

1 **Title: The Social Networks of Neural Progenitor Cells**

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25 **Abstract**

26

27 Quantitative understanding of how neural progenitor cells (NPCs) collectively self-organize into neural
28 networks can provide critical insight into how to optimize neural regenerative strategies. To that end, we
29 characterized the topology of human embryonic NPCs during differentiation by designing and employing
30 a spatial graph-theoretic analysis. Statistical measures of information flow in NPC spatial graphs revealed
31 a shift from topologies with high global efficiency to high local efficiency, around the time mature
32 neuronal phenotypes appeared in culture. These results support the view that network-wide signaling in
33 immature progenitor cells gives way to a more structured, hierarchical form of communication in mature
34 neural networks. We also demonstrate that the evaluation of recurring motif patterns in NPC graphs
35 reveals unique geometric arrangements of cells in neural rosette-like structures at early stages of
36 differentiation. Our approach provides insight into the design of developing neural networks, opening the
37 door for new approaches that modulate neural cell self-organization for therapeutic applications.

38

39 Human embryonic stem cells (hESCs) and other sources of pluripotent stem cells have provided much
40 hope for regenerative medicine, especially in the nervous system (Fred H. Gage and Temple 2013).
41 Recently, stem cell therapies have been reported to treat neurological disorders, including stroke and
42 neurodegenerative diseases like Parkinson's disease (Hallett et al. 2014; Rosado-de-Castro et al. 2013). In
43 addition, neural progenitor cells derived from embryonic stem cells (hNPCs) and dissociated cultures
44 have helped elucidate intrinsic transcriptional control of self-renewal and multipotency (Guillemot 2007;
45 Imayoshi and Kageyama 2014; Shen et al. 2006; Kageyama et al. 2009). The influence of external cues
46 such as morphogen gradients in guiding nervous system development is also well documented (Mason
47 2007; Bertrand and Dahmane 2006). Aside from intrinsic genetic programs and extrinsic cues, a third
48 aspect of stem cell differentiation that merits further study is the collective self-organization of progenitor
49 cells into functional neural networks. Quantitative insight into neural cell self-organization, in addition to
50 knowledge of external and intrinsic cues that guide tissue development, will ultimately be necessary to
51 design effective and targeted stem cell therapies.

52

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

64 immediate cellular neighborhood of a progenitor cell provides an important context in which to place its
65 dynamic behavior.

66

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

83

84 Here we present a detailed characterization of spatial organization of H9-derived human neural progenitor
85 cells during neural differentiation over the course of 14 days. To achieve this characterization, we
86 introduce a new approach that integrates weeks-long live imaging assays, automated image analysis and a
87 graph-theoretic approach based on cell body proximity to quantify the spatial organization of progenitor
88 cells. A schematic representation of our experimental paradigm is shown in Figure 1. We use our method
89 to demonstrate that NPCs transition from topologies with high global efficiency to those with high local

90 efficiency for information flow around the time mature neuronal phenotypes appear in culture. We also
91 show that our graph-based metrics capture the characteristic geometric arrangement of cells in neural
92 rosette-like structures seen at early time points of differentiation.

