Title: The Social Networks of Neural Progenitor Cells

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Abstract

Quantitative understanding of how neural progenitor cells (NPCs) collectively self-organize into neural networks can provide critical insight into how to optimize neural regenerative strategies. To that end, we characterized the topology of human embryonic NPCs during differentiation by designing and employing a spatial graph-theoretic analysis. Statistical measures of information flow in NPC spatial graphs revealed a shift from topologies with high global efficiency to high local efficiency, around the time mature neuronal phenotypes appeared in culture. These results support the view that network-wide signaling in immature progenitor cells gives way to a more structured, hierarchical form of communication in mature neural networks. We also demonstrate that the evaluation of recurring motif patterns in NPC graphs reveals unique geometric arrangements of cells in neural rosette-like structures at early stages of differentiation. Our approach provides insight into the design of developing neural networks, opening the door for new approaches that modulate neural cell self-organization for therapeutic applications.
Human embryonic stem cells (hESCs) and other sources of pluripotent stem cells have provided much hope for regenerative medicine, especially in the nervous system (Fred H. Gage and Temple 2013). Recently, stem cell therapies have been reported to treat neurological disorders, including stroke and neurodegenerative diseases like Parkinson’s disease (Hallett et al. 2014; Rosado-de-Castro et al. 2013). In addition, neural progenitor cells derived from embryonic stem cells (hNPCs) and dissociated cultures have helped elucidate intrinsic transcriptional control of self-renewal and multipotency (Guillemot 2007; Imayoshi and Kageyama 2014; Shen et al. 2006; Kageyama et al. 2009). The influence of external cues such as morphogen gradients in guiding nervous system development is also well documented (Mason 2007; Bertrand and Dahmane 2006). Aside from intrinsic genetic programs and extrinsic cues, a third aspect of stem cell differentiation that merits further study is the collective self-organization of progenitor cells into functional neural networks. Quantitative insight into neural cell self-organization, in addition to knowledge of external and intrinsic cues that guide tissue development, will ultimately be necessary to design effective and targeted stem cell therapies.

Cell-cell communication among progenitor cells is an essential aspect of nervous system development. Neural progenitor cells cluster together in specialized microenvironments or niches where communication with neighboring cells plays an important role in determining cell behavior (ffrench-Constant 2008). Prior to the formation of functional synapses, NPCs display structured intercellular communication that plays a critical role in the spatiotemporal control of self-renewal and differentiation, and also shapes developing neural circuits. Examples of structured cell-cell communication include patterned, spontaneous electrical activity mediated partly through gap junctional coupling (Malmersjö et al. 2013; Spitzer 2006; Blankenship and Feller 2010), maintenance of intercellular configurations through tight junction proteins (Watters et al. 2015) and control of cell differentiation through Notch signaling (Edri et al. 2015; Shimojo, Ohtsuka, and Kageyama 2008). Notably, the predominant forms of communication employed by NPCs can be described as juxtacrine signaling, i.e., requiring direct cell-cell contact. Thus, the
immediate cellular neighborhood of a progenitor cell provides an important context in which to place its
dynamic behavior.

Live-cell imaging approaches have yielded significant insight into the dynamics of multipotent progenitor
cells, particularly when coupled with automated image analysis (Imayoshi et al. 2013; Schroeder 2011;
Cohen et al. 2010). However, most of these studies focus on cell-autonomous mechanisms that guide self-
renewal and differentiation of progenitor cells. Graph theory and network analysis methods are well
suited to uncover the role of structured communication among progenitor cells in guiding their behavior.
Graph-theoretic methods have been used to study functional and anatomical connectivity in the adult
brain, leading to significant insight into brain organization (Bullmore et al. 2009). A number of studies
have also applied graph-theoretic approaches to study the functional and anatomical connectivity of
cultured neuronal circuits (Feldt, Bonifazi, and Cossart 2011; Shefi, Ben-Jacob, and Ayali 2002; de
Santos-Sierra, Sendiña-Nadal, Leyva, Almendral, Anava, et al. 2014; Downes et al. 2012). However,
these studies are often conducted in dissociated cultures of post-mitotic neurons, where the physical
wiring among neurons is important and network-wide information is conveyed primarily through synaptic
contacts. Given the dominance of juxtacrine modes of signaling among progenitor cells, we reasoned that
adapting graph-theoretic approaches to study the spatial organization of neural progenitors during
differentiation would yield insight into the spatial evolution of progenitor cell communities and their
relationship with functional differentiation state.

