SUPPLEMENTAL DATA

Detailed protocol for the purification of human astrocytes from hCS

Key differences between purification of astrocytes from hCS versus purification from human primary CNS tissue (Zhang et al., 2016):

- The trituration step is critically important to ensure maximum yield from hCS.
- To help with yield, it is often sufficient to skip the filtering step (step 16 below) if the trituration was done completely. Filtering will lead to a significant decrease in yield, although it is required if planning to FACS at any point after dissociation.
- The ROCK inhibitor Y-27632 should be added to the panning buffer to promote survival during the immunopanning process.
- Because hCS do not contain myeloid lineage cells, immunpanning skips the anti–CD45 plate and starts with anti–Thy1 and anti–HepaCAM plates.
- hCS require at least 30 units of Papain/ml (and up to 50 units/ml for late-stage hCS), which is on the higher end of what is required for primary brain samples.

Day Before

Prepare panning dishes:
Set up panning plates in 15 cm Petri plates: 25 ml of 50mM Tris-HCl pH 9.5 per plate.
1x Thy1 plate: 60 µl anti-mouse IgG
1x HepaCAM plate: 60 µl anti-mouse IgG

Prepare coverslips (this can also be done on the day of prep):
Wash coverslips once with sterile distilled water, transfer coverslips into 24-well plates, add 10 µg/ml poly-D-lysine to each well, incubate at room temperature for 30 minutes, wash 3x with water, and aspirate residual water to dry.

Day of prep

Solutions to Prepare:
*20ml x1 enzyme stock + Papain (600 units for hCS > 70 days, 900 units for hCS > 300 days) + 0.0032-0.0040 g L-cysteine
**21ml x 2 inhibitor stock + 1.5 ml Low Ovo + 100 µl DNase
**10ml x1 inhibitor stock + 2 ml High Ovo + 20 µl DNase
***60ml x 1 0.2% BSA: 57 ml dPBS + 3 ml 4% BSA + 60 µl DNase
***50ml x 1 0.02% BSA: 45 ml dPBS + 5 ml of 0.2% BSA + 50 µl DNase

1. Aliquot 20 ml of enzyme stock* into a 50 ml Falcon tube, break 2 ml pipette, attach filter on top, bubble CO_2 through until solution turns from red to orange, and put into 34C water bath.
2. Aliquot and bubble 2 x 21ml and 1 x 10ml inhibitor stock** as in #1.
3. Wash each panning dish with PBS 3x then add the following antibodies: 20 µl Thy1 in 12 ml of 0.2% BSA, and 20 µl HepaCAM in 12 ml of 0.2% BSA.
4. Add papain to enzyme stock* bubbled with CO_2 and add 0.0036-0.0042 g of L-

cysteine. Warm up solution mixture in 34°C water bath at least 15 minutes before digestion.

5. Transfer hCS to 6 cm Petri plate, use a No. 10 scalpel blade to chop into < 1 mm^3 pieces. Finer is more ideal (at least 30 seconds). Put ~0.5 g of tissue into each 6 cm Petri plate and use multiple petri dishes for digestion if there are more than 0.5g of tissue.
6. Use 0.22 mm filter to filter and discard 2 ml of enzyme stock, then filter 10 ml into each Petri plate containing finely chopped hCS. Add 200 µl DNase to each Petri plate and swirl dish to mix.
7. Papain digestion: Put the Petri plate on a 34°C heat block, drill a ~0.5 cm diameter hole into the lid of the 6 cm Petri plate with heated forceps, attach tubule from a CO_2 tank to a 0.22 mm filter and put the filter tip into the hole in order for the CO_2 to flow over the enzyme stock solution with tissue pieces. Shake the Petri plate every 15 minutes. Digest for 80–100 minutes. Digestion time should be increased with the age of hCS
8. Equilibrate 20 ml of 30% fetal calf serum (FCS) and 8 ml of EBSS in the incubator
9. After digestion, put digested tissue into a 15 ml Falcon tube, wait for tissue to settle, aspirate supernatant, add 4.5 ml of Low Ovo to cells to wash; wait for tissue to settle, repeat for a total of 3 washes.
10. Triturate. Add 4 ml of Low Ovo into the Universal tube, suck up tissue and Low Ovo solution with a 5 ml serological pipette quickly and release quickly, repeat for 20–40 times. Be careful not to introduce bubbles. Do not lift 5 ml pipette out of solution during solution to minimize introduction of air into the solution. Note*** If there is a small amount of tissue, trituration can be done with P1000 pipette in a smaller volume of Low Ovo solution. Low Ovo will become cloudy, therefore let chunks to settle. Transfer single cells with a 1 ml pipette to a Falcon tube, this is the cloudy solution on top of the chunks. Add additional Low Ovo to the Universal tube and repeat trituration.
11. Count cells by diluting it 1:1 with Trypan Blue.
12. Carefully use a 5 ml pipette to layer 2–3 ml of High Ovo under the single cell suspension. This should lead to a clear layer of liquid beneath a cloudy cell suspension. Degree of opacity in cell suspension will depend on the number of hCS used.
13. Spin cells down through High Ovo at 100 g for 5 mins.
14. Aspirate liquid. One should see a pellet of cells at bottom of Falcon tube.
15. Resuspend cell pellet gently with 9 ml of 0.02% BSA***
16. Filter cell suspension through Nitex mesh to remove chunks. If small number of cells, skip this step
17. Wash each panning dishes with 3x dPBS immediately before use.
18. Add cell suspension to Thy1 plate and incubate at room temperature for 15 minutes. Examine the panning dish under a DIC microscope. If cells start to cluster, triturate gently with a 10 ml serological pipette. Transfer the cells to the next panning plate either after the suggested time in this protocol or when visual examination of the plate indicates there are lots of cells stuck.
19. Shake the Thy1 plate and transfer cell suspension to a HepaCAM plate. Then use 1

ml 0.02% BSA to wash the Thy1 plate and collect the 1 ml of solution from the plate and add to the GalC plate. Incubate for 10 minutes.

