USE OF iPSC-DERIVED HUMAN MOTOR NEURONS IN HIGH-THROUGHPUT PHENOTYPIC SCREENING

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Introduction

There exists a dire need for more effective drugs to treat disorders of the central nervous system (CNS), and new cell culture models that are more relevant to psychiatric and neurological diseases are needed to improve the success rate of drug development programs. One such approach is the use of neurons differentiated from patient induced pluripotent stem cells (iPSCs), which present new opportunities for modeling disease processes and screening drug libraries. We have developed technology to produce very large quantities of highly enriched human neurons from patient iPSCs and to greatly accelerate their maturation. We subsequently developed two high-throughput screening (HTS) systems using human motor neurons derived from individuals with amyotrophic lateral sclerosis (ALS) and spinal muscular atrophy (SMA). Using genome editing techniques on the iPSC lines, the reporter nanoluciferase (Nluc) was fused to endogenous neurofilament light chain (NFL) for ALS and full-length SMN2 for SMA. Nanoluciferase is more sensitive than the traditional luciferase and allows for a simple and quantitative readout of NFL and SMN2 protein levels. The assays were adapted to meet HTS requirements, including: large batch sizes, 1536-well format, minimal well-to-well variation, short-term culture, plating by automated dispenser, and low reagent volumes. Applying a quantitative HTS approach, we screened the LOPAC, NPC, and MIPE libraries (>6,000 compounds) in a dose dependent manner on both motor neuron lines with a hit rate of ~0.5%. This work demonstrates the feasibility of running HTS campaigns using human neurons, which present a more physiologically relevant drug discovery platform.

Materials and Methods

Establishment of Reporter iPSC Lines Human iPSCs lines, WC-30 (wildtype), ALS-SOD1-D90A, and SMA-232 were applied in this study. All iPSCs were cultured on irradiated mouse embryonic fibroblasts (MEFs) as described in the standard protocol (www.wicell.org). We applied CRISPR technology to integrate reporter Nluc or GFP into the disease related gene, neurofilament-light chain (NFL) for ALS. CRISPR guide RNA pairs, Cas9-Nickase and Donor plasmid were introduced into iPSCs by electroporation. Neomycin was added in the culture medium to select the resistant cells. The neomycin-resistant iPSC colonies were picked and screened by PCR to detect the integration of the reporter. The PCR products were sequenced to confirm the correct in-frame fusion. In the donor plasmid, we flanked the neomycin resistant cassette with two loxP sites; therefore, it can be easily removed by Cre recombination. All the reporter iPSC lines were confirmed without mutation in SMN2 or NEFL gene, and off-target sites.

Neuron Differentiation from Human iPSCs Motor Neuron differentiation from human iPSCs based on protocols described previously (Du et al. 2015. Nat Commun. 6:6626). Briefly, human iPSCs were treated with small molecules for 1 week to induce neuroepithelial progenitors (NEPs). The NEPs were split and treated in addition patterning molecules for another 1 week to generate subtype-specific neuron progenitors. These progenitors were expanded with the combination of small molecules and frozen in cell freezing medium. To accelerate maturation after thawing and seeding, neurons were culture in medium supplemented with BrainXell Seeding Supplement for another 1-2 weeks.

Mutlielectrode Array (MEA) Analysis Neurons were seeded at 40,000 cells per electrode area (16 electrodes in the center of each well) in specialized 48-well MEA plates (Axion BioSystems). Plates were previously coated with ploy-D-lysine (PDL). From Day 7 to 12, activity was recorded for five minutes from all wells using a Maestro recording chamber (Axion BioSystems).

Quantitative HTS Screening MNPs were thawed and plated with a liquid handling system (MultiDrop) at 1,200-1,500 cells/well in 4 µL into all-white uncoated 1536-well plates. On Day 1 (24 hours after thawing and plating), compounds in the LOPAC, NPC-A/B, and MIPE libraries were added by pin tool. On Day 3 (for SMN2-Nluc) or Day 4 (for NFL-Nluc), Nluc activity was detected using the Nano-Glo Luciferase Assay kit (Promega). Luminescence signal was measured with a ViewLux system (PerkinElmer). Hits were confirmed using identical conditions in a 96-well format.

