Use of Human Neurons as Model Systems for Neurotoxin Countermeasures and Prophylaxes

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NIH National Center for Advancing Translational Sciences

Military Application

Chemical nerve agents have re-emerged as a battlefield threat to America's warfighters and even as possible weapons of terrorism. BrainXell's technology can provide a system to efficiently and accurately assess potential countermeasures or prophylactic approaches to protect our warfighters before or after exposure.

Introduction

BrainXell's innovative human neuron culture platforms provide a means to model the human brain in a dish. These neurons, which are differentiated from induced pluripotent stem cells (iPSCs), provide a unique opportunity to experiment on the human nervous system without involvement of human subjects. We have developed technology to produce very large quantities of highly enriched neurons from normal or gene-edited iPSCs. Complimentary techniques also allow us to greatly accelerate their maturation such that many experiments can be conducted within one week of cell thawing and plating. These model systems can be used for a variety of purposes, with both civilian and military applications. Importantly, they can be used to assess the how well proposed countermeasures or prophylactic strategies can provide protection from chemical agents, especially neurotoxins. For example, in collaboration with the NIH, we have developed a system to screen chemical libraries for toxicity affecting acetylcholinesterase, which is the enzyme targeted by sarin and VX nerve agents. Our experience using a similar platform for drug screening for neurological and psychiatric disorders reinforces the feasibility of this platform. Nearly identical approaches can be utilized to model neurotoxin exposure and determine the effectiveness of proposed countermeasures and prophylaxes.

Materials and Methods

Establishment of eGFP Reporter iPSC Line The human iPSCs line WC-30 (normal wildtype) was used in this study. iPSCs were cultured on irradiated mouse embryonic fibroblasts (MEFs) as described in the standard protocol (www.wicell.org). We applied CRISPR technology to integrate the CAG-eGFP reporter into the AAVS1 safe harbor site. CRISPR guide RNA pairs, Cas9-nickase, and the 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 imaging to detect the integration of the reporter.

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 one week to induce their fate to neuroepithelial progenitors (NEPs). The NEPs were split and treated with additional patterning molecules for another week to generate subtype-specific neuron progenitors. These progenitors were expanded with a 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.

Culture Conditions for High-Content Imaging Thawed motor neurons were seeded on 384-well plates coated with PDL. Neurons were cultured for two to five days before exposure to the toxin rotenone, an insecticide. Rotenone was applied from 0.02-40 µM with serial dilutions of 2-fold. Exposure time before imaging was 24, 48, or 72 hours. Neurons were imaged live using a PerkinElmer Operetta high-content imaging system. Images were collected using either a 10X or 20X air objective. They were subsequently processed using the Neurite Outgrowth module within the Harmony High-Content Imaging and Analysis software package.

AChE Inhibition Assay Briefly, motor neurons were dispensed at 4000 cells/4 µL/well by using a Multidrop Combi 8-channel dispenser (Thermo Fisher), into 1536-well black-wall/clear-bottom assay plates. The assay plates were incubated at 37°C for 48 h to allow cell attachment to wells. Test compounds or controls (23 nL) were transferred into assay plates via pin tool, and the assay plates were incubated for 1 h at 37°C. Chlorpyrifos oxon was used as the positive control and DMSO was the negative control. For the Ellman assay, 4 µL of detection cocktail solution containing DTNB and acetylthiocholine was added to each well, and the assay plates were incubated at room temperature for 60 min. Absorbance (λ = 410 nm) of TNB was measured using an Envision plate reader (PerkinElmer).

