Supplementary discussion

Overall developmental timeline and brain regionalization in human whole-brain organoids

We first defined the timeline of generation of broadly-defined cell classes in organoids by using immunohistochemistry (IHC) for a small number of informative single-gene markers on organoids derived from the iPSC11a line. We examined markers of neural progenitors (e.g. PAX6), neurons (e.g. MAP2, GABA, TH), and glial cells (e.g. GFAP, OLIG2) over a time course of organoid development spanning 1, 3, 6, and 9 months (Extended Data Figure 1b). PAX6$^+$ neural progenitors emerged first, followed by increased expression of the pan-neuronal marker MAP2 (100% of organoids, n=12 from 2 bioreactors), and markers for glutamatergic (VGLUT1), GABAergic (GABA), and dopaminergic (TH) neuronal identities (Extended Data Figure 1b; VGLUT1 100%; GABA 100%; TH 100% of organoids, n=9-13 each, from 2 bioreactors). Matching the endogenous developmental sequence, GFAP$^+$ astroglia became apparent only at 3 mo, following the main wave of neurogenesis (Extended Data Figure 1b; 100% of organoids, n=14 from 2 bioreactors). We also examined the neural progenitor markers NKX2.1, GBX2, OTX2, EMX1, FOXG1, and VSX2 at 1 mo. Similarly to the endogenous developing brain, 1 mo organoids exhibited early brain regionalization, as shown by the expression of markers for germinal zones of the forebrain (PAX6 and NKX2.1, 100% and 55% of organoids, respectively; n=20, 3 bioreactors), midbrain/retina (OTX2, 100% of organoids, n=16, 3 bioreactors), and hindbrain (GBX2, 100% of organoids, n=20, 3 bioreactors) (Extended Data Figure 1c). Besides the dorsal forebrain, PAX6 is also expressed in the developing retina. Given the overlap in gene expression between developing retina and forebrain, we looked specifically at the development of these two structures by immunostaing with VSX2 (a marker of the retinal field), FOXG1 (a marker of the forebrain) and EMX1 (a marker of the dorsal forebrain) at both 1 mo and 6 mo. As seen *in vivo*, the expression

of these markers was mutually exclusive, with the retinal field present in all organoids analysed (100% of organoids were VSX2$^+$, n=35, 6 bioreactors), and the forebrain present in a subset of organoids (37% of organoids contained both EMX1$^+$ and FOXG1$^+$ cells, n=35, 6 bioreactors; no organoid contained only one or the other) (Extended Data Figure 1d).

Quantification of variability in production of cell clusters and subclusters across organoids

In order to quantify variability among organoids and possible bioreactor-based batch effects, we determined the frequency with which cells from each of the ten main clusters were generated in individual organoids, across cultures from different bioreactors (Figure 1c). We found that 89% of organoids contained cluster c1; 95% cluster c2; 53% cluster c3; 32% cluster c4; 89% cluster c5; 89% cluster c9, and 89% cluster c10 (Extended Data Figure 3). Organoids grown in the same bioreactor were more similar to each other in their ability to make cells of each cluster. This was striking when comparing bioreactor 4, where most of the organoids were less differentiated and contained large numbers of progenitors, to bioreactors 1, 2 and 3, where the vast majority of organoids contained much higher percentages of differentiated cells (e.g., cells in clusters c2, c4 and c5). Similarly, 100% of organoids from bioreactor 3 contained the forebrain cluster (c4), while this cluster was less represented in organoids grown in the other bioreactors sampled. To extend this observation, we used immunohistochemical analysis to examine organoids generated in a separate bioreactor (bioreactor 5), and found that 6 out of 6 organoids expressed multiple markers for progenitors of the forebrain (e.g. FOXG1, NES, SOX2), and for excitatory projection neurons of the cortex (e.g. FOXG1/CTIP2 and FOXG1/SATB2) (Extended Data Figure 5a). This indicates that certain flasks have a markedly greater ability to consistently generate forebrain cell identities, suggesting that the specific environment where organoids are grown is a key factor in controlling cell class identity.

