Effects of 3D culturing conditions on the transcriptomic profile of stem-cell-derived neurons

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Understanding neurological diseases requires tractable genetic systems, and engineered three-dimensional (3D) neural tissues are an attractive choice. Yet how the cellular transcriptomic profiles in these tissues are affected by the encapsulating materials and are related to the human brain transcriptome is not well understood. Here, we report the characterization of the effects of different culturing conditions on the transcriptomic profiles of induced neuronal cells and developed a method for the rapid generation of 3D co-cultures of neuronal and astrocytic cells from the same pool of human embryonic stem cells. By comparing the gene-expression profiles of neuronal cells in culture conditions relevant to the developing human brain, we found that modifying the degree of crosslinking of composite hydrogels can tune expression patterns so that they correlate with those of specific brain regions and developmental stages. Moreover, single-cell-sequencing results showed that our engineered tissues recapitulate transcriptional patterns of cell types in the human brain. Analyses of culturing conditions will inform the development of 3D neural tissues for use as tractable models of brain diseases.

To generate a system that can serve as a proxy for studying the genetics of the human brain, we compared a number of different conditions used for generating 3D neural tissues from induced neuronal (iN) cells from hESCs. We also developed a method for rapidly generating 3D co-cultures of iN and astrocytic cells derived from the same population of hESCs. iN cells can be efficiently produced directly from hESCs through the ectopic expression of transcription factors2–4 and have been used to model neurological diseases by culturing them on 2D surfaces5–7. This approach has also been used to produce 3D neural tissues on an electrospun scaffold, which are then used for transplantation8. We extended these approaches to create a 3D culture of iN cells within Matrigel, a basement membrane matrix that includes components that closely reflect the ECM in the brain9. The effects of the addition of hyaluronic acid (HA) and the formation of composite hydrogels (CHs) of Matrigel and alginate with varying crosslinking density and volume for optimizing the components of these tissues were also explored. We compared the transcriptome of these iN cells to a panel of human brain transcriptomic data and showed that the gene expression of iN cells can be tuned to correlate with specific developmental time points and brain regions by modulating the composition of the 3D matrix. Single-cell-sequencing of cells co-cultured in 3D tissues confirmed their transcriptomic correlations to cell types found in the human brain. Finally, we used gene editing tools to knockout genes in our 3D tissues that are implicated in neurodegenerative diseases to demonstrate the feasibility of combining these technologies.

Results

development and optimization of 3D neural tissues from hESCs. To create a robust, genetically tractable 3D neural tissue system, we
used a transcriptional activation approach to differentiate iN cells from hESCs, which were then encapsulated in a Matrigel matrix (Fig. 1a). First, we tested whether hESCs transduced with constructs that overexpress NGN1 and NGN2 and encapsulated in a Matrigel 3D matrix could directly differentiate into iN cells. However, this approach resulted in aggregation of encapsulated cells within 5 days (Supplementary Fig. 1a), preventing efficient differentiation. To circumvent aggregation, hESCs were first seeded on 2D plates and then induced to form neuronal cells, which were subsequently detached and then encapsulated in Matrigel (Supplementary Fig. 1b). This process led to less aggregation; however, aggregates continued to form over time, with spheroids present at day 30 (Supplementary Fig. 1c,d). Further improvements were made by increasing the selection for constructs expressing NGN1 and NGN2 and introducing a proliferation inhibitor, 1-β-D-arabinofuranosylcytosine (Ara-C), to suppress the proliferation of undifferentiated stem cells. This approach resulted in 3D, pure human neural tissues without cell aggregates (Supplementary Fig. 1e, Supplementary Videos 1–3). For comparison, we also generated 2D cultures of iN cells (Fig. 1a, and Methods).

Characterization of 3D cultures. Characterization of the differences between 2D and 3D cultures of iN cells using global transcriptome analysis demonstrated clear differences between these cultures at both the week 1 and week 5 time points (Fig. 1c, Supplementary Tables 1, 2). As maintaining healthy neural tissues for an extended amount of time promotes neuronal maturity13,22,23, we focused our analysis on tissues at the week 5 time point. Gene set enrichment analysis (GSEA) showed more enriched neurological processes present in 3D cultured iN cells than in 2D ones at 5 weeks, whereas 2D cultures were enriched for apoptotic and oxidative stress markers, indicative of their poor health (Supplementary Fig. 2a). We validated a subset of these genes by quantitative PCR (qPCR) (Supplementary Fig. 2b). This validation was supported by a gene ontology (GO) analysis for upregulated and downregulated genes with P < 0.001 (Fig. 1f). This trend was not significantly affected by batch-to-batch variation (Supplementary Fig. 3d,e). We performed differential expression analysis of our RNA-seq data to Matrigel alone, although the global gene expression profiles of 3D, pure human neural tissues were expressed at high levels in the presence of HA (Supplementary Fig. 7b). To gain a global view of the effect of HA on gene expression patterns, we compared the transcriptomes of our engineered tissues cultured in the presence and absence of HA to the human brain transcriptome of four different subregions at four fetal developmental stages. The following four subregions were compared: V1C: primary visual cortex (striate cortex, area V1/17); DFC: dorsolateral prefrontal cortex; A1C: primary auditory cortex (core); and M1C: primary motor cortex (area M1, area 4). The four fetal developmental stages analysed were 12 post-conceptual weeks (pcw), 16 pcw, 19 pcw and 37 pcw. We found that the presence of HA broadly decreased the correlation between the transcriptome of 3D co-cultured iN cells to the human brain developmental transcriptome, although correlations to 37 pcw did not fit this trend (Fig. 2d).

Transcriptome profiles can be tuned using CHs. The differentiation of neurons in the gene expression profiles of 3D tissues can be affected by the mechanical stiffness of the matrix24–26, providing an avenue for creating tunable engineered tissues. As increasing the concentration of Matrigel had minimal effect, we explored how a CH consisting of alginate and Matrigel affected gene expression profiles (Fig. 3a). Alginate networks can be created in the Matrigel through the addition of calcium as a crosslinker, whereby the concentration of calcium can be increased to produce a stiffer matrix19 (Supplementary Fig. 8a). We performed bulk RNA-seq on co-cultured iN cells at week 5 in Matrigel and in CHs with increasing amounts of crosslinker. Principal component analysis (PCA) showed clear transcriptomic differences in the CHs relative to Matrigel alone, although the global gene expression profiles of CHs with intermediate levels of crosslinker were not strongly separated from each other (Fig. 3b). A global differential expression analysis of the transcriptomes of iN cells in CHs versus those in the Matrigel hydrogel showed four distinct clusters of genes with different patterns of expression. These clusters contained a number of genes relevant to human neurological disease such as amyotrophic lateral sclerosis (ALS) (for example, SOD1) and autism spectrum disorder (ASD) (for example, ADSL) (Fig. 3c, Supplementary Fig. 9, Supplementary Table 5). We also looked at the differential expression of genes involved in forebrain development, axon guidance and neuron development biological processes (Fig. 3c, Supplementary Fig. 8b–d). To analyse the effect of increasing the crosslinker concentration in CHs on the gene expression profile of iN cells, we performed differential expression analysis of our RNA-seq data relative to the CH with the lowest level of crosslinker. In particular,
we focused on differentially expressed genes involved in forebrain development, axon guidance and neuron development biological processes (Fig. 4a, Supplementary Fig. 10). Neuronal transcripts such as DLG4, NFIB and UNC5C were expressed at lower levels in iN cells co-cultured in CHs with high levels of crosslinker than in iN cells co-cultured in other CHs (Fig. 4a, Supplementary Fig. 10). Although increasing the crosslinker concentration in CHs increased the expression of ARHGEF12, GSK3B, SLCA47 and GPM6A, neuronal genes such as ID4 and BAD were more highly expressed in iN cells co-cultured in CHs with intermediate levels of crosslinker than in...
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expression clusters for iN cells co-cultured in Matrigel with or without HA. Two clusters, labelled 1 and 2, were identified (see also Supplementary Table 4).

