Generation and characterisation of functional human hypothalamic neurons

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Significance statement:
Since many human diseases stem from the loss or dysfunction of hypothalamic neurons, *in vitro* disease models built around human stem cell-derived hypothalamic neurons are a potentially powerful tool to understand molecular and cellular disease mechanisms. The protocols described here aim to enable groups to interrogate the functions of human hypothalamic neurons.

Abstract:
Neurons in the hypothalamus orchestrate homeostatic physiological processes and behaviours essential for life. Defects in the function of hypothalamic neurons cause a spectrum of human diseases, including obesity, infertility, growth defects, sleep disorders, social disorders, and stress disorders. These diseases have been studied in animal models such as mice, but the rarity and relative inaccessibility of mouse hypothalamic neurons, and species-specific differences between mice and humans highlight the need for human cellular models of hypothalamic diseases. We and others have developed methods to differentiate human pluripotent stem cells (hPSCs) into hypothalamic neurons and related cell types, such as astrocytes. This protocol builds on published studies by providing detailed step-by-step instructions for neuronal differentiation, quality control, long-term neuronal maintenance, and the functional interrogation of hypothalamic cells by calcium imaging. Together, these protocols should enable any group with appropriate facilities to generate and study human hypothalamic cells.

Keywords: hypothalamus, protocol, human, differentiation, pluripotent stem cell, neuron, POMC, obesity

INTRODUCTION

The hypothalamus is highly conserved brain region responsible for a wide array of homeostatic processes (Swaab et al., 1993). Distinct hypothalamic functions are controlled by unique neuronal cell types, often via neuropeptides that have potent effects on behaviour and physiology. For example, neurons producing the melanocortins agouti-related peptide (AGRP), or pro-opiomelanocortin (POMC), potently stimulate or inhibit feeding behaviour, respectively (Fan et al., 1997; Ollmann et al., 1997; Cone, 2006; Zhan et al., 2013). Neurons that produce corticotropin releasing hormone (CRH) sit at the apex of the hypothalamic-pituitary-adrenal (HPA) axis that regulates stress responses (Zoumakis and Chrousos, 2010; Aguilera and Liu, 2012), and neurons that produce thyrotropin releasing hormone (TRH) regulate energy expenditure by affecting the activity of the thyroid gland (Hollenberg, 2008; Lechan and Fekete, 2006). Melanin concentrating hormone (MCH) is a neuropeptide produced by hypothalamic neurons that regulate sleep and metabolism (Konadhode et al., 2013; Qu et al., 1996; Ferreira et al., 2017), and neurons producing hypocretin/orexin (HCRT) are essential for normal sleep regulation and are lost in the sleep disorder narcolepsy (Nixon et al., 2015; Thannickal et al., 2000; Peyron et al., 2000). There is a pressing need to better understand how the abnormal function of hypothalamic neurons contributes to human disease. For example, obesity, thought to be largely a disease of the brain, affects approximately one third of adults in Western countries, and significantly decreases lifespan (Ng et al., 2014; Locke et al., 2015).

To enable the study of live, human hypothalamic neurons, we (Merkle et al., 2015) and others (Wang et al., 2015) developed protocols to differentiate hPSCs, including human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs), into hypothalamic neurons. These differentiation protocols were designed by applying knowledge of the signalling pathways known to pattern the developing ventral forebrain (Puelles and Rubenstein, 2003; Sussel et al., 1999), and by building upon previously established neuronal directed differentiation protocols (Chambers et al., 2009; Maroof et al., 2013; Shi et al., 2012).

Human stem cell-derived hypothalamic neurons recapitulate many of the essential properties of their counterparts *in vivo* (Merkle et al., 2015; Wang et al., 2015, 2017; Yamada-Goto et al., 2017),

making them an excellent scientific model for a number of reasons. First, hPSCs and their derivatives have diploid genomes broadly representative of those found in human populations (Adewumi et al., 2007; Amps et al., 2011; Thomson et al., 1998), an essential consideration when seeking to model the effect of human genetic variants on cellular phenotypes. The ability to readily modify the human genome using CRISPR/Cas9 and other gene editing tools facilitates the generation of reporter cell lines to study cell types of interest and to generate isogenic disease models (Ran et al., 2013; Merkle and Eggan, 2013; Hendriks et al., 2016; Hockemeyer and Jaenisch, 2016). Second, in contrast to the complex environment encountered in the brain, the reduced complexity of a cell culture model system enables the effects of compounds on neuronal function to be directly tested. The accessibility of cells *in vitro* also facilitates time-lapse imaging studies as well as physiological and optogenetic studies. Finally, hPSCs provide a theoretically limitless supply of hypothalamic neurons, which can be differentiated on a large scale. The generation of large cell numbers enables proteomic studies and facilitates the development of functional screens based on neuronal activity.

The protocols described below seek to complement and extend a recently published protocol for human hypothalamic differentiation (Wang et al., 2016) in a number of ways. First, we provide an updated version of our previously published protocol (Merkle et al., 2015) that utilises a chemically defined medium for greater reproducibility, while enabling the production of a broader spectrum of hypothalamic neurons than reported elsewhere, including POMC and AGRP neurons, as well as the disease-relevant HCRT, CRH and TRH neuron populations. Second, we provide protocols for cortical neuron differentiations to enable direct comparison of hypothalamic differentiation quality, as well as additional control measures to enable novice groups to confirm the success of their differentiation without the need for specialised reagents such as *NKX2.1* reporter cell lines (Goulburn et al., 2011). Third, we provide protocols for the maturation, long-term culture, and functional studies of hypothalamic neurons using calcium imaging. We hope that these protocols will enable more groups to study the basic biology of hypothalamic neurons and develop new disease models to facilitate the development of treatments for a range of human diseases.

BASIC PROTOCOL 1 : **HPSC MAINTENANCE (Figure 1)**

HESCs and hiPSCs should be maintained in conditions that stably promote pluripotency. hPSCs are prone to spontaneous differentiation and genetic instability if not handled appropriately (Ronen and Benvenisty, 2012). The presence of spontaneously differentiated cells in hPSC cultures is likely to interfere with efficient hypothalamic differentiation, and the presence of unwanted mutations will complicate downstream analyses. Detailed methods for hPSC culture are provided elsewhere (Andrews et al., 2010; Ludwig and A. Thomson, 2007; Santos et al., 2016)

Briefly, hPSCs are maintained in the absence of antibiotics under feeder-free conditions, monitored and fed daily with complete media changes, and passaged using EDTA before they reach confluence. Cell lines should be adapted to feeder-free conditions and purified from differentiated cells for at least two passages prior to seeding cells for differentiation. Cell lines should be routinely monitored for pathogens such as mycoplasma, as well as the acquisition in culture of unwanted mutations (Merkle et al., 2017; Amps et al., 2011; Baker et al., 2016). Also note that work with hESCs is restricted in some countries and institutions, but can be replaced by hiPSCs for most applications.

Materials

- Geltrex LDEV-Free Reduced Growth Factor Basement Membrane Matrix [Thermo Fisher Scientific, cat. no. A1413202];
- DMEM/F12 without phenol red [Thermo Fisher Scientific, cat. no. 21041025];
- hiPSC or hESC cell line confirmed to be free of pathogens and unwanted mutations (e.g. WA09 cell line, WiCell, RRID:CVCL_9773)
- Phosphate Buffered Saline, calcium and magnesium-free (DPBS), pH range 7.0-7.3 [Thermo Fisher Scientific, cat. no. 14190250];
- Ethylenediaminetetraacetic acid (EDTA) [Sigma Aldrich, cat. no. 03690] [see Reagents and Solutions];

- mTeSR1 medium [STEMCELL Technologies, see Reagents and Solutions];
- Y-27632 dihydrochloride (Rock inhibitor) [DNSK International, cat. no. DNSK-KI-15-02] [see Reagents and Solutions];
- DNase (vial D2) [Worthington Biochemical, cat. no. LK003172];
- Parafilm [Sigma Aldrich, cat. no. P7793]

* Equipment:
- 6-well plate, 12-well plate, 24-well plate, or 10 cm plate;
- 15 ml and 50 ml V-bottom polypropylene tubes (e.g. Falcon tubes);
- 37°C, 20% O_2, 5% CO_2 humidified incubator;
- Laboratory centrifuge [Eppendorf Centrifuge 5804, rotor A-4-44 or similar];
- Class II biosafety cabinet;
- Inverted phase contrast microscope;
- Vacuum aspiration system;
- Pipettes: 2-10 µl, 20-200 µl, 100-1000 µl and corresponding tips;
- Automated pipetman and serological pipettes (5 ml, 10 ml, 25 ml).

Protocol steps – Step annotation

To ensure cultures are kept sterile, cells should be handled in a Class II biosafety cabinet using standard sterile technique at all times (Mather and Roberts, 1998).

A- Coat 6-well plates or 10 cm plates with thin gel layer of Geltrex, a soluble reduced growth factor basement membrane extract that facilitates cell attachment.

1- Thaw Geltrex matrix on ice overnight at 4°C.
* Note - Rapid Geltrex thawing can cause pre-mature gelling, resulting in uneven coating.

2- Prepare a 0.1% (1:10) Geltrex stock by diluting a 5 ml Geltrex vial in 45 ml ice-cold DMEM/F12 without phenol red, gently pipette up and down to mix. During preparation, keep all reagents on ice. Avoid freeze-thaw cycles.

3- To prepare a working solution of Geltrex for hPSC maintenance, further dilute the stock solution 1:10 in ice cold DMEM/F12 without phenol red to a 0.01% final concentration. Again, keep all reagents on ice during this process.

4- Add 1 ml of cold 0.01% Geltrex per well of a 6-well plate, or 5 ml per 10 cm plate, taking care to avoid bubbles.

