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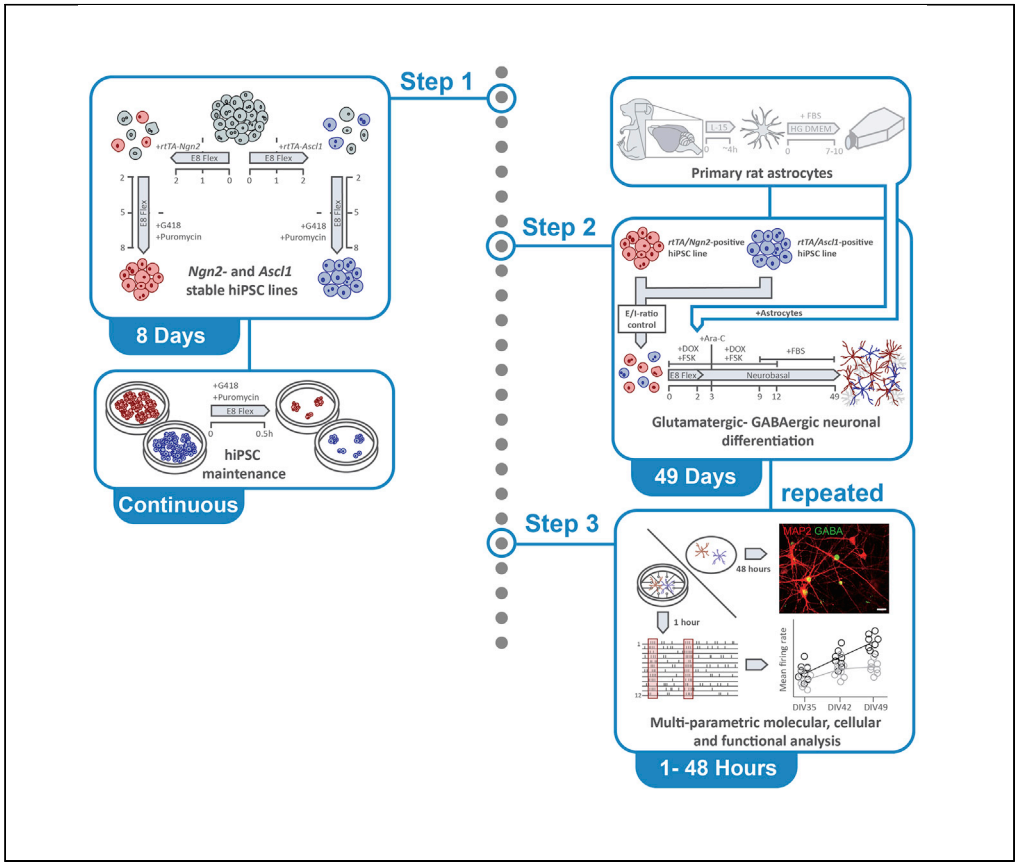
Protocol

Generation of glutamatergic/GABAergic neuronal co-cultures derived from human induced pluripotent stem cells for characterizing E/I balance *in vitro*



Obtaining mechanistic insights into the disruptions of neuronal excitation and inhibition (E/I) balance in brain disorders has remained challenging. Here, we present a protocol for *in vitro* characterization of E/I balance. Using human induced pluripotent stem cells, we describe the generation of glutamatergic excitatory/GABAergic inhibitory neuronal co-cultures at defined ratios, followed by analyzing E/I network properties using immunocytochemistry and multi-electrode array recording. This approach allows for studying cell type-specific contribution of disease genes to E/I balance in human neurons.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Generating human iPSC-derived glutamatergic/GABAergic (E/I) neuronal networks *in vitro*

E/I neuronal networks that develop functionally mature inhibitory GABAergic signaling

Creating networks with definable ratios of glutamatergic and GABAergic neurons

Molecular and functional quantitative analysis of E/I neuronal network development

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Generation of glutamatergic/GABAergic neuronal co-cultures derived from human induced pluripotent stem cells for characterizing E/I balance *in vitro*

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SUMMARY

Obtaining mechanistic insights into the disruptions of neuronal excitation and inhibition (E/I) balance in brain disorders has remained challenging. Here, we present a protocol for *in vitro* characterization of E/I balance. Using human induced pluripotent stem cells, we describe the generation of glutamatergic excitatory/GABAergic inhibitory neuronal co-cultures at defined ratios, followed by analyzing E/I network properties using immunocytochemistry and multi-electrode array recording. This approach allows for studying cell-type-specific contribution of disease genes to E/I balance in human neurons.

For complete details on the use and execution of this protocol, please refer to Mossink et al. (2022)[1] and Wang et al. (2022).[2]

BEFORE YOU BEGIN

Generating *rtTA/Ngn2*- and *rtTA/Ascl1*-positive hiPSCs

Timing: two to three weeks

Before establishing co-cultures of excitatory glutamatergic (E) and inhibitory GABAergic (I) neurons (E/I co-cultures), one needs to generate the relevant lines of *rtTA/Ngn2*- and *rtTA/Ascl1*-positive human induced pluripotent stem cells (hiPSCs). For a detailed protocol for the generation of *rtTA/Ngn2*-positive hiPSCs, please refer to Frega et al.[3] In order to generate *rtTA/Ascl1*-positive hiPSCs, the same procedure can be used as for *rtTA/Ngn2*-positive hiPSCs, when *Ngn2* lentivirus is replaced by *Ascl1* lentivirus.[1]

Maintenance of *rtTA/Ngn2*- and *rtTA/Ascl1*-positive hiPSCs

Timing: Variable duration depends on experimental plan

hiPSCs are cultured on a 6-well plate precoated with 1:15 Matrigel (diluted in DMEM/F-12 medium) in Essential 8 (E8) Flex medium supplemented with primocin, puromycin and G418 at 37°C and 5% CO_2. Medium needs to be refreshed every 2–3 days. When the confluency of hiPSCs reaches 70%–80%, they should be passaged with ReLeSR following the procedure described below.

1. Prepare a Matrigel-coated 6-well plate.
   a. Thaw Matrigel on ice.

   ⚠ CRITICAL: Once it is liquid, use a pre-chilled pipette tip (4°C) to take 1 mL Matrigel and dilute it into 14 mL cold DMEM/F-12 medium in a 50 mL falcon tube.

   b. Coat several wells of a 6-well plate with 1 mL/well Matrigel dilution. Incubate at room temperature (RT) for ≥ 1 h or at 4°C overnight.

   Note: Matrigel-coated plates, sealed with parafilm, can be stored at 4°C for maximum 1 week. RT in our lab is around 20°C.

2. Prewarm E8 Flex medium and DPBS (without Ca^{2+} and Mg^{2+}) to RT.
3. Aspirate the Matrigel dilution from the precoated plate and add 2 mL hiPSC maintenance medium to each well (prepare hiPSC maintenance medium according to the table in the "materials and equipment" section).
4. Check the confluency of hiPSCs under the microscope and decide which well will be passaged (confluency >70%).
5. Remove medium from the well completely.
6. Rinse the well once with 2 mL DPBS (without Ca^{2+} and Mg^{2+}) and aspirate.
7. Add 500 µL ReLeSR to the well and remove it after within 1 min, so that colonies are exposed to a thin film of liquid.
8. Incubate for 2–5 min at RT.