Here we present a detailed characterization of spatial organization of H9-derived human neural progenitor
cells during neural differentiation over the course of 14 days. To achieve this characterization, we
introduce a new approach that integrates weeks-long live imaging assays, automated image analysis and a
graph-theoretic approach based on cell body proximity to quantify the spatial organization of progenitor
cells. A schematic representation of our experimental paradigm is shown in Figure 1. We use our method
to demonstrate that NPCs transition from topologies with high global efficiency to those with high local
efficiency for information flow around the time mature neuronal phenotypes appear in culture. We also show that our graph-based metrics capture the characteristic geometric arrangement of cells in neural rosette-like structures seen at early time points of differentiation.

Figure 1. Schematic illustrating the design of the experiments.
**Results**

**Functional characterization of differentiating hNPC cultures.** The model cell culture system used in this study is neural progenitor cells derived from H9 human embryonic stem cells. These cells were

![Figure 2](image)

**Figure 2. Functional characterization of differentiating hNPCs.** (A) hNPCs at day 0 stain positively for Nestin. Nuclei are labeled by Hoescht. (B) Cells at day 14 stain positive for MAP2. Nuclei are labeled by Hoescht (scale bar = 100μm). (C) Peak inward and outward currents determined through whole-cell patch clamp electrophysiology. Number of cells recorded (N) is shown above the bar for each time period. Error bars represent S.E.M. Student t-test was performed for each pair of samples; * P<0.05. (D) Weak action potentials evoked from a cell at day 14 through current injection. Magnitudes of current injected are -30pA, +20pA and +120pA from holding. (E) Voltage-gated inward and outward currents seen in the same cell. Voltage steps applied were from -60mV to +90mV in 10mV increments.
maintained as undifferentiated, mitotic progenitor cells in the presence of the mitogen basic fibroblast growth factor (bFGF). Withdrawal of bFGF from culture medium was used to induce spontaneous differentiation of hNPCs (F. H. Gage 2000).

We performed immunocytochemistry and whole-cell patch clamp electrophysiology experiments to uncover the time course of functional development in differentiating hNPCs. Cells at day 0 stained positively for Nestin, a Type VI intermediate filament expressed by dividing neural progenitor cells (Figure 2A). Cells at day 14 were positive for microtubule-associated protein-2 (MAP2), a protein associated with dendrite formation in maturing neuronal cells (Figure 2B). Analysis of peak inward and outward currents from voltage-clamp experiments showed that cells at all time points exhibited the same levels of outward currents, but showed increasing magnitudes of inward currents (Figure 2C). Inward currents are typically driven by voltage-gated sodium channels, and their presence indicates a more mature neuronal phenotype. Furthermore, weak action potentials could be elicited from cells showing inward currents at later time points (3/11 cells at day 14) through current injection. Sample current-clamp and voltage-clamp traces are shown for a cell recorded at day 14 in Figure 2D and 2E. These experiments showed that Nestin-positive hNPCs matured over 14 days to MAP2-positive neurons, with neuronal fate commitment occurring between days 4-8 (indicated by the appearance of neuronal phenotypes in that time period).

**Representation of cell community structure using a graph-based approach.** In order to uncover topological changes in differentiating hNPCs, we combined long-term time-lapse microscopy of differentiating cultures with a graph-based approach for quantifying cell community structure. We conducted two biologically independent experiments where cultures were imaged at days 0, 3, 6, 9, 12 and 14 after withdrawal of bFGF. An additional dataset was obtained by imaging differentiating cultures at 1-hour intervals for a total duration of 8 days (Supplemental Video 1). Selected image sequences were
analyzed using custom image-processing algorithms, resulting in the extraction of soma and neurites for each phase-contrast image (Figure 3A-D) (see Methods section for details).
Juxtacrine signaling – signaling through direct cell-cell contact – plays an important role in immature neural circuits. In order to build quantitative representations of hNPC communities that reflect the modes of communication employed by hNPCs, we developed a graph-based approach, where soma are denoted as nodes and spatial proximity of cells is used to assign edges. In this manner, we constructed non-weighted, undirected graphs representing hNPC communities from time-lapse microscope images (Supplemental Video 2).

