Supporting Information

Human pluripotent stem cell-derived neural constructs for predicting neural toxicity

Michael P. Schwartz[a,1], Zhonggang Hou[b,1,2], Nicholas E. Propson[b], Jue Zhang[b], Collin J. Engstrom[c,d], Vitor Santos Costa[e], Peng Jiang[b], Bao Kim Nguyen[b], Jennifer M. Bolin[b], William Daly[a], Yu Wang[b,3], Ron Stewart[b], C. David Page[c,d], William L. Murphy[a,f], James A. Thomson[b,g,h,4]

[a] Department of Biomedical Engineering, University of Wisconsin-Madison, WI 53706

[b] Morgridge Institute for Research, Madison, WI 53715

[c] Department of Biostatistics and Medical Informatics, University of Wisconsin-Madison, WI 53792

[d] Department of Computer Sciences, University of Wisconsin-Madison, WI 53706

[e] Center for Research in Advanced Computing Systems, Institute for Systems and Computer Engineering, Technology and Science, and Department of Computer Science, Faculty of Sciences, University of Porto, Porto 4169-007, Portugal

[f] Department of Orthopedics and Rehabilitation, University of Wisconsin-Madison, WI 53705

[g] Department of Cell and Regenerative Biology, University of Wisconsin-Madison, WI 53705

[h] Department of Molecular, Cellular, and Developmental Biology, University of California-Santa Barbara, CA 93106

[1] Authors contributed equally

[2] Current address: Department of Cell Biology, Harvard Medical School, Boston, MA 02115

[3] Current address: State Key Laboratory of Stem Cell and Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, China

[4] To whom correspondence should be addressed. Email: jthomson@morgridge.org.

Keywords: organoid, machine learning, tissue engineering, differentiation, toxicology

SI Materials and Methods

Human embryonic stem (ES) cell culture.

Essential 8 (E8) medium (1): DMEM/F12 HEPES (Life Technologies, 11330-032), L-ascorbic acid-2-phosphate magnesium (64 mg/L; Sigma-Aldrich, A8960-5G), sodium selenite (14 μg/L; Sigma-Aldrich, S5261), $NaHCO_3$ (543 mg/L), holo-transferrin (10.7 mg/L; Sigma-Aldrich, T0665-1G), insulin (20 mg/L; Sigma-Aldrich, I9278), human recombinant FGF2 (rhFGF2, 100 μg/L), and TGFβ1 (2 μg/L; R&D Systems, 240-B-001MG/CF).

H1 human embryonic stem (ES) cells were maintained in **E8 medium** (1) (Life Technologies) on Matrigel (growth factor reduced, Corning 356230) coated culture plates and were passaged with 0.5 mM EDTA in 1X PBS as previously described (2). Cells were karyotyped within 10 passages and tested negative for mycoplasma contamination.

Human ES cell differentiation into neural progenitor cells (NPCs).

DF3S medium: DMEM/F-12, L-ascorbic acid-2-phosphate magnesium (64 mg/L), sodium selenium (14 μg/L), and $NaHCO_3$ (543 mg/L).

Essential 6 (E6) medium: DMEM/F-12, L-ascorbic acid-2-phosphate magnesium (64 mg/L), sodium selenite (14 μg/L), $NaHCO_3$ (543 mg/L), transferrin (10.7 mg/L), and insulin (20 mg/L).

Neural expansion medium: DF3S medium supplemented with rhFGF2 (5 μg/L), 1X N2 (Life Technologies, 17502-048) and 1X B27 (Life Technologies, 17504-044) supplements.

The procedure for deriving neural progenitor cells was modified from a previously reported protocol (3). H1 ES cells were split using 0.5 mM EDTA in 1X PBS and cultured in **E6 medium supplemented with rhFGF2 (100 μg/L) and SB431542 (TGF-β receptor inhibitor, 10 μM; Sigma-Aldrich)**. After two days, the medium was switched to **E6 medium supplemented with SB431542 (10 μM)** for seven days with daily media exchange to induce the formation of neural rosettes. The neural rosettes were then mechanically dissociated from the culture dish and cultured as floating aggregates in **neural expansion medium** for four days. Aggregates were then dissociated with Accutase (Life Technologies) and plated onto Matrigel (growth factor reduced, Corning 356230) coated plates in **neural expansion medium**. Cells were cultured for an additional 22 days and passaged when confluent, yielding >90% $SOX1^+$/βIII-$tubulin^+$ neural progenitor cells (Fig. S1A, "NPCs"). NPCs were cryopreserved at $1.2x10^7$ cells per vial. Cryopreserved neural progenitor cells were used for subsequent expansion and formation of 3D neural

constructs to ensure a uniform cell source for all experiments (FACS data illustrated in Fig. S1A is for cryopreserved NPCs after thaw).

Human ES cell differentiation into endothelial cells (ECs).

E8BA medium: E8 medium supplemented with BMP4 (5 μg/L) and Activin A (25 μg/L).

E7V medium: E8 medium minus TGFβ1, supplemented with VEGF-A (50 μg/L).

E7BVi medium: E7V supplemented with BMP4 (50 μg/L) and SB431542 (5 μM, TGFβ inhibitor)(4).

H1 ES cells (80-90% confluent) were dissociated using TrypLE (Invitrogen) for three minutes at 37°C and plated 1:3 on vitronectin-coated plates (60 μg / 10 cm dish, VTN-N, Life Technologies)(1). ES cells were first cultured for two days ***(to 100% confluence)*** in **E8BA medium**, which was supplemented with 10 μM Y-27632 for the first day to improve cell survival during attachment. *It is critical to achieve 100% cell confluence by day 2 to ensure highly efficient differentiation*. Cells were then cultured in **E7BVi medium** for an additional three days. Endothelial cells were then isolated with CD34 microbeads (Miltenyi) by autoMACS (Miltenyi) to yield purified populations of $CD34^{+}CD31^{+}$ cells (Fig. S1B, "ECs"). The purified endothelial cells were either cryopreserved immediately or cultured on fibronectin-coated plates in **E7V medium** for one passage before cryopreservation.

Human ES cell differentiation into mesenchymal stem cells (MSCs).

Mesenchymal serum-free expansion medium (M-SFEM)*:* 50% StemLine II serum-free HSC expansion medium (HSFEM; Sigma-Aldrich), 50% human endothelial serum-free medium (ESFM; Invitrogen), GlutaMAX (1/100 dilution; Invitrogen), Ex-Cyte supplement (1/2000 dilution; Millipore), 100 mM monothioglycerol (MTG), and 10 μg/L rhFGF2.

Mesenchymal stem cells (MSCs) were derived from H1 ES cells using a previously published protocol (5). Tissue culture polystyrene plates were coated with human fibronectin (5 mg/mL; Invitrogen) and human collagen I (10 mg/mL; BD Biosciences) in phosphate buffered saline (PBS) for expansion and Accutase (StemPro) was used for passaging. MSCs were expanded for five passages in **M-SFEM** (5), followed by two passages in **pericyte medium** (ScienCell), and then cryopreserved (Fig. S1C, $PDGFRB^{+}CD13^{+}$, "MSCs").

Human ES cell differentiation into microglia/macrophage precursors (MG).

Base Media	Add	Final Concentration
IMDM (Life Technologies, 12440-061)	250 mL	
F12 nutrient mixture (Life Technologies)	250 mL	
L-asorbic acid 2-Phosphate magnesium (Sigma-Aldrich, A8960-5G)	32 mg	64 mg/L
Monothioglycerol (Sigma-Aldrich, 88640-100ML)	20 µL	40 µL/L
Sodium selenite (0.7 mg/mL stock) (Sigma-Aldrich,S5261)	6 µL	8.4 µg/L
Polyvinyl Alcohol (Sigma-Aldrich)		10 mg/L
GlutaMAX 100X (Life Technologies)	5 mL	1X
100X non-essential amino acids (NEAA; Life Technologies, 11140050)	5 mL	1X
100X chemically-defined lipid concentrate (CDLC; Life Technologies)	5 mL	1X
Base Cytokines		
rhInsulin 10 mg/mL (Stem Cell Technologies, 02636)	1 mL	20 mg/L
Human Holo-Transferrin 10.6 mg/mL (R&D Systems, 2914-HT-001G)	500 µL	10.6 mg/L
Differentiation Media 1 (DM1, 12ml)	Add	Final Concentration
Base Media	12 mL	1x
rhFGF2 (100 µg/mL)	6 µL	50 ng/mL
hBMP4 (100 µg/mL; R&D Systems, 314-BP-010/CF)	6 µL	50 ng/mL
hActivin A (100 µg/mL; R&D Systems, 338-AC-010/CF)	15 µL	12.5 ng/mL
LiCl (2M)	12 µL	2 mM
Differentiation Media 2 (DM2, 12ml)	Add	Final Concentration
Base Media	12 mL	1x
rhFGF2 (100 µg/mL)	6 µL	50 ng/mL
rhVEGF (100 µg/mL; R&D Systems, 293-VE-050/CF)	6 µL	50 ng/mL
SB431542 (Sigma)		10 µM
Differentiation Media 3 (DM3, 12ml)	Add	Final Concentration
Base Media	12 mL	1x
rhFGF2 (100 µg/mL)	6 µL	50 ng/mL
rhVEGF (100 µg/mL; R&D Systems, 293-VE-050/CF)	6 µL	50 ng/mL
rhTPO (100 µg/mL; Life Technologies, PHC9514)	6 µL	50 ng/mL
SCF (100 µg/mL; Life Technologies, PHC6034)	6 µL	10 ng/mL
rhIL-6 (100 µg/mL; Life Technologies, 10395HNAE5)	6 µL	50 ng/mL
rhIL-3 (10 µg/mL; Life Technologies, PHC0031)	12 µL	10 ng/mL
Differentiation Media 4 (DM4, 12ml)	Add	Final Concentration
Base Media	12 mL	1x
rhGM-CSF (R&D Systems, 215-GM-050/CF)		200 ng/mL
Differentiation Media 5 (DM5, 12ml)		Final Concentration
IMDM		1x
FBS		10%
rhIL-1B (Peprotech, 200-01B)		10 ng/mL
rhM-CSF (Peprotech, 300-25)		20 ng/mL

Microglia/macrophage precursors were produced using feeder-free conditions by modifying a previous protocol for differentiating H1 ES cells down mesendoderm and hemogenic endothelium lineages (6). 6-well plates were first coated with 40 µg tenascin C overnight at 4^{o} C. Tenascin C plates were rinsed with

PBS, and then seeded with singularized H1 ES cells at a density of 62,500 cells / cm^2 in **E8 medium** + 10 μM Y-27632 (ROCK inhibitor, R&D Systems). Cells were cultured for 24 hours under normoxic conditions.

