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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
All antibodies used for immunohistochemistry in this study, the concentrations used, their source and their identifier are listed on Table S1	N/A	N/A
APC anti-CD56	BioLegend	Cat# 318309
PE anti-CD82	BioLegend	Cat# 342103
Bacterial and Virus Strains		
AAV-DJ-hSYN1::eYFP	Stanford Gene Vector and Virus Core	N/A
G-deleted Rabies Cre-eGFP	Salk Institute Viral Vector Core	N/A
AAV-DJ-EF1a-CVS-G-WPRE-pGHpA	Stanford Gene Vector and Virus Core; Wertz et al., 2015	Addgene plasmid #67528
AAV-DJ-DIO-mCherry	Stanford Gene Vector and Virus Core	N/A
AAV-1-hSYN1-ChrimsonR-tdT	Addgene	Addgene; #59171-AAV1
AAV-1-syn-jGCaMP7s-WPRE	Addgene	Addgene; #104487-AAV1
AAV-8-CAG-FLEX-Rabies G	Stanford Gene Vector and Virus Core	N/A
Chemicals, Peptides, and Recombinant Proteins		
B-27 supplement without vitamin A	Life Technologies	Cat# 12587010
N-2 supplement	Life Technologies	Cat# 17502048
Human recombinant FGF-2	R&D Systems	Cat# 233-FB
Human recombinant EGF	R&D Systems	Cat# 236-EG
Human recombinant BDNF	Peprotech	Cat# 450-02
Human recombinant NT3	Peprotech	Cat# 450-03
Human recombinant IGF-1	Peprotech	Cat# 100-11
L-Ascorbic Acid	Wako	Cat# 321-44823
cAMP	Sigma-Aldrich	Cat# D0627
Dorsomorphin	Sigma-Aldrich	Cat# P5499
SB-431542	Tocris	Cat# 1614
CHIR 99021	Selleckchem	Cat# S1263
SAG	Millipore	Cat# 566660
Retinoic Acid (RA)	Sigma-Aldrich	Cat# R2625
DAPT	STEMCELL Technologies	Cat# 72082
Y-27632	Selleckchem	Cat# S1049
LDN-193189	Selleckchem	Cat# S7507
Recombinant murine HGF	Peprotech	Cat# 315-23
Geltrex LDEV-Free Reduced Growth Factor Basement Membrane Matrix	Life Technologies	Cat# A1413201
(+)-tubocurarine chloride pentahydrate (curare)	Sigma-Aldrich	Cat# 93750
NBQX	Tocris	Cat# 0373
D-AP5 (APV)	Tocris	Cat# 0106
Tetrodotoxin citrate (TTX)	Tocris	Cat# 1069
MNI-caged-L-glutamate	Tocris	Cat# 1490

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Critical Commercial Assays		
P3 Primary Cell 4D-Nucleofector X Kit L	Lonza	V4XP-3024
Chromium Single cell 3′ GEM, Library & Gel Bead Kit v3	10x Genomics	PN: 1000075
Deposited Data		
Processed single cell RNA-seq data; hSpS d53 and hSpS ± DAPT	This study	GEO: GSE123722
Experimental Models: Cell Lines		
Human iPSC line 2242-1	Pașca lab	N/A
Human iPSC line 8858-1	Pașca lab	N/A
Human iPSC line 0307-1	Pașca lab	N/A
Human iPSC line 0524-1	Pașca lab	N/A
Human iPSC line 1205-4	Pașca lab	N/A
Human iPSC line 8119-1	Pașca lab	N/A
Human iPSC line TUBA1B-eGFP	Coriell	AICS-0012 cl.105
Human iPSC line LMNB-eGFP	Coriell	AICS-0013 cl.210
Human iPSC line CAG::EGFP	Coriell; modified in the Pașca lab	CW30261
Human skeletal myoblasts (hSkM)	Thermo Fisher Scientific	A12555, Lot# 1837192
EmbryoMax PMEF	Millipore	Cat# PMEF-N-K
Oligonucleotides		
All primers used for this study are listed on Table S1	N/A	N/A
Recombinant DNA		
lenti-ACTA1::GCaMP6s	Vector Builder; Brennan and Hardeman, 1993	N/A
lenti-Hb9::GFP	Vector Builder; Nakano et al., 2005	N/A
lenti-Hb9::mCherry	Vector Builder; Nakano et al., 2005	N/A
pX330-U6-Chimeric_BB-CBh-hSpCas9	Addgene	Plasmid# 42230
gRNA_AAVS1-T2	Addgene	Plasmid# 41818
AAVS1-CAG-hrGFP	Addgene	Plasmid# 52344
Software and Algorithms		
ImageJ (Fiji)	Schindelin et al., 2012	https://imagej.net/Fiji
Matlab_R2018a	MathWorks	https://www.mathworks.com/help/matlab/ref/rand.html
Cell Ranger v3.0	10x Genomics	https://support.10xgenomics.com/single-cell-gene-expression/software/pipelines/latest/what-is-cell-ranger
Seurat v3.0	Satija Lab; Butler et al., 2018	https://satijalab.org/seurat/
Reference Similarity Spectrum (RSS)	Kanton et al., 2019	https://github.com/quadbiolab/primate_cerebral_organoids
Human ortholog mapping of mouse genes	Skene et al., 2018	https://github.com/NathanSkene/EWCE/blob/master/data/mouse_to_human_homologs.rda
MUSCLEMOTION	Sala et al., 2018	https://github.com/l-sala/MUSCLEMOTION
Other		
10 cm ultra-low attachment plates	Corning	Cat# 3262
24-well ultra-low attachment plates	Corning	Cat# 3473
Silicone wells	Ibidi	Cat# 80369 and 80409

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Cell culture inserts, 0.4 μm pore size	Corning	Cat# 353090
Glass-bottom 96-well plate	Corning	Cat# 4580
Optical fiber-coupled LED	Thorlabs	Cat# M625F2
CYCLOPS LED driver 3.6 with M8 connector	Open Ephys	Cat# f2

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Sergiu P. Paşca (spasca@stanford.edu).

Materials Availability

This study did not generate unique reagents.

Data and Code availability

Processed gene expression data are available in the Gene Expression Omnibus under accession number GEO: GSE123722.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Culture of hiPS cells

The hiPS cell lines used in this study were validated using standard methods as previously described (Paşca et al., 2011; Sloan et al., 2018). A total of nine hiPS cell lines derived from fibroblasts collected from eight healthy subjects were used for experiments (see Table S1 for details of the hiPS cell lines used for each experiment). hiPS cell lines TUBA1B-mEGFP and LMNB1-mEGFP were derived by the Allen Institute and obtained from Coriell. The CAG::EGFP hiPS cell lines was genetically engineered in the lab using the line #CW30261 obtained from Coriell. The sex of each of the hiPS cell lines is indicated in Table S1. Cultures were tested and maintained mycoplasma free. hiPS cells were cultured on inactivated mouse embryonic fibroblast feeders (EmbryoMax PMEF) in hiPS cell medium containing DMEM/F12, knockout serum (20%), non-essential amino-acids (1:100, Life Technologies), GlutaMax (1:200, Life Technologies), β-mercaptoethanol (0.1mM, Sigma-Aldrich), penicillin and streptomycin (1:100, Life Technologies), and supplemented with FGF-2 (10 ng ml^{-1}; R&D Systems). hiPS cell lines used for the generation of skeletal myoblasts and the CAG::EGFP hiPS cell line were maintained and passaged as previously described (Yoon et al., 2019). Briefly, hiPS cells were maintained in 6-well plates coated with recombinant human vitronectin (VTN-N, Life Technologies, A14700) in Essential 8 medium (Life technologies, A1517001). To passage hiPS colonies, cells were incubated with 0.5 mM EDTA for 7 minutes at room temperature, resuspended in Essential 8 medium and distributed in new 6-well plates. Approval for using these lines was obtained from the Stanford IRB panel and informed consent was obtained from all subjects.

Human primary brain tissue

Human brain specimens were obtained under a protocol approved by the Research Compliance Office at Stanford University. PCW17 forebrain tissue was delivered overnight on ice and immediately processed after arrival. Cortical tissue was fixed overnight in 4% PFA, washed three times with PBS and embedded as described below.

METHOD DETAILS

Variability terminology

There are three levels of variability that we accounted for in our study. These are outlined below, together with the steps we have taken to describe it in this study:

- *Line-to-line variability*: hiPS cell lines derived from different individuals; graphs are color-coded so that each cell line used in the study is represented by one color.
- *Experiment-to-experiment (or differentiation) variability*: a differentiation experiment represents a batch of hiPS cells that are differentiated in parallel; graphs are separated per differentiation experiment.
- *Spheroid-to-spheroid or assembloid-to-assembloid variability*: values for individual spheroids or assembloids are shown in graphs, unless specified otherwise.

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Table S1 shows details on how many and which hiPS cell lines were used for each experiment, as well as the number of differentiations the data is generated from, and from how many spheroids or assembloids. This information can also be found in the corresponding Figure legends. RT-qPCR data is generated from 2–3 spheroids pooled per differentiation.

Generation of hCS and hSpS from hiPS cells

The generation of hCS from hiPS cells was performed as previously described (Birey et al., 2017; Paşca et al., 2015; Sloan et al., 2018). Briefly, hiPS cell colonies were lifted from the plates using dispase (0.35 mg ml^{-1}) and transferred into ultra-low attachment plastic dishes (Corning) in hiPS cell medium supplemented with the two SMAD inhibitors dorsomorphin (5 μM; Sigma-Aldrich, P5499) and SB-431542 (10 μM; Tocris, 1614). This medium was replaced daily for the first five days. On the sixth day in suspension, neural spheroids were transferred to neural medium containing neurobasal-A (Life Technologies, 10888022), B-27 supplement without vitamin A (Life Technologies, 12587010), GlutaMax (1:100, Life Technologies), penicillin and streptomycin (1:100, Life Technologies) and supplemented with the growth factors EGF (20 ng ml^{-1}; R&D Systems, 236-EG) and FGF-2 (20 ng ml^{-1}; R&D Systems, 233-FB) until day 24. From day 25 to 42, the neural medium was supplemented with the growth factors BDNF (20 ng ml^{-1}; Peprotech, 450-02) and NT3 (20 ng ml^{-1}; Peprotech, 450-03) with medium changes every other day. From day 43 onward hCS were maintained in neural medium with medium changes every four to six days.

To generate hSpS, hiPS cell medium was supplemented with dual SMAD inhibitors until day 5 and the WNT activator CHIR 99021 (3 μM; Selleckchem, S1263) from day 4 to day 18. On day 6, spheroids were transferred to neural medium supplemented with RA (0.1 μM; Sigma-Aldrich, R2625), EGF (20 ng ml^{-1}; R&D Systems) and FGF-2 (10 ng ml^{-1}; R&D Systems), with addition of the SHH modulator smoothened agonist (SAG, 0.1 μM; Millipore, 566660) from day 11. From day 7, the medium was changed every other day. On day 19, hSpS were transferred to neural medium supplemented with N-2 supplement (Life Technologies, 17502048), BDNF (20 ng ml^{-1}, Peprotech), IGF-1 (10 ng ml^{-1}; Peprotech, 100-11), L-Ascorbic Acid (AA, 200 nM; Wako, 321-44823) and cAMP (50 nM; Sigma-Aldrich, D0627). For hSpS, the Notch pathway inhibitor DAPT (2.5 μM; STEMCELL technologies, 72082) was added on days 19, 21 and 23. From day 43 onward, the medium was changed every four to five days. For the generation of hSpS with the CAG::EGFP hiPS cell line, we used AggreWell 800 (STEMCELL Technologies, 34815) containing 300 microwells as previously described (Yoon et al., 2019). From days 1 to 6 Essential 6 medium supplemented with dorsomorphin (2.5 μM, Sigma-Aldrich) and SB-431542 (10 μM, Tocris) was used, with all other steps performed as described above. A schematic detailing the hSpS recipe is shown in Figure S2A.

For the combinatorial growth factor matrix, small molecules were added on the same days as described above, and the concentrations for dual SMAD inhibitors, CHIR 99021 and EGF were the same as above. Concentrations tested for RA, FGF-2 and SAG are shown in Figure S1C. From day 7, the neural medium was changed every other day until day 20, when spheres were collected. No DAPT was added for this experiment.

