Efficient Derivation of Excitatory and Inhibitory Neurons from Human Pluripotent Stem Cells Stably Expressing Direct Reprogramming Factors

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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) and GABAergic (inhibitory) neurons from human pluripotent stem cells (hPSCs) by using the neurogenic transcription factors Ngn2 and a combination of Ascl1 and Dlx2, respectively. In contrast to the majority of neural transdifferentiation protocols that use transient lentiviral infection, the use of stable hPSC lines carrying doxycycline-inducible transcription factors allows neuronal differentiation to be initiated by addition of doxycycline and neural medium. This article presents a method to generate lentivirus from cultured mammalian cells and establish stable transcription factor–expressing cell lines (Basic Protocol 1), followed by a method for monolayer excitatory and inhibitory neuronal differentiation from the established lines (Basic Protocol 2). The resulting neurons reproducibly exhibit properties consistent with human cortical neurons, including the expected morphologies, expression of glutamatergic and GABAergic 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. © 2021 Wiley Periodicals LLC.

Basic Protocol 1: Lentivirus production and generation of stable hPSC lines
Support Protocol 1: Expansion and maintenance of hPSCs
Basic Protocol 2: Differentiation of EX- and IN-neurons
Support Protocol 2: Experimental methods for validation of EX- and IN-neurons

Keywords: doxycycline inducible tet-O system • excitatory neuron • human pluripotent stem cell • inhibitory neuron • neurogenic transcription factors

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INTRODUCTION

Recent advances of human pluripotent stem cell (hPSC)–derived neuronal differentiation in culture 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 (EX; glutamatergic) and inhibitory (IN; GABAergic) 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; Sun, Yuan, et al., 2016; Yang et al., 2017; Zhang et al., 2013), but 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 needed, and reducing the costs of reagents and manpower associated with making fresh lentiviral preparations. In addition, it eliminates the use of primary murine astrocyte cultures by substituting them with a commercial astrocyte conditioned medium.

Basic Protocol 1 provides a method for generating lentiviral particles that express excitatory and inhibitory neurogenic transcription factors (TFs) and then using lentiviral transduction to establish stable hPSC lines that express these TFs (EX- or IN-hPSCs, respectively). Basic Protocol 2 describes a method for in-parallel differentiation and maturation of EX- and IN-neurons from these hPSCs. The process is outlined in Figure 1. We also provide detailed descriptions for general maintenance of hPSC cultures (Support Protocol 1) and characterization of differentiated EX- and IN-neurons (Support Protocol 2).

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

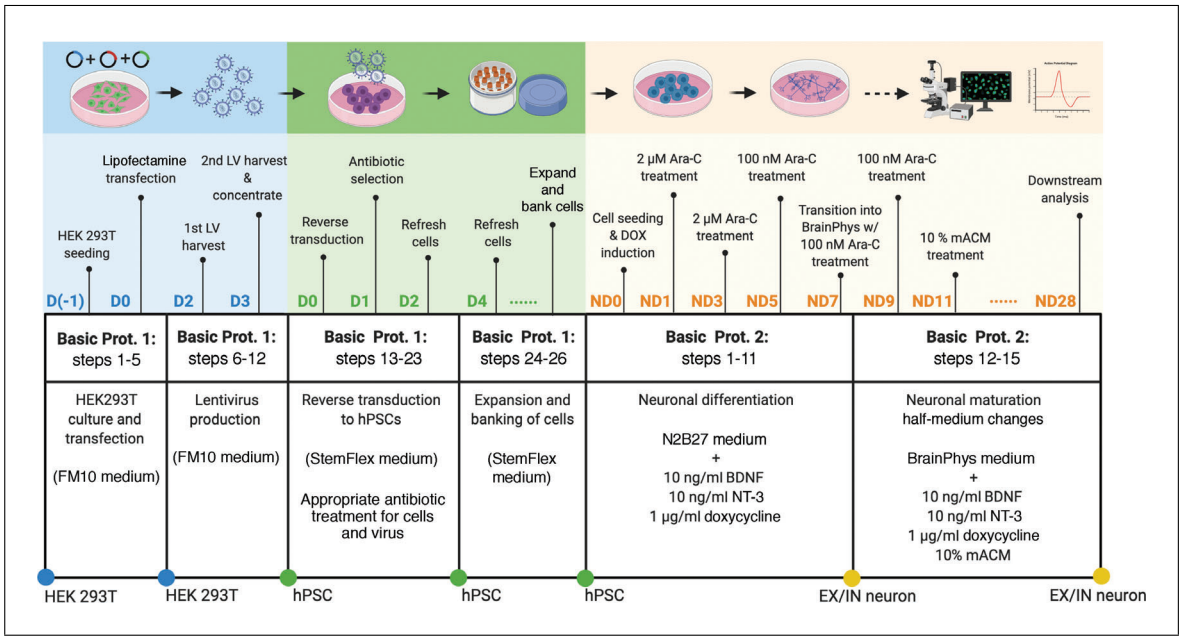


Figure 1 Schematic diagram of two-step differentiation protocol for isolated EX- and IN- neuronal populations using stable lentivirus-integrated human pluripotent stem cells (hPSCs). Basic Protocol 1 describes the production of lentiviruses expressing lineage-specific neurogenic transcription factors in HEK 293 cells, followed by reverse transduction of the lentiviruses into hPSCs to create stable hPSC lines. Basic Protocol 2 focuses on the induction and maturation of hPSCs into distinct populations of excitatory (EX) and inhibitory (IN) neurons. Abbreviations: D, day; ND, day of neuronal differentiation; DOX, doxycycline; LV, lentivirus.

NOTE: All solutions and equipment coming into contact with live cells must be sterile, and proper aseptic technique should be used accordingly. All cell cultures are maintained in a humidified 37°C, 5% CO_2 incubator. All media, reagents, and coated plates should be warmed to 37°C prior to use.

LENTIVIRUS PRODUCTION AND GENERATION OF STABLE hPSC LINES

BASIC PROTOCOL 1

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 a reverse tetracycline-controlled transactivator (rtTA) in HEK 293T cells. Lentiviruses are used for subsequent establishment of stable hPSC lines carrying each virus (Fig. 1). We have found that the plasmid promoters are leaky enough to express sufficient levels of the target and associated antibiotic-resistance transgenes, allowing us to generate stable cell lines without doxycycline induction (Fig. 2). Once these lines are established, they are easy to maintain, propagate, and bank for long-term