20. Wash selection plate, ~8x or until floating contaminating cells are gone with dPBS. For RNA-seq, scrape cells off with Qiazol reagent (Qiagen). For cell culture, go to the next step.
21. Add 200 units of trypsin to 8 ml of equilibrated EBSS, incubate at 37°C for 3–15 minutes. Since the activity of different lots of trypsin can vary, it is important to determine the duration of trypsin digestion empirically. Take the plate out of the incubator after 3 minutes, tap side of the plate, and look under the microscope. Incubate for longer if most cells are still stuck and stop the digestion if about half of the cells are dislodged.
22. Squirt gently around the plate with 10 ml of 30% FCS. Go through every part of the plate. Suck off dislodged cells and add to a 50 ml Falcon tube.
23. Add another 10 ml of 30% FCS to squirt if there are many cells left after the first round of squirting. Add cells to the Falcon tube.
24. Count cells.
25. Add 100 µl of DNase per 10 ml of solution and spin cells down at 130 g for 10 minutes.
26. Aspirate supernatant and resuspend cell pellet in growth media.
27. Pre-plate cells in 50 µl of media onto the center of coverslips. Gently transfer to the incubator, leave for 20 minutes, and carefully add on 450 µl growth medium per 24-well plate well.

Reagents

1x Earle's balanced salt solution (EBSS, Sigma E7510)
ACLAR plastic coverslips (Washed in 10% nitric acid overnight on a shaker at room temperature and then in washed in water 5 times, 30 minute each, and in 75% ethanol once. Store in 75% ethanol)
Brain-derived neurotrophic factor (BDNF, Peprotech, 450-02)
Bovine serum albumin (Sigma, A4161)
Ciliary neurotrophic factor (CNTF, Peprotech 450-13)
Dulbecco's modified eagle medium (DMEM, Invitrogen, 11960-044)
Dulbecco's PBS (dPBS, Gibco)
0.4% DNAse, 12,500 units/ml (Worthington, LS002007)
Fetal Calf Serum (FCS, Gibco, 10437-028)
Forskolin (Sigma F6886)
Goat anti-mouse IgG+IgM (H+L) antibody (Jackson ImmunoResearch, 115-005-044)
Heparin-Binding Epidermal Growth Factor-Like Growth Factor (HBEGF, Sigma E4643)
Insulin (Sigma I-6634)
L-cysteine hydrochloride monochloride (Sigma, C7880)
L-glutamine (Invitrogen, 25030-081)
Mouse anti–HepaCAM antibody (R&D systems, MAB4108)
Mouse anti human Thy1 (CD90) antibody (BD, 550402)
N-Acetyl-L-cysteine (NAC, Sigma, A8199)
Neurobasal (Gibco, 21103-049)

Nitex mesh (Tetko Inc, HC3-20)
NS21-MAX (R&D systems, AR008)
Papain (Worthington, LS 03126)
Penicillin / streptomycin (Invitrogen, 15140-122)
Poly-D-Lysine (Sigma, P6407)
SATO (See below)
Sodium Pyruvate (Invitrogen, 11360-070)
3,3′,5-Triiodo-L-thyronine sodium salt (T3, Sigma T6397)
Trypsin 30,000 units/ml stock (Sigma, T9935)

Solutions Required

Enzyme Stock Solution:
Final Volume = 200 ml

Component	Volume	Final Concentration
10x EBSS	20 ml	
30% D(+)–Glucose	2.4 ml	0.46%
1M $NaHCO_3$	5.2 ml	26 mM
50mM EDTA	2 ml	0.5 mM
ddH_2O	170.4 ml	

Bring to 200 ml with ddH2O and filter through 0.22 μm filter

Inhibitor Stock Solution:
Final Volume = 500 ml

Component	Volume	Final Concentration
10x EBSS	50 ml	
30% D(+)-Glucose	6 ml	0.46%
1M $NaHCO_3$	13 ml	25mM
ddH_2O	431 ml	

Bring to 500 ml with ddH2O and filter through 0.22 μm filter

Low Ovo (10X):
To 150 mL D-PBS, add 3 g BSA (Sigma A8806). Mix well. Add 3 g Trypsin inhibitor (Worthington LS003086) and mix to dissolve. Adjust pH to 7.4; requires the addition of approx. 1mL of 1N NaOH. When completely dissolved bring to 200mL with DPBS and filter through 0.22 μm filter. Make 1.0 mL aliquots and store at -20˚C.

High Ovo (10x):
To 150 mL D-PBS add 6 g BSA (Sigma A8806). Add 6 g Trypsin inhibitor (Worthington LS003083) and mix to dissolve. Adjust pH to 7.4; requires the addition of at least 1.5 mL of 1N NaOH. If necessary, add NaOH until solution no longer too acidic. Bring to 200 mL with DPBS. When completely dissolved, filter through 0.22 μm filter. Make 1.0 mL aliquots and store at –20˚C.

Sato (100X):

To prepare:

Add the following to 80 mL Neurobasal medium:	Final conc.
800 mg transferrin (Sigma T-1147)	100 μg/mL
800 mg BSA	100 μg/mL
128 mg putrescine (Sigma P5780)	16 μg/mL
20 μl progesterone (Sigma P8783)* (stock: 2.5 mg in 100 μl EtOH)	60 ng/mL (0.2 μM)
800 μl sodium selenite (Sigma S5261) (4.0 mg+10 μl 1N NaOH in 10 mL NB)	40 ng/mL

*Do not reuse progesterone and Na selenite stocks; make fresh each time.
Mix well and filter through pre-rinsed 0.22 μm filter.
Make 200 μl or 800 μl aliquots; store at –20˚C

Astrocyte growth media:

50% Neurobasal
50% DMEM
100 units/ml of penicillin
100 μg/ml streptomycin
1mM Sodium Pyruvate
2mM L-glutamine
1x SATO
5 μg/ml NAC
5 ng/ml HBEGF