Generation of CAG::EGFP hiPS cell line

The parental hiPS cell line was obtained from Coriell (#CW30261) and was maintained in 6-well plates using StemFlex medium (Life Technologies, A3349401). Cas9, gRNA and donor plasmids were obtained from Addgene (plasmids #42230, #41818 and #52344, respectively). For nucleofection 3 μg Cas9, 1 μg gRNA and 1 μg donor plasmids were used. On the day of nucleofection, hiPS cells were washed with DPBS and incubated with 1 mL Accutase at 37°C for 10 min, after which 9 mL Essential 8 Medium was added to the well for resuspension. After cell counting, the single cell suspension containing 3 x10^6 cells was centrifuged. The cell pellet was used for nucleofection using the P3 Primary Cell 4D-NucleofectorTM X Kit L (Lonza, V4XP-3024), a 4D-nucleofector core unit and X unit (Lonza) using the nucleofection program DC100. After nucleofection, the cells were immediately seeded into a well of a 6-well plate that was precoated with vitronectin and contained pre-warmed Essential 8 Medium supplemented with the ROCK inhibitor Y-27632 (10 μM; Selleckchem, S1049). While the nucleofected cells recovered to reach 70%–80% confluency, 1 μg ml^{-1} of puromycin was applied for 5 days, after which the media was switched back to StemFlex media. Puromycin-resistant clones became visible after 7 days. Clones were pooled together, expanded, cryopreserved and later sorted into 96-well plates to ensure single clone formation by seeding one cell per well. The sorting was performed on a BD Aria II (Stanford Shared FACS Facility). The clone used in this study was selected based on its morphology and uniform expression of GFP. Validation of hiPS genome integrity was performed by high density SNP arrays.

Generation of hCS-hSpS assembloids

To generate cortico-spinal (hCS-hSpS) assembloids, hCS and hSpS were generated separately, and later assembled by placing them in close proximity with each other in 1.5 mL microcentrifuge tubes for 3 days in an incubator. The neural medium used for assembly was supplemented with BDNF (20 ng ml^{-1}; Peprotech), NT3 (20 ng ml^{-1}; Peprotech), L-Ascorbic Acid (AA, 200 nM; Wako) and cAMP (50 nM; Sigma-Aldrich). Media was carefully changed on day 2 after assembly, and placed in a 24-well ultra-low attachment plate (Corning, 3473) using a cut P1000 pipette tip on the third day. Medium was changed every 3–4 days thereafter. Assembly was performed between days (D) 60 and D120 of hCS and between D30 and D50 of hSpS. For hCS-hCS assembloids, one hCS was D60-D75 and the second hCS was D45 (to match the hSpS age).

Culture of hSkM

Human skeletal myoblasts (hSkM) were obtained from Thermo Fisher Scientific (A12555, Lot# 1837192) and maintained in an undifferentiated state with Skeletal Muscle Cell Growth Medium (ready to use; Promocell, C23060) in 10-cm plates (Primaria Cell Culture

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Dish, Corning). Medium was changed every 2-3 days, and hSkM were passaged using Trypsin (Trypsin-EDTA, 0.25%, phenol red; Life Technologies) when they reached ~80% confluency. hSkM from passages 1 to 4 were used for experiments. For analysis of hSkM differentiation ability, hSkM were plated on wells of 24-well plates (Corning) that had been coated with Geltrex™ (1:50 diluted in DMEM/F12, 1 hour at 37°C; Life Technologies, A1413202). 30,000 hSkM were plated on day 0 in Skeletal Muscle Cell Growth Medium. Medium was replaced the day after plating and every other day after that. When hSkM reached ~90% confluency (2–3 days after plating), Skeletal Muscle Cell Growth Medium was replaced with Skeletal Muscle Cell Differentiation Medium (ready to use; Promocell, C23061). On days 0, 5 and 15, hSkM were washed with PBS once and fixed for 10 minutes with 4% paraformaldehyde (PFA).

Generation of 3D hSkM

For the generation of 3D hSkM cultures, hSkM were dissociated using Trypsin (Trypsin-EDTA, 0.25%, phenol red; Life Technologies) and resuspended in Geltrex™ (Life Technologies) at a density of 3,000 hSkM per μl. Fifty μl of this viscous cell suspension were aliquoted into silicone wells (Ibidi, 80369) located inside 6-well tissue culture plates (Corning), and incubated for 30 minutes at 37°C to allow Geltrex™ gelling, at which point 4 mL of Skeletal Muscle Cell Growth Medium was added. The next day, silicone wells containing hSkM were placed into 6-well ultra-low attachment plates, and medium was changed every 2-3 days. After 7-10 days, medium was changed to Skeletal Muscle Cell Differentiation Medium to allow for differentiation of hSkM with medium changes every 2-3 days. For some experiments, including ACTA1::GCaMP6s imaging, smaller 3D hSkM were generated by resuspending 24,000 cells in 10 μL of Geltrex™ and aliquoting them in small silicone wells (Ibidi, 80409). 3D hSkM were used for assembloid generation 10 to 25 days after the switch to differentiation medium. Figures 5F and S6H show pictures of the 3D hSkM set-up.

Generation of ihSkM and 3D ihSkM

Generation of ihSkM was performed as described in Chal et al., 2016 with some modifications. On day –2, hiPS cells at 65%–80% confluency were detached and plated at a density of 1 x10^5 cells per well in 12-well plates coated with Matrigel (1:25; Corning, 354230) and Essential 8 medium (Life technologies, A1517001) supplemented with Y-27632 (10 μM; Selleckchem, S1049). From day 0–3, DMEM/F12 medium (Life Technologies, 11330032) supplemented with 1% Non-Essential Amino Acids (NEAA; Life Technologies), 1% Insulin-Transferrin-Selenium (ITS; Life Technologies, 25-800-CR), 1% penicillin and streptomycin (Life Technologies), and small molecules CHIR 99021 (3 μM; Selleckchem) and LDN-193189 (500 nM; Selleckchem, S7507) was changed daily. From day 3–6, FGF-2 (20 ng ml^{-1}; R&D Systems) was added in addition to CHIR 99021 and LDN-193189 as described above. From day 6 until sub-culturing, DMEM/F12 with 15% knockout serum (KSR, Life Technologies), 1% penicillin and streptomycin (Life Technologies), 1% Non-Essential Amino Acids (NEAA; Life Technologies), and β-mercaptoethanol (0.1 mM; Sigma-Aldrich) was used. From day 6–9 the basal medium was supplemented daily with LDN-193189 (500 nM; Selleckchem), recombinant FGF-2 (20 ng ml^{-1}; R&D Systems), recombinant HGF (10 ng ml^{-1}; Peprotech, 315-23) and recombinant IGF-1 (2 ng ml−1; Peprotech). From day 9–11 cultures were supplemented only with 2 ng ml^{-1} IGF-1. From day 12 onward, media was supplemented with 2 ng ml^{-1} IGF-1 and 10 ng ml^{-1} HGF every other day. Cultures were sub-passaged or sub-cultured at day 33–34. For sub-culture, four wells of a 12-well plate were washed with DPBS twice and then incubated with 1 mL of TrypLE Express Enzyme each (GIBCO, 12604013) for 9–10 minutes at 37°C. Following incubation, cells were detached, transferred to a 50 mL conical tube with 20 mL of DMEM/F12 with 10% FBS and centrifuged. Cells were then resuspended in Skeletal Muscle Cell Growth Medium (Promocell) and passed through a 70 μm strainer. Cells were resuspended in Skeletal Muscle Cell Growth Medium supplemented with 10 μM Y-27632 and plated at a density of 2.5 x10^5 per well in Matrigel-coated 12-well plates. Medium was changed the following day. These cells, referred as P2, could be maintained and expanded in Skeletal Muscle Cell Growth Medium with daily medium changes and passages every 2–4 days. Starting at P4 cells could be cryopreserved or used for FACS.

The FACS purification of myoblasts was performed as described in Pakula et al. (2019). Briefly, cells were detached using TrypLE Express, resuspended in Hank's Balanced Saline Solution (HBSS; Life Technologies) supplemented with 5% FBS, and counted. The cell suspensions were incubated with APC anti-CD56 and/or PE anti-CD82 at a concentration of 5 μL per 1x10^6 cells (BioLegend, 318309 and 342103, respectively) for 30 minutes on ice, washed with HBSS/FBS and pipetted through the strainer cap of a 5 mL round-bottom FACS tube. Samples were then sorted on a BD Aria II instrument (Stanford Shared FACS Facility) into Skeletal Muscle Cell Growth Medium, and plated on Matrigel-coated 12-well plates for further expansion.

The generation of 3D ihSkM was performed as described above for hSkM. Briefly, 2.5–3.5 x10^5 ihSkM were seeded into silicone wells (Ibidi, 80369) with 50 μL of Geltrex™ (Life Technologies) and maintained in Skeletal Muscle Cell Growth Medium with medium changes every 1–2 days. After 7–10 days, medium was changed to Skeletal Muscle Cell Differentiation Medium with medium changes every 2–3 days.

Generation of hSpS-hSkM and hCS-hSpS-hSkM

To generate neural-muscle assembloids, 3D hSkM that had been in differentiation medium for at least 10 days (see above) were removed from the silicone wells and placed on top of cell culture inserts (0.4 μm pore size; Corning, 353090) that were positioned in 6-well plates containing 2 mL of DMEM/F12 medium supplemented with 1% Non-Essential Amino Acids (NEAA; Life Technologies), 1% Insulin-Transferrin-Selenium (ITS; Life Technologies), 1% penicillin and streptomycin (Life Technologies), L-Ascorbic Acid (AA, 200 nM; Wako) and cAMP (50 nM; Sigma-Aldrich). Next, spheroids (either hSpS or hCS) were placed on the inserts containing 3D hSkM (or ihSkM) and arranged so that they were in contact with one another and were allowed to interact.

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For hCS-hSpS-hSkM assembloids, hSpS-hSkM was assembled first, and hCS was added 1–2 days later. For this combination, sometimes more than one (1–2) hSpS were added. hSpS tend to be smaller in size than hCS, and adding more than one hSpS avoids hCS being in direct contact with hSkM. Only one assembloid was maintained per insert, and half medium changes were performed every other day. Figure 5H shows a schematic detailing this set-up, and Figures 5F and S6H show pictures of the generation of 3D hSkM and the insert set-up.

Imaging of 3D hSkM spontaneous contractions was performed under environmentally controlled conditions (37°C, 5% CO_2) using a 5x objective in a confocal microscope (Leica SP8). Assembloids, still in transwells, were incubated in the environmentally controlled chamber for 20-30 minutes before imaging, and they were imaged for 2 minutes at a frame rate of 14.7 frames/sec. 1–2 fields were imaged per assembloid.

Co-culture of mouse limb and spheroids

For mouse co-culture experiments, timed-pregnant female mice were sacrificed at E11.5, embryos were collected, and limb buds dissected (both forelimbs and hindlimbs were used for this experiment). Limbs and spheroids (at D25) were then assembled together by placing them in close proximity in a 1.5 mL microcentrifuge tube for 3 days in an incubator. One limb and one spheroid were placed per tube. On day 2 medium was carefully changed. Neural medium supplemented with N-2 supplement (Life Technologies, 17502048) was used. After assembly, mouse-spheroid cultures were placed in 24-well ultra-low attachment plates (Corning), and medium was changed every other day. For contraction quantification, assembloids were visualized using brightfield illumination in an EVOS FL Cell Imaging System (Life Technologies), and they were deemed to be contracting if they moved within a time-window of 30 s. Approval for mouse experiments was obtained from the Stanford University's Administrative Panel on Laboratory Animal Care (APLAC).

Viral labeling and rabies-ΔG tracing

Viral labeling of neural spheroids was performed as previously described (Sloan et al., 2018). In brief, spheroids were placed in a 1.5 mL microcentrifuge tube containing 250 μL neural medium with the desired virus and incubated overnight. Fresh medium was added the following day, and spheroids were transferred to ultra-low attachment plates (Corning) the next day. The viruses used for this study are: AAV-DJ-hSYN1::eYFP, lenti-Hb9::GFP or lenti-Hb9::mCherry (Nakano et al., 2005), lenti-ACTA1::GCaMP6s (generated by VectorBuilder) (Brennan and Hardeman, 1993), rabies-ΔG-Cre-eGFP, AAV-DJ-EF1a-CVS-G-WPRE-pGHpA (Addgene, Plasmid #67528) (Wertz et al., 2015), AAV-DJ-DIO-mCherry, AAV-1-hSYN1-ChrimsonR-tdT (Addgene, #59171-AAV1), AAV-1-syn-jGCaMP7s-WPRE (Addgene, Plasmid #104487-AAV1, gift from Douglas Kim & Genie Project) and AAV-8-CAG-FLEX-Rabies G. Lentivirus was generated in-house by transfecting HEK293T cells with Lipofectamine 2000 (Thermo Fisher Scientific, 11668019) and concentrating the supernatant with Lenti-X concentrator (Clontech, 631232) 72 h later. AAVs were generated at the Stanford Gene Vector and Virus Core at Stanford University School of Medicine or acquired from Addgene. Rabies-ΔG viruses were obtained from the Salk institute Viral Vector Core.

