



A Rapid Pipeline to Model Rare Neurodevelopmental Disorders with Simultaneous CRISPR/Cas9 Gene Editing

SCOTT BELL,^a HUASHAN PENG,^a LIAM CRAPPER,^a ILARIA KOLOBOVA,^a GILLES MAUSSION,^a
CRISTINA VASUTA,^a VOLODYMYR YERKO,^a TAK PAN WONG,^b CARL ERNST^{a,b,c}

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^aMcGill Group for Suicide Studies, Douglas Hospital Research Institute, Montreal, Quebec H4H 1R3, Canada; Departments of ^bPsychiatry and ^cHuman Genetics, McGill University, Montreal, Quebec H4H 1R3, Canada

Correspondence: Carl Ernst, Ph.D., Douglas Hospital Research Institute, McGill University, 6875 LaSalle Boulevard, Frank Common Building, Room 2101.2 Verdun, Quebec H4H 1R3, Canada. Telephone: 514-761-6131, ext. 3382; Fax: 514-762-3023; email: carl.ernst@mcgill.ca

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ABSTRACT

The development of targeted therapeutics for rare neurodevelopmental disorders (NDDs) faces significant challenges due to the scarcity of subjects and the difficulty of obtaining human neural cells. Here, we illustrate a rapid, simple protocol by which patient derived cells can be reprogrammed to induced pluripotent stem cells (iPSCs) using an episomal vector and differentiated into neurons. Using this platform enables patient somatic cells to be converted to physiologically active neurons in less than two months with minimal labor. This platform includes a method to combine somatic cell reprogramming with CRISPR/Cas9 gene editing at single cell resolution, which enables the concurrent development of clonal knockout or knock-in models that can be used as isogenic control lines. This platform reduces the logistical barrier for using iPSC technology, allows for the development of appropriate control lines for use in rare neurodevelopmental disease research, and establishes a fundamental component to targeted therapeutics and precision medicine. *STEM CELLS TRANSLATIONAL MEDICINE* 2017;6:886–896

SIGNIFICANCE STATEMENT

Creating stem cell models for neurodevelopmental disorders (NDDs) is time consuming and technically difficult, affecting the type of disorders that are studied. Specifically, common disorders are more likely to be studied because experimental outcomes will affect a greater number of patients. Widening the net of disorders that can be studied requires lowering the barrier to entry to technically challenging stem cell research. We have developed a user-friendly pipeline for the non-expert which can achieve physiologically active forebrain GABA/glutamatergic or midbrain dopaminergic neurons in under two months. This pipeline requires a modest input of reagents and labour, while producing excellent reproducibility across cell lines. We also demonstrate how simultaneous genetic engineering and cell reprogramming can be accomplished to establish clonal control lines, an essential step to ensure experimental accuracy of measured outputs. This pipeline provides a rapid way to make neurons and should prove useful for studying and testing treatments for rare NDDs.

INTRODUCTION

Neurodevelopmental disorders (NDDs) affect approximately 5% of the world's population [1], and have an estimated economic disease burden of up to 200 billion dollars in the United States alone [2]. The majority of these disorders are considered “rare,” with an incidence lower than 1/2,000 persons [1, 3]. Many rare NDDs are genetically defined, which makes them good candidates for the development of effective therapeutics [4]. However, most research attention has been devoted to common NDDs such as Fragile X [5] or Rett syndrome [6], where labor-intensive cell modeling can benefit the most number of

affected individuals. However, even intensely studied NDDs face immense hurdles in the development of effective treatments [7].

Neurological diseases more generally have been historically studied using either immortalized cell lines or animal models [8–10]. While these analogs of disease have been an invaluable research tool, concerns about the translatability of results obtained in these models have been raised since their inception [11]. Immortalized cell lines, while relatively easy to maintain and produce, have a different genetic background than the patients that they purport to model, and their responses may be

influenced by the mutations that allow them to proliferate indefinitely [12]. Transgenic animals have also been used to provide a model in which CNS cells develop in a manner similar to the human brain [13]. However, they are resource intensive to produce and maintain, and species differences coupled with genetic background means that even neurological diseases with defined mutations are sometimes unable to be modeled accurately in animals [12]. Given the difficulty that has been observed in the translation of therapies for very prominent diseases from immortalized cells and animal models [14], the risk of investing research resources to generate models of rare diseases may discourage research into rare NDDs.

What is needed to produce viable treatments for rare NDDs is a model that can be used to move rapidly from genetics to therapeutics. One of the most promising scientific discoveries of the 21st century has been the development of induced pluripotent stem cells (iPSCs) [15]. iPSCs have several advantages over more classical methods of modeling disease. First, they can be derived from patient cells, which eliminates both intraspecies and interspecies variation, and prevents the confounding oncogenic effects observed in immortalized cell lines [16]. Second, they might be able to be used to model the development of human neural cells in a manner similar to in vivo human development [8]. iPSCs have been shown to be a particularly illuminating research tool when paired with genome editing technologies like CRISPR/Cas9 to create isogenic controls or model monogenic disorders [17, 18].

In order for iPSC-based NDD models to become a commonplace technique in rare neurodevelopmental research, there are several logistical hurdles that need to be addressed. The process of generating iPSCs and differentiating them into neurons can be very long and complicated, with some protocols requiring over 10 different media types, each with a complex set of growth factors, and multiple steps of cell plating, aggregation, and dissociation [19, 20]. Increases in protocol steps and time may be one reason for reports of cell line variability, even from neurons generated from the same patient in the same lab [21]. Generating models of NDDs with such a long and complex methodology not only means that generating iPSCs is expensive, but that only researchers with significant experience will be able to successfully carry out the protocol [22], making modeling of rare NDDs impractical [23]. Therefore, simplifying and optimizing the protocols required to generate neural cells from iPSCs is an important step toward increasing the ease of generating iPSC-derived neurons and encouraging the more widespread use of iPSC models.

In the present study, we established a research platform to model rare neurodevelopmental disease in vitro. We have developed and optimized protocols with an emphasis on reducing the required time and complexity of genome editing, iPSC induction, and neuronal differentiation to facilitate the adoption of these techniques by research groups with limited resources, or lacking pluripotent cell culture experience. The set of protocols contained in this platform details the generation of iPSCs from fibroblasts and the differentiation of iPSCs into electrically active neurons rapidly with high efficiency. The optional high-efficiency gene editing protocol can be performed in parallel to patient cell reprogramming with minimal additional time requirements. This platform provides simplified methodologies for developing cell models of NDDs while reducing heterogeneity in final cell output.

MATERIALS AND METHODS

Culturing Skin Fibroblasts

Fibroblasts were obtained from Coriell or from patient biopsies (Supporting Information Table 1). Cells were plated on tissue culture dishes (Corning), after incubation with 0.05% gelatin (Sigma-Aldrich) for 60 minutes at room temperature. Cells were cultured in Dulbecco modified Eagle's medium (DMEM) (ThermoFisher) supplemented with 10% Fetal Bovine Serum (FBS) (ThermoFisher), and 1% Penicillin/Streptomycin (100 U/mL Penicillin and 100 µg/mL Streptomycin) (Mediatech). Cells were passaged when cells reached 80% confluency using 0.05% Trypsin-EDTA (ThermoFisher), and replated at a density of $\sim 2 \times 10^6$ cells per 100 mm² plate.