   Note: Optimal dissociation time may vary depending on the cell line used. When passaging a cell line with ReLeSR for the first time, the optimal dissociation time should be determined. Incubation can be terminated when clear (empty) space can be detected in each clone of the hiPSC culture.

9. Lightly tap the plate to loosen the cells.
10. Add 2 mL E8 Flex medium using a 1,000 µL pipette and loosen the cells from the well by gently pipetting up and down (try to avoid formation of air bubbles).

    Note: Do not pipette up and down more than 5 times, as this may break cell aggregates into a single-cell suspension.

11. Add the appropriate volume of cell suspension (i.e., depending on the required cell density. Split ratio should be between 1:6 and 1:30.) to the wells in the new plate and move the plate in a left-to-right and top-to-bottom motion to distribute the cells evenly over the surface of the well.

    Note: Appropriate split ratio may vary depending on the cell line used and on the intended experiment. Higher split ratio is needed if the hiPSC line grow slow or cells are needed for further experiment shortly.

12. Incubate the cells at 37°C and 5% CO_2.

Astrocytes isolation and culture

Timing: Variable duration depends on experimental plan

To promote synaptogenesis and the maturation of the *in vitro* neuronal network, cortical astrocytes from embryonic (E18) rats need to be prepared for addition to the culture during differentiation. For a detailed protocol of the isolation and culture of rat cortical astrocytes, please refer to Frega et al.[3]

Institutional permissions

Animal experiments were conducted in conformity with the Animal Care Committee of the Radboud University Nijmegen Medical Center, the Netherlands, under DEC application number 2015–0038, and conform to the guidelines of the Dutch Council for Animal Care and the European Communities Council Directive 2010/63/EU.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit anti-MAP2 (1:1000)	Abcam	Ab32454; RRID: AB_776174
Rabbit anti-VGAT (1:500)	Synaptic Systems	131013; RRID: AB_2189938
Rabbit anti-GABA (1:500)	Sigma-Aldrich	A0310; RRID: AB_476667
Mouse anti-Gephyrin (1:1000)	Synaptic Systems	147011; RRID: AB_887717
Guinea pig anti-Synapsin $^{1}/_{2}$ (1:1000)	Synaptic Systems	106004; RRID: AB_1106784
Mouse anti-Homer1 (1:1000)	Synaptic Systems	160011; RRID: AB_2120992
Goat-anti rabbit Alexa 568 (1:1000)	Thermo Fisher Scientific	A11036; RRID: AB_10563566
Goat-anti-mouse Alexa 488 (1:1000)	Thermo Fisher Scientific	A11029; RRID: AB_2534088
Goat anti-guinea pig Alexa Fluor 568 (1:2000)	Thermo Fisher Scientific	A11075; RRID: AB_2534119
Goat anti-guinea pig Alexa Fluor 647 (1:1000)	Thermo Fisher Scientific	A21450; RRID: AB_141882
Recombinant DNA		
pLVX-EF1α-(Tet-On-Advanced)-IRES-G418(R) lentiviral vector	Frega et al.[3] Mossink et al.[1]	N/A
pLV[TetOn]-Puro-TRE3G>mNeurog2 lentiviral vector	Frega et al.[3] Mossink et al.[1]	N/A
pLV[TetOn]-Puro-TRE3G>mAscl1 lentiviral vector	Mossink et al.[1]	N/A
Chemicals, peptides, and recombinant proteins		
Accutase	Sigma	Cat# A6964-100ML
B-27 (50×), serum free	Thermo Fisher Scientific	Cat# 17504001
Boric acid	Sigma	Cat# B9645
Cytosine β-D-arabinofuranoside hydrochloride (Ara-C)	Sigma	Cat# C6645
DMEM/F-12	Thermo Fisher Scientific	Cat# 11320074
DMEM, High Glucose, GlutaMAX	Thermo Fisher Scientific	Cat# 31966021
Doxycycline	Sigma	Cat# D9891-5G
DPBS, No calcium, No magnesium	Thermo Fisher Scientific	Cat# 14190
DPBS, Calcium, Magnesium	Thermo Fisher Scientific	Cat# 14040
DAKO fluorescent mounting medium	DAKO	Cat# S3023
Essential 8 FLEX medium kit	Thermo Fisher Scientific	Cat# A2858501
Fetal Bovine Serum (FBS)	Sigma	Cat# F2442-500ML
Forskolin	Sigma	Cat# F6886-10MG
GlutaMAX supplement	Thermo Fisher Scientific	Cat# 35050038
G418	Sigma-Aldrich	Cat# G8168
Human Recombinant laminin-521	Biolamina	Cat# LN521-05
Hoechst 33342	Thermo Fisher Scientific	Cat# H3570
Mouse laminin	Sigma	Cat# L2020
MEM non-essential amino acid solution (NEAA)	Sigma	Cat# M7145-100ML

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Matrigel	Corning	Cat# 356231
N-2 Supplement (100×)	Thermo Fisher Scientific	Cat# 17502048
Normal goat serum	Thermo Fisher Scientific	Cat# 10000C
Neurobasal	Thermo Fisher Scientific	Cat# 21103049
Nitric acid	Sigma-Aldrich	Cat# 84380
Penicillin/Streptomycin	Sigma	Cat# P4333-100ML
Poly-L-Ornithine	Sigma	Cat# P3655-100MG
Primocin	Thermo Fisher Scientific	Cat# ant-pm-2
Puromycin	Sigma	Cat# P9620
PBS	Sigma	Cat# P5493
Picrotoxin	Tocris	Cat# 1128
Recombinant Human BDNF	PeproTech	Cat# 450-02_10ug
Recombinant Human Neurotrophin 3 (NT3)	Promocell - PromoKine	Cat# C-66425
Revitacell supplement (100×)	Thermo Fisher Scientific	Cat# A2644501
ReLeSR	Stem Cell Technologies	Cat# 05872
Sodium tetraborate	Sigma	Cat# 221732-100G
Sodium chloride	Sigma	Cat# S5886-1KG
Trypsin-EDTA (0.05%), phenol Red	Thermo Fisher Scientific	Cat# 25300054
Triton X-100	Sigma	Cat# 9002-93-1
Experimental models: Organisms/strains		
Wistar WT Rat (Dissociated astrocytes)	Charles River	N/A
Experimental models: Cell lines		
CTR-WTC (Derived from a male, Japanese, 30 years old) (Maximum passage number: 40)	Coriell Institute	https://www.coriell.org/0/Sections/Search/Sample_Detail.aspx?Ref=GM25256&Product=CC
Software and algorithms		
GraphPad Prism	GraphPad software	https://www.graphpad.com/scientific-software/prism/ RRID: SCR_002798
Fiji	Schindelin et al.[4]	https://imagej.nih.gov/ij/download.html RRID: SCR_003070
MATLAB 2014b	Mathworks	RRID: SCR_001622
Multiwell Analyzer	Multichannel Systems, MCS GmbH, Reutlingen, Germany	N/A
Deposited data		
Code	Mossink et al.[5]	https://data.mendeley.com/datasets/bvt5swtc5h/1
Other		
24-well plate	Corning	Cat# 10048760
Coverslips	Thermo Fisher Scientific	Cat# 631-0713
MEA plate	Multichannel Systems	Cat# 24W700

MATERIALS AND EQUIPMENT

hiPSC maintenance medium		
Reagent	Final concentration	Amount
Essential 8 Flex medium	N/A	2 mL
Primocin	1:500	4 µL
Puromycin (1 mg/mL)	0.5 µg/mL	1 µL
G418 (50 mg/mL)	50 µg/mL	2 µL
Total	N/A	**2 mL/well**

Note: hiPSC maintenance medium should be stored at 4°C and can be kept for 1 week.