For viral tracing experiments with rabies-ΔG in hCS-hSpS assembloids, ~d82-88 hCS were labeled with AAV-DJ-DIO-mCherry and ~d46-54 hSpS separately labeled with both rabies-ΔG-Cre-eGFP and AAV-DJ-EF1a-CVS-G-WPRE-pGHpA. Six to seven days after viral infection, hCS and hSpS were thoroughly washed with neural medium, assembled, and maintained in culture with media changes every 3–4 days. For viral tracing experiments with rabies-ΔG in hSpS-hSkM assembloids, 3D hSkM were infected with rabies-ΔG-Cre-eGFP and AAV-8-CAG-FLEX-Rabies G, thoroughly washed, assembled one week later with d37 hSpS, and maintained in culture with half medium changes every other day. After 31 days of fusion (hCS-hSpS) or 18 days of fusion (hSpS-hSkM), assembloids were fixed with 4% paraformaldehyde and processed for immunocytochemistry as described below. Only assembloids with at least 10 GFP^+ cells were included in the analysis. Widespread infection of 3D hSkM with rabies-ΔG-Cre-eGFP and AAV-8-CAG-FLEX-Rabies G was challenging, which resulted in some assembloids having fewer GFP cells for quantification.

Projection imaging in intact hCS-hSpS

The projection of hCS-derived AAV-DJ-hSYN1::eYFP into hSpS was imaged under environmentally controlled conditions (37°C, 5% CO_2) in intact, assembled hCS-hSpS using a confocal microscope with a motorized stage (Leica SP8). Assembloids were transferred to a glass-bottom 96-well plate (Corning) with 200 μL of neural medium, and incubated in the environmentally controlled chamber for 20-30 minutes before imaging. Images were taken using a 10x objective to capture the entire hSpS side at a depth of 50–150 μm. For long-term live imaging of hCS-derived AAV-DJ-hSYN1::eYFP, the same set-up was used, and hCS-hSpS were imaged for 8-12 hours at a rate of 10 min per frame.

hSkM Cal-590 calcium imaging

For calcium imaging co-culture experiments, hSkM were plated on 24-well plates as described above and differentiated with Skeletal Muscle Cell Differentiation Medium. After 6-7 days of exposure to differentiation medium, hSkM were co-cultured with hSpS or hCS. Skeletal Muscle Cell Differentiation Medium was replaced with DMEM/F12 supplemented with 1% Non-Essential Amino Acids (NEAA; Life Technologies), 1% Insulin-Transferrin-Selenium (ITS; Life Technologies), 1% penicillin and streptomycin (Life Technologies), L-Ascorbic Acid (AA, 200 nM; Wako) and cAMP (50 nM; Sigma-Aldrich). hSpS or hCS were placed in the middle of the 24-well plate, taking care not to disrupt the hSkM. One hSpS or hCS were placed per well. hSkM-spheroid co-cultures were

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left undisturbed for two days, and half medium was carefully replaced every other day thereafter. Calcium imaging was performed after 6-7 days of co-culture. Cultures were incubated with Cal-590 AM (10 μM; AAT Bioquest, 20510) and PowerLoad (1:100; Invitrogen, P10020) for 30 minutes at 37°C, washed once for 10 minutes with full medium and then imaged. A Leica SP8 confocal microscope with a resonant scanner was used for imaging. Spontaneous calcium activity was recorded for 2 min (10 frames per second) in 6-9 fields per well, and for each field the distance from the spheroid was measured. (+)-tubocurarine chloride pentahydrate (curare; Sigma-Aldrich, 93750) was used at a final concentration of 100 μM.

Optogenetics and GCaMP imaging in hCS-hSpS

For optogenetic stimulation in hCS-hSpS assembloids, intact hCS-hSpS were placed in a 35 mm Glass bottom dish with a 20 mm micro-well #0 cover glass (Cellvis) and imaged using a 10x objective in a confocal microscope (Leica SP8) under environmentally controlled conditions (37°C, 5% CO_2). GCaMP7s was imaged at a frame rate of 10 frames/sec. A typical stimulation consisted of ten pulses of light (625 nm, 100 ms in duration each and 100-200 s apart) delivered using an optical fiber-coupled LED (400 μm-diameter, 14 mW/mm^2; Thorlabs, M625F2) that was directed toward the hCS. Pulses were generated by a CYCLOPS LED driver coupled with the Leica SP8. NBQX (Tocris, 0373) and APV (Tocris, 0106) were used at a final concentration of 20 μM and 50 μM each.

Glutamate uncaging and optogenetics in hCS-hSpS-hSkM

Intact assembloids were imaged under environmentally controlled conditions (37°C, 5% CO_2) using a 5x or 10x objective in a confocal microscope (Leica SP8). Assembloids, still in transwells, were incubated in the environmentally controlled chamber for 20-30 minutes before imaging. For glutamate uncaging experiments, MNI-caged-L-glutamate (Tocris, 1490) was used at a final concentration of 3.3 mM in culture medium (see above). The FRAP software module of the Leica SP8 confocal microscope was used to uncage glutamate using UV light (405 nm). At a frame rate of 14.7 frames/sec, a typical stimulation experiment consisted of 500 frames acquired during pre-stimulation, 2-5 frames of UV stimulation (in specified region of interest, ROI) and 200 frames acquired during post-stimulation. For optogenetic stimulation, five consecutive pulses of light (625 nm ± 5 nm, 68 or 100 ms in duration each and ~6.8 or ~10 s apart) were delivered using an optical fiber-coupled LED (400 μm-diameter, 14 mW/mm^2; Thorlabs) that was directed toward the hCS. Pulses were generated by a CYCLOPS LED driver (Open Ephys) coupled with the Leica SP8. (+)-tubocurarine chloride pentahydrate (curare; Sigma-Aldrich) was used at a final concentration of 100 μM. NBQX (Tocris) and APV (Tocris) were used at a final concentration of 50 μM each.

We note that optogenetic stimulation experiments depend on at least two factors: *(i)* the duration of light pulses, and *(ii)* the efficiency of opsin delivery. Experiments in this manuscript were performed with stimulations of 68–100 ms for increased sampling speed and to avoid any light-induced toxicity, yet longer stimulations may yield higher success rates. Moreover, because low opsin expression may result in reduced stimulation success, inspection of hCS AAV-1-hSYN1-ChrimsonR-tdT expression before assembly is recommended.

Cryopreservation and immunohistochemistry

Cryopreservation and immunocytochemistry in hCS and hSpS was performed as previously described (Sloan et al., 2018). Briefly, neural spheroids were fixed in 4% paraformaldehyde (PFA in PBS, Electron Microscopy Sciences) for 2 hours. Early spheroids (> 25 days) were fixed for 30 minutes. Fixation was followed by three PBS washes, sucrose cryopreservation (30% sucrose in PBS for 24-48 hours or until samples sank to the bottom), embedding in 1:1, 30% sucrose: OCT (Tissue-Tek OCT Compound 4583, Sakura Finetek) and freezing. For immunocytochemistry, 16 μm thick sections were cut using a cryostat (Leica). PCW17 cryosections were 30 μm thick. Cryosections were then washed with PBS to remove excess OCT, blocked for 1 h at room temperature (10% normal donkey serum (NDS), 0.3% Triton X-100 diluted in PBS), and incubated overnight at 4°C with primary antibodies in blocking solution (Table S1 shows the primary antibodies used in this study). Next day, cryosections were washed with PBS and then incubated with secondary antibodies for 1 h at room temperature. Alexa Fluor secondary antibodies (Life Technologies) diluted in blocking solution at 1:1,000 were used. For neuromuscular junction staining, cryosections were incubated for 30 minutes with anti-bungarotoxin (BTX) conjugated to Alexa Fluor-647 in blocking solution (1:500) after secondary antibody incubation. Following washes with PBS, nuclei were visualized with Hoechst 33258 (Life Technologies). Finally, slides were mounted for microscopy with cover glasses (Fisher Scientific) using Aquamount (Polysciences) and imaged on a Zeiss M1 Axioscope, Keyence fluorescence microscope or Leica TCS SP8 confocal microscope. Images were processed in ImageJ (Fiji) (Schindelin et al., 2012). 3D rendering of neuromuscular junction was performed using the software Imaris. The same procedure was followed for immunocytochemistry of 2D hSkM.

Transmission electron microscopy

hCS-hSpS-hSkM were fixed in 2% glutaraldehyde and 4% PFA in 0.1 M sodium cacodylate buffer (pH 7.4) for 15 minutes at room temperature. After fixation, samples were then moved to 4°C for processing. The fixative was removed and replaced with 1% OsO_4 and samples were then allowed to warm to room temperature rotating for 2 hours. Samples were then washed 3 times with ultrafiltered water. After the 3rd rinse, samples were stained in 1% uranyl acetate for 2 hours while rotating. Samples were then dehydrated in a series of ethanol washes for 20 minutes each at room temperature: 50% ethanol, 70% ethanol (at 4°C overnight), 95% ethanol (while allowing to warm at room temperature) and, lastly 100% ethanol. A 2nd 20-minute rinse in 100% ethanol was performed

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followed by a 15 minute rinse in propylene oxide (PO) at room temperature. Samples were then infiltrated with resin mixed 1:2, 1:1, and 2:1 with PO for 2 hours each, leaving samples in 2:1 resin with PO overnight rotating at room temperature. The following day, the samples were placed in 100% EMbed-812 for 2–4 hours and then placed into molds with fresh resin and placed at 65°C overnight. Sections were picked up on formvar/Carbon coated slot Cu grids, stained for 30 s in 3% uranyl acetate in 50% acetone followed by staining in Sato's Lead Citrate for 3–5 minutes. Sections were inspected using a JEOL JEM-1400 120kV, and images were taken using a Gatan Orius 832 4k X 2.6k digital camera (9 μm pixel).

Real-time quantitative PCR - qPCR

For qPCR analysis of spheroids, at least 2-3 spheroids were pooled per sample. mRNA was isolated using the RNeasy Mini kit and RNase-Free DNase set (QIAGEN), and template cDNA was prepared by reverse transcription using the SuperScript III First-Strand Synthesis SuperMix for qRT–PCR (Life Technologies). qPCR was performed using SYBR Green (Roche) on a ViiA7 machine (Applied Biosystems, Life Technologies). Primers used are listed in Table S1.

Single cell dissociation

Dissociation of hSpS into single cells for transcriptomics analysis was performed as previously described (Birey et al., 2017; Paşca et al., 2015; Sloan et al., 2017, 2018). Briefly, 8-10 hSpS per sample were chopped and incubated in 40 U/ml papain enzyme solution at 37°C for 90 minutes. After digestion, samples were washed with a protease inhibitor solution and gently triturated to achieve a single cell suspension and strained using a 70 μm Flowmi strainer (Bel-Art).

Single cell gene expression - BD Rhapsody

To capture single cell transcriptomic information of hiPS cell derived hSpS, we used the BD Rhapsody system (formerly known as BD Resolve) (BD Biosciences) as previously reported (Birey et al., 2017; Fan et al., 2015). hSpS with or without DAPT exposure were dissociated enzymatically into single cells at day 45 of differentiation and processed on the same day. Ten spheroids for each condition were combined, the proportion of live cells was estimated using a fluorescent assay (~90%) and all cells were used for further processing. Single-cell suspension of ~10,000 cells were captured from all isolated cells, without selection, on an array of > 200,000 microwells through a limited dilution approach. Beads with oligonucleotide barcodes were added to saturation so that a bead was paired with a cell in a microwell. After exposure to cell lysis buffer, poly-adenylated RNA molecules hybridized to the beads. Beads were retrieved into a single tube for reverse transcription. Upon cDNA synthesis, each cDNA molecule was tagged on the 5′ end (that is, the 3′ end of a mRNA transcript) with a molecular index and cell label indicating its cell of origin. Whole transcriptome libraries were prepared from 40% of the captured cells by subsampling the Rhapsody beads that were then subject to second strand cDNA synthesis, adaptor ligation, and universal amplification using twenty-two cycles of PCR. The rest of the beads were archived. Sequencing libraries were prepared using random priming PCR of the whole-transcriptome amplification products to enrich the 3′ end of the transcripts linked with the cell label and molecular indices. The libraries were sequenced on HiSeq4000 (Illumina) using 101 × 2 chemistry.