In order to describe the structure and topology of NPC community graphs, we evaluated a number of metrics derived from graph theory. Table 1 lists 16 metrics that were computed, normalized appropriately to account for network size (Bounova and De Weck 2012). The network metrics provide information on various aspects of the graph structure such as information flow, connectivity and abundance of motifs. Figure 3H shows the covariance matrix of all 16 metrics as a hierarchically clustered heatmap. The heatmap shows several strong positive correlations among degree-related metrics like average degree, average neighbor degree and their variances. Interesting negative correlations include that between...

Figure 3. Image processing and graph representation of time-lapse microscopy images. (A) Representative grayscale image of human NPCs, shown at Day 3 (scale bar = 50 μm). (B) First derivative of the pixel intensity histogram, with a linear fit to the ascending portion shown as a red line. (C) Binary image obtained after thresholding is applied. (D) Separation of linear features through morphological opening of binary image yields cell bodies (blue) and neurites (red). (E) Phase contrast image with soma boundaries overlaid in red, and proximity edges shown in yellow. (F) Inset from panel A showing six soma, of which two pairs (1, 2) and (4, 5) are connected by proximity edges; the intercellular distance for these two pairs are smaller than their average diameter multiplied by a scaling factor S = 2; Soma 3 and 6 are isolated nodes since they are not sufficiently close to any other soma. (G) Degree distribution of the graph in panel E; number of nodes = 317, number of edges = 152, average degree, <k> = 0.96 (H) Correlation heatmap of all metrics obtained by hierarchical clustering of the covariance matrix.
network efficiency and number of connected components, and those between clustering coefficient and all degree-related metrics. In the following sections, we focus on metrics that have intuitive biological interpretations, their trends across time of differentiation, and relationships observed with other metrics that explain these trends. Trends in metrics not discussed in the main text are shown in Supplemental Figure 4.

Table 1. Metrics computed, their descriptions, and mode of normalization to account for the network size. \( n \) = number of nodes, \( m \) = number of edges.