Conversion of Fibroblasts to iPSCs

Fibroblasts were reprogrammed using episomal reprogramming vectors containing Oct4, Sox2, Myc3/4, Klf4, and ShRNA P53 (ALSTEM) and a Neon Transfection System (Invitrogen, Burlington). A total of 5.0×10^5 cells were reprogrammed with 5 µg of episomal reprogramming vectors per reaction. Electroporation parameters were as follows: 11,650 V, 10 ms, 3 pulses. Following transfection, cells were plated on tissue culture plates coated with Matrigel (Corning) in 10% FBS DMEM. The following day, the media was exchanged for fresh 10% FBS DMEM supplemented with 2 µg/mL puromycin (Sigma-Aldrich). Puromycin selection was applied for 48 hours, after which the media was exchanged with fresh TesR-E7 media. During the induction process, TesR-E7 (Stem Cell Technologies, Vancouver), media was changed every day. After approximately 10 days following selection, colonies were observed. These colonies were tracked until they formed robust, distinct cell populations (~ 500 – $1,000$ µm in diameter), at which point cells were detached using ReLeSR media (Stem Cell Technologies, Vancouver). Clumps of floating cells were then picked and plated on Matrigel-coated plates in mTesR1 media (Stem Cell Technologies, Vancouver) supplemented with ROCK inhibitor γ -27632 (Sigma-Aldrich) at a final concentration of 10 µM. Once formed, iPSC colonies were cryopreserved in fetal bovine serum with 10% DMSO (Dimethyl sulfoxide, Sigma-Aldrich), maintained in culture in mTesR1 media with daily media changes, or differentiated into midbrain or forebrain cells.

iPSCs to Forebrain Cells

Direct Generation of Mature Neurons. iPSCs were dissociated using Gentle Cell Dissociation Reagent (Stem Cell Technologies, Vancouver) and resuspended in Neural Induction (NI) media (DMEM/F12 supplemented with N2 [Invitrogen], B27 supplement [Invitrogen], BSA [1 mg/mL], Y27632 [10 µM; AdooQ Bioscience], SB431542 [10 mM; Selleckchem], and noggin [200 ng/mL; GenScript]), onto low-bind plates (Corning) or Petri dishes (Corning). Cells were plated at a density of 2 – 3×10^6 cells per 100 mm² plate.

Cells were cultured in suspension and monitored for the formation of organoids, which occurred approximately 4 days after suspension. Three days after the formation of embryoid bodies (EBs), a 70-µm Falcon cell strainer was used to collect aggregations, which were then resuspended in a fresh low-bind/Petri dish in Neural Progenitor (NP) medium (DMEM/F12 supplemented with N2, B27 supplement, bFGF [20 ng/mL; GenScript], EGF [20 ng/mL; GenScript], laminin [1 µg/mL; Sigma-Aldrich]). The media was exchanged every day for fresh

NP media for 14 days. Following 14 days in NP media, cell aggregations were resuspended in Final Differentiation (FD) medium (DMEM/F12 supplemented with N2, B27 supplement, BDNF [20 ng/mL; GenScript], GDNF [20 ng/mL; GenScript], laminin [1 μ g/mL]). FD media was changed every 2 days for 7 days. Organoids were plated on polyornithine- and laminin-coated tissue culture plates in Neuron Maturation (NM) medium (DMEM/F12 supplemented with N2, B27 supplement). Following attachment, organoids were dissociated with 0.05% trypsin-EDTA, and replated onto fresh polyornithine- and laminin-coated plates in NM media. Half the media was exchanged for fresh media every 3 days.

Generation of Forebrain Neural Progenitor Cells. If neural progenitor cells (NPCs) were desired, the protocol was identical to the One Step method described above, until the point where organoids were resuspended in NP media. Instead of maintaining cells in a three-dimensional (3D) culture (i.e., floating organoids), cells were plated onto polyornithine- and laminin-coated tissue culture plates after 1 week in NP media. Organoids were allowed to attach for 24 hours, then were dissociated and replated on fresh plates. Cells were then maintained in NP media for 7 more days, with the media being changed every third day, before cells adopted a NPC morphology and stained positive for NPC markers. NPCs have been maintained as an NPC population in NP media for 11 passages without any change in cell proliferation rate or morphology. To make mature neurons from these NPC cells, NPCs at 70% confluency were cultured in FD media for 1 week, with media being exchanged every 2 days, followed by culturing in NM media. Half the media was exchanged for fresh NM media every 3 days.

iPSCs to Midbrain Cells

iPSCs were disassociated using Gentle Cell Dissociation Reagent and resuspended in midbrain Neural Induction (mNI) medium (DMEM/F12 supplemented with L-glutamine [2 mM], N2, B27 supplement, bovine serum albumin [1 mg/mL], Y27632 [10 mM; Tocris], SB431542 [10 μ M; Tocris], noggin [200 ng/mL], and SHH C24II [200 ng/mL; Miltenyi Biotec]) at a density of $\sim 1 \times 10^6$ cells per 100 mm² plate. Following EB formation and 3 days after suspension, 50% of the media was changed daily. Organoids were harvested on day 5 and resuspended in midbrain Neural Progenitor 1 (mNI1) medium (DMEM/F12 supplemented with L-glutamine [2 mM], B27, N2 supplement, bovine serum albumin [1 mg/mL], SHH C24II [200 ng/mL]) for 3 days. Aggregations were then suspended in midbrain Neural Progenitor Medium II (mNP2) (DMEM/F12 supplemented with N2, B27 supplement, bFGF [20 ng/mL], EGF [20 ng/mL], laminin [1 μ g/mL], SHH C24II [200 ng/mL], CHIR-99021 [3 μ M; Stem Cell Technologies]) for 3 days, and then switched to Neural Progenitor Medium III (mNP3) (DMEM/F12 supplemented with L-glutamine [2 mM], B27, N2 supplement, bovine serum albumin [1 mg/mL]) for 7 to 14 days. NPCs were plated on polyornithine/laminin plates in midbrain Neural Progenitor Media IV (mNP4) (DMEM/F12 supplemented with L-glutamine [2 mM], B27, N2 supplement, SHH C24II [200 ng/mL], FGF8 [100 ng/mL]) for expansion. When differentiation was desired, Gentle Dissociation Medium was added to cultures of neural rosettes until a large proportion of cell residing at the nucleus of rosettes detached, but cells at the periphery of rosettes remained attached. Media was decanted and cells plated at a density of 1×10^6 cells per 10 cm Petri dish in midbrain Final Differentiation

Medium (mFDM) (DMEM/F12 supplemented with L-glutamine [2 mM], N2, B27 supplement, BDNF [20 ng/mL], GDNF [20 ng/mL], N6, dCAMP [0.5 mM; Sigma-Aldrich], ascorbic acid [200 μ M; Sigma], laminin [1 μ g/mL]). Cell aggregations were observed to form after approximately 3 days in mFDM. Four days after the formation of cell aggregations, aggregations were plated in NM media on polyornithine-/laminin-coated tissue culture plates. Half of the media was exchanged every 3 days.

Direct Conversion of Fibroblasts to Neuronal Cells. Conversion was carried out as previously described [24], using the chemical cocktail VCRFSGY (V, valproic acid 0.5 mM; C CHIR99021 3 μ M; R, Repsox 1 μ M; F, Forskolin 10 μ M; S, SP600125 10 μ M; G, GO6983 5 μ M; Y, Y-27632 5 μ M; all chemicals from Sigma-Aldrich). Fibroblasts were plated in 6-well plates, and specific wells harvested for cell counting on days 1, 3, 5, and 7 following the initiation of differentiation.