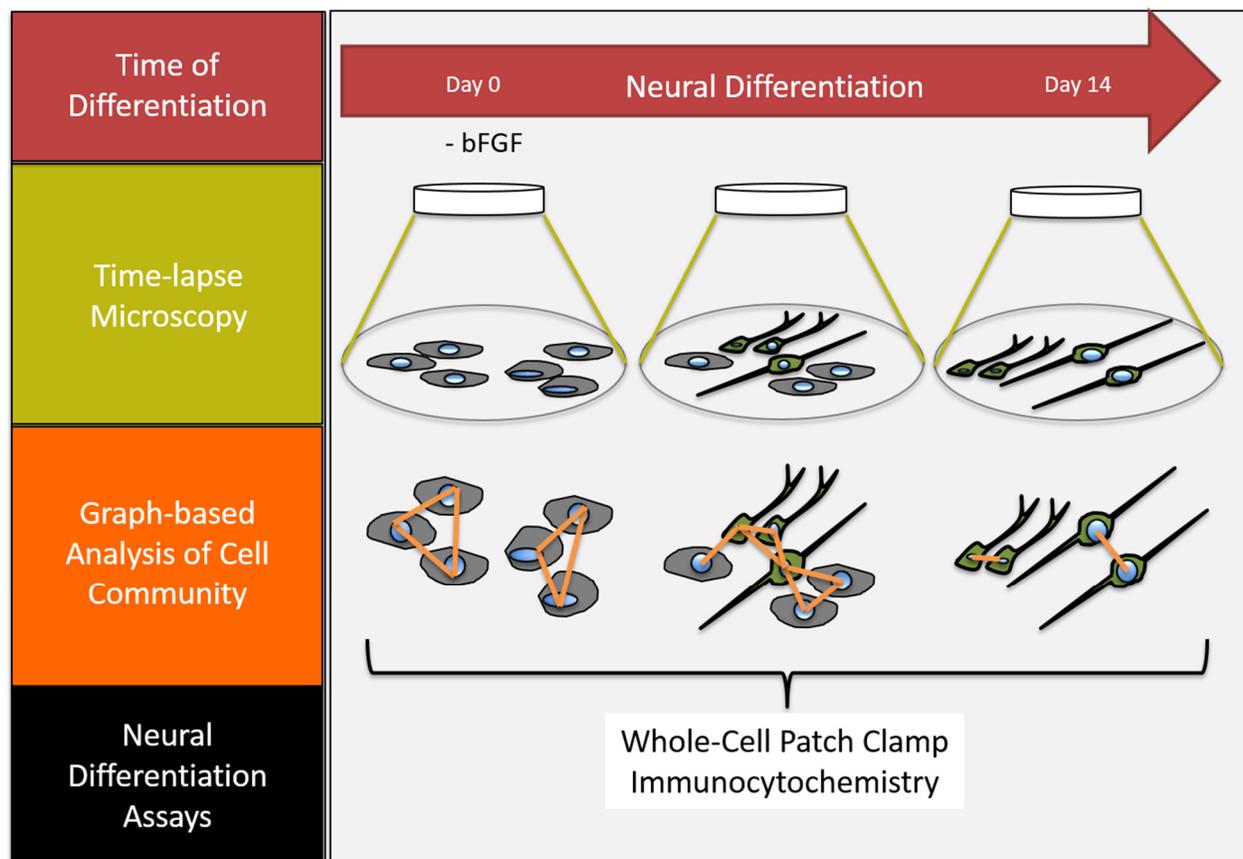


Figure 1. Schematic illustrating the design of the experiments.

93 **Results**

94

95 **Functional characterization of differentiating hNPC cultures.** The model cell culture system used in

96 this study is neural progenitor cells derived from H9 human embryonic stem cells. These cells were

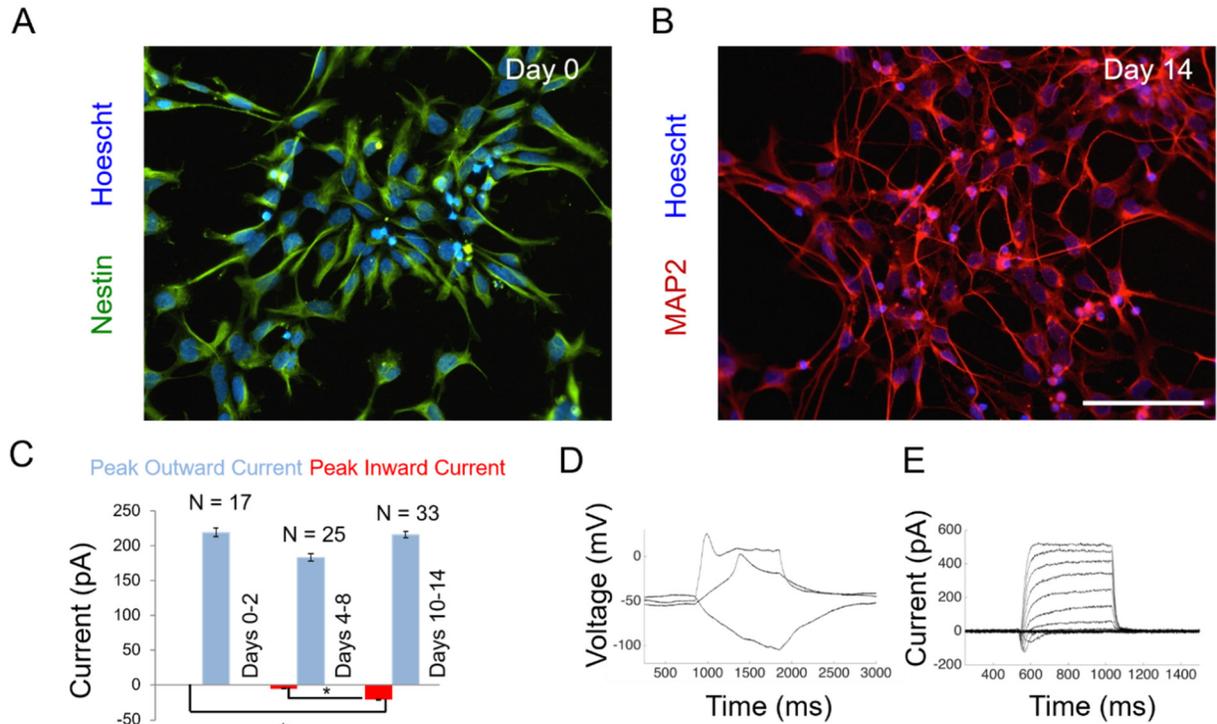


Figure 2. Functional characterization of differentiating hNPCs. (A) hNPCs at day 0 stain positively for Nestin. Nuclei are labeled by Hoescht. (B) Cells at day 14 stain positive for MAP2. Nuclei are labeled by Hoescht (scale bar = 100 μ m). (C) Peak inward and outward currents determined through whole-cell 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.

97 maintained as undifferentiated, mitotic progenitor cells in the presence of the mitogen basic fibroblast
98 growth factor (bFGF). Withdrawal of bFGF from culture medium was used to induce spontaneous
99 differentiation of hNPCs (F. H. Gage 2000).