Neuron growth medium for synapse formation experiments

For rat retinal ganglion cells and human fetal neurons
16 mL DMEM
4 mL dH20
200 μL 0.5mg/ml Insulin
200 μL 100mM Pyruvate
200 μL 100x Penicillin / Streptomycin
200 μL 200mM L-Glutamine
200 μL 100x Sato
200 μL 4 μg/ml Thyroxine (T3)
400 μL NS21-Max
20 μL 5mg/ml NAC
Filter sterilize
Immediately before use add 20 μL per 20 mL each: 4.2 mg/ml forskolin, 50 μg/ml BDNF, 10 μg/ml CNTF

Neuron growth medium for neuron survival experiments

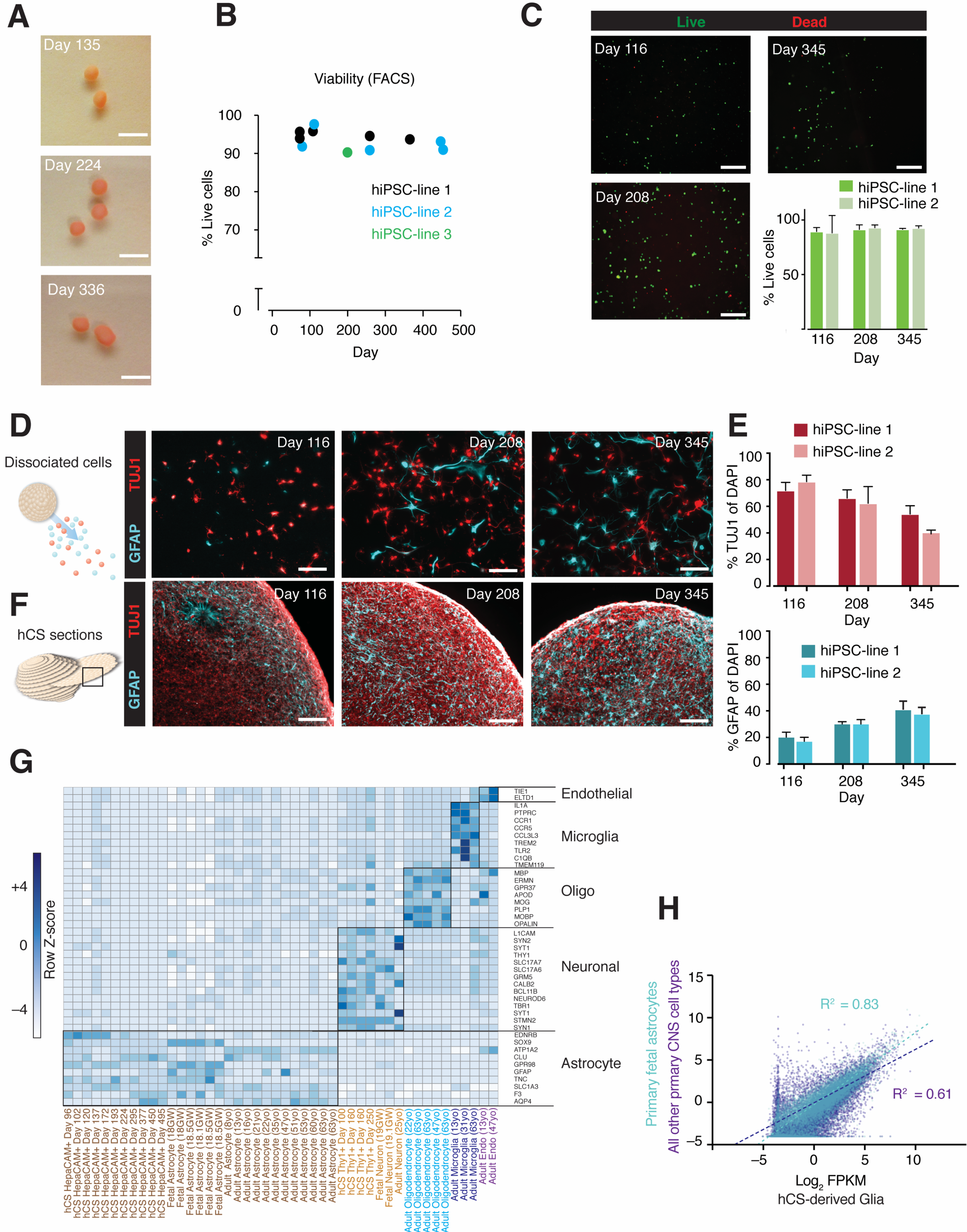
Omit NS21–Max, BDNF and CNTF from the neuron growth medium for synapse formation experiments.

R Code for over-dispersion and t-SNE

```
# Over-dispersion function
sel.by.cv <- function(counts.nodups) {
  require(statmod)# library(pcaMethods); library(fastICA)
  ed <- counts.nodups*1000000/colSums(counts.nodups) # Second pass, no duplicates
  ed <- counts.nodups
  means <- rowMeans(ed)
  vars <- apply(ed,1,var)
  cv2 <- vars/means^2
  winsorize <- function (x, fraction=0.05) {
    if(length(fraction) != 1 || fraction < 0 ||
       fraction > 0.5) {
      stop("bad value for 'fraction'")
    }
    lim <- quantile(x, probs=c(fraction, 1-fraction))
    x[ x < lim[1] ] <- lim[1]
    x[ x > lim[2] ] <- lim[2]
    x
  }
  wed <- t(apply(ed, 1, winsorize, fraction=2/ncol(ed)))
  means = rowMeans(wed); vars = apply(wed,1,var); cv2 <- vars/means^2
  useForFit <- means >= unname( quantile( means[ which( cv2 > .3 ) ], .95 ) )
  fit <- glmgam.fit( cbind( a0 = 1, a1tilde = 1/means[useForFit] ),cv2[useForFit] )
  xg <- exp(seq( min(log(means[means>0])), max(log(means), na.rm=T),
length.out=1000 ))
  afit <- fit$coef["a1tilde"]/means+fit$coef["a0"]
  vfit <- fit$coef["a1tilde"]/xg+fit$coef["a0"]
  varFitRatio <- vars/(afit*means^2)
  varorder <- order(varFitRatio,decreasing=T)
  return(varorder)
}

data <- load("Minibrain_workspace.RData")
data_genes_norm_filtered <-data_genes_norm_log
data <- data_genes_norm_filtered

o <- sel.by.cv(data)
# Filter data set for the top overdispersed genes
data_norm_top1000 <- data[o[1:1000],]
# Calculate correlation distance
dist.cor <- as.dist(1-abs(cor(data_norm_top1000)))

# Perform ViSNE dimensionality reduction on 2D
```