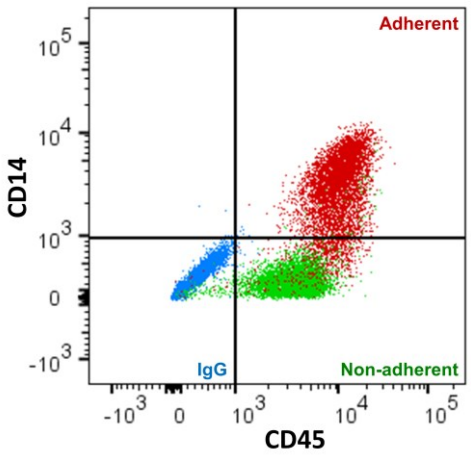
Initiate early mesoderm differentiation. 24 hours after plating H1 ES cells, E8 media was aspirated and replaced with **DM1** + 1 μM Y-27632. Cells were then cultured under ***hypoxic*** conditions (5% O_2) for two days (do not expose cells to normoxia). During the two days of culture, cells will detach and reattach. Take care not to disturb the culture, as cells will aggregate in the middle of the plate, affecting differentiation efficiency.

Continue hematovascular mesoderm differentiation. On day two, check for surviving cell clumps that have not fully reattached. If cells are still non-adherent, use a 10 mL pipette tip to gently pull media off plate, centrifuge non-adherent cells and cell clumps at 300 x g for five minutes to form a pellet, aspirate **DM1** and resuspend in **DM2**, gently plate cells back into same plate, and continue culture in ***hypoxic*** incubator. If only debris is present, aspirate **DM1** and add **DM2** slowly as to not disrupt the adherent cells, and continue culture in ***hypoxic*** incubator.

Differentiate and expand hemogenic endothelial cells into hematopoietic progenitor cells (HPCs). On day 4, aspirate **DM2** medium and replace with **DM3** medium. Continue culture in ***normoxic*** incubator. On day 6 of culture (two days after adding DM3 media), add additional **DM3** media without aspirating media already present. Continue culture in ***normoxic*** incubator. Expand for an additional 3-5 days in **DM3** (longer time required when cells not fully adherent after hematovascular differentiation). If media color indicates a significant pH drop, then pull half the media volume out of the plate and place it into a low attachment dish. Add an additional volume of **DM3** (1:1 mix of old and fresh media) to both culture plates. After 3-5 days, collect spent media containing non-adherent HPCs, and centrifuge at 300 x g for five minutes to pellet.

Myeloid progenitor (MP) differentiation. Continue expansion in myeloid progenitor medium **DM4**, adding $1x10^6$ HPCs / mL to a low attachment culture dish (at this point, cells can be grown in a 10 cm dish), under ***normoxic*** conditions. Expand for 2-5 days in **DM4** medium (at least five days required for proper transition to macrophages, no more than five days), adding media if pH significantly drops (half/half mixture; do not transfer cells). Up to $2x10^7$ cells can be obtained from a 10 cm dish. During expansion in DM4 medium (2-5 days), collect non-adherent cells for sorting to identify $CD34^+$ and $CD45^+$ cells.

Microglia/macrophage precursor (MG) differentiation. After 2-5 days of myeloid progenitor expansion, add $5x10^5$ non-adherent cells to macrophage differentiation medium **DM5** in a 10 cm tissue culture treated dish. Culture cells for three days, then add equivalent volume of **DM5** media (DO NOT aspirate media). After five days (two additional days in **DM5**), ~50-70% of cells will have attached. When cells reach ~70-80% confluence (adherent cells), transfer remaining non-adherent cells to a new 10 cm dish to promote adhesion. Both adherent and non-adherent populations will be $CD45^+$ (see FACS plot, right), but non-adherent cells will be CD14 Low/Negative (see FACS plot, right) and adherent cells will be $CD11b^+CD14^+$ (Fig. S1D, "MG"). On days 5-10, non-adherent cells will begin to attach and transition to $CD11b^+$ and $CD14^+$. Continue culture in **DM5** medium.



Phagocytosis by microglia/macrophage precursors. Aliquots of zymosan A S. cerevisiae BioParticles® (Texas Red® conjugate; Life Technologies) were prepared in PBS. ~$5x10^6$ particles in 500 µL PBS were added to each well of a 6-well plate containing ~400-500K microglia/macrophage precursors in **DM5** media. Phagocytosis was imaged over a 24 hour time period (image capture every 10 minutes) using a Nikon Biostation CT.

Flow cytometry (FACS) analysis.

Flow cytometry analysis was performed on a BD Biosciences FACSCanto II cell analyzer.

Neural Progenitor Cells (NPCs). NPCs were dissociated into single cells with Accutase and fixed with 2% paraformaldehyde in PBS at RT for 10 minutes. Fixed cells were washed once with **FACS buffer I** (2% FBS in PBS) and permeabilized with ice cold 90% methanol in PBS overnight at -20°C. Fixed and permeabilized cells were then washed once with FACS buffer I and stained with SOX1 (1:100 rabbit anti-SOX1, Cell Signaling) and βIII-tubulin (1:200 mouse anti-βIII-tubulin, R&D systems) primary antibodies overnight at 4°C followed by conjugated secondary antibodies at RT for one hour. Stained cells were washed once with FACS buffer I and analyzed by flow cytometry.

Endothelial cells (ECs). ECs were dissociated into single cells with Accutase and washed once with FACS buffer I (2% FBS in PBS). Cells were stained with PE-CD31 (1:100; BD Biosciences, 555446) and

APC-CD34 (1:100; BD Biosciences, 555824) antibodies in FACS buffer I at 4°C for 30 minutes. Stained cells were washed once with FACS buffer I and analyzed by flow cytometry.

Mesenchymal stem cells (MSCs). MSCs were dissociated into single cells with Accutase and washed once with FACS buffer I (2% FBS in PBS). Cells were then stained with fluorescently conjugated PE-PDGFR-β and PE-Cy7-CD13 antibodies in FACS buffer I at 4°C for 30 minutes. Stained cells were then wash once with FACS buffer I and analyzed by flow cytometry.

Microglia/macrophage precursors (MG). Non-adherent cells were first transferred to a conical vial in DM5 medium. Adherent MG were incubated in Accutase, gently removed from the plate using **FACS buffer II** (0.5% BSA in PBS), and added to the conical vial containing non-adherent cells. The cells were centrifuged (5 minutes at 300 x g) and the cell pellet was washed once with FACS buffer II. Cells were then centrifuged (5 minutes at 300 x g), resuspended in FACS buffer II, and incubated at 4°C for 15 minutes for blocking. Cells were centrifuged (5 minutes at 300 x g) and resuspended in FACS buffer II with 1:500 PE-CD11b (BD Biosciences, 555388), Alexa Fluor 488-CD14 (BD Biosciences, 562689) and APC-CD45 (BD Biosciences, 555485) and then incubated at 4°C for 30 minutes (use a shaker plate or invert tube at least three times during incubation). Cells were then washed twice in FACS buffer II and centrifuged (5 minutes at 300 x g). Finally, cells were resuspended in FACS buffer II and analyzed by flow cytometry.

Polyethylene glycol (PEG) hydrogel formulation.

Hydrogel polymerization. Polyethylene glycol (PEG) hydrogels were formed using thiol-ene photopolymerization chemistry with modifications from previously published protocols (7, 8). Stock solutions of 8-arm PEG-norbornene (20,000 MW, JenKem USA, 8ARM (TP)-NB-20K) were prepared at a final concentration of 300 mg/mL by dissolving 300 mg of solid / 0.8 mL PBS to account for volume occupied by 8-arm PEG-norbornene solid, sterile-filtered through a 0.2 μm nylon syringe filter (Fisher Scientific), and stored as frozen aliquots. Matrix metalloproteinase (MMP)-degradable PEG hydrogels were formed using an amino acid sequence modified from a native collagen sequence (9) (KC**GPQG~IWGQ**CK; Active sequence in bold, cleave site = (~); Genscript, >90% purity, C-terminus amidated) with cysteines on each end to crosslink 8-arm PEG-norbornene molecules. Cell adhesion was promoted by incorporating CRGDS peptide (2 mM final monomer solution concentration; Genscript, >90% purity, C-terminus amidated), an amino acid sequence derived from fibronectin (10). Stock MMP-peptide (~75 mM peptide / 150 mM SH) and CRGDS peptide (~100 mM) solutions were prepared and sterile filtered through a 0.22 μm low protein binding PVDF syringe filter (Millex) and the final

concentration was verified after filtration using an Elman's assay (Thermo Scientific; manufacturer's protocol was modified by using PBS to dissolve all reagents).

The final monomer formulation for PEG hydrogels was 40 mg/mL 8-arm PEG-NB, 4.8 mM MMP-peptide crosslinker (9.6 mM cysteine, 60% molar ratio relative to norbornene arms), 2 mM CRGDS, and 0.05% (wt/wt) Irgacure 2959 photoinitiator (BASF Schweiz AG, Basel, Switzerland). Hydrogels were formed by pipetting 30 μL monomer into 24-well BD Transwell inserts (1 μm pores, Fisher Scientific; Quality control experiments) or 40 μL into Corning HTS Transwell-24 well permeable support (0.4 μm pores, Sigma-Aldrich; toxicity experiments). After pipetting, any gaps between the PEG monomer solution and the edge of the insert (due to surface tension) were removed by tilting the insert plate and gently tapping until the solution uniformly covered the bottom of the Transwell insert membrane. Transwell plates containing inserts and monomer solutions were placed on the top shelf of a UVP XX-15 lamp stand (Fisher Scientific) and exposed to ~365 nm centered UV light (UVP XX-15L lamp, Fisher Scientific) for 2.5 minutes. After polymerization, hydrogels were incubated in DF3S medium overnight to allow swelling and equilibration (5% CO_2, 37^{o}C).

Formation of 3D neural constructs.

Seeding H1 ES cell-derived neural progenitor cells (NPCs) on PEG hydrogels. Cryopreserved NPCs were thawed and expanded on 6-well plates coated with Matrigel (growth factor reduced, Corning 356230, 0.5mg per plate for at least one hour) in **neural expansion medium**. One vial of frozen NPCs (~1.2x10^{7} cells) was thawed and plated in three wells of a Matrigel-coated 6-well plate, cultured for 2-3 days (depending on initial confluence) and passaged 1:3 using Accutase. NPCs were passaged 1:3 after two days of additional culture, expanded for 2-3 more days, and used for experiments.

NPCs were removed from the plate using 1 mL Accutase per well, from which an aliquot was removed for counting. After adding the appropriate volume of cell suspension to a conical vial, NPCs were pelleted at 240 x g for four minutes. NPCs were resuspended and seeded in neural expansion medium at a density of 100,000 cells / 24-well insert. NPCs were allowed to attach overnight, and then neural expansion medium was exchanged on day 1 and every two days for the remainder of the experiment. For each media exchange, all media under the insert was aspirated while ~3/4 of the media was removed from the top by sliding the pipette tip down the side of the well to avoid damaging the developing neural tissue constructs.

Differentiation and growth on PEG hydrogels, addition of H1 ES cell-derived endothelial cells (ECs), mesenchymal stem cells (MSCs), and microglia/macrophage precursors (MG). Endothelial cells were

expanded from cryopreserved stocks on fibronectin-coated plates (Life Technologies, 100 μg per plate) in **E7V** media using one vial (~1x10^6 cells) per six wells of a 6-well plate or a single 10 cm dish. ECs were split 1:3 after two days using Accutase, cultured for an additional three days, and then used for experiments. Two days before seeding, MSCs were thawed into **pericyte medium** on fibronectin and collagen-coated tissue culture treated polystyrene plates.