100

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

115

116 **Representation of cell community structure using a graph-based approach.** In order to uncover
117 topological changes in differentiating hNPCs, we combined long-term time-lapse microscopy of
118 differentiating cultures with a graph-based approach for quantifying cell community structure. We
119 conducted two biologically independent experiments where cultures were imaged at days 0, 3, 6, 9, 12
120 and 14 after withdrawal of bFGF. An additional dataset was obtained by imaging differentiating cultures
121 at 1-hour intervals for a total duration of 8 days (Supplemental Video 1). Selected image sequences were

122 analyzed using custom image-processing algorithms, resulting in the extraction of soma and neurites for

123 each phase-contrast image (Figure 3A-D) (see Methods section for details).

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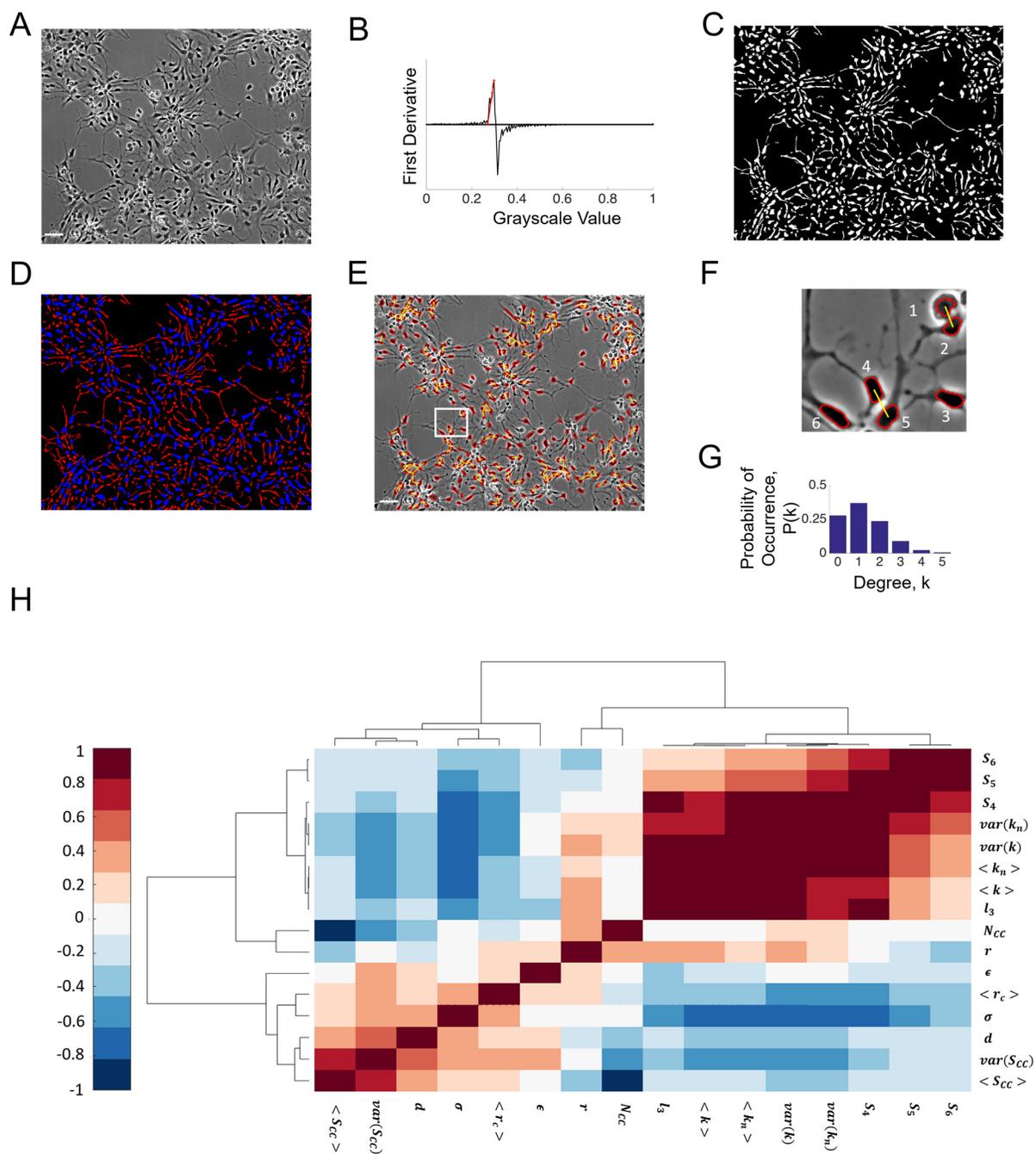


Figure 3. Image processing and graph representation of time-lapse microscopy images.

(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, $\langle k \rangle = 0.96$ (H) Correlation heatmap of all metrics obtained by hierarchical clustering of the covariance matrix.

125

126 neural circuits. In order to build quantitative representations of hNPC communities that reflect the modes
127 of communication employed by hNPCs, we developed a graph-based approach, where soma are denoted
128 as nodes and spatial proximity of cells is used to assign edges. In this manner, we constructed non-
129 weighted, undirected graphs representing hNPC communities from time-lapse microscope images
130 (Supplemental Video 2).

131

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

139 network efficiency and number of connected components, and those between clustering coefficient and all
 140 degree-related metrics. In the following sections, we focus on metrics that have intuitive biological
 141 interpretations, their trends across time of differentiation, and relationships observed with other metrics
 142 that explain these trends. Trends in metrics not discussed in the main text are shown in Supplemental
 143 Figure 4.