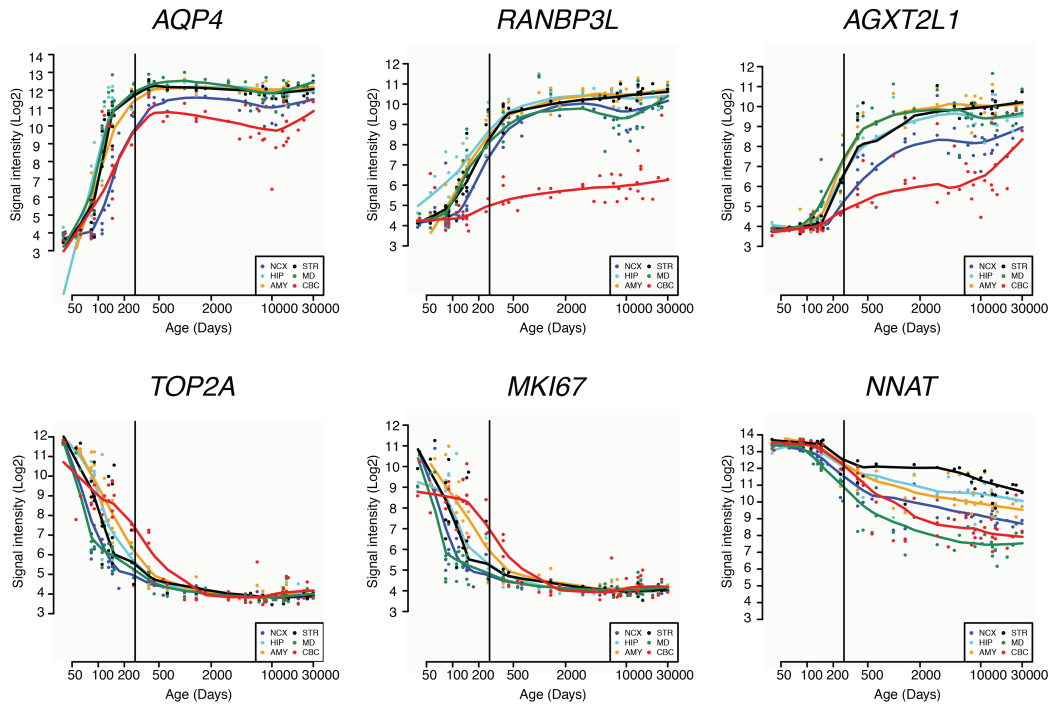
```
my.plot.callback <- function(x) {
  plot(x[,1],x[,2], pch=19)
}
run.tsne <- function(my.dist, plot.callback=my.plot.callback, k, max_iter, perplexity) {
  require(tsne)
  my.tsne <- tsne(my.dist, k=k, initial_dims=50,
epoch_callback=plot.callback,max_iter=max_iter, perplexity=perplexity)
  my.tsne
}

# Perform TSNE
set.seed(123)
my.tsne.2d <- run.tsne(my.dist=dist.cor,k=2, perplexity=50, max_iter=3000) # Last
perplexity used = 15
row.names(my.tsne.2d) <- row.names(as.matrix(dist.cor))
```