5- To coat plates for same-day-use, add Geltrex solution to the wells and incubate plate at 37°C for 2 hours. To coat plates for next-day-use, add Geltrex solution to the wells, wrap plates in Parafilm and store them at 4°C up to 72h. Before use, incubate plate with Geltrex at 37°C for 2 hours.

6- Before plating cells, aspirate excess Geltrex and immediately gently rinse the plate one time with an equal volume of PBS. The coated plate is ready to receive cells for plating as described in step C, point 8.
*After coating the plate, take care not to let it dry out at any time. Avoid bubbles.

B- Feed cells daily with mTeSR1 medium by aspirating old medium and providing a complete media change. Note the morphology of the cultures (Figure 1H). Colonies should be uniform in appearance. Cells should have a high nuclear:cytoplasmic ratio (Figure 1C), contain prominent nucleoli, grow in colonies with well-defined borders, and maintain cell-cell contact with other hSPCs.

* Note - Skipping feedings can stress cells and adversely affect differentiation potential. Do not attempt hypothalamic differentiation if there is evidence of cells with a morphology different from that of hPSCs. Eliminate unwanted differentiated cells by aspirating them at

each feeding or by manually picking hPSC colonies as described elsewhere (Andrews et al., 2010; Ludwig and A. Thomson, 2007; Santos et al., 2016).

C- When hPSCs reach 80-90% confluence (Figure 1A) (approximately 3-4 days after previous split), split cells 1:6

1- Preheat desired volume of EDTA solution in a 37°C water bath for approximately 10 minutes.
2- Wash cells gently and briefly with room-temperature DPBS.
3- Add warm EDTA to cultures, 1 ml per well in 6-well plate, 5 ml per 10 cm plate.
4- Incubate with EDTA for 3-5 minutes at 37°C. After 3 minutes, check for cell detachment on a phase contrast microscope. Cells should start to round up and take a phase-bright appearance (Figure 1D and 1E), but not spontaneously detach from the plate. Once cultures adopt this appearance, gently suck up and dispel ~100 µl of the EDTA solution with a P1000 pipette against the cells. They should easily dislodge and leave a small area devoid of cells. If cells do not dissociate easily, extend EDTA digestion for another minute and repeat this test.
5- Once cells can easily be detached from the plate, but before they spontaneously lift off, gently aspirate EDTA.
6- To dissociate cells, add 1 ml or 5 ml mTeSR1 + Y-27632 for a well of a 6 well plate or 10 cm plate, respectively. Gently pipette this medium over the plate to detach cells and pipette up and down several times dissociate them to suspension of small clumps of cells. Avoid bubbles.
7- Collect cells in 15 ml V-bottom polypropylene tube, and adjust volume with mTeSR1 + Y-27632 if necessary.
8- Plate the desired volume of this cell suspension to achieve a 1:4 to 1:10 split ratio onto Geltrex-coated plates (see above) in mTeSR1 + Y-27632, bringing the final volume to 2 ml per well of a 6 well plate or 10 ml per 10 cm plate. Culture cells in mTeSR1 + Y-27632 for one day at 37°C, 20% O_2, 5% CO_2 humidified incubator, then maintain them by feeding daily with mTeSR1 medium.
   * Note - The split ratio will vary somewhat depending on the growth properties of the cell lines. Cultures should be passaged approximately every 4 days.
9- Depending on experimental design, expand culture to provide sufficient cell numbers for differentiation. A 90% confluent plate 10 cm or 15 cm plate of hPSC yields about 8 x 10^6 or 2 x 10^7 cells, respectively.

BASIC PROTOCOL 2: **HYPOTHALAMIC DIFFERENTIATION (Figure 2)**

The efficient generation of human hypothalamic neurons from hPSCs is based on developmental principles. The signalling pathways that lead to neuralization, forebrain specification, and ventralization are manipulated by small molecule drugs. Specifically, the Wingless-related integration site (WNT), transforming growth factor beta (TGFβ) and bone morphogenetic protein **(**BMP) signalling pathways are inhibited with XAV939, LDN-193189 and SB431542, respectively, followed by activation of the Sonic Hedgehog (SHH) pathway with Purmorphamine and Smoothed agonist (SAG). Later addition of the gamma-secretase inhibitor DAPT promotes neurogenesis by inhibiting NOTCH signalling [Figure 2A] (Merkle et al., 2015; Wang et al., 2015).

This protocol is designed for adherent differentiation in monolayers. Typically, more than 1 x 10^8 neurons are generated per 10 cm plate of hPSCs, providing large numbers of human hypothalamic neurons for functional studies, disease modelling, cellular transplantation, or drug screening. In contrast to previously published studies (Merkle et al., 2015; Wang et al., 2016), the protocol described below omits animal-derived products such as knockout-serum replacement (KOSR) medium and recombinant proteins in favour of chemically defined media and small molecule drugs to ensure greater robustness and reproducibility. Furthermore, the resulting cells can be matured and maintained over long periods of time (at least 6 months) to enable the study of long-term developmental processes.

Materials

- Geltrex LDEV-Free Reduced Growth Factor Basement Membrane Matrix [Thermo Fisher Scientific, cat. no. A1413202];
- Nearly confluent culture of hESCs or hiPSCs (generated in BASIC PROTOCOL 1)
- Phosphate Buffered Saline, calcium and magnesium-free (DPBS), pH range 7.0-7.3 [Thermo Fisher Scientific, cat. no. 14190250];
- TrypLE Express 1X, no phenol red [Life Technologies, cat. no. 12604021];
- mTeSR1 medium [see Reagents and Solutions];
- hPSC wash medium [see Reagents and Solutions];
- Trypan blue [Invitrogen, cat. no. T10282];
- Y-27632 dihydrochloride Rock inhibitor [DNSK International, cat. no. DNSK-KI-15-02] [see Reagents and Solutions];
- N2B27 medium [see Reagents and Solutions];
- Dimethyl Sulfoxide, DMSO [Sigma Aldrich, cat. no. D2650-100ml];
- Bovine Serum Albumin, BSA [Sigma Aldrich, cat. no. A0281];
- XAV939 [Stemgent, cat. no. 04-0046] [see Reagents and Solutions];
- LDN-193189 [Stemgent, cat. no. 04-0074] [see Reagents and Solutions];
- SB431542 [Sigma Aldrich, cat. no. S4317] [see Reagents and Solutions];
- Smoothened agonist (SAG) [Fisher Scientific (EMD Millipore) cat. no. 56-666-01MG, or DNSK International custom order, DNSK-SMO-1] [see Reagents and Solutions];
- Purmorphamine [Calbiochem (EMD Millipore) cat. no. 540220, or DNSK International custom order] [see Reagents and Solutions];
- DAPT [Sigma Aldrich, cat. no. D5942, or DNSK International custom order] [see Reagents and Solutions];
- Brain-derived neurotrophic factor (BDNF) [Pepro Tech, cat. no. 450-02] [see Reagents and Solutions];
- Maturation medium [see Reagents and Solutions];

* Equipment:

- 6-well plate, 12-well plate, 24-well plate, or 10 cm plate;
- 15 ml and 50 ml V-bottom polypropylene tube (e.g. Falcon tubes);
- Automated cell counter and associated consumables [e.g. Life Technologies, Countess II Automated Cell Counter, cat no. AMQAX1000];
- Cell counting slide [Countess Cell Counting Chamber Slides, cat. no. C10228];
- 37°C, 5% CO_2 humidified incubator;
- Laboratory centrifuge [Eppendorf Centrifuge 5804, rotor A-4-44 or similar];
- Class II biosafety cabinet;
- Inverted phase contrast microscope;
- Vacuum aspiration system;
- Pipettes: 2-10 µl, 20-200 µl, 100-1000 µl and corresponding tips;
- Automated pipetman and serological pipettes (5 ml, 10 ml, 25 ml);
- 0.5 or 1.5 ml polypropylene tubes [e.g. Eppendorf tubes].

Protocol steps

A- Coat 6-well plates or 10 cm plates with Geltrex for differentiation as described above (BASIC PROTOCOL 1, A).

B- hPSCs (cultured in BASIC PROTOCOL 1) are dissociated and re-plated for differentiation.

* Note - Before induction of differentiation, hPSCs should lack obvious signs of differentiation or contamination, and be in a rapid growth phase.

1- Aspirate culture medium and briefly and gently wash cell culture in room temperature PBS.
2- Add 37°C TrypLE to cell culture, 1 ml per well in 6-well plate, 5 ml per 10 cm plate.

