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Rapid *Ngn2*-induction of excitatory neurons from hiPSC-derived neural progenitor cells

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Abstract

Since the discovery of somatic reprogramming, human induced pluripotent stem cells (hiPSCs) have been exploited to model a variety of neurological and psychiatric disorders. Because hiPSCs represent an almost limitless source of patient-derived neurons that retain the genetic variations thought to contribute to disease etiology, they have been heralded as a patient-specific platform for high throughput drug screening. However, the utility of current protocols for generating neurons from hiPSCs remains limited by protracted differentiation timelines and heterogeneity of the neuronal phenotypes produced. Neuronal induction via the forced expression of exogenous transcription factors rapidly induces defined populations of functional neurons from fibroblasts and hiPSCs. Here, we describe an adapted protocol that accelerates maturation of functional excitatory neurons from hiPSC-derived neural progenitor cells (NPCs) via lentiviral transduction of *Neurogenin 2* (using both m*Ngn2* and h*NGN2*). This methodology, relying upon a robust and scalable starting population of hiPSC NPCs, should be readily amenable to scaling for hiPSC-based high-throughput drug screening.

Keywords

hiPSC; Directed differentiation; Neuronal induction; iNeuron; Modeling neuropsychiatric disease

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1. Introduction

The brain utilizes neurons and glia to construct neural circuitry, generated and fine-tuned by the formation of numerous plastic synapses. Subtle aberrant changes during development in the spatiotemporal-patterning or synaptic function of specific neuronal subtypes can perturb this complex neuronal connectivity [1], and thus likely contribute to neuropsychiatric diseases such as schizophrenia (SZ) [2,3]. Both human postmortem studies and mouse models of SZ have demonstrated reduced dendritic arborization and synaptic density associated with SZ [4–6]. However, mouse models fail to recapitulate the polygenic effect of disease, and postmortem studies are frequently confounded by factors such as patient medication history, drug/alcohol abuse or environmental stressors and are further limited to the study of the endpoint of disease [7]. Now, human induced pluripotent stem cell (hiPSC)-based models present the ability to generate nearly limitless numbers of patient-derived neurons for the study of disease initiation and progression.

Traditionally, hiPSCs have been differentiated to excitatory neurons via the addition of growth factors and small molecules that modulate developmental signaling pathways. In this process known as “directed differentiation”, hiPSCs are first neuralized by dual SMAD inhibition [8], yielding a transient stage from which it is possible to dissociate and expand neural rosettes as neural progenitor cells (NPCs) [9–11]. These NPCs can be subsequently differentiated into neurons that attain characteristics of functional neurons within two to three months [12]. Such “directed differentiation” protocols are thought to recapitulate *in vivo* development, generating neurons that most resemble fetal forebrain tissue [13,14]. Unfortunately, directed differentiation generally yields heterogeneous neuronal populations that require long-term culture to reach maturity [14–16].

Neuronal induction via the overexpression of the pro-neuronal transcription factors (first demonstrated using the combination of *ASCL1*, *BRN2*, *MYT1L* and *NEUROD1*) can induce human fibroblasts into neurons and is now a viable alternative to directed differentiation [17]. Moreover, further maturity can be achieved by co-expressing microRNA-9/9* and microRNA-124 [18]. Recently, overexpression of mouse *Neurogenin 2* (m*Ngn2*) or human *NEURO-GENIN 2* (h*NGN2*) in hiPSCs, combined with puromycin selection to increase the purity of the cultures, yielded populations of induced neurons (iNs) comprised of more than 90% Microtubule-associated protein 2AB (MAP2AB)-positive neurons within 14 days [19–21]. These m*Ngn2*-iNs express glutamatergic synaptic proteins such as vesicular glutamate transporter 1 (*vGLUT1*), postsynaptic density-95 (*PSD95*) and synapsin1 (*SYN1*) [19]. In addition, iNs exhibited excitatory synaptic function, when co-cultured with mouse cortical neurons, and integrated into the mouse brain following transplantation [19], indicating that this approach is capable of rapidly and efficiently generating highly pure populations of functional excitatory neurons.

Members of the Ngn family are basic-helix–loop–helix transcription factors that regulate many aspects of neural development [22]. *Ngn2* regulates the commitment of neural progenitors to neuronal fate during development [23–25] and is sufficient to induce early postnatal astroglia into neurons [26]. Although overexpression of *Ngn2* in human [19–21] and mouse [27] pluripotent stem cells is sufficient to induce excitatory neurons, *Ngn2* also

has a critical role in specifying motor neuron identity during spinal cord development [28,29]; in fact, overexpression of *Ngn2* together with *Islet-1* and *Lhx3* can induce motor neurons from human pluripotent stem cells [30]. Moreover, overexpression of *Ngn2* and *Sox11* induces fibroblasts into cholinergic neurons [31], while γ -aminobutyric acid (GABA)-ergic neuronal differentiation is inhibited by *Ngn2* overexpression [32].

In this study, we describe a *Ngn2*-mediated neuronal induction protocol that begins with hiPSC-derived NPCs, rather than hiPSCs (Fig. 1A). This approach has a number of advantages, as NPCs proliferate robustly and are relatively straightforward to maintain *in vitro* as they require less frequent feeding and passaging. In addition, NPCs are more amenable to parallel culture of dozens of cell lines and are highly adaptable to automated methods. This makes NPCs an ideal cell source for both the large patient cohort studies required for studying a complex genetic disease, as well as adaptation to high throughput drug and phenotypic screens. Moreover, NPCs are a relatively uniform population committed to forebrain neural fate, potentially reducing methodological variability. Here we report that lentiviral transduction of doxycycline-inducible mouse *Ngn2* (*mNgn2*) or human *NGN2* (*hNGN2*) rapidly yields MAP2AB-positive neurons (Figs. 1 and 2). *Ngn2*-induced neurons exhibit electrical activity within two weeks and show accelerated formation of SYN1-positive puncta and increased expression of glutamatergic genes, even in the absence of co-culture with mouse cortical neurons or glial cells (Figs. 3 and 4). Moreover, transient *mNgn2*-expression is sufficient to accelerate synaptogenesis and induce neuronal activity in *mNgn2*-neurons (Figs. 5 and 6). The method presented here provides a rapid and efficient platform to derive functional human excitatory neurons for the study of – and ultimately high-throughput drug screenings to reverse – the molecular and cellular phenotypes associated with psychiatric disease.