The BD Rhapsody analysis pipeline was used to process sequencing data (.fastq files). Cell labels and molecular indices were identified, and gene identity was determined by alignment against the gencode comprehensive hg19 reference. A table containing molecule counts per gene per cell was the output. Gene expression profiles of 4,175 and 3,173 cells were recovered for hSpS with or without DAPT, respectively, with an average number of reads of ~17,000, ~2,400 molecules and ~1,400 number of genes detected per cell with average molecular index coverage (that is, the number of times a molecule was sequenced) of 4.8.

Analysis of the single cell transcriptome profiles was performed with BD™ Data View as we previously described (Birey et al., 2017). Cells with mitochondrial gene (with a gene symbol starting with *MT*) content > 30%, were discarded, retaining a total of 7,888 cells from both samples. We extracted the expression profiles of the 1,278 genes (Table S1) that define the 10 populations in hSpS and conducted *t*SNE projection on the filtered data.

Single cell gene expression - 10x Genomics

Dissociated cells were resuspended in ice-cold PBS containing 0.02% BSA and loaded onto a Chromium Single cell 3′ chip (with an estimated recovery of 6,000 cells per channel) to generate gel beads in emulsion (GEMs). scRNA-seq libraires were prepared with the Chromium Single cell 3′ GEM, Library & Gel Bead Kit v3 (10x Genomics, PN: 1000075). Libraries from different samples were pooled and sequenced by Admera Health on a NovaSeq S4 (Illumina) using 150 × 2 chemistry. Demultiplexing, alignment, barcode and UMI counting and aggregation were performed using Cell Ranger (v3.0 with default settings). This provided us with a feature by count matrix. Further analysis was performed using the R package Seurat (v3.0) from the Satija Lab (Butler et al., 2018). We excluded cells with more than 6,000 or less than 1,000 detected genes, as well as those with a mitochondrial content higher than 18%. We excluded genes that were not expressed in at least three cells. Due to sex differences between hiPS cell lines we also removed all genes on the X and Y chromosomes from the count matrix. Gene expression was then normalized using a global-scaling normalization method (normalization.method = "LogNormalize," scale.factor = 10000, in Seurat), and the 1,500 most variable genes were then selected (selection.method = "vst," in Seurat) and scaled (mean = 0 and variance = 1, for each gene, as recommended by Seurat) prior to principal component analysis (PCA). The top 18 principal components were utilized to do the clustering with a resolution of 1.1, implemented using the FindNeighbors and FindClusters functions in Seurat. We identified clusters based on expression of known markers (Delile et al., 2019). In some cases, using expression of known markers, we grouped together clusters that were originally

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separate based on the Seurat clustering, namely the 'MN', 'V2a', 'astroglia' and 'other' clusters were composed of 2-4 seurat clusters. Motor neuron subclustering was performed using Seurat as described above using 800 variable genes and the top 10 principal components.

Reference similarity spectrum (RSS) analysis (Kanton et al., 2019) was performed to compare hSpS single cell transcriptomics data to adult mouse brain regions (Zeisel et al., 2018) and developing mouse spinal cord (Delile et al., 2019). Briefly, processed reference data and cell cluster annotations were downloaded from http://mousebrain.org/downloads.html (Zeisel et al., 2018) and https://github.com/juliendelile/MouseSpinalCordAtlas/tree/master/dataset (Delile et al., 2019). Analysis was restricted to non-progenitor cell clusters, neuronal cell clusters, and clusters from central nervous system tissues. For reference datasets, gene expression was averaged for each cell cluster and the top 2000 highly variable genes were identified using the FindVariableFeatures function of the Seurat R package (Stuart et al., 2019) with default parameters. Human ortholog mapping of mouse gene symbols was performed as described in Skene et al. (2018) with the following annotation file: https://github.com/NathanSkene/EWCE/blob/master/data/mouse_to_human_homologs.rda. Pearson correlation between each reference cell cluster and hSpS data (either cell clusters or individual cells) was performed across matched highly variable genes. *z*-Transformation was then applied to get the normalized similarities.

Electrophysiology

Sections of hSpS (day 45-75) for electrophysiology were obtained as previously described (Sloan et al., 2018). In brief, spheroids were incubated in bicarbonate-buffered aCSF at 23 °C and equilibrated with a mixture of 95% O_2 and 5% CO_2. The aCSF solution contained: 126 mM NaCl, 26 mM $NaHCO_3$, 10 mM glucose, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 1 mM $MgSO_4$ and 2 mM $CaCl_2$. Slicing was performed using a Leica VT1200 vibratome. Immediately after sectioning, slices were moved to a circulation chamber containing oxygenated aCSF at room temperature.

Patch-clamp recordings were performed from cells expressing the Hb9::GFP fluorescent reporter using an upright microscope (Slicescope, Scientifica). Recording electrodes of borosilicate glass had a resistance of 7–10 MΩ when filled with internal solution. The internal solution contained: 145 mM K-gluconate, 0.1 mM $CaCl_2$, 2.5 mM $MgCl_2$, 10 mM HEPES, 0.2 mM EGTA, 4 mM Na-phosphocreatine. 4/7 cells were able to fire repetitive action potentials.

To evoke APs in hCS axon terminals while recording from Hb9::GFP cells, we performed whole field illumination via the microscope's 40x objective (Olympus), using a broad-spectrum green-red LED (560 ± 50 nm, CoolLed). Hb9::GFP cells were selected based on the proximity to Chrimson$^+$ fibers. The stimulation duration was set to 5 ms and the stimulation frequency was 0.1 Hz. Chrimson-triggered EPSCs were blocked by bath application of TTX (1 μM; Tocris, 1069).

Data were collected using a 1550A digitizer (Molecular Devices), a 700B patch-clamp amplifier (Molecular Devices) and acquired with pClamp 10.7 software (Molecular Devices). Data were low-pass filtered at 10 kHz and digitized at 20 kHz. Data averaging, digital subtraction of null traces, and current peak detection were performed using clampfit (Molecular Devices).

QUANTIFICATION AND STATISTICAL ANALYSIS

Fluorescence intensity quantification

Fluorescence intensity in cryosections was obtained using the Plot Profile command in ImageJ (Fiji). For each cryosection 3–8 regions were measured.

Projection quantification

hCS-derived AAV-hSYN1::eYFP projections were quantified using ImageJ (Fiji). ROIs were manually drawn to cover the area on the hSpS or hCS to be measured in max projection confocal stacks. Both the brightfield and fluorescent channels were used to draw the ROIs. Following background subtraction (50 rolling ball radius), FeatureJ Hessian filter and contrast enhancement (0.4 saturated), the percentage of YFP$^+$ pixels over total area of hSpS or hCS was calculated in binary images. For the quantification of line length, the ridge detection plugin of ImageJ was used in binary images.

hSkM Cal-590 calcium imaging

Calcium imaging data was processed using ImageJ (Fiji) and custom MATLAB routines. ROIs corresponding to hSkM fibers were automatically generated using the analyze particles plugin on average intensity projections (300 frames) using ImageJ (Fiji). A total number of 798 (hSkM condition), 652 (hCS + hSkM condition), 1006 (hSpS + hSkM condition) and 727 (hSpS + hSkM + curare condition) hSkM fibers were analyzed. Following ROI registration, raw time-series movies were transformed to relative changes in fluorescence: $\Delta F/F(t) = (F(t)-F_0)/F_0$, where F_0 represented the 5th percentile value of the time series of each ROI. To remove slow fluctuations originating from the summation of multiple events, we first high-passed filtered the ROI's ΔF/F(t) functions (ΔF/F(t)'. Calcium candidate events were detected whenever the ROI's ΔF/F(t) crossed a threshold of 7 median absolute deviations (MAD). Active hSkM generated at least 1 calcium event over a 2-minute period. Calcium events are typically characterized by a sharp rise followed by slower decay. To capture these features, we only considered events that follow this behavior. Event time was set to the time the event crossed the threshold.

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hSkM GCaMP6s analysis

GCaMP6s imaging data was processed using ImageJ (Fiji) and custom MATLAB routines as described above. ROIs corresponding to GCaMP6s positive hSkM fibers were generated using standard deviation projections.

GCaMP7s analysis

GCaMP7s imaging data was processed using ImageJ (Fiji) and custom MATLAB routines. For the purpose of this experiment (comparison of stimulation-triggered responses before and after application of NBQX + APV), only fields where cells appeared to be active at baseline were used. We found four out of ten assembloids to have clear responding cells. A cell's ΔF/F amplitude was calculated as the maximum ΔF/F value inside a 6 s window after the LED stimulation time, minus the mean of the baseline 1 s before the stimulation. To eliminate shape-dependent artifacts, the median was used instead of the mean. To demonstrate that responses were time-locked to the stimulation and decouple them from the spontaneous firing, we compared the time-locked ΔF/F response to that of random time-locked ΔF/F.

Contraction analysis

Muscle contraction of 3D hSkM was quantified using the automated, open-source ImageJ plugin MUSCLEMOTION (Sala et al., 2018) (https://github.com/l-sala/MUSCLEMOTION). MUSCLEMOTION quantifies movement by subtracting the summed, absolute changes in pixel intensity between a reference frame and the frame of interest. Because each imaging field consists of a large area containing multiple muscle fibers, several fibers may be moving simultaneously and summation of pixel intensities in these cases may result in non-changing summed values. Therefore, to reduce the chance of subtraction of pixel values, each imaging field (1.8 mm by 1.8 mm in size) was divided into 16 subfields and the analysis was performed in each of the subfields individually (Figure S7A) . For the analysis of spontaneous contractions, event detection was performed using custom MATLAB routines. Events over 5 median absolute deviations (MAD) were counted as a contraction event. Correlation between subfields in a field was calculated in MATLAB by computing a non-normalized covariance calculation. The mean covariance per field was plotted.

For the quantification of stimulation experiments of assembloids, pixel intensity analysis was performed with MUSCLEMOTION as described above. Displacement over time was calculated by normalizing all values to 500 frames preceding stimulation. If different fields (i.e., areas) were stimulated per assembloid, then these were plotted separately. If the same field was stimulated more than once, values were averaged and plotted as one point. For optogenetic stimulation, only the first trial per field was used for quantification, and data values resulting from each of the five pulses of light were averaged.