3- Incubate cell culture for 3-5 minutes at 37°C. After a 3 minute incubation, check to see if cells are detaching. Under a phase contrast microscope, the cells should start to round up and take on a phase-bright appearance, but not spontaneously detach from the plate. Once cultures adopt this appearance, gently suck up and dispel ~100 µl of the TrypLE solution with a P1000 pipette against the cells. They should easily dislodge and leave a small area devoid of cells. If cells do not dissociate easily, extend TrypLE digestion for another minute and repeat this test.
   * Note - Take care to avoid over-digestion, which can cause cell death.
4- Gently aspirate TrypLE.
5- To dissociate cells, add 1 ml or 5 ml mTeSR1 + Y-27632 for a well of a 6 well plate or 10 cm plate, respectively, and gently pipette this medium over the plate to detach cells and dissociate them to a single-cell suspension.
6- Collect cells in 15 ml V-bottom polypropylene tube, and adjust volume with mTeSR1 + Y-27632 (total volume = 10 ml). This wash steps dilutes residual TrypLE to slow further digestion.
7- Centrifuge cells at 160 x g for 3-5 minutes at room temperature. Aspirate supernatant, re-suspend cells in 1 ml hPSC wash medium with a P1000, then bring to 10 ml with wash medium.
8- Centrifuge cells at 160 x g for 3-5 minutes at room temperature. Aspirate supernatant, re-suspend cells in 1 ml mTeSR1 + Y-27632. These wash steps remove any remaining traces of TrypLE.
9- After re-suspending the cell pellet, adjust volume so that the suspension is visibly turbid, but not milky (approximately 1-5 x 10^6 cells/ml).
10- In a 0.5 ml polypropylene tube, mix 10 µl of this cell suspension with 10 µl Trypan blue, transfer 10 µl of that mixture onto cell counting slide. Count cells with automated cell counter.
11- Plate cells onto Geltrex-coated plates in mTeSR1 + Y-27632, at a concentration of 1 x 10^5 cells per cm^2 (corresponding to 9.5 x 10^5 cells per well of a 6-well plate, or 5.5 x 10^6 cells per 10 cm plate). This density corresponds to approximately 80% confluence the following day. Ensure that cells are evenly distributed across the plate by gently shaking the plate left to right, then top to bottom after transferring it to the incubator.
   * Note - If cells are sparser, wait until cells reach the desired density before starting the differentiation. Sparse or over-confluent cells will not pattern well.
12- If cells plated for differentiation are evenly distributed over the plate and at a density of approximately 75% (Figure 2B and 2B'), start differentiation by washing cultures once with PBS and adding Day 0 (D0) medium (see below). Every second day, make full medium changes as follows (5-6 ml per 6-well plate, 10-15 ml per 10 cm plate):
    - Day 0 (D0): N2B27 + 2 µM XAV939 + 100 nM LDN-193189 + 10 µM SB431542
    - Day 2 (D2): N2B27 + 2 µM XAV939 + 100 nM LDN-193189 + 10 µM SB431542 + 1 µM SAG + 1 µM Purmorphamine
    - Day 4 (D4): N2B27 + 1.5 µM XAV939 + 75 nM LDN-193189 + 7.5 µM SB431542 + 1 µM SAG + 1 µM Purmorphamine
    - Day 6 (D6): N2B27 + 1 µM XAV939 + 50 nM LDN-193189 + 5 µM SB431542 + 1 µM SAG + 1 µM Purmorphamine
    - Day 8 (D8): N2B27 + 0.5 µM XAV939 + 25 nM LDN-193189 + 2.5 µM SB431542 + 5 µM DAPT
    - Day 10 (D10): N2B27 + 5 µM DAPT
    - Day 12 (D12): N2B27 + 5 µM DAPT
    - Day 14 (D14): dissociation and re-plating in Maturation medium + 5 µM DAPT + Y-27632 (see Basic Protocol 3)

13- Observe cells daily for changes in morphology. From Days 0-2, the culture should reach confluence and cells should have a simple and uniform hPSC-like morphology (Figure 2C and 2C'). By Day 4, cultures are highly compacted and cells adopt a more rounded appearance (Figure 2D and 2D'). Between Days 4 and 8, the cultures take on a dense neuroepithelial

morphology with identifiable neural ridge-like structures. (Figure 2E and 2E'). A neuroepithelial morphology is still evident before passaging on Day 14 (Figure 2F and F'). For issues that can arise during the differentiation process, please see the Troubleshooting section.

SUPPORT PROTOCOL 1: **CORTICAL NEURON (CONTROL) DIFFERENTIATION**

In order to confirm correct and efficient hypothalamic patterning of hPSCs, it is helpful to compare hypothalamic differentiation to a parallel differentiation of hPSCs to cortical neurons (Shi et al., 2012). To facilitate parallel culture, the time course and basic procedure of this differentiation is similar to hypothalamic differentiation, except that ventralizing factors (SAG and Purmorphamine) are omitted and DAPT is replaced with FGF2.

Materials

- Follow the list of Materials from BASIC PROTOCOL 2;
- FGF2 [Sigma Aldrich, cat.no. F0291] [see Reagents and Solutions].

Protocol steps – Step annotation

Follow the steps from 1 to 10 from BASIC PROTOCOL 2.

11- If cells plated for differentiation are evenly distributed over the plate and at a density of approximately 75%, start the differentiation by washing cultures once with DPBS and adding Day 0 (D0) medium (see below). Every second day, make full media changes as follows:
    - Day 0 (D0): N2B27 + 2 µM XAV939 + 100 nM LDN-193189 + 10 µM SB431542
    - Day 2 (D2): N2B27 + 2 µM XAV939 + 100 nM LDN-193189 + 10 µM SB431542
    - Day 4 (D4): N2B27 + 1.5 µM XAV939 + 75 nM LDN-193189 + 7.5 µM SB431542
    - Day 6 (D6): N2B27 + 1 µM XAV939 + 50 nM LDN-193189 + 5 µM SB431542
    - Day 8 (D8): N2B27 + 0.5 µM XAV939 + 25 nM LDN-193189 + 2.5 µM SB431542
    - Day 10 (D10): N2B27
    - Day 12 (D12): N2B27 + 20 ng/ml FGF2
    - Day 14 (D14): dissociation and re-plating in Maturation medium + 20 ng/ml FGF2 + Y-Y-27632 (see Basic Protocol 3)

BASIC PROTOCOL 3 : **NEURONAL MATURATION**

The aim of this protocol is to create mature hypothalamic neurons that are spontaneously electrically active and that respond to exogenous factors, such as leptin. To promote maturation and induction of target genes such as POMC, cultures are re-plated at 1 x 10^5 cells per cm^2 and 10 ng/ml brain-derived neurotrophic factor (BDNF) is added to the culture medium. In our hands, hypothalamic neurons begin to acquire functional properties after 30 days of culture in the absence of exogenously added primary glia, and mature further over subsequent weeks in culture. Plating dissociated human neurons on a monolayer of glia (3 x 10^4 cells per cm^2) harvested from the newborn mouse or rat brain may accelerate functional maturation and may be particularly valuable for experiments in which neurons are plated at low densities or are to be used for electrophysiological recording (Zuchero and Barres, 2015; Ullian et al., 2001). Protocols for glial isolation and culture are described elsewhere (Foo et al., 2011; Albuquerque et al., 2009). The same maturation protocol can be used for hypothalamic and cortical differentiation.

Materials

- Geltrex LDEV-Free Reduced Growth Factor Basement Membrane Matrix [Thermo Fisher Scientific, cat. no. A1413202];
- Neural progenitor cells after differentiation from BASIC PROTOCOL 2;
- Phosphate Buffered Saline, calcium and magnesium-free (DPBS), pH range 7.0-7.3 [Thermo Fisher Scientific, cat. no. 14190250];

- TrypLE Express 1X, no phenol red [Life Technologies, cat. no. 12604021];
- Trituration medium [see Reagents and Solutions];
- Maturation medium [see Reagents and Solutions];
- Y-27632 dihydrochloride (Rock inhibitor) [DNSK International, cat. no. DNSK-KI-15-02] [see Reagents and Solutions];
- Trypan blue stain 0.4% [Invitrogen, cat. no. AM7962];
- Brain-derived neurotrophic factor (BDNF) [Pepro Tech, cat. no. 450-02] [see Reagents and Solutions];
- Papain [Thermo Fisher Scientific, cat no. 88290] [see Reagents and Solutions];
- Human brain reference total RNA [Thermo Fisher Scientific, cat. no. AM6050];

* Equipment:
- 6-well plate, 12-well plate, 24-well plate, or 10 cm plate;
- 15 ml and 50 ml V-bottom polypropylene tube (e.g. Falcon tubes);
- Automated cell counter and associated consumables [e.g. Life Technologies, Countess II Automated Cell Counter, cat no. AMQAX1000];
- Cell counting slide [Countess Cell Counting Chamber Slides, cat. no. C10228];
- 37°C, 5% CO_2 humidified incubator;
- Laboratory centrifuge [Eppendorf centrifuge 5804, rotor A-4-44 or similar];
- Class II biosafety cabinet;
- Inverted phase contrast microscope;
- Vacuum aspiration system;
- Pipettes: 2-10 µl, 20-200 µl, 100-1000 µl and corresponding tips;
- Automated pipetman and serological pipettes (5 ml, 10 ml, 25 ml);

Protocol steps

A- Coat plates with Geltrex for differentiation as described above (BASIC PROTOCOL 1, A), except use a 0.02% final Geltrex concentration to facilitate neuronal attachment and long-term culture.

B- On Day 14, neural progenitors generated in BASIC PROTOCOL 2 or SUPPORT PROTOCOL 1 are dissociated and re-plated to encourage neurogenesis and neuronal survival and maturation.

1- Wash cell culture gently in PBS.
2- Add TrypLE to cells, 1 ml per well in 6 well plate, 5 ml per 10 cm plate.
3- Incubate cell culture for 3-5 minutes at 37°C. After 3 minutes of incubation, check to see if cells are detaching. Under a phase contrast microscope, the cells should start to round up and take on a phase-bright appearance, but not spontaneously detach from the plate. Once cultures adopt this appearance, gently suck up and dispel ~100 µl of the TrypLE solution with a P1000 pipette against the cells. They should easily dislodge and leave a small area devoid of cells. If cells do not dissociate easily, extend TrypLE digestion for another minute and repeat this test.

   * Note - Take care to avoid over-digestion, which can cause cell death and release of genomic DNA.
4- Gently aspirate TrypLE.
5- To dissociate cells, add 1 ml or 5 ml trituration medium for a well of a 6 well plate or 10 cm plate, respectively, and gently pipette this medium over the plate to detach cells and dissociate them to a single-cell suspension.
6- Collect cells in 15 ml V-bottom polypropylene tube, and adjust volume with trituration medium (total volume = 10 ml).
7- Centrifuge cells at 160 x g for 3-5 minutes at room temperature. Aspirate supernatant, re-suspend cells in 10 ml trituration medium.