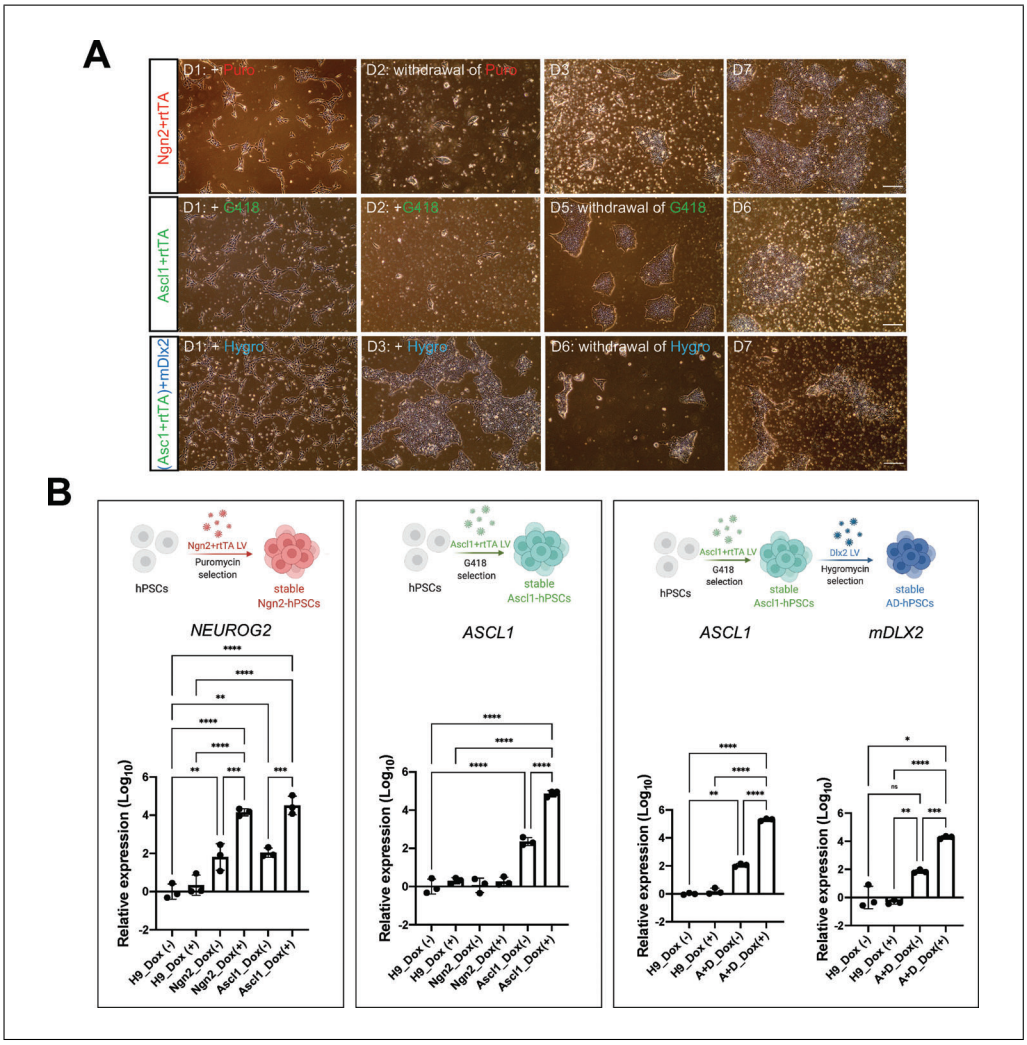


Figure 2 Generation of stable EX- and IN-hPSC lines expressing lineage-specific transcription factors. (**A**) Brightfield time series showing antibiotic selection of stable lines. Top: Puromycin selection of EX-hPSCs (Ngn2 + rtTA in H9 ES cells). Middle: G418 selection of Ascl1-hPSCs (Ascl1 + rtTA in H9 ES cells). Bottom: Hygromycin selection of IN-hPSCs (mDlx2 in Ascl1-hPSCs). Surviving colonies are further expandable. Scale bar, 200 μm. (**B**) qRT-PCR analysis of ectopically expressed transcription factor mRNAs (*NEUROG2*, *ASCL1*, *mDLX2*) in established cell lines after doxycycline (Dox) treatment for 72 hr. All values normalized to *GAPDH*. Bar graphs represent mean ± SD; $N = 3$ per experiment. Scale is $\log_{10}$ of fold change. One-way ANOVA followed by Tukey's multiple comparison test (*$p < .05$, **$p < .01$, ***$p < .001$, ****$p < .0001$).

usage (see Support Protocol 1). We have successfully used this protocol with human embryonic stem cells (WA09/H9 hESCs) and several of our own induced pluripotent stem cell (iPSC) lines.

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. We list key items used routinely in our laboratory, but alternatives are available from multiple vendors.

CAUTION: Biosafety level (BSL) 2+ is appropriate for lentivirus production and transduction experiments. The produced lentivirus, although replication incompetent, can infect human cells. Thus, it is important to decontaminate plasticware (e.g., pipettes, filter units, collection tubes) with bleach to kill the infectious virus at every step following transduction. This protocol uses second-generation lentiviral constructs, but could be adapted to third-generation reagents.

Materials

HEK 293T cells (ATCC, cat. no. CRL-3216)
FM10 medium (see recipe)
Dulbecco's phosphate-buffered saline (DPBS) without $CaCl_2$, $MgCl_2$ (Gibco, cat. no. 14190-044)
0.25% trypsin-EDTA (Gibco, cat. no. 25200-056)
0.4% trypan blue stain (Invitrogen, cat. no. T10282)
Lipofectamine 3000 and P3000 reagent (Invitrogen, cat. no. L3000-015)
Opti-MEM Reduced-Serum Medium (Gibco, cat. no. 31985-070)
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
Bleach
Lenti-X Concentrator (Takara, cat. no. 631232)
Human pluripotent stem cells
StemFlex medium (see recipe)
Accutase cell detachment solution (Innovative Cell Technologies, cat. no. AT-104)
DMEM/F12 medium (Gibco, cat. no. 12320-033)
10 mM Y-27632 (ROCK inhibitor; see recipe)
1 mg/ml puromycin dihydrochloride (Gibco, cat. no. A11138-03)
50 mg/ml G418 sulfate (Gibco Geneticin, cat. no. 10131035)
ReLeSR (Stemcell Technologies, cat. no. 05872)
Freezing medium (see recipe)
50 mg/ml hygromycin B (Gibco, cat. no. 10687010)

15-cm gelatin-coated culture plates (see recipe)
Humidified 37°C, 5% CO_2 incubator
Countess II automated cell counter and counting chamber slides (Invitrogen, cat. nos. AMQAX1000 and C10228)
15- and 50-ml conical tubes (e.g., BD Falcon)
Parafilm
115-ml filter units with SFCA membranes (Thermo Fisher Scientific, cat. no.122-0045)
1.5-ml nuclease-free microcentrifuge tubes (e.g., Thermo Fisher Scientific)

6-well Cultrex-coated plates (see recipe)
2-ml cryovials (Corning, cat. no. 430661)

Prepare lentivirus particles

1. Grow HEK 293T cells in 15-cm gelatin-coated plates containing 20 ml FM10 medium. To passage cells:
   a. Wash cells with 20 ml DPBS.
   b. Add 4 ml of 0.25% trypsin-EDTA and incubate ~5 min until cells are freely floating.
   c. Collect floating cells, deactivate trypsin by adding 11 ml FM10 medium, and centrifuge 4 min at 300 × *g*.
   d. Aspirate medium and resuspend pellet with 5 ml FM10 medium.
   e. Mix 10 µl cells with 10 µl trypan blue and count viable cells by trypan blue exclusion.
   f. Plate the desired number of viable cells into 20 ml FM10.

   For routine passaging, HEK 293T cells can also be split 1:3 without counting, typically three times a week.

   It is important to handle cells gently during washing and trypsinization, as mechanical force easily detaches HEK 293T cells from plastic plates. Washing once with DPBS before adding trypsin-EDTA is necessary because FBS in FM10 medium may block trypsin activity.

   Coating plates with gelatin helps the cells adhere.

2. On the day before transfection (D–1), plate HEK 293T cells on two 15-cm gelatin-coated plates for each virus at a density of 1.8×10^6 cells/cm^2 in 20 ml FM10 medium.

   Cells should be ~80% to 90% confluent on the day of transfection for maximal transfection efficiency.

3. On the day of transfection (D0), prepare enough transfection mix for two plates per virus:
   a. For each viral transfection performed, mix 80 µl Lipofectamine 3000 with 2 ml Opti-MEM in a 15-ml conical tube and vortex for 3 s.
   b. In separate 15-ml conical tubes, prepare plasmid DNA master mixes containing 20 µg target plasmid (Ngn2, Ascl1, Dlx2, or rtTA), 15 µg psPAX2, and 10 µg VSV-G in 2 ml Opti-MEM, then add 80 µl P3000 reagent.
   c. For each target plasmid, prepare a 1:1 mix from both tubes (i.e., 2 ml each from tubes a and b) and incubate at room temperature for 10 min.

   Transfection using Lipofectamine 3000 is performed according to manufacturer's instructions and consistently exhibits high transfection efficiency and reproducibility. The optimum amounts of DNA and reagents for maximum efficiency should be determined by the end-user. Other transfection methods such as calcium phosphate precipitation can be used (see Current Protocols article Kingston, Chen, & Rose, 2003).

4. Gently and slowly pipette the transfection mixture in a dropwise manner onto the cells and incubate at 37°C for 5-6 hr.

5. Replace transfection mixture with 18.5 ml fresh FM10 medium per plate.

   Be careful not to lift cells.

6. Collect the first viral supernatants in 50-ml conical tubes 48 hr after transfection (D2). Close caps tightly, wrap with Parafilm, and keep at 4°C. Feed cells with 18.5 ml fresh FM10 medium.

   Be careful not to introduce bubbles or cell debris to the collection tubes.

7. Harvest the second viral supernatants 72 hr after transfection (D3). Disinfect cell culture plates carefully with bleach and then discard.

   For each virus, you now have two tubes with 36 ml supernatant each.

8. Centrifuge supernatants 3 min at 300 × *g*. Combine the two supernatants for each virus.

9. Pass supernatants through a 115-ml filter by vacuum.

   Apply 2.5 ml DMEM to the filter membrane and turn on the vacuum before applying the supernatants to check that the filter is working and to pre-wet the membrane to prevent loss of virus. Use only cellulose acetate or polyethersulfone filters. Do not use nitrocellulose filters.

10. Transfer filtered supernatant to two new 50-ml conical tubes (36 ml each) and add ⅓ volume (12 ml) Lenti-X Concentrator. Mix by gentle inversion and incubate 2 hr to overnight at 4°C.