For all four subregions tested, we observed a positive trend in the correlation between the transcriptome of co-cultured iN cells to the human brain transcriptome, whereas increasing the cell density led to similar trends (Supplementary Fig. 11). We also investigated how the presence of HA and an increased cell density in the CHs affected gene expression profiles. In agreement with our previous findings, adding HA to the CH decreased the correlation between the transcriptome of co-cultured iN cells and the human brain transcriptome, whereas increasing the cell density led to similar trends (Supplementary Fig. 11). We also investigated the effect of varying the volume of the CH with or without HA on the gene expression profile of co-cultured iN cells. We found that decreasing the volume improved the enrichment of neurological processes while marginally decreasing the correlation to the human brain transcriptome (Supplementary Fig. 12).

To compare broadly all the conditions tested, we performed a differential expression analysis of all conditions relative to stem cells. In agreement with our previous results, we found that stem cells were enriched for cell cycle and cell division processes and depleted for neurogenesis and neuronal developmental processes. 2D cultures showed marginal differences, whereas 3D cultures, with the exception of the HA condition, showed the opposite pattern, whereby neuronal-related processes were enriched for and the expression of cell cycle and cell division genes was reduced (Supplementary Fig. 13, Supplementary Table 14).

Fig. 2 | Incorporating HA within Matrigel leads to enriched non-neuronal biological processes in 3D co-cultured human iN cells and decreases the gene expression correlation to the human brain transcriptome. a, Schematic showing human iN cells and mouse astrocytes (at a concentration of 30 × 10⁶ cells ml⁻¹) encapsulated in Matrigel with or without HA trapped within the Matrigel. b, PCA of gene expression values derived from whole transcriptome sequencing data of 3D co-cultured iN cells in Matrigel with or without HA at week 5 of culture (n = 3 for each condition). c, Gene expression clusters for iN cells co-cultured in Matrigel with or without HA. Two clusters, labelled 1 and 2, were identified (see also Supplementary Table 4). GO terms for genes in each cluster are shown. Differentially expressed genes with P < 0.01 and log₂ (fold change) | VOL 2 | JULY 2018 | 540–554 | www.nature.com/natbiomedeng

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in iN cells co-cultured in CHs with low or high levels crosslinker (Fig. 4a, Supplementary Fig. 10).

We further examined the influence of co-culturing iN cells in Matrigel hydrogel versus in CHs for 5 weeks by comparing their transcriptomes to a panel of human brain transcriptome samples. For all four subregions tested, we observed a positive trend in the correlation between the transcriptome of co-cultured iN cells to the human brain transcriptome at later developmental time points (19 pcw and 37 pcw) when moving from Matrigel to CHs with increasing levels of crosslinker (Fig. 4b). Although there were some exceptions to this trend, in most cases, culturing in CHs was an improvement over culturing in Matrigel alone. At the earliest developmental time point (12 pcw), the gene expression profile of iN cells co-cultured in Matrigel alone correlated more closely with the human data. We then determined which genes were driving the increased correlation to the human brain transcriptome at later developmental time points (19 pcw and 37 pcw) when moving from Matrigel to CHs with 4X crosslinker. To this end, we scored each gene (see Methods) and generated ranked lists for each time point (19 pcw and 37 pcw) and brain region (V1C, DFC, A1C and M1C). High scoring genes exhibited similar expression levels in the human brain developmental transcriptome and the CH with 4X crosslinker condition, but different expression levels in the human brain developmental transcriptome and the Matrigel condition (Supplementary Tables 6–13). The GO analysis for high scoring genes (rank score > 3) for each brain region at 19 pcw showed enrichment for neuron-related processes, whereas high scoring genes (rank score > 3) for each brain region at 37 pcw demonstrated enrichment for ECM-related processes (Supplementary Tables 6–13). Additionally, we investigated how the presence of HA and an increased cell density in the CHs affected gene expression profiles.

To compare broadly all the conditions tested, we performed a differential expression analysis of all conditions relative to stem cells. In agreement with our previous results, we found that stem cells were enriched for cell cycle and cell division processes and depleted for neurogenesis and neuronal developmental processes. 2D cultures showed marginal differences, whereas 3D cultures, with the exception of the HA condition, showed the opposite pattern, whereby neuronal-related processes were enriched for and the expression of cell cycle and cell division genes was reduced (Supplementary Fig. 13, Supplementary Table 14).
CHs modulate the expression levels of individual neuronal genes in 3D co-cultured human iN cells. a. Schematic showing human iN cells and mouse astrocytes (at a concentration of 2 × 10³ cells ml⁻¹) encapsulated in Matrigel (4.6 mg ml⁻¹) or in a CH of Matrigel (4.6 mg ml⁻¹) and alginate (5 mg ml⁻¹) with varying amounts of the crosslinker (CRS) CaCl₂ (1X, 3.125 mM; 2X, 6.25 mM; 4X, 12.5 mM; 8X, 25 mM). b. PCA based on whole transcriptome data of co-cultured iN cells at week 5 of culture (n = 3 for each condition). c. Gene expression clusters for iN cells co-cultured for 5 weeks in Matrigel or CHs. Four clusters, labelled 1, 2, 3 and 4, were identified (see also Supplementary Table 5). Heatmaps show selected neuronal genes in each cluster involved in forebrain development, axon guidance and neuron development, and genes in each cluster associated with the neurological diseases ASD and ALS, and their relative expression among 3D hydrogel conditions (see also Supplementary Figs. 8, 9). Differential expression was performed between co-cultures in CH and co-culture in Matrigel with P < 0.01 and log₂ (fold change) < -1 or log₂ (fold change) > 1 used as the cut-off values.

We next compared the transcriptomes of the 3D tissues under all conditions using PCA, which showed transcriptomic differences among the various conditions (Fig. 5a). A comparison of the mechanical properties of the encapsulating hydrogels also demonstrated storage modulus differences among the various hydrogels used (Fig. 5b, Supplementary Fig. 14). We then profiled the mean correlation between the transcriptomes of the 3D tissues to the human brain transcriptome (Fig. 5c, Supplementary Fig. 15a). Moving from the Matrigel condition to the CH conditions increased the correlation to the four subregions at later developmental time points (19 and 37 pcw) while decreasing the difference between the correlations at the early developmental time point (12 pcw) and late developmental time point (37 pcw). These results suggest that 3D tissues could serve as substrates for studying various neurological diseases while providing transcriptomic correlations to human brain subregions at different developmental time points.