At day 9, ECs and MSCs were seeded on top of the differentiating NPC layer (see Fig. S1F) at a total density of 100,000 cells per well, with a 5:1 ratio of ECs:MSCs. Both ECs and MSCs were harvested using Accutase and counted before centrifugation. Cells were counted and mixed in the appropriate ratio, centrifuged, and resuspended for seeding. **Neural expansion medium** was exchanged on day 11 (2 days after seeding ECs and MSCs). At day 13, MG were harvested and seeded at a density of 25,000 cells per insert. **Neural expansion media** was exchanged on day 14, and then every other day until samples collected for RNA, sorting, or immunofluorescence imaging.

Toxicity screening experiments. For toxicity screening experiments, cells were seeded at densities of 100,000 cells/well for NPCs, 80,000 cells/well for ECs + MSCs (5:1 ratio, ECs:MSCs) and 15,000 cells/well for microglia/macrophage precursors. Neural constructs were treated with non-toxic or toxic compounds starting at day 14, with media exchanged every two days for the day 21 time point. Toxic chemicals were chosen based on previous literature support for neurotoxicity (11-21). All chemicals (including references and vendors) and concentrations used for dosing are provided in Dataset S5.

Immunofluorescence imaging.

Most images represent full neural constructs that were prepared as described in the section "Immunostaining full neural constructs" (data is representative of imaging from at least three separate neural constructs). Images for Fig. 2 and Fig. S3 represent cryosectioned samples that were prepared as described in the section "Immunostaining cryopreserved sections" (representative of at least four sections from two replicate samples for each marker).

Blocking buffer: 0.25 % Triton X-100 and **1% BSA** in PBS.

Incubation buffer: 0.05% Triton X-100 and **1% BSA** in PBS.

Rinse buffer: 0.05% Triton X-100 in PBS.

Host	Primary Antibodies	Vendor	Catalog Number	Dilution
Rabbit	β3-Tubulin	Cell Signaling	5568S	1:500
Rabbit	GABA	Abcam	ab43865	1:200
Rabbit	VGLUT2	Abcam	ab84103	1:100
Rabbit	Sox2	Cell Signaling	3579S	1:200
Rabbit	Bcl-11B/Ctip2	Cell Signaling	12120S	1:200
Rabbit	Calretinin	Abcam	AB137878	1:200
Rabbit	Glial Fibrillary Acidic Protein (GFAP)	DAKO	Z033401-2	1:500
Rabbit	Brn2 (POU3F2)	Cell Signaling	12137S	1:200
Mouse	β3-Tubulin	R&D Systems	MAB1195	1:500
Mouse	Reelin	EMD Millipore	MAB5364	1:100
Mouse	Sox2	Millipore	MAB4343	1:200
Mouse	Vimentin (phospho S55) [4A4]	Abcam	ab22651	1:200
Mouse	MAP2, clone AP20	Millipore	MAB3418	1:500
Mouse	Ki67 (8D5)	Cell Signaling	9449S	1:200
Mouse	CD31, Endothelial Cell, Clone JC70A	DAKO	M082301-2	1:200
Goat	Iba1 (AIF1)	Abcam	ab5076	1:100
Goat	GFAP (C-19)	Santa Cruz Biotechnology	sc-6170	1:100-1:200
Chicken	Tbr1	Millipore	AB2261	1:100-1:200
Host	**Secondary Antibodies**	**Vendor**	**Catalog Number**	**Dilution**
Donkey	Rabbit 488	Life Technologies	A21206	1:200
Donkey	Rabbit 568	Life Technologies	A10042	1:200
Donkey	Rabbit 647	Life Technologies	A31573	1:200
Donkey	Mouse 488	Life Technologies	A21202	1:200
Donkey	Mouse 568	Life Technologies	A10037	1:200
Donkey	Mouse 647	Life Technologies	A31571	1:200
Donkey	Goat 568	Life Technologies	A11057	1:200
Donkey	Goat 647	Life Technologies	A21447	1:200
Goat	Chicken 568	Life Technologies	A11041	1:200

Immunostaining full neural constructs. All steps for immunostaining were performed within the Transwell insert. Neural constructs were fixed for 60 minutes using **2% buffered formalin** and then stored in PBS at 4°C until further processing. Neural constructs were permeabilized and blocked in **blocking buffer** for at least 60 minutes. For some experiments, blocking buffer was used instead of incubation buffer with similar results. The bottom of the Transwell membrane was dried on a paper towel to prevent leakage through the insert before incubation with antibodies. Primary antibodies were prepared in 200-300 µL **incubation buffer**, added to the Transwell insert, and incubated overnight at 4°C. Neural constructs were then rinsed 2 x 60 minutes with **rinse buffer** and at least 60 minutes in **blocking buffer**. Secondary antibodies and 5 µg/mL DAPI (4′,6-Diamidino-2-Phenylindole Dihydrochloride, MP Biomedicals, 157574) were prepared in 200-300 µL **incubation buffer**, added to the neural constructs, and incubated overnight at 4°C or at least four hours at room temperature. Neural constructs were rinsed 2 x 60 minutes in **rinse buffer**, followed by an overnight rinse at 4°C in **rinse buffer**. Samples were then rinsed in PBS (at least 24 hours) and stored until further processing. Neural constructs were removed from

the Transwell insert by cutting the bottom edge of the membrane, separated from the membrane, and mounted in Aqua Polymount solution (Polysciences, Inc.) on the bottom of a 35 mm glass bottom dish (MatTek). To limit bubble formation in the mounting solution, a thin layer was first added to the glass bottom of the 35 mm dish. The neural construct was usually placed face down into the mounting solution (with some samples placed face up), after which a drop of mounting solution was added to cover the construct. A coverslip was then dropped onto the neural construct in mounting solution and allowed to settle, rotating the dish to ensure uniform coverage of the mounting solution under the coverslip. The coverslip was allowed to settle overnight at 4°C and sealed around the edges with fingernail sealant. The mounted samples remained stable for imaging for at least one month.

Immunostaining cryopreserved sections. Neural constructs were fixed in the Transwell insert for 60 minutes using **2% buffered formalin** and rinsed with PBS (overnight at 4°C). The samples were then rinsed in 15% Sucrose/PBS (at least 24 hours at 4°C) followed by 30% Sucrose/PBS (at least 24 hours at 4°C). Neural constructs were removed from the Transwell insert by cutting the bottom edge of the membrane, separated from the membrane, and placed face down into cryogel (Tissue-Tek embedding medium), and stored frozen at -80°C until further processing. Frozen samples were equilibrated to -20°C and sectioned (20-30 μm sections on glass slides). Glass slides containing sectioned samples were soaked in deionized water for at least one hour to remove cryogel. Samples were permeabilized and blocked in **blocking buffer** for 60 minutes, rinsed 2 x 15 minutes with **rinse buffer**, and rinsed in **incubation buffer** for at least 60 minutes at room temperature. Samples were then treated with primary antibodies in **incubation buffer** overnight at 4°C or at least four hours at room temperature. After treating with primary antibodies, samples were rinsed with **wash buffer** (2x15 minutes) and **incubation buffer** (at least 60 minutes at room temperature). Samples were then treated with secondary antibodies and 5 μg/mL DAPI in **incubation buffer** overnight at 4°C or at least two hours at room temperature. Sectioned samples were mounted by adding a drop of Aqua Polymount solution (Polysciences, Inc.) directly onto the tissue section and placing a glass coverslip over the top. The mounted samples were stored overnight at 4°C and then sealed around the edges with fingernail sealant until imaging. The mounted samples remained stable for imaging for several months.

Image processing. Immunofluorescence images were collected using a Nikon A1R laser scanning confocal microscope with Plan Apo 10x, Plan Fluor 20x Ph1 DLL, or Plan Apo 20x DIC M objectives. Images were processed using NIS Elements or ImageJ (22, 23). Some z-stacks were aligned using the "Align Current ND Document" (NIS Elements) or the StackReg plugin (ImageJ)(24) before creating maximum projection images.

RNA-Seq collection and analysis.

RNA isolation, cDNA library preparation and next generation sequencing. The 3D neural constructs were lysed directly in the insert by the addition of 350 µL RLT lysis buffer (Qiagen) and stored at -80°C until being used for RNA isolation. When total RNA was ready to be extracted, the samples were thawed and 150 µL of the cell lysates in buffer RLT were transferred and re-arrayed to an S-block (Qiagen, Cat. No. 19585) to be mixed with one volume of 70% ethanol (the rest of the lysates were stored at -80°C). Total RNA was then isolated using Qiagen's RNeasy 96 kit beginning with step 3 of the manufacturer's protocol (RNeasy 96 Handbook 01/2002, Using Spin Technology) and included the optional DNase treatment.

Quality control studies. Samples used for quality control were prepared for RNA-Seq with Illumina's TruSeq RNA Sample Preparation Kit v2 following the Low-Throughput (LT) protocol (TruSeq RNA Sample Preparation Guide, Part # 15008136, Rev. A) using 100 ng of total RNA as input. The cDNA libraries were pooled and run on Illumina's HiSeq 2500 with a single read of 51 bp and index read of 7 bp.

Toxicity screening study. cDNA preparation for the toxicity screening experiment was previously described in detail (25). Briefly, mRNA is isolated from purified 100ng total RNA using oligo-dT beads (NEB). Isolated mRNA is fragmented in reverse transcription buffer at 85°C for seven minutes, and then reverse transcribed with SmartScribe reverse transcriptase (Clontech) at 23°C for 10 minutes followed by a 30 minute incubation at 42°C with a random hexamer oligo (CCTTGGCACCCGAGAATTCCANNNNNN). After reverse transcription, RNA is removed by RNaseA and RNaseH treatment. A partial Illumina 5' adaptor (/5phos/AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTddC) is then ligated to the single stranded cDNA using RNA ligase 1 (NEB) overnight at 22°C. After purification, ligated cDNA is amplified by 18 cycles of PCR using oligos that contain full Illumina adaptors:

AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT

And index primer (nnnnnnnnnn indicates index nucleotides):

CAAGCAGAAGACGGCATACGAGATnnnnnnnnnnGTGACTGGAGTTCCTTGGCACCCGAGAATTCCA

The indexed cDNA libraries were pooled and sequenced on an Illumina HiSeq2500 with a single 51bp read and a 10bp index read.

RNA-Seq data analysis. FASTQ files were generated by CASAVA (v1.8.2). Reads were mapped to the human transcriptome (RefGene v1.2.3) using Bowtie (26) (v0.12.8) allowing 2-mismatches (--bowtie-n 2)

with seed length 28nt (--seed-length 28) and a maximum of 20 multiple hits (-m 20). The normalized gene expression values (Transcript per Million Reads or TPM) were calculated by RSEM (v1.2.3) (27). RSEM expected read counts for each gene were determined by median normalization (utilizing the median normalization function within EBSeq (version 1.5.3) (28). EBSeq (version 1.5.3) was used to calculate FDR for differentially expressed genes (28).