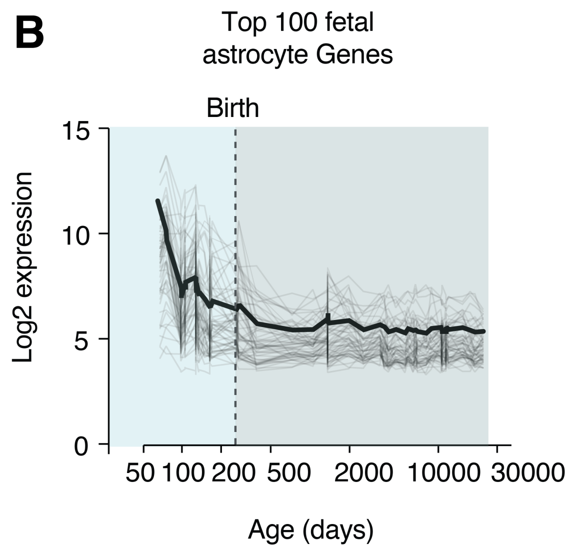
SUPPLEMENTARY FIGURE 1

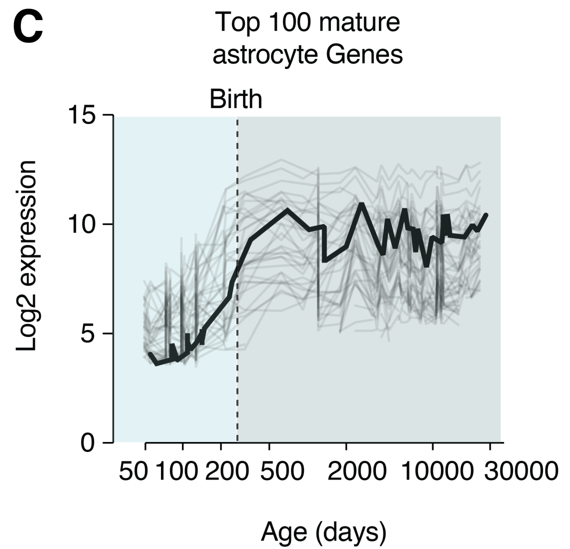


Supplementary Figure 1 (Related to Figure 1).
(**A**) Morphology of hCS at various *in vitro* differentiation stages. Scale bar = 5 mm.
(**B**) Cell viability of dissociated hCS assessed during FACS with the DNA dye DAPI; data from 3 iPSC lines in 9 differentiation experiments. Mean viability ± standard deviation = 94.3% ± 2.3.
(**C**) Viability of hCS immediately after dissociation assessed with the Invitrogen Live/Dead assay using EtBr (red) and Calcein-AM (green) stains. Mean viability ± standard deviation = 90.9% ± 0.7. Representative images of dissociated cells from day 116–345. Cells derived from two iPSC lines in 3 differentiations experiments (6–18 hCS per time-point). Scale bar = 200 μm.
(**D**) Distribution of astrocytes and neurons within hCS at varying *in vitro* differentiation stages assessed by immunocytochemistry with anti– GFAP (cyan) and TUJ1 (red) antibodies in non-immunopanned dissociated hCS (less than 24 hours after plating). Scale bar = 100 μm.
(**E**) Quantification of data in (D); n = 8 fields per time-point. Cells derived from two iPSC lines in 6 differentiation experiments (6–18 hCS per time-point).
(**F**) Distribution of expression of GFAP+ and TUJ1+ in cross-section of hCS at varying stages of *in vitro* differentiation. Scale bar = 100μm.
(**G**) Spearman correlation heatmaps of all hCS-derived and primary human samples. Samples derived from 3 iPSC lines in 1–11 differentiation experiments per line (3–15 hCS per time-point).
(**H**) Scatterplot of the log2 average FPKM for all genes in hCS-derived astrocytes compared with human fetal astrocytes (cyan) or all other human cell types (magenta). Coefficient of determination (R^2) is 0.83 for fetal astrocytes and 0.61 for all other cell types.

SUPPLEMENTARY FIGURE 2

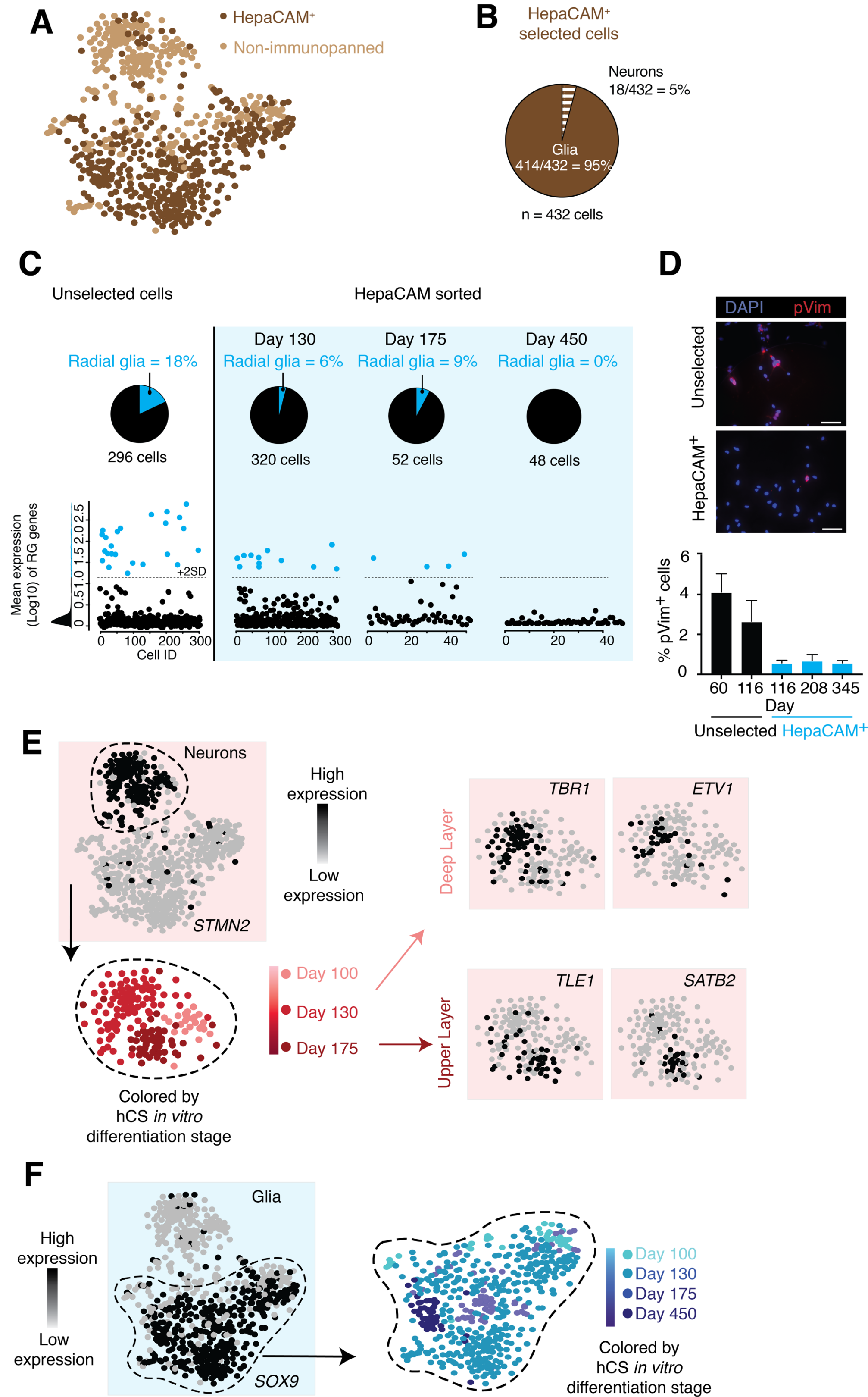






Supplementary Figure 2 (Related to Figure 2).
(**A**) Expression levels of three representative genes highly expressed in mature astrocytes (top) and fetal astrocytes (bottom) across various brain regions throughout development (Kang et al., 2011). NCX = neocortex, HIP = hippocampus, AMY = amygdala, STR = striatum, MD = midbrain, CBC = cerebellum.
(**B, C**) (Light grey) Expression of the top 100 fetal (C) and mature (D) astrocyte genes in dorsal forebrain cortex throughout human development; from (Kang et al., 2011). The dark line in each graph indicates the mean expression of 100 genes throughout developmental time.