Note: E8 Flex medium is made by adding E8 Flex Supplement (10 mL) directly into the freshly opened bottle of E8 Flex Basel medium (500 mL). E8 Flex Supplement is stored at -20°C and should be thawed at RT before use.

⚠ CRITICAL: Do not warm the E8 Flex medium at 37°C and do not thaw the frozen E8 Flex Supplement at 37°C.

Borate buffer		
Reagent	Final concentration	Amount
Sodium tetraborate	0.0125 M	2.52 g
boric acid	0.05 M	3.10 g
Milli-Q	N/A	1 L
Total	**50 mM**	**1 L**

Note: pH needs to be adjusted to 8.4. The solution can be stored at 4°C for at least 6 months.

hiPSC plating medium		
Reagent	Final concentration	Amount
Essential 8 Flex medium	N/A	489 µL
Revitacell	1:100	5 µL
Doxycyclin (1 mg/mL)	4 µg/mL	2 µL
Forskolin (0.5 mg/mL)	10 µM	4.1 µL
Total	N/A	**500 µL/well**

Note: We suggest to make the hiPSC plating medium on the same day when plating cells. This medium can be kept at 4°C and warmed up to RT before use.

Note: Forskolin is a diterpenoid which is not soluble in water due to its polycyclic structure. The stock is diluted in 100% DMSO (5 mg/mL). When diluting the concentrated DMSO stock to water, the drug has to leave the solvent and enter the water at the interface. Sometimes, forskolin can precipitate out and crystalize. To avoid this, add the forskolin stock to a prepared DMEM/F-12 solution with DMSO (e.g., 850 µL DMEM/F-12 + 50 µL DMSO, then vortex, followed by adding 100 µL forskolin stock drop-by-drop). This is the working solution.

Neuronal differentiation medium_1		
Reagent	Final concentration	Amount
DMEM/F-12	N/A	482 µL
N-2	1:100	5 µL
NEAA	1:100	5 µL
NT (10 µg/mL)	10 ng/mL	0.5 µL
BDNF (10 µg/mL)	10 ng/mL	0.5 µL
Primocin	1:500	1 µL
Doxycycline (1 mg/mL)	4 µg/mL	2 µL
Forskolin (0.5 mg/mL)	10 µM	4.1 µL
Total	N/A	**500 µL/well**

Note: We suggest to make fresh "Neuronal differentiation medium_1" directly before use.

Astrocyte medium		
Reagent	Final concentration	Amount
High-glucose DMEM	N/A	4.25 mL
FBS	15% v/v	0.75 mL
Penicillin/streptomycin	1:1000	5 μL
Total	N/A	**5 mL**

Note: Astrocyte medium can be stored at 4°C for 2 weeks.

Neuronal differentiation medium_2		
Reagent	Final concentration	Amount
Neurobasal	N/A	476 μL
B27	1:50	10 μL
Glutamax	1:100	5 μL
Primocin	1:500	1 μL
NT3 (10 μg/mL)	10 ng/mL	0.5 μL
BDNF (10 μg/mL)	10 ng/mL	0.5 μL
Ara-C (2 mM)	2 μM	0.5 μL
Forskolin (0.5 mg/mL)	10 μM	4.1 μL
Doxycycline (1 mg/mL)	4 μg/mL	2 μL
Total	N/A	**500 μL/well**

Note: We suggest to make fresh "Neuronal differentiation medium_2" directly before use. However, Neurobasal medium supplemented with B27, Glutamax and Primocin can be made in a larger volume (e.g., 50 mL), then filtered, and can be stored at 4°C for 2 weeks.

Neuronal differentiation medium_3		
Reagent	Final concentration	Amount
Neurobasal	N/A	238 μL
B27	1:50	5 μL
Glutamax	1:100	2.5 μL
Primocin	1:500	0.5 μL
NT3 (10 μg/mL)	10 ng/mL	0.25 μL
BDNF (10 μg/mL)	10 ng/mL	0.25 μL
Forskolin (0.5 mg/mL)	10 μM	2.1 μL
Doxycycline (1 mg/mL)	4 μg/mL	1 μL
Total	N/A	**250 μL/well**

Note: We suggest to make fresh "Neuronal differentiation medium_3" directly before use. However, Neurobasal medium supplemented with B27, Glutamax and Primocin can be made in a larger volume (e.g., 50 mL), then filtered, and can be stored at 4°C for 2 weeks.

Neuronal differentiation medium_4		
Reagent	Final concentration	Amount
Neurobasal	N/A	232 μL
FBS	2.5% v/v	6.3

(*Continued on next page*)

Continued		
Reagent	Final concentration	Amount
B27	1:50	5 µL
Glutamax	1:100	2.5 µL
Primocin	1:500	0.5 µL
NT3 (10 µg/mL)	10 ng/mL	0.25 µL
BDNF (10 µg/mL)	10 ng/mL	0.25 µL
Forskolin (0.5 mg/mL)	10 µM	2.1 µL
Doxycycline (1 mg/mL)	4 µg/mL	1 µL
Total	N/A	**250 µL/well**

Note: We suggest to make fresh "Neuronal differentiation medium_4" directly before use. However, Neurobasal medium supplemented with B27, Glutamax and Primocin can be made in a larger volume (e.g., 50 mL), then filtered, and can be stored at 4°C for 2 weeks.

Neuronal differentiation medium_5		
Reagent	Final concentration	Amount
Neurobasal	N/A	235 µL
FBS	2.5% v/v	6.3
B27	1:50	5 µL
Glutamax	1:100	2.5 µL
Primocin	1:500	0.5 µL
NT3 (10 µg/mL)	10 ng/mL	0.25 µL
BDNF (10 µg/mL)	10 ng/mL	0.25 µL
Total	N/A	**250 µL/well**

Note: We suggest to make fresh "Neuronal differentiation medium_3" directly before use. However, Neurobasal medium supplemented with B27, Glutamax and Primocin can be made in a larger volume (e.g., 50 mL), then filtered, and can be stored at 4°C for 2 weeks.

STEP-BY-STEP METHOD DETAILS

Plating and maintenance of E/I co-culture

Timing: ≥ 7 weeks

The step-by-step approach describes how to differentiate and maintain E/I co-cultures consisting of 70% glutamatergic and 30% GABAergic neurons, derived from *rtTA/Ngn2*- or *rtTA/Ascl1*-positive hiPSC cultures respectively. The protocol includes 3 main parts:

Part 1: Coating plates (1 day).

Part 2: Plating the cells (1 day).

Part 3: Culture maintenance (> 47 days).