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Supplemental Figures

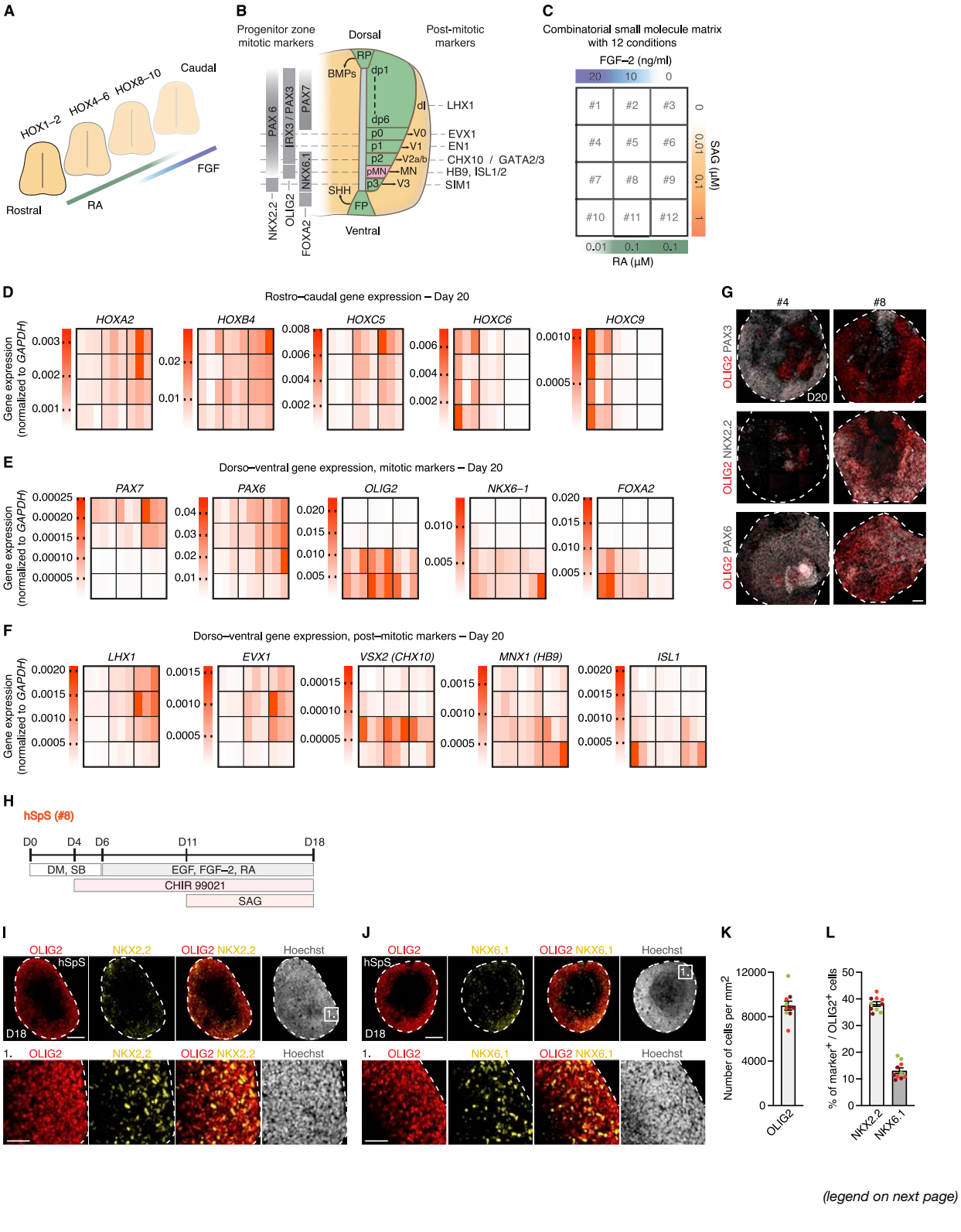

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Figure S1. Combinatorial Analysis to Determine hSpS Culture Conditions and Characterization of Day 18 hSpS, Related to Figure 1

(A and B) Schematics illustrating marker gene expression along the rostro-caudal (A) and dorso-ventral (B) axes of the hindbrain and spinal cord.

(C) Schematic detailing the 12 conditions in the combinatorial growth factor matrix and the molecules and concentrations they receive.

(D–F) Gene expression analysis of genes expressed along the rostro-caudal (D) and dorso-ventral (E), (F) axes in the 12 conditions of the combinatorial matrix at day 20 of *in vitro* differentiation. Each condition shows 3 colored bars representative of n = 3 hiPS cell lines from 1 differentiation (Kruskal-Wallis test $p = 0.002$ for *HOXA2*, $p = 0.008$ for *HOXB4*, $p = 0.003$ for *HOXC9*, $p = 0.002$ for *HOXC9*, $p = 0.0008$ for *PAX7*, $p = 0.02$ for *PAX6*, $p = 0.001$ for *OLIG2*, $p = 0.002$ for *NKX6-1*, $p = 0.0006$ for *LHX1*, $p = 0.007$ for *CHX10*, $p = 0.06$ for *HB9*; one-way ANOVA $p = 0.002$ for *HOXC5*, $p < 0.0001$ for *FOXA2*, $p < 0.0001$ for *EVX1*, $p = 0.01$ for *ISL1*).

(G) Immunocytochemistry in cryosections of hSpS conditions #4 and #8 showing dorsal-like and ventral-like identities. This image was generated by automatic stitching of individual images.

(H) Schematic detailing differentiation conditions used for deriving hSpS.

(I and J) Representative immunocytochemistry images in cryosections of hSpS showing expression of OLIG2 and NKX2.2 (I) or NKX6.1 (J) at day 18 of *in vitro* differentiation.

(K) Quantification of the total number of OLIG2 cells per area (mm^2) in day 18 hSpS (n = 10 hSpS derived from 3 hiPS cell lines from 1 differentiation, with 2 cryosections quantified per hSpS).

(L) Quantification of the percentage (%) of OLIG2 positive cells that co-express either NKX2.2 or NKX6.1 in day 18 hSpS (n = 10 hSpS derived from 3 hiPS cell lines from 1 differentiation, with 2–3 cryosections quantified per hSpS).

Data represent mean ± s.e.m. Scale bars, 50 μm (insets in (I), (J), 100 μm (G), 200 μm (I), (J).

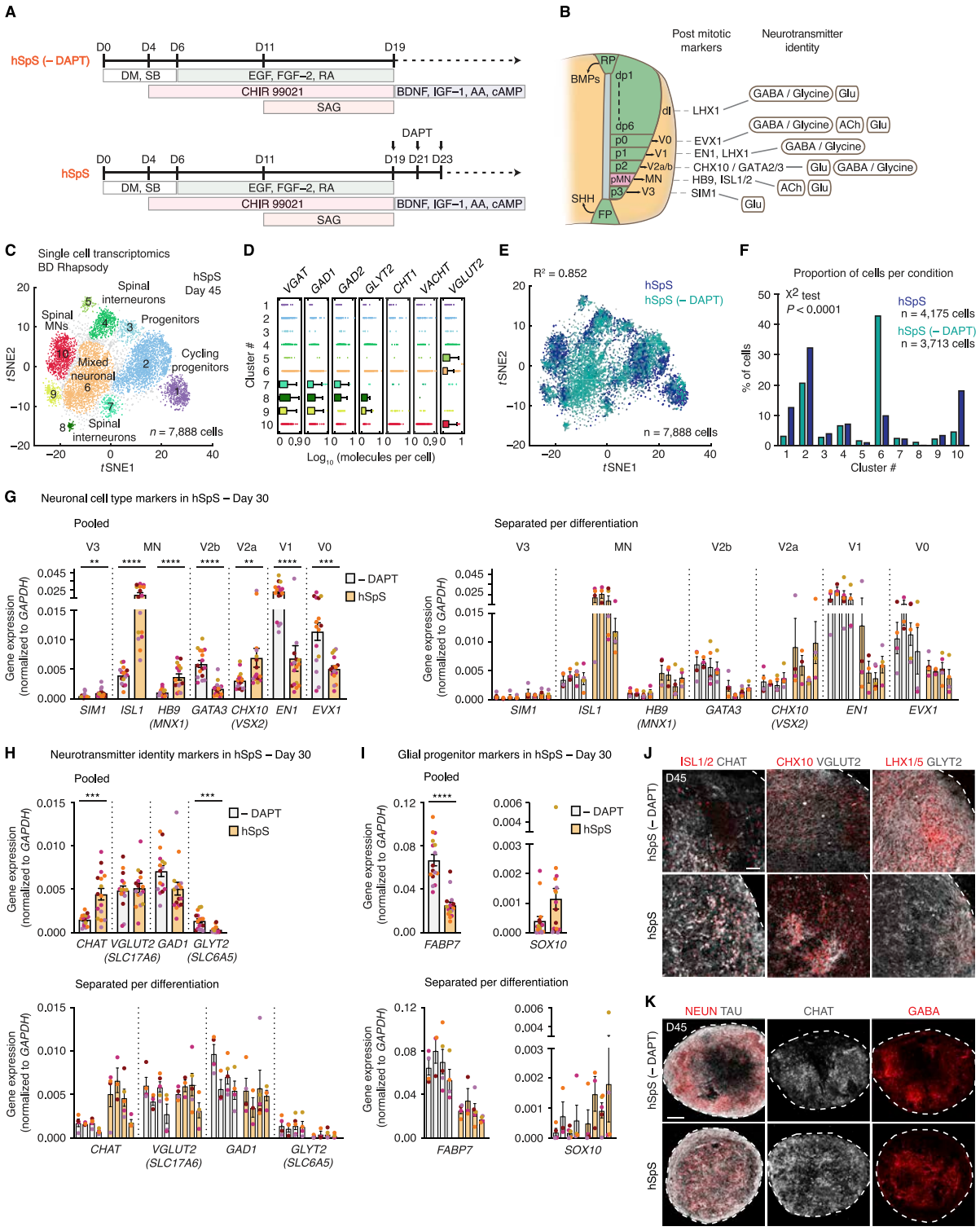

Figure S2. Characterization of the Role of DAPT in hSpS Differentiation, Related to Figure 1

(A) Schematic detailing differentiation conditions used for deriving hSpS with or without DAPT.

(B) Schematic illustrating hindbrain/spinal cord neuronal domains, their corresponding marker genes and their neurotransmitter identities.

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(C) *t*-SNE visualization of single cell gene expression of hSpS with or without DAPT at day 45 of *in vitro* differentiation (n = 7,888 cells; BD™ Rhapsody system) showing 10 main clusters that include a spinal motor neuron (MN) cluster. List of genes for each cluster is included in Table S1.
(D) Boxplots for neurotransmitter-related genes enriched in each of the single cell clusters (*VGAT* also called *SLC32A1*, *GLYT2* also called *SLC6A5*, *CHT1* also called *SLC5A7*, *VACHT* also called *SLC18A3*, *VGLUT2* also called *SLC17A6*).
(E) Distribution of cells in conditions hSpS and hSpS (– DAPT) in *t*-SNE plot. R^2 value shows the correlation between the two hSpS conditions (R^2 = 0.85, p < 0.0001).
(F) Proportion of cells per condition in each of the ten clusters (χ^2 test, hSpS versus hSpS (–DAPT); p < 0.0001).
(G) Gene expression analysis of neuronal cell type markers in hSpS at day 30 of *in vitro* differentiation in 4 separate differentiations that were either pooled (left) or separated (right; n = samples from 5 hiPS cell lines; two-tailed t test: ****p = < 0.0001 for *HB9*, ***p = 0.0003 for *EVX1*; Mann-Whitney test: **p = 0.0014 for *SIM1*, ****p < 0.0001 for *ISL1*, ****p = < 0.0001 for *GATA3*, **p = 0.0045 for *CHX10*, ****p = < 0.0001 for *EN1*).
(H) Gene expression analysis of neurotransmitter identity markers in hSpS at day 30 of *in vitro* differentiation in 4 separate differentiations that were either pooled (left) or separated (right; n = samples from 5 hiPS cell lines; two-tailed t test: ***p < 0.0002 for *CHAT*, p = 0.7 for *VGLUT2*; Mann-Whitney test: p = 0.01 for *GAD1*, ***p = 0.0001 for *GLYT2*).
(I) Gene expression analysis of glial progenitor markers in hSpS at day 30 of *in vitro* differentiation in 4 separate differentiations that were either pooled (left) or separated (right; n = samples from 5 hiPS cell lines; Mann-Whitney test: ****p < 0.0001 for FABP7, p = 0.1 for *SOX10*).
(J) Immunocytochemistry in hSpS and hSpS (– DAPT) cryo-sections at day 45 of *in vitro* differentiation showing expression of different neuronal domain marker genes.
(K) Immunocytochemistry in hSpS and hSpS (– DAPT) cryo-sections at day 45 of *in vitro* differentiation showing expression of different neuronal and neurotransmitter identity markers.
Data represent mean ± s.e.m. Scale bars, 50 μm (J), 200 μm (K).

