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Efficient derivation of excitatory and inhibitory neurons from human pluripotent stem cells stably expressing direct reprogramming factors

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ABSTRACT

It is essential to generate isolated populations of human neuronal subtypes in order to understand cell-type specific roles in brain function and susceptibility to disease pathology. Here we describe a protocol for in-parallel generation of cortical glutamatergic (excitatory; EX) and GABAergic (inhibitory; IN) neurons from human pluripotent stem cells (hPSCs) by using the neurogenic transcription factors (TFs) Ngn2, and the combination of Ascl1 and Dlx2, respectively. In contrast to the majority of neural transdifferentiation protocols which use transient lentiviral infection, this protocol utilizes stable hPSC lines with doxycycline-inducible TFs, which can then be neuronally differentiated by addition of doxycycline and neural media. First, we present a method to generate lentivirus from mammalian cultured cells and establish TFs-incorporated stable cell lines (Basic Protocol 1), and then we describe a monolayer EX- and IN-neuronal differentiation derived from the established hPSCs (Basic Protocol 2). The resulting EX- and IN-neurons reproducibly exhibit properties consistent with human cortical neurons, including the expected morphologies, expression of EX and IN genes, and functional properties. Our approach enables the scalable and rapid production of human neurons suitable for modeling human brain diseases in a subtype-specific manner and examination of differential cellular vulnerability.

Basic Protocol 1: Lentivirus production and creation of stable hPSC lines

Basic Protocol 2: EX-, and IN-neuron differentiation

Support Protocol 1: Expansion and maintenance of hPSCs

Support Protocol 2: Experimental methods for validation of EX- and IN-neurons

Keywords: Human pluripotent stem cell • Excitatory neuron • Inhibitory neuron • Neurogenic transcription factors • Doxycycline inducible Tet-O system

INTRODUCTION

Recent advances of human pluripotent stem cell (hPSC)-derived neuronal culture differentiation allow us to model various human neurological conditions in a more physiologically- relevant system (Garcia-Leon, Vitorica, & Gutierrez, 2019; Jung, Hysolli, Kim, Tanaka, & Park, 2012; Li, Chao, & Shi, 2018). Cortical neural function is finely controlled by the balance and communication between excitatory (glutamatergic; EX) and inhibitory (GABAergic; IN) neurons in the human brain. Understanding subtype-specific roles of neurons is critical to study normal

human development and disease pathology. Here we describe an efficient protocol for generating isolated populations of induced human EX- and IN-neurons as has been done previously (Barretto et al., 2020; Chanda et al., 2014; Frega et al., 2017; Ho et al., 2016; Nicholas et al., 2013; A. X. Sun et al., 2016; Yang et al., 2017; Zhang et al., 2013), in a manner that has distinct advantages compared to prior methods. This protocol does not require repetitive lentiviral transductions for each differentiation, thus reducing risks and extra precautionary measures in addition to the costs of reagents and manpower associated with making fresh lentiviral preparations. In addition, this protocol eliminates the use of primary murine astrocyte cultures by substituting them with astrocyte conditioned media from a commercial source.

This unit begins with a method for the generation of in-house lentiviral particles which express neurogenic transcription factors, and the establishment of stable hPSC lines by lentiviral transduction (Basic Protocol 1), followed by a protocol for in-parallel differentiation and maturation of EX- and IN-neurons from these hPSCs (Basic Protocol 2) (Figure 1). Detailed descriptions for the general maintenance of hPSC culture (Supplementary Protocol 1) and for experimental methods for characterizing differentiated EX- and IN-neurons (Supplementary Protocol 2) are also covered.

NOTE: The following procedures are performed in a Class II biological hazard flow hood or a laminar-flow hood.

NOTE: All solutions and equipment coming into contact with live cells must be sterile, and proper aseptic technique should be used accordingly.

NOTE: All cell cultures are maintained in a humidified 37 °C, 5 % CO_2 incubator.

BASIC PROTOCOL 1

LENTIVIRUS PRODUCTION AND CREATION OF STABLE hPSC LINES

This protocol describes production of concentrated lentivirus from plasmids containing a tetracycline-dependent promoter upstream of neurogenic transcription factors (Ngn2, Ascl1, and mDlx2) along with reverse tetracycline-controlled transactivator (rtTA) in HEK 293T cells. Lentiviruses will be used for the subsequent establishment of stable hPSC lines incorporated with each virus (Figure 1). We found that the promoters of each plasmid are leaky enough to express sufficient levels of the target and associated antibiotic-resistance transgenes which allows us to generate stable cell lines without doxycycline induction (Figure 2). Once these hPSC lines are established, they are easy to maintain, propagate, and bank for long-term usage.

CAUTION: Biosafety level (BSL)-2+ is appropriate to perform lentivirus production and transduction experiments. The produced lentivirus, albeit replication incompetent, can infect human cells. Make sure to decontaminate plasticware such as pipettes, filter units, collection tubes with bleach to kill the infectious virus in every step following transduction. Note that although this protocol uses 2nd generation lentiviral constructs, it could be adapted to 3rd generation reagents.

Materials

HEK 293T cells (ATCC, cat. no. CRL-3216)
HEK 293T culture media (FM10 media; see recipe)
ESGRO 0.1 % Gelatin in distilled water (Sigma-Aldrich, cat. no. SF008)

Lipofectamine 3000 (Invitrogen, cat. no. L3000-015)
Opti-MEM Reduced Serum Media (Gibco, cat. no. 31985-070)
0.25 % Trypsin-EDTA (Gibco, cat. no. 25200-056)
0.4 % Trypan Blue Stain (Invitrogen, cat. no. T10282)
Midi-prepped plasmid DNA:
pLV-TetO-hNGN2-Puro (Addgene #79049 (Ho et al., 2016))
pTight-hASCL1-N174-Neo (Addgene #31876 (Yoo et al., 2011))
TetO-FUW-mDLX2-hygro (Addgene #97330 (Yang et al., 2017))
FUdeltaGW-rtTA (Addgene #19780 (Maherali et al., 2008))
psPAX2 (Addgene #12260), 2nd generation lentiviral packaging plasmid
pCMV-VSV-G (Addgene #8454 (Stewart et al., 2003)), mammalian expression plasmid to express VSV-G envelope
Lenti-X Concentrator (Takara, cat. no. 631232)
Dulbecco's Phosphate-buffered Saline (DPBS) without $CaCl_2$, $MgCl_2$ (Gibco, cat. no. 14190-044)
Human pluripotent stem cells
StemFlex media (Gibco, cat. no. A33494-01; see recipe)
DMEM/F12 media (Gibco, cat. no. 12320-033)
Cultrex Stem Cell Qualified Reduced Growth Factor Basement Membrane Extract (R&D Systems, cat. no. 3434-005-02; see recipe)
ReLeSR (STEMCELL Technologies, cat. no. 05872)
Accutase Cell Detachment Solution (Innovative Cell Technologies, cat. no. AT-104)
Y-27632 (ROCK inhibitor; Selleck Chemicals, cat. no. S1049; see recipe)
1 mg/mL Puromycin Dihydrochloride (Gibco, cat. no. A11138-03)
50 mg/mL Hygromycin B (Gibco, cat. no. 10687010)
50 mg/mL Geneticin™ Selective Antibiotic (G418 Sulfate) (Gibco, cat. no. 10131035)
Freezing media (see recipe)

Purifier Biological Safety Cabinet (*e.g.,* LABCONCO, cat. no. 302488050)
Heracell 150i CO_2 Incubator (*e.g.,* Thermo Scientific, cat. no. 51026529)
Stereomicroscope (*e.g.,* EVOS XL Core, cat. no. AMEX1000)
Refrigerated Centrifuge (*e.g.,* Sorvall Legend RT, cat. no. 75004377)
Countess II Automated Cell Counter (Invitrogen, cat. no. AMQAX1000)
Countess Cell Counting Chamber Slides (Invitrogen, cat. no. C10228)
115-ml Filter Units with SFCA Membrane (Thermo Fisher Scientific, cat. no.122-0045)
15-mL Conical tubes (*e.g.,* BD Falcon)
50-mL Conical tubes (*e.g.,* BD Falcon)
1.5-mL Nuclease-free Microcentrifuge Tubes (*e.g.,* Thermo Fisher Scientific)
6-well Cell Culture Plates (Corning, cat. no. 3516)
Cryovials (Corning, cat. no. 430-661)
Liquid Bleach
Parafilm

Standard equipment and tools for cell culture and cell biology (CO_2 incubator, tissue culture hoods, liquid nitrogen cell storage tank, vacuum aspirator system, centrifuges, electronic pipettors, etc.) are needed. While alternatives are available from multiple vendors, we will list key items we use routinely in our laboratory. Additional reagents and equipment for growing mammalian cells and human pluripotent stem cells on feeder-free are required.

Lentivirus production

1. Grow HEK 293T cells in gelatin-coated 15-cm plates, containing 20 mL of FM10 media. For passaging, wash the cells with 20 mL DPBS, and trypsinize for approximately 5 minutes until cells are freely floating. Collect floating cells, deactivate trypsin by adding FM10 media and centrifuge at 300 x g for 4 min. Aspirate media and resuspend the pellet with FM10 media. Count cells via Trypan blue exclusion procedures.
   Note that a mechanical force easily detaches HEK 293T cells from plastic plates, and thus it is important to handle cells gently during wash and trypsinization. Washing once with DPBS before adding trypsin-EDTA is necessary because FBS in FM10 media may block trypsin activity.

2. On the day before transfection (D-1), plate HEK 293T cells onto a gelatin-coated 15-cm plate at a density of 1.8×10^6 cells/cm^2 in 20 mL of FM10 media.
   Coating a plate with gelatin helps cells to adhere to the well. Prepare two 15-cm culture plates per each virus. Cells should be 80 ~ 90 % confluent on the day of transfection for maximal transfection efficiency.

3. On Day 0, prepare transfection reagents to the standard Lipofectamine 3000 protocol. Mix 80 µL of Lipofectamine 3000 reagent in 2 mL of Opti-MEM to a 15-mL conical tube, and vortex for 3 seconds. In a new 15-mL conical tube, prepare master mix of DNA (20 µg of target plasmids (Ngn2, Ascl1, Dlx2, or rtTA), 15 µg psPAX2, 10 µg VSV-G) by diluting in 2 mL Opti-MEM, then add 80 µL of P3000 reagent. The contents of both 15-mL tubes are mixed together (1:1 ratio) and incubated at room temperature for 10 min.
   Other transfection methods such as calcium phosphate precipitation can be used for transfection (Kingston, Chen, & Rose, 2003). We observed the transfection using Lipofectamine 3000 reagent consistently exhibits high performance of transfection efficiency and reproducible results. Transfections are performed according to manufacturer's instructions. Prepare 2x mix for transfection in two plates for each virus. Optimum amount of DNA and reagents should be determined for the maximum efficiency by end-user.

4. Gently pipette the mix and add the transfection mixture in a dropwise manner to the cells slowly.

5. Incubate cells at 37 °C for 5 - 6 hours.

6. Replace media to 18.5 mL of fresh FM10 media per each plate.
   Be careful not to lift cells.

7. Collect the first viral supernatants 48 hours after transfection (D2). Place supernatants in a 50-mL tube, close cap tightly and wrap with parafilm. Keep in 4 °C. Feed cells with 18.5 mL of fresh FM10 media.
   Be careful not to add bubbles and cell debris to collection tubes.

8. Harvest the 2nd viral supernatants to a 50-mL tube next day 72 hours post-transfection (D3).
   Now you have 36 mL of virus supernatants per each virus in 50-mL collection tubes. Discard cell culture plates carefully after disinfecting with bleaches.

9. Spin down supernatants at 300 x *g* for 3 min.

10. Combine both supernatants and filter supernatants through a 115-mL filter by vacuum.

Apply 2.5 mL of DMEM to a filter membrane prior to pouring supernatants in order to check the filter is working. If filtering, use only cellulose acetate or polyethersulfone (PES) filters. Do not use nitrocellulose filters.

