2D and 3D iPSC-derived platforms for neurotoxicity screening demonstrate compound effects on calcium activity, synapses, viability, and cell proliferation

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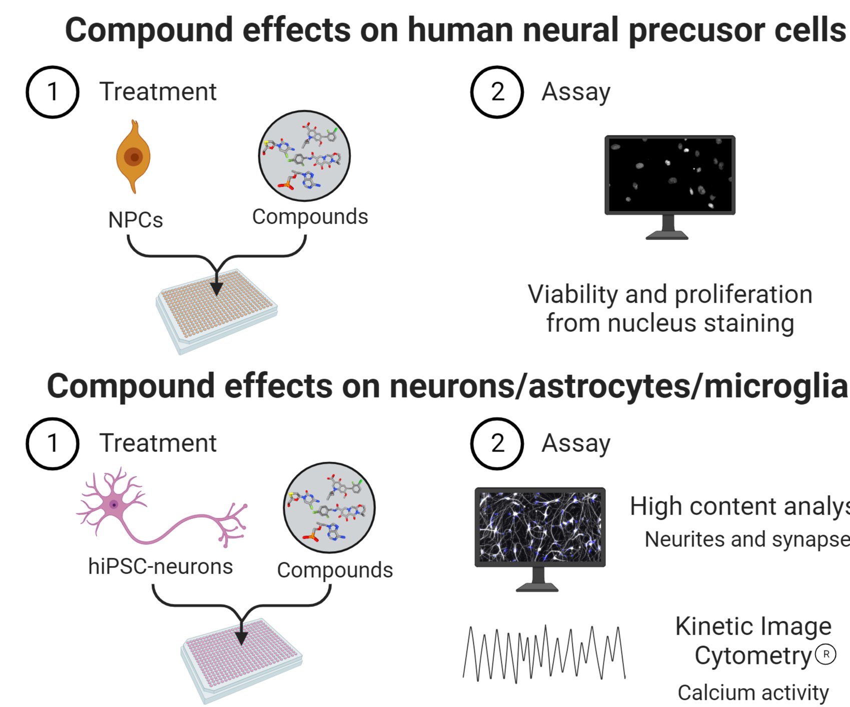
Introduction

Neurotoxicity is a leading cause of drug failures. Neurotoxicity testing in animal models is costly, labor intensive, and low throughput. Human induced pluripotent stem cells (hiPSCs) are a promising avenue for developing cost-effective *in vitro* CNS models with higher throughput that better represent human disease. In this study, we developed and validated three platforms for neurotoxicity screening using iPSC-derived models: neural progenitor cells (NPCs), two-dimensional (2D) neuron-astrocyte-microglia tri-culture models, and three-dimensional (3D) neural spheroid models. We used single cell analysis methods to test compounds such as chemotherapy agents, effectors of channel activity, environmental toxicants, and anti-retroviral drugs on viability, calcium flux, synapse numbers, or cell proliferation. We demonstrate decreased NPC viability and reduced cell proliferation after treatment with anti-retroviral compounds currently prescribed for HIV.

For analysis of calcium activity in our 2D and 3D tri-culture models, we used fluorescent calcium dyes for calcium imaging using Vala Sciences Kinetic Image Cytometer (KIC), followed by single cell analysis of the calcium transients. We observed changes in calcium activity after treatment with a chemotherapy drug (carboplatin) and channel modifying compounds (FPL64176). We routinely analyze synapse count with antibodies recognizing pre- and post-synapses to look at changes in synapses after treatment. We are also developing methods using an AAV construct expressing GFP-tagged PSD-95 to allow for longitudinal investigation of synaptic changes after treatment with compounds, such as the environmental toxin lead. Our platforms can also measure mitochondria or ER toxicity and track neurodevelopment and neural network function.



Methods

Cell Culture

Neural Progenitor cells (Elixirgen Scientific, Quick-Neuron™ Precursor (CW50065)) were maintained in DMEM/F12:Neurobasal (1:1), with glutamax, Pen-Strep, N2, B-27, bFGF, and EGF as recommended by supplier. NPCs were plated in 384-well plates directly from thaw and experiments were initiated without further passaging.

Co-cultures were produced by spontaneous differentiation of the NPC's (Elixirgen Scientific) in T25 flasks in media consisting of 1:1 DMEM:F12 (with glutamax) and Neurobasal, cAMP, ascorbic acid, NEAA, B27, N2, BDNF and GDNF (as in Vadodaria et al, 2019) for 2 weeks. Cells were lifted with Accutase and replated.