144
 145 **Table 1. Metrics computed, their descriptions, and mode of normalization to account for**
 146 **the network size. n = number of nodes, m = number of edges.**

| Graph Metrics | Symbol | Definition | Normalization |
|-----------------------------|-----------------------|---|---|
| Network Density | $\langle k \rangle$ | Average degree of graph, normalized by total maximum possible degree | Maximum possible degree, $(n - 1)$ |
| | | $\langle k \rangle = \frac{2m}{n(n - 1)}$ | |
| Variance in Degree | $var(k)$ | Variance of normalized node degree sequence | Node degree sequence normalized by maximum possible degree, $(n-1)$ |
| Average Neighbor Degree | $\langle k_n \rangle$ | Average degree of node neighborhood, across all nodes | Maximum possible degree, $(n-1)$ |
| Variance in Neighbor Degree | $var(k_n)$ | Variance of the normalized average neighbor degree sequence | - |
| Network Efficiency | ϵ | The average reciprocal of shortest path length across all pairs of nodes, E | Average network efficiency of 100 random graphs generated through degree-preserving rewiring, E_{rand} . Random graph generation is illustrated in Fig. S3. |
| Average Clustering | σ | Fraction of total possible links among the | Average clustering |

| | | | |
|--|--------------------------|--|--|
| Coefficient | | neighbors of a node that are actually present, averaged across all nodes, C | coefficient of 100 random graphs generated through degree-preserving rewiring, C_{rand} |
| Number of connected components | N_{CC} | Number of disconnected sub-graphs in main graph | - |
| Average Size of Connected Components | $\langle S_{CC} \rangle$ | Average number of nodes in each connected component | Total nodes, n |
| Variance in size of connected components | $var(S_{CC})$ | - | - |
| Network Diameter | d | Longest shortest path length of network | Longest possible path, $(n-1)$ |
| Triangular loop count | l_3 | Number of loops of 3 nodes | Total possible number of triplets, ${}^n C_3$ |
| 4-star motif Count | S_4 | Number of star motifs with one hub and three spokes | Total possible number of 4-tuples, ${}^n C_4$ |
| 5-star motif count | S_5 | Number of star motifs with one hub and four spokes | Total possible number of 5-tuples, ${}^n C_5$ |
| 6-star motif count | S_6 | Number of star motifs with one hub and five spokes | Total possible number of 6-tuples, ${}^n C_6$ |
| Rich-Club Metric Average | $\langle r_c \rangle$ | 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 $(n-1)$ | Average Rich-Club Metric of 100 random graphs generated through degree-preserving rewiring, RCM_{rand} |
| Assortativity | r | Pearson correlation coefficient of degrees between pairs of linked nodes (Newman 2002). | - |

148 **Structure and information flow in NPC community graphs.** Network efficiency and clustering
149 coefficient are commonly used measures of efficiency in global and local information flow (see