CRISPR/Cas9 Gene Editing

A double nickase CRISPR/Cas9 gene editing system with gRNA (DNA2.0) targeting a 51 base pair (bp) exonic sequence of *GRIN2B* was generated with a Paprika RFP reporter (DNA 2.0). Five microgram of this construct was added per transfection reaction, and transfection was carried out using the parameters previously described for iPSC induction. Following transfection, cells were plated on Matrigel-coated plates in 10% FBS DMEM for 24 hours. Cells were then detached, and sorted via Fluorescence Activated Cell Sorting (FACS) for RFP+ cells. RFP+ cells were then replated on Matrigel-coated plates, in 10% FBS DMEM supplemented with 2 μ g/mL puromycin. Following 48 hours of selection, cells were dissociated using 0.05% EDTA-Trypsin and plated in Matrigel-coated 6-well tissue culture plates (Corning) in TesR-E7 media at a density of $\sim 1,000$ cells per well. Colony formation, picking, and purification proceeded as described for iPSC induction.

Sequencing

Following the establishment of clonal CRISPR/Cas9 transfected iPSC colonies, DNA was extracted from iPSCs using a Blood & Cell Culture DNA Mini Kit (Qiagen). Primers flanking the targeted region were designed (see Supporting Information), and a PCR preformed using Platinum Taq (ThermoFisher). polymerase chain reaction (PCR) products were loaded into a 1.8% agarose gel and visualized using ethidium bromide (ThermoFisher) to confirm amplification and identify potential knockout or heterozygote colonies. Promising PCR products were then sent to Genome Quebec (Montréal, QC) for Sanger Sequencing on a 3730xl DNA Analyzer (Applied Biosciences).

Quantitative Polymerase Chain Reaction

In order to validate CRISPR/CAS9 knockouts and heterozygotes, quantitative polymerase chain reaction (qPCR) was used to analyze gene expression. To determine the expression level of the non-deleted form of *GRIN2B* mRNA in wild type, in the heterozygous and in the homozygous cells for deletions generated by the CRISPR-Cas9 system, primers were specifically designed to generate an amplicon, which overlapped deleted and nondeleted regions. Reverse transcriptions were done on total RNA fraction in order to obtain cDNA. cDNA synthesis reaction was preformed using 40 μ L solutions containing 1 μ g of total RNA; 0.5 μ g random

primers, 0.5 mM dNTPs, 0.01 M DTT, and 400 U M-MLV RT (Carlsbad, CA). qPCR reactions were performed in 384 well plates using a Quant Studio 6 Flex Real time PCR machine (Life Technology). We used a reference pool of cDNA to generate a standard curve. Serials dilution provided amounts ranging between 0.003052 and 50 ng. Each well included 10 μ L of 2X gene expression master mix (2X Power SYBR Green PCR Master Mix, Applied Biosystems), 1 μ L of 20X primer mix, 3.4 μ L of RNase free water, and 2 μ L of cDNA and RNase free water QSP 20 μ L. GAPDH was used as internal control for normalization.

Immunofluorescence

Cells were washed with phosphate-buffered saline (PBS), then fixed with 3% paraformaldehyde (Sigma-Aldrich) on slides for fifteen minutes. Samples were permeabilized with 0.5% TX-100 (Sigma-Aldrich) in 0.5% PBS-BSA for 15 minutes, and then blocked in 0.5% PBS-BSA for an additional 15 minutes. Primary antibodies were added in appropriate dilutions (Supporting Information Table 2) in 0.5% PBS-BSA and added to samples for 30 minutes. Samples were washed, 0.5% PBS-BSA containing an appropriate dilution of secondary antibody (Supporting Information Table 2) was added to the samples and incubated for 30 minutes in the dark. Samples were washed with 0.5% BSA and visualized on an Apotome Fluorescent Microscope (Zeiss). Images analyzed using ImageJ.

Fluorescent Activated Cell Sorting

Fibroblast cells were detached by Accutase (Millipore) and resuspended in Pre-Sort buffer (BD Biosciences). Red Fluorescent Protein (RFP) positive cells were aseptically sorted in a FACS ARIA Fusion machine (BD Biosciences) using a 130 μ m nozzle at 20 psi. Cells were sorted in a 6-well plate in prewarmed fibroblast growth medium, 2,000 RFP positive cells per well. Determination of RFP negative and RFP positive populations, after doublet discrimination, was based on gating of unelectroporated and electroporated cells in 616/23 and 695/40 filters.

Estimation of populations of forebrain and midbrain cells was done by flow cytometry on BD FACS Aria Fusion. Mature neurons were prepared for FACS as described, stained with DAPI and labeled with a TUJ1 antibody coupled to Alexa488 (TUJ1-Alexa488). Forebrain cells we also labeled with MAP2-Alexa647, and midbrain cells were labeled with TH-Alexa647. Population of single cells was identified by doublet discrimination followed by DAPI positive gating. Neuronal and non-neuronal populations were identified by gating of TUJ1-Alexa 488 positive and negative populations of cells, respectively. MAP2+ and TH+ populations were identified by gating from TUJ1+ population.

Whole Cell Recordings

Cultures were differentiated as described above but on glass coverslips. Differentiated cells attached to glass coverslips were transferred to plates containing a solution for whole cell patch clamp recordings. The extracellular HEPES-based saline contained, in mM: 140 NaCl, 5.4 KCl, 2 CaCl₂, 1 MgCl₂, 15 HEPES, and 10 glucose (pH 7.3–7.4; 295–305 mOsmol). Cells for recordings were identified based on their morphology using an Eclipse E600FN inverted microscope (Nikon), and recordings were performed at room temperature. Whole-cell patch clamp recordings were obtained using borosilicate

pipettes (3–6 M Ω), filled with intracellular solution that contained: 154 mM potassium gluconate, 2 mM EGTA, 1 mM MgCl₂, 10 mM phosphocreatine, 10 mM HEPES, 2 mM Mg-ATP, (pH 7.2–7.3; 275–285 mOsmol). The resistance of the pipettes was determined using Ohm's law ($V = IR$), by injecting a small current in the circuit, to drop the voltage 5 mV from holding. This was done when the pipette was in the bath position before patching. Where indicated, TTX (200 nM) and TEA (50 μ M) were added to the saline solution. Data were acquired using a Multiclamp 700B amplifier (Axon Instruments). Currents were filtered at 2 kHz and digitized at 20 kHz. Responses were analyzed offline using Clampfit (Molecular Devices).

RESULTS

Rapid Conversion of Patient Fibroblasts into iPSCs

We established a pipeline for generating iPSCs and neurons from patient fibroblasts to effectively model genetically defined NDDs (Fig. 1). We cultured skin fibroblasts, then transfected these cells with episomal iPSC induction vectors which also contained a puromycin resistance gene (Fig. 2A). Following transfection, cells were plated on dishes for 24 hours, and exposed to puromycin for 24 hours. From 21 different experiments, 1,900–2,100 cells remained after puromycin selection. These cells were maintained on Matrigel-coated plates in mTesR-E7 for 14 to 20 days until colonies of iPSCs formed. About 18 to 24 colonies were observed per 2,000 cells plated. Colonies were selected by gentle dissociation using ReLeSR media and replated until pure colonies of iPSCs were obtained (usually two-three passages of 5 to 7 days). Pure colonies of iPSCs were consistently generated approximately 25 days after transfection (Fig. 2B). Pluripotency was confirmed by staining colonies for TRA1-60, NANOG, SSEA, and OCT4 (Fig. 2C), and genome ploidy was assessed by karyotype. At present, we have created 21 iPSC lines using this methodology, using both patient and commercial fibroblast lines (Supporting Information Table 1).

