Characterization of Human iPSC-derived Induced Excitatory Neurons for Disease Modeling and Drug Discovery

Ouissame Filali, Robert Bradley, Junyi Ma, Ali Fathi, Rebecca Fiene, Clifford Hogan, Coby Carlson, Simon Hilcove, Scott Schachtele, and Jing Liu

FUJIFILM Cellular Dynamics, Inc., Madison, WI USA

FUJIFILM Cellular Dynamics

Abstract

Human induced pluripotent stem cell (iPSC)-derived neurons are integral for elucidating mechanisms and therapeutic targets underlying neurodevelopmental and neurodegenerative disease. Directed differentiation protocols have largely been employed for generating iPSC-derived neural models, because of the ability to generate specific cortical glutamatergic neurons for use in human drug discovery and screening. While these cells are highly predictive for drug screening, directed differentiation methods are challenged to meet the economies of scale required for high-throughput compound testing within preclinical phases of the drug development pipeline. Induction of neural differentiation using NGN2 forward reprogramming of iPSCs offers a robust method for generating scalable quantities of excitatory neurons with low lot-to-lot variability. In this study we utilize NGN2 overexpression, under a doxycycline (DOX) promoter, to forward program highly pure excitatory glutamatergic neurons from iPSC lines with an apparently healthy normal (AHN) background or heterozygous pathogenic R493X nonsense mutation in the granulin gene (GRN) to model frontotemporal dementia (FTD). We first demonstrate that induced excitatory AHN and GRN (R493X) neurons display gene expression profiles similar to published NGN2 protocols. We further characterized these cells, showing the presence of highly pure neuronal populations (> 90% TUJ1-positive) that can be recovered from cryopreservation to form consistent neural cultures without continued use of DOX. When co-cultured with isogenic iPSC-derived astrocytes, both AHN and GRN (493X) induced excitatory neurons produced robust neural networks on multielectrode array platforms. Last, induced excitatory neurons were co-cultured with iPSC-derived astrocytes on ultra-low adhesion 96-well round-bottom plates to form neurospheres, which developed dynamic calcium oscillations as early as 14 days in culture. These data demonstrate a robust and scalable method using NGN2 forward programming to consistently produce fully differentiated induced excitatory neurons. These cells function across a variety of applications that support high-throughput preclinical drug discovery and compound screening.