Generation of 3D neural tissues with human cell components. To better represent the cell composition of the human brain in our 3D tissues, we developed a method to derive human astrocytic cells directly from hESCs and used this method to engineer 3D tissues of co-cultured human iN and astrocytic cells. A previous study reported that overexpressing NGN1 and NGN2 in stem cells leads to a transient neural progenitor state before the cells turn into iN cells. Thus, we hypothesized that this approach could be exploited to derive human astrocytic cells. We developed a method to induce the formation of astrocytic cells by terminating NGN1 and NGN2 overexpression and adding a morphogen, ciliary neurotrophic factor (CNTF), at day 2, followed by passaging the cells at day 20 to eliminate neuron-like cells (Supplementary Figs. 16a, 17).
The majority of the cells thus derived were positive for GFAP, S100β and vimentin (encoded by VIM) at day 35, comparable to mouse astrocytes (Supplementary Fig. 18a). We also analysed the expression of GFAP, S100B, VIM and ALDH1L1 by qPCR for cells exposed to different derivation protocols for 5, 15 and 30 days without passaging (Supplementary Figs. 16b, 18b). Undifferentiated hESCs, hESCs only exposed to morphogen, and human primary astrocytes were used as controls. GEAP expression was not detectable in undifferentiated hESCs or in hESCs only exposed to morphogen, but when morphogen was added following transcriptional activation, GEAP levels increased similarly. The expression of S100B, VIM and ALDH1L1 gradually increased from day 5 to day 30 during the differentiation protocol (Supplementary Figs. 16b, 18b). The addition of fetal bovine serum (FBS), which is frequently used to derive astrocytes46,47, decreased, but did not abolish, the expression of all genes tested (Supplementary Fig. 18b). However, FBS aided in the passaging steps of these cells for further expansion and was therefore included in the differentiation protocol. We also performed bulk RNA-seq throughout the course of astrocytic cell differentiation and observed that the expression levels of a number of astrocyte marker genes gradually increased starting from day 15, and by day 30 reached levels similar to that of human primary astrocytes. Moreover, these expression levels remained high at day 67 and day 114 in conditions that combined transcription activation, morphogen and FBS, whereas undifferentiated hESCs and hESCs only exposed to morphogen lacked high expression levels for the majority of astrocyte marker genes (Fig. 6a, Supplementary Fig. 18c). Consistent with our qPCR results, the addition of FBS decreased the expression levels of astrocyte marker genes at day 30, whereas expression levels of these marker genes were high at day 67 and day 114 for the same condition (Fig. 6a, Supplementary Fig. 18c). A comparison of single-cell RNA-seq (scRNA-seq) datasets of a fetal human cortex48,49 and a single-nucleus RNA-seq dataset of an adult human brain49 showed similar trends. That is, gradually increasing correlations of astrocytes between the adult human brain (Supplementary Fig. 19a) and the fetal human cortex (Supplementary Fig. 19c) from day 15 to day 30, particularly for conditions with transcription activation and morphogen. The addition of FBS decreased this trend (Supplementary Fig. 19a). We also analysed the expression levels of marker genes of other cell types, such as radial glia (RG), intermediate progenitor cells (IPCs), excitatory neurons and inhibitory neurons, among the samples throughout the course of astrocytic cell differentiation (Fig. 6a, Supplementary Fig. 18c). Transcriptomic correlations between these samples and corresponding cell types in fetal human cortex and adult human brain were then derived (Supplementary Fig. 19a–c). Although these comparisons suggested that there is some heterogeneity in the populations of cells arising from the astrocytic cell differentiation protocols at the transcriptomic level, the immunostaining, qPCR and RNA-seq profiles of marker genes supported the identity of these cells as astrocytic. We therefore co-cultured these cells with iN cells in a 3D system for further experiments (Fig. 6b).

To examine the impact of astrocytic cells on iN cells, we compared the gene expression profiles of iN cells co-cultured with differentiated astrocytic cells or human primary astrocytes, or cultured without astrocytic cells. We performed FACS of iN cells from all cultures at week 5 with minimal cell contamination from astrocytic cells or human primary astrocytes (Supplementary Fig. 19d,e). Gene expression profiling showed that astrocytic cells caused gene expression differences in iN cells similar to that caused by human primary astrocytes (Fig. 6c–e), providing further support that our differentiation protocol can induce astrocytic-like cell fates.
Fig. 5 | Global comparison of effects of culture conditions on human iN cells and mechanical properties of encapsulating hydrogels. a, PCA based on whole transcriptome data of iN cells cultured or co-cultured in a variety of 3D conditions at week 5 (key for the symbols are shown at the bottom of the figure) (n = 3 for each condition). b, Storage modulus at 0.5 Hz of different encapsulating hydrogels (n = 3 for each condition) (see also Supplementary Fig. 14). Open circles represent storage modulus values for each hydrogel condition. Bars show the mean ± s.e.m. c, Pearson’s correlations between RNA-seq data of human iN cells cultured or co-cultured in different 3D conditions at week 5 and human brain transcriptome data of two different subregions (V1C and DFC) at three fetal developmental stages (12, 19 and 37 pcw) (see also Supplementary Fig. 15a). Open circles represent correlation values between a 3D condition and a BrainSpan sample. Each BrainSpan time point–region pair has 1 sample except for 12 pcw, which had 3 samples available for each subregion. Bars show the mean correlation ± s.e.m. d, Expression levels of selected disease-related genes across various 3D conditions encapsulating human iN cells (AD, Alzheimer disease; PD, Parkinson disease) (see also Supplementary Fig. 15b). Colour schemes are based on the Z score distribution.
To further explore the cell fates and transcriptional profiles of the cells in our tissues, we performed single-cell sequencing on 3D co-cultures of human iN cells with mouse astrocytes and with human astrocytic cells cultured in CHs with 4X crosslinker at week 5. We performed clustering on scRNA-seq profiles of human cells in both 3D co-cultures and identified 12 clusters (Fig. 7a, Supplementary Fig. 20a). Using various cell-type marker genes (Supplementary Fig. 20b–d), we classified the cell types into the following five main clusters: neurons, astrocytes, inhibitory neurons, RG, and neuroepithelia (Fig. 7b). iN cells co-cultured with mouse astrocytes contained only neuron cells (only reads aligning to the human genome were analysed), whereas iN cells co-cultured with human astrocytic cells contained cells from clusters of neurons, astrocytes, inhibitory neurons, RG and neuroepithelia (Fig. 7c), suggesting that the astrocytic cell differentiation protocol generates other transcriptionally distinct cell types in addition to astrocytes. This result was also observed in the bulk RNA-seq analysis (Fig. 6a, Supplementary Figs. 18c, 19a–c).