11. Transfer filtered supernatants 36 mL each into two new 50-mL conical tubes and combine 1 volume of Lenti-X Concentrator with 3 volumes of supernatants. Mix them by gentle inversion.
There will be approximately 72 mL of supernatants post-filter. Mix 36 mL of supernatants and 12 mL of Lenti-X concentrator in two 50-mL tubes.

12. Incubate them at 4 °C for 2 hours to overnight.
The cooling of the sample is essential. Incubation times can be longer up to 1 week at 4 °C.

13. Centrifuge concentrated samples at 1,500 x *g* for 45 min at 4 °C. Carefully remove supernatants and place tubes on ice.
Check if a pellet is visible.

14. Gently resuspend pellets with 200 µL of DPBS per each virus (100 µL per each 50-mL tube). Prepare 20-40 µL aliquots into pre-labelled 1.5-mL microcentrifuge tubes.

15. Immediately store at -80 °C.
Small ready-to-use aliquots are useful to avoid multiple freeze-thaw cycles in order to maintain infection efficiency.

Reverse-transduction of lentivirus into hPSCs

16. Grow human pluripotent stem cells (hPSCs) in feeder-free conditions, in StemFlex media on Cultrex-coated 6-well culture plates.
If hPSCs are grown in feeder layer such as mouse embryonic fibroblasts (MEFs), then adapt cells into feeder-free culture condition prior to use for the experiment. If using other stem cell culture media such as E8 media, then follow the manufacturer's standard protocol. StemFlex media generally requires every other day feeding, although cells can skip two days of feeding if at low confluency. Check Support protocol 1 for general hPSC culture maintenance.

17. When cells are confluent, use Accutase for single cell dissociation. Aspirate media and add 1 mL of Accutase to a well, then incubate at 37 °C until cells become detached (which usually takes 5 to 7 min).
Incubation time will vary on density of the cells and cell line used. It is critical to ensure that cells are completely dissociated into single cells. Always equilibrate media and reagents to room temperature prior to use. Warm Cultrex-coated plates at 37 °C 1 hour prior to use.

18. Transfer cells to a 15-mL conical tube with 7 mL of DMEM/F12 media base, with minimal pipetting, and spin for 4 min at 300 x *g* at room temperature.

19. Aspirate Accutase/DMEM/F12 media and resuspend cell pellets gently in StemFlex media supplemented with 10 µM Y-27632 using a P-1000 pipet tip. Count viable cells using Trypan blue stain exclusion assay.
Take 10 µL of cells and mix with 10 µL of Trypan blue for calculating cells. Dissociated cells should yield greater than 90 % of viability. Few million cells from one confluent well of hPSCs from 6-well plate are expected.

20. Dilute cells at a density of 2.6 x 10^5 cells/mL in StemFlex media supplemented with 10 µM Y-27632. Place 2 mL of cells into a 50-mL conical tube and add 5 µL of target virus (*e.g.*, Ngn2 for EX-hPSCs, Ascl1 and mDlx2 for IN-hPSCs) and rtTA virus, for the reverse-transduction.
    20-a. For EX-hPSCs, transduce with 5 µL of Ngn2 and rtTA virus, respectively.
    20-b. For IN-hPSCs, it requires sequential transduction of Ascl1 and mDlx2 virus. Start establishment of Ascl1-hPSCs first by transducing 5 µL of Ascl1 and rtTA virus, respectively.
    Thaw lentiviruses on ice. Reverse-transduction refers to the method of transduction, adding virus into dissociated cells prior to cell plating, rather than adding virus to adherent cells. The authors observe high efficiency of transduction via a reverse-transduction in hPSCs. The day of transduction is referred to as day 0.

21. Seed cell-virus mix into a Cultrex-coated plate and shake plate in order to have the cells spread and attach evenly on the plate. Set up a control well for cells without virus in order to confirm antibiotic selection is working.
    520,000 cells per well in 6-well plate is optimal for making EX-H9 cells, however, cell plating density will need to be optimized for each hESC or hiPSC line and type of target viruses by the end-user.

22. The next day (D1), perform antibiotic selection by changing media with fresh StemFlex media containing antibiotics without Y-27632.
    22-a. For EX-hPSCs, treat with1 µg/mL Puromycin in StemFlex media without Y-27632 for 24 hours (H9 hESCs).
    22-b. For Ascl1-hPSCs, treat with 100 µg/mL G418 in StemFlex media without Y-27632 for 96 hours (H9 hESCs). Refresh media with G418 every other day.
    The concentration and duration of the antibiotics should be determined beforehand by performing a dose response curve for each cell line. Treat with antibiotics until non-transduced cells are all killed. Cells that do not survive selection will be floating.

23. Once non-transduced cells are all dead, refresh with StemFlex media without antibiotic and maintain every other day feeding schedule with StemFlex media until cells get confluent.
    23-a. For EX-hPSCs, refresh with StemFlex media without Puromycin on day 1.
    23-b. For Ascl1-hPSCs, refresh with StemFlex media without G418 on day 5.
    Only a minority of hPSCs will survive selection. These cells will proliferate well enough to be confluent within few days to generate a polyclonal line.

24. To passage the cells, aspirate medium. Add 1 mL ReLeSR/well and incubate 3-5 min at 37 °C. Aspirate ReLeSR and resuspend cells with 1 mL StemFlex media by gently re-suspending cells using a P-1000 pipet tip, then split cell suspension between the desired number of wells. Plate 2 mL/well cells into Cultrex-coated 6-well culture plate.
    Note that ReLeSR passaging is not used for single-cell dissociation. Cell aggregates should be in clumps with relatively uniform size for ReLeSR passaging. Adjust split density based on the cell growth rate of the particular hPSC line. For example, 1:6 is often optimal for H9 hESCs.

25. To freeze cells, perform ReLeSR passaging and resuspend cells in freezing media (see recipe). Freeze one well of a 6-well plate into 2 cryovials, each of which can be thawed back into one well of a 6-well plate.
    We recommend to wait to cryopreserve cells until after at least after two passages from the viral transduction in order to mitigate remaining virus in the culture. Until the second passage, exercise special care.

26. Once Ascl1-hPSCs are made, perform reverse-transduction of 5 µL mDlx2 virus without rtTA virus into Ascl1-hPSCs as described in step 17-23. Following day, start treatment of 50 µg/mL of Hygromycin B until control cells without virus are all killed (72 - 84 hours).
The authors often observed the low cellular viability due to toxicity when all viruses are transduced (co-transduction of Ascl1, mDlx2, and rtTA) at the same time. We highly recommend users to follow sequential-transduction as described, by generating Ascl1-hPSCs then followed by subsequent mDlx2 virus transduction in Ascl1-hPSCs.

SUPPORT PROTOCOL 1

Expansion and maintenance of hPSCs

Materials

Human pluripotent stem cell lines, *e.g.,* we have used both hESCs (WA09/H9) and several of our own iPSC lines; prior to use, adapt cells to StemFlex media
StemFlex media (Gibco, cat. no. A33494-01; see recipe)
Cultrex Stem Cell Qualified Reduced Growth Factor Basement Membrane Extract (R&D Systems, cat. no. 3434-005-02; see recipe)
ReLeSR (STEMCELL Technologies, cat. no. 05872)
Y-27632 (ROCK inhibitor; Selleck Chemicals, cat. no. S1049; see recipe)

6-well Cell Culture Plates (Corning, cat. no. 3516)
15-mL Conical tubes (*e.g.*, BD falcon)

Additional reagents and equipment for human pluripotent stem cells on feeder-free are required.

Feeder-free culture

1. Take out cryopreserved vial of hPSCs from liquid nitrogen storage and thaw immediately in a 37 °C water bath.
Be careful not to wait till complete thawing of the cryovial.

2. Transfer cells into 15-mL conical tube with 7 mL pre-warmed StemFlex media and centrifuge 5 min at 200 × *g* at room temperature.
Warm all reagents, media and Cultrex-coated 6-well plate prior to use.

3. Aspirate supernatants and resuspend cell pellets with 2 mL StemFlex with 10 µM Y-27632. Aspirate Cultrex from one well, add 2 mL cell suspension, making sure to evenly distribute cells in the well, and place plate in a 37 °C incubator.

4. Change media the next day without Y-27632, then repeat every other day until cells are near confluence.
Please follow standard feeding schedules if you are using different hPSC media (e.g., E8 media).

5. To passage the cells, aspirate medium. Add 1 mL ReLeSR/well and incubate 3-5 min at 37 °C. Aspirate ReLeSR and resuspend cells with 1 mL of StemFlex media by gentle pipetting, then split cell suspension between the desired number of wells. Plate 2 mL/well cells into Cultrex-coated 6-well culture plate.

6. Change media every other day until the cells are ready for the transduction.

BASIC PROTOCOL 2

EX-, and IN-neuron differentiation

This protocol describes how to differentiate and mature both EX- and IN- neurons from the stable hPSCs described above. Addition of doxycycline, a derivative of tetracycline which binds to rtTA with high affinity, induces expression of neurogenic transcription factors, which transit cell status from proliferating hPSCs to terminally differentiated neurons. Treatment with neuronal differentiation supplements and small molecules yields efficient production of EX- and IN-neurons. Unified and simplified media conditions and feeding schedules for both types of neurons allows for systemic production of isolated or co-cultured population of EX- and IN-neurons. Cytosine β-D-arabinofuranoside (Cytarabine, Ara-C), a cytosine analog and a potent inhibitor of replicative DNA synthesis, is used to control proliferation (Galmarini, Mackey, & Dumontet, 2001), leading to the removal of remaining proliferating hPSCs in the culture. In this protocol, murine astrocyte co-culture with the neuronal culture is not necessary, and thus subsequent lifting and replating of cells is not required. Therefore, the correct plate size, format, and coating materials should be determined before plating cells. Using this method, we found that neuronal morphology starts to appear within a week after neuronal differentiation and neurons are functionally mature in about 4 weeks. Here, we present validation and characterization data which demonstrate successful EX- and IN-neuron production (Figure 3, 4).

Materials

Stable EX-hPSCs (Ngn2+rtTA), stable IN-hPSCs (Ascl1+mDlx2+rtTA)

Y-27632 (ROCK inhibitor; Selleck Chemicals, cat. no. S1049; see recipe)
DMEM/F12 media base (Gibco, cat. no. 11320-033)
Accutase Cell Detachment Solution (Innovative Cell Technologies, cat. no. AT-104)
Neuronal differentiation media (see recipe)
N2 (Gibco, cat. no. 17502-048)
B27 (Gibco, cat. no. 17504-044)
Cytarabine (Ara-C; TOCRIS, cat. no. 4520; see recipe)
Recombinant human Brain-derived Neurotrophic Factor (BDNF; R&D Systems, cat. no. 248-BDB; see recipe)
Recombinant human NT3 (R&D Systems, cat. no. 267-N3; see recipe)
Cultrex Mouse Laminin I (R&D Systems, cat. no. 3400-010-02)
1 mg/mL Doxycycline Hyclate (Sigma-Aldrich, cat. no. D9891; see recipe)
BrainPhys Neuronal Medium N2-A & SM1 Kit (STEMCELL technologies, cat. no. 05793; see recipe)
Mouse Astrocyte Conditioned Media (mACM; ScienCell Research Laboratories, cat. no. M1811-57)
Polyethylenimine (PEI; Sigma-Aldrich, cat. no. 408727)/laminin-coated plates (see recipe)

Purifier Biological Safety Cabinet (*e.g.,* LABCONCO, cat. no. 302488050)
Heracell 150i CO_2 Incubator (*e.g.,* Thermo Scientific, cat. no. 51026529)
Stereomicroscope (*e.g.,* EVOS XL Core, cat. no. AMEX1000)
Refrigerated Centrifuge (*e.g.,* Sorvall Legend RT, cat. no. 75004377)
Countess II Automated Cell Counter (Invitrogen, cat. no. AMQAX1000)
Countess Cell Counting Chamber Slides (Invitrogen, cat. no. C10228)

15-mL Conical tubes (*e.g.*, BD falcon)
6-well Culture Plates (Corning, cat. no. 3516)

Additional reagents and equipment for growing human pluripotent stem cells under feeder-free conditions and counting viable cells by trypan blue exclusion.

