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Supplementary information

Human brain organoids record the passage of time over multiple years

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SUPPLEMENTARY INFORMATION

Human brain organoids record the passage of time over multiple years

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Supplementary Discussion: detailed discussion of specific experimental procedures, analyses, and results that were summarized in the main text due to length constraints.

Maturation score analysis

To precisely assess transcriptional maturation in our organoid scRNA-seq data, we first identified gene modules correlated with age in the endogenous human cortex. We applied DIALOGUE[13], a computational framework for deriving multicellular programs (MCPs) of co-regulated genes across different cell types, to an *in vivo* human brain cortex single-cell dataset spanning from the first trimester up to 4 years of age (derived from[11,12], **Fig. 1d-f** and **Supplementary Table 2**). This analysis identified five distinct MCPs that correlated with age (**Extended Data Fig. 1a, b**), with MCP4 showing the strongest association (Pearson's r = -0.51, 0.23, -0.03, 0.70, and -0.31 for MCPs 1-5, respectively).

Methylation analysis of organoid timecourse and comparison with locus-specific methylation dynamics in endogenous cortex

To define whether organoids show appropriate age-related epigenetic programs over the years, we next performed whole-genome bisulfite sequencing (WGBS) of single organoids at different ages. DNA methylation, particularly at age-associated CpG sites, serves as a powerful molecular readout of biological age[14]. We sampled organoids at nine time points from 3 months to 5 years in culture (**Fig. 1g-k** and **Extended Data Fig. 1c** and **Supplementary Tables 3-4**). *In vivo*, across multiple tissues, the methylome is dynamic during early development but then reaches stable high levels of DNA methylation that are maintained over time. WGBS profiling of organoids over our extended time-course showed that organoids likewise undergo progressive methylation, and subsequently maintain global stability of DNA methylation patterns (**Fig. 1g**), similar to the high and stable methylation levels observed in postnatal and adult cortical tissue. Pairwise comparisons of genome-wide methylation revealed a strong correlation between adjacent time points (Pearson r = 0.89–0.94), supporting the preservation of the methylation landscape in multi-year cultures (**Extended Data Fig. 1d**). Global CpG methylation remained high across all time points; both partially methylated domains (PMDs), and highly methylated domains (HMDs) retained near-complete methylation at all ages across the 5 years (**Extended Data Fig. 1e**). DNA methylation valleys (DMVs), which often demarcate developmental regulatory regions, remained stable (**Extended Data Fig. 1f**). CpG methylation at repetitive elements similarly remained stable over time, further underscoring the global preservation of the methylome in long-term organoid cultures (**Extended Data Fig. 1g**). Together, these findings indicate that the longitudinal methylation landscape in organoids during extended culture resembles epigenetic features of endogenous tissues.

To determine whether the locus-specific methylation dynamics observed in long-term organoid cultures reflect endogenous postnatal maturation programs, we compared organoid methylomes to cell-type-resolved, age-associated differentially methylated regions (cdDMRs) previously defined in postnatal human cortex[15] from ages 0 – 23 years, based on significant age-associated methylation changes in NeuN+ and NeuN- populations. These cdDMRs capture reproducible methylation gains or losses that occur with age in $NeuN^+$ neuronal and $NeuN^-$ non-neuronal populations *in vivo* and were classified by the authors into six categories based on cell-type specificity and directionality of change (e.g., c2: G0N+ is a class of DMRs that increase in neurons while remaining stable in non-neuronal cells over 0-23 years of postnatal development). We found that a large proportion of the cdDMRs in each category displayed gradual, directional methylation changes across extended culture (**Extended Data Figure 1h, i**), indicating that cell type-specific methylated regions that are variable across human cortical development likewise vary in organoids. Both the fraction of cdDMRs exhibiting significant temporal slopes and the direction of those slopes varied by cdDMR category, in a manner that was largely consistent with their *in vivo* dynamics; e.g., cdDMRs in the c3: G0N- category, which become hypomethylated in $NeuN^+$ neuronal cells but does not change in $NeuN^-$ non-neuronal cells *in vivo*, show hypomethylation in organoids across the 5-year culture window. At the global scale, the cdDMR classes exhibited stable overall mean methylation levels, consistent with early establishment of the cortical methylome (**Extended Data Figure 1j**). Thus, although organoids represent only an early segment of the human postnatal developmental timeline, their progressive epigenetic states resemble endogenous temporal methylation programs.

Genome-view analyses further illustrated progressive, locus-specific remodeling at representative cdDMRs associated with neuronal and glial genes, including activity- and maturation-related loci such as FOSB and MBP (**Extended Data Figure 1k**). These changes

unfolded gradually over the years in culture, despite the absence of continued global CpG methylation shifts. While the limited availability of matched *in vivo* methylomes precludes direct quantification of effect sizes across equivalent developmental windows, the category-specific behavior, directionality, and coordinated temporal progression of cdDMRs in organoids are consistent with regulated endogenous methylation programs rather than stochastic epigenomic drift. Together, these findings show that long-term cortical organoids capture key features of postnatal, cell-type-associated epigenomic changes observed in the human brain.