We next examined whether the cell-type clusters identified in our 3D tissues transcriptionally resemble their counterparts in human brain by comparing our data to scRNA-seq datasets of a fetal human cortex38,39 and to single-nucleus RNA-seq datasets of an adult human brain40. Although we observed some expression of marker genes for IPCs, the transcriptional correlation of our neuron cluster to neurons in the fetal human cortex was higher than its correlation to IPCs in the fetal human cortex (Fig. 7d,e, Supplementary Figs. 20e, 21a,b). Moreover, our neuron cluster showed a high transcriptomic correlation to different types of excitatory neurons in the fetal human cortex data, such as early- and late-born excitatory neurons in the primary visual cortex (EN-V1-2) and prefrontal cortex (EN-PFC2), and in the adult human brain (Fig. 7d,f, Supplementary Figs. 20f, 21a,b). In addition, the gene expression profile of our RG cluster correlated more with RG in the fetal human cortex, such as early RG (RG-early), dividing RG (RG-div2) and medial ganglionic eminence RG (MGE-RG2), which can give rise to inhibitory neurons39 (Fig. 7d,e, Supplementary Figs. 20e, 21a,b). Our inhibitory neuron cluster transcriptionally correlated with both inhibitory neuron types (such as IN-CTX-MGE1; MGE-derived cortex inhibitory neurons) and excitatory neuron types identified in the fetal human cortex (Fig. 7d, Supplementary Fig. 21a,b). By contrast, in the adult human brain, the inhibitory neuron cluster correlated most highly with inhibitory neurons (Fig. 7f, Supplementary Fig. 20f). Similarly, although our astrocyte cluster correlated more highly with RG than with astrocytes in the fetal human cortex (Fig. 7d, Supplementary Fig. 21a,b), its transcriptome correlation with astrocytes in the adult human brain was higher than its correlation to other cell types (Fig. 7f, Supplementary Fig. 20f). This trend was also observed in comparisons between scRNA-seq data from 6-month-old human brain organoids40 and scRNA-seq data from the fetal human cortex39.
As a proof-of-concept, we tested the feasibility of performing CRISPR (clustered regularly interspaced short palindromic repeats)-mediated genome editing in our engineered 3D neural tissues in a disease context. Using Cpf1-mediated genome editing via adeno-associated virus (AAV)-based gene delivery41,42, we targeted SOD1, TARDBP and Tbk1, genes implicated in ALS and frontotemporal dementia43–46. We identified insertions and deletions (indels) in three independently targeted loci and found ~5%, ~14% and ~6% indel formation in SOD1, TARDBP and Tbk1, respectively (see Supplementary Text, Supplementary Figs. 22, 23). These results show that our 3D human neural tissue system can be combined with genome editing and gene delivery tools to perturb genes implicated in neurodegenerative diseases.

**Discussion**

3D neural tissues have the potential to be tractable models for studying the human brain and neurological disorders, but to achieve this potential, they must closely reflect the cell composition, ECM and gene expression profiles of the human brain. Here, we analysed how the transcriptome of iN cells in 3D tissues relates to the human brain transcriptome and is affected by a number of tissue engineering variables. Furthermore, we developed an approach

**Fig. 7|** scRNA-seq reveals that cells in 3D neural tissues reflect their counterparts in the human brain and in human brain organoids. A. A t-distributed stochastic neighbour embedding (tSNE) plot of scRNA-seq profiles from iN cells co-cultured with iN–mAst and from iN cells co-cultured with IN–huAstC. Cells are coloured by condition membership. Both cultures were performed in CHs with 4X CRS (n = 3 for each condition). B, C. A tSNE plot showing identified clusters of distinct cell types with cells coloured by cluster membership (B) and number of cells in each cell type for each condition for scRNA-seq profiles shown in (A) (see also Supplementary Fig. 20) (c). D, Pearson’s correlations between the average gene expression in cell type clusters shown in B for each condition (rows) and cell types defined by scRNA-seq in the human fetal cortex39 (columns) (see also Supplementary Fig. 21a,b). E, Pearson’s correlations between the average gene expression in cell type clusters shown in B for each condition (rows) and cell types (excitatory neurons, inhibitory neurons and astrocytes) defined by DroNC-seq (scRNA-seq with droplet technology) in an adult post-mortem human brain tissue40 (columns) (see also Supplementary Fig. 20f). F, Pearson’s correlations between the average gene expression in cell type clusters shown in B for each condition (rows) and cell types defined by scRNA-seq in 6-month-old human brain organoids41 (columns) (see also Supplementary Fig. 20g). Forebrain subclusters were derived from a forebrain cluster shown in Fig. 7g. Forebrain subclusters were derived from a forebrain cluster shown in the main clusters.

(Supplementary Fig. 21c). To explore this result further, we performed differential expression analysis between astrocytes and RG in the scRNA-seq dataset of the human fetal cortex19. The top nine astrocyte-specific genes and the top nine RG-specific genes based on log fold-change (Supplementary Fig. 21d) were identified. The average expression levels of these genes in the astrocyte and RG clusters in our 3D tissues and in data from the 6-month-old human brain organoids varied among these cell types (Supplementary Fig. 21e,f). This result indicates that in both our 3D tissues and the human brain organoids, astrocytic-like cells are present but they are not transcriptionally homogeneous. Taken together, these results demonstrate that cell types in our 3D tissues transcriptionally resemble their analogues in the fetal human cortex and the adult human brain. Finally, a comparison between scRNA-seq profiles of our 3D tissues and the scRNA-seq dataset of 6-month-old human brain organoids16 revealed that the transcriptomes of our cell-type clusters correlated with their counterparts in 6-month-old human brain organoids (Fig. 7g, Supplementary Fig. 20g).
to generate co-cultured iN and astrocytic cells derived from hESCs in a 3D matrix that can be tuned to reflect different transcriptomic states of the human developing brain transcriptome, which will be helpful for rapidly generating complex neurological disease models.

To gain a global view of the brain-like properties of these engineered tissues, we compared the transcriptomes of cells grown in 3D cultures with those grown in 2D cultures. We found more enriched neuronal biological processes in iN cells cultured in 3D Matrigel than those grown in 2D cultures (Fig. 1f), and this result was not affected by batch-to-batch variations in Matrigel (Supplementary Fig. 3a–c). In addition, transcriptome profiling showed enriched apoptotic and oxidative stress biological processes in cells grown in 2D cultures compared with those grown in 3D cultures, and 3D cultures enabled electrophysiological measurements (Supplementary Figs. 2, 4). These results suggested that iN cells in 3D cultures were healthier than cells in 2D cultures, although we did not directly test cell viability. In addition, we co-cultured iN cells with mouse astrocytes both in 3D and on 2D systems. Although iN cells in both co-cultures were electrophysiologically active at later time points, the transcriptome of 3D co-cultured iN cells was more enriched in neuronal biological processes than that of 2D co-cultured iN cells, indicating that 3D tissues offer a closer approximation to biology than 2D tissues. Varying the cell seeding densities or independent components of the encapsulating matrix (for example, laminin, collagens or synthetic hydrogels) may lead to different characteristics of the iN cells, avenues that could be explored in future functional studies.