2. Material and methods

2.1. Passaging and maintenance of NPCs

2.1.1. Thawing NPCs—NPCs were derived from hiPSCs as previously described [33]; NPCs can be stored indefinitely at -180°C in NPC media supplemented with 10% DMSO.

Plates were coated with Matrigel (BD biosciences, #354230) prior to thawing NPCs. A 1 mg aliquot of Matrigel was thawed for every two plates, by resuspending Matrigel in 24 ml of ice-cold DMEM (ThermoFisher Scientific, #10566-016) and rapidly adding 2 ml of this cold mixture to each well of a 6-well plate. Plates were incubated at 37°C (for at least 1 h, typically overnight).

Immediately prior to thawing NPCs, the Matrigel–DMEM mixture was aspirated and replaced with 2 ml of pre-warmed NPC medium per well (Table 1).

Frozen NPC aliquots were retrieved from liquid nitrogen storage and quickly thawed in a 37°C water bath. NPCs were resuspended in a 15 ml tube containing pre-warmed DMEM and centrifuged ($5\text{ min} \times 1000g$). Following aspiration of DMEM, the cell pellet was gently resuspended in NPC medium (Table 1) and seeded onto Matrigel-coated plates. The plate was gently shaken in order to evenly distribute NPCs and returned to the incubator. NPCs

were fed every second day until the cell density reached ten to fifteen million NPCs in a single well of the 6-well plate.

2.1.2. Passaging NPCs—Prior to beginning, Matrigel-coated plates were prepared as described in Section 2.1.1. NPC medium, DMEM and Accutase (Sigma #A6964) were pre-warmed to room temperature, and the Matrigel–DMEM coating mixture replaced with 2 ml of NPC medium.

NPC medium was aspirated and 1 ml/well of Accutase was added to NPCs for 5–10 min at 37 °C. Following NPC detachment, 2 ml of DMEM was added to the well to dilute the Accutase. NPCs were collected by pipette, transferred to the 15 ml tube containing DMEM and centrifuged (5 min × 1000*g*). After aspirating the DMEM, the cell pellet was gently resuspended by pipetting. NPCs were counted with a hemocytometer and $2.5\text{--}3 \times 10^6$ cells were plated per well of a 6-well plate. The NPCs were then evenly distributed and put back into the incubator. NPCs were typically split 1:3 every 7 days.

2.2. Neuronal induction (Fig. 1A–C)

2.2.1. Seeding NPCs for differentiation

Day-3: Preparing coated plates (or coverslips): 24-well plates were coated with Matrigel, as described in Section 2.1.1.

For multiple electrode array (MEA) assays, plates were first coated with poly-L-ornithine (Sigma #P4957, 10 µg/ml in dH₂O) overnight at 37 °C. Following three washes with dH₂O, Laminin (ThermoFisher Scientific, #23017-015, 1 µg/ml in 1 × PBS) was added again overnight at 37 °C. For confocal microscopy of *mNgn2*-induced neurons, sterile pre-etched coverslips (Fisher Scientific, #12-545-80) were coated the same way but with 50 µg/ml poly-L-ornithine.

Day-2: Seeding NPCs: We recommend that 1 µg/ml Laminin be included in both the NPC medium and neuron medium (Table 1) to promote neurite growth and cell attachment during neuronal induction [34].

Prior to beginning, NPC medium, DMEM and Accutase were pre-warmed. The coating mixture was aspirated and 230 µl of NPC medium was aliquoted into each well of the 24-well plate well.

NPC medium was aspirated and NPCs were incubated with 1 ml/well of Accutase for 5–10 min at 37 °C. Once detached, 2 ml of DMEM was added per well, and NPCs transferred to a 15 ml tube containing DMEM and centrifuged (5 min × 1000*g*). Following aspiration and resuspension in NPC medium, cell concentration was determined with a hemocytometer. Typically, $2.5\text{--}5.0 \times 10^5$ NPCs were seeded into each well of a 24-well and evenly distributed. Cells were incubated overnight.

If the objective is to obtain density-matched populations from multiple NPC lines, it is critical to empirically optimize seeding density for each individual NPC line (see Fig. 7).

2.2.2. Lentiviral transduction

Day-1: Transduction: Note: Third-generation VSV.G pseudotyped HIV-1 lentiviruses were produced by polyethylenimine (PEI, Polysciences #23966-2)-transfection of HEK293T cells and packaged with VSVG-coats using established methods, see [35].

Prior to beginning, NPC medium was pre-warmed in a 37 °C water bath. Lentiviral aliquots of CMV-*rtTA* (Addgene ID: 19780), TetO-m*Ngn2*-P2A-*Puro^R* (Addgene ID: 52047), TetO-*eGFP* (Addgene ID: 30130), TetO-eGFP-*Puro^R* (Addgene ID: 19780) and TetO-*Puro^R* were thawed at room temperature and added to NPC medium. Lentiviral m*Ngn2* transduction was achieved with either CMV-*rtTA*, TetO-m*Ngn2*-P2A-*Puro^R* and TetO-*eGFP* or, CMV-*rtTA* and TetO-m*Ngn2*-P2A-*Puro^R* transduction. As a control, either CMV-*rtTA* and TetO-*eGFP*-T2A-*Puro^R* or CMV-*rtTA* and TetO-*Puro^R* were used. The amount of lentivirus added was adjusted to achieve multiplicity of infection of 1–10.

Note: We recently generated TetO-h*NGN2*-P2A-*Puro^R* and TetO-h*NGN2*-P2A-*eGFP*-T2A-*Puro^R* lentiviral vectors that also efficiently induce neurons from NPCs. This vector can be substituted for TetO-m*Ngn2*-P2A-*Puro^R* as preferred by the investigator (Fig. 1F).