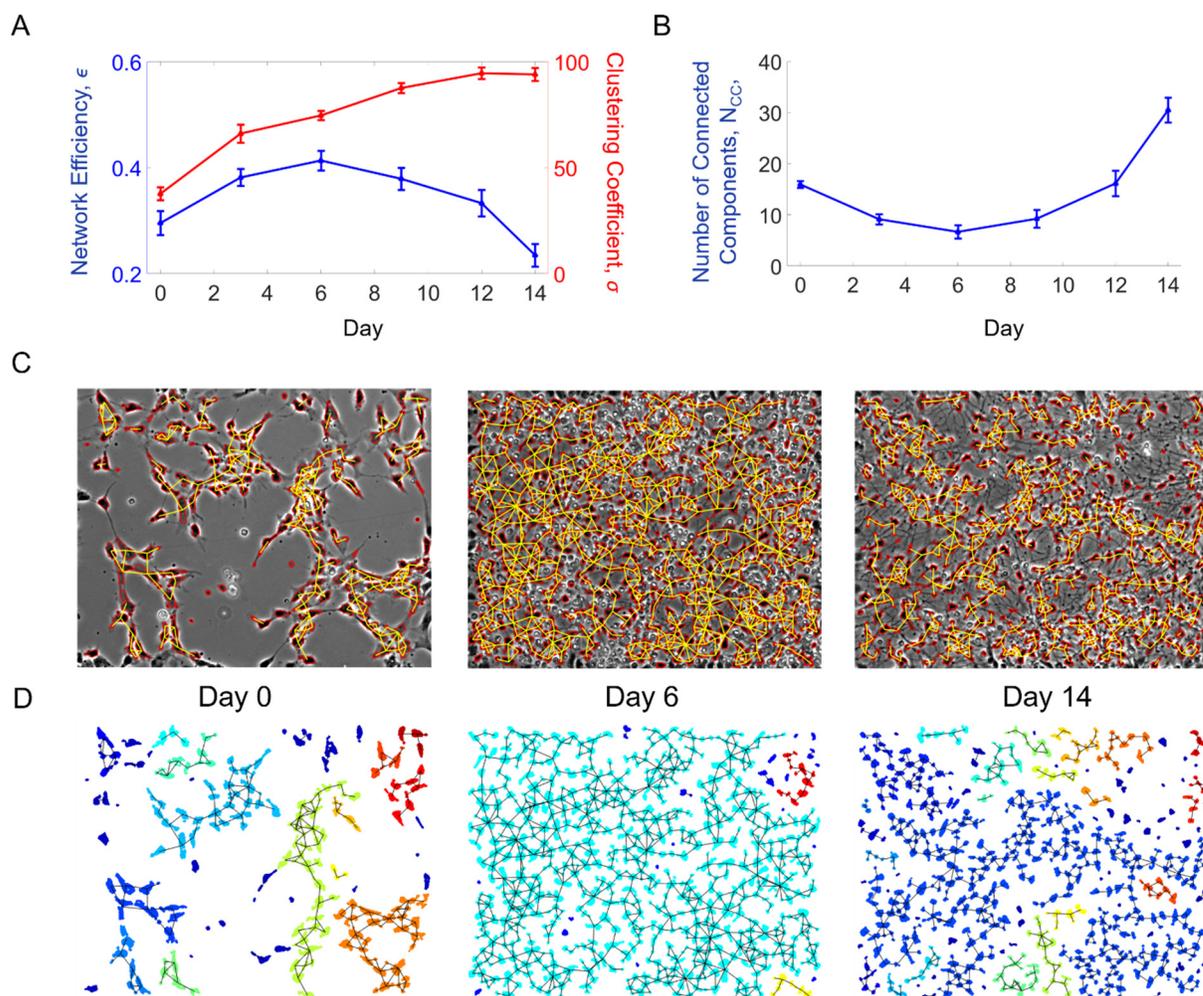


Figure 4. Metrics describing graph connectivity and information flow. (A) Network Efficiency (ϵ) and Clustering Coefficient (σ) across time. Note that the values reported are normalized by corresponding random graph values (see Table 1). (B) Number of connected components (N_{CC}) and network density ($\langle k \rangle$) 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

150 Table 1). When applied to the NPC networks, these metrics describe the efficiency of information
151 exchange at the network-wide and local neighborhood levels through cell body proximity
152 (compared to random graphs obtained through degree-preserving rewiring (Supplemental Figure
153 3)). In this context, information exchange could include flow of ions or growth factors between
154 cells through cell-adjacent means like gap junctions. Evaluation of these metrics in NPC networks
155 sampled across 30 different locations from two biologically independent experiments showed that
156 network efficiency increased from day 0 to day 6, and then decreased from days 6 to 14, while
157 clustering coefficient rose constantly from day 0 to 14 (Figure 4B). Thus, there appears to be a
158 transition from topologies favoring global information flow to those favoring a more structured,
159 hierarchical form of communication, occurring from day 6 – 14 of differentiation.

160

161 The correlation heatmap in Figure 3H shows a strong negative correlation between network
162 efficiency and number of connected components in the graph. The number of connected
163 components is a count of the number of disconnected sub-graphs in the main network and is a
164 measure of the connectivity of the graph – a graph with a high number of connected components
165 has a low connectivity. NPC networks at day 0, 6, and 14 are shown in Figure 4D and the
166 corresponding connected components are shown in Figure 4E. The formation of a giant connected
167 component due to cell proliferation up to day 6 leads to an increase in the connectivity of the
168 network, which in turn causes an increase in network efficiency. The subsequent disaggregation
169 of the large component into smaller modules from days 6 to 14 contributes to the decrease in
170 efficiency seen in that time period.

171 **Motif counts indicative of rosette-like topologies.** We observed relatively high motif counts
172 (star-patterns and loops) at early time points of culture (Supplemental Figure 4). Neuroepithelial
173 cells are known to self-organize *in vitro* into rosette structures reminiscent of cross-sections of the