Engineered neural tissues can be made more brain-like by adding other ECM components, such as HA, which has been shown to promote better replication of the brain microenvironment. Under the conditions we tested, however, the incorporation of HA in our 3D Matrigel co-cultures of iN cells did not strongly improve the correlation to the transcriptome of the human developing brain, although some individual neuronal transcripts and genes associated with neurological diseases were upregulated (Fig. 5, Supplementary Fig. 7b). We chose to use a high molecular mass HA and incorporated it in a high concentration of Matrigel (which showed a storage modulus closed to Matrigel without HA due to the uncrosslinked state of HA in Matrigel) to minimize its diffusion from the hydrogel structures while maintaining its natural state. However, other variables (such as chemical modifications and the concentration and source of HA) or shorter culture times (such as 1 week) that were not tested may have a greater impact on gene expression. Given that high molecular mass HA is thought to inhibit remyelination following central nervous system injury, introducing HA into hydrogels could impede the expression of genes involved in neuronal development. Matrix stiffness in engineered tissues can also affect cell properties and can be increased in a pure Matrigel hydrogel simply by increasing the concentration of Matrigel. Alternatively, in a CH, stiffness can be increased by increasing the amount of crosslinker while maintaining the concentration of alginate and Matrigel constant. We therefore investigated whether developing 3D co-cultures of iN cells within CHs of Matrigel and alginate improved the transcriptomic correlation to human brain samples. Alginate is a naturally occurring polysaccharide composed of mannuronic acid and guluronic acid with no cell adhesion ligands, and can be crosslinked to form a network within Matrigel through the addition of divalent cations such as calcium. We demonstrated that increasing the crosslinking of the alginate network in CHs (which led to an increased storage modulus) can tune the correlation of the transcriptome of 3D co-cultured iN cells to the transcriptome of particular subregions of the human brain at specific developmental stages. Furthermore, modulating the amount of crosslinker and/or the volume of the CH led to gene expression changes in specific neuronal transcripts, including DLG4, GRN3A and SOD1, as well as changes in the expression levels of genes associated with neurological diseases (Figs. 3c, 5d). As HA has been previously integrated in cross-linked alginate hydrogels, we incorporated HA in CHs of Matrigel and alginate and, in agreement with our other results, found it had little effect. It will be informative to analyse the effects of changing the hydrogel volume or removing the Matrigel on the cellular state to better understand how these parameters affect gene expression.

To better model the cell composition in the human brain within our 3D tissues, we first developed a method to derive human astrocytic cells directly from hESCs. We exploited a previously reported transient neural progenitor state of stem cells induced via the overexpression of neurogenin to differentiate cells towards an astrocytic phenotype. Immunostaining, qPCR and bulk RNA-seq analyses showed that the derived astrocytic cells expressed astrocyte markers and that this expression increased over time. By day 15, we could detect the expression of marker genes for astrocytic cells, inhibitory neurons and RG, thus demonstrating the potential for rapidly creating 3D tissues with controlled composition of these cell types by generating reporter cell lines using these markers. Using scRNA-seq, we evaluated the gene expression profiles of the cells arising from our differentiation protocols and compared these to published transcriptional datasets of fetal and adult human brain as well as human brain organoids. We found that the cells present in our 3D co-cultured tissues broadly reflected their counterparts in the human brain, and we observed interesting similarities between the gene expression profiles in our system and in human brain organoids as they relate to the human brain data. Overall, these results suggest that we were able to generate relevant cell types, but further functional studies are required to fully characterize these astrocytic cells and inhibitory neurons in our system. Moreover, it will be informative to test additional differentiation protocols (such as the use of other transcription factors or small molecules) to further expand the cell types that can be studied in this system.

Our method of deriving both iN and astrocytic cells from the same pool of hESCs enables the rapid creation of engineered tissues with an isogenic background. These 3D tissues, composed of iN cells (at day 35) and astrocytic cells (at day 118), exhibited transcriptional profiles that correlated with relevant cell types in the human brain as well as with 6-month-old human brain organoids, suggesting that this system may be a faster alternative to organoids. To demonstrate the potential for studying the genetics of neurodegenerative diseases in our 3D neural tissues, we perturbed three genes implicated in ALS and frontotemporal dementia using Cpf1 directly in iN cells, and observed at least 5% indel formation rates for each gene. Directly injecting AAV mixtures within 3D tissues instead of mixing with culture medium could be tested in future studies to improve indel formation rates. This approach could be extended by independently targeting astrocytic cells within 3D tissues by using the GFAP promoter in gene editing constructs.

Despite the potential for this approach as a scalable method for interrogating the genetics of brain disorders, there are a number of limitations and challenges. All 3D tissue models are limited in their ability to recapitulate complex environmental features, such as the interplay between the immune system and the central nervous system, vasculature and the signals that are distributed throughout this network, and ageing. Nevertheless, as the technology and our understanding of the brain advances, it should be possible to develop increasingly complex tissues that contain multiple cell types that develop over time.

Outlook

By varying the parameters of the 3D neural matrix (for example, adjusting the composition), we have shown that our engineered tissues can be tuned to express different levels of neuronal transcripts as well as genes associated with neurological diseases and to reflect specific stages of the human brain developmental transcriptome.
Single-cell sequencing revealed that our 3D tissues contain cell types that transcriptionally resemble their analogues in the human brain, further supporting the utility of this system. In combination with genome editing tools, which can be used to precisely disrupt specific genes in a cell-type specific manner, these tissues offer an adaptable and genetically tractable system for studying neurodevelopmental disorders. In particular, the ability to rapidly model polygenic diseases such as ALS, Alzheimer disease and Parkinson disease in a controllable environment will be particularly beneficial for unraveling these complex diseases. This goal may be achieved by either independently targeting gene function in neuronal and astrocytic cells or developing 3D tissues directly from hESCs carrying the desired mutations and utilizing scRNA-seq or single-nucleus RNA-seq methodologies in combination with functional studies.

3D cultures and co-cultures of iN cells. 3D cultures and co-cultures of iN cells were performed by encapsulating them within hydrogels. These hydrogels were made of pure GFR Matrigel, GFR Matrigel and HA, GFR Matrigel and alginate, or GFR Matrigel, alginate and HA. For the encapsulation experiments, the following preparations were performed: GFR Matrigel was kept on ice, sodium alginate (3%) in a medium made of a 1:3 mixture of mTeSR and neurobasal medium (without supplements) with 150 mM sodium chloride (Sigma) and this solution was then incubated for 6 h at 37 °C to enable further dissolution of the alginate. This alginate stock solution was passed through a 0.22 μm filter (EMD Millipore) and kept on ice. Calcium chloride (CaCl2) solution (1 M in water, Sigma) was diluted in a medium made of a 1:3 mixture of mTeSR and neurobasal medium (without supplements) at concentrations of 25 mM, 12.5 mM, 6.25 mM and 3.125 mM. These CaCl2 solutions were individually passed through 0.22 μm filters and kept on ice. HA sodium salt (Sigma, #53747) was dissolved at 1% (10 mg ml−1) concentration under sterile conditions in a medium made of a 1:3 mixture of mTeSR and neurobasal medium (without supplements) for 6 h at 37 °C to enable further dissolution of the HA and frequently vortexed. This HA stock solution was kept on ice. A sheet of Parafilm and a microcentrifuge tube rack were sprayed with 70% ethanol and kept in a biohood under UV light for 30 min. Parafilm dimples were formed by placing a sheet of Parafilm on the microcentrifuge tube rack and pressing gently on Parafilm. Serum-free DMEM was kept on ice.