To transduce NPCs, the media was aspirated and replaced with NPC medium containing the lentiviruses. The plate was then incubated at 37 °C for >15 min.

Spinfection was used to increase transduction efficiency [36]. The 24-well plate was placed in the plate centrifuge and spun for 1 h at 1000g, 25 °C. Cells were then incubated at 37 °C. Viral medium was replaced with fresh NPC medium after 3–4 h of transduction.

2.2.3. Doxycycline addition and puromycin selection

Day 0: Doxycycline treatment: Doxycycline (Sigma, #D9891) was diluted (1 µg/ml) in NPC medium and 500 µl added to each well of a 24-well plate.

24 h following doxycycline addition, GFP fluorescence can be observed in most NPCs (usually >90%, Fig. 1D).

Day 1: Puromycin selection: Media was replaced with fresh NPC medium containing 1 µg/ml puromycin (Sigma, #P7255) and 1 µg/ml doxycycline for 24 h.

2.2.4. Puromycin withdrawal and switch to neuron medium

Day 2: Puromycin withdrawal and switch to neuron medium: Media was replaced with neuron medium (Table 1) containing 1 µg/ml doxycycline (500 µl per well of a 24-well plate).

Day 4–20: Regular half medium changes: Doxycycline can be withdrawn at any time during the protocol. We recommend a 50% reduction in the total doxycycline concentration every 48 h, which will result in a complete removal from the culture medium by day 7–8 (Fig. 5A).

To achieve this, 250 μ l of medium per well was removed and replaced with 280 μ l of fresh neuron medium (the added volume is greater than the removed volume in order to prevent gradual loss of the media volume by evaporation). Cells were fed every second day by half medium change until the day of assay.

Day 6–20: Ara-C treatment: Neuron medium can be supplemented with 50 nM Cytosine β -D-arabinofuranoside hydrochloride (Ara-C) (Sigma, #C6645) to reduce the proliferation of non-neuronal cells in the culture (Fig. 4F–H).

2.3. Analysis of mNgn2- and hNGN2-induced neurons

2.3.1. Immunostaining of mNgn2- and hNGN2-neurons—For immunostaining, neurons were first cooled to room temperature for 5 min. 250 μ l of medium was removed per well, leaving approximately 220 μ l, to which 32 μ l of 32% paraformaldehyde (Electron Microscopy Sciences, #15714) was directly added, for an effective concentration of 4% paraformaldehyde (PFA). The PFA/neuron medium solution was gently mixed and incubated for 30 min at room temperature.

Following aspiration of the PFA/medium mix, ice-cold blocking/ permeabilization buffer (PBS containing Ca^{2+} and Mg^{2+} ThermoFisher Scientific (21300-058) supplemented with 0.1% Triton-X (Sigma, #T8787) and 5% Donkey serum (Jackson, #017-000-121) was gently added. Neurons were incubated with blocking/permeabilization buffer on ice for 30 min, followed by an additional 30 min at room temperature. It is critical to perform this step on ice to retain neurite morphology during permeabilization and to prevent fragmentation of neurites.

During blocking and permeabilization, the primary antibody (Table 2) solution was prepared in ice-cold blocking/permeabilization buffer. Neurons were incubated with the primary antibody solution overnight at 4 °C.

Following incubation, the primary antibody solution was removed and the wells were washed with ice-cold $\text{PBS}^{\text{Ca}^{2+}+\text{Mg}^{2+}}$ three times as described above.

Neurons were then incubated with secondary antibody (Table 2) prepared in ice-cold blocking/permeabilization buffer for two hours at room temperature, followed by two washes with $\text{PBS}^{\text{Ca}^{2+}+\text{Mg}^{2+}}$. Nuclei were counterstained with 0.5 μ g/ml DAPI (Sigma, #D9542) for 10 min and then neurons were washed with PBS three additional times.

2.3.2. Preparation of neurons for confocal microscopy—AquaPolymount mounting solution (Polysciences Inc., #18606-20) was equilibrated to room temperature. One drop (~20 μ l) of mounting solution was placed onto each slide and the coverslips was gently lowered onto the slide by facing neuron side down. Mounted coverslips were air-dried in the dark overnight at room temperature. Coverslips were imaged with a Zeiss LSM 780 microscope.

2.3.3. Analysis for MAP2AB intensity and SYN1 puncta—mNgn2-induced neurons and control GFP transduced NPCs at two or three weeks of culture were immunostained

with MAP2AB and DAPI and mounted, as described in Sections 2.3.1 and 2.3.2. MAP2AB and DAPI images were captured (five images each from three biological replicates per condition and cell line) using an epifluorescence microscope (Olympus IX51) at 100× total magnification. DAPI images were thresholded, and cell segmentation was achieved using the watershed separation feature in NIH Image J (<http://imagej.nih.gov/ij/>), so that DAPI-positive nuclei could be counted using NIH Image J. The MAP2AB area was measured using NIH Image J, and then divided by DAPI-positive nuclei numbers in order to calculate MAP2AB levels normalized to cell count.

mNgn2-induced neurons and GFP-NPCs at two and three weeks of culture were immunostained with SYN1 and MAP2AB as described in Sections 2.3.1 and 2.3.2. The images were acquired (five images each from three biological replicates per condition and cell line) using a confocal microscope (LSM 780, Zeiss) at 400× total magnification. After uniform thresholding of all SYN1 and MAP2AB images, SYN1 puncta were counted, and the MAP2AB-positive area of the thresholded images was measured using NIH Image J. Total SYN1 puncta per image were divided by that image's respective MAP2AB-positive area in order to calculate SYN1 puncti normalized to MAP2AB levels.