SUPPLEMENTARY FIGURE 3



Supplementary Figure 3 (Related to Figure 3).

(**A**) t-SNE representation of all single cells colored by their selection method (HepaCAM selection in dark brown, unselected in light brown).

(**B**) Percentage of HepaCAM-selected cells that are classified as glia (95%) and neurons (5%) based on unsupervised hierarchical clustering in Figure 3D.

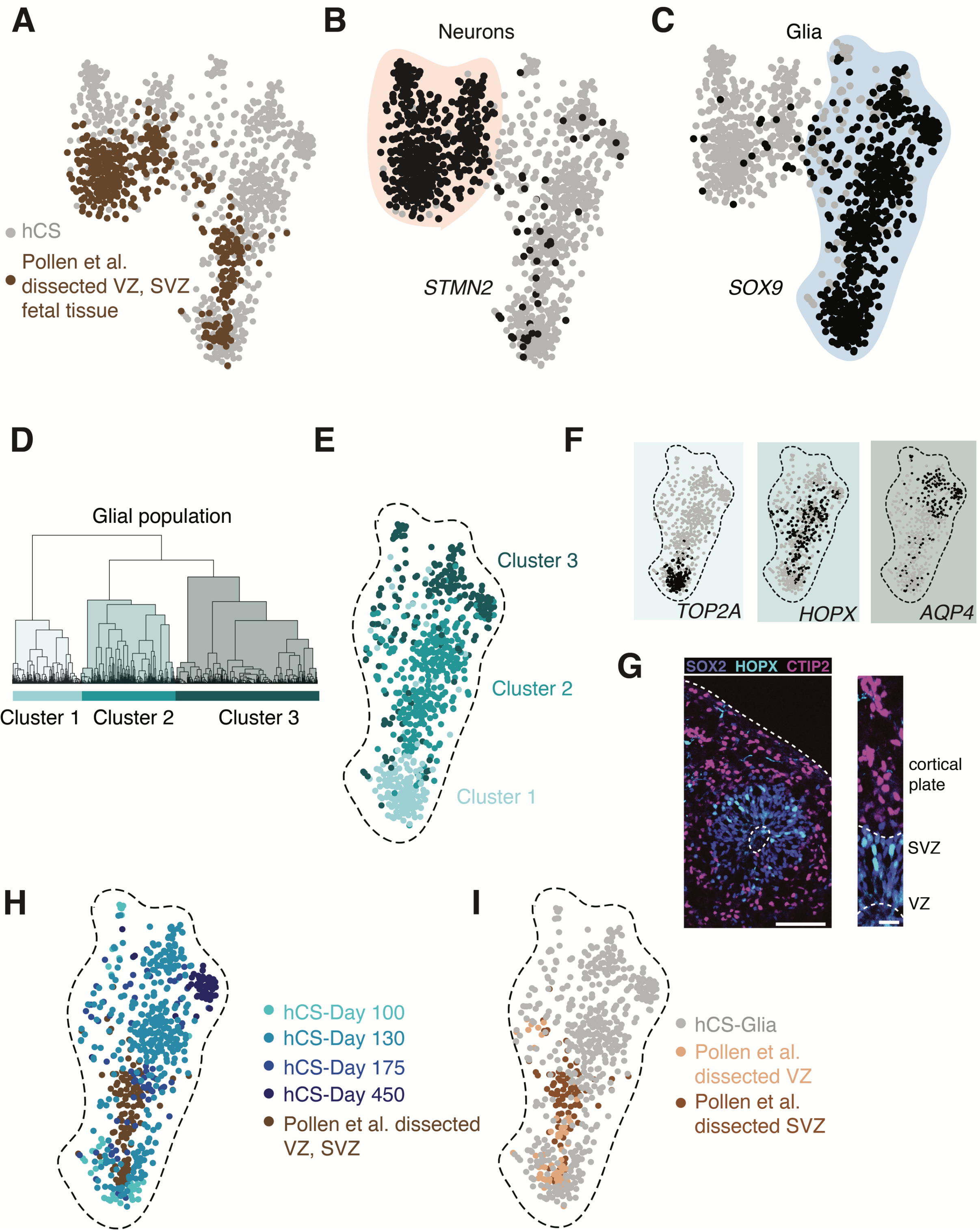
(**C**) Distribution and percentage of ventral radial glia in unselected versus HepaCAM-selected single cells at multiple *in vitro* differentiation stages. Cells from each population were queried for their expression of the top 50 ventral radial glia-specific genes (as determined by DEseq from (Pollen et al., 2015)). A cutoff of 2 standard deviations above the mean (Z = 2.0) was used to identify ventral radial glia (blue dots).

(**D**) (Upper) Phospho-Vimentin (pVIM) immunostaining (at one day after plating) of unselected (top) and HepaCAM$^+$ immunopanned cells (bottom) at day 116 of differentiation. (Lower) Quantification of pVIM$^+$ cells (out of DAPI$^+$ cells) in unselected versus HepaCAM immunopanned cell populations. Cells derived from 2 iPSC lines in 4 differentiation experiments (7–15 hCS per time-point).