Coating plates

Day -1: Coat plates with poly-L-ornithine and laminin

Timing: 1 day

Table 1. Required volume per well of poly-L-ornithine dilution in filtered borate buffer

Culture dish	Required poly-L-ornithine dilution per well
24-well plate	400 µL
24-well plate with coverslips	800 µL
24-well MEA (Multichannel Systems)	150 µL

Before plating hiPSCs, the plate needs to be coated with poly-L-ornithine and laminin-521. The following section describes how to prepare the coating for culturing E/I co-cultures. Based on the research question or downstream analysis, certain types of cell culture formats can be chosen. hiPSCs can be plated in 24-well plates with coverslips. Coverslips are pretreated with nitric acid and autoclaved) for immunocytochemistry or single-cell patch clamp recording. Additionally, hiPSCs can be cultured in multi-electrode array (MEA) plates for measuring the neuronal network activity. The volumes of reagents described here are tailored to a 24-well plate and MEA plate.

1. Dilute poly-L-ornithine in filtered borate buffer (50 mM) to a final concentration of 50 µg/mL.
   a. Add poly-L-ornithine dilution in the corresponding volumes as presented in Table 1 depending on the culturing format.

   Note: The coverslips need to be pretreated in nitric acid at least overnight before being extensively washed by Milli-Q water for 20 times. For the washing step, the Milli-Q water should immerse the coverslip in a beaker. Gently shake the beaker each time for at least 1 min. Take the coverslips out of the water by using a tweezer, and distribute the coverslips evenly in a petri dish without overlapping before autoclaving. Coverslips are then ready to use.

   Note: The pore size of the Polyethersulfone filter used for sterilization is 0.22 µm.

2. Incubate at 37°C and 5% CO_2 for at least 3 h.
3. Thaw laminin-521 slowly at 4°C.
4. Aspirate the diluted poly-L-ornithine from the wells.
5. Wash the wells 3 times with 500 µL filtered Milli-Q water in each well.
6. Dilute laminin-521 (100 µg/mL) 20 times in DPBS$^{Ca2+/Mg2+}$ to a final concentration of 5 µg/mL. (concentration of Ca^{2+} and Mg^{2+} in DPBS$^{Ca2+/Mg2+}$ is 100 mg/L).
   a. Add laminin-521 dilution in the corresponding volumes as presented in Table 2 depending on the culturing format.
7. Incubate at 4°C overnight.

   Note: The coated plates with laminin-521 dilution can be stored at 2–8°C for up to 2–4 weeks when sealed and kept sterile.

Plating the cells

Day 0: Plating of single cells dissociated from rtTA/Ngn2- and rtTA/Ascl1-positive hiPSC lines

Timing: 1 day

Table 2. Required volume per well of laminin-521 dilution in DPBS$^{Ca2+/Mg2+}$

Culture dish	Required laminin-521 dilution per well
24-well plate	400 µL
24-well plate with coverslips	400 µL
24-well MEA (Multichannel Systems)	150 µL

To obtain the E/I neuronal networks (E/I plating ratio 70:30), hiPSC colonies need to be dissociated into single cells for plating. The following section describes how to plate *rtTA/Ngn2*- and *rtTA/Ascl1*-positive hiPSC lines for direct conversion to neurons. All following volumes are corresponding to culturing conditions of hiPSCs in a 6-well plate.

Note: When changing the E/I plating ratios to 85:15, 80:20 or 60:40, the number of glutamatergic neurons present in the culture should be kept at a similar density whereas the number of GABAergic neurons is altered. This allows to make sure that the baseline electrophysiological activity remains consistent.[1] We determined these ratios by combining the defined number of glutamatergic and GABAergic neurons for the plating at DIV0. However, during culturing a certain number of neurons do not survive or do not converse, the proportion of which is typically slightly higher for GABAergic neurons. To this end for each plating ratio at DIV0, we provide also the real E/I ratios after 49 days in culture (Figure 2B).

8. Calculate the volume of medium required: 500 µL per number of 24-well plate well or MEA well for culturing the cells and 2 mL per hiPSC line per 6-well plate well for resuspending the cells. Compose the hiPSC plating medium according to the table in the "materials and equipment" section and warm up to RT.
9. Prewarm 10 mL of DMEM/F-12 and 1 mL of Accutase per hiPSC line to RT.
10. Check the confluency of *rtTA/Ascl1*-positive hiPSCs under the microscope and decide which well will be dissociated.

Note: For plating of single cells, the confluency of hiPSCs should reach at least 70%. Troubleshooting 1.

11. Remove medium from the well completely.
12. Rinse the well once with 2 mL DPBS (without Ca^{2+} and Mg^{2+}) and aspirate.
13. Add 1 mL of Accutase to the *rtTA/Ascl1*-positive hiPSC line.
14. Incubate 3–5 min at 37°C and check under the microscope whether the cells are detaching.

Note: Incubation time may vary depending on the cell line used. When cells become rounded, the incubation can be terminated. Increase the incubation time in case the line is difficult to be detached.

15. Add 2 mL DMEM/F-12, and gently suspend the cells using a 1,000 µL pipette, then transfer the cell suspension to a 15 mL falcon tube.
16. Add 8 mL DMEM/F-12 to the cell suspension in the falcon tube.
17. Spin at 200 × *g* for 5 min at RT.
18. Aspirate the supernatant and resuspend the cell pellet in 2 mL of the hiPSC plating medium (i.e., supplemented with Revitacell, doxycycline, forskolin as shown in step 8). Resuspend the hiPSCs into single cells by putting the tip of the 1,000 µL pipette against the side of the 15 mL falcon tube and resuspend the cells gently.
19. Check under the microscope whether the cells are single cells. If not, continue resuspending the cells. If so, take a sample for counting. Troubleshooting 2.
20. Count the cells using a counting chamber.
21. Aspirate the diluted laminin of the precoated plate and add 500 µL of E8 Flex medium per 24-well plate well, and 150 µL per MEA well.

⚠ CRITICAL: Do not aspirate the laminin if the cells are not ready for plating.

⚠ CRITICAL: Prewarm the precoated plates to RT before use.

Note: Due to the special structure of the MEA plate (Multichannel Systems, 24W700), 150 μL culturing medium needs to be first added into the wells to make sure all the added cells can attach to the bottom.

22. Plate the dissociated cells in the prepared plates at a density of 8000 *rtTA/Ascl1*-positive hiPSCs per well. Troubleshooting 3.
23. Incubate the *rtTA/Ascl1*-positive cells for 1–2 h to allow the cells to attach to the bottom of the plate at 37°C and 5% CO_2.

Optional: In case separation of two neuronal cell types is needed, the *rtTA/Ascl1*-positive line can be transduced with AAV2-hSyn-mCherry (UNC Vector Core). After 4 h of virus incubation, cultures need to be washed twice with 500 μL DMEM/F-12 after which the *rtTA/Ngn2*-positive hiPSC line can be plated into the well.

24. After incubation of the *rtTA/Ascl1*-positive hiPSC line, repeat steps 10–20 for the *rtTA/Ngn2*-positive hiPSC line.
25. Plate 18,000 *rtTA/Ngn2*-positive hiPSCs per well. Troubleshooting 3.

Note: After adding the cells to the MEA plate, incubate at 37°C and 5% CO_2 for 1 h, then refill the well with fresh medium to reach 500 μL medium volume per well.