Gene Ontology analysis. Gene ontology (GO) terms were identified using the DAVID Bioinformatics Database (version 6.7) Functional Annotation Tool (29, 30). GO terms were identified by analyzing differentially expressed genes with (FDR $\leq$ 0.005) and >3-fold upregulated expression for 3D neural constructs relative to H1 ES cells (see Dataset S2). The following settings were used for DAVID analysis using differentially expressed genes: Gene Ontology category GOTERM_BP_5; Benjamini corrected p-value $\leq$ 0.05; Threshold options: Counts = 10, EASE = 0.01.

Comparisons to Allen Brain Atlas data. Gene expression values (RPKM) were downloaded from the Allen Brain Atlas (RNA-Seq data only, samples: 8 PCW – 40 years). Pairwise Spearman's rank correlation (Dataset S1) was calculated for neural constructs and Allen Brain Atlas data (RPKMs for RNA-Seq data only; samples: 8 PCW – 40 years) (31, 32).

Code availability (EBSeq). The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (33) and are accessible through GEO Series accession number **GSE63935** (**http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=wfepekigrfqbfot&acc=GSE63935**). Details about the EBSeq algorithm were previously described in detail (28). EBSeq download and documentation is available at:

http://www.bioconductor.org/packages/devel/bioc/html/EBSeq.html

Additional files and instructions for downloading the user interface are also available online, and include instructions for installing the EBSeq toolshed for Galaxy (34-36):

https://www.biostat.wisc.edu/~kendzior/EBSEQ/

Machine Learning.

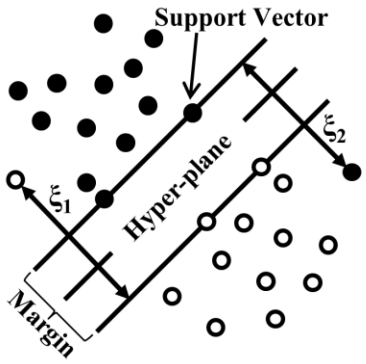
We employed linear support vector machines (SVMs) to construct our predictive models (37-41). Previous studies have demonstrated that linear SVMs are robust to the kind of high-dimensional (or "wide") data commonly found with human gene expression data sets with far more features (genes) than data points (subjects), as exemplified in the data graphic below (37, 38). Simulation experiments indicate that at least 15 samples of each class are needed to have sufficient power to estimate predictive accuracy of our models to within plus or minus five percentage points of the true value via cross-validation. We employed SVMs for the following task specification (Dataset S6):

Given: RNA-Seq gene expression measurements for ~19,000 genes on one day or on several different days following exposure to various drugs, together with a neurotoxicity label on each drug, as illustrated in the graphic below.

Do: Construct a model that can accurately identify if the drug is neurotoxic from the same type of expression data on a new drug.

Compound	Gene 1	…	Gene 19K	Class
MeHg	3.42	…	5.39	Toxic
Caffeine	2.04	…	4.98	Control

Evaluations of the model, including estimates of accuracy and receiver operating characteristic (ROC) curves, were all by hold-out testing; either leave-one-out cross-validation or use of a blinded trial with a single hold-out set. A 2-dimensional linear SVM is illustrated in the plot shown below, where the hyper-plane reduces to a line that separates examples of the two classes (represented by either a filled or open circle) and maximizes the margin between the closest points of the different classes. The support vectors are the examples that fix the position and orientation of the hyper-plane. The $\mathbf{x_i}$s are the example (circles below; gene expression for the current study), the $\mathbf{y_i}$s are their labels (filled or open circles below;



Soft-margin support vector machine (SVM)
Given $\mathbf{x_i}$ for all $1 \leq i \leq \ell$, find $\mathbf{w}$ and b to:

$$\min \quad \tfrac{1}{2} \| \mathbf{w} \|^2 + \mathbf{C} \sum_{\mathbf{i}=1}^{\ell} \xi_{\mathbf{i}}$$
$$\text{s.t.} \quad y_i(\mathbf{w}'\mathbf{x_i} - \mathbf{b}) \geq \mathbf{1} - \xi_{\mathbf{i}} \quad \forall \mathbf{i} = \mathbf{1} \ldots \ell$$
$$\xi_i \geq 0$$

toxic or non-toxic for the current study), and **w** is the weight vector, or vector of coefficients on the features (the dimensions). Soft margin SVMs (41) that allow for errors in the training set were used here. The effect of a misclassified example $\mathbf{x_j}$ is measured through its distance to the hyper-plane (ξ_j). The extensions required for the soft margin version of the SVM are highlighted in the equation (red background), which minimizes the sum of the distances between incorrectly classified training points (ξ_i) in addition to the margin, and is used when the data is not linearly separable (41). The SVM is trained to minimize the sum of the margin and errors (weighted by parameter C). The parameter C is tuned with an inner loop of cross-validation and repeated on every fold of the outer, evaluative cross-validation procedure using only the training data for each fold. As noted in other studies (37, 38), tuning parameters in this way usually yields better results than arbitrarily choosing a single default parameter value, and also gives a much fairer estimate of future performance than performing cross-validation with multiple values of a parameter and then "cherry picking" the value that yields the best result. The linear SVM's output is the weight vector (**w)** and the other coefficient (**b)**. To make a prediction, the SVM outputs the number $\mathbf{w'x_i - b}$ and outputs the label 0 (non-toxic for our application) if this number is less than 0 or 1 otherwise. While the numerical output does not have a probabilistic interpretation like that of the output of logistic regression, it is common to build a logistic regression model with one input variable (the SVM's output) from the same training set to output a probability, which was done here (for probability of toxic).

Leave-one-out cross-validation. For leave-one-out cross-validation, there are N data points (compounds) in a training set, and the method proceeds in N steps. In each step, a different data point is held out of the training set and the SVM is trained on the remaining data points. The model then makes a prediction on the held-aside data point. Hence, every data point is a test case exactly once for a model trained without that data point. Results are aggregated over all the folds (or test cases) to estimate how well the SVM model trained on all the data will perform on a new data point (a new compound for the present study). Because we had two replicates for each compound, we held out both replicates of a given compound for testing on each fold, as leaving one replicate in the training set and the other in the test set could give overly-optimistic estimates of future predictive accuracy. So, in effect, we performed "leave-one-compound-out" cross-validation by taking the average of the two output probabilities from the two replicates as the probability of the compound being toxic. Finally, we also averaged predictions for each compound from both days 16 and 21 to produce a further improved prediction of toxicity. The evaluation of this approach also proceeded in the leave-one-compound-out fashion.

Cross-validation methodology was used to compute the number of true positive (TP), false positive (FP), true negative (TN), and false negative predictions (FN), where positive represents toxic and negative represents non-toxic. Then the accuracy and sensitivity of these predictions can be computed (true positive

rate; TP/[TP+FN]), specificity (TN/[TN+FP]), and precision (or positive predictive value; TP/[TP+FP]), along with other metrics such as F-measure and the negative predictive value. Nevertheless, all of these metrics depend not only on the model that produces probabilistic predictions for toxicity, but also on the probability threshold at which we make positive predictions (such as 0.5). Hence, it is common in machine learning and statistical classification to report "thresholdless" curves and/or metrics, the most popular being the ROC curve (See Fig. 5 and Fig. S7) and the area under this curve (AUC). The ROC curve plots the true positive rate on the y-axis against the false positive rate (1-specificity) on the x-axis as the threshold is varied. Random uniform guessing produces a diagonal from the lower left corner to the upper right corner (AUC = 0.5), while perfect prediction produces a graph that goes up to the upper left corner and then across (AUC = 1.0). The ROC curve is produced by ranking the examples by their predicted probability of being toxic and then varying the threshold.

Blinded trial. In addition to constructing the SVM model, an estimate of how well the model predicts neurotoxicity for compounds not in the training set was made, since merely reporting its accuracy on the training set would be overly optimistic. For the unbiased method, RNA-Seq data was collected for a set of 10 blinded compounds that were not in the training set. The predictive model was tested on the blinded samples after the model had been constructed and optimized using the training set. This prediction is the blinded trial since the researchers running the SVM did not know the identity of the chemicals, their ground truth labels, or the number of toxins within the blinded set. Predictions were made using a probability threshold of 0.5. While 0.5 is the most common threshold to use, a threshold could have alternatively been chosen based on the training data using the threshold that maximizes accuracy for the ROC curve. The toxicity assignment was revealed for the blinded chemicals only after the SVM's predictions were made.

Code availability for machine learning. For those wishing to replicate machine learning experiments, all runs were performed using LibSVM (42) within the publicly available Weka machine learning toolkit version 3.6.1.0 (43). We used all default parameter settings, including normalization, with the following three natural exceptions that we have already described and justified: We selected (1) the linear kernel; (2) CVParameterSelection, in order to correctly tune parameters in the manner specified above; and (3) logistic regression, in order to produce the final output probabilities based on the SVM output. We implemented scripts that use the R-system ROCR package for ROC curve visualization (44). Commands were executed on a system running Scientific Linux 6.6 with four six-core processors running at 2.4 GHz and 132 GB of main memory.

Gene selection by recursive feature elimination. Genes were selected by using the Weka toolbox (43) to perform recursive feature elimination (45), a wrapper-based feature selection algorithm. The methods are available as the metaClassifier AttributeSelectedClassifier. Features (genes) were evaluated by Weka's SVMAttributeEval, set to discard the lowest scoring 10% of the genes at each iteration and to stop at 1000 genes. The Weka's Ranker method was used to select genes.

SUPPLEMENTAL REFERENCES

1. Chen GK, *et al.* (2011) Chemically defined conditions for human iPSC derivation and culture. *Nat Methods* 8(5):424-429.
2. Beers J, *et al.* (2012) Passaging and colony expansion of human pluripotent stem cells by enzyme-free dissociation in chemically defined culture conditions. *Nat Protoc* 7(11):2029-2040.
3. Chambers SM, *et al.* (2009) Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling. *Nat Biotechnol* 27(3):275-280.
4. Inman GJ, *et al.* (2002) SB-431542 is a potent and specific inhibitor of transforming growth factor-beta superfamily type I activin receptor-like kinase (ALK) receptors ALK4, ALK5, and ALK7. *Mol Pharmacol* 62(1):65-74.
5. Vodyanik MA, *et al.* (2010) A mesoderm-derived precursor for mesenchymal stem and endothelial cells. *Cell Stem Cell* 7(6):718-729.
6. Uenishi G, *et al.* (2014) Tenascin C promotes hematoendothelial development and T lymphoid commitment from human pluripotent stem cells in chemically defined conditions. *Stem Cell Rep* 3(6):1073–1084.
7. Fairbanks BD, *et al.* (2009) A versatile synthetic extracellular matrix mimic via thiol-norbornene photopolymerization. *Adv Mater* 21(48):5005-5010.
8. Hansen TD, *et al.* (2014) Biomaterial arrays with defined adhesion ligand densities and matrix stiffness identify distinct phenotypes for tumorigenic and non-tumorigenic human mesenchymal cell types. *Biomater Sci* 2(5):745-756.
9. Nagase H & Fields GB (1996) Human matrix metalloproteinase specificity studies using collagen sequence-based synthetic peptides. *Biopolymers* 40(4):399-416.
10. Pierschbacher MD & Ruoslahti E (1984) Cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule. *Nature* 309(5963):30-33.
11. Crofton KM, *et al.* (2011) Developmental neurotoxicity testing: Recommendations for developing alternative methods for the screening and prioritization of chemicals. *ALTEX-Altern Anim Exp* 28(1):9-15.
12. Radio NM, Freudenrich TM, Robinette BL, Crofton KM, & Mundy WR (2010) Comparison of PC12 and cerebellar granule cell cultures for evaluating neurite outgrowth using high content analysis. *Neurotoxicol Teratol* 32(1):25-35.
13. Han Y, *et al.* (2009) Identification by automated screening of a small molecule that selectively eliminates neural stem cells derived from hESCs but not dopamine neurons. *PLoS One* 4(9):e7155.
14. Wang X, *et al.* (2007) Thiamine deficiency induces endoplasmic reticulum stress in neurons. *Neuroscience* 144(3):1045-1056.
15. Verdaguer E, *et al.* (2002) Kainic acid-induced neuronal cell death in cerebellar granule cells is not prevented by caspase inhibitors. *Br J Pharmacol* 135(5):1297-1307.
16. Cooper MK, Porter JA, Young KE, & Beachy PA (1998) Teratogen-mediated inhibition of target tissue response to Shh signaling. *Science* 280(5369):1603-1607.