To increase resolution and assess the distribution across organoids of distinct cell types from the forebrain cluster, we examined the forebrain sub-clusters in organoids from bioreactor 3, in which all organoids generated forebrain cells, in more detail. We found that among organoids from this bioreactor, cortical interneurons and corticofugal projection neurons were present in 50% of the organoids, while callosal projection neurons were present in all organoids sampled. Among forebrain progenitors, radial glia cells were very abundant and present in all organoids, while only 50% of organoids contained intermediate progenitor cells (Extended Data Figure 5b). This is likely an overestimation of the true variability, given the relatively small number of cells in each sub-cluster sampled from individual organoids, and the likelihood that different cell types will vary in robustness to dissociation and collection. This conclusion is supported by the observation that IHC for known marker genes indicates a much higher frequency of co-development of the same putative populations of forebrain cells (Extended Data Figure 5a).

In order to determine the reproducibility with which the retina cluster was generated in different organoids, we examined organoids from bioreactors 1, 2 and 3, where all organoids generated retinal cells (Figure 2d). Of the neuronal subtypes, photoreceptors were present in 73% of the organoids, while the other neuronal subtypes (retinal ganglion cells, bipolar cells and amacrine cells) were each present in approximately 55% of the organoids. Muller glia were found in 36% of the organoids. Pigmented epithelial cells, on the other hand, were found in most of the organoids (91%). Again, this is likely an underestimation of the prevalence of these cell types, given that by IHC, markers for the same putative populations of retinal cells were detected in every organoid examined (Extended Data Figure 5c).

Synapse density in comparison to human fetal brain

We performed serial EM reconstruction of a region about 70 μm below the surface of an 8 mo organoid, and identified a total of 129 synapses in this volume (0.088 synapses per μm^3). In the developing human brain, the number of synapses per volume depends strongly on age and brain region (see for example [31]). Our measure of 0.088 synapses per cubic micron is in the same range as the values reported for human fetal cortex (approximately 0.03 - 0.12 synapses per cubic micron at 200 days of gestation, depending on region[31]).

Reproducibility of the electrophysiology data

We validated electrophysiological profile in a second organoid line generated from the hES cell line HuES66 (Extended Data Figure 7c,d).

Supplementary Table 1 Primary antibodies employed in this study.

Antibody	Host animal	Company	Catalog number	Dilution
AP2α	Mouse	U of Iowa	3B5	1:100
CTIP2	Rat	Abcam	AB18465	1:100
CUX1	Mouse	Abcam	AB54583	1:350
DCX	Goat	Santa Cruz	SC8066	1:300
EMX1	Rabbit	Sigma	HPA006421	1:50
FOXG1	Rabbit	Abcam	AB18259	1:300
GFAP	Mouse	Sigma	G3893	1:400
GABA	Rabbit	Sigma	A2052	1:1000
GS	Rabbit	Abcam	AB73593	1:200
HIF1α	Mouse	Abcam	AB16066	1:200
ISL1	Rabbit	Abcam	AB109517	1:2000
MAP2	Chicken	Abcam	AB5392	1:5000
MITF	Mouse	Exalpha	X2398M	1:500
Nestin	Mouse	Abcam	AB6142	1:100
NKX2.1	Rabbit	DSBS	74.5A5	1:250
OTX2	Rabbit	Abcam	AB21990	1:100
PAX6	Rabbit	Biolegend	901301	1:400
SYN1	Mouse	SYSY	106 001	1:100
TH	Rabbit	Millipore	AB152	1:5000
TUJ1	Rabbit	Sigma	T2200	1:1000
GBX2	Goat	Abcam	AB109726	1:200
OLIG2	Rabbit	IBL	18953	1:200
VGLUT1	Rabbit	SYSY	135302	1:1000
HOMER1	Rabbit	SYSY	160003	1:700
c-FOS	Rabbit	Abcam	AB134122	1:500
RHODOPSIN	Mouse	Abcam	AB5417	1:800
VSX2	Sheep	Millipore	AB9016	1:300