    Cooling of the sample is essential. Incubation times can be longer (up to 1 week) at 4°C.

11. Centrifuge samples for 45 min at 1500 × *g*, 4°C. Carefully remove supernatants and place on ice.

    A lack of a visible pellet indicates poor virus production and the lentiviral production should be repeated.

12. Gently resuspend pellets with 100 µl DPBS per tube. Prepare 20- to 40-µl aliquots in labeled 1.5-ml microcentrifuge tubes and store immediately at –80°C.

    Small ready-to-use aliquots are useful to avoid multiple freeze-thaw cycles in order to maintain infection efficiency.

Transduce lentivirus into hPSCs

13. Grow hPSCs in feeder-free conditions in StemFlex medium on 6-well Cultrex-coated culture plates (see Support Protocol 1).

    If hPSCs are growing on a feeder layer, such as mouse embryonic fibroblasts, they should be adapted to feeder-free conditions prior to the experiment. If using other stem cell culture media, such as E8 medium, follow the manufacturer's standard protocol.

    StemFlex medium generally requires feeding every other day, although two days of feeding can be skipped if cells are at low confluency. See Support Protocol 1 for general hPSC culture maintenance.

    Always equilibrate medium and reagents to room temperature prior to use.

14. When cells are confluent, treat with Accutase for single-cell dissociation. Aspirate medium, add 1 ml prewarmed Accutase per well, and incubate at 37°C until cells become detached (usually 5-7 min).

    Incubation time will vary depending on cell density and the cell line used. It is critical to ensure that cells are completely dissociated into single cells.

15. Transfer cells to a 15-ml conical tube with 7 ml DMEM/F12 medium with minimal pipetting.

16. Centrifuge 4 min at 300 × *g*, room temperature. Aspirate supernatant.

17. Gently resuspend cell pellet in StemFlex medium supplemented with 10 µM Y-27632 using a P-1000 pipet tip.

    Treatment with Y-27632 prevents cell death of hPSCs when dissociating to single cells (Watanabe et al., 2007).

18. Mix 10 µl cells with 10 µl trypan blue and count viable cells by trypan blue exclusion.

    Dissociated cells should yield >90% viability. A few million cells are expected from one confluent well of hPSCs from the 6-well plate.

19. Dilute cells to 2.6×10^5 cells/ml in StemFlex medium supplemented with 10 µM Y-27632. Transfer 2 ml to three 50-ml conical tubes for each antibiotic being selected for (two for transductions and one for a non-transduced control).

20. Thaw lentiviruses on ice. Perform reverse transduction by adding 5 µl target virus (Ngn2 for EX-hPSCs; Ascl1 for IN-hPSCs) plus 5 µl rtTA virus to two of the tubes.

    IN-hPSCs require sequential transduction with Ascl1 and mDlx2, because we often observe low cellular viability due to toxicity following co-transduction with all three viruses (Ascl1, mDlx2, and rtTA). We highly recommend generating Ascl1-hPSCs and then transducing these with mDlx2 virus.

    Reverse transduction refers to the method of adding virus to dissociated cells prior to cell plating, rather than adding virus to adherent cells. We observe high transduction efficiency in hPSCs via this method. The day of transduction is referred to as D0.

21. Seed cell/virus mixtures on a 6-well Cultrex-coated plate and shake to spread the cells evenly in the wells. Include a control well without virus to confirm antibiotic selection. Incubate overnight.

    Plating density should be optimized for each cell line and target virus. For EX-H9 cells, 520,000 cells per well in 6-well plate is optimal.

22. The next day (D1), perform antibiotic selection by changing medium with fresh StemFlex medium containing antibiotics without Y-27632.

    For EX-hPSCs, treat with 1 µg/ml Puromycin for 24 hr.
    For Ascl1-hPSCs, treat with 100 µg/ml G418 for 96 hr. Refresh medium with G418 every other day.

    The concentration and duration of 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. For EX-hPSCs, it typically takes 24-48 hr for non-transduced H9 hESCs to be killed by Puromycin. For Ascl1-hPSCs, it takes 96-120 hr for non-transduced H9 hESCs to be killed by G418. Cells that do not survive selection will be floating.

23. Once non-transduced cells are all dead, refresh with StemFlex medium without antibiotics and maintain an every-other-day feeding schedule with StemFlex medium until cells are confluent.

    For EX-hPSCs, refresh with StemFlex medium without Puromycin on D2.
    For Ascl1-hPSCs, refresh with StemFlex medium without G418 on D5.

    Only a minority of hPSCs will survive selection. These cells will proliferate well enough to be confluent within a few days to generate a polyclonal line.

24. To passage cells:

    a. Aspirate medium and replace with 1 ml ReLeSR/well. Incubate 3-5 min at 37°C.
    b. Aspirate ReLeSR and gently resuspend cells in 1 ml StemFlex medium using a P-1000 pipet tip.
    c. Split suspension between the desired number of wells. Plate 2 ml/well into a 6-well Cultrex-coated 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.

Split density should be based on the growth rate of the particular hPSC line. For example, 1:6 is often optimal for H9 hESCs. In general, hPSCs should be passaged once the cells are 80-90% confluent and split in a 1:6-1:10 ratio (~4 or 5 days).

25. To freeze cells, perform ReLeSR passaging and resuspend cells in freezing medium. Freeze cells from one well of a 6-well plate in two cryovials, each of which can be thawed back into one well of a 6-well plate.

    We recommend cryopreserving cells after at least two passages in order to mitigate remaining virus in the culture. Until the second passage, exercise special care.

26. Once Ascl1-hPSCs have been generated and several backup vials cryopreserved, repeat reverse transduction using 5 µl mDlx2 virus without rtTA virus. The following day, treat with 50 µg/ml Hygromycin B until control cells without virus are all killed (72-84 hr).

SUPPORT PROTOCOL 1

EXPANSION AND MAINTENANCE OF hPSCs

Before transduction, hPSCs are expanded and adapted to StemFlex medium as described here. All materials are listed in Basic Protocol l.

Plate cells

1. Quickly remove a vial of hPSCs from liquid nitrogen and thaw immediately in a 37°C water bath until just a small bit of ice remains in the vial (1-2 min).

   The DMSO in freezing medium is toxic to cells after they have thawed. It is important to thaw the cells rapidly in order to facilitate the effective dilution and removal of DMSO.

2. Transfer cells to a 15-ml conical tube with 7 ml StemFlex medium and centrifuge 5 min at 200 × *g*, room temperature.

3. Aspirate supernatant and resuspend cell pellet with 2 ml StemFlex medium with 10 µM Y-27632.

4. Aspirate Cultrex from one well and add 2 ml cell suspension, making sure to distribute the cells evenly in the well. Place in a 37°C incubator overnight.

5. The next day, replace medium with fresh StemFlex medium without Y-27632. Repeat every other day until cells are near confluence.

   Follow standard feeding schedules if you are using a different hPSC medium (e.g., E8 medium).

Passage cells

6. Aspirate medium and add 1 ml ReLeSR/well. Incubate 3-5 min at 37°C.

7. Aspirate ReLeSR and resuspend cells in 1 ml StemFlex medium by gentle pipetting.

8. Split cell suspension between the desired number of wells. Plate 2 ml/well cells in a Cultrex-coated 6-well culture plate.

9. Change medium every other day until the cells are ready for transduction.

BASIC PROTOCOL 2

DIFFERENTIATION OF EX- AND IN-NEURONS

This protocol describes how to differentiate and mature EX- and IN-neurons from the stable hPSCs described above. Addition of doxycycline, a derivative of tetracycline that binds to rtTA with high affinity, induces expression of the neurogenic transcription factors, which transit the cells 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 medium conditions

and feeding schedules for both types of neurons allow for systemic production of isolated or co-cultured populations of EX- and IN-neurons. Cytosine β-d-arabinofuranoside (cytarabine, Ara-C), a cytosine analog and potent inhibitor of replicative DNA synthesis, is used to control proliferation (Galmarini, Mackey, & Dumontet, 2001), leading to removal of any proliferating hPSCs remaining 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 cells are plated. Using this method, neuronal morphology starts to appear within 1 week after differentiation, and neurons are functionally mature in ∼4 weeks.