Development and Characterization of iPSC-derived Induced Neurons

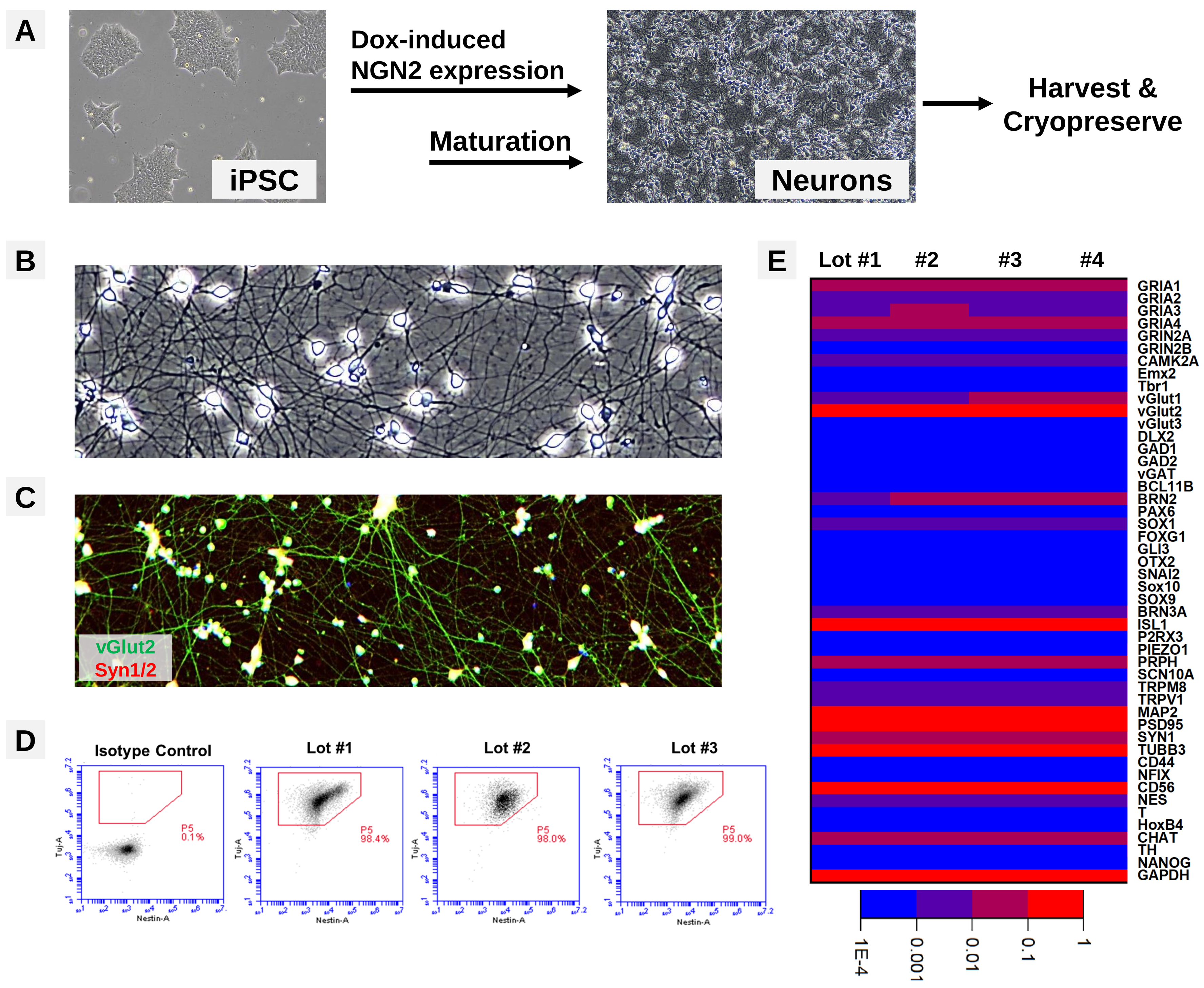
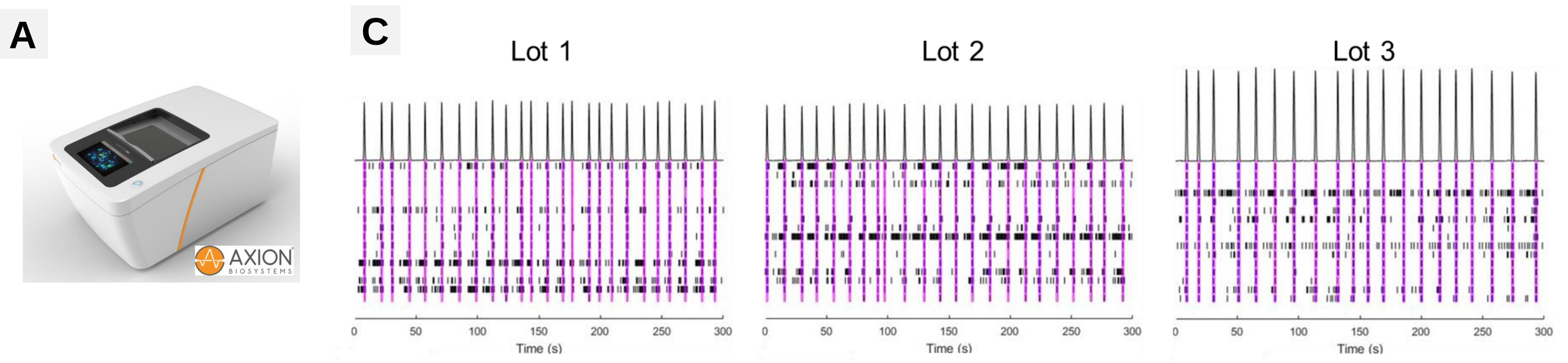


Figure 1. Development of iCell® Induced Excitatory Neurons. (A) Human iPSC lines were genetically engineered to contain a doxycycline (Dox)-inducible NGN2 construct at the AAVS1 safe harbor site. Our NGN2-iPSC differentiation protocol was optimized for highly consistent and reproducible neuronal cultures, the ability to cryopreserve the cells, and a product that yields functionally mature excitatory neurons upon thaw and reanimation. (B) Brightfield image of the cells on Day 3 post-thaw shows extensive neurite outgrowth and no visible impurities. (C) Immunostaining for vGlut2 and Synapsin 1/2 on Day 7 post-thaw highlights glutamatergic synapses containing VGLUT2 transporters. (D) Flow cytometry marker analysis (β3-Tubulin vs. Nestin) demonstrates that cells are a highly purified (>98%) TUJ1-positive population of neurons. (E) Post-thaw gene expression analysis across 4 lots of iCell Induced Excitatory Neurons reveals a primarily excitatory glutamatergic neuronal population and a high degree of lot-to-lot consistency. Worth noting that increased expression of peripheral neuron markers (PRPH, P2RX3) is observed, as in published results from NGN2-induced neurons (Lin, H. C. et. al. (2021). https://doi.org/10.1016/j.stemcr.2021.07.006).