(**E**) (Left) Neuronal population colored by *in vitro* differentiation stage. (Right) Expression of lower layer (*CTIP2*, *ETV1*) and upper layer (*SATB2*, *TLE1*) cortical genes.

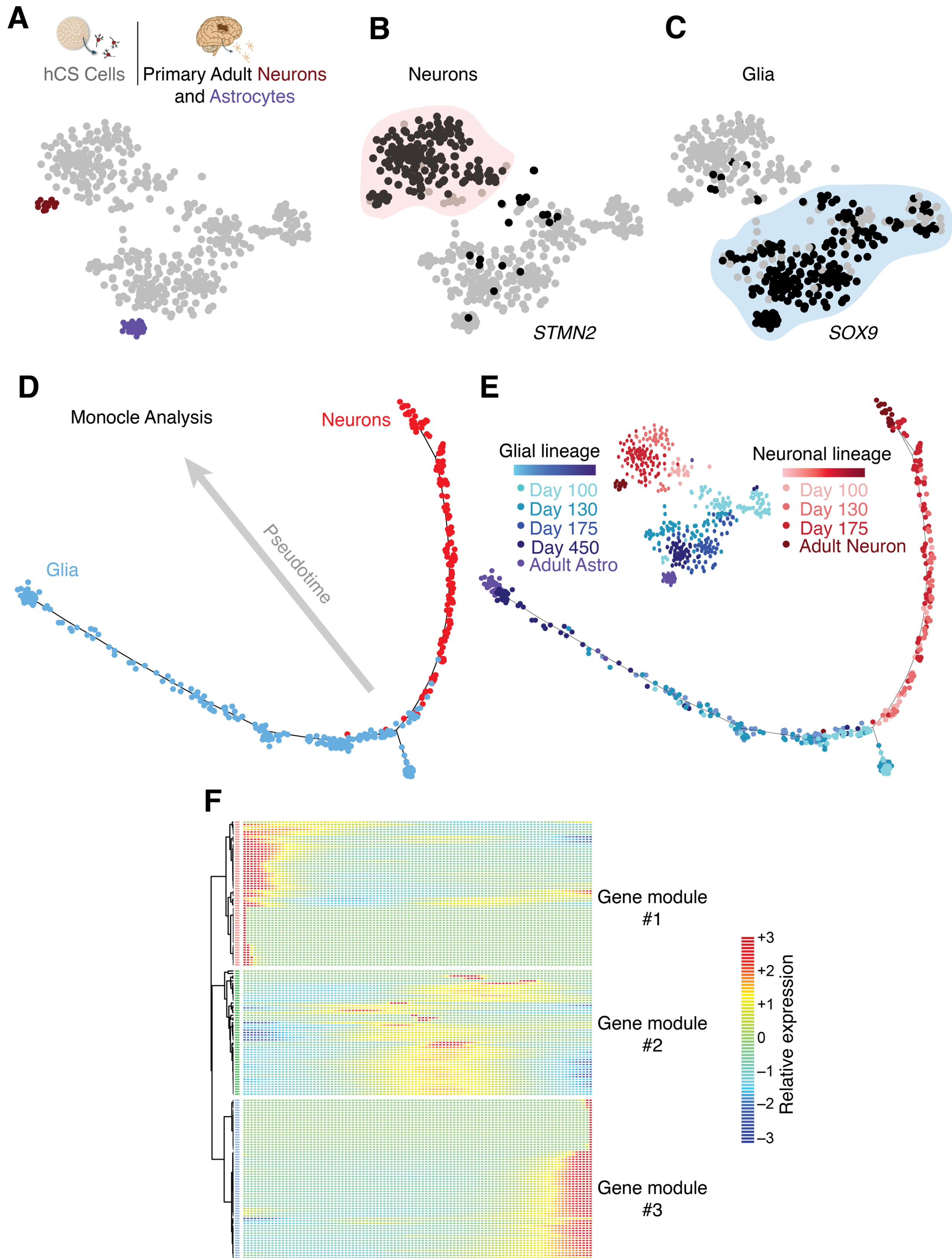
(**F**) Glial population colored by hCS *in vitro* differentiation stage.