<table>
<thead>
<tr>
<th>Graph Metrics</th>
<th>Symbol</th>
<th>Definition</th>
<th>Normalization</th>
</tr>
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<tbody>
<tr>
<td>Network Density</td>
<td>(&lt; k &gt;)</td>
<td>Average degree of graph, normalized by total maximum possible degree</td>
<td>Maximum possible degree, ((n-1))</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(&lt; k &gt; = \frac{2m}{n(n-1)})</td>
<td></td>
</tr>
<tr>
<td>Variance in Degree</td>
<td>(var(k))</td>
<td>Variance of normalized node degree sequence</td>
<td>Node degree sequence normalized by maximum possible degree, ((n-1))</td>
</tr>
<tr>
<td>Average Neighbor Degree</td>
<td>(&lt; k_n &gt;)</td>
<td>Average degree of node neighborhood, across all nodes</td>
<td>Maximum possible degree, ((n-1))</td>
</tr>
<tr>
<td>Variance in Neighbor Degree</td>
<td>(var(k_n))</td>
<td>Variance of the normalized average neighbor degree sequence</td>
<td>-</td>
</tr>
<tr>
<td>Network Efficiency</td>
<td>(\epsilon)</td>
<td>The average reciprocal of shortest path length across all pairs of nodes, (E)</td>
<td>Average network efficiency of 100 random graphs generated through degree-preserving rewiring, (E_{rand}). Random graph generation is illustrated in Fig. S3.</td>
</tr>
<tr>
<td>Average Clustering</td>
<td>(\sigma)</td>
<td>Fraction of total possible links among the</td>
<td>Average clustering</td>
</tr>
<tr>
<td>Metric</td>
<td>Description</td>
<td>Formula/Note</td>
<td></td>
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<tr>
<td>-------------------------------</td>
<td>-----------------------------------------------------------------------------</td>
<td>------------------------------------------------------------------------------</td>
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<tr>
<td>Coefficient neighbors of a node that are actually present, averaged across all nodes, ( \mathcal{C} )</td>
<td>coefficient of 100 random graphs generated through degree-preserving rewiring, ( \mathcal{C}_{\text{rand}} )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of connected components</td>
<td>( N_{cc} )</td>
<td>Number of disconnected sub-graphs in main graph</td>
<td></td>
</tr>
<tr>
<td>Average Size of Connected Components</td>
<td>( &lt;S_{cc}&gt; )</td>
<td>Average number of nodes in each connected component</td>
<td></td>
</tr>
<tr>
<td>Variance in size of connected components</td>
<td>( \text{var}(S_{cc}) )</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Network Diameter</td>
<td>( d )</td>
<td>Longest shortest path length of network</td>
<td></td>
</tr>
<tr>
<td>Triangular loop count</td>
<td>( l_3 )</td>
<td>Number of loops of 3 nodes</td>
<td></td>
</tr>
<tr>
<td>4-star motif Count</td>
<td>( S_4 )</td>
<td>Number of star motifs with one hub and three spokes</td>
<td></td>
</tr>
<tr>
<td>5-star motif count</td>
<td>( S_5 )</td>
<td>Number of star motifs with one hub and four spokes</td>
<td></td>
</tr>
<tr>
<td>6-star motif count</td>
<td>( S_6 )</td>
<td>Number of star motifs with one hub and five spokes</td>
<td></td>
</tr>
<tr>
<td>Rich-Club Metric</td>
<td>( &lt;r_c&gt; )</td>
<td>Measure of the tendency of hub nodes (nodes with high number of links) to be well connected among each other (Colizza et al. 2006); Computed for threshold degrees between 1 and (n-1)</td>
<td>Average Rich-Club Metric of 100 random graphs generated through degree-preserving rewiring, ( RCM_{\text{rand}} )</td>
</tr>
<tr>
<td>Assortativity</td>
<td>( r )</td>
<td>Pearson correlation coefficient of degrees between pairs of linked nodes (Newman 2002).</td>
<td>-</td>
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</table>
Structure and information flow in NPC community graphs. Network efficiency and clustering coefficient are commonly used measures of efficiency in global and local information flow (see Figure 4). Metrics describing graph connectivity and information flow. (A) Network Efficiency ($\epsilon$) and Clustering Coefficient ($\sigma$) across time. Note that the values reported are normalized by corresponding random graph values (see Table 1). (B) Number of connected components ($N_{CC}$) and network density ($<k>$) across time. (C) Graph representations of images taken at day 0, day 6 and day 14. Soma are outlined in red and edges are shown in yellow. (D) Cell bodies from the images in panel D, with each connected component labeled with distinct colors. Values are reported as the mean across $N = 30$ networks $\pm$ S.E.M.
Table 1). When applied to the NPC networks, these metrics describe the efficiency of information exchange at the network-wide and local neighborhood levels through cell body proximity (compared to random graphs obtained through degree-preserving rewiring (Supplemental Figure 3)). In this context, information exchange could include flow of ions or growth factors between cells through cell-adjacent means like gap junctions. Evaluation of these metrics in NPC networks sampled across 30 different locations from two biologically independent experiments showed that network efficiency increased from day 0 to day 6, and then decreased from days 6 to 14, while clustering coefficient rose constantly from day 0 to 14 (Figure 4B). Thus, there appears to be a transition from topologies favoring global information flow to those favoring a more structured, hierarchical form of communication, occurring from day 6 – 14 of differentiation.