1. Grow stable EX- and IN-hPSC cell lines generated above, in StemFlex media in a Cultrex-coated 6-well plate until 80 % confluency.
   Or if you freeze cells, thaw cells accordingly (Support Protocol 1) and passage cells at least once before proceeding to downstream process.

2. On the first day of neuronal differentiation (ND0), add 1 mL/well of Accutase to cells and incubate at 37 °C for 5-7 min, until cells release with gentle tapping of the bottom of the plate.
   Warm the reagents, media, and coated-plates in advance.

3. Collect cells with a P-1000 pipet tip into a 15-mL tube with 7 mL of DMEM/F12 media base, and centrifuge 4 min at 300 x *g*, at room temperature. Remove supernatants by aspiration.

4. Resuspend cell pellets with 1 mL neuronal differentiation media containing 10 ng/mL BDNF, 10 ng/mL NT3, 1 µg/mL doxycycline, 1 µg/mL laminin, and 10 µM Y-27632. Resuspend gently. Count viable cells by trypan blue exclusion assay.
   Add supplements freshly to media prior to use.

5. Seed cells onto PEI/laminin-coated 12-well plate at 1.3 x 10^5 cells/cm^2 (2.6 x 10^5 cells/mL in 12-well) and return plate to 37 °C incubator.
   260,000 cells per well in 12-well plate is optimal for making EX, and IN-neurons (H9), however, cell plating density varies for each hESC or hiPSC line and types of target virus used by the end-user. The authors recommend to determine the optimal cell plating density by testing a few different cell densities before scaling up experiments.

   Cells can be cultured on tissue culture-treated plasticware or on glass coverslips for the downstream experimental purposes (see recipe).

   Option A) For biochemical applications requiring large number of neurons, plate cells on PEI/laminin-coated 12-well or 6-well plates.
   Option B) For immunocytochemistry or electrophysiology applications, plate cells on 12-mm (or 15-mm) size round glass coverslips in 24-well plates with PEI/laminin coating.

6. Next day (ND1), start to treat Ara-C in order to get rid of any remaining proliferating cells. Change media with neuronal differentiation media containing 10 ng/mL BDNF, 10 ng/mL NT3, 1 µg/mL doxycycline, 1 µg/mL laminin, and 2 µM Ara-C.
   On this day, you will start to observe the transition to neurons by morphological changes. High dose of Ara-C (such as 2 µM) is helpful to get rid of remaining hPSCs. You will observe significant of cell death the next day. If you observe too much of cell death after treating Ara-C, then delay treatment of Ara-C until ND2 (in this case, media should be still refreshed with full neuronal differentiation media supplemented with BDNF, NT3 and doxycycline without Y-27632 on ND1). Authors recommend to conduct a smaller scale pilot experiment in order to determine how cell lines respond to Ara-C treatment.

7. Two days later (ND3), change media with neuronal differentiation media containing 10 ng/mL BDNF, 10 ng/mL NT3, 1 µg/mL doxycycline, and 2 µM Ara-C.

8. On day 5 (ND5), refresh media with neuronal differentiation media containing 10 ng/mL BDNF, 10 ng/mL NT3, 1 µg/mL doxycycline, and 100 nM Ara-C.
   We recommend to treat with a high dose of Ara-C (2 µM) at least two times and change to a lower dose of Ara-C (100 nM) on subsequent feeds.

9. On day 7 (ND7), change basal media to BrainPhys neuronal differentiation media. Add 100 nM Ara-C along with 10 ng/mL BDNF, 10 ng/mL NT3, and 1 µg/mL doxycycline.
   Add low dose of Ara-C (100 nM) to the culture few days until any remaining proliferating cells are observed. Usually, a week treatment is sufficient. The authors recommend to add laminin (1 µg/mL) to the culture once per a week in order to help neuronal attachment. Transition into BrainPhys media is recommended for functional assays such as whole-cell patch electrophysiology for improved neuronal function (Bardy et al., 2015).

10. On day 9 (ND9), refresh with a half-medium change with BrainPhys neuronal media supplemented with 10 ng/mL BDNF, 10 ng/mL NT3, 1 µg/mL doxycycline and 100 nM Ara-C.
    For the half-media change in 12-well plate, remove 500 µL of old media and add fresh 500 µL media to the culture. Modify the duration and times of Ara-C treatment based on culture condition. Stop treatment of Ara-C if you do not observe any non-neuronal cells.

11. On day 11 (ND11), start to add 10 % mACM to the BrainPhys neuronal medium supplemented with 10 ng/mL BDNF, 10 ng/mL NT3 and 1 µg/mL doxycycline in order to support the growth of neurons.
    Adding mACM to the medium is an alternative way to co-culture with murine primary astrocyte culture. Addition of mACM to the culture supports the survival and promotes the functional maturity of neurons (Yan, Tan, & Huang, 2013). You will observe bigger somas and healthier neurons after addition of mACM over a period of time.

12. For the rest of the differentiation, perform a half-medium change with BrainPhys neuronal medium supplemented with 10 ng/mL BDNF, 10 ng/mL NT3 and 1 µg/mL doxycycline every 3-4 days. Neurons have been cultured for over 30 days using this protocol.
    Doxycycline might be withdrawn after 2 weeks of neuronal differentiation, depending on the user's preference. Upon reaching the end of the desired culture period, cells can be processed for downstream applications such as immunocytochemistry, electrophysiology, RNA, or protein isolation.

SUPPORT PROTOCOL2

Experimental methods for validation of EX- and IN-neurons

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

RNA was extracted using TRIzol (Invitrogen, cat. no. 15596026)/chloroform method, followed by sodium acetate and ethanol precipitation. 0.1 (v/v) of 3 M sodium acetate (pH 5.5) (Invitrogen, cat. no. AM9740) with 1 (v/v) of room temperature isopropanol were added and vortexed to mix thoroughly. 1 µg/mL of Glycogen (Thermo Scientific, cat. no. R0551) was added to aid visualization of precipitants. The mix was precipitated at room temperature for 20 min and then centrifuged at 13,000 × *g* for 10 min. Pellet was washed twice with ice-cold 70 % ethanol, spinning for 1 min each time. Ethanol was removed, and the tubes spun quickly to remove the trace amount of ethanol. Air dry the pellet and resuspend in an appropriate volume of nuclease-free water. RNA concentration was

measured using a NanoDrop instrument. Normalized RNA (90 ng) was used for cDNA synthesis. cDNA synthesis was prepared with the Maxima First Strand cDNA Synthesis Kit (Thermo Scientific, cat. no. K1672). Real-time PCR was conducted with Fast SYBR Green Master Mix (Applied Biosystems, cat. no 4385612) using a QuantStudio 7 Flex thermocycler (Applied Biosystems, cat. no. A43183). The $-\Delta\Delta Ct$ method was used to determine the relative expression of each gene with *GAPDH* gene as a reference. The primers used to amplify target genes by qRT-PCR are as follows (see Supplementary Table 1).

Category	Target gene	Sequence	Ref
Housekeeping gene	*GAPDH*	Frw: 5'-TGAAGGTCGGAGTCAACGGATTTGG-3' Rev: 5'-CATGTAGGCCATGAGGTCCACCAC-3'	(Riera et al., 2019)
Pluripotent stem cell marker	*SOX2*	Frw: 5'-AGAAGGATAAGTACACGCTGC-3' Rev: 5'-TCCAGCCGTTCATGTGC-3'	In this study
Pan-neuronal markers	*MAP2*	Frw: 5'-AAACTGCTCTTCCGCTCAGACACC-3' Rev: 5'-GTTCACTTGGGCAGGTCTCCACAA-3'	(Ho et al., 2016)
	MAPT	Frw: 5'-AGGCGGGAAGGTGCAAATAG-3' Rev: 5'-TCCTGGTTTATGATGGATGTT-3'	(Lacovich et al., 2017)
Cajal-Retzius cell marker	*RELN*	Frw: 5'-TGAGAGCCAGCCTACAGGA-3' Rev: 5'-TCGTTCCACATTCTGTACCAA-3'	(Baek et al., 2015)
Synaptic markers	*SYN1*	Frw: 5'-GCAAGGACGGAAGGGATCACATCA-3' Rev: 5'-CCTGAGCCATCTTGTTGACCACGA-3'	(Ho et al., 2016)
	PSD95	Frw: 5'-GGCAGCCCTGAAGAACACGTATGA-3' Rev: 5'-CCCAGGTAGCTGCTGTGACTGATC-3'	(Ho et al., 2016)
Neurogenic transcription factors	*NEUROG2*	Frw: 5'-CAAGCTCACCAAGATCGAGACC-3' Rev: 5'-AGCAACACTGCCTCGGAGAAGA-3'	(Kim et al., 2014)
	ASCL1	Frw: 5'-GCGGCCAACAAGAAGATGAG-3' Rev: 5'-AGTCGTTGGAGTAGTTGGGG-3'	In this study
	mDLX2	Frw: 5'-GCCTCAACAATGTCTCCTACTC-3' Rev: 5'-ATTTCAGGCTCAAGGTCTTCC -3'	In this study
	hDLX2	Frw: 5'-GCACATGGGTTCCTACCAGT-3' Rev: 5'-TCCTTCTCAGGCTCGTTGTT-3'	(Park et al., 2017)
GABAergic neuronal markers	*NKX2-1*	Frw: 5'-AGCACACGACTCCGTTCTC-3' Rev: 5'-GCCCACTTTCTTGTAGCTTTCC-3'	(Y. Sun et al., 2016)
	GAD1	Frw: 5'-TTGCACCAGTGTTTGTCCTCATGG-3' Rev: 5'-CCGGGAAGTACTTGTAGCGAGCAG-3'	(Barretto et al., 2020)
	GAD2	Frw: 5'-CTATGACACTGGAGACAAGGC-3' Rev: 5'-CAAACATTTATCAACATGCGCTTC-3'	(Barretto et al., 2020)
	SLC32A1	Frw: 5'-CACGACAAGCCCAAAATCAC-3' Rev: 5'-CGGCGAAGATGATGAGAAACAAC-3'	(Barretto et al., 2020)
	GABRA2	Frw: 5'-GTTCAAGCTGAATGCCCAAT -3' Rev: 5'-ACCTAGAGCCATCAGGAGCA -3'	(Park et al., 2017)
	CALB2	Frw: 5'-CTCCAGGAATACACCCAAA -3' Rev: 5'-CAGCTCATGCTCGTCAATGT -3'	(Park et al., 2017)
	SST	Frw: 5'-GCTGCTGTCTGAACCCAAC -3' Rev: 5'-CGTTCTCGGGGTGCCATAG -3'	(Park et al., 2017)
Glutamatergic neuronal markers	*SLC17A7*	Frw: 5'-CGCATCATGTCCACCACCAACGT-3' Rev: 5'-GAGTAGCCGACCACCAACAGCAG-3'	(Ho et al., 2016)
	SLC17A6	Frw: 5'-TCAACAACAGCACCATCCACCGC-3' Rev: 5'-GTTTCCGGGTCCCAGTTGAATTTGG-3'	(Ho et al., 2016)

[Supplementary Table 1]

Immunocytochemistry and imaging

Cells grown in coverslips were gently washed 1x PBS and fixed with 4 % (v/v) paraformaldehyde (Alfa Aesar, cat. no. J61899AK) for 15 min on ice. Fixed cells were blocked for 15 min in SuperBlock (PBS) Blocking Buffer (Thermo Scientific, cat. no. 37515) with 0.3 % Triton X-100 (Alfa Aesar, cat. no. A16046AE), and incubated with primary antibodies at 4 °C overnight. The information of primary antibodies and their dilutions used in this study are as follows: MAP2 (Chicken, 1:1,000, Aves Labs, AB_2313549), FOXG1 (Rabbit, 1:500, abcam, ab18259), NGN2 (Rabbit, 1:250, Cell Signaling Technology, 13144S), vGLUT1 (Guinea pig, 1:500, Sigma-Aldrich, AB5905), DLX2 (Rabbit, 1:100, Invitrogen, 702009), GABA (Rabbit, 1:400, Sigma-Aldrich, A2052), and vGAT (Rabbit, 1:500, Synaptic Systems, 131 003). The next day, cells were washed three times with PBS and incubated with fluorescently conjugated secondary antibodies (Chicken fluorescein (Aves Labs, F-1005), Rabbit Alexa Fluor 568 (Invitrogen, A10042), and Guinea Pig Alexa Fluor 594 (Invitrogen, A11076)) for 1 hour at room temperature. Cells were washed with 1x PBS for 5 times and mounted onto a glass slide using ProLong Diamond Antifade Mountant with DAPI (Molecular Probes, cat. no. P36971). Images were acquired with a EVOS BX53 microscope (Olympus) 40x objective for immunostained cells, and with a EVOS XL Core 10x, and 20x objective for brightfield images. Images were processed with ImageJ (v1.53a NIH, USA).

