

Small-world networks of spontaneous Ca²⁺ activity

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Synchronized network activity among groups of interconnected cells is essential for diverse functions in the brain. However, most studies have been made on cellular networks in the mature brain when chemical synapses have been formed. Much less is known about the situation earlier in development. When studying neural progenitors derived from embryonic stem cells and neural progenitors from mice embryos, we found networks of gap junction coupled cells with vivid spontaneous non-random calcium (Ca²⁺) activity driven by electrical depolarization that stimulated cell growth. Network activity was revealed by single-cell live Ca²⁺ imaging and further analyzed for correlations and network topology. The analysis revealed the networks to have small-world characteristics with scale-free properties. Taken together, these results demonstrate that immature cells in the developing brain organize in small-world networks that critically regulate neural progenitor proliferation.

Molecular Mechanism of Spontaneous Ca²⁺ Activity in Neural Progenitor Cells

Cell signaling driven by Ca²⁺ is essential for all cell types¹ since Ca²⁺ signals can activate important cell programs.^{2,3} Spontaneous Ca²⁺ activity (i.e., without externally applied stimuli) has been shown to regulate developmental events, including axon outgrowth and path-finding, synaptic connectivity and maturation of neuronal signaling properties.⁴⁻⁷ To understand how immature cells communicate

with each other we performed time-lapse microscopy measuring intracellular Ca²⁺ levels in differentiating neurons. Neural progenitors derived from mouse embryonic stem cells exhibited clusters of vivid spontaneous Ca²⁺ activity.⁸

Several previous studies have described molecular mechanisms of intercellular Ca²⁺ waves in different cell types and tissues (for review see Leybaert and Sanderson).⁹ Two commonly described mechanisms involve release of ATP into the extracellular space through connexin hemichannels or intercellular diffusion of inositol-1,4,5-trisphosphat through gap junctions, which both rely on Ca²⁺ release from intracellular stores. Interestingly, the spontaneous Ca²⁺ activity we have described in neural progenitors is mainly driven by a different mechanism that seems to be largely independent of intracellular Ca²⁺ stores.

Using pharmacological inhibitors and gene knockdown we determined that the spontaneous Ca²⁺ activity in neural progenitors was critically dependent on gap junctions (*Connexin 43*) and voltage-dependent Ca²⁺ channels. Electrophysiological recordings in vitro and in vivo confirmed that neural progenitors were capable of transmitting depolarizing currents through gap junctions. Inhibiting the functional networks by blocking gap junctions not only abolished the spontaneous Ca²⁺ activity but also reduced the proliferation rate in vitro and in vivo. In mouse embryos, blocking gap junctions decreased proliferation, which lead to brains smaller in size and significantly reduced cortical thickness. Interestingly, a few cells maintained their

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Ca²⁺ activity during the pharmacological inhibition, indicating the existence of “trigger cells” driving the network activity via gap junctions.

Three components were necessary for the spontaneous Ca²⁺ activity: gap junctions connecting neighboring cells within the network, functional voltage-dependent Ca²⁺ channels in the plasma membrane and “trigger cells” producing depolarizing currents which are spread through gap junctions, resulting in the activation of voltage-dependent Ca²⁺ channels and the subsequent increase of cytosolic Ca²⁺. The spontaneous Ca²⁺ activity described in neural progenitors was absent in embryonic stem cells. This is not due to lack of connectivity through gap junctions, since embryonic stem cells have been shown to express functional gap junctions,¹⁰ but is rather explained by the absence of functional voltage-dependent Ca²⁺ channels.¹¹ As the embryonic stem cells are differentiated toward neurons they gain functional voltage-dependent Ca²⁺ channels and around the same point in time the networks of spontaneous Ca²⁺ activity emerges. However, it is not clear if the “trigger cells” start to generate the depolarizing currents at a similar time point as the voltage-dependent Ca²⁺ channels become functional or even earlier during the differentiation process.

Neural Progenitors Organize in Small-World Networks

By applying mathematical cross-correlation analysis to single cell Ca²⁺ recordings we investigated if developing neurons were interconnected creating networks with small-world/scale-free properties. An example of a scale-free small-world network is how airlines connect the world through nodes of airports.¹² A random disruption to one of the thousands of airport around the world would usually not disturb the flow of travelers, but a shutdown of a hub, such as Chicago O’Hare, could severely harm the network. Hence a

scale-free small-world network has a good tolerance for random deletion of nodes, but low tolerance for a directed attack to a hub. Graph theory predicts that such network designs are effective for biological systems, since they enable efficient information transfer and robustness against failure of single cells.¹³ Cross-correlation analysis of our single cell Ca²⁺ data uncovered highly correlated clusters of cells. Additional analysis of network parameters revealed highly connected “hub cells” (scale-freeness), as well as high clustering (small-worldness). The spontaneous Ca²⁺ activity stimulated neural progenitor proliferation, which suggests that the network of connected cells would expand as the participating cells are more likely to divide compared with cells which are not part of the network. However, this is not a ubiquitous mechanism for cell proliferation, since we demonstrate that embryonic stem cells proliferate at a high rate without vivid spontaneous Ca²⁺ activity.⁸

The highly connected “hub cells” may be a different population of cells from the “trigger cells” driving the network activity. Recently, it was shown that the nodes one should control in order to control the entire network are usually not the hubs, but other less well connected nodes.¹⁴ The mechanisms of how the “trigger cells” generate the depolarizing currents and how they are maintained over time is not yet known.

In conclusion, our results demonstrate that immature cells in the developing brain organize in small-world networks through gap junctions and that these network circuits critically regulate neural progenitor proliferation. Our data underscore the critical role of intricate cell signaling during embryonic development and show that complex network formations between immature cells in the brain exist well before birth.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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