2.4. Quantitative Real Time PCR

Cells were harvested with RNABee (Amsbio, CS-105B) at room temperature for 5 min. Lysates were transferred to a 1.5 ml micro-tube. Chloroform was mixed with RNABee at one fifth of the total volume. Samples were incubated on ice for 5 min then centrifuged for 15 min at 4 °C at 12,000g. The aqueous phase was collected and an equivalent volume of isopropanol was added, mixed and centrifuged again for 15 min at 4 °C at 12,000g. The RNA pellet was washed two times with RNase-free 75% ethanol and dissolved in DEPC-treated water.

RNA was treated with DNase kit (Ambion, AM1907) and quantified with a Nanodrop. 1 µg of total RNA was converted into cDNA using the Superscript III reverse transcription kit (ThermoFisher Scientific, 11752-050); cDNA was diluted to 2.5 ng/µl.

qPCR was performed in 384-well format using 8 µl total volume: 300 nM forward and reverse primers, 5 ng of cDNA and Fast SYBR green master mix (Applied Biosystems, #4385612) using PCR conditions: 1 min at 95 °C, followed by 40 cycles of 5 s at 95 °C and 30 s at 60 °C in a LightCycler®480 System (Roche). Primer sequences are listed in Table 3.

2.5. Electrophysiology

2.5×10^5 NPCs were seeded onto poly-ornithine/Laminin-coated acid-etched coverslips (cultured in 24-well plates), which were then used for recordings. Neurons were recorded using conventional patch-clamp electrophysiology techniques in whole-cell configuration. Cells were visualized on an Olympus BX51WI upright microscope using IR-DIC imaging with an Olympus Oly-140 IR camera. Signals were sampled at 20 kHz through a Multi-Clamp 700B amplifier and digitized using a Digidata 1440 digitizer (Molecular Devices, Sunnyvale, CA) and filtered between 1000–10,000 Hz. Neurons were bathed at 32 °C in artificial cerebral spinal fluid (ACSF) containing (in mM): sodium chloride, 119; calcium chloride, 2; potassium chloride, 2.5; magnesium chloride, 1.3; D-glucose, 11; sodium

bicarbonate, 26.2; sodium phosphate, 1. All chemicals were purchased from Sigma–Aldrich Co. (St. Louis, MO), unless otherwise stated. Patch pipettes were made from borosilicate capillary tubes (World Precision Instruments, Sarasota, FL) pulled on a vertical gravity puller (Narishage International USA, East Meadow, NY) to a final pipette resistance of 3–5 M Ω . Neurons were patched with pipettes containing an internal solution containing (in mM): potassium-D-gluconate, 140; sodium chloride, 4; magnesium chloride, 2; EGTA, 1.1; HEPES, 5; Na₂ATP, 2; sodium creatine phosphate, 5; Na₃GTP, 0.6. Neurons were chosen at random for patch-clamp recording and analyzed for evoked activity in current-clamp as shown in Fig. 3. For voltage-clamp recordings, voltage-steps from –100 mV to +70 mV were used to elicit voltage-gated Na⁺ currents and K⁺ currents.

2.6. Axion Multielectrode Array (MEA)

1.25–5 $\times 10^5$ NPCs (per well) were seeded onto poly-L-ornithine/Laminin-coated 12- or 48-well MEA plate (Axion), respectively. Transduction, spinfection, doxycycline addition, puromycin selection and Ara-C treatment were performed as described above. NPC medium was replaced with neuron medium, and half media changes, every second day were completed thereafter. Doxycycline was withdrawn at various time points, as indicated (Fig. 6B). After 14 days of induction, the MEA plate was loaded in the Axion Maestro MEA reader, the electrical activity of *mNgn2*-induced neurons was recorded and analyzed by the AxIS 2.0 software.

To investigate pharmacological effects, synaptic antagonists were directly added to the MEA wells containing *hNGN2*-neurons. The GABA_A receptor antagonist picrotoxin (PTX) (Tocris #1128), the AMPA/kainate receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (Alomone labs, #C-140) and sodium ion/channel antagonist tetrodotoxin (TTX) (Alomone labs, #T-550) were used at final concentration of 100 μ M, 60 μ M and 1 μ M, respectively. Spontaneous recordings were obtained for 3–5 min before and after drug administration (Fig. 6C).

3. Results

3.1. Lentiviral *Ngn2*-overexpression accelerated neuronal differentiation from NPCs

Although we validated this method predominantly using isogenic comparisons of the effect of *Ngn2*-overexpression in one NPC line (NSB553-3-C); transduction efficiencies are comparable across NPCs derived from independent hiPSC lines from the same individual, as well as from six independent individuals (Fig. 1E). Over the time course of neuronal induction (described in Fig. 1C), exogenous expression of *mNgn2* and *hNGN2* in NPCs resulted in accelerated acquisition of neuronal morphology relative to NPCs transduced with GFP alone (Fig. 1F); this effect was robust in NPCs derived from six independent individuals (Fig. 7). After two weeks of induction, MAP2AB levels in *mNgn2*- and GFP-transduced (as well as *hNGN2*- and GFP-transduced) NPCs were compared. Immunofluorescence analysis showed increased MAP2AB staining with an enhanced neuronal morphology (1.3-fold, $p < 0.001$) in *mNgn2*-induced NPCs (Fig. 2A and B). Consistent with this, we observed increased *MAP2AB* mRNA expression in *mNgn2*-induced NPCs, relative to GFP-transduced NPCs (2.3-fold, $p < 0.001$) (Fig. 2C). Moreover, three-

week-old *mNgn2*-induced neurons showed elevated MAP2AB protein relative to two-week-old *mNgn2*-induced neurons (1.4-fold, $p < 0.001$), indicating that neuronal differentiation increased with continued culture (Fig. 2D and E).

