ALS Drug Discovery via High-Throughput Phenotypic Screening Using iPSC-Derived Human Motor Neurons

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Introduction

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease primarily affecting motor neurons. Unfortunately, there are only two drugs approved to treat the condition, neither of which increases patient survival by more than a few months. This sobering reality highlights the urgent need for new ALS therapeutic development, which has been plagued by high failure rate of drug candidates during clinical trials. This high failure rate suggests that pre-clinical screening strategies need to be re-evaluated. One of the markers of disease in ALS patients is the aberrantly low expression of neurofilament light chain (NFL) in motor neurons. Further, recovery of NFL to normal levels prevents hallmark phenotypic changes in ALS neurons. Therefore, we wanted to establish a clinically relevant screening platform to identify compounds that return expression of NFL to normal levels in ALS patient derived motor neurons. At BrainXell, we established new technologies to rapidly differentiate ALS patient induced pluripotent stem cells (iPSCs) into large quantities of neurons. We then used genome editing techniques to endogenously fuse NFL with a nanoluciferase (NLuc) reporter, thus enabling a high-throughput screening (HTS) system that monitors the expression levels of NFL after 72 h exposure to each compound. The assay was 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. Compounds that increase NFL expression by >30% (to approximately normal levels) were considered hits. From these screens we identified 80 hit compounds that are currently going through secondary validation. Preliminary data look promising. For example, two of these hits restore normal expression of NFL with no observed toxicity.

ALS Patient iPSC-Derived Motor Neurons

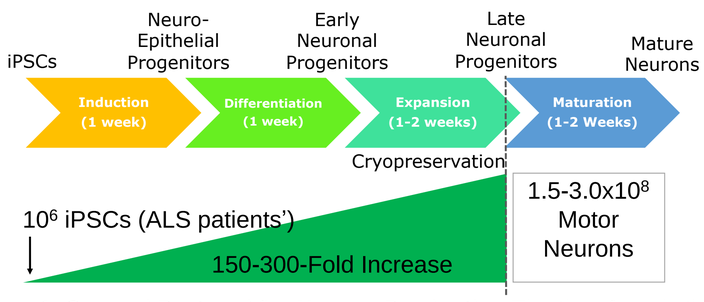


Figure 1. General Protocol for Neuron Production The time from initiation of iPSC/ESC culture until cryopreservation is 4-6 weeks. Neurons mature 1-2 weeks after plating with BrainXell maturation supplements.

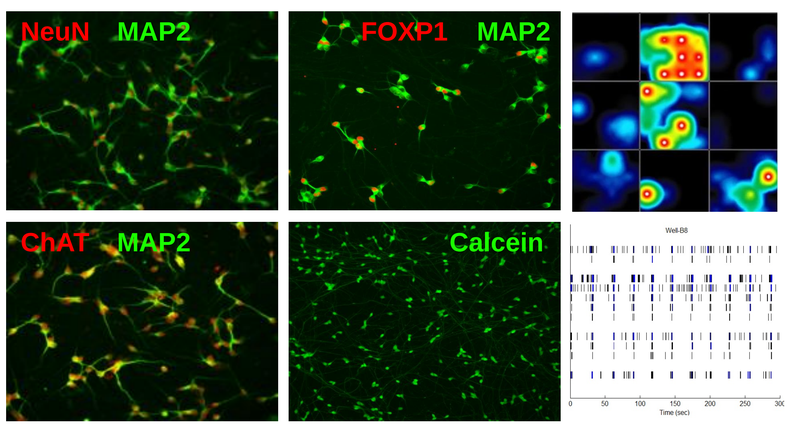


Figure 2. ALS patient iPSC-derived Motor Neuron Characterization *(A-D)* Neurons express markers associated with spinal motor neuron identity, including MAP2 (>90%), NeuN (>90%), FoxP1 (70%), and ChAT (85%). (E-F) By day 12 neurons display robust spontaneous activity, including spikes, bursts, and synchronous network activity as measured by multielectrode array (MEA).

Materials and Methods

Establishment of Reporter iPSC Lines Human iPSCs lines, WC-30 (wildtype) and ALS-SOD1-D90A 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 neurofilament-light chain (NFL). 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 was easily removed by Cre recombination. All the reporter iPSC lines were confirmed without mutation in NEFL gene or off-target sites.

Neuron Differentiation from Human iPSCs Motor Neuron differentiation from human iPSCs was 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 additional 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 cultured in medium supplemented with BrainXell Seeding Supplement for another 1-2 weeks.

Multielectrode 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 poly-D-lysine (PDL). From maturation 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 (23 nL). On Day 4 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.