Materials

Stable EX-hPSCs (Ngn2+rtTA) and IN-hPSCs (Ascl1+mDlx2+rtTA) (see Basic Protocol 1)
StemFlex medium (see recipe)
Accutase cell detachment solution (Innovative Cell Technologies, cat. no. AT-104)
DMEM/F12 medium (Gibco, cat. no. 11320-033)
Neuronal differentiation medium (see recipe)
20 μg/ml BDNF (see recipe)
10 μg/ml NT-3 (see recipe)
1 mg/ml doxycycline (see recipe)
1 mg/ml Cultrex mouse laminin I (R&D Systems, cat. no. 3400-010-02)
10 mM Y-27632 (ROCK inhibitor; see recipe)
0.4% trypan blue stain (Invitrogen, cat. no. T10282)
5 mM Ara-C (see recipe)
BrainPhys neuronal medium (see recipe)
Mouse astrocyte conditioned medium (mACM; ScienCell Research Laboratories, cat. no. M1811-57)

6-well Cultrex-coated plate (see recipe)
Humidified 37°C, 5% CO_2 incubator
15-ml conical tubes (e.g., BD Falcon)
Countess II automated cell counter and chamber slides (Invitrogen, cat. nos. AMQAX1000 and C10228)
12-well PEI/laminin–coated plate (see recipe)

Initiate differentiation

1. Grow stable EX- and IN-hPSCs in StemFlex medium in a 6-well Cultrex-coated plate to 80% confluency.

   If using frozen cells, thaw and passage at least once before proceeding (see Support Protocol 1).

2. On the first day of neuronal differentiation (ND0), add 1 ml/well prewarmed Accutase to cells and incubate at 37°C for 5-7 min, until cells release with gentle tapping on the bottom of the plate.

3. Collect cells with a P-1000 pipet tip and transfer to a 15-ml tube with 7 ml DMEM/F12 medium.

4. Prepare 1 ml prewarmed neuronal differentiation medium containing the following:

   10 ng/ml BDNF
   10 ng/ml NT-3
   1 μg/ml doxycycline
   1 μg/ml laminin

10 μM Y-27632

Supplemented medium should be prepared immediately before use.

5. Centrifuge cells 4 min at 300 × g, room temperature.
6. Aspirate supernatant and gently resuspend cell pellet with 1 ml supplemented neuronal differentiation medium.
7. Mix 10 μl cells with 10 μl trypan blue and count viable cells by trypan blue exclusion.
8. Seed cells on a 12-well PEI/laminin–coated plate at 1.3×10^5 cells/cm^2 (2.6×10^5 cells/ml in 12-well plate) and incubate at 37°C.

   Cell plating density varies for each hESC or hiPSC line and target virus, and the optimal cell plating density should be determined by testing a few different densities before scaling up experiments. In our hands, 260,000 cells/well in 12-well plate is optimal for making EX- and IN-neurons from H9 cells.

   Cells can be cultured on tissue culture–treated plasticware or on glass coverslips for downstream experiments. For biochemical applications requiring large numbers of neurons, cells should be plated on 12-well or 6-well plates. For immunocytochemistry or electrophysiology applications, cells should be pated on 12-mm or 15-mm round glass coverslips in 24-well plates.

Remove proliferating cells

9. The next day (ND1), begin treating with Ara-C. Replace medium with freshly prepared neuronal differentiation medium containing:

   10 ng/ml BDNF
   10 ng/ml NT-3
   1 μg/ml doxycycline
   1 μg/ml laminin
   2 μM Ara-C.

   On ND1, you will start to observe the transition to neurons by morphological changes. On ND2, significant cell death will be observed due to Ara-C treatment. If too much cell death is observed after treating with Ara-C (i.e., to the point where the density does not support a healthy culture), begin again and delay treatment with Ara-C until ND2. In this case, the medium should still be refreshed on ND1 using supplemented neuronal differentiation medium without Y-27632. It is recommended to perform a small-scale pilot experiment to determine how cells respond to Ara-C.

   A high dose of Ara-C (2 μM) is helpful to remove remaining hPSCs. We recommend treating with a high dose at least twice and then changing to a lower dose (100 nM) in subsequent feeds.

10. On ND3, replace medium with neuronal differentiation medium containing:

    10 ng/ml BDNF
    10 ng/ml NT-3
    1 μg/ml doxycycline
    2 μM Ara-C.

    Laminin is not required with each change of medium, but should be included once a week (1 μg/ml) to help neuronal attachment. If preferred, it can be included each time.

11. On ND5, replace medium with neuronal differentiation medium containing a lower dose of Ara-C:

    10 ng/ml BDNF
    10 ng/ml NT-3

1 µg/ml doxycycline
100 nM Ara-C.

12. On ND7, change to BrainPhys neuronal medium containing the same supplements.

    A transition to BrainPhys medium is recommended for functional assays such as whole-cell patch electrophysiology to improve neuronal function (Bardy et al., 2015).

    A low dose of Ara-C (100 nM) may be needed for a few more days, until no remaining proliferating cells are observed. Usually, a week of treatment is sufficient. Laminin should be included at this point.

13. On ND9, refresh with a half-medium change of BrainPhys neuronal medium containing the same supplements.

    For a half-medium change in a 12-well plate, replace 500 µl old medium with 500 µl fresh medium.

    The duration and times of Ara-C treatment should be adjusted based on culture conditions. Treatment is stopped when no non-neuronal cells are observed.

Switch to medium supporting neuronal maturation

14. On ND11, replace Ara-C with 10% mACM to support the growth of neurons.

    Using mACM is an alternative to co-culture with murine primary astrocytes. Addition of mACM supports neuronal survival and promotes functional maturity (Yan, Tan, & Huang, 2013). Larger somas and healthier neurons will be observed after treatment with mACM over a period of time.

15. For the rest of differentiation, perform half-medium changes every 3-4 days with BrainPhys neuronal medium containing mACM, BDNF, NT-3, and doxycycline.

    Neurons have been cultured for over 30 days using this protocol. After the desired culture period, process cells for downstream applications such as immunocytochemistry, electrophysiology, or isolation of RNA or protein.

    Doxycycline can be withdrawn after 2 weeks of differentiation, depending on the user's preference.

EXPERIMENTAL METHODS FOR VALIDATION OF EX- AND IN-NEURONS

SUPPORT PROTOCOL 2

This protocol provides brief descriptions for validation by quantitative reverse-transcription PCR (qRT-PCR) and immunocytochemistry of ectopically expressed neurogenic transcription factors as well as electrophysiological characterization by whole-cell patch clamp. Figures 3 and 4 show validation and characterization data that demonstrate successful production of EX- and IN-neurons.

qRT-PCR

Materials

EX- and IN neurons (see Basic Protocol 2)
TRIzol reagent (Invitrogen, cat. no. 15596026)
Chloroform
3 M sodium acetate, pH 5.5 (Invitrogen, cat. no. AM9740)
Ethanol
Glycogen (Thermo Scientific, cat. no. R0551)
Maxima First Strand cDNA Synthesis Kit (Thermo Scientific, cat. no. K1672)
PCR primers (Table 1)
Fast SYBR Green Master Mix (Applied Biosystems, cat. no 4385612)

Nanodrop
QuantStudio 7 Flex thermocycler (Applied Biosystems, cat. no. A43183)