8- After re-suspending the cell pellet, adjust volume so that the suspension is visibly turbid, but not milky (approximately 1-5 million cells/ml).
9- In a 0.5 ml polypropylene tube, mix 10 µl of this cell suspension with 10 µl Trypan blue, transfer 10 µl of that mixture onto cell counting slide. Count cells with automated cell counter.
10- Centrifuge cells at 160 x g for 3-5 minutes at room temperature. Aspirate supernatant, re-suspend cells in a desired volume of maturation medium + Y-27632 to enable plating at the desired density.
    * Note - If desired, cultures can be frozen at this point for later thawing as progenitors/immature neurons using the same procedure used for freezing hPSCs (Santos et al., 2016).
11- Plate cells onto Geltrex-coated plates in maturation medium + Y-27632 at a concentration of 1 x 10^5 cells per cm^2 (corresponding to 9.5 x 10^5 cells per well of a 6 well plate, or 5.5 x 10^6 cells per 10 cm plate).
12- On Day 15, aspirate medium and feed with maturation medium.
13- On Day 16, aspirate medium and add twice the normal volume of maturation medium (e.g. 4 ml per well of a 6 well plate, 20 ml per 10 cm plate) for neuronal maintenance. This larger volume helps ensure that neurons are exposed to a relatively constant supply of nutrients.
14- Change 75% of media volume every second day. BDNF should be added fresh at each feeding. On Day 30, repeat the dissociation procedure outlined in steps 1-11, with the following exception: resuspend 1 vial of Papain to 10 ml of TrypLE at step 2. Papain aids in neuronal dissociation and will ensure significantly higher survival upon re-plating.
    * Note - It may be desirable to perform re-plating slightly earlier (such as Day 25 when genes such as POMC become robustly expressed) or later (such as Day 40 when genes such as HCRT become robustly expressed).
15- After replating neurons at a later time point such as Day 30, aspirate medium and add twice the normal volume of maturation medium (e.g. 4 ml per well of a 6 well plate, 20 ml per 10 cm plate) for neuronal maintenance. Cell cultures can be maintained in this manner for 6 months or more, although cell detachment can become an issue (see Troubleshooting).
    * Note - If long-term culture is desired, consider culturing cells at lower density (2 x 10^4 cell per cm^2) on a monolayer of primary astrocytes plated on Geltrex-coated plastic. Also note that non-neuronal cells maycontinue to proliferate and can lead to culture overgrowth. Although cultures can be maintained for long periods of time, many experiments can be executed between Day 30-80.

SUPPORT PROTOCOL 2: **QUALITY CONTROL – CONFIRMATION OF HYPOTHALAMIC PATTERNING AND NEUROGENESIS**

To confirm successful hypothalamic patterning and generation of hypothalamic neurons, we recommend performing quality control experiments based on immunostaining and RT-qPCR before performing further experiments. Hypothalamic patterning can be gauged by the expression of regionally-expressed transcription factors (Figure 3A and 3B), and hypothalamic neurogenesis can be confirmed by testing for neuropeptides that are highly enriched in the hypothalamus (Figure 3C, 3D, 3F) (Tables 1-3).

Table 1. Proteins detectable by immunofluorescence in differentiated cultures by Day 14 or by Day 40.

EXPRESSION	HYPOTHALAMIC NEURONS	CORTICAL NEURONS
Day 14	FOXG1 (few cells) NKX2.1 (most cells)	FOXG1 (most cells) NKX2.1 (few cells) OTX1/2 (many cells) PAX6 (many cells)
Day 40	TUJ1 (most cells)	TUJ1 (most cells)

	MAP2 (most cells) POMC (many cells) AGRP (some cells) HCRT (some cells) PMCH (some cells)	MAP2 (most cells) POMC (negative) AGRP (negative) TBR1 (many cells) CTIP2 (many cells)

Table 2. List of primary antibodies.

ANTIGEN	SUPPLIER, catalog number, RRID	SPECIES	DILUTION
AGRP	Acris Antibodies GmbH Cat# GT15023-100 Lot# RRID:AB_1611787	goat	1:300
CTIP2	Abcam Cat# ab18465 Lot# RRID:AB_2064130	rat	1:500
FOXG1	Santa Cruz Biotechnology Cat# sc-18583 Lot# RRID:AB_2294176	goat	1:100
MAP2	Abcam Cat# ab5392 Lot# RRID:AB_2138153	chicken	1:2,000
NKX2.1	Innovative Research Cat# 18-0221 Lot# RRID:AB_86728	mouse	1:500
OTX1/2	Millipore Cat# AB9566 Lot# RRID:AB_2157186	rabbit	1:300
PAX6	Covance Research Products Inc Cat# PRB-278P-100 Lot# RRID:AB_291612	rabbit	1:500
POMC	Thermo Fisher Scientific Cat# MA5-16319 Lot# RRID:AB_2537838	mouse	1:200
PMCH	Sigma-Aldrich Cat# M8440 Lot# RRID:AB_260690	rabbit	1:1,000
TBR1	Abcam Cat# ab31940 Lot# RRID:AB_2200219	rabbit	1:500
TUJ1	BioLegend Cat# 845502 Lot# RRID:AB_2566589	rabbit	1:2,000

Table 3. Primers used for gene expression analysis.

GENE	Expected expression in hypothalamic cultures	Expected expression in cortical cultures	Taqman Assay ID (Thermo Fisher)
AGRP	high	low	Hs00361403_g1
EMX1	low	high	Hs00417957_m1
FOXG1	low	high	Hs01850784_s1
NKX2.1	high	low	Hs00968940_m1
POMC	high	low	Hs01596743_m1
OTP	high	low	Hs01888165_s1
SIM1	high	low	Hs00231914_m1

Materials

- Geltrex LDEV-Free Reduced Growth Factor Basement Membrane Matrix [Thermo Fisher Scientific, cat. no. A1413202];
- Glass coverslip [Thermo Scientific, cat. no. MENZCB00120RAC20];
- Hydrochloric acid (HCl) [Fisher Chemicals, cat. no. MFCD00011324 – 2.5 L];
- Cultures generated in BASIC PROTOCOL 3;
- Paraformaldehyde [Sigma Aldrich, cat. no. 158127 – see Reagents and Solutions, or 4% Paraformaldehyde in PBS, Santa Cruz, cat. no. 30525-89-4];
- Normal Donkey Serum [Jackson Immunoresearch Labs Inc., cat. no. 017-000-121];
- Triton X-100 [Sigma Aldrich, cat. no. T8787];
- Tris Buffered Saline (TBS) [Sigma Aldrich, cat. no. T5030];
- Primary antibodies [see Reagents and Solutions, Table 2];
- 4’,6-Diamidine-2’-phenylindole dihydrochloride (DAPI) [Sigma Aldrich, cat. no. 10236276001];

- Phosphate Buffered Saline, calcium and magnesium-free (DPBS), pH range 7.0-7.3 [Thermo Fisher Scientific, cat. no. 14190250];
- ProLong Diamond Antifade Mountant [Thermo Fisher Scientific, cat. no. P36965];
- RNA isolation kit [Qiagen, cat. no. 74104];
- Primers [see Reagents and Solutions, Table 3];
- Ethanol [Sigma Aldrich, cat. no. V0T0041] [see Reagents and Solutions];
- Parafilm [Sigma Aldrich, cat. no. P7793];
- Sodium Azide [Sigma Aldrich, cat. no. 769320] [see Reagents and Solutions];
- Human brain total RNA [Thermo Fisher Scientific, cat. no. AM7962].

* Equipment:
- 6-well plate, 12-well plate, 24-well plate, or 10 cm plate;
- 15 ml V-bottom polypropylene tube (e.g. Falcon tubes);
- Pipettes: 2-10 µl, 20-200 µl, 100-1000 µl and corresponding tips;
- Automated pipetman and serological pipettes (5 ml, 10 ml, 25 ml);
- Transfer pipettes [Biologix, cat. no. 30-0135A1];
- Fine forceps for handling glass coverslips;
- Chemical hood;
- Adhesive film [e.g. Bio Rad, cat. no. MSB1001].

Protocol steps – Step annotation

A- Prepare coverslips for cell plating.

1- Incubate glass coverslips in 5 M HCl at 65°C overnight, preferably in a rolling or shaking incubator to enable even acid etching of coverslips.
2- Carefully remove acid and transfer coverslips to a clean glass beaker.
3- Thoroughly wash cover slips 5x with large volumes of distilled water.
4- Cover and store cover slips in 70% ethanol. Cover with Parafilm.
5- In a class II Biosafety cabinet, transfer one acid-etched and ethanol-sterilised glass coverslip per well of a 24-well plate using a sterile pair of forceps. Transfer a sufficient number of coverslips to analyse desired number of antibodies for each cell line and differentiation protocol, keeping in mind biological and technical replicates.
6- Once coverslips have completely dried, coat them with Geltrex as described in BASIC PROTOCOL 1, A.
   * Note - At this point it is useful to coat a sufficient number of wells with Geltrex for RT-qPCR analysis as described below if this analysis is to be carried out, especially for analysis at later time points such as Day 40.
7- Plate differentiated neurons, dissociated as described in BASIC PROTOCOL 3, B, step 1-12, onto 24-well plate filled with glass cover slips.
8- Allow cells to recover for 24-48 hours.
9- Gently aspirate medium and wash cells with PBS, taking care to avoid cell detachment.
10- In a chemical hood, gently add 4% Paraformaldehyde (PFA) and incubate cells 10 minutes at room temperature.
    * Note - If using PFA from a frozen stock, ensure that PFA is completely thawed but not warm.
11- In a chemical hood, collect PFA for waste disposal, but take care not to let cells dry.
12- Wash cells 3 times with 1 x TBS, again taking care not to let cells dry.
    * Note - To ensure gentle washing of cells, it is advisable to use a plastic transfer pipette capped with a P200 pipette tip. Fixed cells can be stored in TBS at this point at 4˚C for up to 72 hours before staining.