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Despite the overall stability of methylation in endogenous tissues, regulation of methylation state at specific developmentally-relevant genes are required for cell fate specification and overall development. To determine whether developmentally-regulated loci undergo regulated changes over time in organoids, we examined CpG methylation at key cortical genes involved in fate acquisition and layer specification—*FEZF2* and *TBR1* for deep-layer projection neurons, *POU3F2* for upper-layer neuron identity, and *NEUROD2* for broader excitatory neuron differentiation. Each of these loci showed progressive, time-dependent methylation gains, particularly within CpG islands and DMVs (**Fig. 1h and Supplementary Table 3**). To examine temporal changes over organoid development more broadly, we used differential methylation analysis between the 3-month and 5-years timepoints to identify differentially methylated regions (DMRs, **Extended Data Fig. 2a, b**). DMRs highlight parts of the genome where methylation changes between conditions; in this case, between early and late cultures. We found 213 age-correlated DMRs, including both hyper-DMRs, which gained methylation over this period, and hypo-DMRs, which lost methylation. We then examined the behavior of these DMRs over the intermediate timepoints, and found that rather than arising abruptly or stochastically, these methylation shifts emerged continuously over the culture period, suggesting a tightly regulated epigenetic program (**Fig. 1i**). The relatively small number of DMRs and their gradual emergence at intermediate time points suggest coordinated regulation that is non-fluctuating rather than random drift. Functional annotation revealed that DMRs were enriched in epigenetic features of known developmental importance, including CpG islands, DNA methylation valleys, and gene regulatory elements (**Extended Data Fig. 2b**). Notably, the genes associated with these DMRs include many known to play key roles in the developing neocortex, such as *PAX6, HES6, EMX1, EMX2, NES, TBR1, EOMES, SATB2, NEUROD2, FEZF2, DLX1, DLX2, OLIG2, GAD1, GAD2, SLC32A1, DLX5, DLX6* and *POU3F2* (**Fig. 1i** and **Extended Data Fig. 2c, d** and **Supplementary Table 3**). While it is

difficult to quantify the exact number of methylation changes across comparable *in vivo* developmental windows due to the limited *in vivo* data available in the literature, the number and nature of DMRs observed in organoids, and the fact that they are enriched in developmentally relevant loci and show coordinated progression, are consistent with regulated *in vivo* methylation programs rather than stochastic methylation drift.

Finally, we examined CpA methylation (mCA), a non-CpG modification that is a hallmark of neuronal maturity[16]. In neurons, mCA accumulates postnatally and is believed to contribute to long-term gene silencing and transcriptional fine-tuning, making it a valuable molecular marker of neuronal maturation[16]. Global mCA levels increased steadily in organoids over the first year (**Extended Data Fig. 2e, f**) and remained stable thereafter. Importantly, when we examined a set of 1383 human fetal forebrain super-enhancers that gain mCA during postnatal neuron development *in vivo*[16], we found progressive mCA at these sites over time in organoids. This indicates that neuron-specific, non-CpG methylation pathways are not only active in our *in vitro* system, but are temporally regulated at appropriate target loci (**Extended Data Fig. 2f, g)**. Together, these results imply that the ongoing methylation dynamics observed in organoids over extended culture may reflect *in vitro* recapitulation of endogenous development and maturation epigenetic programs.

Analysis of ultrastructural and morphological complexity in APM vs CDM4 organoids

To investigate whether APM promoted ultrastructural features associated with neuronal maturity and activity, we examined synapse density and synaptic spine formation by quantitative electron microscopy (EM). We performed EM on organoids cultured in either CDM4 or APM at 6 and 12 months (n=3 organoids from each of n=2 genetic backgrounds at 6 months: H1 and 11a, and n=6 organoids from each of n=3 genetic backgrounds at 12 months: H1, 11a, and PGP1, for both CDM4 and APM conditions). We acquired large 2D panoramic

images (approximately 350 µm x450 µm) that covered a representative portion of each organoid, spanning from the surface to the core, to examine structures at different depths (**Fig. 2e**).

To quantify synapse density across these large sections, we refined an existing deep learning algorithm[25] for the automatic detection of synapses in each section. While no statistically significant differences in synaptic density were observed between groups at 6 months (Wilcoxon rank-sum test *P* value = 0.7), at 12 months, APM organoids exhibited higher synaptic densities (*P* = 0.002) in comparison with CDM4 organoids (**Fig. 2e-g** and **Supplementary Table 6** and **Supplementary Video 1-2)**.