3.2. *Ngn2*-neuronal induction accelerated synaptogenesis and neuronal activity of *Ngn2*-induced neurons

To ascertain the neuronal activity of *mNgn2*-induced neurons, we conducted electrophysiological recordings of *mNgn2*-induced neurons. By 2.5 weeks of induction, *mNgn2*-induced neurons had resting potentials near -70 mV, exhibited repetitively firing action potentials in current-clamp, and showed gating of putative voltage sensitive Na^+ and K^+ channels in voltage-clamp (Fig. 3A and B).

mNgn2-induced neurons demonstrated elevated mRNA and protein levels of excitatory synaptic markers, relative to GFP-transduced NPCs. At 3 weeks of induction, *mNgn2*-induced neurons showed increased SYN1-puncta (relative to MAP2AB area; 1.9-fold, $p < 0.05$) (Fig. 4A and B) and *SYN1* mRNA expression (2.3-fold, $p < 0.001$) (Fig. 4C). Neither vGLUT1 nor PSD95 puncta could be detected in our cultures, which lacked either mouse cortical neuron or human astrocyte co-culture. *mNgn2*-induced neurons demonstrated elevated *vGLUT1* (4.5-fold, $p < 0.001$) and *vGLUT2* (3.8-fold, $p < 0.001$) mRNA expression, but not increased *PSD95*, relative to GFP-transduced NPCs (Fig. 4C).

3.3. *Ngn2*-neuronal induction yields a population of primarily excitatory neurons

To examine the purity of iN cultures, we tested for the presence of inhibitory GABAergic neurons, dopaminergic neurons and astrocytes via expression-based and immunohistochemical approaches. Three-week-old *mNgn2*-induced neurons showed reduced *GAD67* (and comparably low levels of *vGAT* expression) relative to six-week-old hiPSC forebrain neurons (1.5-fold, $p < 0.005$), lower TH levels (6.3-fold, $p < 0.005$), and comparably low *TPH1* (Fig. 4D). *mNgn2*-induced neurons also showed reduced levels of GFAP and S100 β (13.6-fold, $p < 0.001$ and 3.2-fold, $p < 0.001$, respectively) (Fig. 4D). By immunohistochemistry, we failed to detect GAD65/67 or TH-positive neurons, or GFAP-positive astrocytes, in *mNgn2*-induced cultures (Fig. 4E). Taken together, these data suggest that, as predicted from the hiPSC data [19], *mNgn2* overexpression primarily induces excitatory neurons.

To study population-wide changes in neuronal activity following pharmacological manipulation, we used an Axion MEA to record the firing rate of h*NGN2*-neurons. First, we treated with the GABA_A receptor antagonist PTX, which did not increase the firing activity in 21-day-old h*NGN2*-neurons (Fig. 6C), consistent with the low levels of GAD65/67 we observed in our cultures (Fig. 4D and E). Second, when we applied the AMPA/kainate glutamate receptor antagonist CNQX, the firing rate was decreased (Fig. 6C). Third, treatment with TTX eliminated all firing (Fig. 6C). In aggregate, this data implies that our h*NGN2*-induced neurons are comprised primarily of excitatory neurons.

During *Ngn2*-neuronal induction, a non-neuronal population appears within the first week of induction and rapidly expands. Expansion of these cells decreases the purity of neurons, a particular concern when performing biochemical, transcriptomic, epigenomic or other

population-wide analyses. Ara-C is a DNA analog that prevents cellular replication while not interfering with gene expression. It has been widely used to eliminate glia, when culturing primary neurons isolated from rodent brains [37]. We tested the ability of Ara-C to reduce contamination by proliferative cells during extended *Ngn2*-induction protocols from NPCs (Fig. 4F–H).

A dose–response curve of h*NGN2*-induced neurons treated with Ara-C from days 6–20 of our protocol demonstrated that low concentrations (10 nM) of Ara-C was insufficient to eliminate Ki67-positive replicative cells in our culture, while substantial cell death was observed in cells treated with Ara-C concentrations of 1 μ M (Fig. 4F). We observed robust reduction of replicative cells across all NPC lines tested with 50–100 nM Ara-C treatment from days 6–20 (more than 5-fold, $p < 0.05$) (Fig. 4G and H). Thus, our recommendation is to include 50 nM Ara-C whenever experimental design requires excluding proliferative cells, although it is may be possible to reduce the length of Ara-C treatment.

3.4. Transient *Ngn2*-induction is sufficient to yield functional neurons

During embryonic and adult neurogenesis *in vivo*, *Ngn2* is highly expressed at the neuroblast stage, rather than in mature excitatory neurons [38]. Moreover, the Allen Brain Span Atlas shows high *NGN2* expression in early fetal human cortical tissue with gradual decreases during embryonic development [39]. Thus, we hypothesized that transient induction of m*Ngn2* would be sufficient to induce functional excitatory neurons from hiPSC forebrain NPCs. To test this, we removed doxycycline at different time points, and then assayed three-week-old m*Ngn2*-induced neurons (Fig. 5A) by immunofluorescence and qPCR.

Withdrawal of doxycycline at any time point had no significant effect on MAP2AB signal intensity at three weeks of culture (Fig. 5B and C); quantified across biological triplicates from one NPC line. Moreover, no statistically significant difference in the number of SYN1-positive puncta was observed (Fig. 5D and E); quantified across biological triplicates from one NPC line. Strikingly, doxycycline addition for as little as two days was sufficient to increase expression of the synaptic genes *SYN1*, *vGLUT1* and *vGLUT2* relative to GFP-transduced NPCs (Fig. 5F); quantified across biological triplicates from one NPC line.

Finally, we confirmed the sufficiency of a transient m*Ngn2* pulse to induce neuronal activity. Again, we used the Axion MEA to compared neuronal activity of *Ngn2*-neurons with increasing lengths of doxycycline administration to induce m*Ngn2*-induction (Fig. 6A). Using MEA, we compared the percentage of active electrodes and the percentage of wells showing bursting activity (Fig. 6B) of m*Ngn2*-induced neurons with different lengths of doxycycline treatment. In two-week-old m*Ngn2*-induced neurons, transient doxycycline addition for as little as two days increased the rate of neuronal activity relative to uninduced controls (Fig. 6B). Thus, transient m*Ngn2*-induction seems to be sufficient to accelerate the maturation of functional neurons from NPCs.