Neuron Production

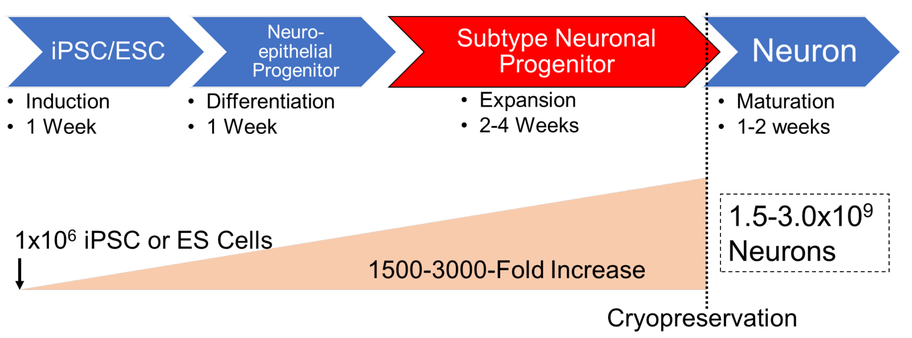

Figure 1. General Protocol for Neuron Production Scheme showing the general production protocol. The time from initiation of iPSC/ESC culture until cryopreservation is 4-6 weeks. Neurons mature 1-2 weeks after plating with BrainXell maturation supplements.

Use of Human Motor Neurons for Neurotoxicity Assessment

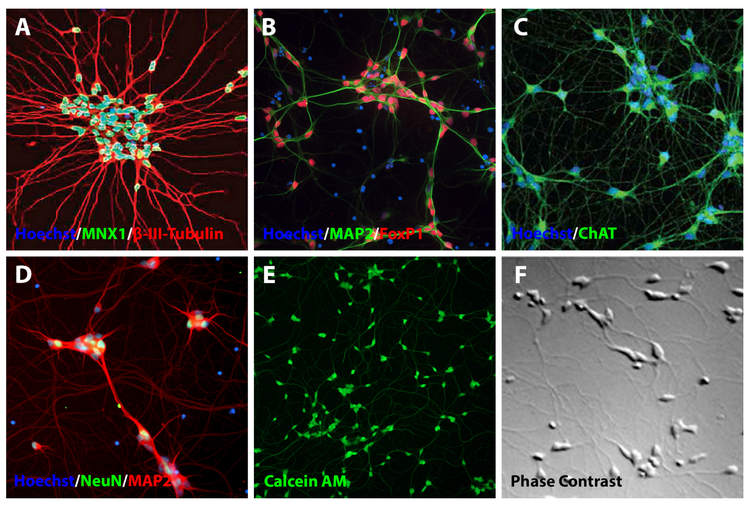

Figure 2. Expression of Subtype-Specific Neuronal Markers *(A-C)* Neurons express markers associated with spinal motor neuron identity, including MNX1, FoxP1, and ChAT. *(D)* Approximately 60-65% of motor neurons are positive for the mature neuronal marker NeuN. *(E)* Extensive neurite outgrowth, beginning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

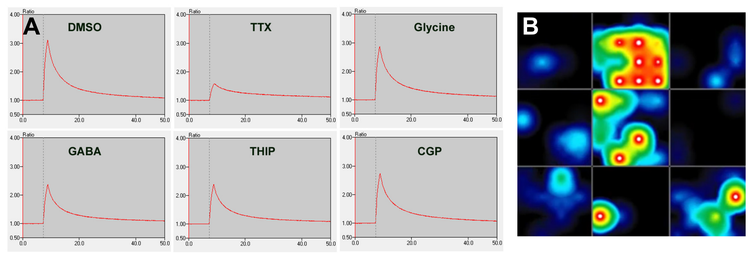

Figure 3. Functional Activity *(A)* Calcium changes after electrical stimulation at Day 10 in the presence of DMSO (no compound), TTX (sodium channel blocker), glycine, GABA, THIP (selective GABA-A receptor agonist), and CGP64626 (selective GABA-B antagonist). *(B)* As measured by multielectrode array (MEA), neurons display robust spontaneous activity, including spikes, bursts, and synchronous network activity. Shown is a single time-point activity map at Day 12.