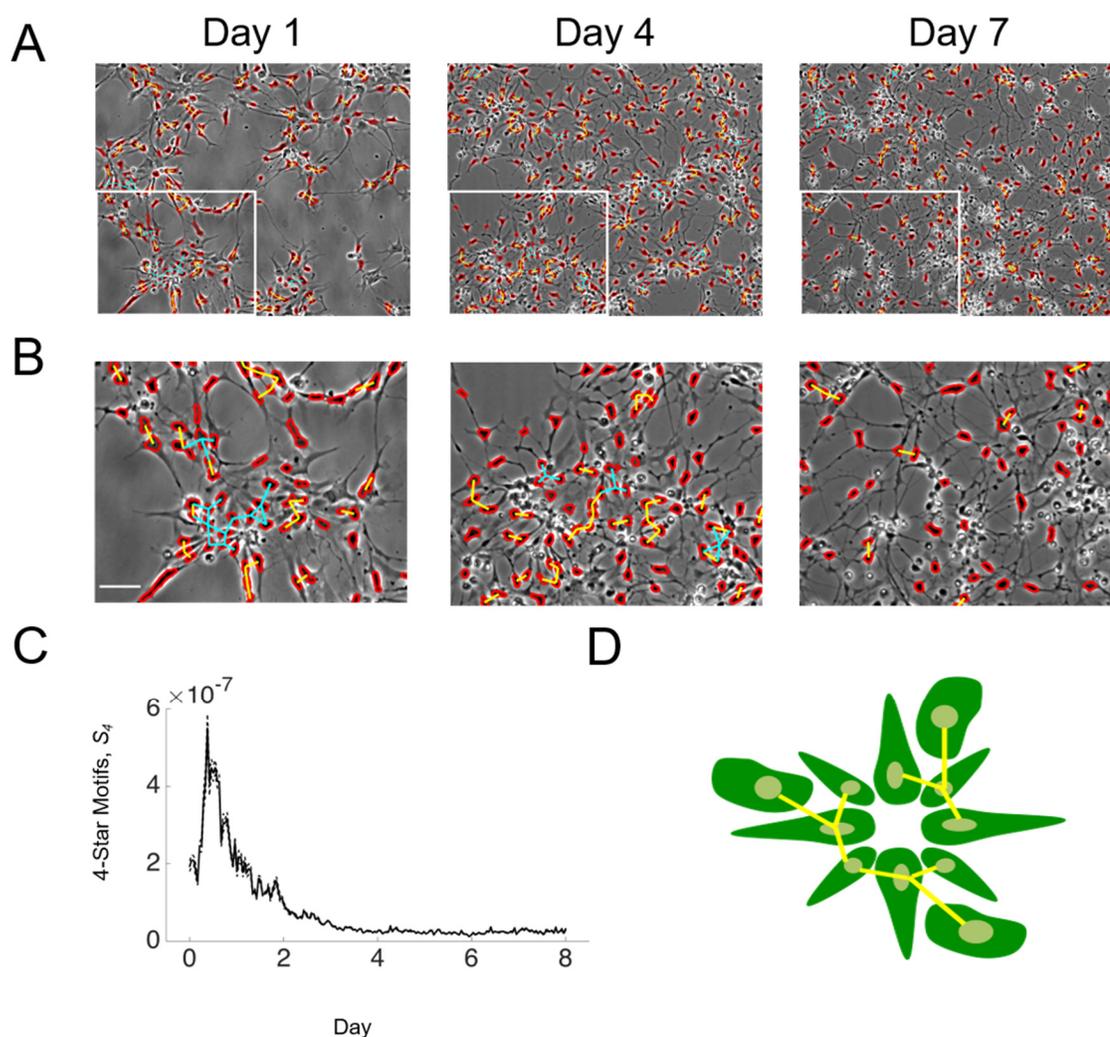


Figure 5. Motif counts indicate the presence of rosette-like structures. (A) Graph representations of NPC networks at day 1, 4 and 7; soma outlines are shown in red, proximity edges in yellow and edges part of a 4-star motif are highlighted in cyan. (B) Insets from corresponding images in panel A. (C) 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 \pm S.E.M.

174 embryonic neural tube (Wilson and Stice 2006). We hypothesized that the high number of loops
175 and star-motifs at early time points was caused by rosette-like formations in culture. In order to
176 investigate this more closely, we collected an additional dataset where differentiating cultures
177 were continuously monitoring for 8 days at 1-hour intervals (Supplemental Video 1 and 2). We
178 observed radial arrangements of columnar cells 1-2 days after removal of bFGF followed by
179 disaggregation of these structures after day 4 (Figure 5A, 5B). 4-star motifs – topological patterns
180 with 1 central hub cell and 4 spoke cells – are highlighted in Figure 5B and the normalized counts
181 are shown in Figure 5C. 5- and 6-star motif counts showed similar trends (data not shown). Thus,
182 the relative abundance of high-degree motifs at early time points can be attributed to the unique
183 spatial arrangement of cells in a rosette-like structure (Figure 5D) and the low count at later time
184 points corresponds to the disaggregation of these structures and more regular arrangements of
185 cells.

186

187 **Discussion**

188 Because chemical and electrical signaling between neural progenitor cells often involves direct
189 contact between adjacent cells, we expect spatial cell organization to be an important aspect of
190 neural development. Two examples where spatial organization would play a role include the
191 Notch/Delta signaling and gap junctional communication between cells. The Notch/Delta
192 signaling pathway, which influences the proliferation and neuronal fate commitment of
193 progenitor cells (Zhou et al. 2010; Androutsellis-Theotokis et al. 2006), is an example of
194 juxtacrine chemical signaling. The canonical Notch signaling pathway functions through the
195 binding of a transmembrane ligand on one cell with the transmembrane receptor on a contacting
196 cell, resulting in the release of the notch intracellular domain (NICD) to initiate downstream
197 signaling cascades in the contacting cell (Andersson, Sandberg, and Lendahl 2011). In addition,
198 immature neural circuits are known to display spontaneous electrical activity, which is an

199 important aspect of their proper development (Spitzer 2006; Blankenship and Feller 2010). Gap
200 junctions or electrical synapses allow direct access between cells and result in exchange of ions
201 and growth factors, and these are known to be important in the propagation of spontaneous
202 electrical activity. Indeed, neural progenitor cells have been shown to display structured and
203 synchronous calcium activity, dependent on gap junctions and which promotes cell proliferation
204 (Malmersjö et al. 2013). More broadly, structured cell-cell communication has been implicated in
205 coordinated chemosensing (Sun et al. 2012) and migration during development (Friedl and
206 Gilmour 2009). Thus, the methods of cell-cell communication employed by immature neural cells
207 indicate the significance of spatially organized electrical and chemical signaling.

208

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

219

220 We use our graph-based approach to analyze trends in information flow in NPC networks. Global
221 network efficiency rises from day 0 – 6, and then falls from day 6 – 14. The trend in network
222 efficiency is intuitively explained by its negative correlation with the number of connected
223 components, a measure of graph connectivity. Cell proliferation from day 0 – 6 leads to the
224 merging of many disconnected clusters of cells into a giant connected component, leading to a

225 rise in the overall connectivity and reduction in the average path length. The reorganization of the
226 giant component into smaller modules from day 6 – 14 leads to a reduction in network efficiency.
227 Overall, our data indicates that the topologies observed at intermediate stages of differentiation
228 would facilitate cellular behavior requiring network-wide coordination, like chemosensing,
229 migration and proliferation (Figure 6).