The correlation heatmap in Figure 3H shows a strong negative correlation between network efficiency and number of connected components in the graph. The number of connected components is a count of the number of disconnected sub-graphs in the main network and is a measure of the connectivity of the graph – a graph with a high number of connected components has a low connectivity. NPC networks at day 0, 6, and 14 are shown in Figure 4D and the corresponding connected components are shown in Figure 4E. The formation of a giant connected component due to cell proliferation up to day 6 leads to an increase in the connectivity of the network, which in turn causes an increase in network efficiency. The subsequent disaggregation of the large component into smaller modules from days 6 to 14 contributes to the decrease in efficiency seen in that time period.
**Motif counts indicative of rosette-like topologies.** We observed relatively high motif counts (star-patterns and loops) at early time points of culture (Supplemental Figure 4). Neuroepithelial cells are known to self-organize *in vitro* into rosette structures reminiscent of cross-sections of the

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![Figure 5. Motif counts indicate the presence of rosette-like structures.](image)

**(A)** Graph representations of NPC networks at day 1, 4 and 7; soma outlines are shown in red, proximity edges in yellow and edges part of a 4-star motif are highlighted in cyan. **(B)** Insets from corresponding images in panel A. **(C)** 4-star motif counts, normalized by the total possible 4-tuples. Note that 5-star and 6-star motif counts displayed similar trends (data not shown). **(D)** Schematic representation of rosette-like structures as a source of 4-star motifs. Values are reported as the mean across \(N = 10\) networks ± S.E.M.
embryonic neural tube (Wilson and Stice 2006). We hypothesized that the high number of loops and star-motifs at early time points was caused by rosette-like formations in culture. In order to investigate this more closely, we collected an additional dataset where differentiating cultures were continuously monitoring for 8 days at 1-hour intervals (Supplemental Video 1 and 2). We observed radial arrangements of columnar cells 1-2 days after removal of bFGF followed by disaggregation of these structures after day 4 (Figure 5A, 5B). 4-star motifs – topological patterns with 1 central hub cell and 4 spoke cells – are highlighted in Figure 5B and the normalized counts are shown in Figure 5C. 5- and 6-star motif counts showed similar trends (data not shown). Thus, the relative abundance of high-degree motifs at early time points can be attributed to the unique spatial arrangement of cells in a rosette-like structure (Figure 5D) and the low count at later time points corresponds to the disaggregation of these structures and more regular arrangements of cells.

Discussion

Because chemical and electrical signaling between neural progenitor cells often involves direct contact between adjacent cells, we expect spatial cell organization to be an important aspect of neural development. Two examples where spatial organization would play a role include the Notch/Delta signaling and gap junctional communication between cells. The Notch/Delta signaling pathway, which influences the proliferation and neuronal fate commitment of progenitor cells (Zhou et al. 2010; Androutsellis-Theotokis et al. 2006), is an example of juxtacrine chemical signaling. The canonical Notch signaling pathway functions through the binding of a transmembrane ligand on one cell with the transmembrane receptor on a contacting cell, resulting in the release of the notch intracellular domain (NICD) to initiate downstream signaling cascades in the contacting cell (Andersson, Sandberg, and Lendahl 2011). In addition, immature neural circuits are known to display spontaneous electrical activity, which is an
important aspect of their proper development (Spitzer 2006; Blankenship and Feller 2010). Gap
junctions or electrical synapses allow direct access between cells and result in exchange of ions
and growth factors, and these are known to be important in the propagation of spontaneous
electrical activity. Indeed, neural progenitor cells have been shown to display structured and
synchronous calcium activity, dependent on gap junctions and which promotes cell proliferation
(Malmersjö et al. 2013). More broadly, structured cell-cell communication has been implicated in
coordinated chemosensing (Sun et al. 2012) and migration during development (Friedl and
Gilmour 2009). Thus, the methods of cell-cell communication employed by immature neural cells
indicate the significance of spatially organized electrical and chemical signaling.

Our study provides quantification of the spatial organization of immature neural cells during
differentiation, using a unique application of graph theory. The experimental paradigm presented
here enabled us to uncover relationships between spatial topology of NPC communities and
functional maturation of developing neural circuits, and allowed us to develop hypotheses about
the role of certain topologies on NPC function. It is to be noted that not all metrics derived from
graph theory have a ready biological interpretation, especially in the context of spatial graphs. For
example, interpretation of metrics like degree-degree correlations and rich-club metric
(Supplemental Figure 4) are limited, due to the implicit limit in the type of connections that are
possible in spatial graphs. Keeping this in mind, we analyzed metrics with an intuitive biological
interpretation, i.e., information flow and connectivity.