Use of Human Motor Neurons in Quantitative HTS

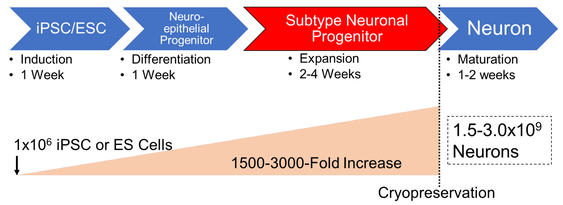


Figure 1. General Protocol for Neuron Production Scheme showing the general production protocol. The time from initiation of iPSC/ESC culture until cryopreservation is 4-6 weeks. Neurons mature 1-2 weeks after plating with BrainXell maturation supplements.

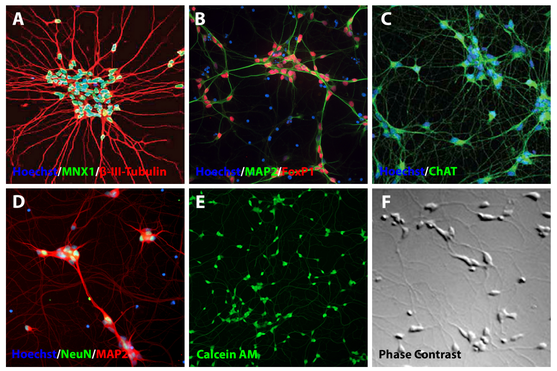


Figure 2. Expression of Motor Neuron Markers *(A-C)* Neurons express markers associated with spinal motor neuron identity, including MNX1, FoxP1, and ChAT. *(D)* Approximately 60-65% of motor neurons are positive for the mature neuronal marker NeuN. *(E-F)* Extensive neurite outgrowth, which begins within a day of plating, is shown by calcein AM staining and phase microscopy.

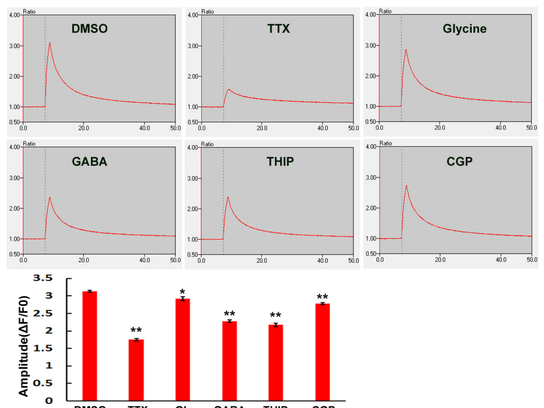


Figure 3. Functional Activity: Calcium Influx Calcium changes after electrical stimulation at Day 10 in the presence of DMSO (no compound), TTX (sodium channel blocker), glycine, GABA, THIP (selective GABA-A receptor agonist) and CGP64626 (selective GABA-B antagonist). Bottom bar graph shows a comparison of response amplitude for all conditions.

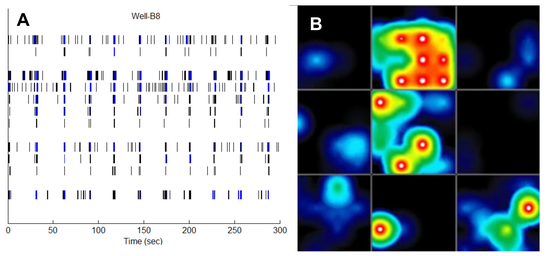


Figure 4. Functional Activity: MEA As measured by multielectrode array (MEA) on Day 12, neurons display robust spontaneous activity, including spikes, bursts, and synchronous network activity. *(A)* Activity at all electrodes in one well over five minutes. *(B)* Single time-point activity map for all wells.

