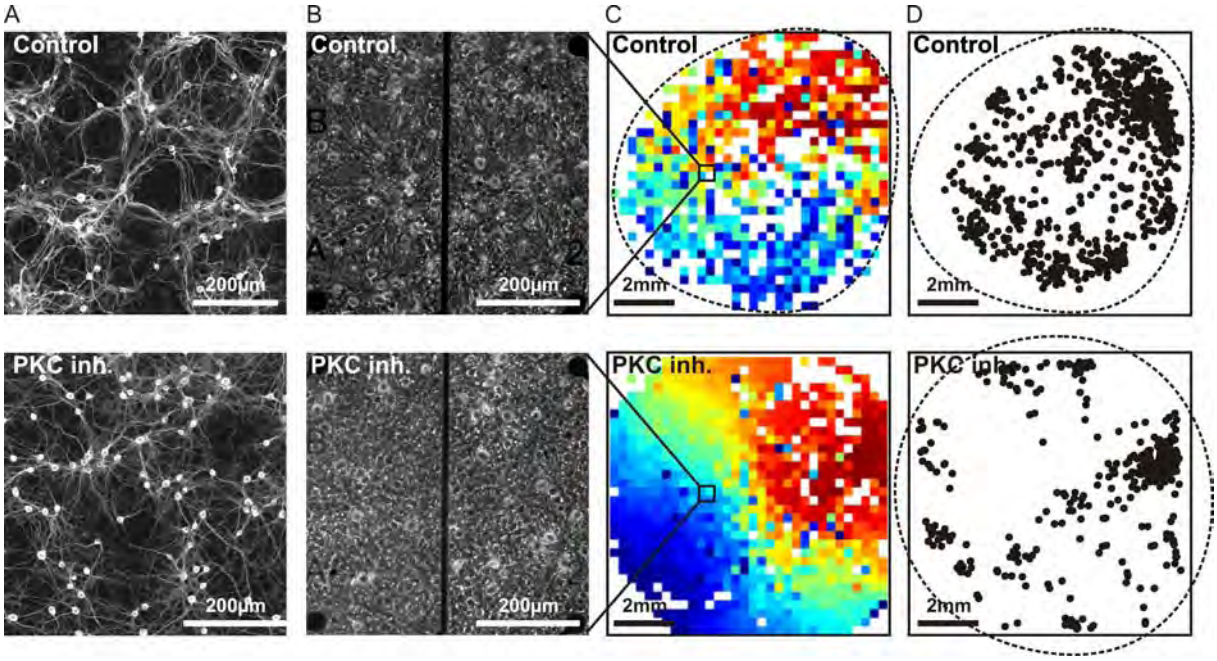

Fig. 1. Structure and dynamics in cortical cell cultures

A) MAP2 Staining against dendrites and somata in low density cultures (~200 neurons/mm2) after 42DIV. PKC inhibition impaired neuronal clustering and reduced dendritic fasciculation in developing networks which suggests more homogeneous connectivity.

B) Dense cultures (~2000 neurons/mm2) were grown on MEAs and documented by phase contrast microscopy. More homogenous networks had formed under impaired PKC activity at 44DIV.

C) Propagation of activity during SBE was assessed with 1024 electrode MEAs (32x32) that spanned almost the entire area of cultured networks (corners lack neurons). The pseudo-color map depicts the first spike rank order (from early in red to late blue; white indicates no activity) of during exemplary SBEs. PKC inhibited networks showed more regular propagation patterns indicating more homogeneous connectivity. The zoom-in gives an impression of the size of neurons, networks and MEA.

D) SBE initiation zones were identified as median coordinate position of the first percent of active sites during single SBEs, respectively. Network boundaries (dashed circle) were generally more susceptible to initiate SBEs. More homogeneous networks forming under impaired PKC embedded fewer initiation sites than controls and triggered SBEs at lower rates (Controls N=807 SBEs in 60min; PKC inhibited N=676 SBEs in 140min).

A simple but plausible culture model recreates fast burst propagation and predicts their persistence

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Abstract
Present in the dynamics of cultured networks are periods of strongly synchronized spiking, termed 'bursting', whose role is not understood but dominates network dynamics and, due to their presence in cultures regardless of cell origin and their resistance to attempts to remove them, have been suggested to be an inherent feature in culture dynamics. Bursts have been demonstrated to contains distinct spatiotemporal motifs, repudiating the possibility that they are random or chaotic activity. However, the speeds of these propagating wavefronts has been measured as 5-100mm/s, and hence much faster than can be accounted for by local connectivity.
In attempting to represent cultured networks using 2D network models, typical connectivity models, such as random connectivity, prove to be insufficient for recreating some of the distinct phenomena associated with the dynamics of cultured networks, noticeably the fast propagation speeds.

1 Methods

Here, we introduce a simple but biologically plausible connectivity model that is able to reproduce phenomena. The relative simplicity of the connectivity scheme allows us to simulate a network of 12,500 neurons within a 1.5mm radius circular area on a 2D plane, resulting in a model that is comparable in density and size to experimental networks. We then extend this model to incorporate some of the subtle structural inhomogeneities observed experimentally, such as clustering of soma positions, to investigate their implications for network dynamics.

2 Results

We demonstrate that our simple but biologically plausible representation of network connectivity is able to emulate burst propagation with speeds comparable to those observed experimentally. Furthermore, the inclusion of structural inhomogeneities strongly facilitate burst propagation as well as form the emergence of distinct burst motifs. Importantly, our model confirms that bursts are indeed an inherent feature of such networks, as they are an inescapable by-product of network connectivity and structure.