Functional Characterization of Induced Excitatory Neurons on MEA



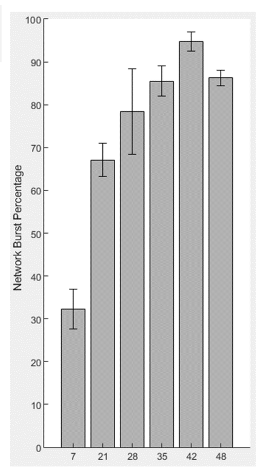


Figure 2. iCell Induced Excitatory Neurons are highly functional on MEA. We evaluated electrophysiological properties of these electrically active iPSC-derived induced neurons across various platform technologies, including multi-electrode array (MEA). (A) Using the Maestro Pro MEA system, we developed a neuron MEA assay that features co-culture with iCell Astrocytes 2.0 in 48-well MEA plates. (B) Cells develop synchronous network bursting activity over time as indicated by an increase in Network Burst Percentage over 7 weeks in culture. (C) Raster plots of MEA recordings at Day 28 post-thaw (typical peak time) show how consistent the neural network activity is across 3 different manufacturing lots of iCell Induced Excitatory Neurons.

Analysis of Induced Excitatory Neurons on High-density MEA

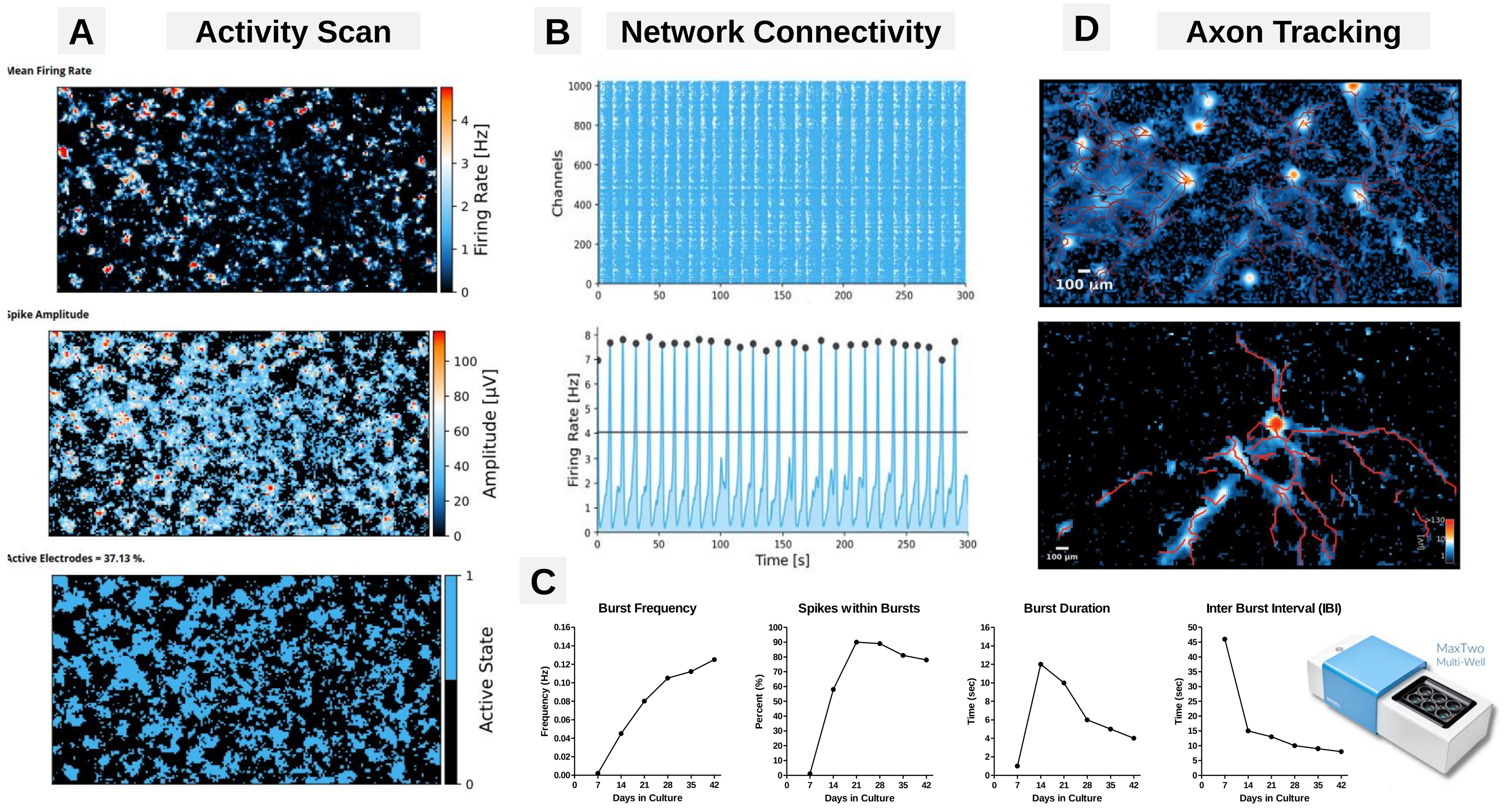


Figure 3. HD-MEA analysis of iCell Induced Excitatory Neurons. Neurons were plated together with iCell Astrocytes 2.0 on MaxTwo 24-well plates and co-cultured continuously. (A) The Activity Scan Assay was used to acquire a whole-sample electrical image to assess electrical activity of the neurons, with outputs such as mean firing rate (Hz), spike amplitude (µV), and active area. (B) Network dynamics and development were observed over time with the Network Connectivity Assay. The raster plot and binned