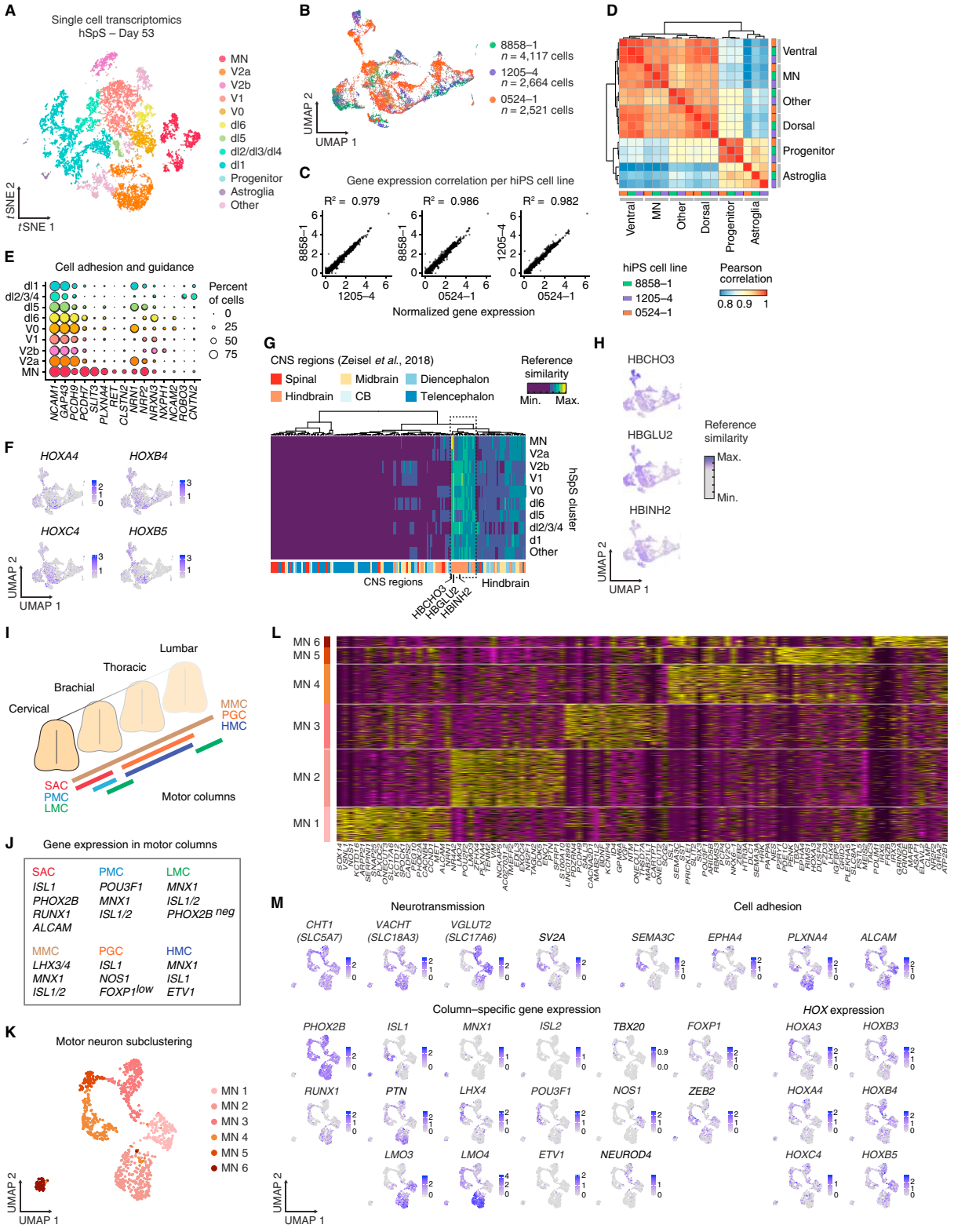

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Figure S3. Single-Cell Gene Expression of hSpS at Day 53 of *In Vitro* Differentiation, Related to Figure 1

(A) *t*-SNE visualization of single cell gene expression of hSpS at day 53 of *in vitro* differentiation (n = 9,302 cells; 10x Genomics) showing 12 main clusters that include a spinal motor neuron (MN) cluster. See Figure 1F for visualization using UMAP.

(B) UMAP showing cells in hSpS colored by the hiPS cell line they were derived from.

(C) Plots showing the Pearson correlation of the normalized average gene expression, log (counts per 10,000) between each of the three hiPS cell lines used for this experiment. R^2 shows the Pearson correlation value for each.

(D) Heatmap showing the Pearson correlation of the ventral, dorsal, progenitor, astroglia and other domains between hiPS cell lines used for this experiment. Hierarchical clustering of sample correlations is displayed with a dendogram.

(E) Dot plot showing expression of cell adhesion and neuronal guidance genes in each of the neuronal clusters in hSpS. The size of the circle represents the percent of cells expressing each gene per cluster.

(F) UMAP plots showing average gene expression of selected HOX genes.

(G) Hierarchical clustering showing RSS analysis of hSpS clusters to single-cell RNA-seq clusters from adult mouse central nervous system (CNS) regions in Zeisel et al. (2018). CB, cerebellum; HBCHOL3, hindbrain cholinergic neuron, HBGLU2: hindbrain excitatory neuron; HBINH2, hindbrain inhibitory neuron.

(H) hSpS UMAP plots colored by RSS to selected single cell RNaseq clusters from adult mouse CNS from Zeisel et al. (2018).

(I and J) Schematic illustrating hindbrain/spinal cord motor columns (I) and their corresponding marker gene expression (J). SAC, spinal accessory column; PMC, phrenic motor column; LMC, lateral motor column; MMC, medial motor column; PGC, preganglionic column; HMC, hypaxial motor column. Figure adapted from Dasen and Jessell (2009) and Stifani (2014).

(K) UMAP plot showing sub-clustering of the motor neuron cluster.

(L) Heatmap showing the top 20 differentially expressed genes in each motor neuron cluster (MN 1–6).

(M) UMAP plots showing average gene expression of selected markers, including neurotransmission-related genes, column-specific genes, cell adhesion genes and HOX genes.

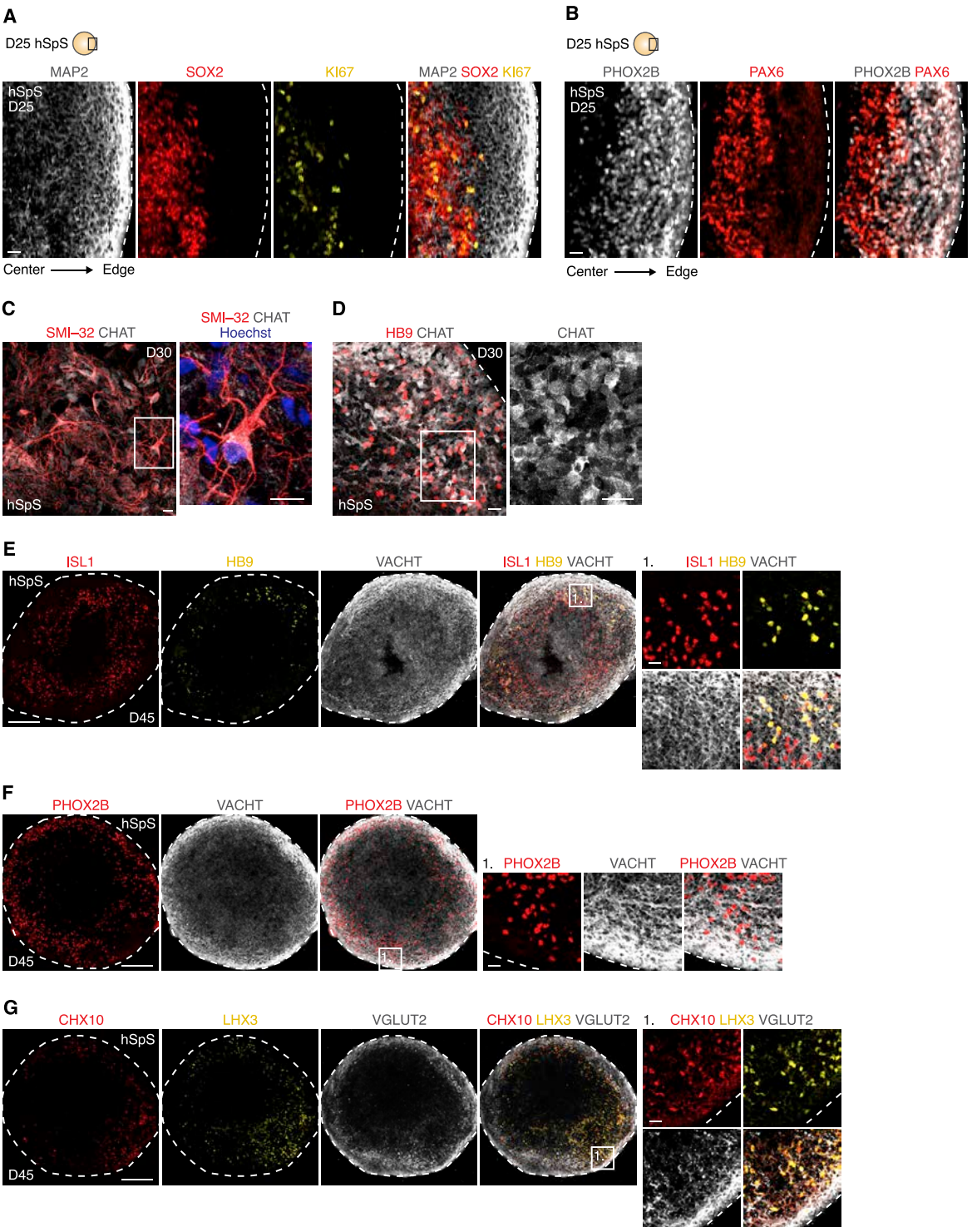

Figure S4. Characterization of Neuronal Domains in hSpS, Related to Figure 2

(A) Representative immunohistochemistry images in cryosections of day 25 hSpS showing expression of post-mitotic neurons expressing MAP2 at the edge of hSpS and proliferating progenitors expressing SOX2 and KI67 in the center.

(B) Representative immunohistochemistry images in cryosections of day 25 hSpS showing expression of post-mitotic motor neurons expressing PHOX2B at the edge of hSpS and medial-dorsal progenitors expressing PAX6 in the center.

(legend continued on next page)

(C and D) Immunocytochemistry in cryosections of hSpS showing motor neuron markers at day 30 of *in vitro* differentiation.
(E and F) Representative immunohistochemistry images in cryosections of day 45 hSpS showing expression of motor neuron markers ISL1, HB9 (F) and PHOX2B (G).
(G) Representative immunohistochemistry images in cryosections of day 45 hSpS showing localization of spinal neuronal domain V2a represented by CHX10 and LHX3 glutamatergic cells.
Scale bars, 10 μm (C), 20 μm (A), (B), (D), insets in (E), (F), (G), 200 μm (E), (F), (G).

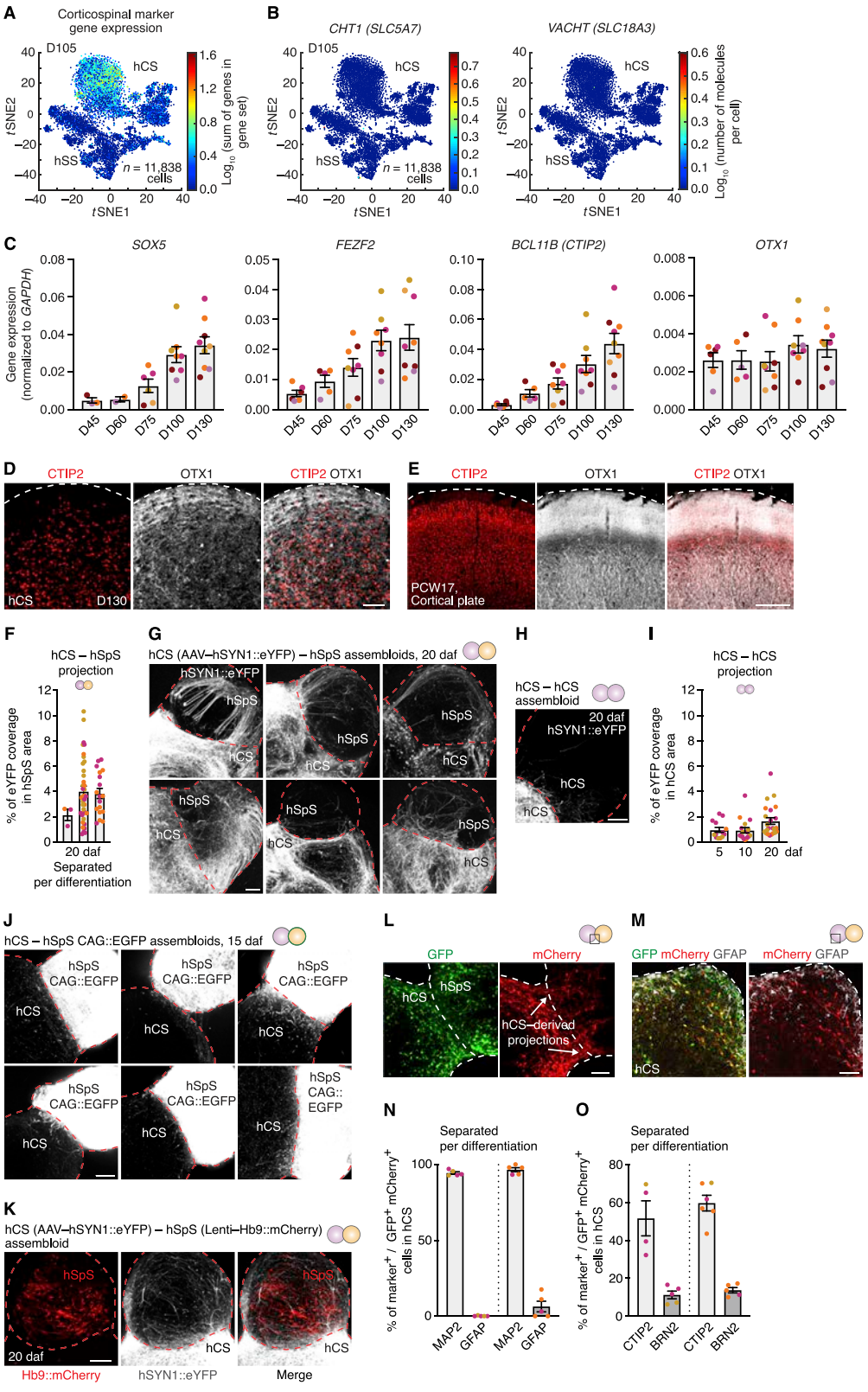

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Figure S5. Characterization of Cortico-Spinal Neurons in hCS and Cortico-Spinal Projections in hCS-SpS Assembloids, Related to Figure 3

(A) *t*-SNE visualization of single cell gene expression of hCS and hSS (human subpallial spheroids) at day 105 of differentiation (n = 11,838 cells; BD™ Resolve system, from Birey et al. [2017]) showing the distribution of expression of a set of genes associated with cortico-spinal neuronal identity (*SOX5*, *FEZF2*, *BCL11B* or *CTIP2*, *BHLHE22*, *LDB2*, *CRIM1* and *OTX1*).