Whole-cell patch clamp

Electrophysiological recordings were carried out using conventional whole-cell current using neurons at Day 28 (ND28), as previously described (Jacko et al., 2018). Briefly, the external recording solution contained 145 mM NaCl, 5 mM KCl, 10 mM HEPES, 10 mM glucose, 2 mM $CaCl_2$ and 2 mM $MgCl_2$, pH 7.3, osmolality 325 mOsm. The pipette solution contained 130 mM CH_3KO_3S, 10 mM CH_3NaO_3S, 1 mM $CaCl_2$, 10 mM EGTA, 10 mM HEPES, 5 mM MgATP and 0.5 mM Na_2GTP (pH 7.3, 305 mOsm). A -14 mV liquid junction potential correction was applied before the experiment. During recordings, current (< 100 pA) was manually injected to hold the cells at approximately −60 mV. Action potentials were evoked using 1 s duration current steps incrementally increasing by 5 pA. Experiments were performed at room temperature.

REAGENTS AND SOLUTIONS

For culture recipes and steps, use sterile tissue culture-grade water. For other purposes, use deionized, distilled water or equivalent in recipes and protocol steps.

Gelatinized plates (for HEK 293T culture)

Coat plates with enough 0.1 % (w/v) gelatin solution (Sigma-Aldrich, cat. no. SF008) to cover the surface and incubate at 37 °C. Remove gelatin after 15 min and the plates can be used immediately.

FM10 media (for HEK 293T culture)

For a total volume for 500 mL:
Dulbecco's modified Eagle's media (DMEM; Gibco, cat. no. 11965-092)
10 % (v/v) heat-inactivated fetal bovine serum (FBS; Gibco, cat. no. 16140-071): 50 mL
1x GlutaMAX (Gibco, cat. no. 35050-061): 5 mL
1x 10 mM non-essential amino acids (Gibco, cat. no.11140-050): 5 mL
1x Penicillin/streptomycin (Gibco, cat. no. 15140-122): 5mL
0.1 mM β 2-mercaptoethanol (Gibco, cat. no. 21985-023): 900 µL
Store up to 3 weeks at 4 °C.

Cultrex-coated plates

It is recommended to keep Cultrex on ice in a refrigerator during thawing process. Working quickly,resuspend 250 μL of Cultrex in 25 mL of cold DMEM/F12 media base. Add 1 mL/well of a 6-well plate, and incubate inside the culture hood for at least 1 hour. Swirl plate for an even distribution, and ensure the entire surface is covered. Seal plate with Parafilm and store up to 3 weeks at 4 °C. Plates must be incubated at 37 °C for at least 30 min prior to usage.

StemFlex media (for feeder-free hPSC culture)

For a total volume for 500 mL:
StemFlex media (Gibco, cat. no. A33494-01)
10x StemFlex supplement (Gibco, cat. no. A33494-01): 50 mL
1x Penicillin/streptomycin (Gibco, cat. no. 15140-122): 5 mL
Store up to 3 weeks at 4 °C.

Freezing media

For a total volume for 10 mL:
45 % (v/v) pluripotent stem cell media (or any culture media): 4.5 mL
45 % (v/v) KnockOut serum replacement (Gibco, cat. no. 10828-028): 4. 5 mL
10 % (v/v) dimethyl sulfoxide (DMSO; Sigma-Aldrich, cat. no. D2650): 1 mL
Store up to 3 weeks at 4 °C.

0.1 % Polyethylenimine (PEI) solution (1x)

Mix 2 mL of 20x Polyethylenimine (PEI; Sigma-Aldrich, cat. no. 408727), 8 mL of 0.5 M sodium borate (Alfa Aesar, cat. no. J62902AP), and 30 mL of sterile water. Filter and store up to a week at 4 °C. For 20x PEI, dilute PEI in 0.1 M borate solution (1:1000) and store up to a month at 4 °C. Please note that PEI is very viscous.

PEI/laminin-coated plates

Dispense 1x PEI solution into each well of a plate (0. 5 mL for 12-well, 24-well, and 1 mL for 6-well plate) and incubate at 37 °C for 1 hour. Aspirate PEI and wash with sterile water five times and completely air-dry in the biosafety cabinet. Dispense laminin (10 μg/mL) solution in DPBS and store at 4 °C. Coated plates can be kept in the incubator right after the addition of laminin for 6 hours to overnight. Alternatively, seal plates with Parafilm and store up to 1 week at 4 °C. Plates must be incubated at 37 °C incubator overnight prior to use. For immunostaining and whole-cell patch clamp purposes, prepare the coatings on a glass coverslip. Use sterile forceps to dip a glass coverslip into 70 % ethanol and place in each well of a 24-well plate. Let them dry for 15 min. After complete dry of ethanol, proceed with PEI/laminin coating procedure as described above. Ensure that coverslips are completely submerged in the coating solution, as they tend to float. If this happens, use a sterile plastic pipette tip to push the coverslip to the bottom of the well.

Neuronal differentiation media

For a total volume for 500 mL:
DMEM/F12 media base (Gibco, cat. no. 11320-033)
1x N2 supplement (Gibco, cat. no. 17502-048): 5 mL
1x B27 supplement (Gibco, cat. no.17504-044): 10 mL
1x Penicillin/streptomycin (Gibco, cat. no. 15140-122): 5 mL
Store up to 3 weeks at 4 °C.

BrainPhys neuronal media (for neuronal maturation)

For a total volume for 500 mL:
BrainPhys Neuronal Medium N2-A & SM1 Kit (STEMCELL technologies, cat. no. 05793)
BrainPhys neuronal medium (STEMCELL technologies, cat. no. 05790)
1x SM1 supplement (STEMCELL technologies, cat. no. 05711): 10 mL
1x N2 supplement-A (STEMCELL technologies, cat. no. 07152): 5 mL
1x Penicillin/streptomycin (Gibco, cat. no. 15140-122): 5 mL

Store up to 3 weeks at 4 °C.

10 mM ROCK inhibitor Y-27632 (1000x stock)

Reconstitute Y-27632 to a final concentration of 10 mM in DMSO (3.122 mL for 10 mg) and stored in aliquots at -20 °C. Avoid multiple freeze-thaw cycle. Aliquots in use can be maintained for 2 weeks at 4 °C.

20 µg/mL BDNF (2000x stock)

Resuspend lyophilized BDNF (R&D Systems, cat. no. 248-BDB) to a final concentration of 20 µg/mL in PBS containing 0.1 % (w/v) bovine serum albumin (BSA). Store at -80 °C after reconstitution. Avoid multiple freeze-thaw cycle. Aliquots in use can be maintained for 2 weeks at 4 °C.

10 µg/mL NT3 (1000x stock)

Resuspend lyophilized NT3 (R&D Systems, cat. no. 267-N3-005) to a final concentration of 10 µg/mL in PBS containing 0.1 % (w/v) BSA. Store at -80 °C for long-term storage. Avoid multiple freeze-thaw cycle. Aliquots in use can be maintained for 2 weeks at 4 °C.

5 mM Ara-C (2500x stock)

Resuspend Ara-C (TOCRIS, cat. no. 4520) to a 25 mM concentrated stock (10 mL for 60.8 mg) in sterile water. Filter-sterilize and dilute to 5 mM to obtain 2500x aliquots for daily use. Further dilute to 100 µM for the low-dose treatment. Aliquot and store at -20 °C. Aliquots in use can be maintained for 3 weeks at 4 °C.

1 mg/mL Doxycycline (1000x stock)

Resuspend Doxycycline hyclate (Sigma-Aldrich, cat. no. D9891) to a 1 mg/mL in sterile water. Filter-sterilize and store them away from light. Store aliquots at -20 °C for long-term storage. Aliquots in use can be maintained for 3 weeks at 4 °C.

COMMENTARY

Background Information

Human neuronal cultures derived from hPSCs provide a unique opportunity to investigate previously inaccessible aspects of human brain biology in health and disease and can provide functional cell types for neuronal disease modeling and cell therapy approaches (Dolmetsch & Geschwind, 2011; Sproul, 2015). Many neurodevelopmental and neurodegenerative diseases have selective cellular vulnerability in different brain regions or cell types (Fu, Hardy, & Duff, 2018). Therefore, the need arises for the capacity to efficiently generate isolated population of excitatory and inhibitory neurons in order to understand the underlying mechanisms of this differential vulnerability and pathogenesis.

There has been substantial progress in directed differentiation of hPSCs into functional neurons using stepwise differentiation approaches that employ small molecules, growth factors, and extracellular signals to recapitulate key aspects of neurogenesis *in vivo* (Kirwan et al., 2015; Shi, Kirwan, & Livesey, 2012). However, often these direct differentiation protocols require long maturation times and/or generate heterogeneous cell populations that contain both excitatory and inhibitory neurons, along with emergence of astrocytes in the culture, hence complicating experimental setup and data interpretation.

Recent studies have introduced the use of the neurogenic transcription factor Ngn2 by lentivirus transduction into hPSCs or human neuronal progenitor cells (hNPCs), leading to direct conversion of hPSCs into functional EX-neurons within 4 weeks (Barretto et al., 2020; Chanda et al., 2014; Ho et al., 2016; Nehme et al., 2018; Nicholas et al., 2013; A. X. Sun et al., 2016; Yang

et al., 2017; Zhang et al., 2013). In a similar approach, several studies use the neurogenic transcription factors Ascl1 and Dlx2, resulting hPSC or hNPCs into functional IN-neurons (Barretto et al., 2020; Chanda et al., 2014; Nicholas et al., 2013; A. X. Sun et al., 2016; Yang et al., 2017). Except for a study from Frega et al. (Frega et al., 2017), these transient protocols require transduction of viruses every differentiation, which is cumbersome and more challenging to scale up neuronal culture for large scale assays such as drug screens. It can also lead to different viral batch effects on differentiation efficiency.

We have observed that a minority of undifferentiated stem cells after lentivirus transduction survive antibiotic selection without treatment of doxycycline (Figure 2). This leaky expression of transcription factors and antibiotic resistance genes are sufficient to establish stable hPSCs that harbor the neurogenic transgene. Hence, we have built on conventional protocols which use transient transduction of lentivirus for both EX (Ngn2) and IN (Ascl1, Dlx2) to undifferentiated hPSCs, followed by antibiotic selection in order to remove non-transduced cells. In order to make stable IN-hPSCs, it requires sequential combinatorial generation of stable cell lines (Ascl1-hPSC, followed by Dlx2 transduction) due to high cell toxicity while selecting for multiple antibiotics in parallel. After expanding the surviving clones, we confirmed that these cells can be successfully differentiated into EX- and IN-neurons, respectively, by using defined and standard culture conditions, which allows for both isolated EX and IN populations as well as co-cultures of the two subtypes of neurons at the same time (Figure 3, 4).