Isogenic tri-cultures were produced as follows: Excitatory cortical neurons were generated using the Quick-Neuron™ Excitatory kit (Elixirgen Scientific). Astrocytes were generated using lentiviral over-expression of the transcription factors Sox9 and Nfib (Canals et al, 2018). hiPSC-derived microglia were made using a protocol modified from Muffat et al *Nature Medicine* (2016). Cells were combined in 96 or 384-well plates at a ratio of 70:20:10 (Neuron:Astrocyte:Microglia), and maintained in Brainphys (StemCell Technologies) supplemented with B27, N2, BDNF, GDNF, IL-34, and M-CSF. Cultures are maintained for 31 days before beginning experiments.

Neural progenitor cells were made in house from iPSC line using the protocol in Vessoni et al (2016). Cryopreserved NPCs were thawed and expanded in media containing DMEM:F12 and Neurobasal, Glutamax, N2, Gem12, bFGF. NPCs were lifted and were mixed with hiPSC-derived microglia (as above) and placed in 6-well dishes in media same media, supplemented with IL-34 and M-CSF on a shaker at 90pm. The following day, media was changed to fresh media without bFGF. Media was changed ~5 days and matured on the shaker for weeks. Before experimentation, 384-well plates were coated with laminin, and spheroids were plated in the wells. Many spheroids maintain shape, and some develop 2D processes that extend into the well.

Procedures

NPC Viability and Proliferation Experiments:

Directly after thaw, NPC's were plated in 384-well dishes at 2,000 cells/well. After 72hrs, HIV anti-retroviral compounds (see Table 1 for details) were added for an additional 72 hours. Click-iT EdU kit (ThermoFisher) was used to label the dividing cells for 3 hours, followed by Click-iT detection protocol with nuclear staining (Hoechst), and imaging on Vala Sciences IC200-KIC®. Images were analyzed using Vala Sciences CyteSeer® analysis platform to detect total nuclei, distinguish live or dead nuclei, and detect EdU signal in the nuclei to quantify diving cells. N=6 wells per condition.

Calcium Experiments:

After 4 weeks (co-cultures) or 31 days (tri-cultures) in culture, cultures were treated with compounds for indicated time. Cultures were loaded with calcium imaging dye Calbryte 590 (AAT Bioquest) and a nuclear dye in compound supplemented phenol free Brainphys imaing media (StemCell Technologies). Plate was loaded onto the IC200-KIC® and imaged for calcium activity at 4 frames per second for 2 minutes (co-culture) or 12 frames per second for 5 minutes (tri-culture). The calcium videos were analyzed using CyteSeer® to identify live nuclei and calcium transients of each cell. The calcium transients are analyzed to identify putative neuron spikes from the calcium data, and quantified for measurements including mean spike integral per ms (a measurement proportional to number of action potentials), mean spikes per second (Hz, a measure of event frequency, and mean spike amplitude.

Compounds Tested

Drug concentrations and combinations:

Dolutegravir (DTG): 10µM (NPC), 8µM and 0.8µM (tri-culture)
Elvitegravir (EVG): 10µM
Tenofovir disproxyl fumerate (TDF): 10µM
Emtricitabine (FTC): 10µM
Carboplatin (CBPN): 5µM
FPL64176: 5µM
Lead Chloride ($PbCl_2$): 1µM, 0.5µM, 0.1µM

Imaging with IC200-KIC® and CyteSeer® Image Analysis

For these studies, the IC200-KIC® was equipped with a 20X/0.75 NA objective to acquire both live movies and single-timepoint images from fixed cells.

CyteSeer®, Vala's powerful high-content imaging analysis software, can analyze single cells from static images and kinetic time series.

IC200-KIC® and CyteSeer® can measure compound effects on up to thousands of cells simultaneously to discern curative or detrimental effects with high sensitivity.

Contact

For more information on the IC200-KIC®, CyteSeer®, or high content screening contract/collaborative research opportunities, please visit www.valasciences.com.