26. Incubate at 37°C and 5% CO_2.

Note: When cell type separation is not needed, you can also plate the *rtTA/Ascl1*-positive and *rtTA/Ngn2*-positive hiPSCs at the same time by making a cell suspension with two lines mixed and plate directly.

Culture maintenance

Day 1: Refresh medium

Timing: 1 day

To induce the proper differentiation of the hiPSCs, certain supplements are added into the medium. The following section describes the initial medium change after plating the *rtTA/Ngn2*- and *rtTA/Ascl1*-positive cell lines in a 24-well plate or MEA plate.

27. Calculate the volume of medium required: 500 μL per number of 24-well plate well or MEA well. Compose the "Neuronal differentiation medium_1" according to the table in the "materials and equipment" section.
28. Prewarm the medium to 37°C.
29. Filter the resulting medium and add mouse laminin to a final concentration of 0.2 μg/mL.
30. Aspirate the medium from the wells.
31. Add 500 μL of the prepared medium per well.
32. Incubate at 37°C and 5% CO_2.

⚠ CRITICAL: When aspirating the medium, be very gentle to avoid detaching the cells. When adding the medium, be very gentle to avoid touching the cells or electrodes.

Day 2: Add astrocytes to the culture

Timing: 1 day

To promote the neuronal synaptogenesis, rat astrocytes are added to the neuronal culture. The following section describes the procedure of adding primary astrocytes to the co-cultures of *rtTA/Ngn2*- and *rtTA/Ascl1* hiPSC-derived neurons.

33. Prewarm 0.05% trypsin-EDTA to RT.
34. Prewarm the DMEM/F-12 medium and DPBS (without Ca^{2+} and Mg^{2+}) to 37°C.
35. Aspirate the medium of the astrocytes.
36. Add 5 mL (in case of a T75 culture flask) DPBS (without Ca^{2+} and Mg^{2+}) and rock the flask gently.
37. Aspirate the DPBS (without Ca^{2+} and Mg^{2+}).
38. Add 5 mL of 0.05% trypsin-EDTA, and rock the flask gently.
39. Incubate for 5–10 min at 37°C and 5% CO_2.
40. Check under the microscope whether the cells are detaching. Detach the last cells by tapping the flask a few times.
41. Add 5 mL of astrocyte medium (see table in the "materials and equipment" section) to the flask.
42. Triturate the cells gently inside the flask with a 10 mL pipette.
43. Collect 10 mL suspension in a 15 mL falcon tube.
44. Spin the tube of cells at 200 × *g* for 8 min.
45. Aspirate the supernatant and resuspend the cells in 1–3 mL of DMEM/F-12.
46. Triturate repeatedly with a 1,000 μL pipette 10–15 times against the side of the tube to get a single cell suspension.
47. Count the cells using a counting chamber and add the astrocytes to the well in a 1:1 ratio compared to the number of hiPSCs present. That is, add 26,000 astrocytes per well. Troubleshooting 4.
48. Add 2 million cells back into the flask in 10 mL of high-glucose DMEM with 15% (v/v) FBS and penicillin/streptomycin (1:1000) for expansion.
49. Incubate at 37°C and 5% CO_2.

Note: Rat astrocytes can be used from passage number 1 and they can be maintained in culture for at least two months. However, we advise against using astrocytes beyond two months of age since their purity may decrease.

Note: Due to the special structure of the MEA plate (Multichannel Systems, 24W700), 350 μL culturing medium needs to be first aspirated then astrocytes can be added to make sure all the astrocytes can attach to the bottom. After adding the astrocytes, incubate at 37°C and 5% CO_2 for 1 h, then refill the well with fresh medium to reach 500 μL medium volume per well.

Day 3: Refresh the medium

Timing: 1 day

Cytosine β-D-arabinofuranoside hydrochloride (Ara-C) is added to remove non-differentiated hiPSCs left in the culture and inhibit the proliferation of astrocytes. The following section describes the medium change of day 3.

50. Calculate the volume of medium required: 500 μL per number of 24-well plate well or MEA well. Compose the "Neuronal differentiation medium_2" according to the table in the "materials and equipment" section.
51. Prewarm the medium to 37°C.
52. Aspirate the medium from the wells.
53. Add 500 μL of the prepared medium per well.
54. Incubate at 37°C and 5% CO_2. Troubleshooting 5.

Note: When aspirating the medium, be very gentle to avoid detaching the cells. When adding the medium, be very gentle to avoid touching the cells or electrodes.

Day 4–8: Refresh 50% of the medium

Timing: 5 days, once every other day

Only 50% of the medium is refreshed once every two days to maintain neuron conditioned medium and promote the neuronal differentiation and maturation. The following section describes the medium preparation from day 4 till day 8.

55. Calculate the volume of medium required: 250 μL per number of 24-well plate well or MEA well. Compose the "Neuronal differentiation medium_3" according to the table in the "materials and equipment" section.
56. Prewarm the medium to 37°C.
57. Aspirate 230 μL of the medium from each well.
58. Add 250 μL of the prepared medium per well.
59. Incubate at 37°C and 5% CO_2.

Note: Due to the evaporation that occurs during the incubation at 37°C, take 230 μL medium out whereas add 250 μL.

Day 9–12: Refresh 50% of the medium supplemented with FBS

Timing: 4 days, once every other day

Only 50% of the medium is refreshed once every two days. The medium is supplemented with FBS to support astrocytes. The following section describes the medium change from day 9 till day 12.

60. Calculate the volume of medium required: 250 μL per number of 24-well plate well or MEA well. Compose the "Neuronal differentiation medium_4" according to the table in the "materials and equipment" section.
61. Prewarm the medium to 37°C.
62. Aspirate 230 μL of the medium from each well.
63. Add 250 μL of the prepared medium per well.
64. Incubate at 37°C and 5% CO_2.

Day 13–49: Refresh 50% of the medium without doxycycline and forskolin

Timing: 37 days, once every other day

From day 13 onwards, 50% of the Neurobasal medium is refreshed once every 2 days and the medium is supplemented with 2.5% (v/v) FBS to support the astrocytes. Forskolin and doxycycline are removed to promote the maturation of GABAergic neurons.

65. Calculate the volume of medium required: 250 μL per number of 24-well plate well or MEA well. Compose the "Neuronal differentiation medium_5" according to the table in the "materials and equipment" section.
66. Prewarm the medium to 37°C.
67. Aspirate 230 μL of the medium from each well.
68. Add 250 μL of the prepared medium per well.
69. Incubate at 37°C and 5% CO_2.

Analysis of E/I network

Timing: Variable duration depends on the type of analysis

After plating the cells, the developing hiPSC-derived E/I network can be utilized for downstream quantitative multi-parametric analyses. The analysis approaches typically include molecular assays, such as RNAseq or qPCR, structural analyses (neuronal reconstructions, immunohistochemistry) and functional analyses (single cell electrophysiology, calcium imaging or MEA recordings). For downstream analysis the age of the culture can vary depending on the particular research question. In healthy control networks spontaneous activity starts to occur reproducibly at around 7 days in culture, spontaneous synchronized activity (i.e., bursts) after 14 days. In this section, we provide two relevant examples of structural and functional downstream analyses that in combination allow an assessment of the quality and viability/activity of the E/I co-cultures: Immunocytochemistry for characterizing the cellular and synaptic composition within the E/I network, and MEA recordings for characterizing neuronal network activity at days *in vitro* (DIV) 35 and DIV 49.