We also found high efficiency of transduction by adding virus into dissociated hPSCs prior to cell plating, so called reverse-transduction, and it does not require polybrene, which often has high cellular toxicity. We use cytosine β-D-arabinofuranoside (Ara-C) during the early neuronal differentiation period, a cytosine analog and a potent inhibitor of replicative DNA synthesis, in order to remove any remaining proliferating hPSCs in the culture as well as other potential contaminating cycling cells. In addition, this protocol does not require murine astrocytes in the neuronal culture, hence any further dissociation of neurons is unnecessary once they are plated on the original plates. With the currently described protocol, we were able to observe neuronal morphology within a week, and these neurons can be further maintained for several additional weeks for functional maturation.

Critical Parameters and Troubleshooting

It is essential to have good quality starting material for successful stable hPSC cell line establishment. In order to obtain high quality of lentivirus, HEK 293T cells should be split regularly to prevent overgrowth and should be near but not fully confluent before transfection using Lipofectamine 3000 reagent. Ultra-pure plasmid DNAs is also required for the successful transfection and production of virus. If hPSCs for transduction are not growing well, consider thawing a new vial of cells, changing to a new batch of StemFlex media, or a new batch of Cultrex. We recommend sub-passaging hPSC culture a maximum of ten times in order to avoid genomic instability. We have tested 10 times of sub-passaging after incorporating virus into hPSCs, and these cells still produce robust functional neurons. We recommend banking early passaged reverse-transduced hPSCs into cryogenic storage, sub-passaging less than 10 times, and thawing earlier passage cells if the differentiation efficiency start to decrease.

The correct initial cell plating density is important for the successful neuronal differentiation. Since this protocol does not require further lifting and re-plating of cells, it is important to plate the proper number of cells initially. If the cultures are too dense, neurons have limited space for connecting and spreading out their processes with other neurons. If cultures are too sparse, the survival rate decreases. We found that 2.2 - 2.8 x 10^5 cells/well in 12-well plates is adequate for the most of downstream applications such as immunocytochemistry, electrophysiology and RNA isolation, although this density can be modified based on user preference. We also suggest to use robust coating substrates (PEI/laminin) and plate format (12-mm or 15-mm coverslips, 12-

well or 24-well plate) depending on your experiments. Treatment of Ara-C can be a bit challenging, as there are cell line-specific differences. We treat with a high dose of Ara-C in initial stage of differentiation in order to remove remaining hPSCs, but discontinue Ara-C in later stages of differentiation to preserve neuronal health. Hence, we propose to treat twice with a high dose of Ara-C (2 μM) and subsequently lower the dose to 100 nM for a week. We highly recommend performing a pilot experiment to determine appropriate cell plating density and Ara-C treatment conditions before scaling up to larger and longer culture duration.

In order to improve neuronal maturity, we recommend switching basal media to BrainPhys neuronal media and associated supplements after a week of doxycycline induction. To do so, we recommend gradually transitioning the basal media from DMEM/F12 to BrainPhys media via half-medium changes. We add mACM to neuronal culture in order to support the survival and promote functional maturity of neurons without exogeneous cell addition (Yan et al., 2013).

Step	Problem	Possible cause(s)	Solution(s)
Basic **Prot. 1; Step 1 - 15**	Low yield of lentivirus	• Unhealthy HEK 293T cells • Low efficiency of Lipofectamine transfection • Unsuccessful concentration of virus	• Thaw new batch of cells. • Test the transfection efficiency by fluorescence expressing plasmid (*e.g.*, GFP). • Check the purity of plasmid DNA and reagents. • Chill concentrated virus supernatants longer at 4 °C before the final centrifugation.
Basic **Prot. 1; Step 16 - 26**	Low efficiency of transduction to hPSCs	• Unhealthy hPSCs • Low yield of lentivirus particles	• Thaw new batch of cells. • Work with early passage cells. • Calculate the virus titer using quantitative methods such as qPCR.
Basic **Prot. 1; Step 22 - 26**	No surviving cells after antibiotic selection	• Poor/no transduction into hPSCs • Wrong dose/duration of antibiotic	• Check the transduction efficiency by GFP viral transduction. • Determine the kill curve of antibiotics before conducting experiments. Set up control wells with no virus and treat with antibiotics until complete death of cells.
Basic Prot. **1; step 17-20, Basic Prot. 2; Step 2-5**	Low viabiliy of cells after Accutase passaging	• Incubation with Accutase is too long • Too much pipetting during resuspension	• Determine the proper duration of Accutase treatment and perform gentle trituration after harvesting cells.
Throughou t the process	No neurons are made	• Transduction issue • Reagent issue	• Transduction of virus into hPSCs is low. • Make fresh media. • Ensure that neuronal differentiation reagents are working and switch to new aliquots of reagents if needed.
Basic Prot. **2; Step 6 - 10**	Too much cell death after Ara-C treatment	• Too much Ara-C treatment	• Carefully observe cell status after treatment of Ara-C. Shorten the period of treatment if no flat cells are observed in the culture.
Basic Prot. **2; Step 5 - 12**	Cells lifting from plates	• Insufficient coating issue • Physical force during media change	• Fresh prepare coating materials (*e.g.* 1x PEI). • Be gentle during media change.

Basic Prot. **2;** **Step 1 - 12**	Neurons are not made as efficiently	• Viral integration to hPSCs is reduced/promoter is turned off	• Thaw earlier passage hPSCs.
Basic **Prot. 2;** **Step 8 - 12**	Neurons get unhealthy	• Media issue • Ara-C treatment • Cell lifting	• Remake medium if necessary. Ensure all reagents are working and used at the correct concentration. • Withdraw Ara-C treatment once no undifferentiated cells are observed in the culture. • Add laminin at least once per week.

[Table 1] Troubleshooting guide

Understanding Results

This protocol generates functional human cortical EX- and IN-neurons. With the current protocol, a single (EX-hPSC) or two-step virus transduction (IN-hPSC) into hPSCs allows continual maintenance of hPSCs to feed into a neuronal differentiation paradigm (Figure 1). qRT-PCR analysis showed the relative mRNA expression of transcription factors from established hPSCs upon doxycycline treatment (Figure 2B). As expected, expression of all transcription factors was highly increased due to doxycycline-induced activation. Since the *DLX2* promoter for IN-hPSCs is derived from the mouse *DLX2* sequence, we confirmed the increased expression of mDLX2. Note increased levels of *NEUROG2* (a 1.8-fold increase in Log_{10} scale compared to ES cells) in EX-hPSCs and *ASCL1* (a 2.3-fold increase in Log_{10} scale) and *DLX2* (a 1.9-fold increase in Log_{10} scale) in IN-hPSCs even without doxycycline, supporting our observation that target and antibiotic genes are expressed in a leaky manner without doxycycline induction. Interestingly, we also observed upregulated expression of *NEUROG2* in Ascl1-hPSCs (a 2-fold increase in Log_{10} scale compared to ES cells) but not *vice versa*, which is in line with previous studies showing a hierarchical role of Ascl1 in regulating expression of *NEUROG2* (Figure 2B) (Aydin et al., 2019; Chanda et al., 2014; Vasconcelos & Castro, 2014).

Most differentiated neurons exhibit neuronal morphology, such as a polarized cell shape with distinctive axon and dendrite structures and expression of pan-neuronal marker (MAP2) (Figure 3A-D). We assessed characteristics of EX- and IN-neurons by target gene expression of standard marker genes and immunostaining against to these markers by employing molecular and functional assays. We found glutamatergic neuronal markers (NGN2, vGlut1) were stained positive in EX-neurons, while GABAergic neuronal markers (DLX2, GABA, vGAT) were detected in most IN-neurons (Figure 3C, D). Furthermore, we assessed expression of multiple standard marker genes including a pluripotent stem cell marker (*SOX2*), neurogenic transcription factors (*NEUROG2*, *ASCL1*, *mDLX2*, *hDLX1*), pan-neuronal markers (*MAP2*, *MAPT)*, Cajal-Retzius cells marker (*RELN*), synaptic markers (*SYN1*, *PSD95*), glutamatergic (*SLC17A7*, *SLC17A6*), and GABAergic neuronal markers (*NKX2-1*, *GAD1*, *GAD2*, *SLC32A1*, *CALB2*, *GABRA2, SST*) at the mRNA level from day 14 and 28 of EX- and IN-neurons, respectively (Figure 3E, F). Consistently, qRT-PCR also revealed gene expression patterns of isolated populations of EX- and IN-neurons for glutamatergic, and GABAergic neuronal genes, respectively. Notably, dramatic increases of GABAergic neuronal markers in IN-neurons were found for multiple target genes. We also confirmed the increased expression of human *DLX2* gene as well as mouse *DLX2* gene in IN-hPSCs, supporting that these neurons are committed to GABAergic neuronal fate. Samples were harvested at two different time-points (ND14 and 28) and there were no evident differences of gene expression changes by the differentiation duration among most of the target genes tested here. Consistent with increased level of *NEUROG2* in Ascl1-hPSCs (Figure 2B), this feature seems to be extended in differentiated neurons, showing increase of gene expression of glutamatergic neuronal markers (*SLC17A7*, *SLC17A6*) in IN-neurons (a 1.9-fold increase in EX14,

a 1.2-fold increase in IN14, a 1.8-fold increase in EX28, and 1.5-fold increase in IN28 for *SLC17A7* gene, all in Log_{10} scale), though the expression level change is higher in EX-neurons than IN-neurons. Meanwhile, GABAergic neuronal markers (*GAD1*, *GAD2*, *SLC32A1*, *CALB2*, *GABRA2*) were mostly exclusively expressed in IN-neurons, but not in EX-neurons (Figure 3E, F). These results indicate that the successful development of stable hPSCs into isolated population of neurons with molecular and cellular features of EX- and IN-neurons. Recently, two studies reported multiple cell populations in their human NGN2 derived induced neurons (iN) (similar to our EX-neuron), including those positive for sensory neuronal markers *PRPH* (intermediate neurofilament peripherin), and homeodomain transcription factors (*POU4F1*/*BRN3A*) and *PHOX2B* from single-cell RNA-seq analysis. This suggests that NGN2 can also induce the differentiation of PSC into nociceptive sensory neurons (H.-C. Lin et al., 2020; Schornig et al., 2021), as well as EX neurons with a mixture of sensory and cortical transcriptional profiles. However, it is noteworthy that the majority of neurons were found to express cortical markers and only a minority expressed a sensory neuron lineage marker at the protein level. We also found some evidence that a sensory neuron transcriptional signature might be present in this and other published NGN2-iN studies by assessment by gene expression of sensory marker genes from RNA-seq analysis (Chen et al., 2020; Y. T. Lin et al., 2018; Nehme et al., 2018). The use of additional transcription factors and/or patterning agents may improve the transcriptional fidelity of these neuronal cell types in future studies.

In order to determine whether these cells have characteristics of functional neurons, we assessed the excitability of EX- and IN-neurons using whole-cell patch-clamp recording. Over maturation, these neurons present functional electrophysiological properties such as a development of stable resting membrane potentials and action potential (AP) generation (Figure 4). The relatively high capacitance, hyperpolarized membrane potential, and low membrane resistance, as well as the ability to fire repetitive APs, is consistent with the properties of a mature neuron. This result suggests that EX- and IN-neurons made by the current protocol function as mature neurons without murine astrocyte co-culture, although astrocyte co-culture might further accelerate synaptic activity by a contact-dependent mechanism (Chung, Allen, & Eroglu, 2015; Johnson, Weick, Pearce, & Zhang, 2007).