(B) *t*-SNE visualization of single cell gene expression of day 105 hCS and hSS showing expression of *CHT1* (also called *SLC5A7*) and *VACHT* (also called *SLC18A3*).

(C) Gene expression analysis of cortico-spinal-related genes in hCS over time (n = 4-6 hiPS cell lines from 1–2 differentiations; one-way ANOVA interaction: p = 0.001 for *FEZF2*, p < 0.0001 for *BCL11B*, p = 0.5 for *OTX1*; Kruskal-Wallis test interaction: p = 0.002 for *SOX5*).

(D and E) Immunocytochemistry in hCS cryosections at day 130 of *in vitro* differentiation showing expression of cortico-spinal markers CTIP2 and OTX1 (D). Antibodies were validated in slices of human cortical tissue at post-conception week 17 (PCW17, E).

(F) Quantification of hCS-derived eYFP fluorescence coverage in hSpS areas in hCS-hSpS assembloids (at 20 daf) with samples separated by differentiation (n = 3 hiPS cell lines; one-way ANOVA interaction: p = 0.3). Figure 3D shows data combined.

(G) Images of intact hCS-hSpS assembloids 20 days after fusion (daf) showing hCS-derived hSYN1::eYFP projections.

(H and I) Representative image of intact hCS-hCS assembloid (H) and quantification of the extent of projection of hSYN1::eYFP (I) (n = 3 hiPS cell lines from 1–2 differentiations; Kruskal-Wallis test interaction p = 0.01, with Dunn's multiple comparison test: p > 0.9 for 10 versus 5 daf, p = 0.06 for 20 versus 5 daf). See Figure 3D for quantification of the projection in hCS-hSpS assembloids.

(J) Representative images of intact hCS-hSpS$^{CAG::EGFP}$ assembloids showing some reciprocal projections from hSpS to hCS and limited cell migration.

(K) Representative image of intact hCS-hSpS assembloid where hCS was infected with AAV-hSYN1::eYFP and hSpS with lenti-Hb9::mCherry. No hSpS-derived Hb9::mCherry projections are observed in hCS (similar results were observed in 15 assembloids from 2 differentiations and 3 hiPS cell lines).

(L) Representative immunocytochemistry image of hCS-hSpS assembloid in retrograde viral tracing experiment at 31 daf showing GFP expression in hSpS and colocalization of GFP and mCherry in hCS. mCherry projections from hCS to hSpS can also be seen.

(M) Representative immunocytochemistry image of GFP, mCherry and the glial marker GFAP on the hCS side of hCS-hSpS assembloid at 31 daf.

(N and O) Quantification of the percentage (%) of GFP$^+$ and mCherry$^+$ cells on the hCS side of hCS-hSpS assembloids that co-express either the neuronal marker MAP2 or the glial marker GFAP (O) or the cortical layer-specific markers CTIP2 or BRN2 (N) separated by differentiation (n = 10 assembloids from 3 hiPS cell lines, with 2–3 cryosections quantified per assembloid). Figures 3J and 3M show quantification with both differentiations combined.

Data represent mean ± s.e.m. Scale bars, 50 μm (D), 100 μm (L), (M), 200 μm (G), (H), (J), (K), 500 μm (E).

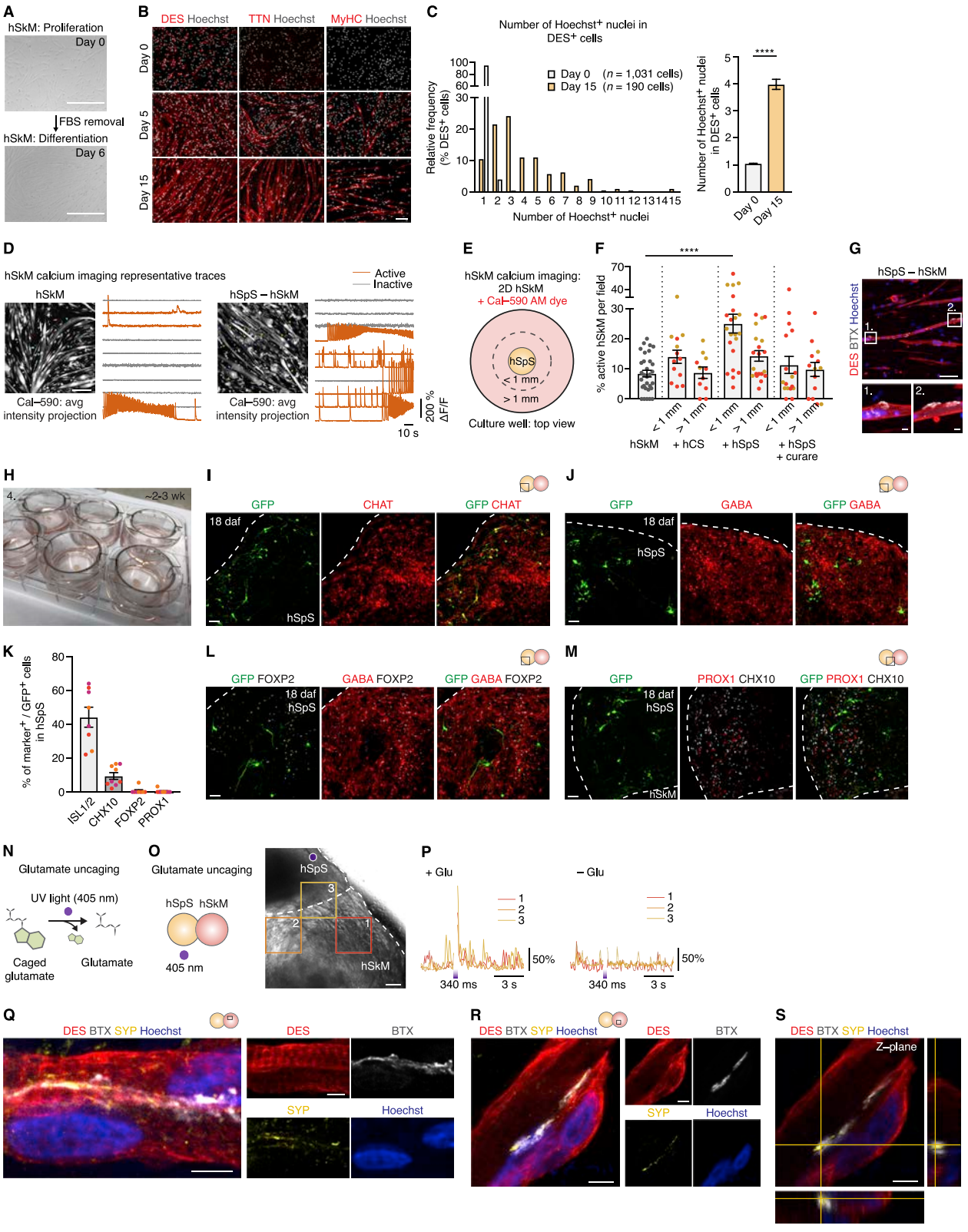

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Figure S6. Characterization of hSkM and hSpS-hSkM Assembloids, Related to Figure 5

(A and B) Characterization of human skeletal myoblast (hSkM) differentiation. hSkM differentiate upon removal of FBS from the culture medium (A). Immunocytochemistry images for desmin (DES), titin (TTN) and the heavy myosin chain (MyHC) show differentiation of myoblasts over time (B).

(C) Distribution and mean number of Hoechst^{+} nuclei in desmin cells at day 0 and day 15 of hSkM differentiation. Data represent mean ± s.e.m. (n = 1,031 desmin^{+} cells at day 0, and n = 190 desmin^{+} cells at day 15; data was collected in each case from 5 fields within a culture well in 2 hSkM differentiation replicates; Mann-Whitney test: ****p < 0.0001).

(D) Representative calcium imaging for hSkM and hSpS-hSkM. Images show average (avg) intensity projections of Cal-590 AM in a field (average intensity projections were used to delineate cells for quantification). Traces show examples of active and inactive hSkM cells in either hSkM alone or hSpS-hSkM cultures. ΔF/F indicates the fluorescence intensity over baseline fluorescence. Arrows show cells analyzed.

(E) Schematic detailing hSkM analyzed for calcium imaging experiment. Spontaneous calcium activity was recorded in 6-9 fields per culture well. Of these fields some were within 1 mm of the spheroid, and some were further than 1 mm from the spheroid.

(F) Quantification of spontaneous calcium imaging activity in hSkM over a period of 2 minutes. Fields were separated according to their distance from the spheroid (hCS or hSpS). Data represent mean ± s.e.m. (n = 2 hiPS cell lines from 1–2 differentiations; one-way ANOVA interaction: p = < 0.0001, with Dunnett's multiple comparison test ****p < 0.0001).

(G) Representative immunocytochemistry image showing BTX binding in hSkM that had been co-cultured with hSpS.

(H) Step number 4 in the generation and assembly of hSpS-3D hSkM. After 2-3 weeks in differentiation medium, 3D hSkM can be co-cultured with hSpS and/or hCS. For this, 3D hSkM and spheroids are placed on insert wells with 2 mL of medium per well.

(I and J) Representative immunohistochemistry images in cryosections for rabies-derived GFP and the cholinergic markers CHAT (I) and GABA (J) on the hSpS side of hSpS-hSkM assembloids at 18 daf.

(K) Quantification of the percentage (%) of GFP positive ($^{+}$) cells on the hSpS side of hSpS-hSkM assembloids that co-express either ISL1/2, CHX10, FOXP2 or PROX1 (n = 8 to 9 assembloids derived from 3 hiPS cell lines and 1 differentiation, with 3–6 cryosections quantified per assembloid).

(L and M) Representative immunohistochemistry images in cryosections for rabies-derived GFP and either GABA and FOXP2 (L) or PROX1 and CHX10 (M) on the hSpS side of hSpS-hSkM assembloids at 18 daf.

(N) Schematic illustrating the glutamate uncaging approach. Caged glutamate is added to the culture medium and uncaged upon UV light stimulation (405 nm).

(O and P) Glutamate uncaging of hSpS in hSpS-hSkM assembloid (H). Displacement normalized to baseline over time is shown for 3 subfields in the presence (I) or absence (J) of caged glutamate in the medium.

(Q and R) Representative immunohistochemistry in cryo-sections of hSpS-hSkM assembloids 31 days after fusion showing desmin (DES), BTX, synaptophysin 1 (SYP) and Hoechst.

(S) Single Z-plane and orthogonal views of projection in (R) showing YZ (right) and XZ (bottom) planes of stack and indicating apposition of BTX and SYP. Yellow lines indicate the point in the stack being visualized.

Data represent mean ± s.e.m. Scale bars, 10 μm (Q), (R), (S), insets in (G), 50 μm (I), (J), (L), (M), 100 μm (G), 200 μm (B), (D), (O), 400 μm (A).