230

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

241

242 The fall of network efficiency and rise of clustering coefficient (compared to random networks)
243 from day 6 -14 indicates a shift from topologies favoring global information flow to those
244 favoring information flow in more restricted neighborhoods. It is interesting to note that this shift
245 occurs around the time mature neuronal phenotypes appear in culture. We believe this indicates a
246 shift in the mode of communication from network-wide signaling to a more structured,
247 hierarchical form of communication in mature neuronal networks. As discussed previously,
248 network-wide communication is implicated in facilitating behaviors like cell proliferation,
249 migration and coordinated chemosensing, while the reorganization of more mature neuronal

250 cultures into highly clustered cell bodies likely serves the purpose of increasing the wiring
251 efficiency. Conceptually, these ideas are represented in the schematic in Figure 6.

252

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

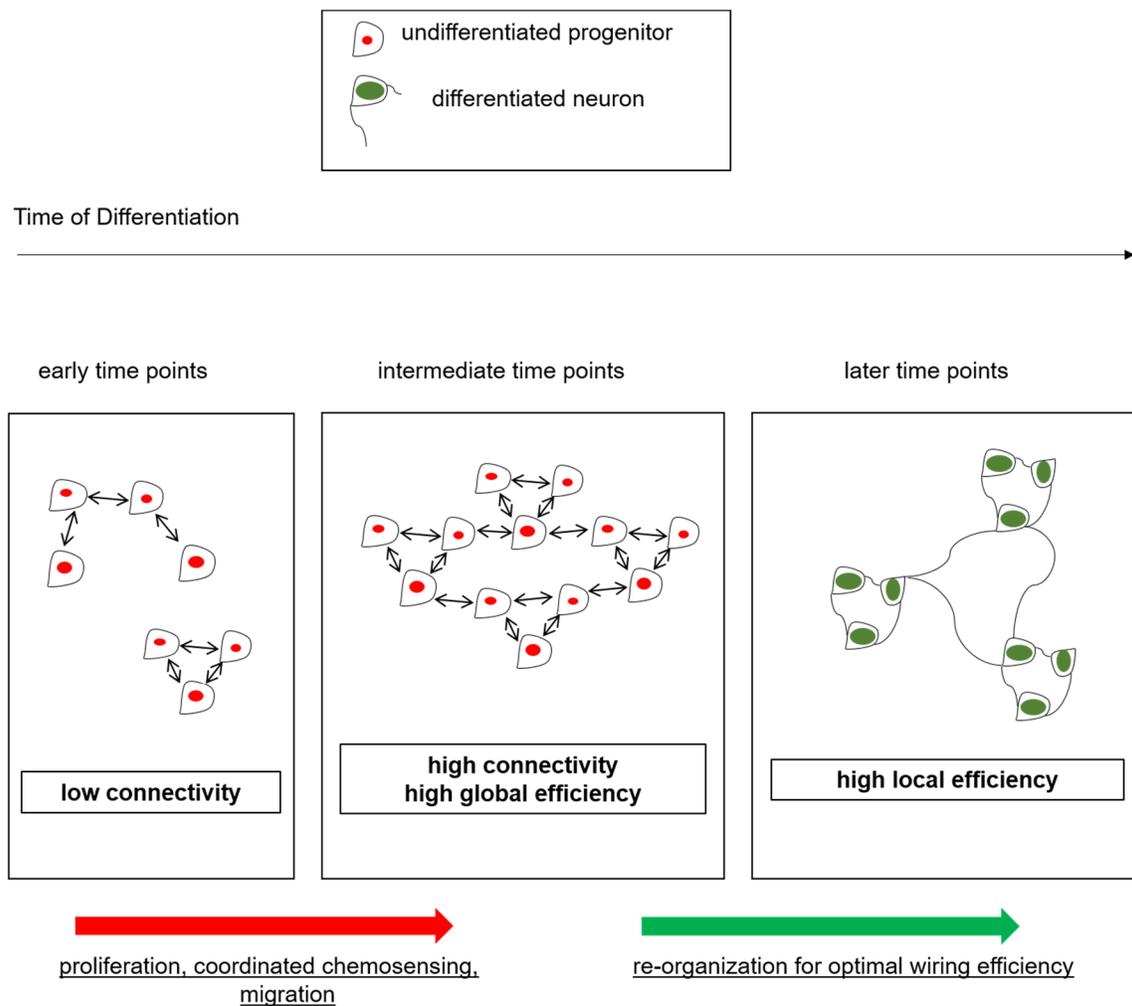


Figure 6. Schematic illustrating the observed changes in neural cell information flow during differentiation.

258 points (within 1-2 days of the differentiation stimulus) due to the unique geometric arrangement
259 of cells forming rosette-like structures. We also observed a decrease in star-motif counts
260 corresponding with the disaggregation of these structures after day 4. These results further
261 highlight the useful biological information that can be obtained through topological analysis of
262 differentiating hNPCs.