3.5. Variable intra- and inter-individual efficiencies in *Ngn2*-induction between NPC lines

The advantage of using this scalable platform is that it should be possible to induce *Ngn2*-neurons from independent NPC lines derived from several hiPSCs from many individuals in

parallel. To test this, we first evaluated the efficacy of CMV-*rtTA* and TetO-h*NGN2*-P2A-*eGFP*-T2A-*Puro*^R lentiviral transduction across ten NPC lines (three NPC lines each from two controls: NSB553-1-1, NSB553-2-3, NSB553-3-C; NSB2607-1-4, NSB2607-3-1, NSB2607-4-1), and one NPC line each from a third control (NSB690-1-4) as well as from three schizophrenia patients (NSB581-1-2, NSB1442-4-3, NSB2513-1-4). Even without puromycin selection, we robustly achieved >80%-GFP positive cells by fluorescent activated cell sorting (Fig. 1E). Although minor variations between NPC lines were observed, lentiviral transduction efficiency was not diagnosis-dependent, showing at least as much intra-individual variation as inter-individual variation.

Nonetheless, when we tested the ability of h*NGN2* overexpression to robustly induce neurons from these same six individuals (three controls and three schizophrenia patients), we initially failed to produce suitable populations for comparisons (Fig. 7). While we did not attempt to discern whether this variation arose from altered levels of replication, cell death or induction efficiencies in transduced NPCs, it was adequately addressed by varying the initial seeding density of individual NPC lines (Fig. 7). Once optimal plating densities are empirically established, these values appear to be relatively robust from experiment-to-experiment. Therefore, if achieving a consistent neuronal density at the end-point is required, we recommend that the protocol be specifically tailored with respect to starting cell density for each NPC line used.

4. Discussion

Here we report a novel adaptation of *Ngn2*-induction strategies, whereby hiPSC derived NPCs can be rapidly induced to functional neurons within two weeks. Moreover, transient exogenous transgene expression is sufficient to accelerate neuronal differentiation, synaptogenesis and neuronal activity. This protocol provides a rapid and scalable platform for inducing functional neurons from NPCs, one that is highly amenable to high throughput drug screenings. It remains unresolved why neuronal induction methods, in which pro-neuronal transcription factors are overexpressed, so greatly exceed neuronal differentiation protocols in terms of purity and speed, but we speculate that this may reflect: (i) increased expression levels of key neuronal genes via overexpression relative to levels achieved by growth factor signaling; (ii) positive selection for cells with transgene expression; (iii) reduced heterogeneity owing to spontaneous growth factor secretion by poorly patterned cells; (iv) inconsistent dosing or batch effects by biological protein and small molecules with; (v) variable growth factor receptor expression across independent hiPSC lines derived from different individuals. This is not to say that we believe that there is an inherent biological limitation in directed differentiation protocols, only that in this case, *Ngn2*-overexpression improves the purity, yield and rate of maturation of excitatory neurons from NPCs.

A few striking differences between our NPC-based and previous hiPSC-based neuronal induction methods should be noted. First, we were able to identify SYN1-positive synaptic puncta in m*Ngn2*-induced neurons without astrocyte co-culture, perhaps indicating that prior neural commitment of NPCs, unlike hiPSCs, permits enhanced neuronal maturation via m*Ngn2*-induction. Nonetheless, we expect that co-culture of *Ngn2*-induced neurons with

human astrocytes would enhance synaptogenesis of *Ngn2*-induced neurons from NPCs, perhaps permitting detection of puncta positive for additional synaptic markers. Second, we found it surprising that our *Ngn2*-induced neurons showed elevated expression of presynaptic proteins such as *SYN1* and *vGLUT1*, but not the postsynaptic marker *PSD95*, contrary to previous hiPSC-based induction studies [19–21] and may suggest that *Ngn2*-induction primarily accelerates the gene networks relevant to presynaptic maturation or that astrocyte co-culture is required for postsynaptic maturation. Third, neuronal induction methods to date maintain transgene expression throughout neuronal maturation [17,19]. *Ngn2* decreases during neurogenesis *in vivo* [38]; here we demonstrate that transient *Ngn2*-induction, for a period as short as two days, is sufficient to induce functional neurons.

The ability to induce functional neurons via only transient transgene expression may be a critical proof-of-concept for those considering adapting this method for the modeling of neuropsychiatric disease. By eliminating the need for sustained overexpression of *Ngn2* throughout neuronal induction, we believe we have reduced the likelihood that by employing induction strategies, rather than directed differentiation protocols, disease-related phenotypes or gene expression signatures unique to patient derived hiPSC neurons will be masked or obfuscated. Moreover, by combining induction with puromycin selection and Ara-C treatment, we have described a method that, with minimal optimization for cell density, can produce nearly pure populations of excitatory neurons from NPCs derived from independent hiPSC lines and different individuals.

In this study, we described a method by which mouse or human *Ngn2* transduction can rapidly induce hiPSC-derived NPCs into functional excitatory neurons. We have now applied this method to hiPSC derived NPCs from more than twenty individuals (cases and controls). Although we have observed some variation in both the efficiency of lentiviral transduction and *Ngn2*-induction between individuals, we believe that this is a scalable method that can be routinely applied to a robustly proliferative starting population. We hope that this will be a useful platform for modeling neuropsychiatric disorders with patient-derived hiPSCs and for developing high throughput drug screens to identify novel therapeutics for the treatment of these diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

hiPSCs	human induced pluripotent stem cells
NPCs	neural progenitor cells

<i>Ngn2</i>	<i>Neurogenin 2</i>
SZ	schizophrenia
GFP	green florescent protein
<i>Puro^R</i>	puromycin resistance

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ymeth.2015.11.019>.

Fig. 1.