We have generated 9 EX-hPSC lines (7 from hES, and 2 from hiPSCs, including CRISPR-Cas9 engineered mutant lines) and 3 IN-hPSC lines (2 from hES, and 1 from hiPSCs) using the current protocol, and have confirmed that all lines produce robust and reproducible EX- or IN-neurons up to 10 sub-passages by following standard and defined culture conditions.

We believe that this protocol will be a robust tool for molecular profiling and drug screening assays due to its scalability and rapid neuronal maturation without the need of murine glia. In addition, this isolated culture protocol can be further adapted to new applications such as co-culture of EX- and IN-neurons to mimic a more physiologically relevant neuronal environment. For example, EX-hPSCs expressing a fluorescent protein (eg. Ngn2-2A-GFP) may be plated with IN-hPSCs and differentiated into neurons in parallel. The two types of neurons can be easily distinguishable by fluorescent microscopy, making it as a useful platform for investigating selective neuronal vulnerability in a physiologically-relevant environment.

Time Considerations

Basic Protocol 1 consists of two parts: an in-house production of lentivirus from HEK 293T cells, requiring a week at minimum, and 2 weeks of establishment of hPSCs including reverse-transduction, selection, expansion, and banking. To make IN-hPSCs, it takes about 3-4 weeks since it requires two-step sequential transduction. Once a batch of lentivirus is made, it should be aliquoted and stored at -80 °C for long-term storage. Note that it only requires 5 μL of concentrated lentivirus in order to establish stable and inducible hPSC lines. Once the EX- and IN-hPSC lines are generated, they can be expanded further and banked for long term use. We recommend to

freeze early passaged cells, maintain hPSC culture below 10 passages and thaw early passaged cells if efficiency of differentiation decreases. Basic Protocol 2 is a longer protocol but relatively low-maintenance, requiring 1 week of induction upon doxycycline treatment, and then 3 weeks of differentiation, and requires the continual maintenance of neurons. Neuronal morphological assay such as immunostaining analysis and gene expression analysis such as qRT-PCR are possible within two weeks of neuronal differentiation, however it requires maintaining of cultures up to 4 weeks in order to observe mature characteristics of neuronal activity by whole-cell patch clamp analysis.

Conflict of Interest: The authors have no conflicts of interest to disclose.

Data Availability: All data supporting the current study are provided in Support Protocol 2.

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Literature Cited

Aydin, B., Kakumanu, A., Rossillo, M., Moreno-Estelles, M., Garipler, G., Ringstad, N., . . . Mazzoni, E. O. (2019). Proneural factors Ascl1 and Neurog2 contribute to neuronal subtype identities by establishing distinct chromatin landscapes. *Nat Neurosci, 22*(6), 897-908. doi:10.1038/s41593-019-0399-y

Baek, S. T., Copeland, B., Yun, E. J., Kwon, S. K., Guemez-Gamboa, A., Schaffer, A. E., . . . Gleeson, J. G. (2015). An AKT3-FOXG1-reelin network underlies defective migration in human focal malformations of cortical development. *Nat Med, 21*(12), 1445-1454. doi:10.1038/nm.3982

Bardy, C., van den Hurk, M., Eames, T., Marchand, C., Hernandez, R. V., Kellogg, M., . . . Gage, F. H. (2015). Neuronal medium that supports basic synaptic functions and activity of human neurons in vitro. *Proc Natl Acad Sci U S A, 112*(20), E2725-2734. doi:10.1073/pnas.1504393112

Barretto, N., Zhang, H., Powell, S. K., Fernando, M. B., Zhang, S., Flaherty, E. K., . . . Brennand, K. J. (2020). ASCL1- and DLX2-induced GABAergic neurons from hiPSC-derived NPCs. *J Neurosci Methods, 334*, 108548. doi:10.1016/j.jneumeth.2019.108548

Chanda, S., Ang, C. E., Davila, J., Pak, C., Mall, M., Lee, Q. Y., . . . Wernig, M. (2014). Generation of induced neuronal cells by the single reprogramming factor ASCL1. *Stem Cell Reports, 3*(2), 282-296. doi:10.1016/j.stemcr.2014.05.020

Chen, M., Maimaitili, M., Habekost, M., Gill, K. P., Mermet-Joret, N., Nabavi, S., . . . Denham, M. (2020). Rapid generation of regionally specified CNS neurons by sequential patterning and conversion of human induced pluripotent stem cells. *Stem Cell Res, 48*, 101945. doi:10.1016/j.scr.2020.101945

Chung, W. S., Allen, N. J., & Eroglu, C. (2015). Astrocytes Control Synapse Formation, Function, and Elimination. *Cold Spring Harb Perspect Biol, 7*(9), a020370. doi:10.1101/cshperspect.a020370

Dolmetsch, R., & Geschwind, D. H. (2011). The human brain in a dish: the promise of iPSC-derived neurons. *Cell, 145*(6), 831-834. doi:10.1016/j.cell.2011.05.034

Frega, M., van Gestel, S. H., Linda, K., van der Raadt, J., Keller, J., Van Rhijn, J. R., . . . Nadif Kasri, N. (2017). Rapid Neuronal Differentiation of Induced Pluripotent Stem Cells for Measuring Network Activity on Micro-electrode Arrays. *J Vis Exp*(119). doi:10.3791/54900

Fu, H., Hardy, J., & Duff, K. E. (2018). Selective vulnerability in neurodegenerative diseases. *Nat Neurosci, 21*(10), 1350-1358. doi:10.1038/s41593-018-0221-2

Galmarini, C. M., Mackey, J. R., & Dumontet, C. (2001). Nucleoside analogues: mechanisms of drug resistance and reversal strategies. *Leukemia, 15*(6), 875-890. doi:10.1038/sj.leu.2402114

Garcia-Leon, J. A., Vitorica, J., & Gutierrez, A. (2019). Use of human pluripotent stem cell-derived cells for neurodegenerative disease modeling and drug screening platform. *Future Medicinal Chemistry, 11*(11), 1305-1322. doi:10.4155/fmc-2018-0520

Ho, S. M., Hartley, B. J., Tcw, J., Beaumont, M., Stafford, K., Slesinger, P. A., & Brennand, K. J. (2016). Rapid Ngn2-induction of excitatory neurons from hiPSC-derived neural progenitor cells. *Methods, 101*, 113-124. doi:10.1016/j.ymeth.2015.11.019

Jacko, M., Weyn-Vanhentenryck, S. M., Smerdon, J. W., Yan, R., Feng, H., Williams, D. J., . . . Zhang, C. (2018). Rbfox Splicing Factors Promote Neuronal Maturation and Axon Initial Segment Assembly. *Neuron, 97*(4), 853-868 e856. doi:10.1016/j.neuron.2018.01.020

Johnson, M. A., Weick, J. P., Pearce, R. A., & Zhang, S. C. (2007). Functional neural development from human embryonic stem cells: accelerated synaptic activity via astrocyte coculture. *J Neurosci, 27*(12), 3069-3077. doi:10.1523/JNEUROSCI.4562-06.2007

Jung, Y. W., Hysolli, E., Kim, K. Y., Tanaka, Y., & Park, I. H. (2012). Human induced pluripotent stem cells and neurodegenerative disease: prospects for novel therapies. *Curr Opin Neurol, 25*(2), 125-130. doi:10.1097/WCO.0b013e3283518226

Kim, Y. J., Lim, H., Li, Z., Oh, Y., Kovlyagina, I., Choi, I. Y., . . . Lee, G. (2014). Generation of multipotent induced neural crest by direct reprogramming of human postnatal fibroblasts with a single transcription factor. *Cell Stem Cell, 15*(4), 497-506. doi:10.1016/j.stem.2014.07.013

Kingston, R. E., Chen, C. A., & Rose, J. K. (2003). Calcium phosphate transfection. *Curr Protoc Mol Biol, Chapter 9*, Unit 9 1. doi:10.1002/0471142727.mb0901s63

Kirwan, P., Turner-Bridger, B., Peter, M., Momoh, A., Arambepola, D., Robinson, H. P., & Livesey, F. J. (2015). Development and function of human cerebral cortex neural networks from pluripotent stem cells in vitro. *Development, 142*(18), 3178-3187. doi:10.1242/dev.123851

Lacovich, V., Espindola, S. L., Alloatti, M., Pozo Devoto, V., Cromberg, L. E., Carna, M. E., . . . Falzone, T. L. (2017). Tau Isoforms Imbalance Impairs the Axonal Transport of the Amyloid Precursor Protein in Human Neurons. *J Neurosci, 37*(1), 58-69. doi:10.1523/JNEUROSCI.2305-16.2016

Li, L., Chao, J., & Shi, Y. (2018). Modeling neurological diseases using iPSC-derived neural cells : iPSC modeling of neurological diseases. *Cell Tissue Res, 371*(1), 143-151. doi:10.1007/s00441-017-2713-x

Lin, H.-C., He, Z., Ebert, S., Schörnig, M., Santel, M., Weigert, A., . . . Treutlein, B. (2020). Ngn2 induces diverse neuronal lineages from human pluripotency. *bioRxiv*, 2020.2011.2019.389445. doi:10.1101/2020.11.19.389445

Lin, Y. T., Seo, J., Gao, F., Feldman, H. M., Wen, H. L., Penney, J., . . . Tsai, L. H. (2018). APOE4 Causes Widespread Molecular and Cellular Alterations Associated with Alzheimer's Disease Phenotypes in Human iPSC-Derived Brain Cell Types. *Neuron, 98*(6), 1294. doi:10.1016/j.neuron.2018.06.011

Maherali, N., Ahfeldt, T., Rigamonti, A., Utikal, J., Cowan, C., & Hochedlinger, K. (2008). A high-efficiency system for the generation and study of human induced pluripotent stem cells. *Cell Stem Cell, 3*(3), 340-345. doi:10.1016/j.stem.2008.08.003

Nehme, R., Zuccaro, E., Ghosh, S. D., Li, C., Sherwood, J. L., Pietilainen, O., . . . Eggan, K. (2018). Combining NGN2 Programming with Developmental Patterning Generates Human Excitatory Neurons with NMDAR-Mediated Synaptic Transmission. *Cell Rep, 23*(8), 2509-2523. doi:10.1016/j.celrep.2018.04.066

Nicholas, C. R., Chen, J., Tang, Y., Southwell, D. G., Chalmers, N., Vogt, D., . . . Kriegstein, A. R. (2013). Functional maturation of hPSC-derived forebrain interneurons requires an extended timeline and mimics human neural development. *Cell Stem Cell, 12*(5), 573-586. doi:10.1016/j.stem.2013.04.005

Park, J., Lee, N., Lee, J., Choe, E. K., Kim, M. K., Lee, J., . . . Chang, M. S. (2017). Small molecule-based lineage switch of human adipose-derived stem cells into neural stem cells and functional GABAergic neurons. *Sci Rep, 7*(1), 10166. doi:10.1038/s41598-017-10394-y

Riera, M., Patel, A., Bures-Jelstrup, A., Corcostegui, B., Chang, S., Pomares, E., . . . Sparrow, J. R. (2019). Generation of two iPS cell lines (FRIMOi003-A and FRIMOi004-A) derived from Stargardt patients carrying ABCA4 compound heterozygous mutations. *Stem Cell Res, 36*, 101389. doi:10.1016/j.scr.2019.101389

Schornig, M., Ju, X., Fast, L., Ebert, S., Weigert, A., Kanton, S., . . . Taverna, E. (2021). Comparison of induced neurons reveals slower structural and functional maturation in humans than in apes. *Elife, 10*. doi:10.7554/eLife.59323

Shi, Y., Kirwan, P., & Livesey, F. J. (2012). Directed differentiation of human pluripotent stem cells to cerebral cortex neurons and neural networks. *Nat Protoc, 7*(10), 1836-1846. doi:10.1038/nprot.2012.116

Sproul, A. A. (2015). Being human: The role of pluripotent stem cells in regenerative medicine and humanizing Alzheimer's disease models. *Mol Aspects Med, 43-44*, 54-65. doi:10.1016/j.mam.2015.06.007

Stewart, S. A., Dykxhoorn, D. M., Palliser, D., Mizuno, H., Yu, E. Y., An, D. S., . . . Novina, C. D. (2003). Lentivirus-delivered stable gene silencing by RNAi in primary cells. *RNA, 9*(4), 493-501. doi:10.1261/rna.2192803

Sun, A. X., Yuan, Q., Tan, S., Xiao, Y., Wang, D., Khoo, A. T., . . . Je, H. S. (2016). Direct Induction and Functional Maturation of Forebrain GABAergic Neurons from Human Pluripotent Stem Cells. *Cell Rep, 16*(7), 1942-1953. doi:10.1016/j.celrep.2016.07.035

Sun, Y., Pasca, S. P., Portmann, T., Goold, C., Worringer, K. A., Guan, W., . . . Dolmetsch, R. E. (2016). A deleterious Nav1.1 mutation selectively impairs telencephalic inhibitory neurons derived from Dravet Syndrome patients. *Elife, 5*. doi:10.7554/eLife.13073

Vasconcelos, F. F., & Castro, D. S. (2014). Transcriptional control of vertebrate neurogenesis by the proneural factor Ascl1. *Front Cell Neurosci, 8*, 412. doi:10.3389/fncel.2014.00412

Yan, J. W., Tan, T. Y., & Huang, Q. L. (2013). Protective effect of astrocyte-conditioned medium on neurons following hypoxia and mechanical injury. *Chin J Traumatol, 16*(1), 3-9.