3D cultures of iN cells were performed by encapsulating them within 200 μl hydrogels of Matrigel at 10×10^6 cells ml−1. iN cells generated within 3 days were detached as previously described and filtered through a 40 μm cell strainer (Corning), and pelleted at the desired amount. The final concentration of iN cells used in each condition was either 10^6 cells ml−1. Matrigel stock concentration varied from batch to batch (8.5 mg ml−1 to 10 mg ml−1), different amounts of iN culture medium were used to adjust the final concentration of Matrigel in hydrogels and kept on ice until used. A desired amount of Matrigel stock solution was placed in a 1.5 ml centrifuge tube and kept on ice. A pipette tip was chilled by pipetting cold serum-free DMEM. This pipette tip was then used to resuspend the iN cell pellet in pre-chilled 1.3 culture medium. These resuspended iN cells were then mixed with the Matrigel in the 1.5 ml centrifuge tube. The final concentration of iN cells in this cell-gel solution was 10×10^6 cells ml−1, and the final concentration of Matrigel in this solution was either 4.6 mg ml−1 or 7.36 mg ml−1. The cell-gel solution was vortexed for 10 s and then kept on ice while chilling a plate. Once the plate was free of pipetting cold solutions the Matrigel stock concentration was adjusted by the human EF1A (hEF1A) promoter, and doxycycline-inducible expression of human NGN1 (hNGN1) and NGN2 (hNGN2), NGN1 (human) and NGN2 (human) were linked to hNGN1 and hNGN2 by a P2A linker to enable selection. Lentivirus-infected hESCs were plated at ~15,000 cells cm−2 in 15 cm tissue culture dishes pre-coated with 5 μg ml−1 doxycycline. One-third of the entire culture was then incubated for 6 h at 37 °C to enable further dissolution of the alginate. This alginate stock solution was passed through a 0.22 μm filter (EMD Millipore) and kept on ice. A sheet of Parafilm and a microcentrifuge tube rack were sprayed with 70% ethanol and kept in a biohood under UV light for 30 min. Parafilm dimples were formed by placing a sheet of Parafilm on the microcentrifuge tube rack and pressing gently on Parafilm. Serum-free DMEM was kept on ice.
3D co-cultures of iN cells with human astrocytic cells and with human primary astrocytes. Astrocytic cells derived following the morphogen-FBS protocol cultured until day 52 (passage 5) and day 61 (passage 7) were detached from culture plates with Accutase and pooled for 3D cultures and co-cultures with iN cells. For 3D co-cultures of iN cells with human astrocytic cells, a 1:1 mixture at a final cell concentration of 20×10^6 cells ml^-1 were encapsulated in 100 μl of hydrogels (7.36 mg ml^-1 Matrigel). 3D co-cultures of iN cells with human primary astrocytes (passage 5) were carried out by encapsulating a 1:1 mixture (at a final cell concentration of 20×10^6 cells ml^-1) within 100 μl of hydrogels (7.36 mg ml^-1 Matrigel). 3D cultures of iN cells alone and 3D cultures of human astrocytic cells alone were performed by encapsulating them (at a final cell concentration of 10×10^6 cells ml^-1) within separate 100 μl of hydrogels (7.36 mg ml^-1 Matrigel). After UCl3 encapsulations, protocols were as described above with the following changes: the culture medium did not contain doxycycline and the final concentration of Ara-C in each culture well was 1 μM. Six days after the formation of 3D cultures and co-cultures, the 3D cultures and co-cultures of iN cells were infected with AAV U6-hsyn1-mCherry-KASH-iHGH vectors encoding non-targeting single guide RNA to enable FACS of iN cells. At week 5 of culture, 3D cultures and co-cultures of iN cells were disassociated by first individually immersing 3D tissues in Accutase, incubating at 37 °C for ~8 min, and then disrupting the 3D matrix by pipetting in Accutase. Each disassociated tissue sample was resuspended in neural culture medium and centrifuged at 200 × g for 5 min. The pellet was then resuspended in a 1:3 DharmaFECT transfection filter (Dharmacon) to remove potential cell clumps and hydrogel residue before cell sorting. Cell suspensions were sorted using a Beckman Coulter MoFlo Astrios EQ cell sorter (Broad Institute Flow Cytometry Core). Each 3D culture and co-culture was analysed in triplicate, and a population of 1×10^4 mCherry-positive iN cells was collected from each disassociated 3D tissue into DNA/RNA Shield (Zymo Research). 3D cultures of human astrocytic cells were individually immersed in DNA/RNA Shield without cell sorting.

RNA isolation. For 2D cultures of iN cells and their 2D co-cultures with mouse astrocytes and for 2D cultures of only mouse astrocytes, 2D cultured hESCs, and day 3 iN cells, 300 μl of RNA lysis buffer (Zymo Research) was used to lyse cells in each well of a 6-well plate. Each lysate was then transferred to a 1.5 ml centrifuge tube with this CaCl2 solution on a Parafilm dimple. For 3D cultures of iN cells and their 3D co-cultures with mouse astrocytes, each hydrogel was transferred using an RNase-free spatula (Corning) from a culture well to a 1.5 ml centrifuge tube and immersed in 300 μl of RNA lysis buffer. All centrifuge tubes were placed on dry ice for rapid freezing and then stored at −80 °C. Total RNA isolation was performed using a Zymo RNA QuickPrep Mini kit (Zymo Research) following the manufacturer’s protocol with the following modifications. 3D culture hydrogels were homogenized using a hand-held pestle (Fisher Scientific) in a 1.5 ml Eppendorf tube containing 300 μl lysis buffer with a few strikes to break down the large pieces. Lysates were transferred to a 1.5 ml tube and centrifuged at 14,000 g for 5 min at room temperature. The supernatant was used for RNA isolation using a standard procedure. For CHs containing HA, the cell concentration was 30×10^6 cells ml^-1. CaCl2 was added to each hydrogel solution with this CaCl2 solution on a Parafilm dimple. 3D cultures of human astrocytic cells were individually immersed in DNA/RNA Shield without cell sorting.

For CHs containing HA, the cell concentration was 30×10^6 cells ml^-1. CaCl2 was used to crosslink alginate in the CHs. Each 200 μl droplet of CH was made by mixing 150 μl cell–gel solution with 50 μl CaCl2 solution on a Parafilm dimple. The cell–gel solution was prepared accordingly so that in each 200 μl CH, the final concentration of Matrigel was 4.6 mg ml^-1 and that of alginate was 0.5%. For each 200 μl droplet of CH containing HA, the final HA concentration was either 1 mg ml^-1 or 1.5 mg ml^-1. To prepare gel solutions, a desired volume of Matrigel was placed in a centrifuge tube with a pre-chilled pipette tip and then the desired volume of 4% alginate mixed with Matrigel in the centrifuge tube with a pre-chilled pipette tip, vortexed for 10 s, and kept on ice. For CHs containing HA, a desired volume of 10 mg ml^-1 HA was mixed with Matrigel and alginate solution, vortexed for 10 s, and kept on ice. A volume of 1:3 culture medium required to adjust the concentration of components in gel solution was used to resuspend 1:1 mixture of iN cells and mouse astrocytes. These resuspended cells were then mixed with gel solution and vortexed for 10 s. Each 200 μl droplet of CH was made by first placing 50 μl CaCl2 solution (at concentrations of 25 mM, 12.5 mM, 6.25 mM and 3.125 mM) on a Parafilm dimple and then rapidly mixing 150 μl cell–gel solution with this CaCl2 solution on a Parafilm dimple with a pre-chilled pipette tip without generating bubbles. Droplets (50 μl) of CHs were generated in the same manner by adjusting volumes. To allow Matrigel gelling, all droplets were then placed at 37 °C for 1 h. After forming the hydrogels, the same protocol as described above was followed for the remainder of the experiment, except the final concentration of Ara-C in each well was at 2–5 μM, which was added with the neural culture medium in each well on the second day of encapsulation.

Rheological measurements. Hydrogels (100 μl) volume without cells were formed as described above. The mechanical properties of the hydrogels were characterized using a AR 2000 rheometer (TA Instruments) fitted with a Peltier stage set to 37 °C. Oscillatory frequency sweep measurements were conducted at a 0.5% strain amplitude. All measurements were performed in triplicates using a 8 mm 4° cone and 200 μm gap size, and the results analysed using TRIOS software (TA instruments).