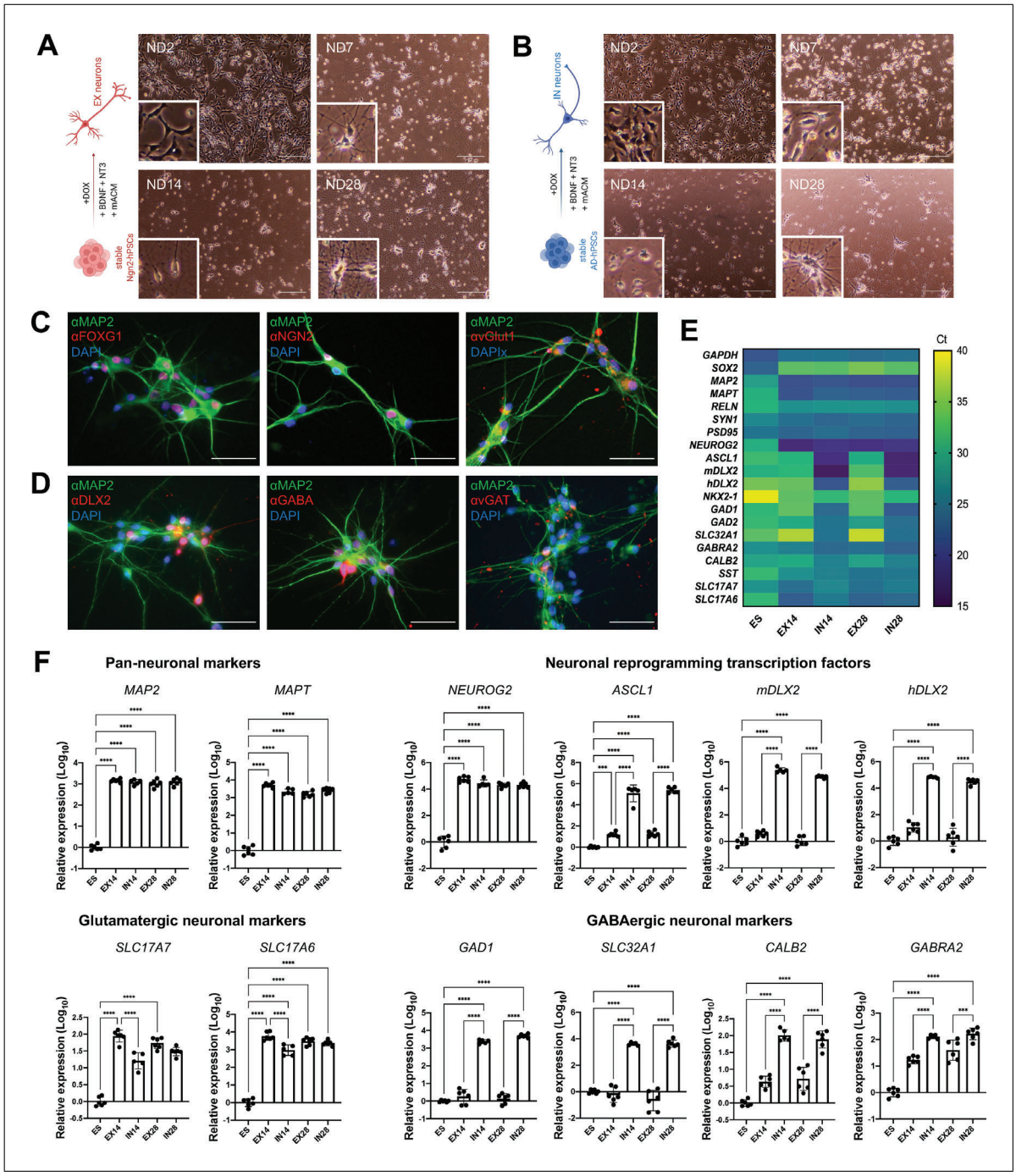


Figure 3 Validation of differentiated human glutamatergic EX-neurons and GABAergic IN-neurons. (**A**) Brightfield images of EX-neuron differentiation upon doxycycline treatment of stable EX-hPSCs. (**B**) Brightfield images of IN-neuron differentiation upon doxycycline treatment of stable IN-hPSCs. Scale bars, 200 μm (**A**,**B**). (**C**) Representative images of EX-neurons at neuronal differentiation day 28 (ND28). Cells were stained with a general neuronal marker (αMAP2), forebrain marker (αFOXG1), excitatory neuronal markers (αNGN2, αvGlut1), and nuclear marker (DAPI). (**D**) Representative images of IN-neurons at ND28. Cells were stained with αMAP2, GABAergic neuronal markers (αDLX2, αGABA, αvGAT), and DAPI. Scale bars, 50 μm (**C**,**D**). (**E**) Heatmap of qRT-PCR analysis in human ES cells and EX- and IN-neurons on ND14 and ND28. Expression levels of genes indicated on the left (expressed as Ct values) are color-coded as shown on the right. 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* in ES cells and EX- and IN-neurons on ND14 and ND28. Bar graphs represent mean ± 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 (*$p < .05$, **$p < .01$, ***$p < .001$, ****$p < .0001$).

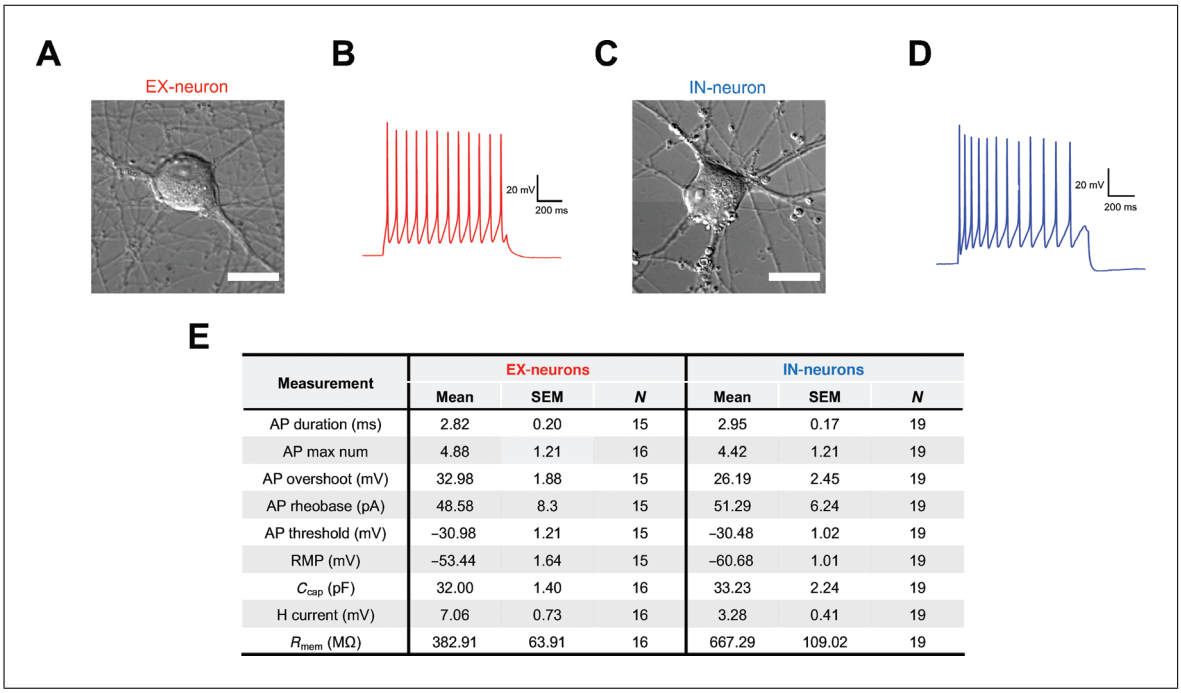
Table 1 Primers for qRT-PCR of EX- and IN-Neuron Markers

Category	Target gene	Sequence	Reference
Housekeeping gene	*GAPDH*	Forward: 5′-TGAAGGTCGGAGTCAACGGATTTGG-3′ Reverse: 5′-CATGTAGGCCATGAGGTCCACCAC-3′	Riera et al. (2019)
Pluripotent stem cell marker	*SOX2*	Forward: 5′-AGAAGGATAAGTACACGCTGC-3′ Reverse: 5′-TCCAGCCGTTCATGTGC-3′	This study
Pan-neuronal markers	*MAP2*	Forward: 5′-AAACTGCTCTTCCGCTCAGACACC-3′ Reverse: 5′-GTTCACTTGGGCAGGTCTCCACAA-3′	Ho et al. (2016)
	MAPT	Forward: 5′-AGGCGGGAAGGTGCAAATAG-3′ Reverse: 5′-TCCTGGTTTATGATGGATGTT-3′	Lacovich et al. (2017)
Cajal-Retzius cell marker	*RELN*	Forward: 5′-TGAGAGCCAGCCTACAGGA-3′ Reverse: 5′-TCGTTCCACATTCTGTACCAA-3′	Baek et al. (2015)
Synaptic markers	*SYN1*	Forward: 5′-GCAAGGACGGAAGGGATCACATCA-3′ Reverse: 5′-CCTGAGCCATCTTGTTGACCACGA-3′	Ho et al. (2016)
	PSD95	Forward: 5′-GGCAGCCCTGAAGAACACGTATGA-3′ Reverse: 5′-CCCAGGTAGCTGCTGTGACTGATC-3′	Ho et al. (2016)
Neurogenic transcription factors	*NEUROG2*	Forward: 5′-CAAGCTCACCAAGATCGAGACC-3′ Reverse: 5′-AGCAACACTGCCTCGGAGAAGA-3′	Kim et al. (2014)
	ASCL1	Forward: 5′-GCGGCCAACAAGAAGATGAG-3′ Reverse: 5′-AGTCGTTGGAGTAGTTGGGG-3′	This study
	mDLX2	Forward: 5′-GCCTCAACAATGTCTCCTACTC-3′ Reverse: 5′-ATTTCAGGCTCAAGGTCTTCC -3′	This study
	hDLX2	Forward: 5′-GCACATGGGTTCCTACCAGT-3′ Reverse: 5′-TCCTTCTCAGGCTCGTTGTT-3′	Park et al. (2017)

(*Continued*)