Hypothesis

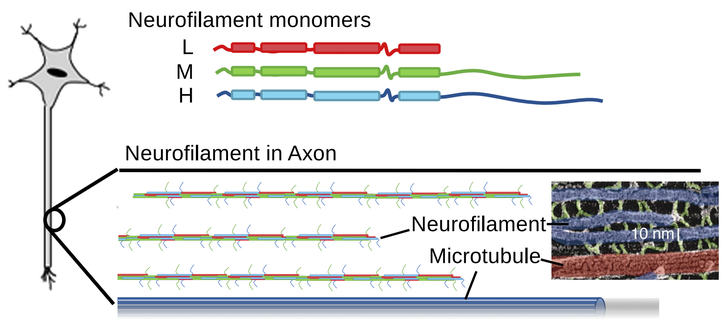
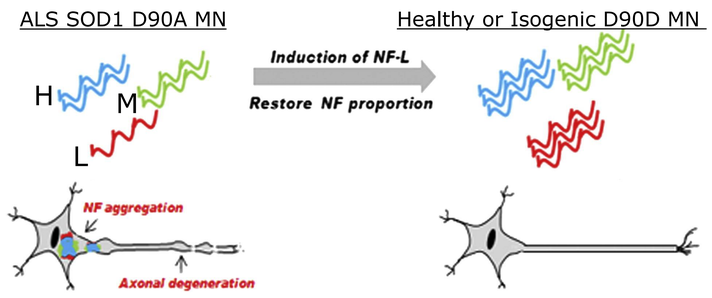


Figure 4. Neurofilaments in Motor Neurons Cartoon representation of Light (L), Medium (M) and Heavy (H) neurofilament monomers. Neurofilaments helps maintain axon width, electrophysiology, and integrity.



Hypothesis: Small molecule induction of NF-L expression will restore NF proportion and recover ALS disease phenotype. *(Demonstrated in ref.[1] and in SOD1 mouse model in ref. [2])*

NFL-NLuc Reporter

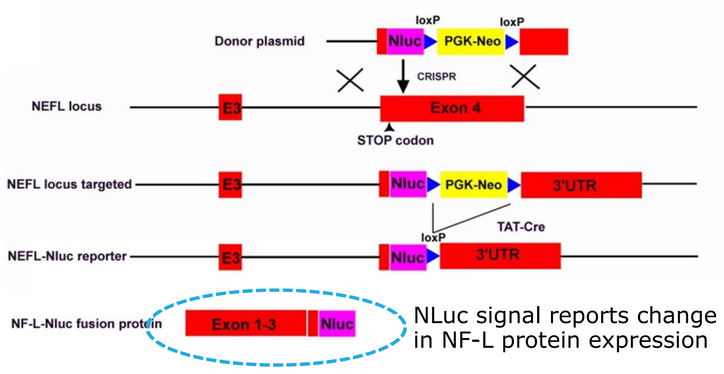


Figure 5. Reporter Construction Schematics showing the strategy to establish NFL-NLuc reporter lines by CRISPR as a single copy at the endogenous locus.

Quantitative HTS (qHTS) Assay

Table 1. Assay Optimization

Parameter	Range tested	Condition chosen
Cell Density	9,000-75,000 MNs/cm^2	30,000 MNs/cm^2
Plate coating	PEI, PDL, Matrigel	Matrigel or PDL + matrigel
Assay duration	4-20 days	4 days for qHTS 10 days rescreen
Interventions	0-3 medium changes	0 medium changes
Nano-Glo	2-10X dilution	2X dilution

Table 1. Assay was optimized to decrease %CV, optimize signal intensity, and simplify handling.

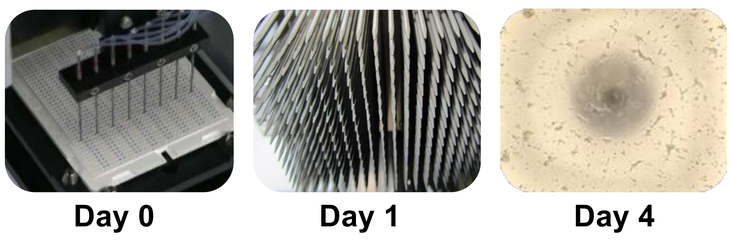
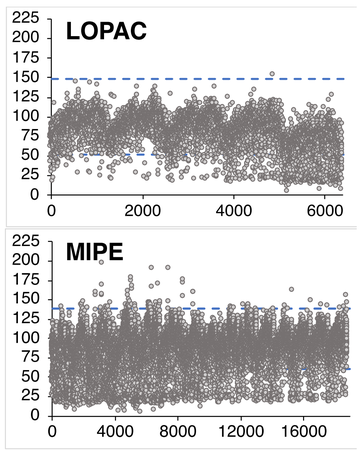


Figure 6. Final protocol for HTS in 1536-well plates. Simple three-step protocol: 1) Plate neurons in medium containing BrainFast maturation supplement, 2) Pin-transfer compound library, 3) Dispense NanoGlo reagent for analysis.



Library	Library Size	Threshold (% increase)	Hits
LOPAC	1280	40%	21
NPC-A	1408	30%	22
NPC-B	1408	30%	10
MIPE	1920	30%	18

Figure 7. qHTS Screening Data Summary Screening campaigns were run with NF-L-NLuc motor neurons using the LOPAC, NPC-A/B, and MIPE libraries. Luminescence in each well after 72 h treatment (grey dots). Also shown is the mean ± 2 standard deviations of DMSO control (dashed lines). Table summary of library screen and number of putative hits identified.