Structural assay: Immunocytochemistry

Timing: 2 days

Depending on the research question, immunohistochemistry can be performed at various time points during network formation and maturation. In the following we provide an example of histochemistry performed on relatively mature E/I network at DIV49, which represents a moment where in heathy control networks GABAergic signaling has become inhibitory.

70. Fix the cells seeded on coverslips with 4% paraformaldehyde (PFA, dissolved in PBS) supplemented with 4% sucrose for 15 min at RT.

Note: In case the experiment is performed in a 24-well plate, use 300 µL reagent (e.g., PFA, Triton, goat serum, second antibody dilution and Hoechst 33342 dilution) per well to cover the coverslips. For washing step, 500 µL PBS can be used per well.

71. Aspirate PFA and wash the cells at least 3 time with PBS.
72. Add 0.2% Triton (diluted in PBS) for 10 min at RT.
73. Aspirate Triton and add 5% normal goat serum (diluted in PBS) for 1 h at RT.
74. Aspirate the normal goat serum and incubate the cells with 80 µL primary antibody dilution (dilutions shown in the key resources table) at 4°C overnight.

Note: Use a tweezer to take out the coverslip from the 24-well plate and place the coverslip face-up on a plate wrapped by parafilm. Gently distribute the antibody dilution over the coverslip. The solution will be contained on the coverslip by the hydrophobic parafilm. This way can reduce the volume of primary antibody being used.

75. The second day, remove the primary antibody dilution. Wash the cells at least 3 time with PBS.
76. Incubate the cells with secondary antibodies (dilutions shown in the key resources table) for 1 h at RT.
77. Remove the secondary antibody and wash the cells at least 3 time with PBS.
78. Add Hoechst 33342 dilution (diluted 1:10000 in PBS) for 10 min at RT.
79. Aspirate Hoechst 33342 dilution and wash the cells 3 times with PBS.
80. Add a single drop (5 µL) of fluorescent mounting medium to the center of a slide, and place the coverslip on top of the mounting media using a tweezer.
81. The mounted slides can be imaged using a fluorescent microscope (Figure 2).

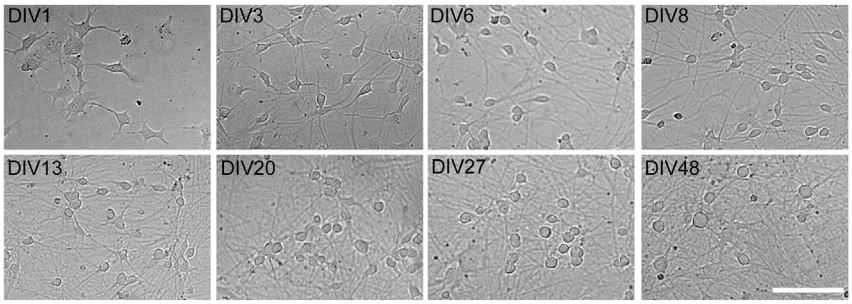

Figure 1. Bright-field microscopy images showing differentiation of hiPSCs into E/I network at different developmental stages

After DIV2, these networks were co-cultured with rat astrocytes. Typically, after 1 day in culture neurites begin to sprout, which elongate and branch over the course of 1 week; after DIV6-8 simple but arborized dendritic trees and axons become detectable, which both increase further in complexity over the course of the following 6 weeks *in vitro*. Scale bar = 100 μm.

Note: Mounted slides can be stored for short term at 4°C or long term at -20°C.

Note: Fluorescent images can be analyzed using FIJI software. Synapse puncta can be counted manually and normalized to the length of the dendritic branch where they reside.

Functional assay: MEA recordings of neuronal population activity

Timing: 1 h

MEA recordings typically allow non-invasive functional assessments of the networks in closed (sterile) conditions and thus can be performed repetitively on the same culture over time. Intervals can vary between 1 day and 1–2 weeks, depending on the developmental time line of interest. Here we present data obtained before and after GABAergic signaling typically becomes inhibitory in healthy control E/I co-cultures, i.e., DIV 35 and DIV 49.

82. Take the MEA plate out of the incubator, and put the plate in the recording chamber which is constantly maintained at 37°C and at 5% CO_2 level. Depending on the available/installed equipment or infrastructure one can apply carbogen gas (5% CO_2 / 95%O_2) or by mixing pure CO_2 with air with a CO_2 level detector.
83. Before recording, cultures on MEA are allowed to adapt for 10 min in the recording chamber.
84. Record 10 min of electrophysiological activity of neurons cultured on MEAs.

Note: The recording is sampled at 10 kHz, and filtered with a high-pass filter with a 100 Hz cut-off frequency and a low-pass filter with a 3,500 Hz cut-off frequency. The spike detection threshold is set at ± 4.5 standard deviations. Spike, burst and network burst detection is recognized by a built-in algorithm in Mulitwell Analzer software (Multichannel Systems), and a custom-made MATLAB (The Mathworks, Natrick) code to extract parameters characterizing network activity. For more details, please refer to Mossink et al.[1]

EXPECTED OUTCOMES

Using this protocol, we generated human E/I co-culture *in vitro* in 7 weeks. Starting from hiPSCs, the cells gradually differentiated into neurons with typical neuronal dendritic morphology (Figure 1). After 7 weeks, similar to the hiPSC plating ratio, there were around 30% GABA positive cells (Figures 2A and 2B), including calbindin and somatostatin expressing neurons.[1] In addition, both glutamatergic and GABAergic synapses can be observed in the culture (Figures 2C and 2D). Our previous data showed existence of glutamatergic and GABAergic synaptic input and mature intrinsic properties. Importantly,