Table 1 Primers for qRT-PCR of EX- and IN-Neuron Markers, *continued*

Category	Target gene	Sequence	Reference
GABAergic neuronal markers	*NKX2-1*	Forward: 5′-AGCACACGACTCCGTTCTC-3′ Reverse: 5′-GCCCACTTTCTTGTAGCTTTCC-3′	Sun, Pasca, et al. (2016)
	GAD1	Forward: 5′-TTGCACCAGTGTTTGTCCTCATGG-3′ Reverse: 5′-CCGGGAAGTACTTGTAGCGAGCAG-3′	Barretto et al. (2020)
	GAD2	Forward: 5′-CTATGACACTGGAGACAAGGC-3′ Reverse: 5′-CAAACATTTATCAACATGCGCTTC-3′	Barretto et al. (2020)
	SLC32A1	Forward: 5′-CACGACAAGCCCAAAATCAC-3′ Reverse: 5′-CGGCGAAGATGATGAGAAACAAC-3′	Barretto et al. (2020)
	GABRA2	Forward: 5′-GTTCAAGCTGAATGCCCAAT -3′ Reverse: 5′-ACCTAGAGCCATCAGGAGCA -3′	Park et al. (2017)
	CALB2	Forward: 5′-CTCCAGGAATACACCCAAA -3′ Reverse: 5′-CAGCTCATGCTCGTCAATGT -3′	Park et al. (2017)
	SST	Forward: 5′-GCTGCTGTCTGAACCCAAC -3′ Reverse: 5′-CGTTCTCGGGGTGCCATAG -3′	Park et al. (2017)
Glutamatergic neuronal markers	*SLC17A7*	Forward: 5′-CGCATCATGTCCACCACCAACGT-3′ Reverse: 5′-GAGTAGCCGACCACCAACAGCAG-3′	Ho et al. (2016)
	SLC17A6	Forward: 5′-TCAACAACAGCACCATCCACCGC-3′ Reverse: 5′-GTTTCCGGGTCCCAGTTGAATTTGG-3′	Ho et al. (2016)



E

Measurement	EX-neurons			IN-neurons		
	Mean	SEM	*N*	Mean	SEM	*N*
AP duration (ms)	2.82	0.20	15	2.95	0.17	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_{cap} (pF)	32.00	1.40	16	33.23	2.24	19
H current (mV)	7.06	0.73	16	3.28	0.41	19
R_{mem} (MΩ)	382.91	63.91	16	667.29	109.02	19

Figure 4 EX- and IN-neurons exhibit functional properties consistent with maturity at ND28. (**A,C**) Differential interference contrast (DIC) images of an EX- and IN-neuron during whole-cell patch clamp recording. Scale bar, 20 μm. (**B,D**) Representative membrane potential recordings showing multiple action potentials (APs) evoked by 1-s current step in an EX- and IN-neuron. (**E**) Summary of membrane properties in EX- and IN-neurons. AP characteristics as well as passive membrane properties such as resting membrane potential (RMP), membrane capacitance (C_{cap}), and input resistance (R_{mem}) were measured. Values represent mean ± SEM; N = 15-19 cells.

1a. Extract RNA using TRIzol/chloroform according to manufacturer's instructions.

2a. Precipitate RNA using 0.1 vol. of 3 M sodium acetate, pH 5.5, 1 vol. room temperature ethanol, and 1 μg/ml glycogen. Vortex thoroughly and let sit at room temperature for 20 min.

Glycogen aids visualization of precipitants.

3a. Centrifuged 10 min at 13,000 × *g*.

4a. Wash pellet twice with ice-cold 70% ethanol, spinning for 1 min each time. Remove ethanol.

5a. Spin briefly to remove any trace amount of ethanol and air-dry the pellet.

6a. Resuspend in an appropriate volume of nuclease-free water.

7a. Measure RNA concentration using a NanoDrop instrument.

8a. Synthesize cDNA using the Maxima First Strand cDNA Synthesis Kit according to manufacturer's instructions using a normalized RNA samples of 90 ng.

9a. Perform real-time PCR using the Fast SYBR Green Master Mix on a QuantStudio 7 Flex thermocycler. For primers, see Table 1.

10a. Use the −ΔΔCt method to determine the relative expression of each gene with *GAPDH* as a reference.

Immunocytochemistry

Materials

EX- and IN-neurons on PEI/laminin–coated coverslips (see Basic Protocol 2)
1× PBS (Invitrogen, cat. no. AM9625)

4% (v/v) paraformaldehyde (Alfa Aesar, cat. no. J61899AK)
SuperBlock (PBS) Blocking Buffer (Thermo Scientific, cat. no. 37515)
Triton X-100 (Alfa Aesar, cat. no. A16046AE)
Primary antibodies: DLX2 (rabbit, Invitrogen, 702009), NGN2 (rabbit, Cell Signaling Technology, 13144S), GABA (rabbit, Sigma-Aldrich, A2052), FOXG1 (rabbit, Abcam, ab18259), vGAT (rabbit, Synaptic Systems, 131 003), vGLUT1 (guinea pig, Sigma-Aldrich, AB5905), MAP2 (chicken, Aves Labs, AB_2313549)
Fluorescently conjugated secondary antibodies: Fluorescein anti-chicken (Aves Labs, F-1005), Alexa Fluor 568 anti-rabbit (Invitrogen, A10042), and Alexa Fluor 594 anti–guinea pig (Invitrogen, A11076)
Anti-fade medium with DAPI (e.g., ProLong Diamond Antifade Mountant, Molecular Probes, cat. no. P36971)

Glass slides
Fluorescence (e.g., Olympus EVOS BX53) and brightfield (e.g., Olympus EVOS XL Core) microscopes with appropriate filters and objectives
Imaging software (e.g., ImageJ v1.53a, NIH)

1b. Wash neurons gently with 1× PBS.

Alternatively, 1× DPBS can be used throughout the protocol. 1× PBS or DPBS should be diluted from 10× in molecular-purified water.

2b. Fix with 4% (v/v) paraformaldehyde for 15 min on ice.

3b. Block for 15 min in SuperBlock (PBS) Blocking Buffer with 0.3% Triton X-100.

4b. Incubate with primary antibodies at 4°C overnight using the following dilutions in blocking buffer:

1:100 rabbit anti-DLX2
1:250 rabbit anti-NGN2
1:400 rabbit anti-GABA
1:500 rabbit anti-FOXG1
1:500 rabbit anti-vGAT
1:500 guinea pig anti-vGLUT1
1:1000 chicken anti-MAP2

5b. Wash cells three times with PBS.

6b. Incubate with the appropriate fluorescently conjugated secondary antibodies for 1 hr at room temperature using dilutions of 1:750 to 1:1000 in blocking buffer:

Alexa Fluor 568 anti-rabbit
Alexa Fluor 594 anti–guinea pig
Fluorescein anti-chicken

7b. Wash cells five times with PBS and mount on glass slides using an anti-fade medium with DAPI.

8b. Acquire images fluorescence and brightfield images and process as desired.

Whole-Cell Patch Clamp

Electrophysiological recordings were carried out using conventional whole-cell current methods on Day 28 (ND28) neurons as previously described (Jacko et al., 2018). 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, 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 current steps that were increased incrementally 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.

Ara-C, 5 mM (2500×)

Resuspend Ara-C (cytarabine; TOCRIS, cat. no. 4520) at 25 mM in sterile water (10 ml for 60.8 mg). Filter-sterilize and dilute to 5 mM to give 2500× aliquots for daily use at the high dose. Store long term at –20°C. Once thawed, store up to 3 weeks at 4°C. Dilute to 100 μM for 1000× at the low dose.

Brain-derived neurotrophic factor (BDNF), 20 μg/ml (2000×)

Resuspend lyophilized recombinant human BDNF (R&D Systems, cat. no. 248-BDB) to a final concentration of 20 μg/ml in DPBS (Gibco, cat. no. 14190-044) containing 0.1% (w/v) bovine serum albumin (BSA). Store aliquots long term at –80°C. Avoid multiple freeze-thaw cycles. Once thawed, store up to 2 weeks at 4°C.

BrainPhys neuronal medium

BrainPhys neuronal medium (Stemcell Technologies, cat. no. 05790)
1× SM1 supplement (Stemcell Technologies, cat. no. 05711)
1× N2 supplement-A (Stemcell Technologies, cat. no. 07152)
1× penicillin/streptomycin (Gibco, cat. no. 15140-122)
Store up to 3 weeks at 4°C

The three Stemcell components are also sold together as the BrainPhys Neuronal Medium N2-A & SM1 Kit (Stemcell Technologies, cat. no. 05793).