Establishment of a Direct Method for the Generation of Forebrain Neurons from iPSCs

To create a more efficient methodology for the differentiation of iPSCs into forebrain neurons, we experimented with directly differentiating forebrain neurons from organoids (Fig. 3A), using previous 3D culture protocols as our guide [25]. iPSCs were disassociated and resuspended in NI media to allow aggregations to form. Aggregations were maintained in NI media for approximately 15 days, followed by 5 days in NP media, and finally 5 days in FD Media. These aggregations were then plated onto a poly-orinithine/laminin coated plate (Fig. 3B). Following 24 hours of attachment, these aggregations were dissociated and replated onto polyornithine/laminin plates in NM Media. Both attached organoids and dissociated cells were shown to uniformly express both MAP2 and TUJ1 (Fig. 3C). FACS sorting of forebrain neurons 4 weeks after the initiation of differentiation identified 94.5% of cells expressed TUJ1, with 100% of TUJ1+ cells expressing MAP2 (Supporting Information Fig. 1). After a further 3 weeks in culture following dissociation of organoids, TUJ1 continued to be expressed, and mature neuronal markers GABA α 1, GLUR1, and SYT1 were observed (Fig. 3D).

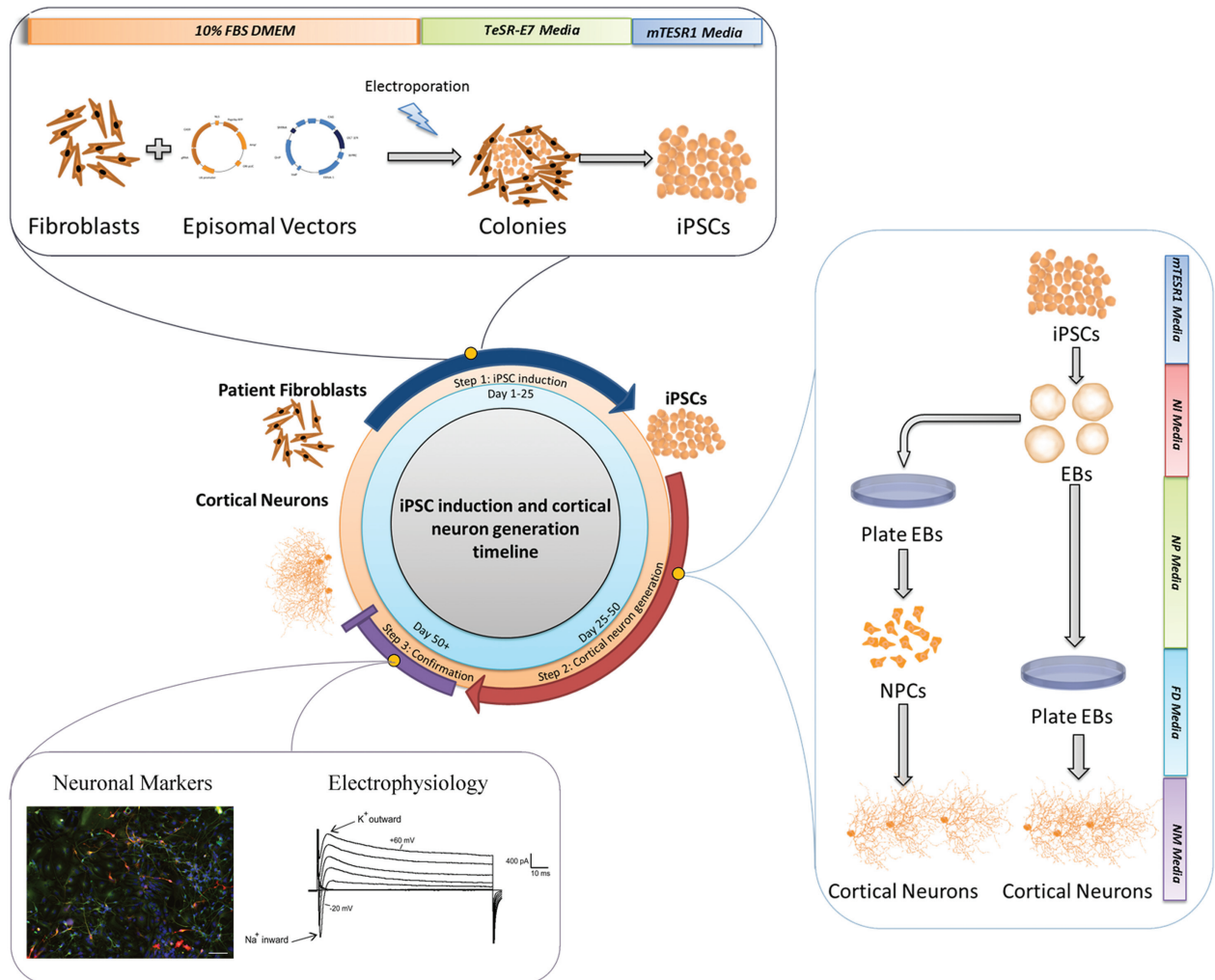


Figure 1. iPSC and forebrain neuron generation timeline. Patient fibroblasts are transfected with iPSC episomal vectors using electroporation to form colonies of iPSCs, which are purified through successive passages until pure iPSC colonies are achieved. Organoids are generated from iPSCs, and are either maintained in an aggregate state or plated as NPCs as different media are utilized to guide the differentiation of the cells into forebrain neurons. Once putative forebrain neurons have been generated, they are validated using immunocytochemistry and electrophysiology. Scale bar indicates 30 μm . Abbreviations: EBs, embryoid bodies; FBS, Fetal Bovine Serum; DMEM, Dulbecco modified Eagle's medium; iPSC, induced pluripotent stem cell; NPC, neural progenitor cells.

This protocol generates physiologically active cells, which respond to GABA and glutamate activating drugs, results consistent with expression GABA and glutamate receptors. Differentiated cells also show a time-course dependent increase in cell properties consistent with neuronal maturation, for example, in increased firing rate as cells mature (Fig. 4 A–4M).

We cryosectioned organoids 25 days following initiation of differentiation, and found that they contain distinct populations of TUJ1+/MAP2+ and TUJ1-/MAP2+ cells (Supporting Information Fig. 2). This suggests that organoids themselves show variation as patterns of staining of TuJ1 and Map2 differed across organoids. This variation has implications for cellular assays for mature neuronal cells that may wish to be performed in case and control cells.

This protocol has several important features: (a) organoids are maintained in the same wells while media is changed, avoiding potential disruption; (b) the maturation position of each cell is maintained from NPC to neuron, which reduces

potential heterogeneity from breaking organoids and allowing differentiation in two-dimensional (adherent) cultures, where cells differ from each other in distance to neighbouring cells; and (c) this 3D (floating, nonadherent) method generates cells with good physiological activity consistent with maturing neurons.