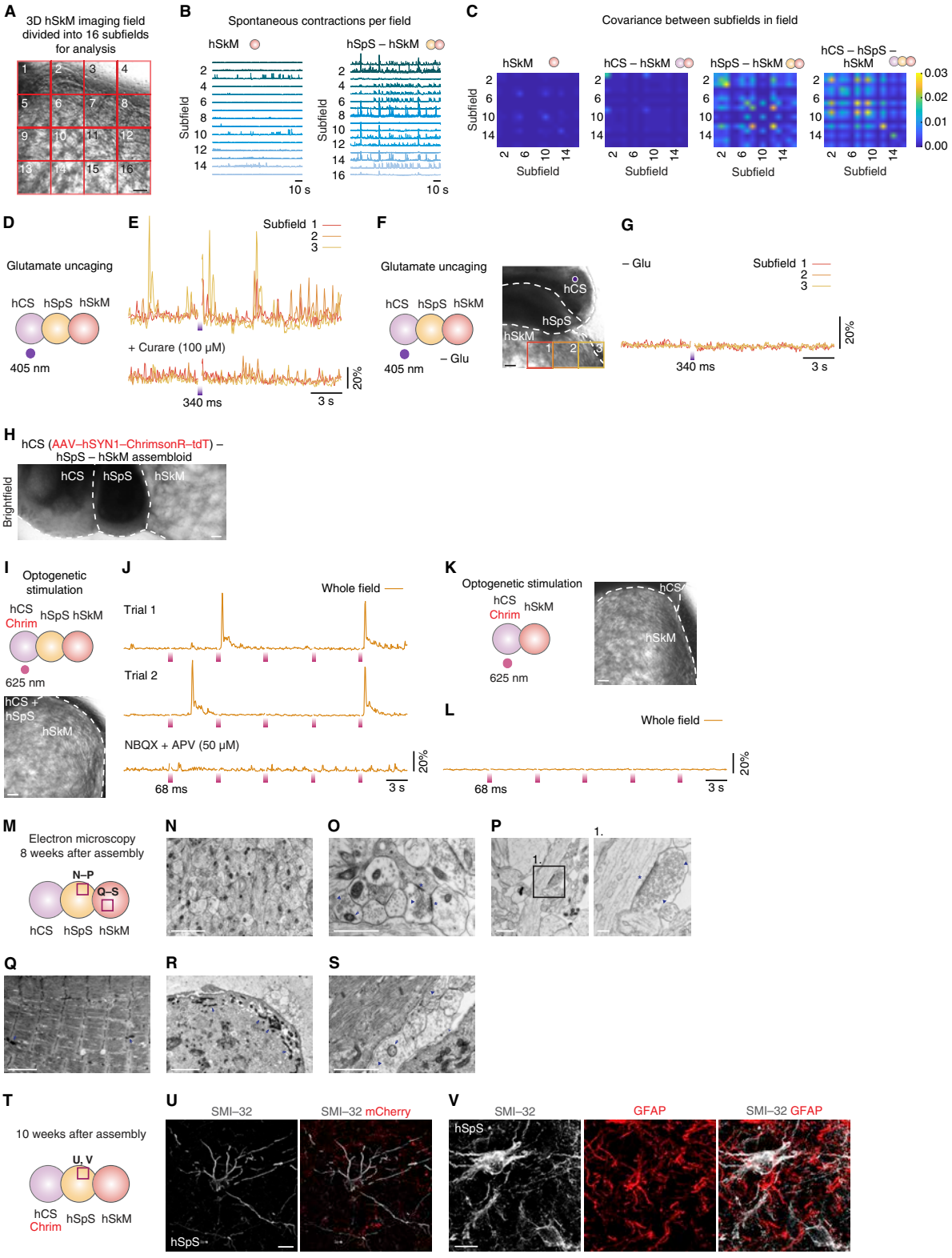

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Figure S7. Characterization of hCS-hSpS-hSkM Assembloids, Related to Figures 6 and 7

(A) Imaging fields (1.8 mm x 1.8 mm in size) are divided into 16 subfields for analysis. Only subfields containing hSkM are analyzed.

(B and C) Representative spontaneous contraction traces in subfields of hSkM or hSpS-hSkM assembloids (B). The correlation of displacements between subfields in a field is quantified using covariance analysis. Heatmaps show representative examples of covariance between subfields in a field of hSkM, hCS-hSkM, hSpS-hSkM or hCS-hSpS-hSkM assembloids (C). Video S4 shows live imaging of spontaneous contractions in an hCS-hSpS-hSkM assembloid.

(D and E) Glutamate uncaging in hCS-hSpS-hSkM assembloids. UV light (405 nm) uncages glutamate specifically on hCS (D). Displacement normalized to baseline over time is shown for 3 subfields without or with addition of curare (100 μM, E).

(F and G) Glutamate uncaging of hCS in hCS-hSpS-hSkM assembloid. Displacement normalized to baseline over time is shown for 3 subfields in the absence of caged glutamate in the medium (see Figures 6F and 6G for glutamate uncaging in this field in the presence of caged glutamate, and see Table S1 for details of all stimulation experiments).

(H) Representative brightfield image showing an intact hCS-hSpS-hSkM assembloid. Figure 6K shows AAV-hSYN1-ChrimsonR-tdT in hCS.

(I and J) Representative example of optogenetic stimulation in hCS-hSpS-hSkM assembloids (five consecutive 68 ms pulses). hCS were infected with AAV-hSYN1-ChrimsonR-tdT (Chrim). Traces of displacement in the whole field in (I) are shown normalized to the pre-stimulation baseline. Two trials for the same assembloid are shown. Addition of NBQX (50 μM) and APV (50 μM) abolished muscle contraction upon light-induced hCS stimulation (J).

(K and L) Optogenetic stimulation in hCS-hSkM assembloid (five consecutive 68 ms pulses). hCS were infected with AAV-hSYN1-ChrimsonR-tdT (K). Displacement over time normalized to pre-stimulation baseline is shown for the whole field in (L). Similar results were obtained in n = 5 hCS-hSkM assembloids derived from 2 hiPS cell lines.

(M) Schematic detailing location of electron microscopy images within hCS-hSpS-hSkM assembloids.

(N–P) Representative images showing axons and synapses in hCS-hSpS-hSkM assembloids with synaptic vesicles (arrow heads), post-synaptic densities (asterisks) and mitochondria (arrows). Inset in (P) shows an axo-axonic synapse.

(Q) Representative images within hSkM in hCS-hSpS-hSkM assembloids showing skeletal muscle fibers with actin and myosin filaments and mitochondria (arrows).

(R and S) Representative images within hSkM in hCS-hSpS-hSkM assembloids showing points of contact between hSpS neurons and hSkM. Neuron terminals are vesicle-laden (arrow heads) and present mitochondria (arrows). hSkM are surrounded by a basal lamina (hollow arrowhead).

(T) Schematic detailing location of images within $hCS^{AAV\text{-}Chrim}$-hSpS-hSkM assembloids.

(U and V) Representative immunohistochemistry images in cryosections of $hCS^{AAV\text{-}Chrim}$-hSpS-hSkM assembloids showing the presence of SMI-32^{+} motor neurons in hSpS with hCS-derived mCherry-positive projections (U) and astrocytes (V).

Scale bars, 200 nm (inset in P), 1 μm (O), (P), (S), 2 μm (N), (Q), (R), 20 μm (U), (V), 200 μm (A), (F), (H), (I), (K).

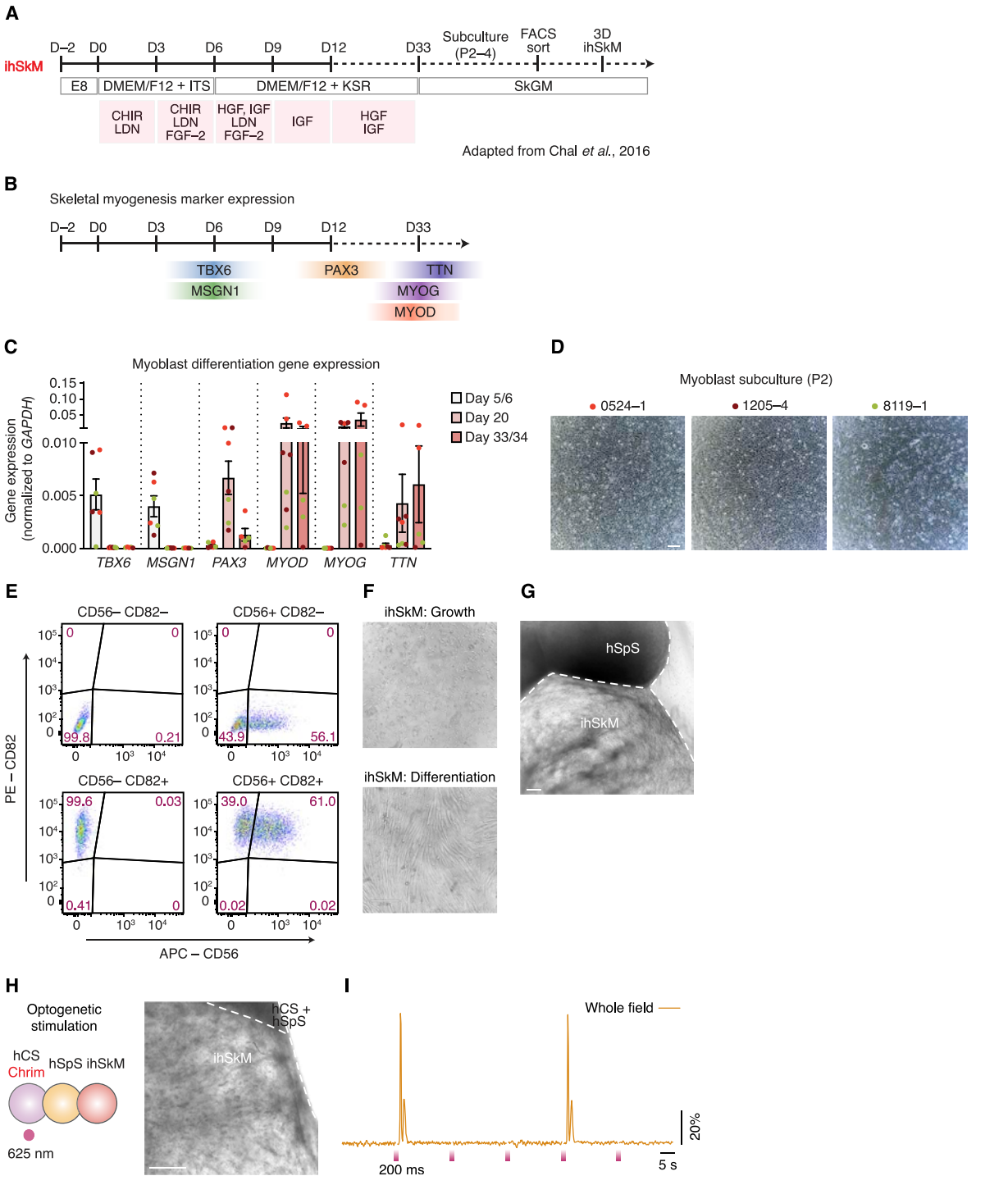

Figure S8. Generation and Characterization of hiPS Cell-Derived Skeletal Myoblasts (ihSkM), Related to Figure 6

(A) Schematic detailing the differentiation condition used for generating hiPS cell-derived skeletal myoblasts. Protocol adapted from Chal et al. (2016).

(B) Schematic indicating marker genes expressed during the *in vitro* generation of myoblasts from hiPS cells.

(C) Gene expression analysis of skeletal myogenesis markers at days 5/6, 20 and 33/34 of *in vitro* myoblast differentiation (n = 3 hiPS cells lines from 2–3 differentiations).

(D) Representative images of myoblasts derived from 3 hiPS cell lines 2 to 4 days after subculture at day 33 of *in vitro* differentiation.

(E) FACS plots showing purification of the $CD56^{+}$ / $CD82^{+}$ myoblast population.

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(F) Representative images of $CD56^+$ / $CD82^+$ myoblasts under either growth conditions or differentiation conditions.
(G) Representative image of an hSpS-3D ihSkM assembloid.
(H and I) Optogenetic stimulation in hCS-hSpS-ihSkM assembloids. hCS were infected with AAV-hSYN1-ChrimsonR-tdT (Chrim) before assembly. Five consecutive pulses of light (625 nm, 200 ms in duration each and 20 s apart) were delivered using a fiber-coupled LED directed toward the hCS (H). Traces of whole-field muscle displacement are shown after normalization to the pre-stimulation baseline.
Scale bars, 200 μm (D), (G), (H), 210 μm (F).