Cultrex-coated plates

Thaw Cultrex stem-cell-qualified, reduced-growth-factor, basement membrane extract (R&D Systems, cat. no. 3434-005-02) on ice in a refrigerator. Working quickly, resuspend 250 μl Cultrex in 25 ml cold DMEM/F12 medium (Gibco, cat. no. 12320-033). Add 1 ml/well to a 6-well plate (Corning, cat. no. 3516) and swirl for even distribution, ensuring that the entire surface is covered. Incubate in a culture hood for at least 1 hr. Seal with Parafilm and store up to 3 weeks at 4°C. Before use, warm in a 37°C incubator for at least 30 min and aspirate Cultrex immediately before plating.

Doxycycline, 1 mg/ml (1000×)

Resuspend doxycycline hyclate (Sigma-Aldrich, cat. no. D9891) to a 1 mg/ml in sterile water. Filter-sterilize and store long term in aliquots away from light at –20°C. Once thawed, store up to 3 weeks at 4°C.

FM10 medium

Dulbecco's modified Eagle's medium (DMEM; Gibco, cat. no. 11965-092)
10% (v/v) heat-inactivated fetal bovine serum (FBS; Gibco, cat. no. 16140-071)
1× GlutaMAX (Gibco, cat. no. 35050-061)
1× non-essential amino acids (Gibco, cat. no.11140-050)
1× penicillin/streptomycin (Gibco, cat. no. 15140-122)
0.1 mM β2-mercaptoethanol (Gibco, cat. no. 21985-023)
Store up to 3 weeks at 4°C

Freezing medium

45% (v/v) pluripotent stem cell medium (or any culture medium)
45% (v/v) KnockOut serum replacement (Gibco, cat. no. 10828-028)
10% (v/v) dimethyl sulfoxide (DMSO; Sigma-Aldrich, cat. no. D2650)
Store up to 3 weeks at 4°C

Gelatin-coated culture plates

Coat plates with enough ESGRO 0.1% (w/v) gelatin solution (Sigma-Aldrich, cat. no. SF008) to cover the surface and incubate 15 min at 37°C. Remove gelatin and use immediately or store up to 3 weeks at 4°C. After storage, warm to 37°C before use.

Neuronal differentiation medium

DMEM/F12 medium (Gibco, cat. no. 11320-033)
1× N-2 supplement (Gibco, cat. no. 17502-048)
1× B27 supplement (Gibco, cat. no.17504-044)
1× penicillin/streptomycin (Gibco, cat. no. 15140-122)
Store up to 3 weeks at 4°C

Neurotrophin-3 (NT-3), 10 µg/ml (1000×)

Resuspend lyophilized recombinant human NT-3 (R&D Systems, cat. no. 267-N3-005) to a final concentration of 10 µg/ml in DPBS (Gibco, cat. no. 14190-044) containing 0.1% (w/v) BSA. Store aliquots long term at –80°C. Avoid multiple freeze-thaw cycles. Once thawed, store up to 2 weeks at 4°C.

Polyethylenimine (PEI), 0.1% (1×)

Prepare 20× PEI by diluting stock (Sigma-Aldrich, cat. no. 408727) 1:1000 in 0.1 M sodium borate (Alfa Aesar, cat. no. J62902AP). Store up to 1 month at 4°C. For 1× PEI solution, dilute in 0.1 M sodium borate, filter, and store up to 1 week at 4°C.

Note that PEI is very viscous.

Polyethylenimine (PEI)/laminin–coated plates and coverslips

For plates: Dispense 1× PEI solution (see recipe) to all wells of the plate (1 ml for 6-well plates; 0.5 ml for 12- or 24-well plates) and incubate at 37°C for 1 hr. Aspirate PEI and wash with sterile water five times. Air-dry completely in a biosafety cabinet. Dispense 10 µg/ml laminin (R&D Systems, cat. no. 3400-010-02) in DPBS (Gibco, cat. no. 14190-044) to all wells at half the feed volume (e.g., 1 ml/well for 6-well plates). Place in a 37°C incubator right after addition of laminin for 6 hr to overnight before use. Alternatively, seal with Parafilm and store up to 1 week at 4°C. Warm refrigerated plates overnight at 37°C prior to use.

For coverslips: Use sterile forceps to dip glass coverslip in 70% ethanol and place one per well in a 24-well plate. Let dry completely for 15 min. Proceed with coating procedure as described above. Ensure that coverslips are completely submerged in coating solution, as they tend to float. If they do float, use a sterile plastic pipette tip to push them to the bottom of the well.

StemFlex medium

StemFlex medium (Gibco, cat. no. A33494-01)
10% (v/v) StemFlex supplement (Gibco, cat. no. A33494-01)

1× penicillin/streptomycin (Gibco, cat. no. 15140-122)
Store up to 3 weeks at 4°C

Y-27632, 10 mM (1000×)

Reconstitute Y-27632 (Selleck Chemicals, cat. no. S1049) to a final concentration of 10 mM in DMSO (3.122 ml for 10 mg) and store in aliquots at –20°C. Store up to 2 weeks at 4°C. Avoid multiple freeze-thaw cycles.

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, there is a need to efficiently generate isolated populations 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 in vivo neurogenesis (Kirwan et al., 2015; Shi, Kirwan, & Livesey, 2012). However, these direct differentiation protocols often require long maturation times and/or generate heterogeneous cell populations that contain both excitatory and inhibitory neurons and even have 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 the cells 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; Sun, Yuan, et al., 2016; Yang et al., 2017; Zhang et al., 2013). In a similar approach, several studies have used neurogenic transcription factors Ascl1 and Dlx2, resulting in differentiation of hPSCs or hNPCs into functional IN-neurons (Barretto et al., 2020; Chanda et al., 2014; Nicholas et al., 2013; Sun, Yuan, et al., 2016; Yang et al., 2017). Except in the case of Frega et al. (2017), which also used a permanent line approach for EX-neurons, these transient protocols require transduction of viruses for every differentiation, which is cumbersome and more challenging when scaling up the neuronal cultures for large-scale assays such as drug screens. It can also lead to various viral batch effects on differentiation efficiency.

We have observed that a minority of undifferentiated stem cells after lentivirus transduction survive antibiotic selection without doxycycline treatment (Fig. 2). This leaky expression of transcription factors and antibiotic resistance genes is sufficient to establish stable hPSCs that harbor the neurogenic transgene. Hence, we have built on conventional protocols that use transient transduction of undifferentiated hPSCs with Ngn2 lentivirus (for EX-hPSCs) or Ascl1/Dlx2 lentiviruses (for IN-hPSCs), followed by antibiotic selection to remove non-transduced cells. Making stable IN-hPSCs requires sequential combinatorial generation of stable cell lines (i.e., generation of Ascl1-hPSCs followed by Dlx2 transduction) due to the high cell toxicity when 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 using defined and standard culture conditions, allowing for isolation of separate EX and IN populations as well as co-cultures (Figs. 3 and 4).

We observed high transduction efficiency by adding virus to dissociated hPSCs prior to cell plating (so-called reverse transduction). This method does not require the use of polybrene, which often has high cellular toxicity. During the early neuronal differentiation period, we use cytosine β-D-arabinofuranoside (Ara-C, a cytosine analog and potent inhibitor of replicative DNA synthesis) to remove any remaining proliferating hPSCs and any 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 this protocol, we have been able to observe neuronal morphology within 1 week and

to maintain the neurons for several additional weeks for functional maturation.

Critical Parameters and Troubleshooting

It is essential to have good-quality starting material to successfully establish hPSC cell lines. To obtain high-quality 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 DNA is also required for successful transfection and production of virus. If hPSCs for transduction are not growing well, consider thawing a new vial of cells or changing to new batches of StemFlex medium or Cultrex. To avoid genomic instability, we recommend sub-passaging hPSC cultures no more than ten times. In our tests, hPSCs carrying these lentiviruses produce robust functional neurons after sub-passaging ten times. We recommend banking early-passage reverse-transduced hPSCs in cryogenic storage, sub-passaging less than ten times, and thawing earlier passage cells if the differentiation efficiency starts to decrease.

The correct initial cell plating density is important for successful neuronal differentiation. Since this protocol does not require further lifting and replating of cells, it is important to plate the proper number of cells initially. If the cultures are too dense, neurons have limited space for spreading out processes and connecting with other neurons. If cultures are too sparse, the survival rate decreases. In our experience, $2.2–2.8 \times 10^5$ cells/well in 12-well plates is adequate for most downstream applications, such as immunocytochemistry, electrophysiology, and RNA isolation, but this density can be modified based on user preference. We suggest using robust coating substrates (PEI/laminin) and an appropriate plate format (12- or 15-mm coverslips, 12- or 24-well plates) depending on your experiments.