network activity plot was generated from cells on Day 28 post-thaw. (C) Neuronal bursting features such as Burst Frequency, Spikes within Bursts, Burst Duration, and IBI were extracted and plotted against Days in Culture, illustrating robust synchronous network development. (D) Finally, the Axon Tracking Assay was used to identify individual axonal branches from iCell Induced Excitatory Neurons and quantify metrics like neuron conduction velocity, longest branch length, and amplitude at initiation site (data not shown here). All MEA recordings were performed on a MaxTwo HD-MEA system and co-culture data was recorded, processed, and analyzed with MaxLab Live Software.

Calcium Waveform Oscillations from Induced Neuron 3D Neurospheres

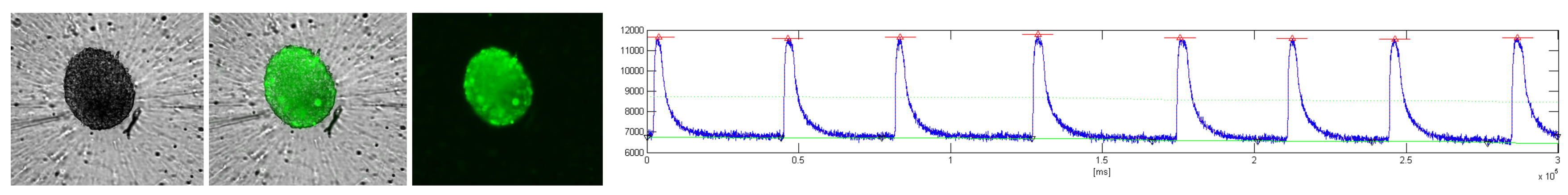


Figure 4. Formation and testing of 3D neurospheres. iCell Induced Excitatory Neurons (20K cells) can be mixed with iCell Astrocytes 2.0 (5K cells) in defined ratios (4:1) in PrimeSurface® 3D Culture Spheroid ULA plates to form functional neurospheres. After 3 weeks of culture in complete BrainPhys medium, neurospheres were ready for calcium assay on the FDSS/µCell instrument. Baseline Ca^{2+} waveform oscillations were recorded for 10 minutes. Factors such as culture media, calcium dye, ratio of neurons to astrocytes, total number of cells, and number of days post-thaw all contributed significantly to the observed phenotype (i.e., peak count, amplitude, and width).