SUPPLEMENTARY FIGURE 4



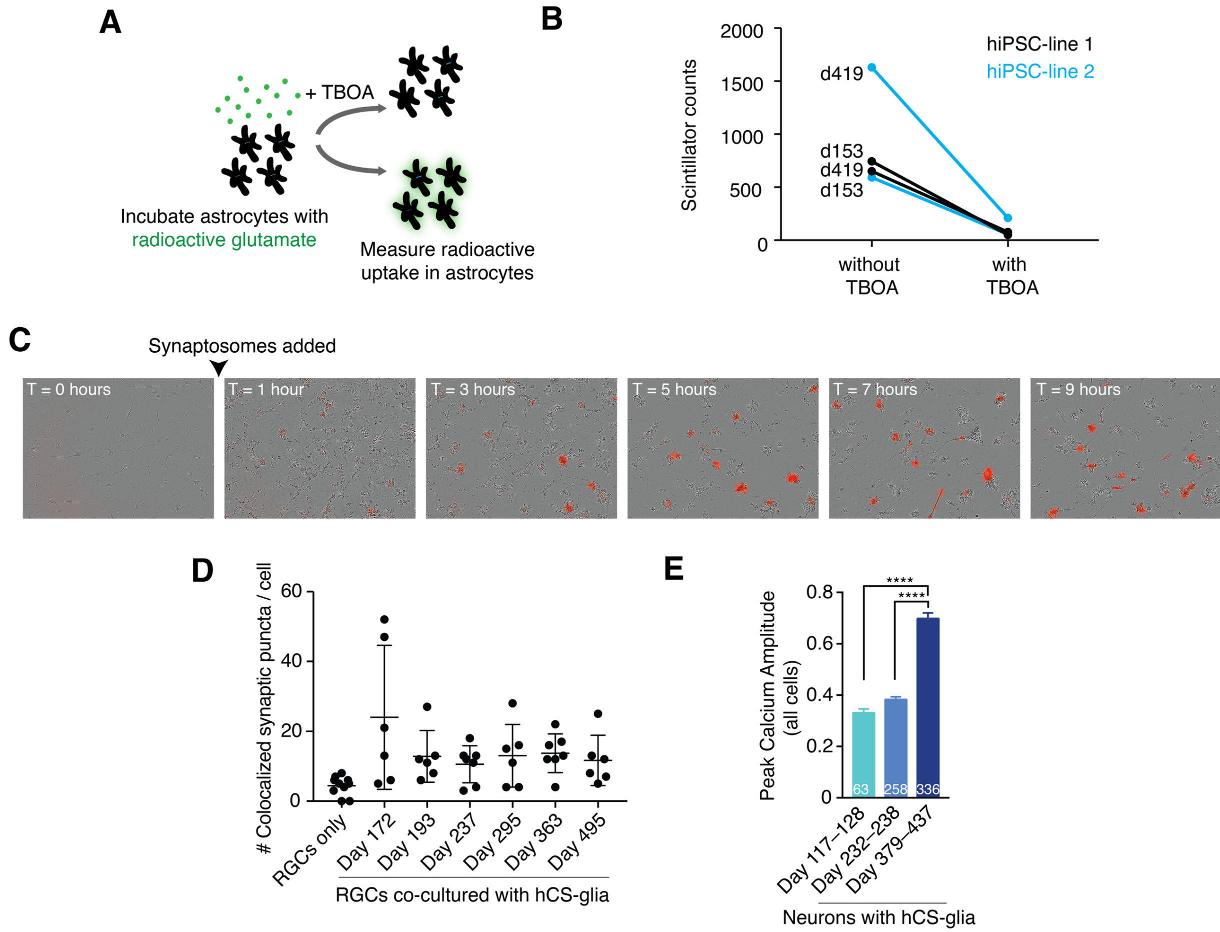
Supplementary Figure 4 (Related to Figure 3).
(**A**) t-SNE representation of all hCS-derived single cells (grey) combined with single cells from primary fetal tissue at GW16–18 (Pollen et al., 2015) (brown).
(**B**, **C**) Neuronal and glial populations as indicated by expression of *STMN2* and *SOX9* in single cells from both hCS and primary fetal tissue (Pollen et al., 2015).
(**D**) Unsupervised hierarchical clustering of glial population in (C) using the top 1000 over-dispersed genes.
(**E**) Glial population from (C) pseudo-colored using the Cluster identity in (D).
(**F**) Expression of various maturation-specific markers in hCS single cells and primary fetal cells from (Pollen et al., 2015).
(**G**) (Left) Immunocytochemistry of hCS at day 125 of differentiation showing HOPX^{+} cells (cyan) at the outer edge of the SOX2^{+} proliferative zone (blue) and below CTIP2^{+} neurons in a cortical plate-like region (magenta). Scale bar = 100 μm. (Right) Inset showing of SOX2, HOPX, and CTIP2 expression in hCS. Scale bar = 20 μm.
(**H, I**) Glial population from (C), which includes hCS and primary cells from Pollen et al. colored by source (hCS versus primary fetal tissue), *in vitro* differentiation stage (day 100–450), and dissection location (VZ, SVZ versus hCS).

SUPPLEMENTARY FIGURE 5



Supplementary Figure 5 (Related to Figure 3).
(**A)** t-SNE representation of all hCS-derived cells (grey) combined with single cells from primary adult brain tissue (dark blue for astrocytes, dark red for neurons) (Darmanis et al., 2015)
(**B, C**) Neuronal and glial populations as indicated by expression of *STMN2* and *SOX9* in single cells from both hCS and adult brain tissue.
(**D**) Monocle lineage tree of all hCS-derived cells and primary adult brain cells, colored by whether the cells were originally classified as astrocytes (blue) or neurons (red) in (C).
(**E**) Same Monocle lineage tree and t-SNE plots (as in A–D) colored by *in vitro* differentiation stage, with astrocyte lineage cells in shades of blue, and neuronal lineage in shades of red.
(**F**) Heatmap identifying three gene modules with pseudotime-specific patterns of expression ($P < 1e^{-4}$) in the glial branch of the lineage tree shown in D.

SUPPLEMENTARY FIGURE 6



Supplementary Figure 6 (Related to Figure 4).
(**A**) Schematic of glutamate uptake assays in hCS-derived $HepaCAM^+$ cells.
(**B**) Scintillator counts of radioactive glutamate in hCS-astrocytes in the presence or absence of TBOA (n = 4 samples from 2 iPSC lines at two *in vitro* differentiation stages; two-tailed paired t-test, P = 0.04)
(**C**) Representative images of synaptosome phagocytosis over a 9-hour imaging period. Scale bar = 100 µm.
(D) Number of co-localized synapse puncta in RGCs cultured with hCS-derived astrocyte lineage cells of various *in vitro* differentiation stages.
(**E**) Peak calcium amplitude of all responding cells following KCl induced depolarization.

Supplementary Table 1(Related to Figure 1).
Top 50 differentially expressed genes between bulk adult human primary astrocytes and hCS-derived astrocytes at day 250–495 (ranked by P-value), along with GO term analysis using all significant differentially expressed genes (cutoff of $P < 0.05$).

Supplementary Table 2 (Related to Figure 3).
Top 50 significantly enriched genes in each Cluster (1–3) of the hCS-glial t-SNE (ranked by P-value).

Supplementary Table 3 (Related to Figure 3).
Differentially expressed genes ($P < 1e^{-4}$) between the three pseudotime-related gene modules.

Supplementary Movie 1 (Related to Figure 4).
Synaptosome assay in iPSC-derived astrocytes isolated from hCS (day 172 of differentiation). Movie duration = 24 hours. Synaptosomes are labeled with pHrodoRed and fluoresce in acidic compartments when engulfed.