Derivation of human astrocytic cells. Each well of a 6-well plate was coated with 1.5 ml of GFR Matrigel diluted in DMEM at 1:100 ratio and incubated for 30 min at 37°C. The culture medium required to adjust the concentration of components in gel solution was used to resuspend a 1:1 mixture of iN cells and mouse astrocytes. These resuspended cells were then mixed with gel solution and vortexed for 10 s. Each 200 μl droplet of CH was made by first placing 50 μl CaCl2 solution (at concentrations of 25 μM, 12.5 μM, 6.25 μM and 3.125 μM) on a Parafilm dimple and then rapidly mixing 150 μl cell–gel solution with this CaCl2 solution on a Parafilm dimple with a pre-chilled pipette tip without generating bubbles. Droplets (50 μl) of CHs were generated in the same manner by adjusting volumes. To allow Matrigel gelling, all droplets were then placed at 37°C for 1 h. After forming the hydrogels, the same protocol as described above was followed for the remainder of the experiment, except the final concentration of Ara-C in each well was at 2–5 μM, which was added with the neural culture medium in each well on the second day of encapsulation.
percentage human reads. The STAR bams output by RSEM were used to filter the original fastq files with seqtk (https://github.com/felix/seqtk). In particular, all reads that mapped to the mouse transcriptome were removed, as were unmapped reads. The filtered fastq files were run on sequencing reads using the human hg19 UCSC genome to estimate gene expression values, which were then used to create a non-normalized count matrix. This count matrix was used for further analysis involving co-culture conditions of iN cells with mouse astrocytes. For the data analysis of culture conditions of iN cells without mouse astrocytes, RSEM with default parameters was run on fastq files of iN cells using the human hg19 UCSC genome to estimate gene expression values, which were then used to form a non-normalized count matrix. Similarly, RSEM with default parameters was used on sequencing reads of conditions involved in the derivation of astrocytic cells using the human hg19 UCSC genome to estimate gene expression values, which was then used to generate a non-normalized count matrix.

Further bulk RNA-seq data analysis was performed using DESeq2 package54 and R v.3.2. Following DESeq2 protocols58, significant genes from the differential expression analysis were identified using DESeq2's own two-sided statistical test and taking the false discovery rate (FDR) adjusted P value cut-off of 0.05 for all analyses in this study. As previously described54, DESeq2 relies on the negative binomial distribution and utilizes Benjamini–Hochberg adjustment. PCA was carried out using DESeq2 package and R. Database for Annotation, Visualization and Integrated Discovery v6.7 and v6.8 and the Molecular Signatures Database (MSigDB) were used to reveal enriched biological processes for differentially expressed genes (upregulated or downregulated in one condition). GSEA was performed using GSEA software59 v.2.2.3 using default parameters to find enriched biological processes in GO and enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways in compared conditions. The k-means function in R was used to cluster groups of genes. Groups were generated by first performing differential expression analysis between a reference condition and other conditions and then filtering the resulting differentially expressed genes by setting log(2, fold change) and P cut-off values.

To compare the transcriptomes of 3D cultures of iN cells to the human brain development transcriptome, gene level expression values were obtained from the BrainSpan database (http://www.brainspan.org/static/download.html). To our knowledge, raw data for the BrainSpan transcriptome profiling dataset in the form of fastq or BAM files are not available for download. Therefore, in an effort to minimize technical differences between the experimental and reference datasets, RNA-seq data of 3D cultures of iN cells were reproced to more closely match that of BrainSpan following the alignment and gene quantification protocol as detailed. Briefly, filtered fastq files were then used as default parameters with TopHat v.2.0.14 using Bowtie v.0.12.9 and samtools v.0.1.9. To further match the BrainSpan dataset, the data were processed using Gencode v.10. RSEQtools, which was utilized by the BrainSpan group, to obtain gene level expression values. This process included converting the reproced BAM files into MRG files, and using the mrqQuantifier function to obtain the final gene expression matrix. The BrainSpan region expression matrix between development stages of 8 pcw and 1 year old was filtered to maximize the proportion of genes with high expression (RPKM > 5) and variance (> 1). Pearson correlations based on these 11,972 genes were obtained for each 3D culture condition of iN cells and developmental brain region–time point pair. All plots represent the mean correlation (± s.e.m.) of three replicates of 3D culture conditions of iN cells with a specific developmental brain region and time point.

For profiling expression levels of disease-associated genes in iN cells cultured in 3D conditions, ASD-associated genes were obtained from the Gene Family Atlas (https://www.sfari.org/auxdb/GSGenetLat.do?cs=S, ALS-associated genes were obtained from the ALS Online Database60 (http://ashod.rop.kcl.ac.uk/misc/download.aspx?C1) and the ALS Gene Database61 (http://www.alsgene.org/top_results), Alzheimer disease-associated genes were obtained from a published study62, and Parkinson disease-associated genes were obtained from the PD Gene Database63 (http://www.pdgene.org/top_results). To determine which genes are driving the increased correlation in expression with BrainSpan data in iN cells co-cultured in the G0 condition with 4x10^5 iN cells co-cultured in Matrigel, we calculated both the squared log fold-change between iN cells co-cultured in Matrigel and BrainSpan data and the squared log fold-change between iN cells co-cultured in the CH with 4x10^5 crosslinker and BrainSpan data (using RPKM normalized data), and took the difference between the two values to obtain our final score.

scRNA-seq. 3D co-cultures of iN cells with mouse astrocytes and with human astrocytes were loaded onto a Chromium Instrument (10^6 cells ml–1) in corresponding CHs and cultured as described above. Astrocytic cells derived using the morphogen–FBS protocol were cultured until day 83 in passage 5, and were detached in passage 6 from culture plates with Accutase and pooled. A 1:1 mixture of iN cells and astrocytic cells was encapsulated at a final concentration of 30 × 10^6 cells ml–1 in corresponding CHs and cultured as described above, with the following change: the culture medium did not contain doxycycline. At week 5 of culture, cell dissociation from 3D tissues was performed utilizing a previously described protocol64 with the following modifications. Briefly, each 3D tissue was cut into small pieces with a blade and immersed in 1 ml of 20:1 mix of papain solution (PAP2, Worthington) and DNase solution (D2, Worthington) in a 15 ml tube, which was then incubated at 37°C for 20 min and shaken by hand every 5 min. After 20 min, pieces of 3D tissues in this solution were pipetted for further dissociation and then incubated at 37°C for 10 min. A volume (1 ml) of Earl's balanced salt solution (EBSS, Worthington) was mixed with the solution of dissociated tissue. The cloudy cell suspension was transferred to a new 15 ml tube and mixed with 1.9 ml of inhibitor solution (OLBSA, Worthington). This final solution was then centrifuged at 300g for 5 min. Cell pellets from three biological replicates of each 3D co-culture condition (iN cells with mouse astrocytes and iN cells with human astrocytic cells) were pooled by resuspension in the cell pellets in ice-cold DPBS with 0.2% BSA (Sigma), which was then passed through a 30 μm filter (Sysmex). Cell suspensions for each condition, 3D co-cultures of iN cells with mouse astrocytes and 3D co-cultures of iN cells with human astrocytic cells, were loaded onto a 10x Chromium Instrument (10x Genomics) through two independent channels to generate single-cell gel bead in emulsion. scRNA-seq libraries were constructed using 10x Chromium 3' Solution (10x Genomics) following the manufacturer’s protocol and sequenced on a NextSeq 500 instrument (Illumina) with 26 bases for read and 57 bases for read2.
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**Author contributions**