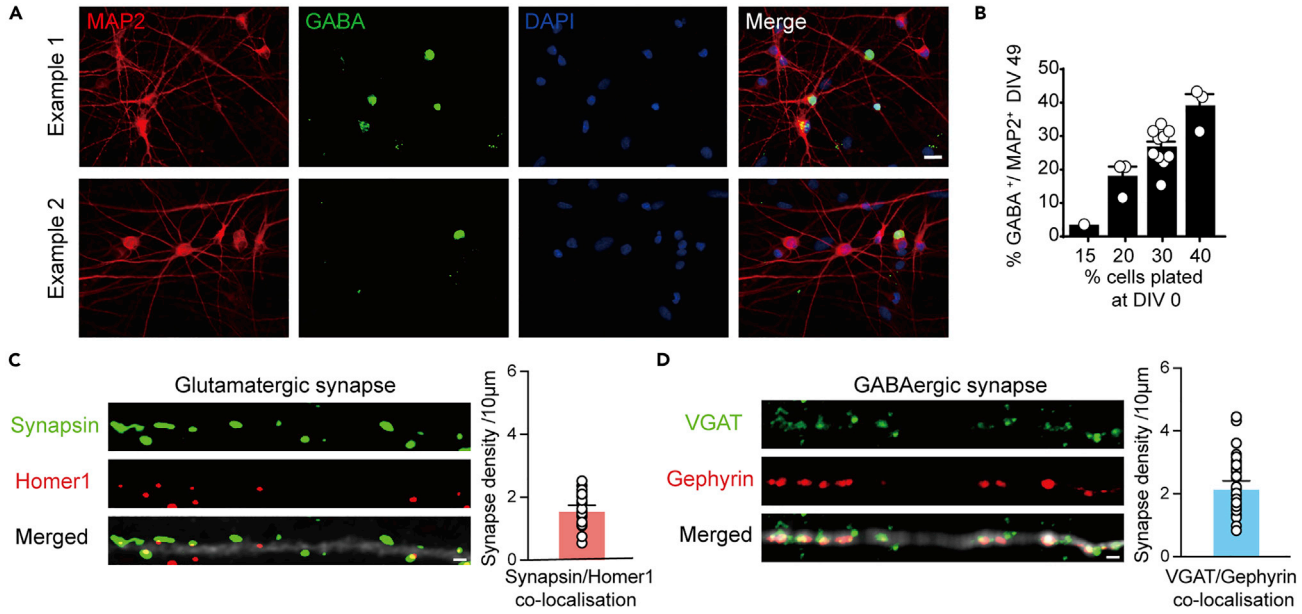

Figure 2. GABAergic neurons and glutamatergic/GABAergic synapses in hiPSC derived E/I networks

(A) Immunostaining for GABA (green) and the general (somatodendritic) neuronal marker MAP2 (red) in the mature E/I network at DIV49. Scale bar = 10 µm.

(B) Quantification of percentage of GABAergic neurons in E/I co-cultures at DIV49 plotted against the plated percentage of GABAergic neurons at DIV0. Each dot represents data collected from one image from independent culture.

(C) Representative images of immunocytochemistry stained for glutamatergic synapses (synapsin as a presynaptic marker and Homer1 as a postsynaptic marker) and dendrites (MAP2 in gray) and quantification of the density of co-localized Synapsin/Homer1 puncta.

(D) Representative images of immunocytochemistry stained for GABAergic synapses (VGAT as a pre-synaptic marker and Gephyrin as a post-synaptic marker) and dendrites (MAP2 in gray) and quantification of the density of co-localized VGAT/Gephyrin puncta. (B) was adapted from Mossink et al.[1] (C) and (D) were adapted from Wang et al.[2] Scale bar = 2 µm. Synapsin/Homer1: n = 25; VGAT/Gephyrin n = 19. Data are represented as mean ± SEM. Each dot represents data collected from one image from independent culture.

the hyperpolarizing shift of GABA reversal potential occurs at around DIV42.[1] In accordance, we found that acute treatment of the $GABA_A$ receptor antagonist Picrotoxin (PTX) on DIV35 networks did not influence the mean firing rate (MFR), network burst rate (NBR) and network burst duration (NBD). However, PTX treatment led to a significant increase of MFR and NBD on DIV49 networks (Figures 3A–3D). This indicates that GABAergic neurons in our culture exert robust inhibitory control by DIV49.

LIMITATIONS

One limitation of this protocol is that the accelerated neuronal differentiation skips certain early developmental stages, such as the neuron progenitor cell stage. However, the defined and controllable cellular composition of human neuronal networks allows investigation of core molecular and cellular mechanisms of neuronal network formation and maturation, also in the context of E/I balance. Furthermore, even though we could reliably detect a small percentage of soma targeting parvalbumin expressing (PV^+) neurons, it should be noted that in the co-cultures, we found a prominent proportion of MEF2C expressing ($MEF2C^+$) GABAergic neurons,[1] which are considered as immature PV^+ GABAergic neruons.[6] Our typical time frame for inspecting network maturation indicates that full functional maturity of these $MEF2C^+$ neurons into fast spiking PV^+ GABAergic neurons will not be reached until DIV72. However, in control neuronal networks, mature GABAergic signaling is developing ealier: after DIV49 the GABAergic signaling will switch from immature depolarizing to mature hyperpolarizing/shunting effects on postsynaptic neurons.[1] Currently, the protocol includes the addition of rat astrocytes to the culture for trophic support as well as for promoting synaptogenesis and neuronal network maturation. Alternatively, in the future, hiPSC-derived astrocytes could be utilized to establish a pure human *in vitro* culture assay for disease modeling.

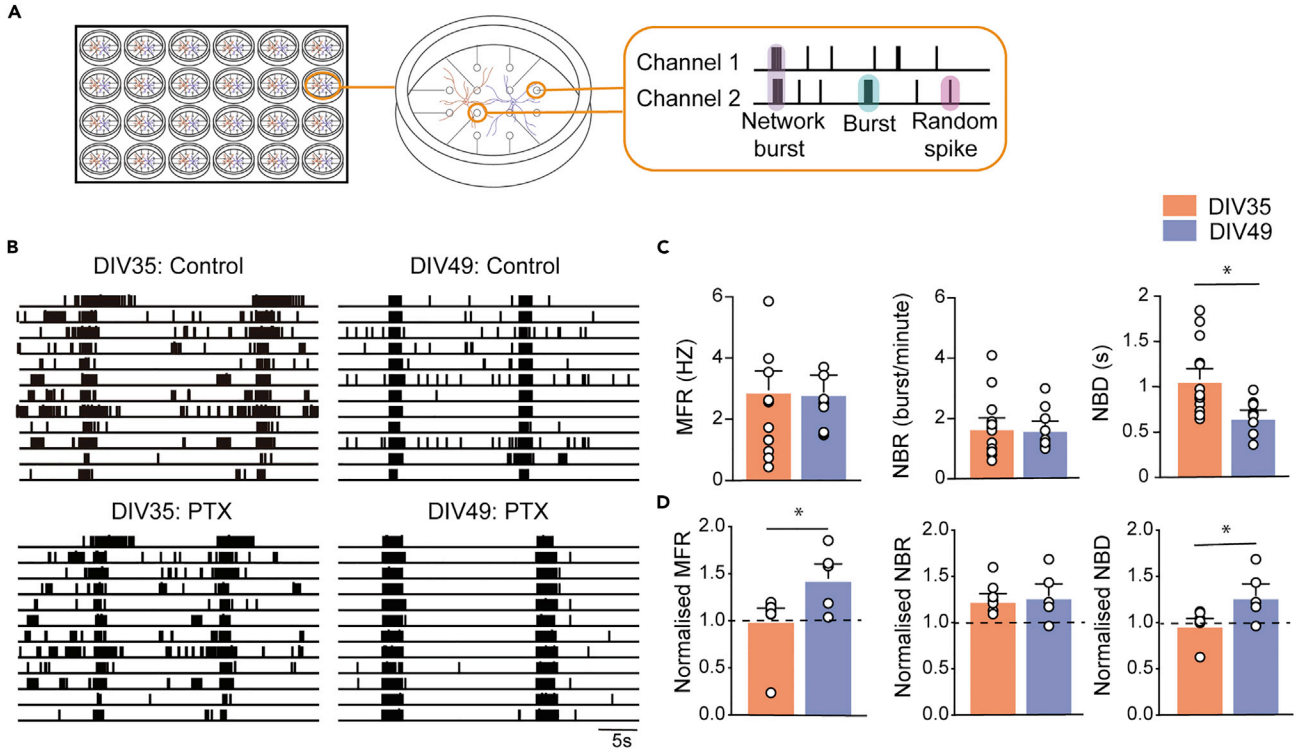

Figure 3. Functional GABAergic inhibition in in hiPSC derived E/I networks at DIV49

(A) Schematic illustration of electrophysiological recordings from neurons cultured on multi-well MEAs and the extraction of basic network activity parameters.