Establishment of a Method for the Generation of Forebrain NPCs from iPSCs

In many cases, it is helpful to have NPCs derived from an iPSC colony because these can be frozen down and expanded at a later date. To generate NPCs, we next attempted to develop a simple addendum to our One Step protocol that would allow differentiation to be halted at a NP stage of development, but which would allow differentiation to forebrain neurons to be rapidly resumed (Supporting Information Fig. 3). As previously described, we allowed aggregations to form iPSCs in NI media for 1 week, and kept them in

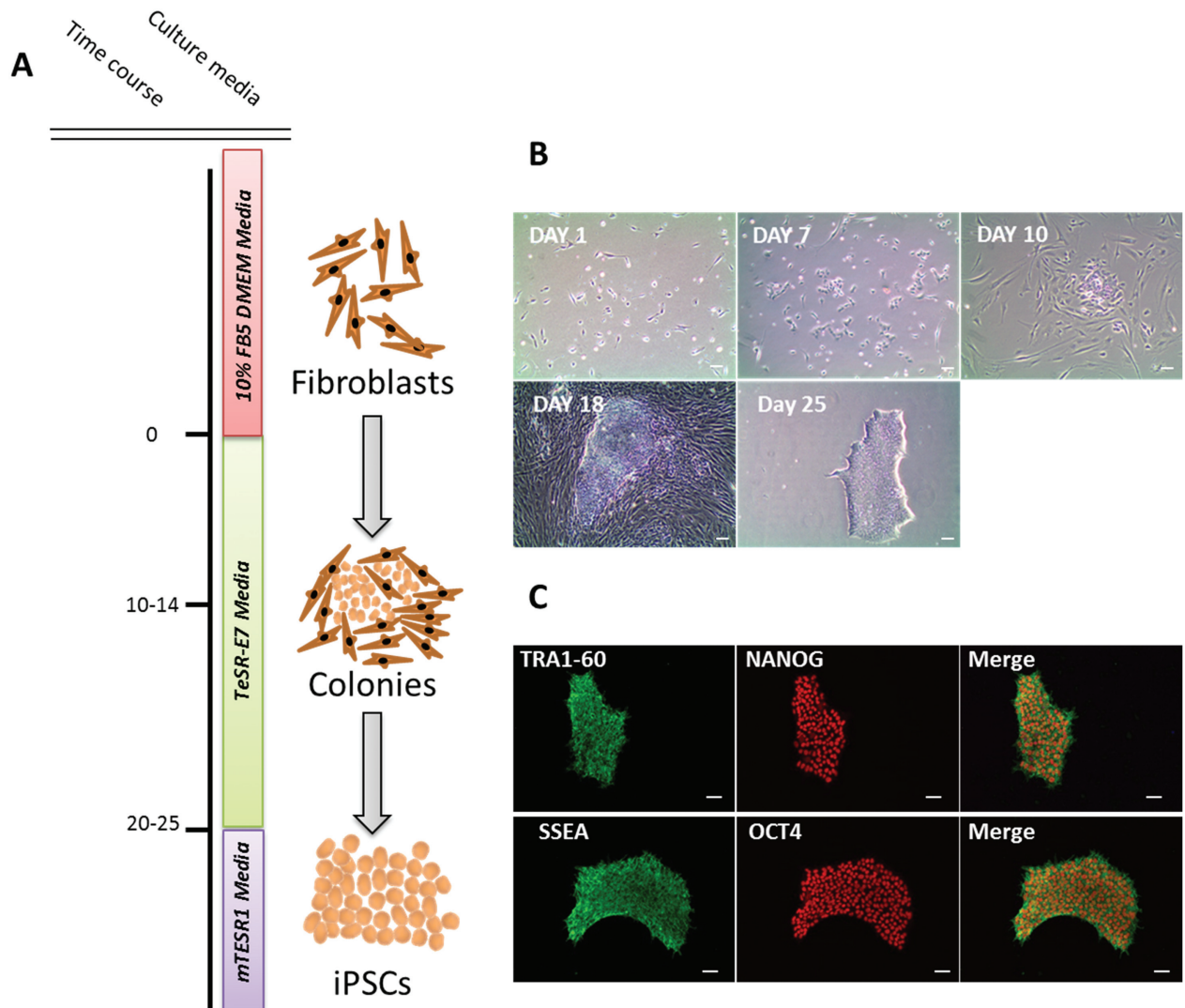


Figure 2. Induction of iPSCs from fibroblasts. **(A):** Schematic illustrating the steps of differentiation, media used and time course. Days are measured with respect to the end of selection. **(B):** Brightfield images showing different timepoints in the process of induction from fibroblasts to iPSCs. Scale bar indicates 30 μm . **(C):** Staining of iPSC colonies demonstrates all cells express the pluripotent markers TRA1-60, Nanog, SSEA, and OCT4. Scale bar indicates 30 μm . Abbreviations: FBS, Fetal Bovine Serum; DMEM, Dulbecco modified Eagle's medium; iPSC, induced pluripotent stem cell.

NP media for another week before disassociating the cells and plating them on polyornithine/laminin plates. These dissociated cells multiplied rapidly, displayed NPC morphology, did not express pluripotent markers, and were positive for the NPC markers NESTIN, SOX1, and PAX6 (Supporting Information Fig. 3). These NPCs could be maintained in culture without noticeable decreases in replication for at least 11 passages (Supporting Information Fig. 4). When differentiation into neurons was desired, cells were cultured in FE media for 1 week, followed by 1 week of culture in NM media. Forebrain neuron phenotypes were confirmed identical to the One Step protocol.

Simultaneous CRISPR/CAS9 Genome Editing and iPSC Induction

CRISPR/Cas9 gene editing technology can be used to create genetic knockouts or genetic knockouts or to correct patient mutations. For rare NDD research, this is an extremely

important tool because it allows for the generation of either isogenic controls or control lines generated directly from patient cells. One key concern when introducing CRISPR/CAS9 gene editing is heterogeneity. Although the CAS9 enzyme cleaves DNA at a very specific point, the repair of double-stranded breaks introduces random mutations into the cut site, which can produce very different mutations in different cells, which consequently have different phenotypes [26]. It is therefore desirable to make a CRISPR-CAS9 gene edited cell line in which all cells are descended from a single gene-edited cell.

We developed a protocol to make clonal cell lines from a CRISPR/Cas9 edited cell (Fig. A). We tested this approach performing a targeted editing of *GRIN2B*, a gene implicated in rare NDDs [27]. Importantly, the nature of this protocol allows for the creation of heterozygous cell lines, which will be an invaluable tool for those NDDs caused by reduced dosage, including *GRIN2B* deletion syndrome. This is because some cells will by chance be cut at only one allele. Fibroblasts from healthy subjects were transfected