We use our graph-based approach to analyze trends in information flow in NPC networks. Global
network efficiency rises from day 0 – 6, and then falls from day 6 – 14. The trend in network
efficiency is intuitively explained by its negative correlation with the number of connected
components, a measure of graph connectivity. Cell proliferation from day 0 – 6 leads to the
merging of many disconnected clusters of cells into a giant connected component, leading to a
rise in the overall connectivity and reduction in the average path length. The reorganization of the giant component into smaller modules from day 6–14 leads to a reduction in network efficiency. Overall, our data indicates that the topologies observed at intermediate stages of differentiation would facilitate cellular behavior requiring network-wide coordination, like chemosensing, migration and proliferation (Figure 6).

We observed a constant rise in clustering coefficient, a measure of efficiency in local information flow. Previous studies on dissociated cultures have shown the prevalence of clustering of neuron cell bodies during maturation in culture (Shefi et al. 2002; de Santos-Sierra, Sendiña-Nadal, Leyva, Almendral, Ayali, et al. 2014), indicating a common pattern in neuron self-organization and wiring. High clustering facilitating local computations, accompanied by long-range neurite connections to connect different clusters has been hypothesized to facilitate increased wiring efficiency in mature neural circuits. The characteristic “small-world” network topology is also hypothesized to be the organizing principle in the human brain (Bullmore et al. 2009). Thus, the reorganization of cell bodies into highly clustered units in our study supports this view of maturing neural circuits.

The fall of network efficiency and rise of clustering coefficient (compared to random networks) from day 6-14 indicates a shift from topologies favoring global information flow to those favoring information flow in more restricted neighborhoods. It is interesting to note that this shift occurs around the time mature neuronal phenotypes appear in culture. We believe this indicates a shift in the mode of communication from network-wide signaling to a more structured, hierarchical form of communication in mature neuronal networks. As discussed previously, network-wide communication is implicated in facilitating behaviors like cell proliferation, migration and coordinated chemosensing, while the reorganization of more mature neuronal
cultures into highly clustered cell bodies likely serves the purpose of increasing the wiring efficiency. Conceptually, these ideas are represented in the schematic in Figure 6.

Our method also detected the formation of neural rosette-like structures. Neural rosettes are known to harbor specific phenotypes such as apico-basal polarity, active Notch signaling and interkinetic nuclear migration, all of which are involved in the proper sequential production of neurons and glia (Abranches et al. 2009; Wilson and Stice 2006). We found that high-degree star-motifs (comprising of a hub cell with 4-6 spoke cells) were present in high numbers at early time points.

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**Figure 6.** Schematic illustrating the observed changes in neural cell information flow during differentiation.
points (within 1-2 days of the differentiation stimulus) due to the unique geometric arrangement of cells forming rosette-like structures. We also observed a decrease in star-motif counts corresponding with the disaggregation of these structures after day 4. These results further highlight the useful biological information that can be obtained through topological analysis of differentiating hNPCs.

In this study, we show that network analysis provides unique information about the structure of neural progenitor cell communities at the local and global levels. It remains to be seen whether spatial topology of developing cultures is predictive of synaptic connectivity in mature neuronal networks. Several in vivo studies have provided evidence for a structure-functional relationship between adult neuronal wiring and the spatiotemporal origin of the constituent neurons. For example, sister excitatory neurons in the neocortex are more likely to develop synapses with each other rather than with other cells (Yu et al. 2009), and the electrophysiological phenotypes of GABAergic interneurons have been shown to be dependent on the time and place of their birth (Butt et al. 2005). Thus, the analysis of spatial topology in developing neuronal circuits in a controlled setting has the potential to uncover structure-function relationships in the resulting mature neural circuits.

The present study also lays the foundation for analysis of the role of cellular neighborhood on cell fate determination of individual progenitor cells. The expression of cell-fate determination factors such as bHLH transcription factors like Hes1 and Ngn2, and proteins involved in cell-cell communication pathways such as Notch/Delta proteins, have been shown to be tightly coupled with each other (Shimojo, Ohtsuka, and Kageyama 2008; Kageyama et al. 2009). Computational modeling studies have predicted that Notch-Hes1 intercellular signaling affects differentiation and cell cycle progression of individual cells and this signaling is important for the maintenance of an optimal balance between differentiating cells and self-renewing progenitor cells (Pfeuty
2015). The spatial dynamics of cell-cell signaling and its impact on single-cell differentiation status is an intriguing subject for future study.