B- Staining

1- After fixing cells, remove TBS and add primary antibody solution (10% Normal Donkey Serum re-suspended in TBS + 0.1 % Triton + primary antibodies) to fixed cells and incubate them at 4°C overnight (see Table 2).
2- Wash cells with 1 x TBS, 3 times briefly and gently, and then once for at least 30 minutes.
3- Add secondary antibody solution (10% Normal Donkey Serum re-suspended TBS + 0.1% Triton + secondary antibodies), incubate cells 2h at room temperature.
4- Wash cells 1x TBS, 3 times briefly and gently, and then once for at least 30 minutes.
5- Add 360 nM DAPI solution and incubate cells 5 minutes at room temperature in darkness.
6- Wash cells 3x times with 1 x TBS.
7- Store plates at 4°C in TBS with 0.1% sodium azide, protect from light.
* Note - If cells are on coverslips, mount them onto glass slides as described below. If cells are on plates, add an appropriate volume of TBS with 0.1% sodium azide for the size of the well and seal the plate with adhesive film to prevent evaporation.

C- Mounting slides

1- Add 1 drop of mounting medium (ProLong Diamond Antifade Mountant) onto a glass slide, taking care to avoid introducing air bubbles.
2- Remove excess liquid from cover slip by dabbing on a paper towel.
3- Place cover slip sample-side down onto mounting medium on the glass slide.
4- Cure for 24 hours at room temperature, protect from light.
5- Gently clean cover slips with 70% ethanol and image.

D- Analysis of cells by RT-qPCR

1- Collect approximately 1 x 10^6 cells per condition from freshly dissociated cells or from adherent cultures dissociated with TrypLE as described above.
2- Wash and pellet cells as described above.
3- Carefully aspirate all supernatant from cell pellet and resuspend in 350 µl RLT buffer from the Qiagen RNeasy Mini Kit.
4- Isolate RNA according to manufacturer's instruction using the Qiagen RNeasy Mini Kit.
5- Analyse gene expression by TaqMan RT-qPCR as described elsewhere (Toyohara et al., 2015). As a positive control, we use commercially available human whole-brain RNA [See materials].

SUPPORT PROTOCOL 3 **FURA-2 IMAGING OF HYPOTHALAMIC CULTURES**

Calcium imaging can be performed on hPSC-derived hypothalamic neurons, allowing for functional physiological analysis of multiple neurons in parallel. Compared to conventional whole cell electrophysiology, calcium imaging enables the analysis of larger cell numbers in a shorter amount of time, albeit with lower temporal precision or insights into cellular membrane properties. A number of different calcium indicators are available for different applications (Russell, 2011; Ikegaya et al., 2005). We describe here the use of the bulk-loaded ratiometric indicator Fura-2AM, which should be broadly applicable. Ratiometric indicators minimise the effects of photobleaching and other artefacts such as uneven loading into cells. Fura-2AM fluoresces more brightly at 340 nm and more weakly at 380 nm in the presence of calcium, enabling the 340:380 nm fluorescence intensity ratio of Fura20AM to indicate changes in intracellular calcium levels (Takahashi et al., 1999). In cultures at least 30 days old, calcium imaging can be used to investigate how the hPSC-derived neurons respond to stimuli. Neurons older than Day 50 tend to be more active, and are better suited to studying synaptic activity (Kirwan et al., 2015; Shi et al., 2012; Prè et al., 2014).

Materials:

- Geltrex LDEV-Free Reduced Growth Factor Basement Membrane Matrix [Thermo Fisher Scientific, cat. no. A1413202];

- hPSC-derived hypothalamic neurons Day 30+ (preferably Day 40-Day 60) [generated in BASIC PROTOCOL 3];
- TrypLE Express 1X, no phenol red [Thermo Fisher Scientific, cat. no. 12604021];
- DMEM/F12 without phenol red [Thermo Fisher Scientific, cat. no. 21041025];
- Artificial cerebrospinal fluid (ACSF) [see Reagents and Solutions];
- Fura2-AM [Fisher Scientific, cat. no. F1221];
- Dimethyl Sulfoxide, DMSO [Sigma Aldrich, cat. no. D2650-100ml];
- Pluronic F127 [Sigma-Aldrich, cat. no. P2443];
- Papain [cat.no.].[Thermo Fisher Scientific, cat no. 88290];

* Equipment:
- 35 mm Ibidi dishes [Thistle Scientific, cat. no. 80136];
- 37°C, 5% CO_2 humidified incubator;
- Class II biosafety cabinet;
- Pipettes 2-10 µl, 20-200 µl, 100-1000 µl and corresponding tips;
- Automated pipetman and serological pipettes (5 ml, 10 ml, 25 ml);
- Inverted microscope, e.g. Eclipse TE2000U (Nikon)
- Epifluorescence illuminator and monochromator to provide excitation wavelengths at 340 nm, 360 nm, 380 nm. e.g. Optoscan (Cairn Research Ltd);
- Emission filter: 510nm;
- Objective: 40x Nikon Oil immersion, N.A. 1.3;
- EMCCD camera, e.g. Orca-ER-1394 (Hamamatsu)
- Perfusion system; we use a custom-built gravity perfusion system, however commercial systems are available, e.g. MPS-2 (World Precision Instruments);
- Imaging Software: e.g. Metafluor (Molecular devices, RRID: SCR_014294);

Protocol steps – Step annotation

A- Preparation of cells for Calcium imaging.

1- Seed neurons at 1 x 10^5 cells per cm^2 onto Geltrex coated 35 mm Ibidi dishes using Papain with TrypLE (see Basic Protocol 3, step 14).
   * Note - Cells can be imaged as soon as 24 hours after plating onto 35 mm dishes, depending on the experiment. To detect spontaneous and synaptic activity, it is recommended that cells are allowed to recover for at least 1 week after plating.
2- Aspirate culture media from cells and add 1 ml of Fura2-AM of loading solution per 35 mm dish (Figure 4A).
3- Transfer cells to tissue culture incubator at 37˚C and 5% CO_2 for 45 minutes.
4- Remove loading solution from the cells and wash 2 times with ACSF.
5- Transfer cells to incubator for a further 30 minutes before imaging.

B- Calcium Imaging

1- If perfusion is required, prepare during the final incubation after loading the cells with Fura2-AM. Setup so that perfusion flows at a rate of 50 µl per second. Turn on heating if required.
   * Note - If studying synaptic activity, it is recommended that cells are recorded at 37˚C.
2- Transfer cells to imaging setup and begin perfusion of ACSF onto cells. Allow 10 minutes for perfusion and temperature to stabilise.
3- Check for fluorescence under UV illumination.
4- Navigate to a field where the cell density allows for clear resolution of single cells and which also enables the measurement of background intensity (Figure 4B)
5- Use software to set up acquisition at 340 nm, 360 and 380 nm excitation, and detection of emitted light at 515 nm.

* Note: The 360 nm channel is used to determine the extent to which photobleaching is occurring. This is done comparing the first and last image in the 360 nm channel from the dataset, checking whether fluorescence intensity was lost during the experiment.

6- Focus the cells and adjust the gain and exposure settings so that the signal is not saturated. We recommend exposures of 50-200 milliseconds to minimise bleaching.

7- Use the imaging software to highlight cell bodies of interest and a background area. Use the background area to perform a background signal subtraction.
* Note - Observations of neuronal activity and background subtraction can also be performed post-acquisition by exporting the acquired image sets using commercial software or custom scripts (e.g. MATLAB, RRID, SCR_001622).

8- Acquire time-lapse recordings at 1-10 Hz depending on the experiment. Generally, activity that produces subtle, fast changes in calcium influx should be recorded at 10 Hz, while activities that produce large, robust calcium influxes can be recorded at 1 Hz. The length of recording will depend on the application, i.e. drug perfusions, number of treatments, and latency of response, but is typically 30-50 minutes.

9- Near the end of each recording, perfuse cultures with ACSF containing 30 mM KCl. This will depolarise the cells and act as a benchmark for the electrical maturity and general health of the cells. An example recording is shown in Figure 4C.

REAGENTS AND SOLUTIONS (in alphabetical order)

ACSF

- 129 mM NaCl [Sigma Aldrich, cat. no. 31434],
- 5 mM KCl [Sigma Aldrich, cat. no. 31248],
- 1 mM $CaCl_2$ [Sigma Aldrich, cat. no. C5670],
- 1 mM $MgCl_2$ [Promega, cat. no. A3511],
- 25 mM HEPES [Thermo Fisher Scientific, cat. no. 15630056],
- 11.1 mM Glucose [Sigma Aldrich, cat. no. G7021],

Check that pH is 7.2-7.4 and adjust if necessary.

* Note - Stock solutions of each reagent are made up in sterile distilled water.

ACSF, 30 mM KCl

- 104 mM NaCl [Sigma Aldrich, cat. no. 31434],
- 30 mM KCl [Sigma Aldrich, cat. no. 31248],
- 1 mM $CaCl_2$ [Sigma Aldrich, cat. no. C5670],
- 1 mM $MgCl_2$ [Promega, cat. no. A3511],
- 25 mM HEPES [Thermo Fisher Scientific, cat. no. 15630056],
- 11.1 mM Glucose [Sigma Aldrich, cat. no. G7021],

Check that pH is 7.2-7.4 and adjust if necessary.

* Note - Stock solutions of each reagent are made up in sterile distilled water.

BDNF

Reconstitute powder in DPBS with 0.1% BSA (sterile filtered) to 100 µg/ml, aliquot, and store aliquots at -80°C. Use at 1:1,000 for a 10 ng/ml final concentration.

DAPI

Reconstitute powder in sterile distilled water to 360 µM (1 µg/ml) (in the dark at room temperature) and store aliquots at -20°C. Use at 1:10,000 for a 360 nM final concentration.

DAPT

Reconstitute powder in DMSO to generate a 50 mM stock, aliquot, and store aliquots at -20°C. Use at 1:10,000 for a 5 µM final concentration.