Treatment with Ara-C can be a bit challenging, as there are cell line–specific differences. We treat with a high dose of Ara-C in the initial stage of differentiation to remove remaining hPSCs, then lower the dose, and finally discontinue Ara-C in later stages to preserve neuronal health. We propose treating twice with at 2 μM and then lowering the dose to 100 nM for a week. We highly recommend performing a pilot experiment to determine the appropriate cell plating density and Ara-C treatment conditions before scaling up and increasing culture duration.

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

Additional troubleshooting can be found in Table 2.

Understanding Results

This protocol generates functional human cortical EX- and IN-neurons. The one-step (EX-hPSCs) and two-step (IN-hPSCs) virus transductions into hPSCs allow continual maintenance of hPSCs to feed into a neuronal differentiation paradigm (Fig. 1). qRT-PCR analysis demonstrates relative mRNA expression of transcription factors in established hPSCs upon neuronal induction with doxycycline (Fig. 2B). As expected, expression of all transcription factors was highly increased by doxycycline. Since the *DLX2* promoter for IN-hPSCs is derived from mouse, we confirmed the increased expression of mDLX2. Note that there are increased levels of *NEUROG2* in EX-hPSCs (1.8-fold in $\log_{10}$ scale compared to ES cells) and of both *ASCL1* and *DLX2* in IN-hPSCs (2.3- and 1.9-fold, respectively) 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 (2-fold) but not vice versa (Fig. 2B), which is in line with previous studies showing a hierarchical role of Ascl1 in regulating expression of *NEUROG2* (Aydin et al., 2019; Chanda et al., 2014; Vasconcelos & Castro, 2014).

Most differentiated neurons exhibit neuronal morphological characteristics, such as a polarized cell shape, distinctive axon and dendrite structures, and expression of the pan-neuronal marker MAP2 (Fig. 3A-D). We further evaluated EX- and IN-neuron differentiation by analyzing mRNA expression and immunostaining of standard marker genes. Glutamatergic neuronal markers (NGN2, vGlut1) were stained in EX-neurons, and GABAergic neuronal markers (DLX2, GABA, vGAT) were detected in most IN-neurons (Fig. 3C,D). Furthermore, we assessed expression of multiple standard marker genes at the mRNA level on days 14 and 28

Table 2 Troubleshooting Guide

Protocol	Problem	Possible cause(s)	Solution(s)
Basic Protocol 1	Low lentivirus yield	Unhealthy HEK 293T cells	Thaw new batch of cells
		Low Lipofectamine transfection efficiency	Test transfection efficiency using a fluorescence-expressing plasmid (e.g., GFP); check purity of plasmid DNA and reagents
		Unsuccessful concentration of virus	Chill concentrated viral supernatants longer at 4°C before final centrifugation
	Low transduction efficiency	Unhealthy hPSCs	Thaw new batch of cells; use early passage cells
		Low yield of lentivirus particles	Determine viral titer using quantitative methods such as qPCR; see above
	No surviving cells after antibiotic selection	Poor/no transduction into hPSCs	Check transduction efficiency by GFP viral transduction
		Wrong dose/duration of antibiotic	Determine the antibiotic kill curve before conducting experiments; set up control wells with no virus and treat with antibiotics until complete death of cells
Basic Protocols 1 & 2	Low viability after Accutase passaging	Incubation is too long	Determine proper duration of treatment
		Too much pipetting during resuspension	Triturate gently after harvesting cells
Basic Protocols 1 & 2	No neurons are made	Transduction issue	Transduction into hPSCs is low (see above)
		Reagent issue	Make fresh medium; ensure that neuronal differentiation reagents are working and switch to new aliquots of reagents, if needed
Basic Protocol 2	Too much cell death after Ara-C treatment	Overtreatment with Ara-C	Carefully observe cell status after Ara-C treatment; shorten treatment time if no flat cells are observed
Basic Protocol 2	Cells lift from plates	Insufficient coating	Fresh prepare coating materials (e.g., 1× PEI)
		Excessive physical force during medium changes	Be gentle during medium changes
Basic Protocol 2	Neurons are not made efficiently	Viral integration to hPSCs is reduced; promoter is turned off	Use earlier passage hPSCs
Basic Protocol 2	Neurons are unhealthy	Medium issue	Prepare fresh medium; ensure that all reagents are working and are used at correct concentration
		Ara-C treatment	Withdraw Ara-C once no undifferentiated cells are observed
		Cells are lifting	Add laminin at least once per week

of neuronal differentiation (Fig. 3E,F). These markers included a pluripotent stem cell marker (*SOX2*), neurogenic transcription factors (*NEUROG2*, *ASCL1*, *mDLX2*, *hDLX1*), pan-neuronal markers (*MAP2*, *MAPT)*, Cajal-Retzius cell marker (*RELN*), synaptic markers (*SYN1*, *PSD95*), glutamatergic neuronal markers (*SLC17A7*, *SLC17A6*), and GABAergic neuronal markers (*NKX2-1*, *GAD1*, *GAD2*, *SLC32A1*, *CALB2*, *GABRA2, SST*). qRT-PCR revealed the expected gene expression patterns of glutamatergic and GABAergic neurons in isolated EX- and IN-neurons, respectively. Notably, dramatic increases of GABAergic neuronal markers in IN-neurons were found for multiple target genes. We also confirmed the increased expression of both human and mouse *DLX2* in IN-hPSCs, supporting the commitment of these cells to a GABAergic neuronal fate. There were no evident differences in gene expression changes between ND14 and ND28 for most of the target genes tested. Consistent with the increased level of *NEUROG2* in Ascl1-hPSCs (Fig. 2B), this feature seems to be extended in differentiated neurons, showing increase of gene expression of glutamatergic neuronal markers (*SLC17A7*, *SLC17A6*) in IN-neurons (1.9-fold in EX14, 1.2-fold in IN14, 1.8-fold in EX28, and 1.5-fold in IN28 for *SLC17A7*, all in $\log_{10}$ scale), though the expression change was higher in EX-neurons than IN-neurons. Meanwhile, GABAergic neuronal markers (*GAD1*, *GAD2*, *SLC32A1*, *CALB2*, *GABRA2*) were almost exclusively expressed in IN-neurons (Fig. 3E,F). These results indicate the successful development of stable hPSCs into isolated populations with molecular and cellular features of EX- and IN-neurons.

Recently, two studies reported multiple cell populations in human NGN2-derived induced neurons (iNs; similar to our EX-neurons), including those positive for sensory neuronal markers (intermediate neurofilament peripherin, *PRPH*) and homeodomain transcription factors (*POU4F1*/*BRN3A* and *PHOX2B*) by single-cell RNA-seq analysis. This suggests that NGN2 can also induce differentiation of PSCs into nociceptive sensory neurons (Lin et al., 2020; Schornig et al., 2021) as well as EX-neurons with a mixture of sensory and cortical transcriptional profiles. It is noteworthy, however, 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 of sensory marker gene expression from RNA-seq analysis (Chen et al., 2020; 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 excitability using whole-cell patch-clamp recording. Over the course of maturation, EX- and IN-neurons presented functional electrophysiological properties such as development of stable resting membrane potentials and generation of action potentials (APs; Fig. 4). The relatively high capacitance, hyperpolarized membrane potential, low membrane resistance, and ability to fire repetitive APs are consistent with the properties of a mature neuron. This result suggests that EX- and IN-neurons made using this method 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).

Using this approach, we have generated nine EX-hPSC lines (seven from hES cells, two from hiPSCs, including CRISPR-Cas9-engineered mutant lines) and three IN-hPSC lines (two from hES cells, one from hiPSCs), and have confirmed that all lines produce robust and reproducible EX- or IN-neurons up to ten 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 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 (e.g., Ngn2-2A-GFP) may be plated with IN-hPSCs and differentiated into neurons in parallel. The two types of neurons can be easily distinguished by fluorescence microscopy, making this 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.

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Author Contributions

Saera Song: Conceptualization, formal analysis, investigation, methodology, writing of original draft. **Archana Ashok:** Investigation, methodology, review and editing of manuscript. **Damian Williams:** Data curation, formal analysis, investigation, writing of original draft, review and editing of manuscript. **Maria Kaufman:** Investigation, methodology, review and editing of manuscript. **Karen Duff:** Funding acquisition, review and editing of manuscript. **Andrew Sproul:** Conceptualization, funding acquisition, investigation, supervision, review and editing of manuscript.

Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

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

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