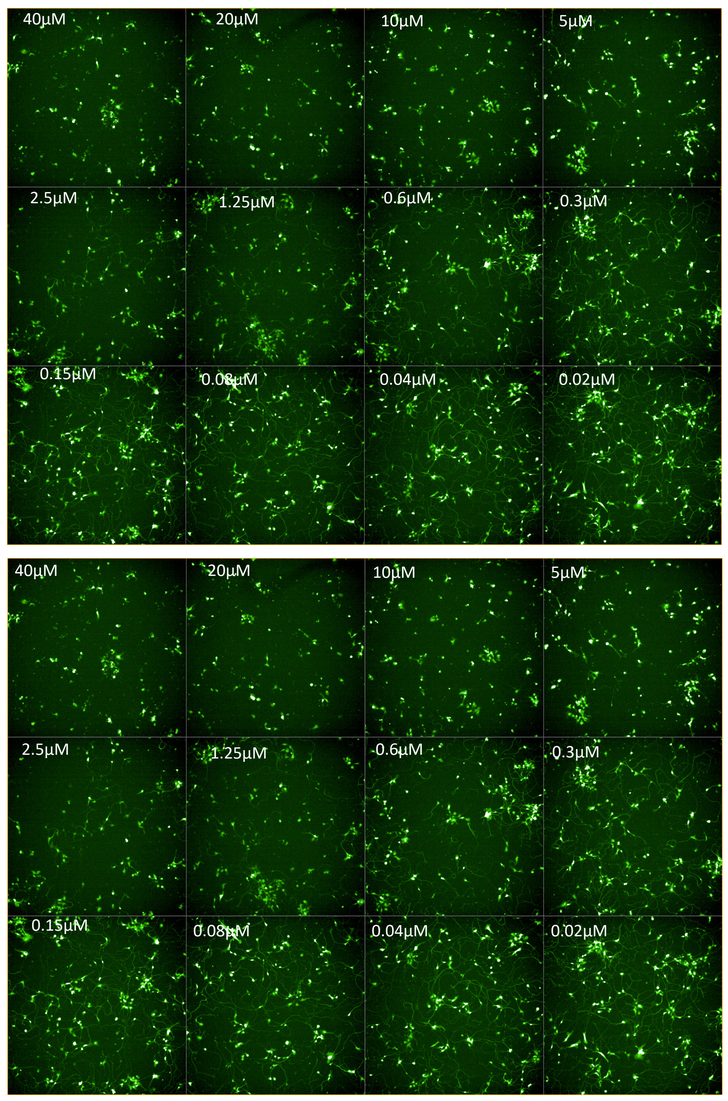

Figure 4. Rotenone Dose-Response Imaging Assay. Images of neurite deterioration at 24 hours (top) and 48 hours (bottom) with exposure to increasing concentrations of rotenone.