EDTA

Purchased at a stock concentration of 0.5 M. Dilute 1:1,000 in tissue culture grade Ca^{2+} and Mg^{2+} free PBS and sterile filter. Store at room temperature or at 4°C for up to a year.

Ethanol 70% (100 ml)

- 30 ml sterile distilled water,
- 70 ml ethanol (technical grade 99%)

FGF2

Reconstitute powder according to manufacturer's directions to generate a 20 ng/ml stock, aliquot, and store aliquots at -80°C.

Fura2

Prepare Fura2-AM stock by dissolving 50 µg in 48 µl DMSO and 2 µl 10% w/v pluronic acid. This will give a 1 mM stock solution. Aliquot and freeze at -20˚C.

Fura2 Loading solution

Add 1 µl of 1 mM Fura2-AM stock per 3 ml of ACSF (final concentration of 333 nM). Prepare 1ml per 35 mm dish to be loaded.

hPSC wash medium (30 ml)

- 30 ml mTeSR1,
- 30 µl Y-27632 dihydrochloride Rock inhibitor stock solution (1000x) to a final concentration of 10 µM,
- 1 vial D2 DNAse [Worthington, cat. no LK003170].

LDN-193189

Reconstitute powder in DMSO according to manufacturer's directions to generate a 1 mM stock, aliquot, and store aliquots at -20°C. Use at 1:10,000 for a 100 nM final concentration.

Maturation Medium (MAT) (1 L)

- 1 L N2B27 medium,
- BDNF to a final concentration of 10 ng/ml, added fresh at each feeding.

mTeSR1 (0.5 L)

- 400 ml mTeSR1 medium, [STEMCELL Technologies, cat. no 05851],
- 100 ml mTeSR1 5x Supplements, [STEMCELL Technologies, cat. no 05852].

N2B27 (1.07 L)

- 500 ml Neurobasal-A, (LS), [Thermo Fisher Scientific, cat. no 10888022],
- 500 ml DMEM/F12 with GlutaMAX, (LS), [Thermo Fisher Scientific, cat. no 31331093],
- 10 ml Glutamax, [Thermo Fisher Scientific, cat. no 35050038],
- 10 ml Sodium Bicarbonate, [Thermo Fisher Scientific, cat. no 25080-094],
- 5 ml MEM Nonessential Amino Acids, [Thermo Fisher Scientific, cat. no 11140035],
- 1 ml Ascorbic Acid (200 mM, use 1:1,000), [Sigma Aldrich, cat. no A4403],
- 10 ml Penicillin-Streptomycin [Thermo Fisher Scientific, cat. no. 15140122],

Sterile filter, then add the following supplements:

- 20 ml B27 supplement (x50), [Thermo Fisher Scientific, cat. no 17504044],

- 10 ml N2 supplement (x100), [Thermo Fisher Scientific, cat. no 17502048].

Papin with TrypLE

Dissolve 1 vial of Papain to 10 ml of TrypLE, directly before dissociation procedure warm it up to 37°C.

Paraformaldehyde, PFA

Prepare 4% Paraformaldehyde as described previously (Fox et al., 1985), aliquot and store at -20°C.

Purmorphamine

Reconstitute powder in DMSO to generate a 10 mM stock, aliquot, and store aliquots at -80°C. Use at 1:10,000 for a 1 µM final concentration.

SB431542

Reconstitute powder in DMSO to generate a 10 mM stock, aliquot, and store aliquots at -80°C. Use at 1:1,000 for a 10 µM final concentration.

Sodium Azide:

Dissolve sodium azide powder to 10% w/v in distilled water. Make to 0.1% w/v by diluting 1 in 100 in TBS.

Smoothed agonist (SAG)

Reconstitute powder in DMSO to generate a 10 mM stock, aliquot, and store aliquots at -80°C. Use at 1:10,000 for a 1 µM final concentration.

TBS (Tris buffered saline)

Dissolve the tablets in sterile distilled water according to manufacturer's directions, adjust the pH value to 7.4 with Hydrochloric acid.

Trituration medium (30 ml)

- 30 ml Maturation medium,
- 30 µl Y-27632 dihydrochloride Rock inhibitor stock solution (1,000x) to a final concentration of 10 µM,
- 1 vial D2 DNAse [Worthington, cat. no LK003170].

XAV939

Reconstitute powder in DMSO according to manufacturer's directions to generate a 10 mM stock, aliquot, and store aliquots at -20°C. Use at 1:5,000 for a 2 µM final concentration.

Y-27632 dihydrochloride Rock inhibitor

Reconstitute powder in sterile distilled water to generate a 10 mM stock, aliquot, and store aliquots at -20°C. Use at 1:1,000 for a 10 µM final concentration.

DISCUSSION

The aim of this collection of protocols is to enable research groups with adequate facilities and expertise to generate hPSC-derived hypothalamic neurons. Complemented with *in vivo* validation in animal models, the utility of this hPSC-system should prove a powerful tool to study the basic biology of hypothalamic neurons and to model diseases of hypothalamic origin with the aim of developing improved treatments.

A principal advantage of this culture system is its scalability. Since each 10 cm plate of stem cells yields ~1 x 10^8 neurons or more, even neuron types that are produced relatively inefficiently (~1%) can be produced in large numbers (~1 x 10^6 per plate). This enables human hypothalamic neurons to be deeply characterised, used in disease modelling studies, and studied in high-throughput imaging assays and analyses, paving the way for small molecules screens for modulators of hypothalamic neuron activity and function.

Depending on the application, experiments can be carried out at relatively early time points. For example, many hypothalamic neurons generated by this protocol respond to depolarising stimuli as early as D30 (unpublished observations). This allows for the study of certain cell-intrinsic responses in contrast to other culture system in which electrophysiological maturation can take several months (Hartfield et al., 2014; Kirwan et al., 2015; Shi et al., 2012).

Another key advantage of the hPCS system is the ability to perform gene editing using CRISPR/Cas9 (Cong et al., 2013; Santos et al., 2016). Gene-editing technology should provide a powerful platform to probe key aspects of human hypothalamic biology and for investigating the mechanisms of metabolic disorders. Together, these techniques could allow for a robust investigation of the molecular and cellular pathways that are important for hypothalamic health and disease.

Critical parameters

hPSC culture: It is critical that hPSCs are passaged routinely and are not allowed to become over-confluent or to become contaminated with differentiated cells. Since hPSCs accumulate genetic defects over time in culture (Amps et al., 2011; Merkle et al., 2017; Ronen and Benvenisty, 2012) it is advisable to use cell lines with low to moderate passage number (<P50). Avoid splitting cells at ratios exceeding 1:10, as sharp reductions in the size of a cell population which can lead to unwanted selection events. We also recommend working with cell lines that have been rigorously genetically characterised by sequencing, and to routinely karyotype and/or sequence cell lines to test for the acquisition of unwanted mutations.

Cell density and feeding frequency: A major cause of cell death or poor differentiation can be cell overgrowth or inadequate nutrient availability. First, ensure that initial plating densities are accurate by using automated cell counters that adjust for the number of live cells. There is some variability in growth rate between cell lines. The protocols described here should be appropriate for most cell lines, but if you notice significant cell death or that your media visibly acidifies within 24 hours if phenol red is present as an indicator, feed cells more frequently or with larger media volumes.

Cell passaging: Cell passaging can be a major source of cell death. It is essential to gently handle cells when mechanically triturating them, particularly stem cells and neurons. Minimise the formation of bubbles by aspirating and dispelling less than the full media volume. Do not use excessive force when pipetting, and ensure that cells easily wash off of the plate before attempting to dissociate the culture, increasing the time that cells are incubated in EDTA, TrypLE, or TrypLE+Papain, if necessary. Ensure that DNase is present in the trituration medium.

Troubleshooting

Poor neural specification: At Day 10 to Day 14, the majority of cells (above 80%) should stain positive for the ventral forebrain progenitor transcription factor NKX2.1. Between Day 15 and 30, neurons should become the dominant cell type in the cultures. If this is not the case, there was likely a defect in neuronal patterning and specification. The likely causes are:

1. Poor quality or differentiated hPSCs: Make sure that hPSCs are maintained appropriately, lack differentiated cells, and are in rapid growth phase when they are dissociated and plated for differentiation (refer to Critical Parameters and BASIC PROTOCOL 1)
2. Low starting density: Make sure the density of hPSCs is not less than 60%.
3. Reagent problem: Make sure the differentiation media is fresh. Avoid using N2B27 medium older than 2 weeks. Add small molecule inhibitors and recombinant proteins on the day of use. Avoid repeated freeze-thaw cycles of aliquots and other reagents.

Cell detachment: Detachment can sometimes occur during the early phase of hypothalamic differentiation. Ensure that cell density is approximately 75% confluence when Day 0 medium is added. Poor Geltrex coating can also result in detachment. Make sure Geltrex is always thawed at 4˚C and generate single-use aliquots. Do not use Geltrex that contains visible clumps, and use plates immediately after coating. Detachment can also occur in older cultures (> Day 50). Make sure that the density at the terminal plating is not below 5 x 10^4 cells per cm^2, or above 2 x 10^5 cells per cm^2. Dissociate and re-plate cells if necessary with TrypLE+Papain.

Neurons do not attach after passaging: Make sure TrypLE is fully washed out, since residual enzyme can prevent attachment. Ensure that plates are properly coated with Geltrex (see above). If plating on glass, make sure the glass is thoroughly acid-etched. Poor neuronal survival can also indicate over-digestion in TrypLE or TrypLE/papain, or rough mechanical dissociation (see Critical Parameters).

Contaminating cell types during differentiation: Some non-neuronal cell-types can undergo significant proliferation and become dominant in older cultures. If this is the case based on immunostaining and phase contrast microscopy, extend DAPT-treatment to Day 20. Avoid passaging cultures older than Day 40 if possible, as neurons survive passaging best before Day 30 whereas non-neuronal cell-types will preferentially survive passaging, even in older cultures. Avoid re-plating cells below 5 x 10^4 cells per cm^2. Neurons can also be purified by FACS if a reporter line is available, or using a neuron-specific antigen such a NCAM.