17. Adams J (1993) Structure-activity and dose-response relationships in the neural and behavioral teratogenesis of retinoids. *Neurotoxicol Teratol* 15(3):193-202.
18. Sogaard U*, et al.* (1990) A tolerance study of single and multiple dosing of the selective dopamine uptake inhibitor GBR 12909 in healthy subjects. *Int Clin Psychopharmacol* 5(4):237-251.
19. Weiss JH, Koh J-y, & Choi DW (1989) Neurotoxicity ofβ-N-methylamino-l-alanine (BMAA) andβ-N-oxalylamino-l-alamine (BOAA) on cultured cortical neurons. *Brain Res* 497(1):64-71.
20. Khera KS (1987) Neuronal degeneration caused by ethylenethiourea in neuronal monocell layers in vitro and in fetal rat brain in vivo. *Teratology* 36(1):87-93.
21. Gorman ALF & Marmor MF (1974) Long-term effect of ouabain and sodium pump inhibition on a neuronal membrane. *J Physiol-London* 242(1):49-60.
22. Schneider CA, Rasband WS, & Eliceiri KW (2012) NIH Image to ImageJ: 25 years of image analysis. *Nat Meth* 9(7):671-675.
23. Rasband WS (1997-2012) (Image J, U.S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/).
24. Thevenaz P, Ruttimann UE, & Unser M (1998) A pyramid approach to subpixel registration based on intensity. *IEEE Trans Image Process* 7(1):27-41.
25. Hou Z*, et al.* (2015) A cost-effective RNA sequencing protocol for large-scale gene expression studies. *Sci Rep* 5:9570.
26. Langmead B, Trapnell C, Pop M, & Salzberg S (2009) Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biol* 10(3):R25.
27. Li B & Dewey CN (2011) RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinformatics* 12:323.
28. Leng N*, et al.* (2013) EBSeq: an empirical Bayes hierarchical model for inference in RNA-seq experiments. *Bioinformatics* 29(8):1035-1043.
29. Huang DW, Sherman BT, & Lempicki RA (2008) Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protocols* 4(1):44-57.
30. Ashburner M*, et al.* (2000) Gene Ontology: tool for the unification of biology. *Nature Genet* 25(1):25-29.
31. Miller JA*, et al.* (2014) Transcriptional landscape of the prenatal human brain. *Nature* 508(7495):199-206.
32. Hawrylycz MJ*, et al.* (2012) An anatomically comprehensive atlas of the adult human brain transcriptome. *Nature* 489(7416):391-399.
33. Edgar R, Domrachev M, & Lash AE (2002) Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. *Nucleic Acids Res* 30(1):207-210.
34. Goecks J, Nekrutenko A, Taylor J, & Galaxy T (2010) Galaxy: a comprehensive approach for supporting accessible, reproducible, and transparent computational research in the life sciences. *Genome Biol* 11(8):R86.
35. Giardine B*, et al.* (2005) Galaxy: A platform for interactive large-scale genome analysis. *Genome Res* 15(10):1451-1455.
36. Blankenberg D*, et al.* (2001) Galaxy: a web-based genome analysis tool for experimentalists. *Curr Protoc Mol Biol* 89:19.10.1-19.10.21.
37. Struyf J, Dobrin S, & Page D (2008) Combining gene expression, demographic and clinical data in modeling disease: a case study of bipolar disorder and schizophrenia. *BMC Genomics* 9:531.
38. Hardin J*, et al.* (2004) Evaluation of multiple models to distinguish closely related forms of disease using DNA microarray data: an application to multiple myeloma. *Stat Appl Genet Mol Biol* 3(1):e10.
39. Furey TS*, et al.* (2000) Support vector machine classification and validation of cancer tissue samples using microarray expression data. *Bioinformatics* 16(10):906-914.
40. Vapnik VN (1998) *Statistical Learning Theory* (Wiley, New York).
41. Cortes C & Vapnik V (1995) Support-Vector Networks. *Mach Learn* 20(3):273-297.

42. Chang C-C & Lin C-J (2011) LIBSVM: A library for support vector machines. *ACM Trans Intell Syst Technol* 2(3):1-27.
43. Hall M*, et al.* (2009) The WEKA data mining software: an update. *SIGKDD Explor Newsl* 11(1):10-18.
44. Sing T, Sander O, Beerenwinkel N, & Lengauer T (2005) ROCR: visualizing classifier performance in R. *Bioinformatics* 21(20):3940-3941.
45. Guyon I, Weston J, Barnhill S, & Vapnik V (2002) Gene selection for cancer classification using support vector machines. *Mach Learn* 46(1-3):389-422.

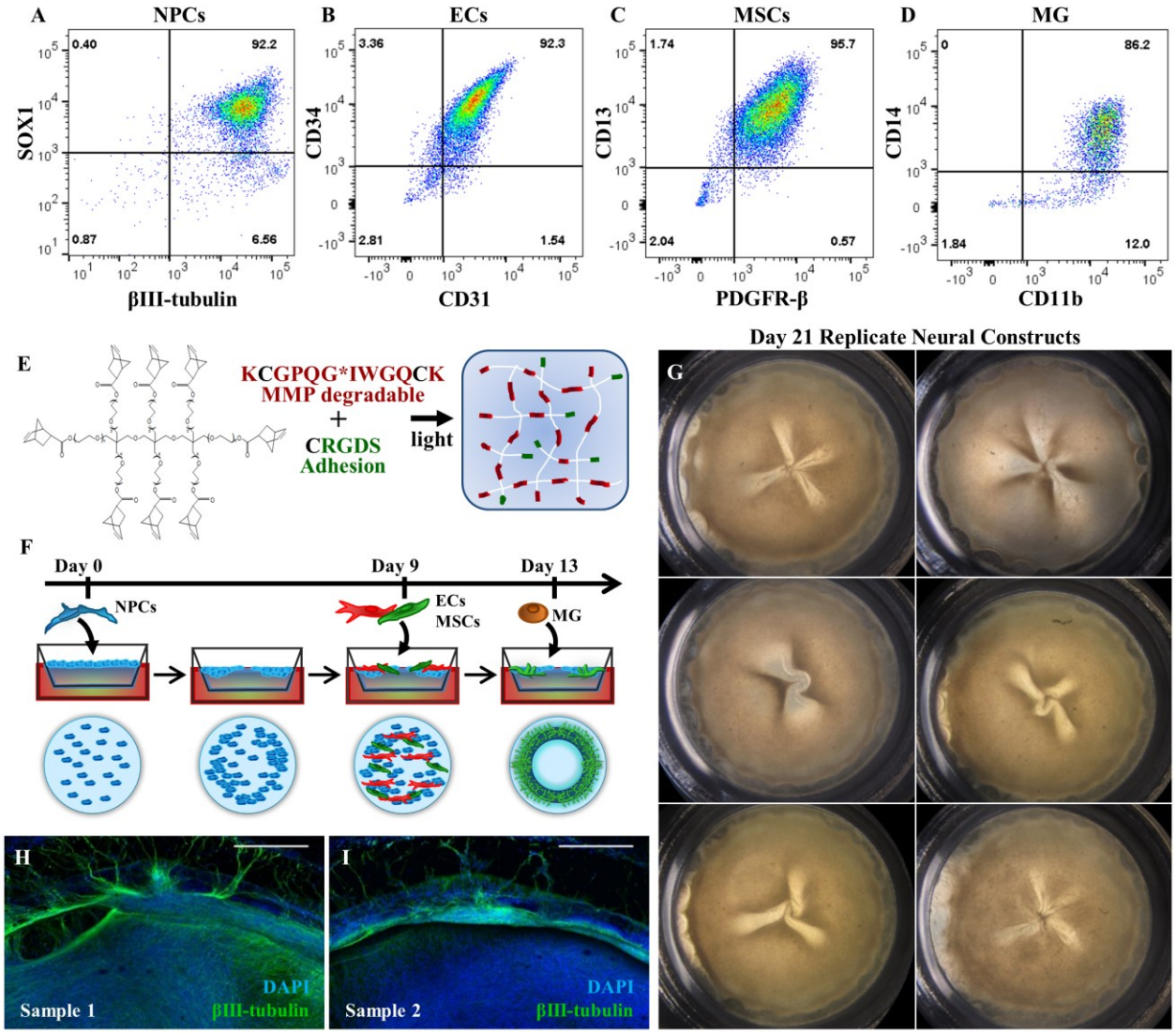


Fig. S1. Protocol for producing 3D neural constructs. FACS analysis for H1 human embryonic stem cell-derived precursors: **(A)** Neural progenitor cells (***NPCs***; SOX1^{+}βIII-tubulin^{+}); **(B)** Endothelial cells (***ECs***; CD31^{+}CD34^{+}); **(C)** Mesenchymal stem cells (***MSCs***; PDGFRβ^{+}CD13^{+}); **(D)** Microglia/macrophage precursors (***MG***; CD11b^{+}CD14^{+}). See ***SI Appendix, SI Materials and Methods*** for additional details. **(E)** Hydrogels were formed using "thiol-ene" photopolymerization to crosslink 8-arm poly(ethylene glycol)-norbornene (***PEG-NB***) molecules with cysteine-flanked matrix metalloproteinase (MMP)-degradable peptide molecules, while pendant Cys-Arg-Gly-Asp-Ser (CRGDS) peptide molecules were incorporated to promote cell adhesion (See Fairbanks *et al. Adv. Mater.* 2009). **(F)** Human embryonic stem cell-derived precursor cells were cocultured on PEG hydrogels in 24-well Transwell inserts. NPCs were seeded on synthetic PEG hydrogels at day 0, followed by ECs and MSCs at day 9, and MG at day 13. For neurotoxicity screening, test chemicals were added starting at day 14 and samples collected after 2 and 7 days of continuous exposure (days 16 and 21 of culture on PEG hydrogels). **(G)** Brightfield microscopy images for replicate neural constructs in 24-well Transwell inserts (Corning) after 21 days of culture on PEG hydrogels (as described in *F*). **(H,I)** Maximum intensity projection immunofluorescence images illustrating βIII-tubulin (neurons, green) and DAPI (nuclei, blue) expression for two separate neural constructs (Scale bars, 250 μm). The full neural construct for the image in *H* is illustrated in Fig. 1.