Scheme of *Ngn2*-neuronal induction from human NPCs and the accelerated neuronal morphology of *Ngn2*-NPCs. (A) Schematic of *mNgn2* and *hNGN2* neuronal induction, starting from hiPSC-derived NPCs. (B) Representative bright-field images of hiPSCs, NPCs and *mNgn2*-induced neurons. Scale bar 100 μ m. (C) Timeline of *mNgn2* and *hNGN2*-neuronal induction strategy. (D) GFP images of NPCs transduced with GFP-Puro^R lentivirus (images taken before and 24 h after doxycycline treatment). Scale bar 100 μ m. (E) FACS quantification of the percentage of GFP-positive cells across ten NPC lines transduced with *hNGN2*-eGFP-Puro^R (presented as the average of three NPC lines each from two controls, and one NPC line each from a third control as well as three schizophrenia patients). (F) GFP images of live GFP-, *mNgn2*- and *hNGN2*-transduced NPCs at various induction time points, showing that *Ngn2*-NPCs acquire neuronal morphology faster than GFP-NPCs. Scale bar 50 μ m. (G) Averaged MAP2AB fluorescent intensity of GFP-, *mNgn2* and *hNGN2*-induced neurons at three weeks induction. Error bars are SEM (Standard Error of the Mean). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Fig. 2.

Accelerated increase in MAP2AB protein and RNA by *Ngn2*-transduction. (A) Representative images of *mNgn2*-induced neurons and GFP-NPCs at two weeks immunostained with neuronal dendrite marker MAP2AB (red), GFP (green) and DAPI-stained nuclei (blue). Scale bar 30 μ m. (B) Averaged MAP2AB fluorescent intensity of GFP-NPCs and *mNgn2*-induced neurons at two weeks induction. MAP2AB intensity was normalized to total DAPI-positive nuclei per image, and further standardized to GFP-NPCs; *mNgn2*-induced neurons showed a 1.3-fold increase in MAP2AB intensity over GFP-NPCs. (C) Real-time qPCR analysis of *MAP2AB* mRNA expression from GFP-NPCs and *mNgn2*-induced neurons at two weeks of age. The expression level is normalized to GAPDH, and further standardized to GFP-NPCs; *mNgn2*-neurons showed 2.4-fold increase in *MAP2AB*. (D) Representative images of two-week-old and three-week-old *mNgn2*-induced neurons stained with MAP2AB (red) and DAPI (blue). Scale bar 30 μ m. (E) Averaged MAP2AB fluorescent intensity of two-week-old and three-week-old *mNgn2*-induced neurons, normalized to two-week-old *mNgn2*-induced neurons; there was a 1.35-fold increased MAP2AB intensity in three-week-old *mNgn2*-induced neurons. Error bars are SEM (Standard Error of the Mean). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Fig. 3.

Evoked activity recorded from *Ngn2*-induced neurons. (A) Voltage clamp recording shows putative voltage-gated Na^+ (inward) and K^+ (outward) currents. Voltage pulses shown below (-100 to $+70$ mV). Holding potential was -70 mV. (B) Current clamp recording from the same neuron shows evoked action potentials. Dashed line indicates -60 mV. Current injection pulses shown below (-16 pA to 48 pA).

Fig. 4.

Accelerated synaptic differentiation of *Ngn2*-induced neurons by neuronal induction. (A) Representative images of *mNgn2*-neurons and GFP-NPCs at three weeks, immunostained with presynaptic marker SYN1 (red) and MAP2AB (magenta). Scale bar 4 μ m. (B) SYN1 puncta count, normalized to MAP2AB⁺ area, of *mNgn2*-induced neurons and GFP-NPCs at three weeks (gradual doxycycline withdrawal from day 2). The SYN1 puncta ratio was increased 1.9-fold relative to matched GFP-NPCs. (C) Real-time qPCR analysis of *SYN1*, *vGLUT1*, *vGLUT2* and *PSD95* mRNA expression from GFP-NPCs and *mNgn2*-induced neurons at three weeks, normalized to GAPDH, relative to GFP-NPCs. *mNgn2*-neurons showed increased *SYN1* (2.3-fold), *vGLUT1* (4.5-fold), *vGLUT2* (3.8-fold); there no significant difference in *PSD95* expression. (D) qPCR analysis of *TH*, *GAD67*, *VGAT*, *TPH1*, *GFAP* and *S100 β* mRNA expression between three-week-old *mNgn2*-induced neurons and 6-week-old hiPSC forebrain (FB) neurons derived by directed differentiation; *Ngn2*-induced neurons showed decreased *TH* (6.3-fold), *GAD67* (1.5-fold), comparably low levels of *VGAT* and *TPH1*, and decreased *GFAP* (13.6-fold) and *S100 β* (3.2-fold) expression relative to 6-week-old forebrain neurons. (E) Representative images of 3-week-old *mNgn2*-induced neurons and *Puro^R*-NPCs, stained with the dopaminergic marker TH, the GABAergic marker GAD65/67 and the astrocyte marker GFAP. DAPI-stained nuclei (blue). Scale bar 30 μ m. (F) Representative bright-field images of 3-week-old hNGN2-induced neurons treated with 0 M, 10 nM, 50 nM, 100 nM and 1 μ M Ara-C from days 6–20. Scale bar 30 μ m. (G) Representative images of 3-week-old hNGN2-induced neurons (from two independent controls) treated with 0 nM, 10 nM, 50 nM, and 100 nM Ara-C from days 6–

20, stained with the replication marker Ki67. DAPI-stained nuclei (blue). Scale bar 30 μm .
(H) Percentage of Ki67-positive cells in three-week-old *Ngn2*-induced neuron populations treated with between 0 and 100 nM Ara-C. Error bars are SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Fig. 5.

Transient *Ngn2* is sufficient to generate induced neurons. (A) Timeline of mNgn2-neuronal induction strategy with different doxycycline lengths. Doxycycline was withdrawn at day two (D2), day eight (D8), day fourteen (D14) or not at all (No W/D). (B) Representative images of three-week-old mNgn2-induced neurons immunostained with MAP2AB (red) and DAPI (blue). Scale bar 30 μ m. (C) Average MAP2AB fluorescent intensity at three weeks. (D) Representative images mNgn2-induced neurons immunostained with the presynaptic marker SYN1 (red) and MAP2AB (magenta). Scale bar 4 μ m. (E) SYN1 puncta count, normalized to MAP2AB⁺ area at three-weeks. (F) Real-time qPCR analysis of *SYN1*,

vGLUT1, *vGLUT2* and *PSD95* mRNA expression at three-weeks. Error bars are SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Fig. 6.