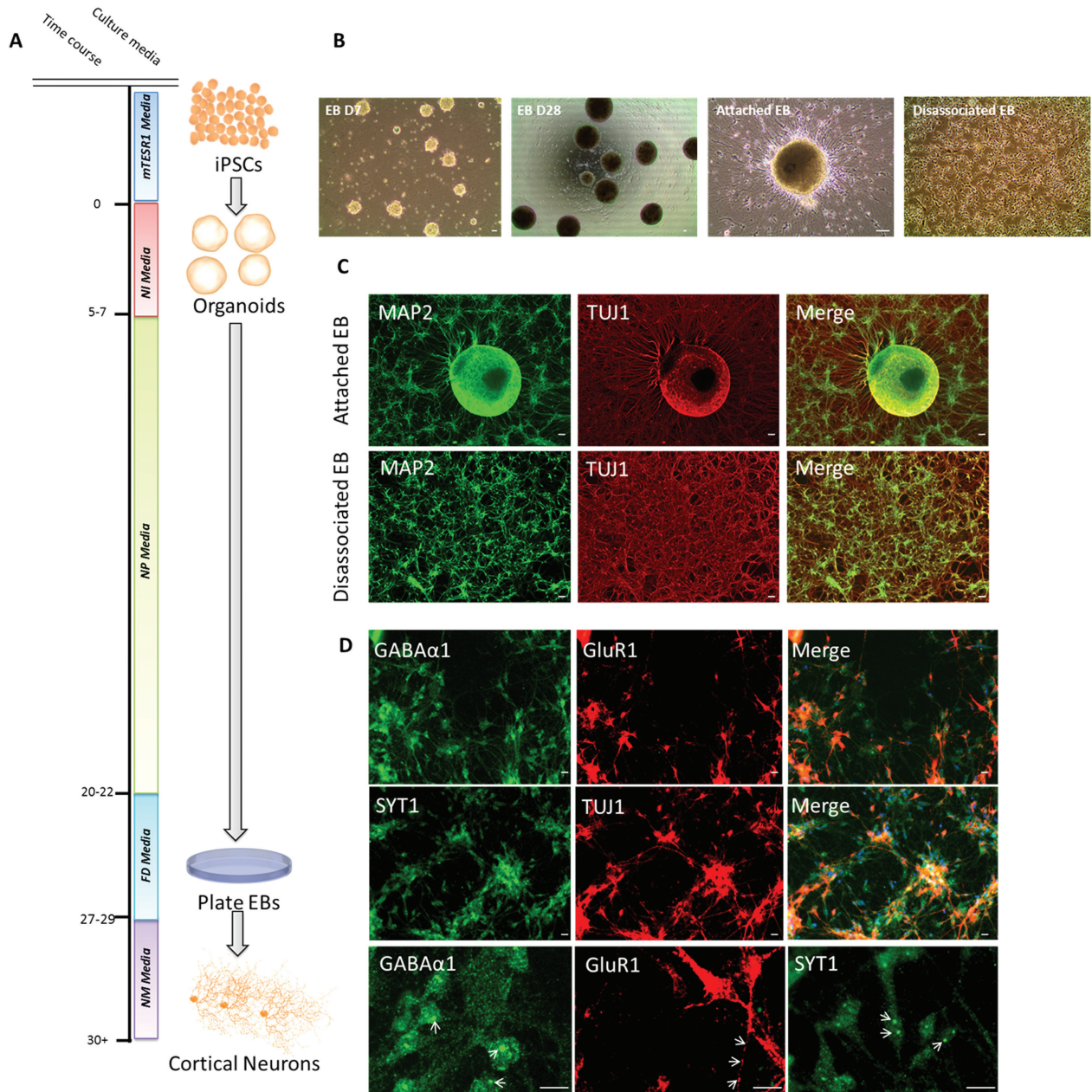


Figure 3. Direct method of differentiating iPSCs into forebrain neurons. **(A):** Schematic illustrating the steps of differentiation, media used, and time course. Days are measured with respect to dissociation of iPSCs. **(B):** Brightfield images of organoids at 1 week and 4 weeks after dissociation of iPSC colonies. Image of an EB attached to a plate immediately before dissociation, and the resulting culture 5 days after replating cells. Scale bar indicates 30 μ m. **(C):** Staining of attached organoids and dissociated cells 5 days after plating reveals cell in both conditions to uniformly express both MAP2 and TUJ1. Scale bar indicates 30 μ m. **(D):** Top: Staining of putative forebrain neurons 4 weeks after plating from EBs shows all cells express SYT1 and TUJ1, and that both GABAergic and glutamatergic neurons are present. Bottom: Punctate staining is present for GABA α 1, GluR1, and SYT1. Scale bars indicate 30 μ m. Abbreviations: EB, embryoid body; FD, final differentiation; iPSC, induced pluripotent stem cell; NI, neural induction; NM, neuron maturation; NP, neural progenitor.

with an episomal CRISPR/CAS9 construct containing a gRNA targeting the gene *GRIN2B* and *RFP* marker gene, as well as episomal iPSC vectors containing a puromycin resistance gene (Fig. 5A). Electroporation was performed using identical electroporation parameters to iPSC transfection. After transfection, cells were plated for 24 hours, then sorted using FACS into RFP⁺ and RFP⁻ single cell fractions (Supporting Information Fig. 5). From 21 cell lines tested, we observed 1,900-2,100 RFP⁺ cells from 100,000 initial cells transfected. RFP⁺ cells were replated, puromycin

selection was applied, and colonies allowed to form, which were found to be uniformly RFP⁺ initially, but gradually became RFP⁻ as induction proceeded (Fig. 5B). Pure iPSC colonies were achieved in the same time and using the same methodology described above (Fig. 5A). From 21 cell lines, we observed 18 to 24 colonies form per cell line tested. Once pure colonies formed, DNA was extracted, and PCR performed to identify potential knockouts. We used this methodology to produce a heterozygote and knockout cellular model for *GRIN2B* (Fig. 5D), which