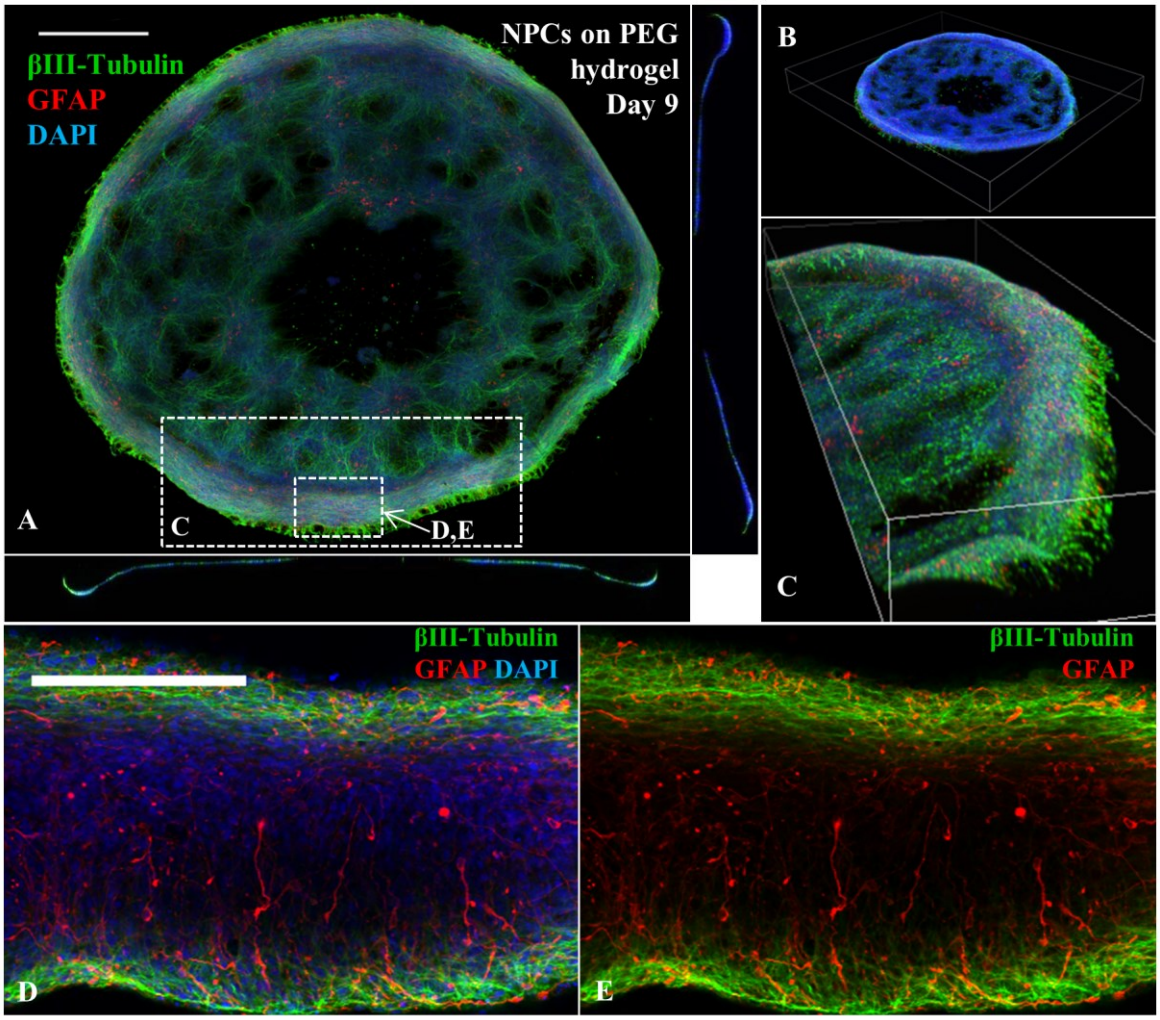


Fig. S2. Neural constructs were characterized by layered structure and radial organization after nine days of culture on poly(ethylene glycol) (PEG) hydrogels. Maximum intensity projection immunofluorescence images illustrating βIII-tubulin (neurons, green), GFAP (glial cells, red), and DAPI (nuclei, blue) expression for neural constructs nine days after H1 human embryonic stem cell-derived neural progenitor cells (NPCs) were seeded onto a PEG hydrogel. **(A)** Maximum intensity projection image for a complete neural construct. Cross section views through the center of the neural construct are also illustrated (right and bottom; thickness = 600 μm). **(B,C)** Volume View (NIS Elements) images showing 3D organization of neural constructs. The image shown in *B* represents the Volume View for the neural construct shown in *A*. The image shown in *C* represents the Volume View for the larger boxed region in *A*. **(D-E)** Maximum intensity projection image (10 μm thickness) for the smaller boxed region in *A* illustrating stratification and radial orientation of early neural and glial populations. **Scale bars**: **(A)** 1000 μm; **(D, E)** 200 μm (shown in *E*).

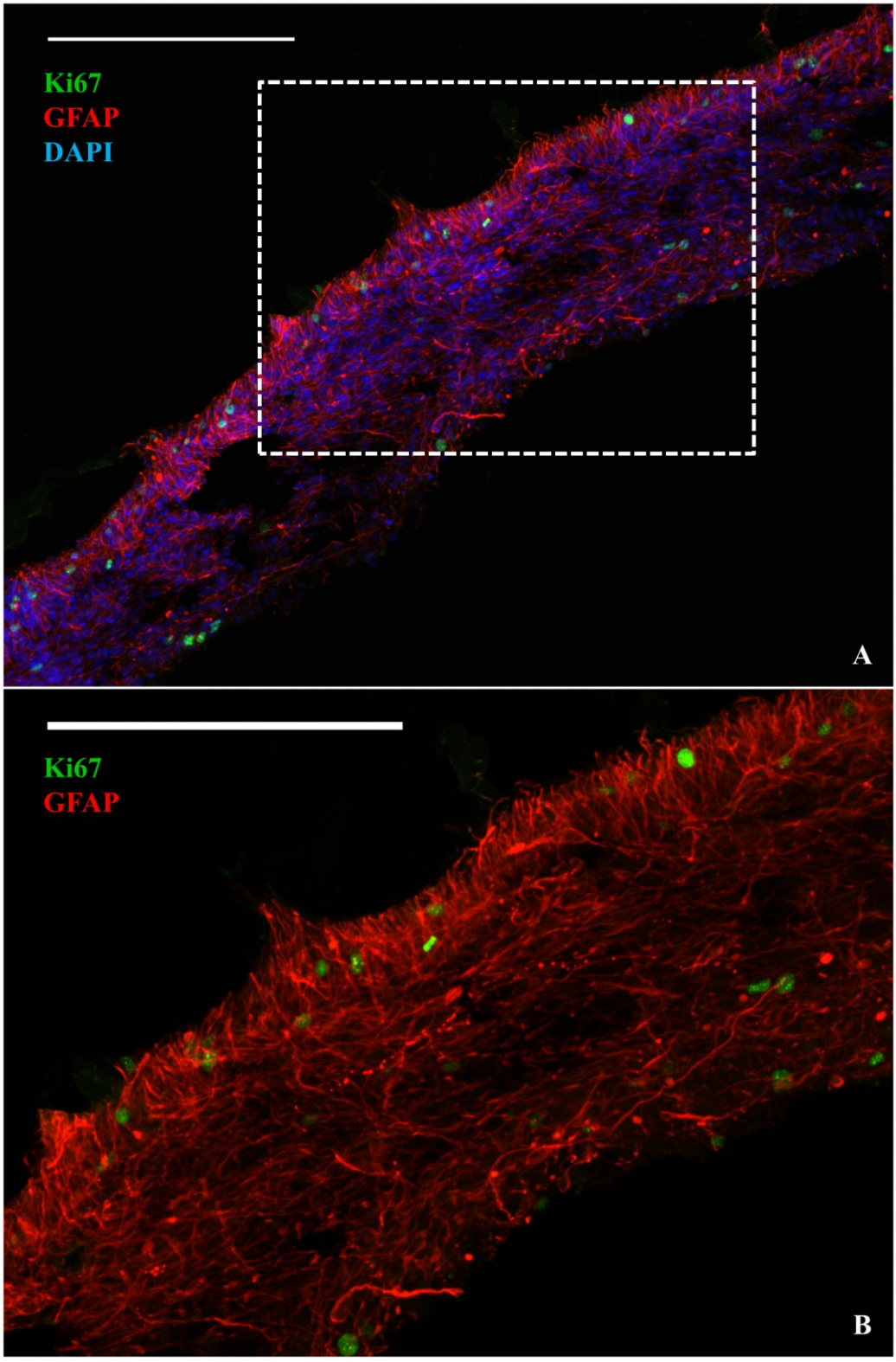


Fig. S3. Proliferation within a neural construct. **(A)** A cryosectioned day 21 neural construct (10 μm thickness) illustrating GFAP (glial cells, red), Ki67 (proliferative cells, green), and DAPI (nuclei, blue). **(B)** Boxed region shown in *A* without DAPI. **Scale bars: (A)** 250 μm; **(B)** 200 μm.