Neural Progenitor Cell Models: Cell Viability and Proliferation

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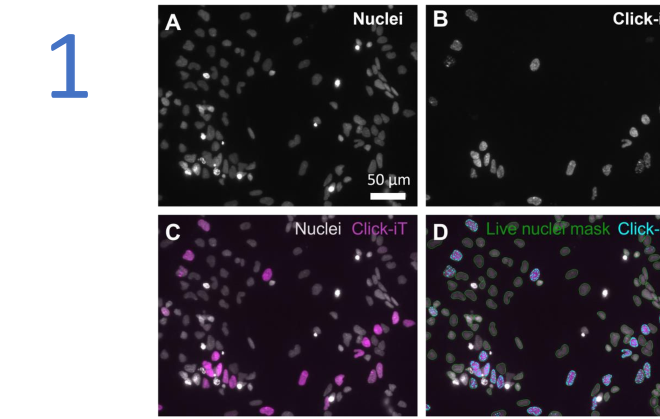

Figure 1. NPC Viability and Proliferation Assay. Images of NPC nuclei stained with Hoechst (greyscale – A,C,D) and Click-iT EdU (magenta – B,C,D). Live nuclei are masked in green (D) and Click-iT puncta in live nuclei are masked in cyan. CyteSeer® quantifies the number of live nuclei and the percent of live nuclei with Click-iT puncta.

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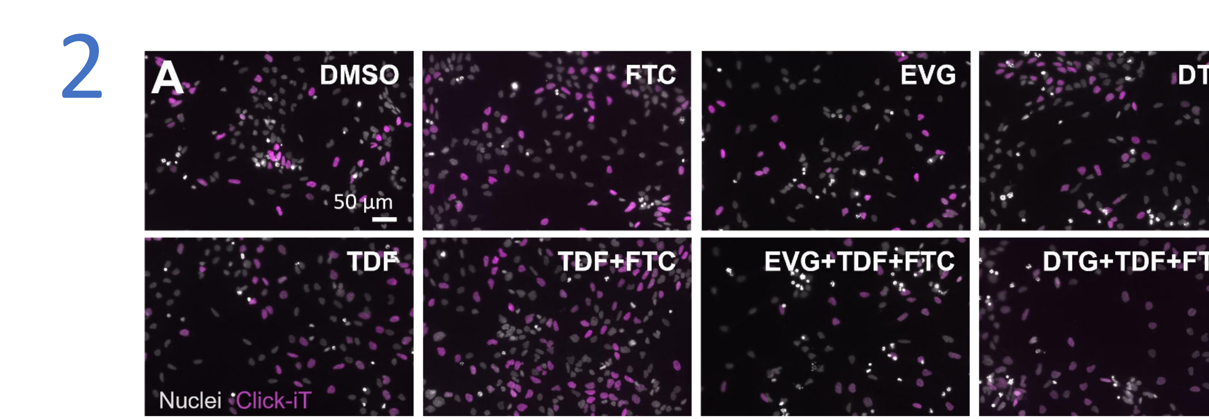