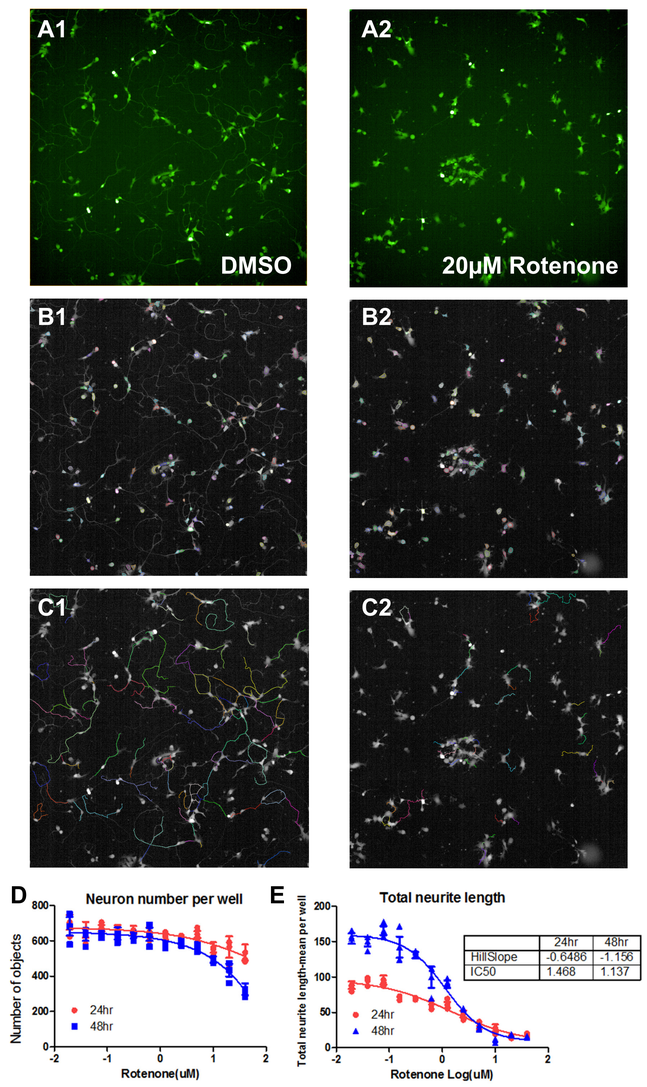

Figure 5. Detection of Neurons and Neurites. Original images (A) were processed to detect cell bodies (B) and neurites (C) for the control (DMSO) and 20 µM rotenone conditions. Dose-response curves for both neuron number (E) and total neurite length per well (F) were measured.

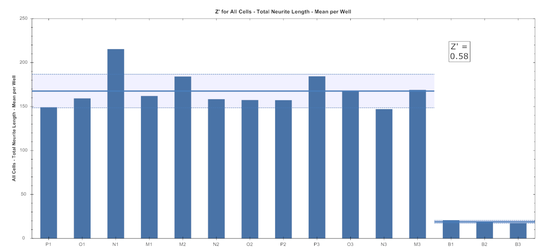

Figure 6. Z-Prime Determination for Imaging Assay. A Z-prime value of 0.58 was determined for 48 hours of treatment with 20 µM rotenone. (Value at 24 hours was 0.28.)

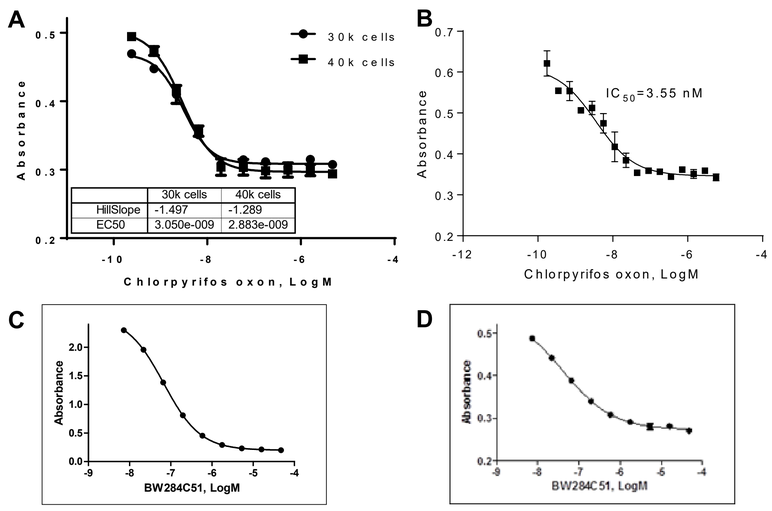

Figure 7. Measurement of Acetylcholinesterase (AChE) Inhibition. (A) Seeding density was compared in 96-well format with 30K or 40K neurons per well using the AChE inhibitor chlorpyrifos oxon (B) Similar results were obtained when the assay was adapted to 1536-well format. Additionally, AChE activity was compared between two cells types: (C) SH-SY5Y neuroblastoma cells and (D) motor neurons. For all dose-response curves, decreased absorbance indicates decreased activity.