Yang, N., Chanda, S., Marro, S., Ng, Y. H., Janas, J. A., Haag, D., . . . Wernig, M. (2017). Generation of pure GABAergic neurons by transcription factor programming. *Nat Methods, 14*(6), 621-628. doi:10.1038/nmeth.4291

Yoo, A. S., Sun, A. X., Li, L., Shcheglovitov, A., Portmann, T., Li, Y., . . . Crabtree, G. R. (2011). MicroRNA-mediated conversion of human fibroblasts to neurons. *Nature, 476*(7359), 228-231. doi:10.1038/nature10323

Zhang, Y., Pak, C., Han, Y., Ahlenius, H., Zhang, Z., Chanda, S., . . . Sudhof, T. C. (2013). Rapid single-step induction of functional neurons from human pluripotent stem cells. *Neuron, 78*(5), 785-798. doi:10.1016/j.neuron.2013.05.029

Figure Legends

Figure 1. Schematic diagram of two-step differentiation protocol for isolated EX and IN-neuronal populations using stable LV-integrated human pluripotent stem cells (hPSCs). Basic Protocol 1 describes the protocol for generating stable hPSC lines by reverse-transduction of lentivirus (LV) expressing lineage-specific neurogenic transcription factors. It describes how to produce LV from HEK293T cells and to perform reverse-transduction of LV into hPSCs. Basic Protocol 2 focuses on the induction and maturation of hPSCs into excitatory

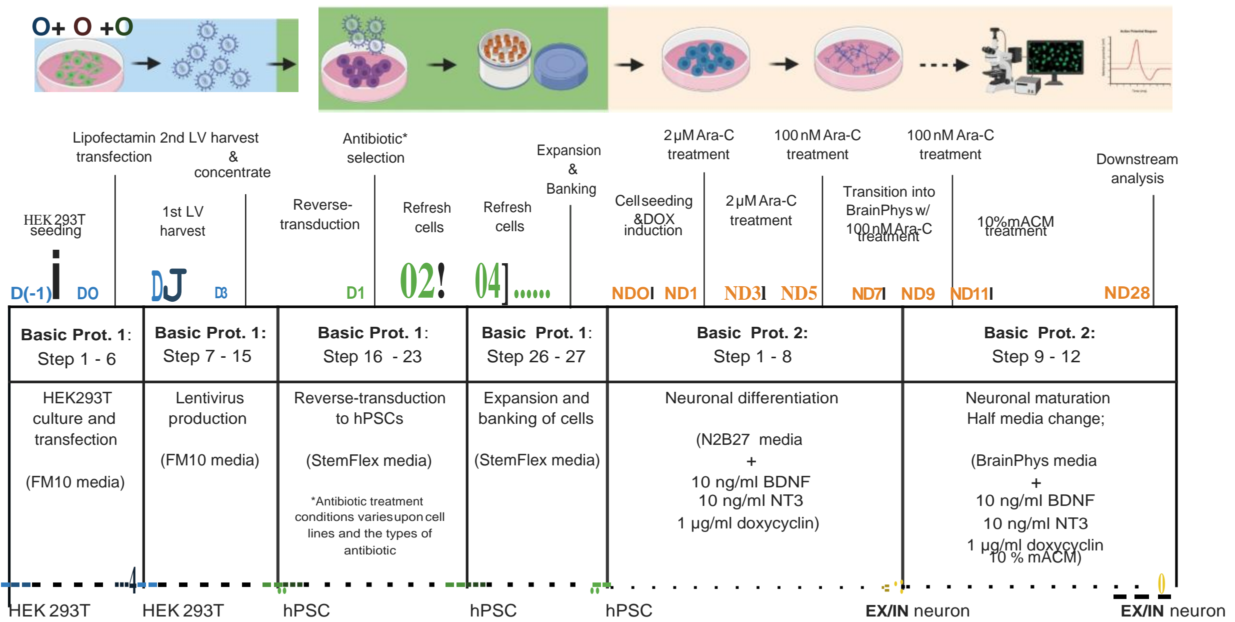
(EX) and inhibitory (IN) neurons, resulting in terminal differentiation into distinct populations of neurons. D refers to a day, ND refers to a day of neuronal differentiation.

Figure 2. Generation of stable EX- and IN-hPSC lines expressing lineage-specific transcription factors. (A) Time-series demonstration of generation of stable hPSCs expressing transcription factors by specific antibiotic selection. Bright field images show stable cell lines. Top row: EX-hPSC with Puromycin selection; Ngn2 with rtTA-integrating line into H9 ES cells. Middle row: Ascl1-hPSC with G418 selection; Ascl1 with rtTA-integrating line into H9 ES cells. Bottom row: IN-hPSC; sequential transduction of mDlx2 lentivirus into Ascl1-hPSCs followed by Hygromycin selection. Surviving colonies for each antibiotic are further expandable. Scale bar, 200 μm. (B) qRT-PCR analysis for mRNA levels of ectopic expression of transcription factors (*NEUROG2*, *ASCL1*, and mouse *DLX2* (*mDLX2)*) normalized to *GAPDH* from established cell lines upon doxycycline (Dox) treatment for 72 hours. Bar graphs represent mean values ±SD. N = 3 per each experiment. Scale is $\log_{10}$ of fold change. One-way ANOVA followed by Tukey's multiple comparison test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

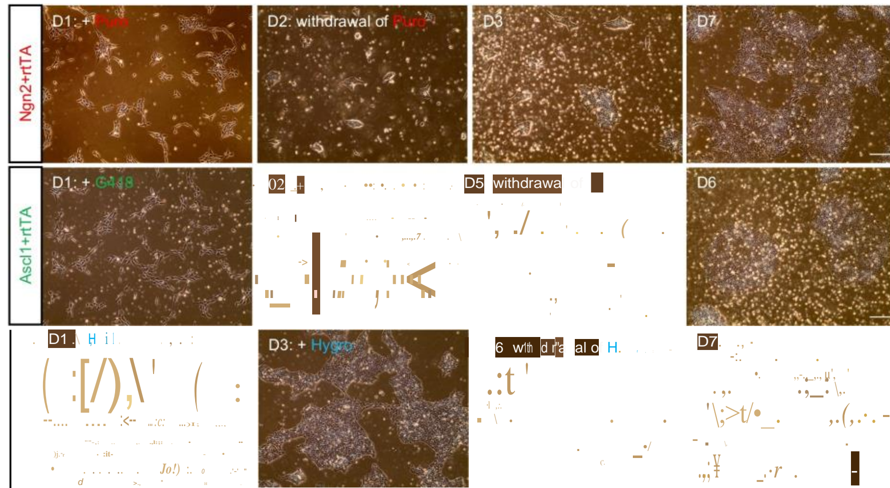
Figure 3. Validation of differentiated human glutamatergic EX- and GABAergic IN-neurons. (A) Brightfield image of time-course differentiation of EX- neurons from stable EX-hPSCs upon doxycycline treatment. (B) Brightfield image of time-course differentiation of IN-neurons from stable IN-hPSCs upon doxycycline treatment. Scale bar, 200 μm (A, B). (C) Representative images of EX-neurons at neuronal differentiation day 28. Cells were stained with general neuronal marker (αMAP2), forebrain marker (αFOXG1), excitatory neuronal markers (αNGN2, αvGlut1), and counter-stained with DAPI for nuclei. (D) Representative images of IN-neurons at neuronal differentiation day 28. Cells were stained with general neuronal marker (αMAP2), GABAergic neuronal markers (αDLX2, αGABA, αvGAT), and counter-stained with DAPI for nuclei. Scale bar, 50 μm (C, D). (E) Heatmap of qRT-PCR analysis in human embryonic stem cells (ES), EX-, and IN-neurons cultured for 14 and 28 days (EX14, IN14, EX28, and IN28), respectively for genes indicated on the left. Expression levels (expressed as Ct values) are color-coded as shown at the right (color scales). Ct, crossing threshold. All experiments were performed in H9-ES cells. (F) Individual bar plots showing relative mRNA expression of target genes normalized to GAPDH from ES, EX, and IN-neurons of 14 and 28 days of differentiation. Graphs are grouped in each category. Bar graphs represent mean values ±SD. N = 5-6 from three independent differentiation experiments (E, F). Scale is $\log_{10}$ of fold change. One-way ANOVA followed by Tukey's multiple comparison test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$) was performed.

Figure 4. EX- and IN-neurons exhibit functional properties consistent with maturity at 28 days after differentiation. (A) Differential interference contrast (DIC) image of an EX-neuron during whole-cell patch clamp recording. Scale bar, 20 μm (A, C). (B) Representative membrane potential recording showing multiple action potentials (APs) evoked by 1 s current step in an EX- neuron. (C) DIC image of an IN-neuron during whole-cell patch clamp recording. (D) Representative membrane potential trace multiple action potentials (APs) are evoked by 1 s current step in an IN-neurons. (E) Summary table of membrane properties EX- and IN-neurons.

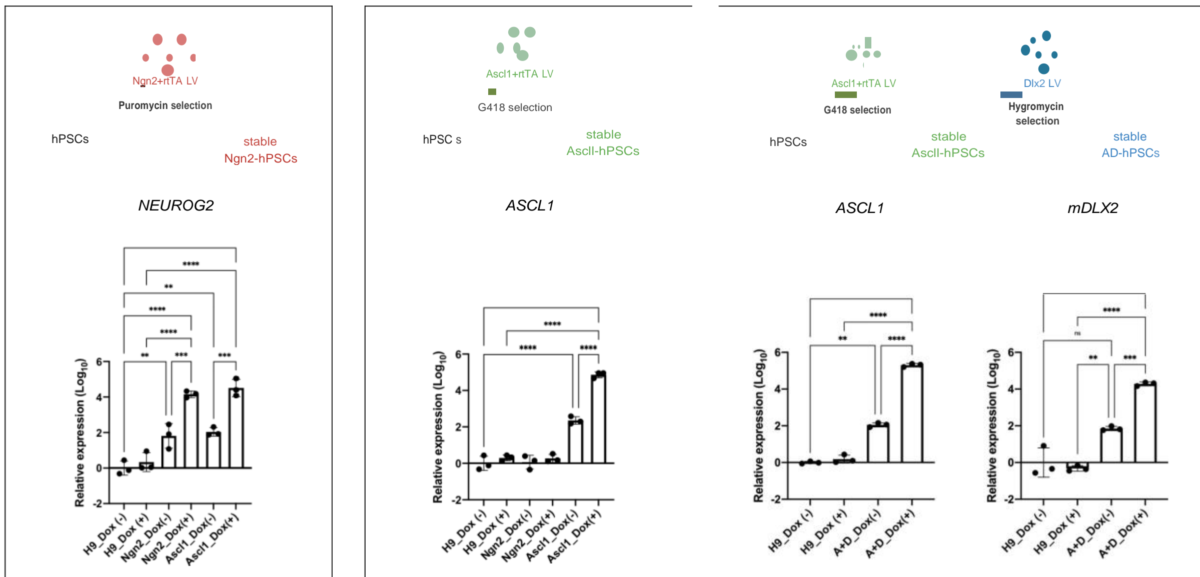
Passive membrane properties such as resting membrane potential (RMP), membrane capacitance (C_{cap}), and input resistance (R_{mem}) were measured. AP characteristics such as number of maximum AP, duration of AP, overshoot of AP, and threshold of AP were analyzed. Parameters represent mean values ±SEM. N = 15-19 cells.