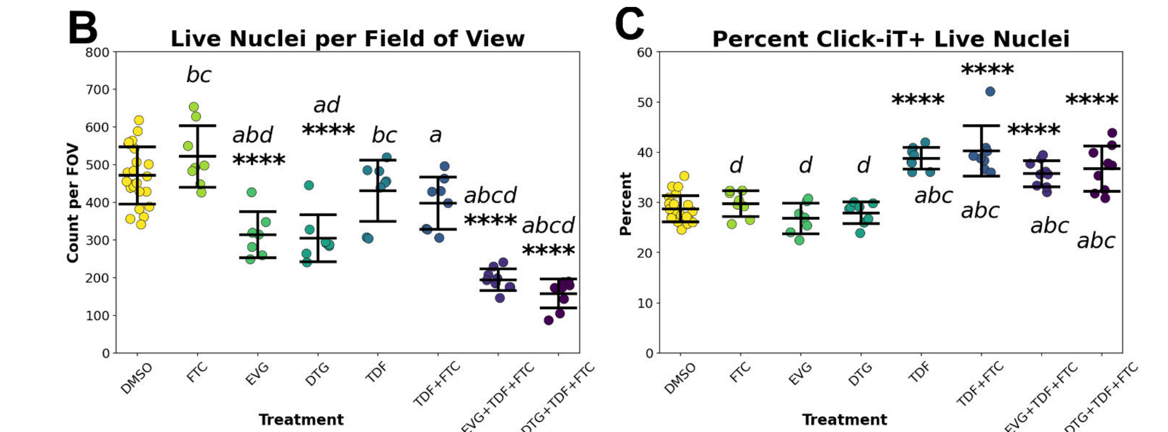

Figure 2. Exposure to EVG and DTG alone or in combination decreases neural progenitor cell count, while exposure to TDF alone or in combination increases the percent of cells replicating DNA. NPCs were treated with 0.2% DMSO (vehicle control) and 10 µM of FTC, EVG, and DTG alone, or 10 µM of each ARV in clinically relevant combinations for 72 hours. **A)** Representative portions of a field of view of NPC nuclei stained with Hoechst (greyscale) and Click-iT EdU (magenta). **B)** Mean number of live NPC nuclei identified by CyteSeer in each field of view (four fields of view per well). C) Percent of live NPC nuclei imaged per well identified as Click-iT positive (four fields of view per well). (B,C) Bars represent mean ± SD. Each point represents one value per well. DMSO N=25 wells; FTC: n=8; EVG: n=7; DTG: n=8; TDF: n=8; TDF+FTC: n=8; EVG+TDF+FTC: n=9; DTG+TDF+FTC: n=9. Statistical comparisons by one-way ANOVA followed by Tukey's multiple comparison's test. Asterisks denote statistically significant differences from cells treated with single ARVs: a:FTC, b:EVG, c:DTG, d:TDF.

2D models: Co-culture and Tri-culture

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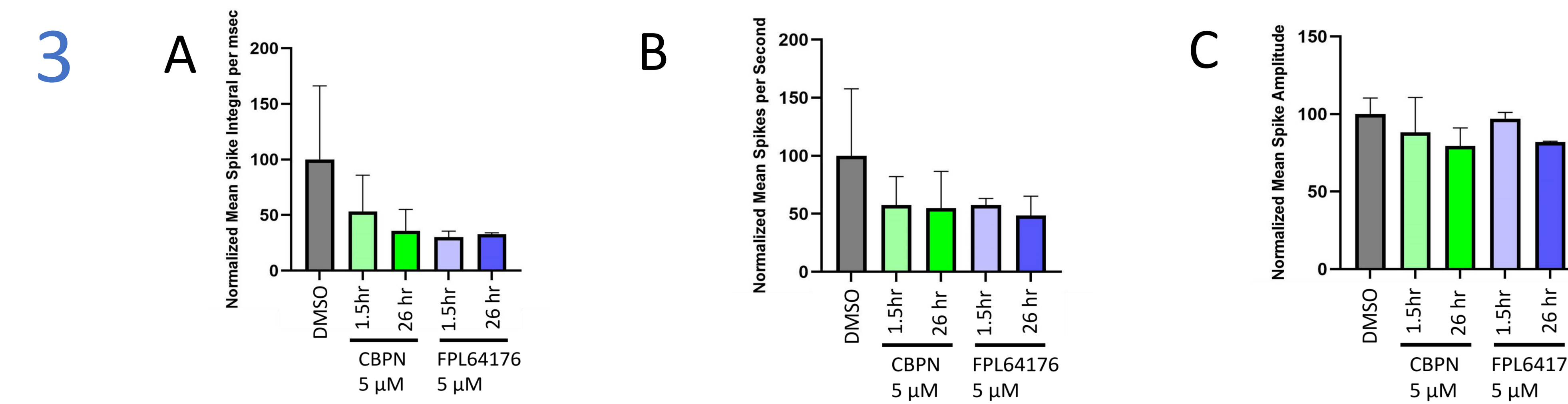

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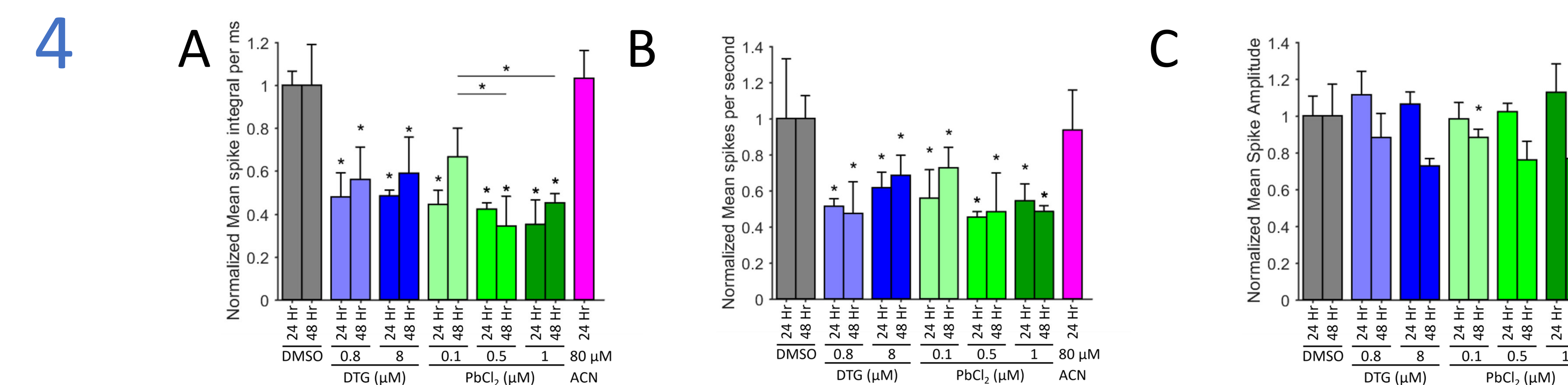