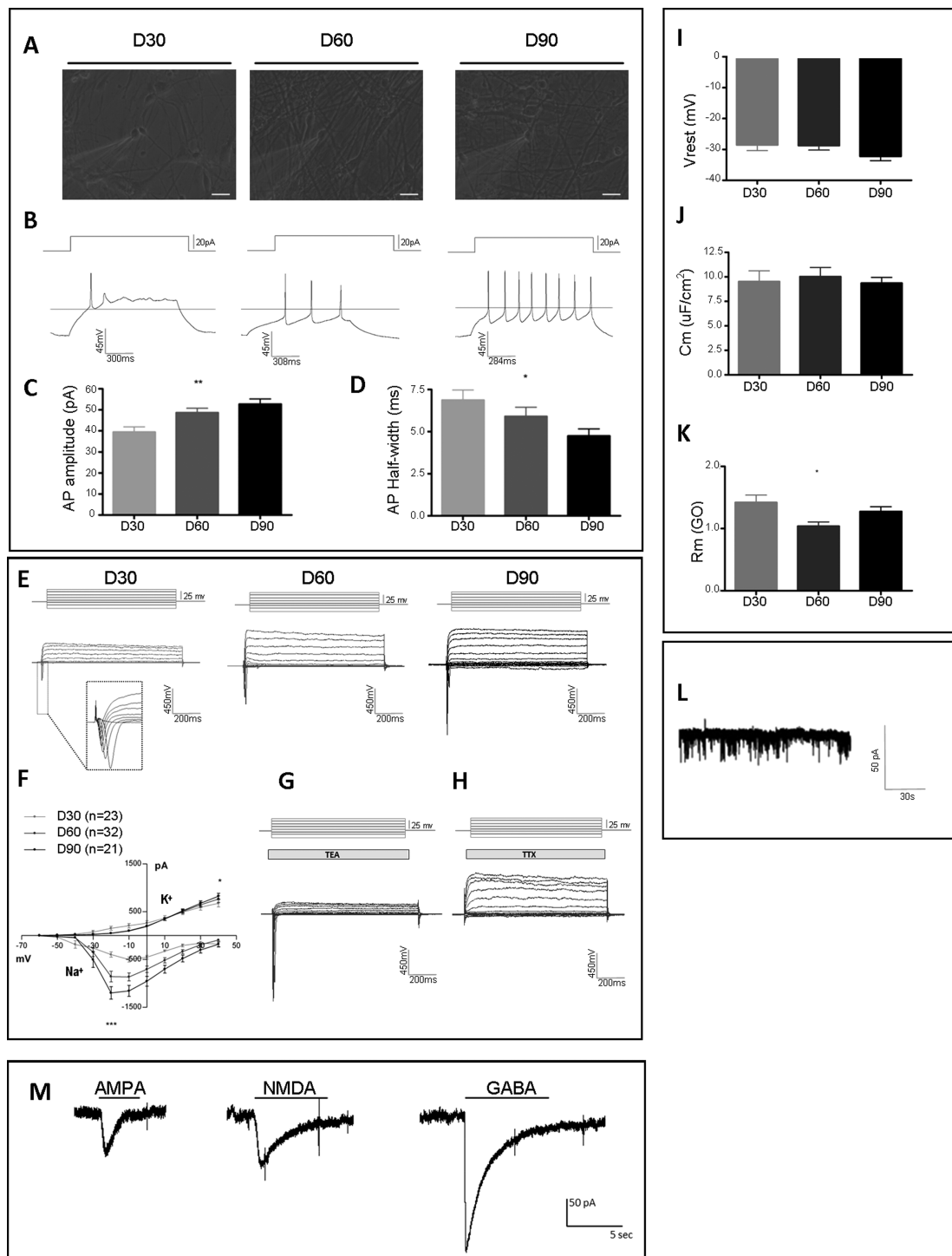


Figure 4. Electrophysiological characterization of forebrain neurons derived from induced pluripotent stem cells (iPSCs). **(A):** Sample phase images of forebrain neurons in culture at D30, D60, and D90 postdifferentiation. Scale bars indicate 25 μm . **(B):** Representative recordings of AP in current-clamp mode, induced by somatic current injection ($\Delta I = 20 \text{ pA}$, from membrane potential of -70 mV) from forebrain neurons at D30, D60, and D90 postdifferentiation. **(C, D):** AP amplitude and AP half-width measures in forebrain neurons over development. Stars denote statistical significance of change in AP parameters as a function of time spent differentiating cells ($* \leq 0.05$, $** \leq 0.01$). **(E):** Experimental voltage pulse-step protocol (top) and representative voltage-clamp recording traces (from a holding potential $V_{\text{hold}} = -60 \text{ mV}$) from forebrain neurons at D30 (including expanded view of Na currents [dashed boxes, insert]), D60, and D90, postdifferentiation. **(F):** Average Na and K currents recorded from iPSC-NPC1 at D30, D60, and D90 postdifferentiation, plotted as a function of step voltage amplitudes. Stars indicate significance of change in average currents as a function of time spent in differentiation ($* \leq 0.05$, $*** \leq 0.001$). **(G, H):** Representative voltage clamp traces of forebrain neurons at D90 postdifferentiating in the presence of the sodium channel block TTX and the potassium channel blocker TEA. **(I):** Membrane voltage at rest, determined immediately after establishing the whole-cell configuration, without current injection. **(J):** Membrane capacitance determined from the compensatory circuit in voltage-clamp. **(K):** Membrane resistance, while in the GOhm range, decreases during development. **(L):** Representative trace of miniature EPSCs from a forebrain neuron held at -60 mV . **(M):** Representative traces of macroscopic currents elicited by puffs of agonist-containing solution targeting AMPA receptors, NMDA receptors, and GABA_A receptors. Abbreviations: AMPA, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; AP, action potentials; NMDA, *N*-methyl-D-aspartate; GABA, γ -aminobutyric acid.

We suggest that this methodology is efficient in the sense that we required only one attempt to get the desired clones of interest. We have approximately a 2% success rate in Yamanaka vector transfection, and a 2% efficiency of colony formation. Gene editing does not appear to have any effect on transformation efficiency of cells.

Generation of Midbrain Cells

While forebrain cells offer many advantages in modeling many neurodevelopmental diseases, many NDDs would be better modeled in other types of neuronal cells. Therefore, in order to test the flexibility of our research platform, we examined how easily our protocols could be shifted to generate alternative varieties of neurons. Due to their critical role in many NDDs, we focused our efforts on generating midbrain cells. By altering a select few supplements in the NI stage of development based on previously described protocols [28–30], and employing a second round of organoid formation, in which selection was based on cell adherence, we were able to generate cellular cultures that were TUJ1+ and TH+ (Supporting Information Fig. 6B). Furthermore, staining of midbrain NPCs showed cells to be uniformly positive for the midbrain floorplate midbrain markers FOXA2, OTX2, and LMX1 (Supporting Information Fig. 6A). FACS of midbrain cells 4 weeks after the initiation of differentiation found 95.8% to be TUJ1+, with all TUJ1+ cells expressed TH, meaning that 95.8% of the total cellular population expressed key markers of midbrain neurons (Supporting Information Fig. 1). This is, to our knowledge, the highest proportion of TH+ neurons derived from iPSCs yet reported [30, 31]. These results indicate that this research platform is well suited to generating cellular midbrain models, and suggests that this research platform is flexible enough to be adapted to generate a wide variety of neuronal models.

Direct Conversion of Fibroblasts to Neurons

Speed and efficiency are critical for modeling rare NDDs and any technology that can decrease time spent without decreasing quality should be evaluated. In this context, we tested several parameters of a recently published method whereby skin cells could be directly converted to neurons [24] to determine if this would be a viable method for modeling rare NDDs (Supporting Information Fig. 7). If successful, this methodology would enable the generation of neuronal cells from fibroblasts without generating iPSCs, saving time and lowering costs. However, while we could observe neuronal conversion of fibroblasts early in the induction process of the protocol, we observed very high rates of cell death (even after changing multiple parameters—see Supporting Information). While this method may be appropriate for some uses, the high rate of cell death (>90%) makes it currently impractical for rare NDD research.

DISCUSSION

iPSCs have tremendous potential in modeling neurological disorders. However, the logistical and technical challenges of generating neurological models of disease from iPSCs present a significant barrier, particularly for rare NDDs. This research platform offers relatively fast and low complexity methodologies to generate neurological models of disease. Moreover, the induction of iPSCs can be combined with CRISPR/Cas9 genome editing with minimal increase to the length, cost, and complexity of the induction protocol.

In the described protocols, we utilized episomal vectors in our transfection to induce patient fibroblasts into iPSCs. Episomal vectors were utilized over other methods of iPSC induction, such as Sendai Virus reprogramming, due to its relatively high efficiency and inability to integrate into the host genome of transfected cells [22]. Utilizing a vector with puromycin selection enabled much faster induction of iPSCs, as the rate limiting step in the induction process is the generation of pure iPSC colonies [32]. Performing genome editing in fibroblasts combined with FACS allows for single-cell work, unlike iPSCs, which mostly require cell–cell contact to remain in a pluripotent state [33]. Having neurons derived from a single, edited fibroblast ensures genotypically homogenous cells—essential for establishing control cell lines.

This platform allows for the simultaneous integration of CRISPR/Cas9 genome editing and iPSC induction. CRISPR editing of iPSCs allows for the modeling of monogenic diseases even when patient tissue is unavailable, and gives the option to test potential genetic contributions to genetically complex diseases. In contrast to existing protocols that combine genome editing with iPSC-based models [34–36], our methodology does not extend the timeline of generating iPSCs, due to our dual transfection of episomal iPSC vectors and CRISPR/Cas9 construct, and efficient selection of double transfected cells.

To generate forebrain and midbrain neurons from iPSCs, we generated organoids, clusters of differentiating cells floating in suspension, before plating them as immature neurons. We utilized this direct approach out of several other protocols established in the literature, such as embedding iPSCs into supportive substrate and culturing on a spinning bioreactor [37], or differentiating cells plated as a monolayer [38], due to the diminished complexity of the procedure and increased homogeneity of the cells produced [25]. It seems plausible that the increased homogeneity of neuronal cells produced from an organoid as opposed to a monolayer may be due in part to the organoid providing a more analogous developmental environment for immature neuronal cells.

Recognizing the convenience that rapidly proliferating NPC populations provide in a variety of applications, such as high-throughput assays or collection of biological reagents [38], we have incorporated a simple addendum to the direct differentiation of iPSCs into forebrain neurons that enables the generation of NPCs as an intermediate step toward differentiated neurons.

Heterogeneity is a constant concern in models, and iPSC models in particular are noted for producing variable phenotypes depending on what protocol is followed [39]. We have found that differentiating cells in organoids gives superior homogeneity. Using the protocol described here, 94.8% of the cells in organoids plated after 4 weeks in culture were found to express mature forebrain neuronal markers. As mentioned above, we suspect this is due to the more consistent cell–cell contact that organoids offer cells. That the protocol is adaptable to successfully generate midbrain neurons with a similarly high yield by only altering a few key media compositions is an indicator of the flexibility of the platform for generating neural models of disease from different cell types.