Electrical activity of *Ngn2*-induced neurons from NPCs. (A) 5-min MEA raster plot of spontaneous activity from a neural network of 14-day-old neurons, with 0-days (left panel) or 14-days (right panel) doxycycline treatment to induce mNgn2 expression. The response over multiple electrodes is a measurement of network connectivity. Each thin black line is representative of an action potential. Bursts of electrical activity are indicated with solid blue blocks. (B) The percentage of active electrodes per well (left panel) or percentage of spikes in bursts (right panel) following either 0 days (white bar), 2 days (pink bar), 8 days (light red) or 14 days (dark red) of doxycycline treatment to induce mNgn2 expression in 14-day-old neurons. (C) Total number of spikes (left panel) or mean firing rate (Hz) (right panel)

following treatment with vehicle control (DMSO), picrotoxin (PTX), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and tetrodotoxin (TTX) on 21-day-old h*NGN2*-neurons. Error bars are SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Fig. 7. Inter-individual efficiencies in *Ngn2*-induction between NPC lines. Representative bright-field images of hNGN2-neurons from six individuals (three controls and three schizophrenia patients) over three independent experiments. Varying the initial seeding density of individual NPC lines (NPC plating densities inset) is required if achieving a consistent neuronal density at the end-point is desired.

Table 1

Composition of NPC and neuron medium.

Media	Reagents
NPC	DMEM/F12 (Life Technologies, #10565)
	1 × N2 (Life Technologies, #17502-048)
	1 × B27-RA (Life Technologies, #12587-010)
	20 ng/ml FGF2 (R&D, #233-FB-10)
	1 mg/ml Natural Mouse Laminin (Life Technologies, #23017-015)
Neurons	DMEM/F12 (Life Technologies, #10565)
	1 × N2 (Life Technologies, #17502-048)
	1 × B27-RA (Life Technologies, #12587-010)
	1 mg/ml Natural Mouse Laminin (Life Technologies, #23017-015)
	20 ng/ml BDNF (Peprotech, #450-02)
	20 ng/ml GDNF (Peprotech, #450-10)
	500 µg/ml Dibutyl cyclic-AMP (Sigma, #D0627)
	200 nM L-ascorbic acid (Sigma, #A0278)

Table 2

List of antibodies.

Antibody	Species	Dilution ratio	Company (Catalog #)
MAP2AB	Mouse	1:500	Sigma–Aldrich (M1406)
MAP2AB	Chicken	1:2000	Abeam (ab5392)
GAD65/67	Rabbit	1:500	Millipore (AB1511)
GFAP	Chicken	1:1000	Aves (GFAP)
GFP	Chicken	1:500	Aves (GFP-1020)
Ki67	Rabbit	1:1000	Abeam (abl5580)
SYNAPSIN1	Rabbit	1:500	Millipore (574778)
SYNAPSIN1	Mouse	1:500	Synaptic Systems (106 011)
TH	Mouse	1:1000	Immunostar (22941)
Alexa 488 anti-chicken	Donkey	1:400	Jackson immuno. (703-545-155)
Alexa 568 anti-mouse	Goat	1:600	Life Technologies (A-11004)
Alexa 568 anti-Rabbit	Goat	1:600	Life Technologies (A-11011)
Alexa 647 anti-mouse	Donkey	1:400	Jackson immuno. (715–605-150)
Alexa 647 anti-chicken	Donkey	1:400	Jackson immuno. (703-605-155)

Table 3

List of qPCR primer sequences.

<i>GAPDH</i>	Forward	AGGGCTGCTTTTAACTCTGGT
	Reverse	CCCCACTTGATTTTGGAGGGA
<i>β-ACTIN</i>	Forward	AAACTGGAACGGTGAAGGTG
	Reverse	AGAGAAGTGGGGTGGCTTTT
<i>βIII-TUBULIN</i>	Forward	CCCGTTATCCCAGCTCCAATATGCT
	Reverse	ATGGCTTGACGTGCGTACTTCTCC
<i>MAP2AB</i>	Forward	AAACTGCTCTTCCGCTCAGACACC
	Reverse	GTTCACTTGGGCAGGTCTCCACAA
<i>SYNAPSIN1</i>	Forward	GCAAGGACGGAAGGGATCACATCA
	Reverse	CCTGAGCCATCTTGTGACCACGA
<i>vGLUT1</i>	Forward	CGCATCATGTCCACCACCAACGT
	Reverse	GAGTAGCCGACCACCAACAGCAG
<i>vGLUT2</i>	Forward	TCAACAACAGCACCATCCACCGC
	Reverse	GTTTCCGGGTCCCAGTTGAATTGG
<i>PSD95</i>	Forward	GGCAGCCCTGAAGAACACGTATGA
	Reverse	CCCAGGTAGCTGCTGTGACTGATC
<i>GFAP</i>	Forward	GGTTGAGAGGGACAATCTGGCACA
	Reverse	CTATAGGCAGCCAGGTGTTCTCGG
<i>S100b</i>	Forward	GGAAGGGAGGGAGACAAGCACAAG
	Reverse	TTCGCCGTCTCCATCATTGTCCAG
<i>GAD67</i>	Forward	TTGCACCAGTGTTCCTCATGG
	Reverse	CCGGGAAGTACTTGTAGCGAGCAG
<i>vGAT</i>	Forward	CACGACAAGCCAAAATCAC
	Reverse	CGGCGAAGATGATGAGAAACAAC
<i>TPH1</i>	Forward	GAGACACAGTTCAGATCCCTTC
	Reverse	CTTGGGAGAATTGGGCAAAAC
<i>TH</i>	Forward	CCGAGCTGTGAAGGTGTTGA
	Reverse	CGGGCCGGGTC TCTAGAT