To determine whether synapses were located on spines, we classified a subset of synapses (103 predicted synapses in each section) based on their postsynaptic target. Synapses were categorized as being located on dendritic spines (henceforth spines) if they were either clear, well-defined spines attached to a dendritic shaft, or putative spines—structures with morphology consistent with spines observed in 2D EM sections (**Fig. 2e, f**). Synapses that did not meet these criteria were classified as being located on non-spine elements. This quantification revealed a significant increase of spine proportion in the APM-treated organoids at 12 months (**Fig. 2f,** 25% vs 52% in CDM4 and APM, respectively; two-sided Fisher's exact tests: 6mo: p = 0.51, OR = 0.88, 95%CI 0.60 to 1.30; 12mo: p = 9.5e-8, OR = 4.67, 95%CI 2.49 to 9.23). Altogether, these findings suggest that APM treatment supports the maintenance of mature ultrastructural features in neurons over time. Notably, the higher proportion of synapses formed on dendritic spines in APM-treated organoids is consistent with enhanced synaptic maturation and stabilization, features that are critical for the long-term integrity of synaptic circuits.

We next asked whether neuronal complexity (i.e., arborization and process outgrowth), which is a clear measure of cortical neuron maturation *in vivo*, was enhanced by culture in APM. We

adapted epitope tag barcoding[26–29] as a novel tracing strategy[30] to reconstruct the morphology of individual neurons in densely-packed organoid tissue (**Fig. 2h, i and Extended Data Fig. 5a**). This method leverages stochastic expression of distinct epitope tag combinations in the form of antigenically orthogonal spaghetti monster fluorescent proteins (smFPs)[31] to ensure robust cytosolic barcode filling (see **Methods**). Combined with the Magnify Expansion Microscopy strategy[32], optimized for robust multi-round epitope tag read-out (see **Methods**), this approach enables high-resolution detection of individual neurons and tracing of their fine processes in densely labeled tissue.

We infected organoids with an adeno-associated virus (AAV) mix for three weeks (n=3 organoids per condition, at 7 months in culture). Each of the nine AAVs in the mix contained a plasmid for the expression of an smFP, with eight copies of either V5, Myc, Flag, Ollas, E, S, HSV, Protein C or Ty1 epitope tag, and performed iterative post-expansion immunostaining of the expanded organoid sections to read out epitope tag expression and reconstruct 30 individual neurons for each condition. APM-treated neurons exhibited significantly greater total neurite length, longer maximal process extension, and a higher number of terminal branches, revealing that both increased branching behavior and greater process outgrowth into the tissue contribute to enhanced neuronal complexity under APM culture (**Fig. 2j, k**). Strahler order analysis further showed that APM neurons achieved significantly higher maximum Strahler numbers, confirming greater complexity of neuronal arborization (**Fig. 2l)**. Notably, the distribution of lower Strahler indices remained comparable between conditions, indicating that increased arborization in APM organoids still follows the same principles of hierarchical neuronal architecture (**Fig. 2m, n)**.

Analysis of metabolic health in organoid timecourse

To determine whether extended culture may negatively affect global metabolic health of long-term organoid cultures, we analyzed cell type–specific variation in metabolic processes using curated gene sets from MSigDB across our scRNA-seq timecourse. We observed an overall increase in metabolic and cellular stress signatures over time (LMEM: P = 9.6e-35, 2.78e-6, and 3.25e-26 for Apoptotic, Glycolytic, and Hypoxic signatures, respectively) (**Extended Data Fig. 8a)**. Stratification by cell type revealed that this increase was primarily driven by progenitor populations, whereas other cell types, including neurons, did not show as large or as significant of an increase over time (LMEM, see **Supplementary Table 11** for results) (**Extended Data Fig. 8b-d)**. As an independent measure of tissue oxygenation, we performed hypoxia labeling using the pimonidazole detection reagent. Pimonidazole signal was largely confined to the organoid core, consistent with previous observations[6], while a large proportion of peripheral tissue remained non-hypoxic, even after 1 year *in vitro* (**Extended Data Fig. 8e)**.

Mouse temporal chimeroid model

To test whether progenitors in chimeroids retain a memory of their time in culture, we developed an *ex vivo* mouse chimeroid system using progenitors of different ages derived from the embryonic mouse brain. This system gives the distinct advantage of fast development and endogenous origin of the progenitor cells. We dissected the cerebral cortex from E11.5 mouse embryos and seeded single cells in suspension to generate endogenous mouse cortical organoids (**Extended Data Fig. 11a-d)**. After two to three weeks in culture, these organoids predominantly expressed astrocytic markers as well as the CPN marker SATB2 (**Extended Data Fig. 11d)**. This 2-to-3-week time-point represents the “old” progenitor stage for our experiments.

To determine whether neuronal cells in mono-chronic and hetero-chronic chimeroids are generated *de novo* within the culture, or are carried over through dissociation and re-

aggregation of chimeroids, we infected endogenous organoids shortly after initial aggregation with adeno-associated virus (AAV) PHP.eB, which exhibits neuronal tropism[38–40], and cultured them for three weeks, followed by dissociation and reaggregation. GFP^+ cells were readily detectable before dissociation but were not detected after re-aggregation, indicating that the majority of pre-existing neuronal cells are lost after re-aggregation (**Extended Data Fig. 11e, f**). This finding is consistent with our previous results showing that human cortical chimeroid cultures immediately after remix are predominantly composed of progenitors[9].