Cell death in older cultures: When cell density increases, particularly as the cultures become older, the culture media can acidify and become depleted of nutrients. Feed cultures daily or with a larger media volume, or re-plate if necessary.

Statistical analysis

To allow for thorough statistical analysis, all experiments are performed with a minimum of three replicate wells. In addition, experiments should be repeated in independent differentiations, preferably with 3 genetically distinct cell lines. The statistical analysis performed will vary depending on the experimental design.

Understanding results

If the procedures for quality control are followed, it should be straightforward for users to determine if they have succeeded in generating hypothalamic neurons. Although the degree to which stem cell-derived hypothalamic neurons accurately mimic their *in vivo* counterparts has yet to be fully explored, they do appear to share a number of key features (Merkle et al., 2015; Wang et al., 2015, 2017).

Time Considerations

Expanding hPSCs for differentiation typically takes 1-2 weeks. Differentiation to NKX2.1 positive progenitors takes 8-10 days. Hypothalamic neurons are generated after 14-20 days. POMC immunopositive cells are clearly visibly by D25. Functional studies such as calcium imaging can be carried out as soon as D30, although further culturing cells to D60 may increase neuronal maturation and functional responsiveness. With careful maintenance, cultures can be maintained for well over 100 days.

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FIGURE LEGENDS

Figure 1) hPSC maintenance

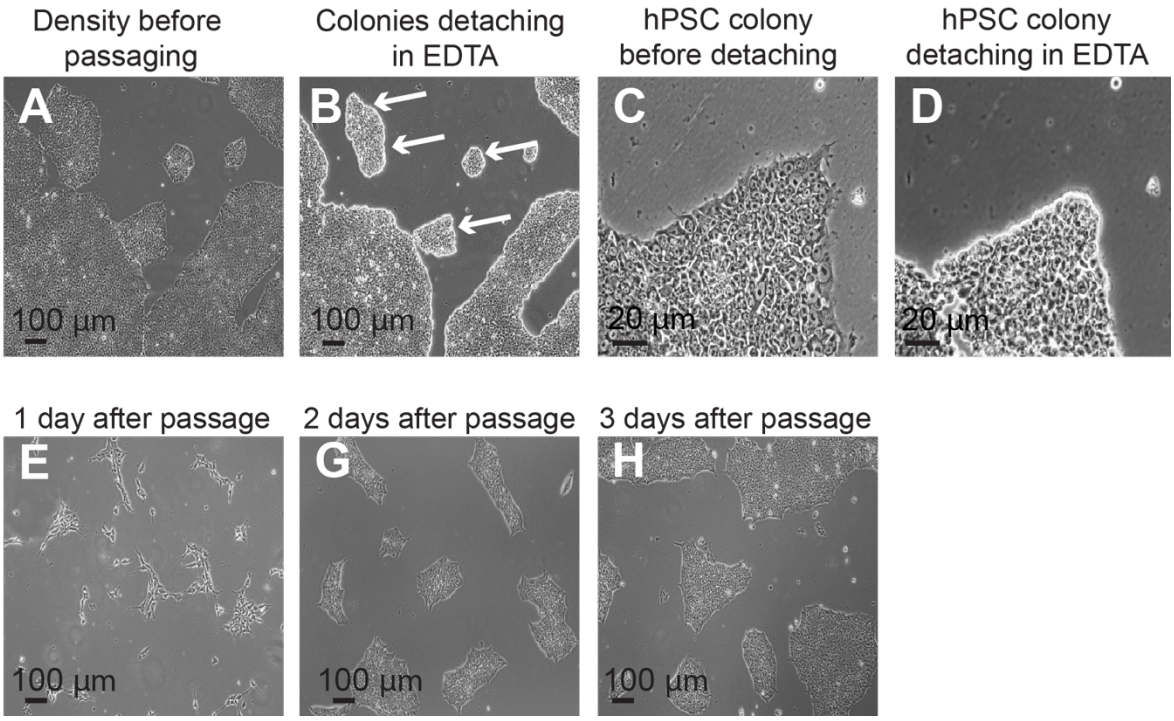

Figure 1: hPSC culture and maintenance. **(A)** HPSCs at 80% confluence before passaging. **(B)** After brief incubation in EDTA, the edges of the colonies begin to detach, taking on a phase bright appearance (arrows). **(C)** 40x magnification image of a hPSC colony before passaging. **(D)** After incubation in EDTA, borders between individual hPSCs should become clear, phase bright, and well-separated. **(E)** HPSCs on the day following a passage, showing a typical density after passaging. Note that hPSCs extend more processes in the presence of Rock inhibitor, Y-27632, giving colonies a jagged appearance. **(G)** Colonies acquire smoother borders upon withdrawal of Y-27632 the following day. **(H)** Expansion of hPSC colonies is evident 2 days after passaging. Colonies of undifferentiated hPSCs should have cells that are uniform in morphology.

Figure 2

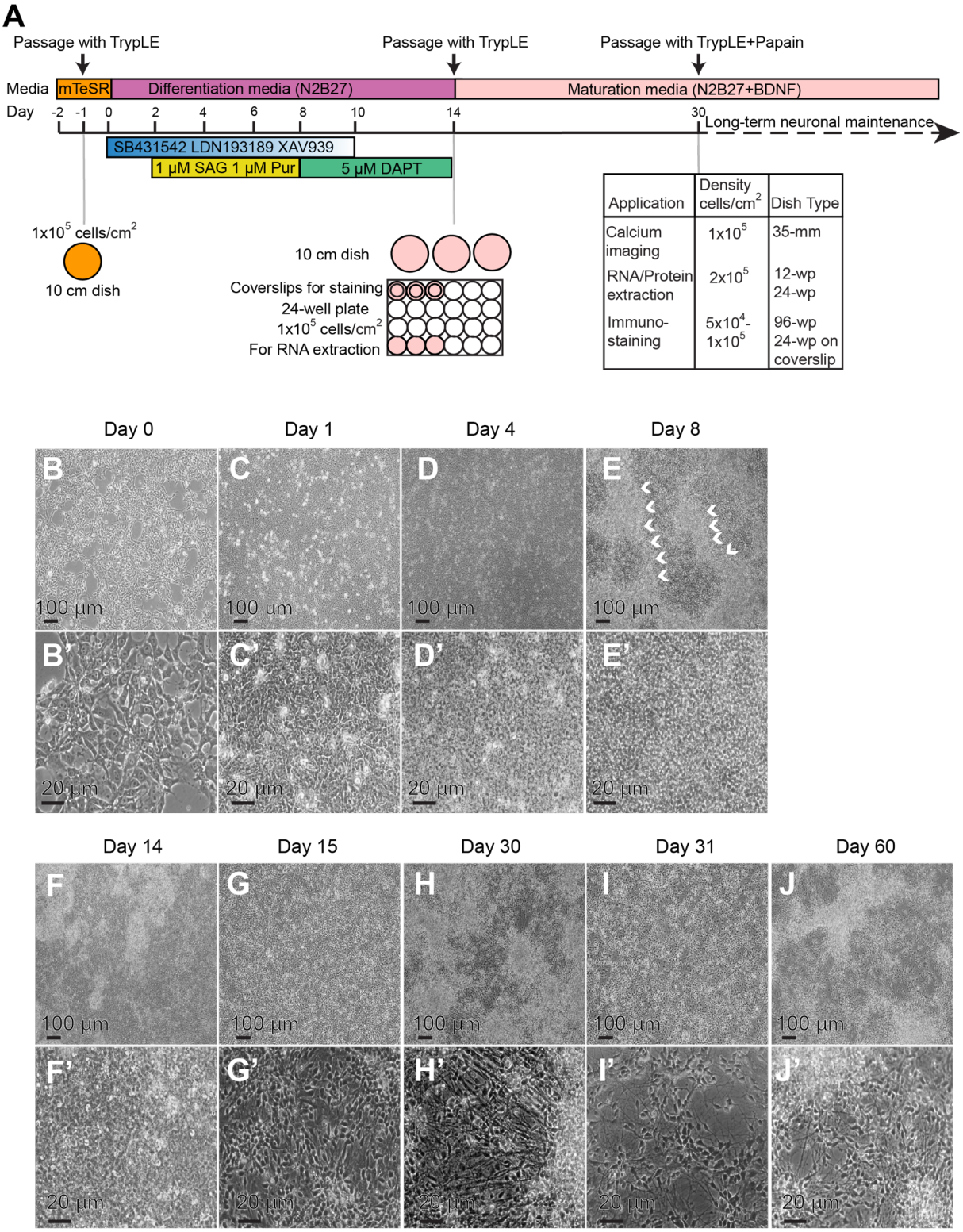

Figure 2: Differentiation of hPSCs to hypothalamic neurons. **(A)** Schematic of hypothalamic differentiation protocol. **(B,B')** Dissociated hPSCs that were plated at a concentration of 1 x 10^5 cells per cm^2 on Day 1 should be at approximately 75% on Day 0. Differentiation medium (N2B27) with SMAD inhibitors (10 µM SB431542, 100 nM LDN193189) and a Wnt inhibitor (2 µM XAV939) is then added to induce a forebrain fate. **(C, C')** Cultures should reach confluence and adopt a uniform

appearance by Day 1. **(D, D')** By Day 4, cell density is dramatically increased but cells are still largely a single adherent layer. The concentrations of the SMAD inhibitors and Wnt inhibitors are reduced to 75%, 50% and 25% of the Day 0-4 concentration on Days 4-6, 6-8 and 8-10 respectively and cultures are ventralised from Day 2-8 by adding 1 µM Smoothened Agonist (SAG) and 1 µM Purmorphamine (Pur). **(E, E')** By Day 8, cultures adopt a clear neuroepithelial morphology, as evidenced by the formation of neural-ridge like structures (arrows). At this time point, 5 µM of the NOTCH inhibitor DAPT is added until Day 14 to encourage neurogenesis. **(F, F')** On Day 14, cultures have become over-confluent with progenitors and newborn neurons, and are passaged 1:3. At this time point, it is prudent to plate a fraction of the differentiated cells in a 24-well plate for quality control. After Day 14, cells are maintained in maturation media: N2B27 supplemented at each feeding with 10 ng/mL BDNF. **(G, G')** After passaging, most cells have a neuronal morphology. **(H, H')** By Day 30, cultures contain a high percentage of cells with neuronal morphology but may also have some cell-dense regions. It is advisable to re-plate cultures again at this time point to purify cell types of interest and decrease cell density to ensure that neurons receive adequate nutrients. **(I, I')** Culture morphology at Day 31 after re-plating at $1x10^5$ cells per cm^2. **(J, J')** Cellular morphology of Day 60 cultures re-plated at $1x10^5$ cells per cm^2 on Day 30. Abbreviations: 12-wp, 12 well plate; 24-wp, 24 well plate; 96-wp, 96 well plate.