CONCLUSION

iPSCs represent a powerful and demanding platform for modeling disease. With this platform, we hope to demonstrate how the logistical barrier to using iPSCs can be lowered and

encourage more widespread use of iPSCs, particularly in modeling rare NDDs.

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AUTHOR CONTRIBUTIONS

S.B. and C.E.: collection and assembly of data, data analysis and interpretation, manuscript writing; H.P., L.C., I.K., G.M., C.V., V.Y., and T.W.: collection and assembly of data, data analysis and interpretation.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicated no potential conflicts of interest.

REFERENCES

- Mitchell KJ. *The Genetics of Neurodevelopmental Disorders*. Hoboken, NJ: Wiley-Blackwell, 2015.
- Szpir M. New thinking on neurodevelopment. *Environ Health Perspect* 2006;114:A100–A107.
- Griggs RC, Batshaw M, Dunkle M et al. Clinical research for rare disease: Opportunities, challenges, and solutions. *Mol Genet Metab* 2009;96:20–26.
- Wong GK, Chiu AT. Gene therapy, gene targeting and induced pluripotent stem cells: Applications in monogenic disease treatment. *Biotechnol Adv* 2011;29:1–10.
- Kumari D, Swaroop M, Southall N et al. High-throughput screening to identify compounds that increase fragile X mental retardation protein expression in neural stem cells differentiated from fragile X syndrome patient-derived induced pluripotent stem cells. *STEM CELLS TRANSLATIONAL MEDICINE* 2015;4:800–808.
- Djuric U, Cheung AY, Zhang W et al. MECP2e1 isoform mutation affects the form and function of neurons derived from Rett syndrome patient iPSC cells. *Neurobiol Dis* 2015;76:37–45.
- Hamilton DR. Treatment of neurodevelopmental disorders: Targeting neurobiological mechanisms. *J Dev Behav Pediatr* 2015;36:425.
- Marchetto MC, Brennand KJ, Boyer LF et al. Induced pluripotent stem cells (iPSCs) and neurological disease modeling: Progress and promises. *Hum Mol Genet* 2011;20:R109–R115.
- Watabe K, Sakamoto T, Kawazoe Y et al. Tissue culture methods to study neurological disorders: Establishment of immortalized Schwann cells from murine disease models. *Neuropathology* 2003;23:68–78.
- Chesselet MF, Carmichael ST. Animal models of neurological disorders. *Neurotherapeutics* 2012;9:241–244.
- Kaiser T, Feng G. Modeling psychiatric disorders for developing effective treatments. *Nat Med* 2015;21:979–988.
- Marchetto MC, Winner B, Gage FH. Pluripotent stem cells in neurodegenerative and neurodevelopmental diseases. *Hum Mol Genet* 2010;19:R71–R76.
- Pappas SS, Leventhal DK, Albin RL et al. Mouse models of neurodevelopmental disease of the basal ganglia and associated circuits. *Curr Top Dev Biol* 2014;109:97–169.
- Albani S, Prakken B. The advancement of translational medicine—from regional challenges to global solutions. *Nat Med* 2009;15:1006–1009.
- Takahashi K, Tanabe K, Ohnuki M et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 2007;131:861–872.
- Liu J, Koscielska KA, Cao Z et al. Signaling defects in iPSC-derived fragile X premutation neurons. *Hum Mol Genet* 2012;21:3795–3805.
- Smith C, Gore A, Yan W et al. Whole-genome sequencing analysis reveals high specificity of CRISPR/Cas9 and TALEN-based genome editing in human iPSCs. *Cell Stem Cell* 2014;15:12–13.
- Smith C, Abalde-Atristain L, He C et al. Efficient and allele-specific genome editing of disease loci in human iPSCs. *Mol Ther* 2015;23:570–577.
- Marchetto MC, Carroue C, Acab A et al. A model for neural development and treatment of Rett syndrome using human induced pluripotent stem cells. *Cell* 2010;143:527–539.
- Zhou YY, Zeng F. Integration-free methods for generating induced pluripotent stem cells. *Genomics Proteomics Bioinformatics* 2013;11:284–287.
- Nityanandam A, Baldwin KK. Advances in reprogramming-based study of neurologic disorders. *Stem Cells Dev* 2015;24:1265–1283.
- Deng XY, Wang H, Wang T et al. Non-viral methods for generating integration-free, induced pluripotent stem cells. *Curr Stem Cell Res Ther* 2015;10:153–158.
- Bishop DV. Which neurodevelopmental disorders get researched and why? *PLoS One* 2010;5:e15112.
- Hu W, Qiu B, Guan W et al. Direct conversion of normal and Alzheimer's disease human fibroblasts into neuronal cells by small molecules. *Cell Stem Cell* 2015;17:204–212.
- Pasca AM, Sloan SA, Clarke LE et al. Functional cortical neurons and astrocytes from human pluripotent stem cells in 3D culture. *Nat Methods* 2015;12:671–678.
- Wang P, Lin M, Pedrosa E et al. CRISPR/Cas9-mediated heterozygous knockout of the autism gene CHD8 and characterization of its transcriptional networks in neurodevelopment. *Mol Autism* 2015;6:55.
- Talkowski ME, Rosenfeld JA, Blumenthal I et al. Sequencing chromosomal abnormalities reveals neurodevelopmental loci that confer risk across diagnostic boundaries. *Cell* 2012;149:525–537.
- Kriks S, Shim J-W, Piao J et al. Dopamine neurons derived from human ES cells efficiently engraft in animal models of Parkinson's disease. *Nature* 2011;480:547–551.
- Boyer LF, Campbell B, Larkin S et al. Dopaminergic differentiation of human pluripotent cells. *Curr Protoc Stem Cell Biol* 2012:1H. 6.1–1H. 6.11.
- Hartfield EM, Yamasaki-Mann M, Fernandes HJR et al. Physiological characterization of human iPSC-derived dopaminergic neurons. *PLoS One* 2014;9:e87388.
- Hallett PJ, Deleidi M, Astradsson A et al. Successful function of autologous iPSC-derived dopamine neurons following transplantation in a non-human primate model of Parkinson's disease. *Cell Stem Cell* 2015;16:269–274.
- Beers J, Gulbranson DR, George N et al. Passaging and colony expansion of human pluripotent stem cells by enzyme-free dissociation in chemically defined culture conditions. *Nat Protoc* 2012;7:2029–2040.
- Stadtfeld M, Hochedlinger K. Induced pluripotency: History, mechanisms, and applications. *Genes Dev* 2010;24:2239–2263.
- Song B, Fan Y, He W et al. Improved hematopoietic differentiation efficiency of gene-corrected beta-thalassemia induced pluripotent stem cells by CRISPR/Cas9 system. *Stem Cells Dev* 2014;24:1053–1065.
- Xie F, Ye L, Chang JC et al. Seamless gene correction of β -thalassemia mutations in patient-specific iPSCs using CRISPR/Cas9 and piggyBac. *Genome Res* 2014;24:1526–1533.
- Li HL, Fujimoto N, Sasakawa N et al. Precise correction of the dystrophin gene in duchenne muscular dystrophy patient induced pluripotent stem cells by TALEN and CRISPR-Cas9. *Stem Cell Rep* 2015;4:143–154.
- Lancaster MA, Renner M, Martin CA et al. Cerebral organoids model human brain development and microcephaly. *Nature* 2013;501:373–379.
- Pasca SP, Panagiotakos G, Dolmetsch RE. Generating human neurons in vitro and using them to understand neuropsychiatric disease. *Annu Rev Neurosci* 2014;37:479–501.
- Brennand KJ, Marchetto MC, Benvenisty N et al. Creating patient-specific neural cells for the in vitro study of brain disorders. *Stem Cell Rep* 2015;5:933–945.



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