Disease Modeling with Induced Neurons: GRN R493X HZ KO

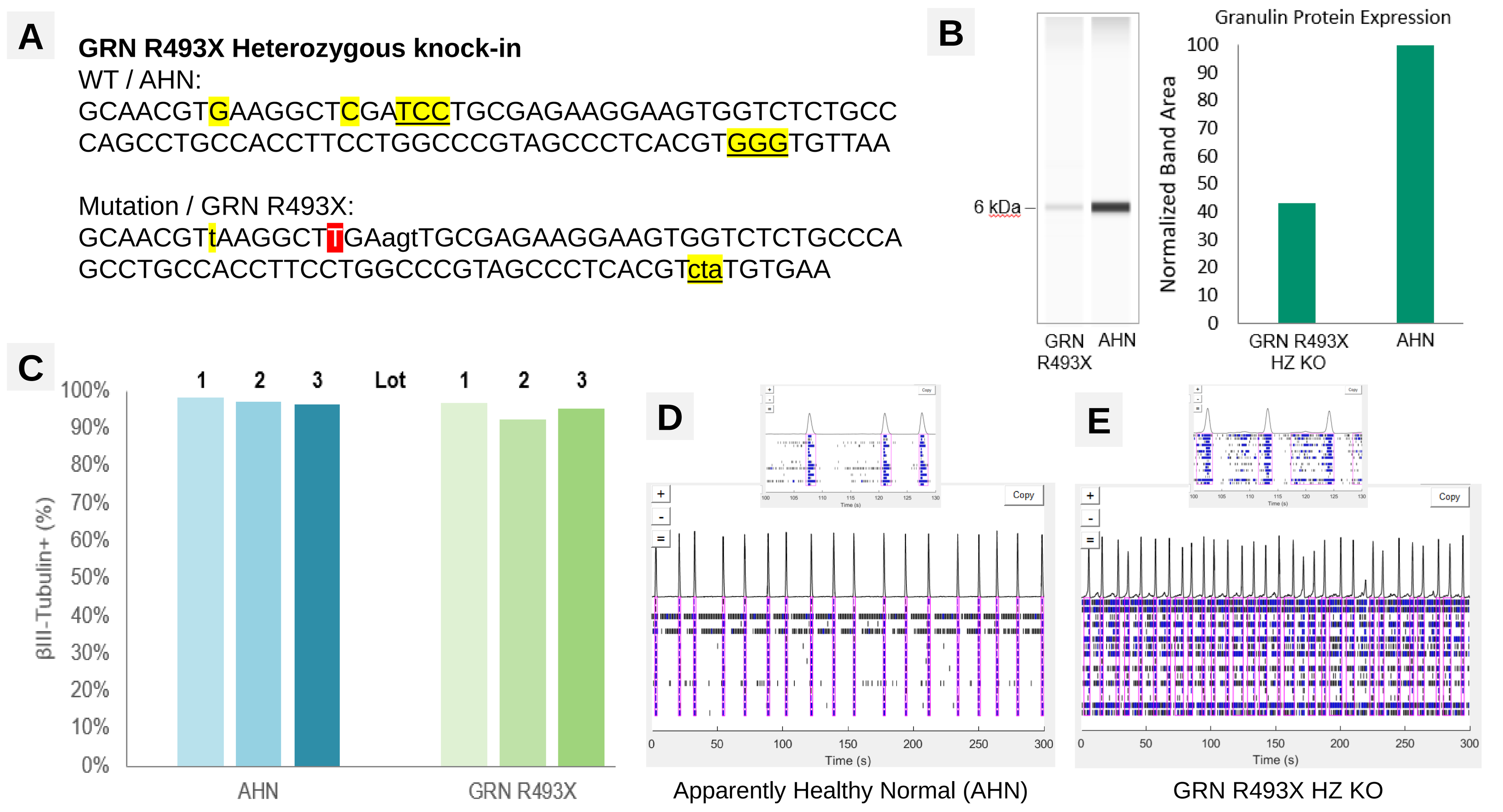
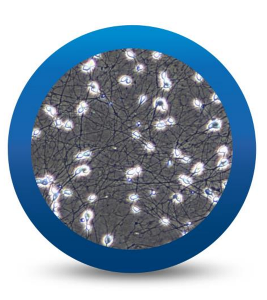


Figure 5. iCell Induced Excitatory Neurons enable rapid generation of disease models. (A) iPSCs were engineered using CRISPR-Cas9 technology to contain a heterozygous mutation in the granulin (GRN) gene resulting in a R493X nonsense mutation replicating a relatively severe form of frontal-temporal lobe dementia (FTD). (B) Cells were analyzed by Western blot for granulin protein expression, and the cleaved GRN fragments of ~6 kDa were much more lightly expressed in the HZ GRN neurons as compared to AHN control. (C) Manufacturing analysis of 3 different lots of iCell Induced Excitatory Neurons from the study showed high purity of both cell types (>90%). (D) Finally, baseline MEA analysis of AHN vs. GRN neurons cultured with iCell Astrocytes 2.0 on the Maestro Pro MEA system. Recordings at 21 days post-thaw revealed unique baseline architecture of network bursting events. MEA recording (300 sec) illustrate the robustness of these cell lines to produce consistent electrical activity. Observed differences in burst firing rates and synchronous activity between healthy and diseased iCell Induced Excitatory Neurons suggest an aberrant phenotype in the GRN R493X cell line.

Summary



These data demonstrate a highly consistent and easy-to-use induced ("forward programmed") neuron that allows for high-throughput analysis of toxicity and neuronal function. iCell Induced Excitatory Neurons will function without additional differentiation by the user. We also show these cells can be combined with CRISPR engineering to create robust disease models within glutamatergic neurons.