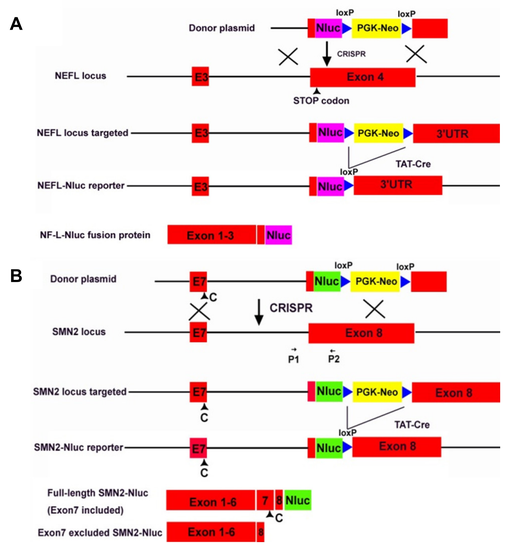


Figure 5. Reporter Construction Schematics showing the strategy to establish *(A)* NFL-Nluc and *(B)* SMN2-Nluc reporter lines by CRISPR. Each reporter is a single copy expressed at the endogenous level.

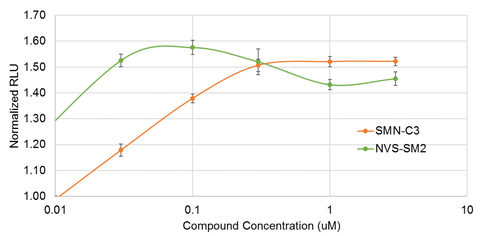
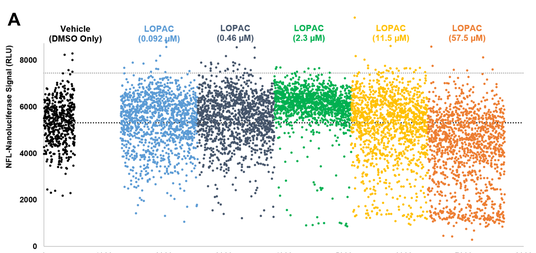
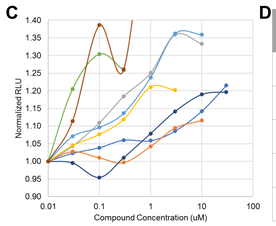


Figure 6. Validation with Positive Controls The SMN2-Nluc reporter system responds to two compounds, SMN-C3 and NVS-SM2, in development to treat SMA. Both compounds have a maximum efficacy of 50-60% although differ by more than ten-fold in potency. The assay has a Z'=0.24 for 96-well format. Note that no positive controls were available for the NFL-Nluc reporter system.





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Library	1 µM SMN-C3 %↑	%CV (DMSO)	%CV (SMN-C3)
LOPAC	24.1%	7.4%	8.2%
NPC-A	36.6%	5.1%	6.0%
NPC-B	38.4%	7.7%	5.7%
MIPE	36.7%	11.6%	11.6%
Mean:	***37.0%***	***8.1%***	***7.8%***

Figure 7. Example Screening Data Screening campaigns were run with motor neurons containing both reporter systems using the LOPAC, NPC-A/B, and MIPE libraries. (*A*) Example screening data with LOPAC for NFL-Nluc. (*B*) Example screening data with NPC-A for SMN2-Nluc. (*C*) Dose-response curves showing increased NFL-Nluc signal from eight confirmed hits. (*D*) Table of SMN2-Nluc signal increases from positive control SMN-C3 and %CVs.

Conclusions

Summary

Although the window size for both systems is relatively small, because of a very low false-positive rate, we were able to uncover several compounds that increase NFL-Nluc signal, and one of these is currently undergoing additional studies. Although no compounds with activity similar to the positive control were discovered for the SMN2-Nluc system, we are currently screening the NCATS Genesis library (~100,000 compounds) for new hits. *Overall, our results demonstrate the feasibility of using iPSC-derived human neurons to conduct HTS campaigns on a platform more relevant to diseases of the human CNS.*

Acknowledgement

Support from NINDS/NIH Grant 1R43NS097080-01A1.

Contact

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