In conclusion, we present a multiplexed approach integrating long-term live imaging, automated image analysis, and a unique graph-based analysis to quantify the spatial organization of neural progenitors during neuronal differentiation. Our method introduces a tangible means to test theories about spatially-dependent forms of neural cell communication. Insights from this study help further our understanding of the design principles involved in the development of functional neural networks. Applications of this work can help pave the way for systematic modulation of neural cell self-organization for therapeutic purposes.

Methods

hNPC culture. Human neural progenitor cells (hNP1™) derived from H9 human embryonic stem cells were obtained from ArunA Biomedicals (Athens, GA). Cells were expanded on tissue culture flasks pre-coated with either fibronectin (Sigma-Aldrich) or Matrigel (BD Biosciences), in proliferation medium consisting of AB2™ basal neural medium, ANS™ neural supplement (both supplied by manufacturer), 10 ng/ml leukemia inhibitory factor (LIF; EMD Millipore), 20 ng/ml basic fibroblast growth factor (bFGF; R&D Systems) and 2 mM GlutaMAX™ supplement (Life Technologies). For neuronal differentiation experiments, cells were plated on different substrates pre-coated with Matrigel and cultured in differentiation medium (proliferation medium lacking bFGF).

Electrophysiology. For whole-cell patch clamp experiments, cultures were maintained in extracellular recording solution containing 119 mM NaCl, 5 mM KCl, 10 mM HEPES, 2 mM CaCl2 and 1 mM MgCl2, titrated to a pH of 7.3. Pipettes (5-10 MΩ) were pulled from standard
borosilicate glass capillaries and back filled with intracellular recording solution containing 8 mM NaCl, 10 mM KCl, 5 mM HEPES, 0.06 mM CaCl$_2$, 5 mM MgCl$_2$, 130 mM potassium gluconate and 0.6 mM EGTA, titrated to a pH of 7.4. Recordings were performed using a MultiClamp 700A amplifier and a Digidata 1550 Data Acquisition System coupled with Clampex 10.4 software (Molecular Devices). Traces were analyzed in MATLAB.

In voltage-clamp experiments, cells were held at a holding potential of -50 mV and given a series of voltage steps from -90 to +100 mV. In current-clamp experiments, cells were held at approximately -70 mV through minimal current injection before application of a series of current steps ranging from -40 to +120 pA. Magnitudes of the current steps were modified according to the input resistance. Peak outward current amplitude was measured 40 ms after the initiation of the voltage sweep. Peak inward current was defined as the maximum transient negative current at any command voltage.

**Immunocytochemistry.** For immunostaining experiments, hNPCs were plated on Matrigel-coated 12 mm glass coverslips and differentiated as described above. Cultures were fixed with 4% paraformaldehyde for 20 min, permeabilized with 0.2% Triton-X for 5 min and blocked with 6% goat serum for 45 min. Primary antibodies used were mouse Nestin (1:200) and rabbit MAP2 (1:500). Secondary antibodies were Alexa Fluor 488 goat anti-mouse (1:1000), Alexa Fluor 594 goat anti-rabbit (1:1000) and cell nuclei were stained using Hoescht dye.

**Time-lapse Microscopy.** For all time-lapse imaging experiments, hNPCs were plated at approximately 50% confluence on 12-well plates pre-coated with Matrigel and switched to differentiation medium 24 hours post-plating. Two datasets (biological replicates) were obtained by imaging the well plates at days 0, 3, 6, 9, 12 and 14 after withdrawal of bFGF from culture medium, using an automated stage Nikon Eclipse Ti-E Microscope. At the start of the
experiment, 5 locations were chosen arbitrarily for each well, and the same locations were imaged at each time point. Imaging sessions lasted about 10 minutes and the plates were returned to the incubator after imaging. A third dataset was obtained through continuous imaging, for which the well plate was mounted on the stage of the microscope in a bold line cage incubator (Okolabs) equipped with temperature control and gas flow rate control enabling a 37°C 5% CO₂ environment. For this dataset, images were acquired at 1-hour intervals for 8 days (Supplemental Video 1). In all imaging experiments, 8-bit phase contrast images were acquired through a 10X objective and at a resolution of 1280 x 1080 pixels. Physical pixel size was 0.64μm.