Hit Verification and Validation

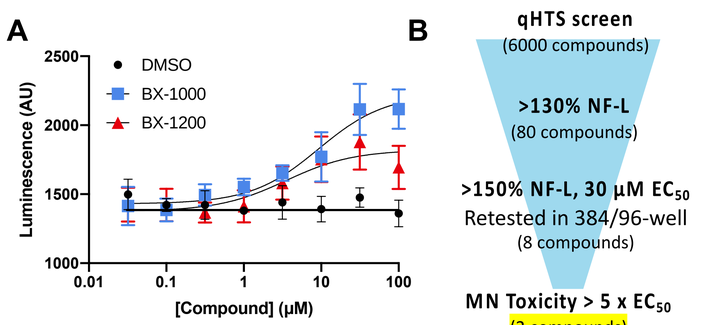


Figure 8. Hits from qHTS were verified in-house. We retested the 80 hit compounds that met qHTS threshold criteria. From rescreen, 2 compounds are considered true hits.

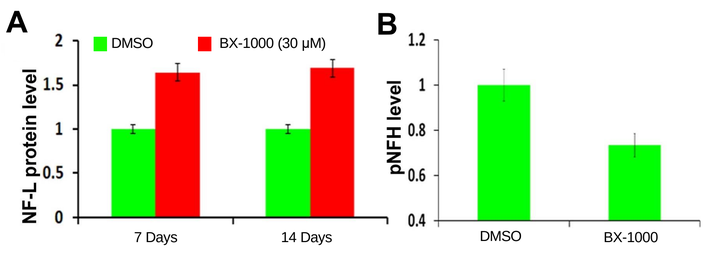


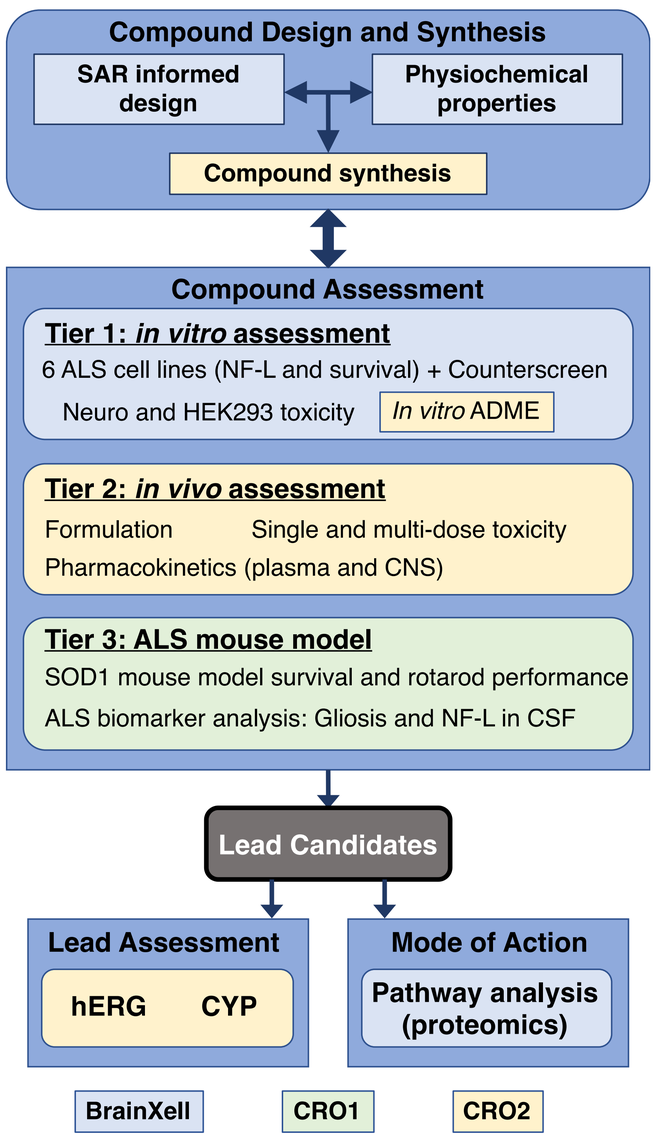
Figure 9. ELISA Validation of Hit Compound. (A) Increase in NF-L expression was validated by ELISA in a non-reporter SOD1 ALS cell line. NF-L increase persisted up to 2 weeks after treatment. (B) Concurrent decrease in pNF-H indicates less NF-H is accumulating in MN soma.

Next Steps

Ongoing SAR-by-catalog

Tested 86 compounds structurally similar to BX-1000

- **10 were active** in NF-L-NLuc assay
- **2** increased potency >2-fold
- **0** increased efficacy



Future Directions

Increase hit number and diversity

- Screen 95,000 compound Genesis library (NCATS)

Optimize hit

- Continue SAR (catalog and custom synthesis
- Test activity of other compounds that hit same target

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References:

1. Chen, H., et al. (2014). Cell Stem Cell **14**(6): 796-809.
2. Kong, J. and Z. Xu (2000). Neuroscience Letters **281**(1): 72-74.