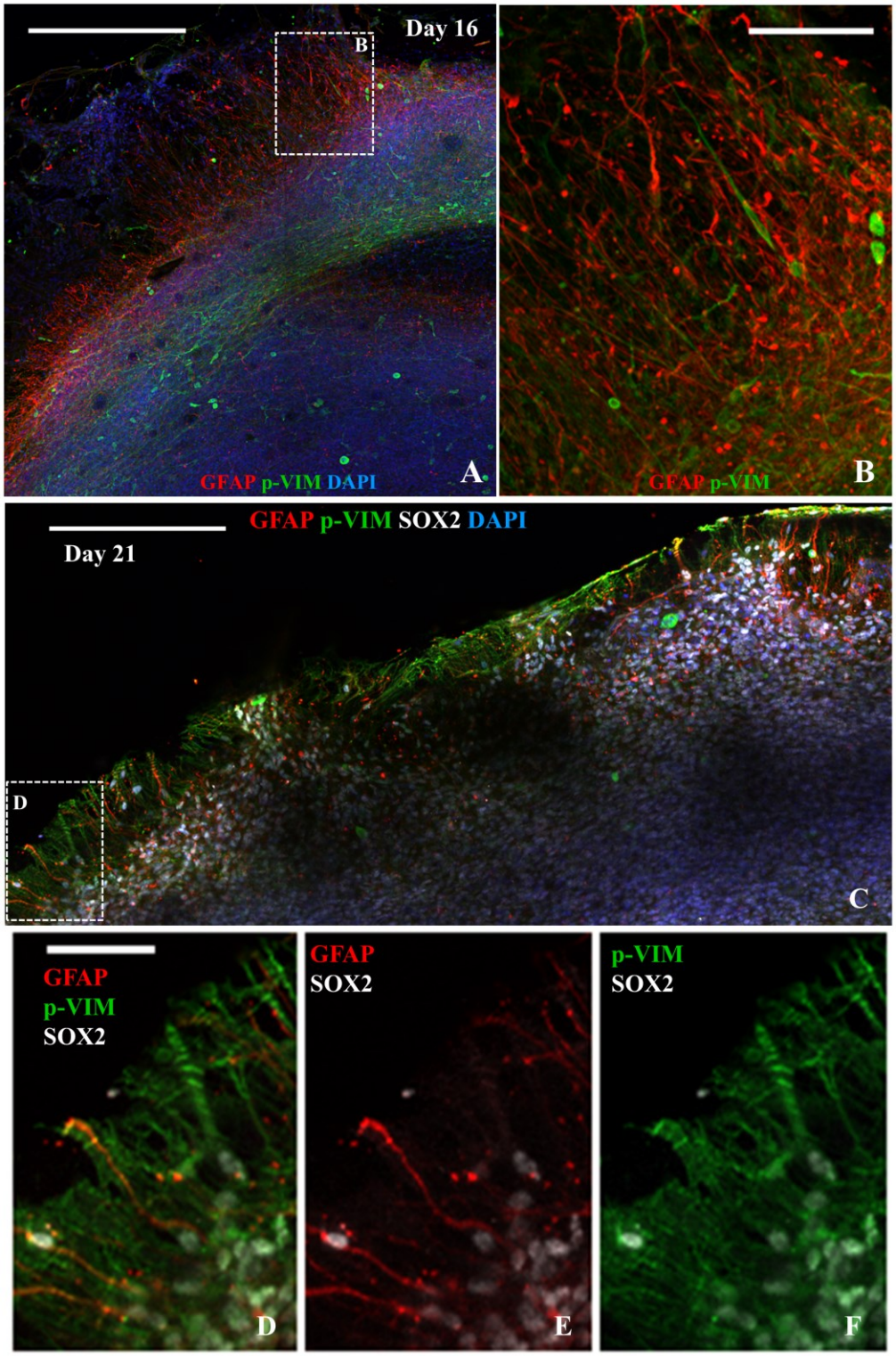


Fig. S4. Radially organized glial cells within day 16 and day 21 neural constructs. (A) Maximum intensity projection immunofluorescence images illustrating phosphorylated vimentin (p-VIM, green), GFAP (glial cells, red), and DAPI (nuclei, blue) expression for a day 16 neural construct. **(B)** Boxed region in *A* without DAPI. **(C)** Phosphorylated vimentin (p-VIM, green), GFAP (red), SOX2 (white), and DAPI (blue) expression for a day 21 neural construct. **(D)** Phosphorylated vimentin (p-VIM, green), GFAP (red), and SOX2 (white) expression for the boxed region shown in *C*, and the same region with **(E)** $SOX2^{+}/GFAP^{+}$ and **(F)** $pVIM^{+}/GFAP^{+}$ cells illustrated separately. **Scale bars:** (**A**) 500 μm; (**B**) 100 μm; (**C**) 250 μm; (**D-F**) 50 μm (shown in *D*).

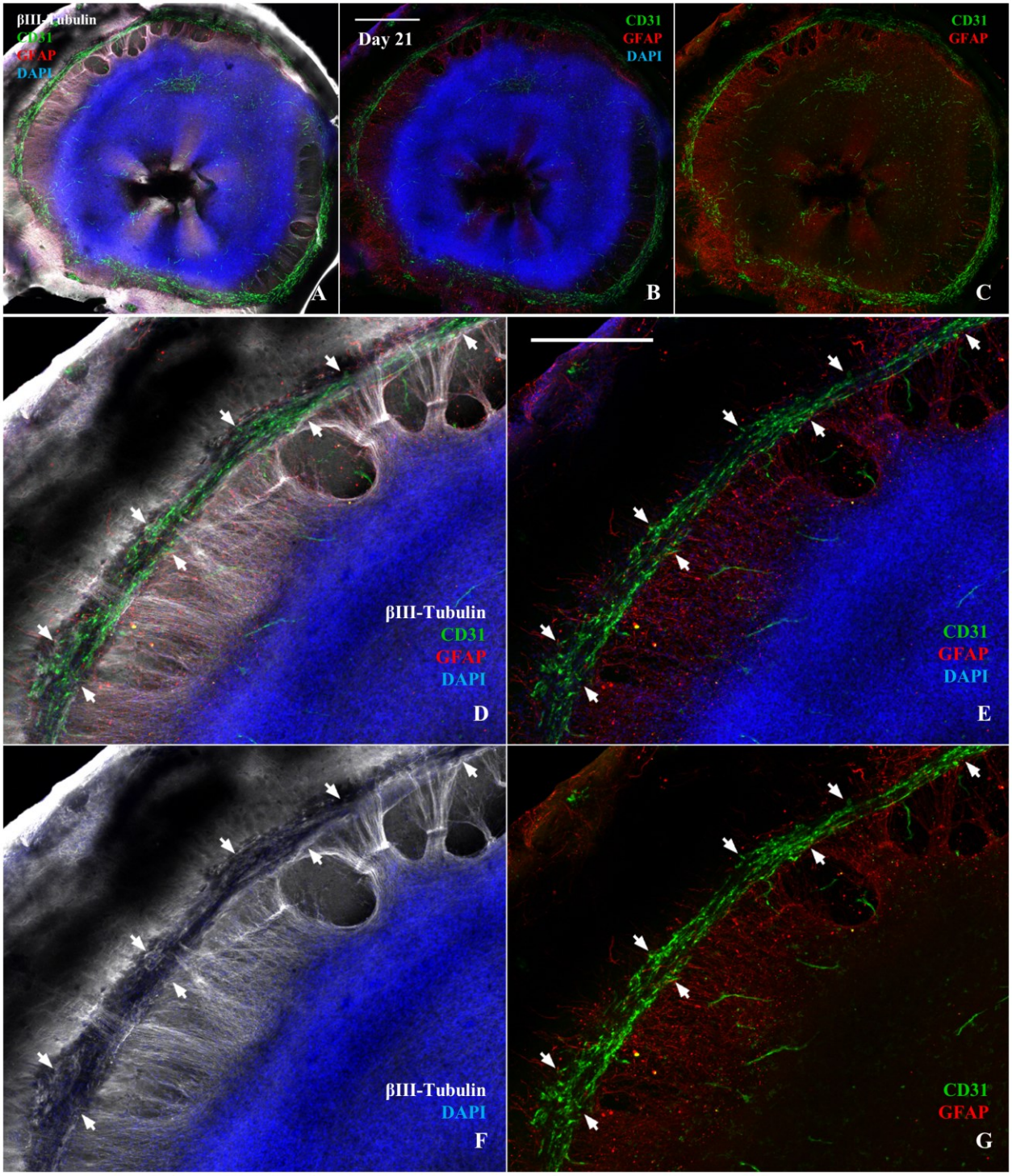


Fig. S5. Vascular networks are interconnected and penetrate into neural layers. Maximum intensity projection immunofluorescence images illustrating CD31 (endothelial cells, green), GFAP (glial cells, red), βIII-tubulin (neurons, white), and nuclei (DAPI, blue) within a day 21 neural construct. Different channels are shown for the same neural construct to highlight penetration of vascular networks into neural layers. **(A-C)** Interconnected vascular networks form around the circumference of the neural construct and penetrate into the interior regions. **(D-G)** The top, left region of the neural construct shown in *A-C*. Arrows highlight dense vascular networks within evolving neural layers. **Scale bars: (A-C)** 1000 μm (Shown in *B*); **(D-G)** 500 μm (shown in *E*).

A. Expressed Microglia Genes (Toxicity Experiments)		Day 16	Day 21
AIF1/IBA1	Allograft inflammatory factor 1	12.1±3.3	15.6±8.8
TREM2	triggering receptor expressed on myeloid cells 2	6.7±2.2	7.0±5.0
ITGAM/CD11b	Integrin alpha M	2.3±0.6	1.2±1.2
PTPRC/CD45	Protein tyrosine phosphatase, receptor type, C	4.1±0.9	3.6±3.3
CX3CR1	CX3C chemokine receptor 1	3.8±0.8	3.7±4.6
CD68	Cluster of Differentiation 68	17.3±4.0	23.8±18.9
CD14	Cluster of Differentiation 14	1.8±1.6	3.0±1.4
CD86	CD86 molecule	3.6±1.1	2.9±0.8
CD4	CD4 molecule	3.8±0.9	3.2±2.0
Normalized expression (Avg. TPM ± S.D.; N = 4)			