**Image Processing.** Image sequences were chosen for analysis based on the ability of a human observer to distinguish cellular features in the images. Images with large amounts of debris occluding cells were discarded manually. In this manner, a total of 16, 14 and 10 image sequences for each of the 3 independent datasets were chosen for analysis.

The selected time-lapse image sequences were analyzed using custom-written MATLAB code. Grayscale images were pre-processed by applying a median filter with a neighborhood of 3x3 pixels to remove noise and segmented using an unbiased intensity-gradient thresholding approach (Curl et al. 2004). Starting from the grayscale image, the first derivative of the pixel intensity histogram was calculated. Fitting a linear function to the ascending portion of the first derivative and extrapolating to the x-axis resulted in a grayscale threshold, which was used to generate a binary image distinguishing cellular features from the background. Morphological operations performed on the binary image were:

1. Small objects of size lesser than 50 pixels were removed to filter out noise and other imaging artifacts.
2. Morphological opening was performed using a disk structuring element of radius 4 pixels. This was done to separate linear features (neurites), and circular features (cell bodies).

3. Neurites were skeletonized using the bwmorph function in MATLAB to obtain neurite length statistics.

4. Cell bodies were separated using connected component labeling using the default 8-connected neighborhood.

5. Cell body objects smaller than 150 pixels and those touching the image border were removed.

All parameters used in image processing are listed in Table S1.

In order to quantify the accuracy of our image processing algorithms, we compared the results with manual tracing of soma. These results showed a close agreement between the numbers of cells detected by our algorithm and by manual tracing at different time points (Supplemental Figure 1).

Graph Representation of Microscope Images. For each pair of soma, a threshold distance for proximity was defined as the average of the two soma diameters, multiplied by a scaling factor (S). If the Euclidean distance between the soma centroids was lower than the threshold distance computed, then the pair of soma was connected with a “proximity edge” (Figure 3E-G).

In order to capture changes in network structure across a 14-day period of differentiation, we chose to use label-free phase contrast imaging (Weber et al. 2013). Immunostaining or the use of fluorescent reporters would have resulted in images with better signal-to-noise to aid in image analysis, but we chose to avoid these methods due to the inability to perform longitudinal imaging without harming cell health. Due to the limits of our imaging method, automated image
segmentation often detected a smaller region than the exact cell boundary. In order to compensate for this, we used a scaling factor to define spatial proximity for graph representations. Small values of the scaling factor (1<S<2) resulted in sparse graphs, where interpretation of metrics became difficult. Higher values of the scaling factor (2<S<3) resulted in qualitatively similar results (Supplemental Figure 2), and we therefore chose S = 2 as the default scaling factor.

**Metric Computation.** All the network metrics described in Table 1 were computed using custom-written code, building upon the routines provided in (Bounova and De Weck 2012). In addition, some parameters like connected components and path lengths were computed using built-in MATLAB functions.

Random graphs were constructed through degree-preserving rewiring, maintaining the degree distribution of the original graph (Supplemental Figure 3). Each link (edge) belonging to any given node in the original graph was randomly re-assigned to a node that was chosen from all possible nodes with uniform probability. Metrics computed for random graphs were averaged across 100 different realizations of the random graphs. This mode of random graph generation was chosen to eliminate finite-size effects inherent in other models of random graphs such as Erdős-Rényi random graphs.

To ensure robustness of the network metrics, we tested varying fields of view for the images, and confirmed the trends remained consistent (Supplemental Figure 5).

**Statistical Analyses.** Data are presented as means ± S.E.M. of a minimum of 3 experiments, unless indicated otherwise. Student t-test was performed to analyze peak currents in electrophysiology experiments, with significance accepted at *P < 0.05.
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Author Contributions

All authors designed the experiments. A.S.M. performed the experiments. A.A.Q. and A.S.M. analyzed the data. All authors contributed to writing the manuscript. A.A.Q. and J.T.R. supervised the work.

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