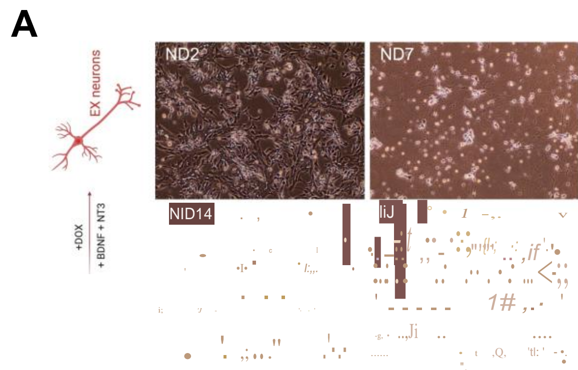

Lipofectamin transfection
2nd LV harvest & concentrate
Antibiotic* selection
Expansion & Banking
2 μM Ara-C treatment
100 nM Ara-C treatment
100 nM Ara-C treatment
Downstream analysis
HEK 293T seeding
1st LV harvest
Reverse-transduction
Refresh cells
Refresh cells
Cell seeding &DOX induction
2 μM Ara-C treatment
Transition into BrainPhys w/ 100 nM Ara-C treatment
10%mACM treatment
D(-1)
DO
D1
NDO
ND1
ND3
ND5
ND7
ND9
ND11
ND28
Basic Prot. 1: Step 1 - 6
HEK293T culture and transfection
(FM10 media)
Basic Prot. 1: Step 7 - 15
Lentivirus production
(FM10 media)
Basic Prot. 1: Step 16 - 23
Reverse-transduction to hPSCs
(StemFlex media)
*Antibiotic treatment conditions varies upon cell lines and the types of antibiotic
Basic Prot. 1: Step 26 - 27
Expansion and banking of cells
(StemFlex media)
Basic Prot. 2: Step 1 - 8
Neuronal differentiation
(N2B27 media + 10 ng/ml BDNF 10 ng/ml NT3 1 μg/ml doxycyclin)
Basic Prot. 2: Step 9 - 12
Neuronal maturation Half media change;
(BrainPhys media + 10 ng/ml BDNF 10 ng/ml NT3 1 μg/ml doxycyclin 10 % mACM)
HEK 293T
HEK 293T
hPSC
hPSC
hPSC
EX/IN neuron
EX/IN neuron

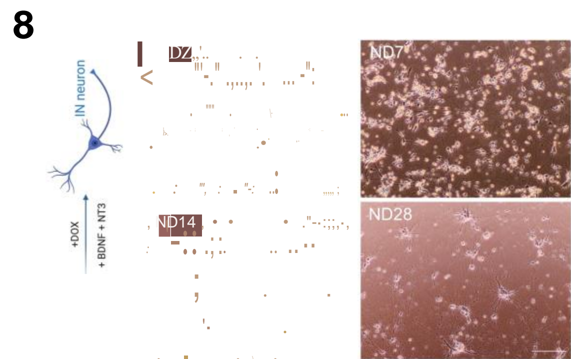
A

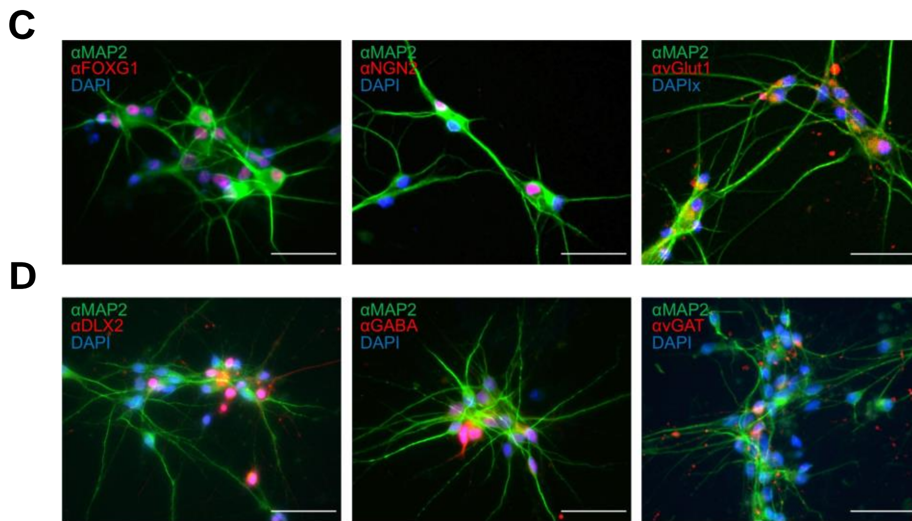


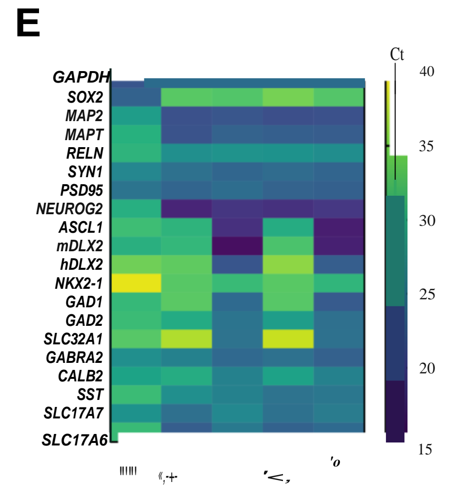
B

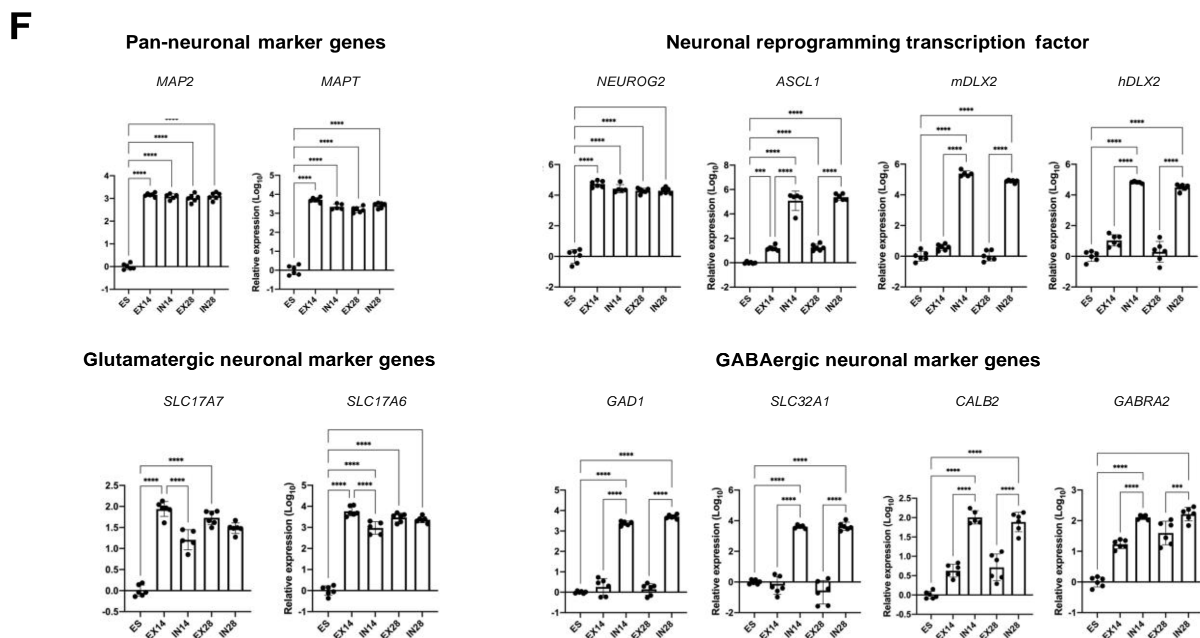


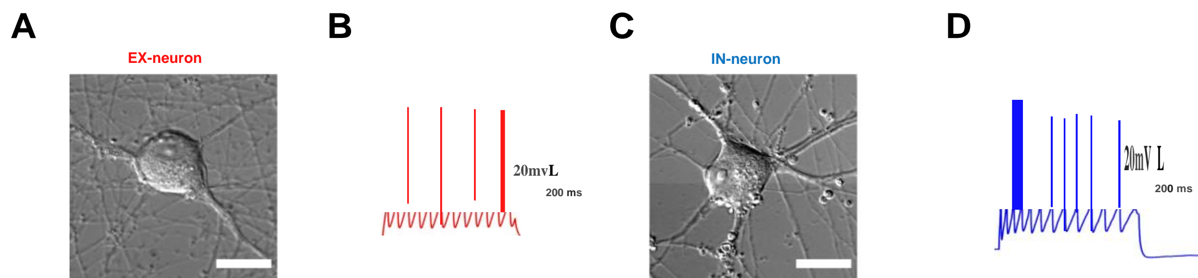

A
EX neurons
ND2
ND7
ND14
+DOX
+ BDNF + NT3


B
IN neuron
ND7
ND14
ND28
+DOX
+ BDNF + NT3


C
αMAP2
αFOXG1
DAPI
αMAP2
αNGN2
DAPI
αMAP2
αvGlut1
DAPI
D
αMAP2
αDLX2
DAPI
αMAP2
αGABA
DAPI
αMAP2
αvGAT
DAPI


E
GAPDH
SOX2
MAP2
MAPT
RELN
SYN1
PSD95
NEUROG2
ASCL1
mDLX2
hDLX2
NKX2-1
GAD1
GAD2
SLC32A1
GABRA2
CALB2
SST
SLC17A7
SLC17A6
Ct
40
35
30
25
20
15


F
Pan-neuronal marker genes
MAP2
MAPT
Neuronal reprogramming transcription factor
NEUROG2
ASCL1
mDLX2
hDLX2
Glutamatergic neuronal marker genes
SLC17A7
SLC17A6
GABAergic neuronal marker genes
GAD1
SLC32A1
CALB2
GABRA2
Relative expression (Log10)
ES
EX14
IN14
EX28
IN28



E

Measurement	EX-neuron			IN-neuron		
	Mean	SEM	N	Mean	SEM	N
AP durat ion (ms)	2.82	0.20	15	2.95	0.1 7	19
AP max num	4.88	1.21	16	4.42	1.21	19
AP overshoot (mV)	32.98	1.88	15	26.19	2.45	19
AP rheobase (pA)	48.58	8.3	15	51.29	6.24	19
AP threshold (mV)	-30.98	1.21	15	-30.48	1.02	19
RMP(mV)	-53.44	1.64	15	-60.68	1.01	19
C,,, (pF)	32.00	1.40	16	33.23	2.24	19
H current (mV)	7.06	0.73	16	3.28	0.41	19
Rmom (MO)	382.91	63.91	16	667.29	109.02	19