Figure 3

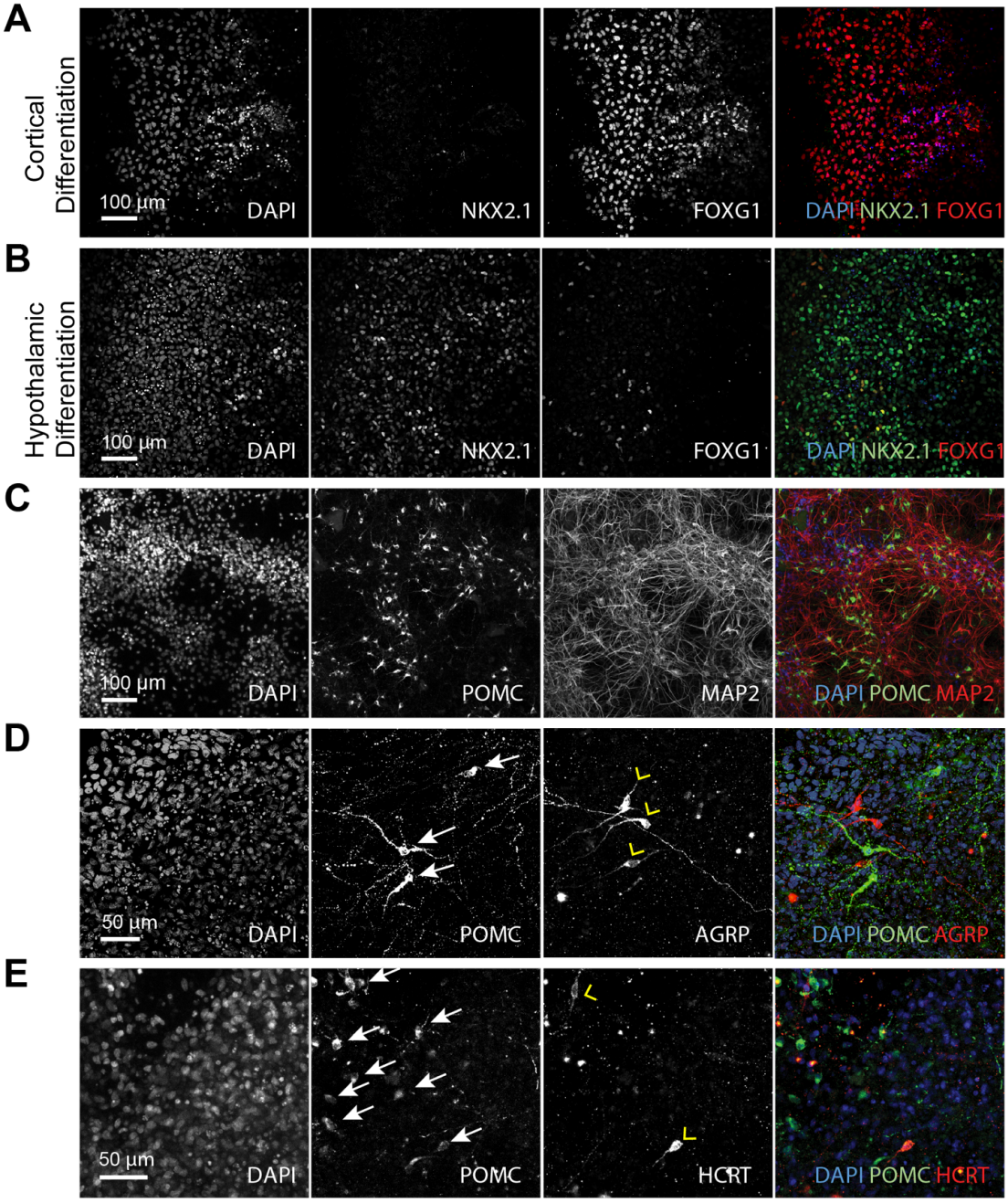

Figure 3: Quality control of hPSC hypothalamic differentiations by immunocytochemistry. **(A)** Immunofluorescent staining on Day 10 of hES cells differentiated to dorsal forebrain (cortical) cells. The vast majority of cells in these cultures express the transcription factor FOXG1 that is expressed in the telencephalon in vivo, and few cells expressing the transcription factor NKX2.1 that is expressed in the ventral forebrain. DNA is stained with DAPI to visualise cell nuclei. **(B)** On Day 10 of hypothalamic differentiation, the vast majority of cells express NKX2.1, and only a few express FOXG1. **(C)** On Day 24 of hypothalamic differentiation, some hPSC-derived cells express POMC and many cells express microtubule-associated protein 2 (MAP2), a dendritic marker indicative of neuronal identity. **(D,E)** On Day 119 of differentiation, hypothalamic cultures retain numerous neurons expressing POMC (arrows), Agouti-related peptide (AGRP) (arrowheads) and Hypocretin/orexin (HCRT) (arrowheads).

Figure 4

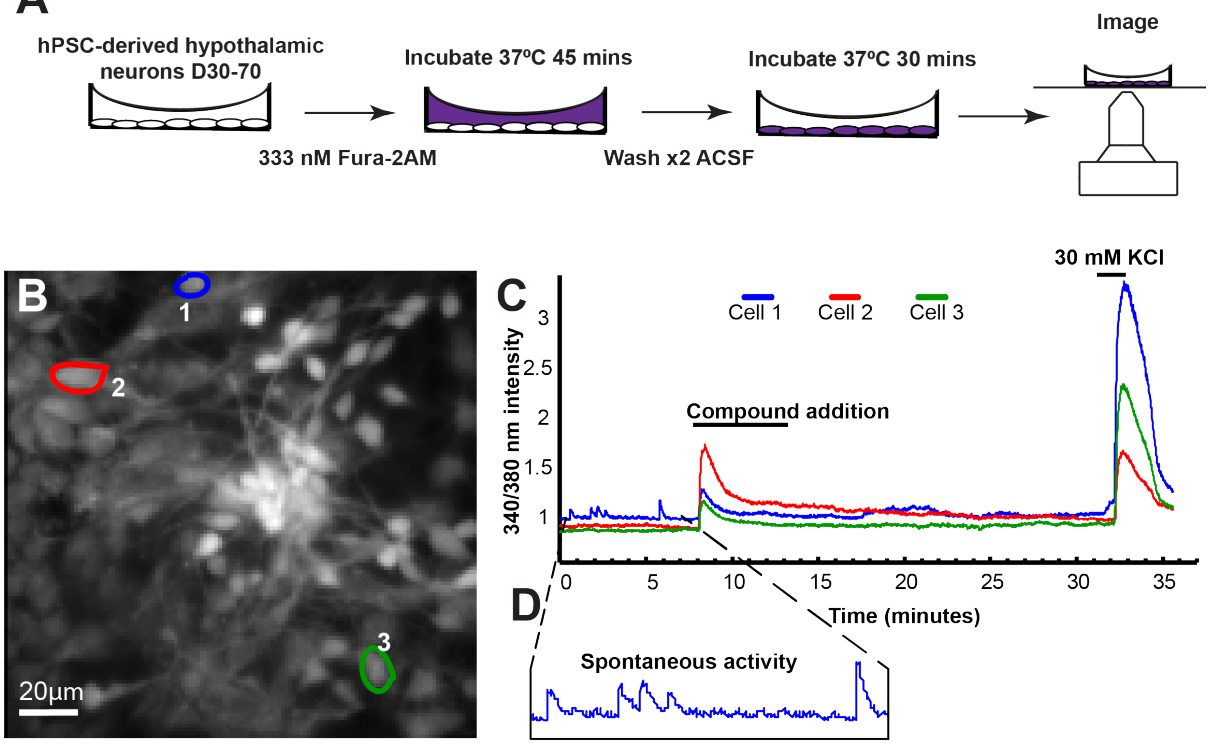

Figure 4: Calcium imaging of hPSC-derived hypothalamic neurons. **(A)** Schematic showing Fura-2AM loading of neurons. Cells are incubated in 333 nM Fura-2AM for 45 minutes in an incubator at 37˚C. After gently washing cultures twice with ACSF, cells are incubated for a further 30 minutes before performing the imaging. **(B)** A typical field of Fura-2AM-loaded hypothalamic neurons at Day 77 post-differentiation with cells of interest highlighted (1, 2 and 3). **(C)** Trace of the 340 nm / 380 nm intensity ratio over time in the three cells highlighted in (B), showing a depolarisation in the cells after perfusion of an exogenous compound and a large depolarisation when perfused with ACSF containing 30 mM potassium chloride (KCl). **(D)** Spontaneous calcium transients in the absence of stimulation.