(B–D) E/I co-cultures show altered network activity patterns when treated with PTX ($GABA_A$ receptor antagonist) at DIV49, but not at DIV35. (B) Representative raster plot of E/I co-cultures before and during treatment with PTX at DIV35 and DIV49. (C) Mean firing rate (MFR), Network burst rate (NBR) and Network burst duration (NBD) at DIV35 and DIV49. (D) MFR, NBR and NBD of 100 μM PTX treated E/I networks when normalized to their respective baseline recording at DIV35 and DIV49. DIV35, control: n = 13; PTX n = 8; DIV 49, control: n = 13; PTX n = 5. Data are represented as mean ± SEM. * $p < 0.05$.

TROUBLESHOOTING

Problem 1

Cell viability is not sufficient after plating (step 10).

Potential solution

- When dissociating the hiPSC lines into single cells (or when passaging hiPSCs), we suggest to wait until the hiPSC lines reach at least 70%–80% confluency (Figure 4). Dissociation of cultures from sparse hiPSC colonies might lead to high rate of cell death.

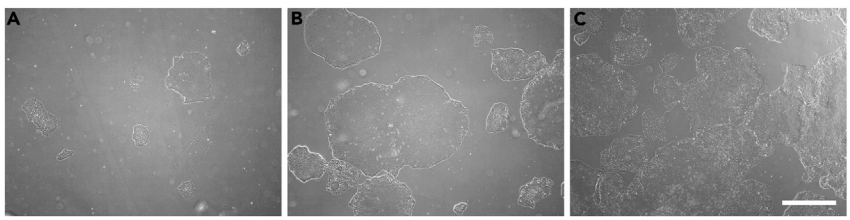

Figure 4. Images showing different confluency of hiPSC cultures

(A–C) Culture from (A) is too sparse. Confluency in between of (B to C) reached the proper level for further split or dissociation. Scale bar = 100 μm.

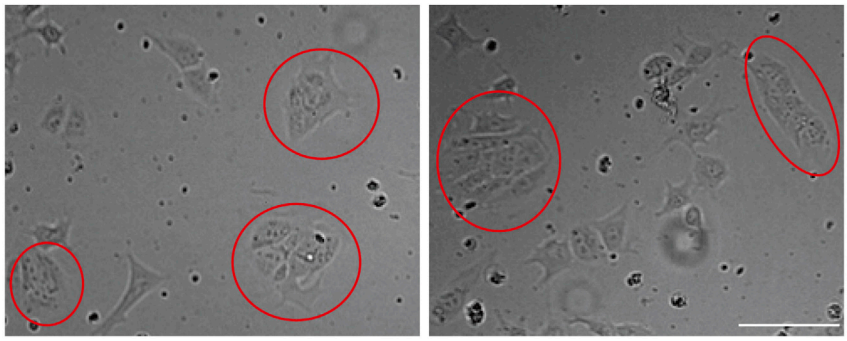

Figure 5. Cells tend to grow into colonies after plating
Scale bar = 100 μm.

- Poly-L-ornithine is a toxic reagent for cells. For coating the plate, make sure to wash off poly-L-ornithine with Milli-Q water 3 times.
- When dissociating the hiPSCs using Accutase, do not leave the cells in Accutase for too long. A prolonged period with Accutase can lead to more floating cells, which eventually impairs cell viability.

Problem 2
Cells are not single and tend to grow into colonies after plating (Figure 5) (step 19).

Potential solution

- When dissociating the hiPSC lines into single cells, make sure to check under microscopy that they are indeed single cells without clustering. Make sure to mix the cell suspension especially right before adding the cells into the wells to avoid cells cluster together.

Problem 3
The density of the *rtTA/Ngn2*- or *rtTA/Ascl1*-positive cells is too sparse or too dense during differentiation (steps 22 and 25).

Potential solution

- For the *rtTA/Ngn2*- or *rtTA/Ascl1*-positive hiPSC lines, the differentiation efficiency can differ from line to line. Before starting real experiments, we suggest to first try a series of different plating density to get an idea about the efficiency, and choose the proper starting cell density for later experiments. Especially when comparing control E/I co-cultures to the one from patient or CRISPR/Cas9 edited lines, it is pivotal to keep these cultures in a similar density to exclude any confounding factors from the culture itself.

Problem 4
The neuronal cultures cluster over time (Figure 6) (step 47).

Potential solution

- Clustered cultures could be resulted from the poor quality of rat cortical astrocytes. In this case, we suggest to check the purity of the astrocytes to make sure the dissection and culturing of the astrocytes is proper. Poor handling in meningeal removing during dissection could potentially increase the contamination level in astrocyte culture.

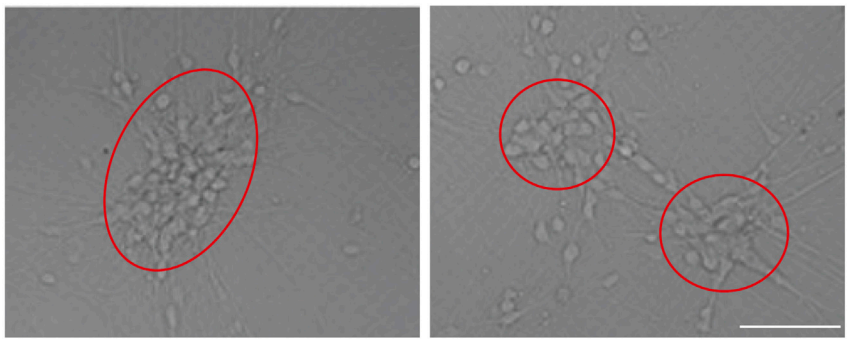

Figure 6. Neuronal clusters in the culture during differentiation
Scale bar = 100 μm.

- It could be that the number of astrocytes added to the culture is less than expected. Make sure to properly mix the cell suspension before aspirating the certain amount to add into the well.

Problem 5
High rate of cell death after adding Ara-C (step 54).

Potential solution

- This indicates the *Ngn2*- or *Ascl1*-positive hiPSCs might express a low level of *Ngn2* or *Ascl1*. We strongly recommend to test the expression of *Ngn2* or *Ascl1* to make sure there is overexpression of the relevant gene. It may be required to generate new *Ngn2*- or *Ascl1*-positive hiPSC lines.

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact Dirk Schubert d.schubert@donders.ru.nl.

Materials availability
This study did not generate novel unique reagents. The plasmids used in this study can be made available on request, but we may require a payment and/or a completed Materials Transfer Agreement if there is a potential for commercial application.

Data and code availability
All original code has been deposited at: Mendeley Data: https://doi.org/10.17632/bvt5swtc5h.1. The authors confirm that the data supporting the findings of this study are available within the article. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

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AUTHOR CONTRIBUTIONS

S.W., R. H., B. M., D.S., and N.N.K. conceived and designed all the experiments. D.S. and N.N.K. supervised the study.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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