263

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

275

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

284 2015). The spatial dynamics of cell-cell signaling and its impact on single-cell differentiation
285 status is an intriguing subject for future study.

286

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

294

295 **Methods**

296

297 **hNPC culture.** Human neural progenitor cells (hNP1TM) derived from H9 human embryonic
298 stem cells were obtained from Aruna Biomedicals (Athens, GA). Cells were expanded on tissue
299 culture flasks pre-coated with either fibronectin (Sigma-Aldrich) or Matrigel (BD Biosciences), in
300 proliferation medium consisting of AB2TM basal neural medium, ANSTM neural supplement (both
301 supplied by manufacturer), 10 ng/ml leukemia inhibitory factor (LIF; EMD Millipore), 20 ng/ml
302 basic fibroblast growth factor (bFGF; R&D Systems) and 2 mM GlutaMAXTM supplement (Life
303 Technologies). For neuronal differentiation experiments, cells were plated on different substrates
304 pre-coated with Matrigel and cultured in differentiation medium (proliferation medium lacking
305 bFGF).

306

307 **Electrophysiology.** For whole-cell patch clamp experiments, cultures were maintained in
308 extracellular recording solution containing 119 mM NaCl, 5 mM KCl, 10 mM HEPES, 2 mM
309 CaCl₂ and 1mM MgCl₂, titrated to a pH of 7.3. Pipettes (5-10 MΩ) were pulled from standard

310 borosilicate glass capillaries and back filled with intracellular recording solution containing 8 mM
311 NaCl, 10mM KCl, 5 mM HEPES, 0.06 mM CaCl₂, 5 mM MgCl₂, 130 mM potassium gluconate
312 and 0.6mM EGTA, titrated to a pH of 7.4. Recordings were performed using a MultiClamp 700A
313 amplifier and a Digidata 1550 Data Acquisition System coupled with Clampex 10.4 software
314 (Molecular Devices). Traces were analyzed in MATLAB.

315

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

323

324 **Immunocytochemistry.** For immunostaining experiments, hNPCs were plated on Matrigel-
325 coated 12 mm glass coverslips and differentiated as described above. Cultures were fixed with
326 4% paraformaldehyde for 20 min, permeabilized with 0.2% Triton-X for 5 min and blocked with
327 6% goat serum for 45 min. Primary antibodies used were mouse Nestin (1:200) and rabbit MAP2
328 (1:500). Secondary antibodies were Alexa Fluor 488 goat anti-mouse (1:1000), Alexa Fluor 594
329 goat anti-rabbit (1:1000) and cell nuclei were stained using Hoescht dye.

330

331 **Time-lapse Microscopy.** For all time-lapse imaging experiments, hNPCs were plated at
332 approximately 50% confluence on 12-well plates pre-coated with Matrigel and switched to
333 differentiation medium 24 hours post-plating. Two datasets (biological replicates) were obtained
334 by imaging the well plates at days 0, 3, 6, 9, 12 and 14 after withdrawal of bFGF from culture
335 medium, using an automated stage Nikon Eclipse Ti-E Microscope. At the start of the

336 experiment, 5 locations were chosen arbitrarily for each well, and the same locations were imaged
337 at each time point. Imaging sessions lasted about 10 minutes and the plates were returned to the
338 incubator after imaging. A third dataset was obtained through continuous imaging, for which the
339 well plate was mounted on the stage of the microscope in a bold line cage incubator (Okolabs)
340 equipped with temperature control and gas flow rate control enabling a 37°C 5% CO₂
341 environment. For this dataset, images were acquired at 1-hour intervals for 8 days (Supplemental
342 Video 1). In all imaging experiments, 8-bit phase contrast images were acquired through a 10X
343 objective and at a resolution of 1280 x 1080 pixels. Physical pixel size was 0.64µm.

344

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

349

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

358 1. Small objects of size lesser than 50 pixels were removed to filter out noise and other
359 imaging artifacts.

- 360 2. Morphological opening was performed using a disk structuring element of radius 4
361 pixels. This was done to separate linear features (neurites), and circular features (cell
362 bodies).
- 363 3. Neurites were skeletonized using the `bwmorph` function in MATLAB to obtain neurite
364 length statistics.
- 365 4. Cell bodies were separated using connected component labeling using the default 8-
366 connected neighborhood.
- 367 5. Cell body objects smaller than 150 pixels and those touching the image border were
368 removed.

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

370

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

375

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

380

381 In order to capture changes in network structure across a 14-day period of differentiation, we
382 chose to use label-free phase contrast imaging (Weber et al. 2013). Immunostaining or the use of
383 fluorescent reporters would have resulted in images with better signal-to-noise to aid in image
384 analysis, but we chose to avoid these methods due to the inability to perform longitudinal imaging
385 without harming cell health. Due to the limits of our imaging method, automated image

386 segmentation often detected a smaller region than the exact cell boundary. In order to compensate
387 for this, we used a scaling factor to define spatial proximity for graph representations. Small
388 values of the scaling factor ($1 < S < 2$) resulted in sparse graphs, where interpretation of metrics
389 became difficult. Higher values of the scaling factor ($2 < S < 3$) resulted in qualitatively similar
390 results (Supplemental Figure 2), and we therefore chose $S = 2$ as the default scaling factor.

391

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

396

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

404

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

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

410

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419

420 **Author Contributions**

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

424

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