Figure 3. Exposure of 2D co-culture (neuron + astrocyte) to Carboplatin and FPL64176 decrease the activity by reducing neuron spikes and spike frequency. NPC's differentiated into neuron-astrocyte cultures were treated with DMSO (vehicle control), CBPN, and FPL64176 with concentrations as shown on the graph for 1.5 or 26 hours before testing. Measurements of neuron spikes shown are A) Normalized Mean spike integral per ms, B) Normalized Mean Spikes per Second, and C) Normalized Mean Spike Amplitude for each compound compared to DMSO control. N=9 wells (DMSO), N=3 (all compounds).

Figure 4. Exposure of tri-cultures to Dolutegravir and lead decrease the activity by reducing neuron spikes and spike frequency. Neuron, astrocyte, and microglia tri-cultures were treated with DMSO (vehicle control), CAN (negative control), DTG, and lead ($PbCl_2$) with concentrations as shown on the graph for 24 or 48 hours before testing. Measurements of neuron spikes shown are A) Normalized Mean spike integral per ms, B) Normalized Mean Spikes per Second, and C) Normalized Mean Spike Amplitude for each compound compared to DMSO control. Bars represent mean ± SD. N=3 wells.

3D spheroid models

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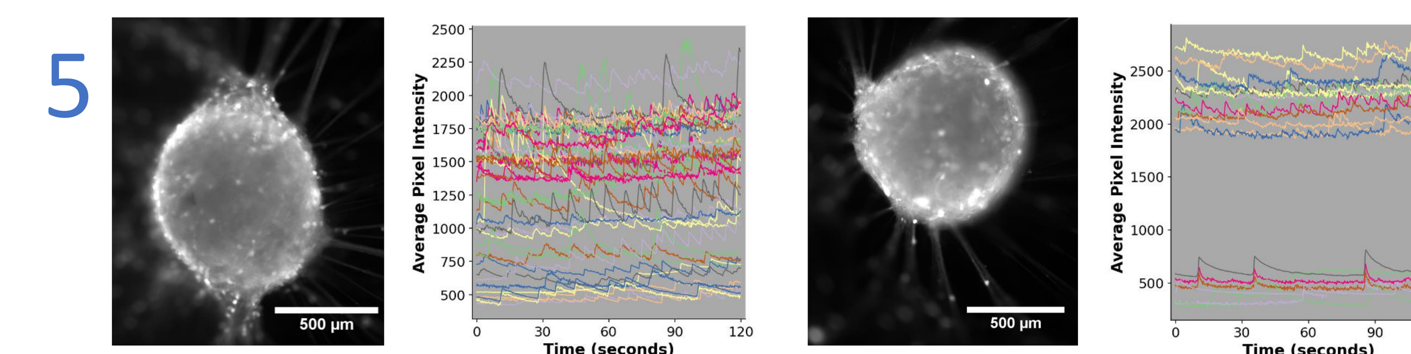

Figure 5. 3-D neurospheres with microglia show single cell activity. 10 week old spheriods were plated on a flat bottomed 384-well plate and loaded with calcium dye and imaged for 2 minutes at 4 frames per second. Single-cell calcium transients from cells in the spheroid are shown in rainbow plots to the right of the image.

Future goals

- Screen multiple classes of compounds in the isogenic tri-culture platform with hiPSC-derived neurons, astrocytes and microglia using voltage and calcium indicators.
- Perform neurite length and synapse detection experiments after the calcium study to increase information on toxicity in the culture models.
- Continue to develop the 3-D culture models and monitor calcium transients after compound treatment.

Acknowledgements

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Citations

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