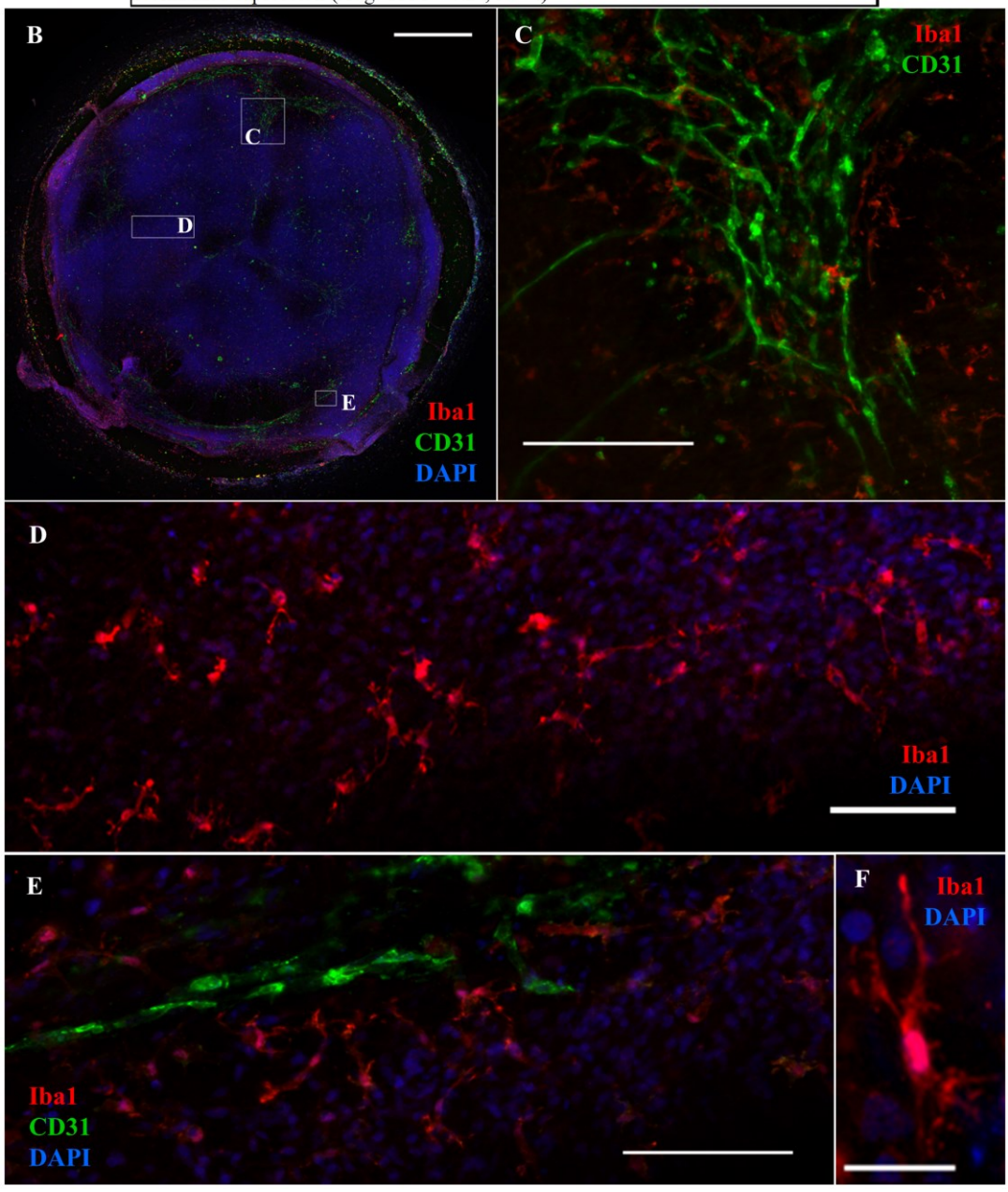


Fig. S6. Identification of microglia within neural constructs. (A) Expression of microglia genes (TPM ± S.D., N = 4) for day 16 and day 21 neural constructs (untreated control samples, toxicity screening experiment). **(B)** Maximum intensity projection immunofluorescence images illustrating Iba1 (microglia, red), CD31 (endothelial cells, green), GFAP (glial cells, red), and DAPI (nuclei, blue) for a day 21 neural construct. **(C-E)** The boxed regions shown in *B*. **(F)** Iba1 (red) and DAPI (blue) expression for a microglial cell with ramified morphology. **Scale bars: (B)** 1000 μm; **(C)** 250 μm; **(D)** 100 μm; **(E)** 50 μm ; **(F)** 25 μm.

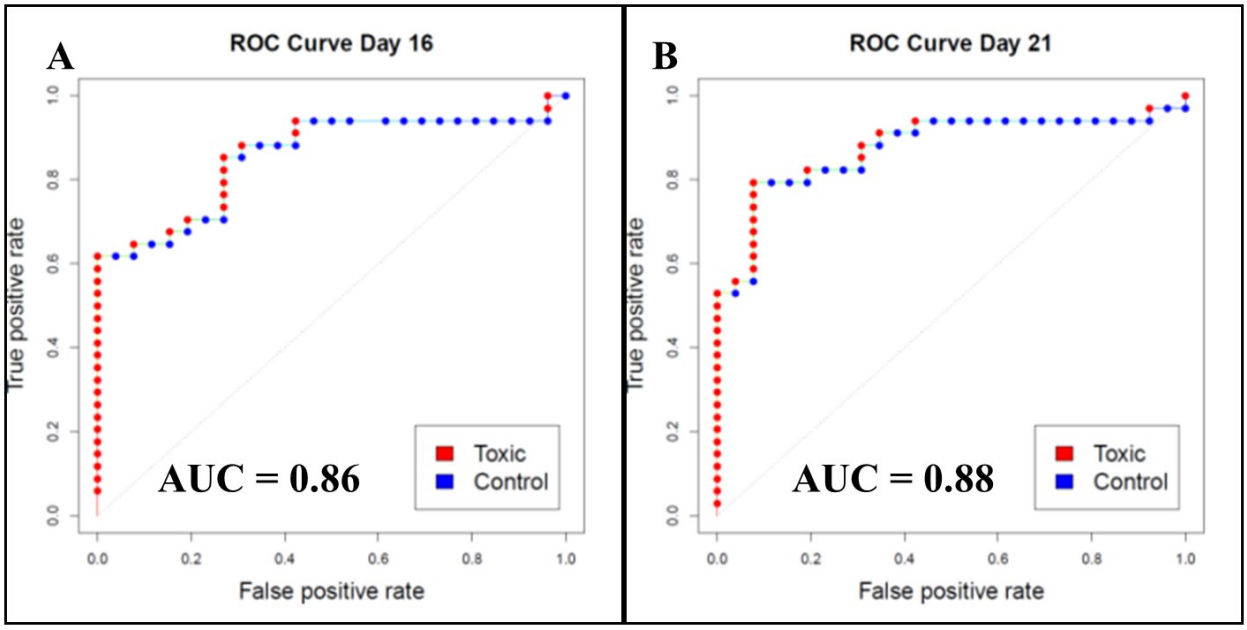


Fig. S7. Machine learning results for day 16 and day 21 training sets. Leave-one-out cross-validation was used to generate ROC curves for **(A)** day 16 (2 days dosing) and **(B)** day 21 (7 days dosing) neural constructs. The ROC curve for averaged data is provided in Fig. 5 (see Dataset S6 for ranked values). The ROC curve plots true positive rate on the y-axis against the false positive rate (1 – specificity) on the x-axis as the threshold is varied. See Fig. 5 text and *SI Appendix*, *SI Materials and Methods* for additional details.

Dataset Captions

Dataset S1. **Spearman's rank correlation for day 16 and day 21 3D neural constructs compared to samples from the Allen Brain Atlas**.
Replicate 3D neural constructs at days 16 and 21 of growth (N=4 for each) were compared to RNA-Seq data downloaded from the Allen Brain Atlas [Miller, J.A. *et al.* (2014) *Nature* 508: 199-206; Hawrylycz, M.J. *et al.* (2012) *Nature* 489: 391-399]. Spearman's rank correlation coefficients were determined for the normalized data from the Allen Brain Atlas compared to normalized expression for samples from the current study (TPM).

Dataset S2: Differential gene expression and Gene Ontology (GO) analysis for day 21 neural constructs and H1 ES cells.
DE Genes tab. Differentially expressed genes with FDR ≤ 0.005 for H1 ES cells (N=4) and day 21 neural constructs (N=4). EBSeq (version 1.5.3) [Leng, N. *et al.* (2013) *Bioinformatics* 29: 1035-1043] was used to calculate FDR for differentially expressed genes using the mean normalized expected counts for each sample. FDR values < 10^{-6} are reported as 0. ***GO Analysis tab***. The DAVID Bioinformatics Database Functional Annotation Tool [Huang, D.W. *et al.* (2008) *Nat. Protocols* 4: 44-57; Ashburner, M. *et al.* (2000) *Nature Genet.* 25: 25-29] was used to identify GO terms from differentially expressed genes for H1 ES cells and day 21 neural constructs using FDR ≤ 0.005 and 3-fold upregulated mean expected counts as cutoffs (Column A; See ***DE Genes tab*** for all differentially expressed genes). The following settings were applied for generating GO terms in DAVID (Columns F-N): Gene Ontology category GOTERM_BP_5; Benjamini corrected p-value < 0.05; Threshold options: Counts = 10, EASE = 0.01. DAVID was also used to generate a functional annotation clustering table for the same set of genes (Columns P-AB; Top 75 clusters).

Dataset S3: Normalized gene expression for control neural constructs.
Normalized expression expressed as transcripts per million (TPM) for select genes associated with different phenotypes present within neural constructs (Columns A-V) and full datasets for all control samples (Columns X-AS). Gene expression data was collected using TruSeq ("Quality Control Experiments") or an in house protocol ("Toxicity Experiment") [Hou, Z., *et al.* (2015) *Sci. Rep.* 5: Article Number 9570], as described in *SI Appendix*, SI Materials and Methods. Replicate samples from the quality control experiment include day 13 constructs formed using neural progenitor cells only as a control (N=3), day 21 samples formed without microglia/macrophage precursors (N=4), and day 21 neural constructs produced using all cell types (N=4). Day 16 and day 21 neural constructs (N=4 both days) analyzed using the in house protocol were untreated control samples collected from the toxicity screening experiment. All samples except day 13 neural progenitor cell control were collected from two separate 24-well plates (2 samples/plate). Key: Neural progenitor cells (NPC); Endothelial cells (EC); Mesenchymal stem cells (MSC); Microglia/macrophage precursor cells (MG).

Dataset S4: Mean normalized gene expression (TPM) for day 16 and day 21 neural constructs and genes with TPM > 16.
Gene expression data was collected using an in house protocol [Hou, Z., *et al.* (2015) *Sci. Rep.* 5: Article Number 9570], as described in *SI Appendix*, SI Materials and Methods. Columns A-C: Mean normalized

gene expression (TPM) for day 16 and day 21 untreated control samples from the toxicity screening experiment (Individual samples shown in Dataset S3, Columns AK-AS). All genes with an average TPM > 16 are provided for day 16 (Column E) and day 21 (Column F) neural constructs. Genes with average TPM > 16 are shown within select gene ontology (GO) categories for day 21 neural constructs (Columns H-R).

Dataset S5: Toxic and non-toxic control chemicals used for machine learning predictions.
Toxic and non-toxic chemicals, stock concentrations, dosing concentrations, vendor information, and references are provided. T1-34: Toxic chemicals used for the training set. C1-C26: Non-toxic controls used for the training set. B1-B5: Blinded toxins. B6-B10: Blinded non-toxic controls.

Dataset S6: Machine learning analysis for predicting neurotoxicity.
Ranked Probabilities tab. Averaged probabilities from RNA-Seq data for duplicate samples collected on day 16 or day 21 (2 or 7 days after adding chemicals). Columns A-F: Averaged probabilities for the blinded set. Columns H-L: Averaged probabilities for the training data separated by label (1 = toxic; 0 = non-toxic) at each time point (Days 16 and 21) or combined from both days. Columns N-X: Ranked data used for generating the ROC curves on day 16 (Columns N-P), day 21 (Columns R-T), or combined from both days (Columns V-X). ***RFE tab.*** Genes were selected by recursive feature elimination (RFE) using the Weka toolbox [Hall M, et al. (2009) SIGKDD Explor. Newsl. 11(1):10-18] to perform recursive feature elimination (RFE) [Guyon I et al. (2002) Mach. Learn. 46(1-3):389-422], a wrapper-based feature selection algorithm. The methods are available as the metaClassifier AttributeSelectedClassifier. Features (genes) were evaluated by Weka's SVMAttributeEval, set to discard the lowest scoring 10% of the genes at each iteration and to stop at 1000 genes. The Weka's Ranker method was used to select genes.

Dataset S7: Normalized gene expression (TPM) for day 16 neural constructs after dosing with toxic or non-toxic chemicals (two days of exposure). Gene expression data was collected using an in house protocol [Hou, Z., *et al.* (2015) *Sci. Rep.* 5: Article Number 9570], as described in *SI Appendix*, SI Materials and Methods. Samples were exposed to chemicals beginning on day 14 and RNA was collected on day 16 (2 days of exposure). Duplicate samples from separate plates were exposed for each chemical. See Dataset S5 for the full list of chemicals and corresponding sample number.

Dataset S8: Normalized gene expression (TPM) for day 21 neural constructs after dosing with toxic or non-toxic chemicals (seven days of exposure). Gene expression data was collected using an in house protocol [Hou, Z., *et al.* (2015) *Sci. Rep.* 5: Article Number 9570], as described in *SI Appendix*, SI Materials and Methods. Samples were exposed to chemicals beginning on day 14 and collected on day 21 (7 days of exposure). Media was exchanged every two days. Duplicate samples from separate plates were exposed for each chemical. See Dataset S5 for the full list of chemicals and corresponding sample number.