H.T. and F.Z. conceived the study. H.T. designed the experiments, developed 3D and 2D cultures and 3D co-cultures of iN cells and the method to derive astrocytic cells, analysed the RNA-seq data, performed qPCR, immunostaining and imaging. S.S. filtered mouse reads from bulk RNA-seq data of iN cells co-cultured with mouse astrocytes. B.C. and H.T. performed and interpreted the comparisons between the transcriptomes of 3D cultures and co-cultures of iN cells and the human brain developmental transcriptome. D.D. constructed scRNA-seq libraries and C.C.H. performed sequencing. J.Z.L. aligned the scRNA-seq data to reference genomes. S.S. and H.T. analysed and interpreted the scRNA-seq data. L.G., S.R.C. and M.H. cloned the DNA constructs. L.G. and H.T. tested single guide RNAs in HEK cells. S.R.C. produced the AAVs. H.T. developed 3D tissues of iN cells and astrocytic cells, performed AAV infection of iN cells in 3D tissues and isolated targeted iN cells by FACS. J.Z.L. performed electrophysiology experiments. V.Y. and H.T. performed the mechanical characterization of the hydrogels. N.E.S. and X.S. developed hESCs with inducible expression of NGN1 and NGN2. C.L. isolated and expanded mouse glia. H.T. wrote the paper with input from all authors.

**Competing interests**

H.T. and F.Z. are co-inventors in a patent application relating to work in this manuscript. The remaining authors declare no competing interests.

**Additional information**

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**Experimental design**

1. **Sample size**
   Describe how sample size was determined.
   
   Where possible, we used 3 biological replicates as standard practice (see Replication section for details).

2. **Data exclusions**
   Describe any data exclusions.
   
   For single-cell RNA-sequencing analysis, we removed low quality cells (expressing less than 1000 human genes or more than 300 mouse genes) from downstream analysis.

3. **Replication**
   Describe the measures taken to verify the reproducibility of the experimental findings.
   
   We used three replicates for all experiments where possible. Results were reproduced between biological replicates. For iN cells day-3 conditions, we used 2 replicates (given the limited nature of the analysis of this condition). For electrophysiology measurements, we used 6 cells, but we do not perform any statistical tests on these results; they are included to demonstrate the feasibility of this approach. For genome editing experiments, 2 biological replicates of 3D tissues were used (per condition). For the targeting gRNA, DNA from two biological replicates was used for NGS analysis. For the non-targeting control, DNA from two biological replicates was pooled for NGS analysis. From each tissue, 1000 mCherry+ cells were isolated for analysis of genome editing efficiency. For qPCR comparison of human astrocytic cells to primary human astrocytes, for the control (primary human astrocytes), all 3 replicates were derived from a single pool of astrocytes. For 3D co-cultures of iN cells with human astrocytic cells and with human primary astrocytes experiments, each 3D culture/co-culture had triplicates and a population of 1000 mCherry+ iN cells was collected from each disassociated 3D tissue.

4. **Randomization**
   Describe how samples/organisms/participants were allocated into experimental groups.
   
   This is not relevant to this study. Samples were not randomized.

5. **Blinding**
   Describe whether the investigators were blinded to group allocation during data collection and/or analysis.
   
   This is not relevant to this study. Samples were not blinded.

Note: all in vivo studies must report how sample size was determined and whether blinding and randomization were used.
6. Statistical parameters

For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

| n/a | Confirmed |
|-----|-----------|
| ☐   | ☒ The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.) |
| ☐   | ☒ A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly |
| ☐   | ☒ A statement indicating how many times each experiment was replicated |
| ☐   | ☒ The statistical test(s) used and whether they are one- or two-sided |
| ☐   | ☒ Only common tests should be described solely by name; describe more complex techniques in the Methods section. |
| ☐   | ☒ A description of any assumptions or corrections, such as an adjustment for multiple comparisons |
| ☐   | ☒ Test values indicating whether an effect is present |
| ☐   | ☒ Provide confidence intervals or give results of significance tests (e.g. P values) as exact values whenever appropriate and with effect sizes noted. |
| ☐   | ☒ A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range) |
| ☐   | ☒ Clearly defined error bars in all relevant figure captions (with explicit mention of central tendency and variation) |

See the web collection on statistics for biologists for further resources and guidance.

Software

7. Software

Describe the software used to analyze the data in this study.

Graphpad Prism 7 was used to generate plots for qPCR, electrophysiology, and imaging experiments, gene ontology analyses, and representing indel percentages for genome-editing experiments. Data for characterization of mechanical properties of hydrogels were analyzed by using TA instruments TRIOS software and Graphpad Prism 7. Bulk RNA-seq analyses were performed using DESeq2 package and R. Gene Set Enrichment Analysis (GSEA) was performed using GSEA software v2.2.3. Cellranger was used to map fastq files generated by single-cell RNA-seq to the joint hg19 and mm10 transcriptome. Single-cell RNA-seq analysis was performed using Seurat v1.3 in R.

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). Nature Methods guidance for providing algorithms and software for publication provides further information on this topic.

Materials and reagents

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a third party.

No restrictions.

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

We used commercially available antibodies.

Mouse anti-Map2 (M4403, Sigma) and citing articles: http://www.sigmaaldrich.com/catalog/product/sigma/m4403?lang=en&region=US

Rabbit anti-Pax6 (901301, BioLegend) and citing articles: https://www.biolegend.com/en-us/products/purified-anti-pax-6-antibody-11511

Chicken anti-GFAP (ab4674, Abcam) and citing articles: http://www.abcam.com/gfap-antibody-ab4674-references.html

Mouse anti-S100β (ab11178, Abcam) and citing articles: https://www.citeab.com/antibodies/779833-ab11178-anti-s100-beta-antibody-sh-b1/publications

Rabbit anti-Vimentin (5741, Cell Signaling) and citing articles: https://www.cellsignal.com/products/primary-antibodies/vimentin-d21h3-xp-rabbit-mab/5741
10. Eukaryotic cell lines
   a. State the source of each eukaryotic cell line used. Human Embryonic Stem Cell (hESC) line (HUES66) was obtained from the Harvard Stem Cell Institute (https://ipscore.hsci.harvard.edu) and used with Embryonic Stem Cell Research Oversight committee (ESCRO) approval from the Broad Institute (BRR #32); human embryonic kidney 293FT (HEK293FT) cells were obtained from ATCC.
   b. Describe the method of cell line authentication used. No authentication method was used.
   c. Report whether the cell lines were tested for mycoplasma contamination. Not tested.
   d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use. No commonly misidentified cell lines were used.

## Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals
   Provide all relevant details on animals and/or animal-derived materials used in the study. Newborn C57 mice were used in the study to isolate glial cells from their cortex in procedures carried out in accordance with Animal Care and Use regulations at the Broad Institute, with protocol (0008-06-14) approved by the Broad Institute’s Institutional Animal Care and Use Committee (IACUC).

Policy information about studies involving human research participants

12. Description of human research participants
   Describe the covariate-relevant population characteristics of the human research participants. The study did not involve human research participants.