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Induced Pluripotent Stem Cell-Derived Human Glutamatergic Neurons as a Platform for Mechanistic Assessment of Inducible Excitotoxicity in Drug Discovery

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Additional information is available at the end of the chapter

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Abstract

Since the guiding principles of Replace, Reduce, and Refine were published, wider context-of-use for alternatives to animal testing have emerged. Induced pluripotent stem cell-derived human glutamatergic-enriched cortical neurons can be leveraged as 2- and 3-dimensional platforms to enable candidate drug screening. Uniquely so, 2-dimensional models are useful considering that they exhibit spontaneous firing, while, 3-dimensional models show spontaneous synchronized calcium transient oscillations. Here, the limitations of selected induced acute seizure models as well as the early utilization of fully differentiated glutamatergic neuron models for interrogation of inducible excitotoxicity following exposure to neuromodulators will be described. The context of use for candidate biomarkers of inducible seizure is also discussed.

Keywords: GlutaNeurons, microelectrode array, fluorometric imaging plate reader

1. Introduction

Animal models are often leveraged to evaluate neuronal function within an intact system [1]; yet, the “gold standard” of ex vivo evaluation of brain pathophysiology is “the brain slice assay” [2]. Brain slice models lend to reduced numbers of animals for the conduct of neuroscience research. While primary neuronal cell cultures derived from animals have supported *in vitro* neuroscience studies for decades, their utility often leave to question possible translation to humans [3]. Human embryonic stem cell models were envisioned to provide more

translatable context [4]. However, ethical bias towards stem cell procurement from embryos and the lack of access to physiologically-relevant adult human brain tissues further encouraged scientists to focus on the advancement of human induced pluripotent stem cell (iPSC)-derived neuronal models [5]. Several iPSC-derived neurons have been developed to recapitulate network behavior and signaling cascades on-a-dish reminiscent of phenotypes observed in humans. Comprising the predominant excitatory network are glutamatergic neurons throughout the cortex, cerebellum, hippocampus, striatum, thalamus, hypothalamus, and visual/auditory system. Neuronal vesicles contain glutamate and migrate to the synapse where they release glutamate into the synaptic gap following Na^+/K^+ exchange (depolarization). More recently, iPSC-derived human glutamatergic neurons were generated to support cell-based research strategies [6]. Hence, researchers continue to learn and integrate these cutting-edge technologies for early safety assessment. This chapter will spotlight the validation of glutamatergic neurons as a platform for mechanistic assessment of inducible excitotoxicity in drug discovery.

2. Challenges when leveraging iPSC-derived human glutamatergic neurons

The most important tasks in using iPSC-derived human glutamatergic neurons is achieving large-scale generation of human iPSC-derived neural stem cells/early neural progenitor cells in spite of several challenges related to the differentiation procedures [7]. Terminal differentiation coupled with preliminary cell model qualification steps often reveal how different iPSC lines made from a single donor exhibit similar phenotypic marker expression as well as sensitivity to selected neurotoxicants. Yet, a limitation of iPSCs, in general, is lot-to-lot variability, including optimizations of culturing conditions (e.g. medium, supplements), following advanced understanding of how to optimally use such models to assess compound-related effects *in vitro*. The iCell® GlutaNeurons (Cellular Dynamics International, a Fujifilm Company, cat. no. GNC-301-030-001) were developed to provide a more physiologically relevant *in vitro* platform for de-risking potentially translatable compound-induced neuronal toxicity early in the drug discovery process. Notably, iCell® GlutaNeurons represent post-mitotic and highly pure populations ($\geq 90\%$) of human cortical glutamatergic neurons (**Figure 1**). According to the manufacturer, the iPSC cell lines were generated from human peripheral blood through ectopic expression of reprogramming factors by episomal transfection. The iPSC cell clones were engineered using nuclease-mediated methodologies to exhibit neomycin resistance under the control of a neuronal-specific promoter.

When considering the impact of epigenetic factors state in neuronal development and differentiation and that neurons derived from iPSCs offer a consistently reliable source of cells from a single donor, baseline gene expression analysis profiles for novel iPSC-derived human glutamatergic neurons represents another paramount validation step. Baseline single cell gene transcription analyses was determined from iCell® GlutaNeurons at DIV7 and DIV21 using a Biomark HD platform and custom assay reagents purchased from Fluidigm (**Figure 2**).

In general, a major challenge when leveraging hiPSC-derived GlutaNeurons is the lack of endogenous mechanisms for detoxification as well as the ability to evaluate the impact of different routes of administration (e.g., intramuscular and/or intraperitoneal injections) and the inability to evaluate relevant co-morbidities which are observed in animal models and/or

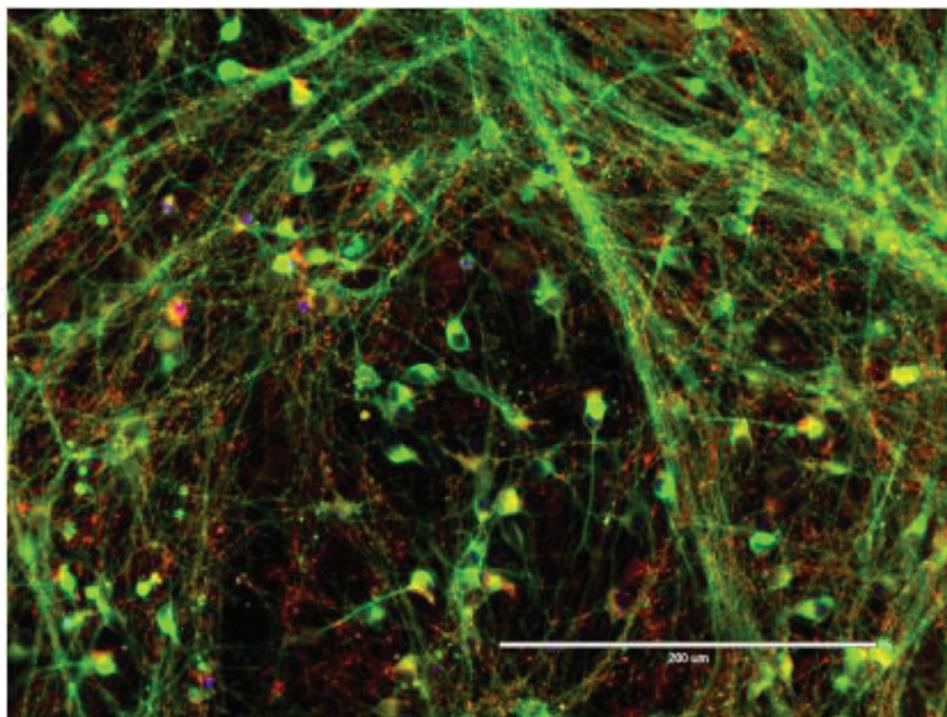


Figure 1. Human iCell[®] GlutaNeurons at DIV14. Fluorescence immunolabeling for phenotypic marker indicated neurons (β III-tubulin stain, green); non-neoplastic neurons (synaptophysin stain, red) and non-neuronal cells (4',6-diamidino-2-phenylindole, DAPI stain, blue). Branched networks were observed rapidly (after 24 h) and remained viable and adherent for an extended period in culture.

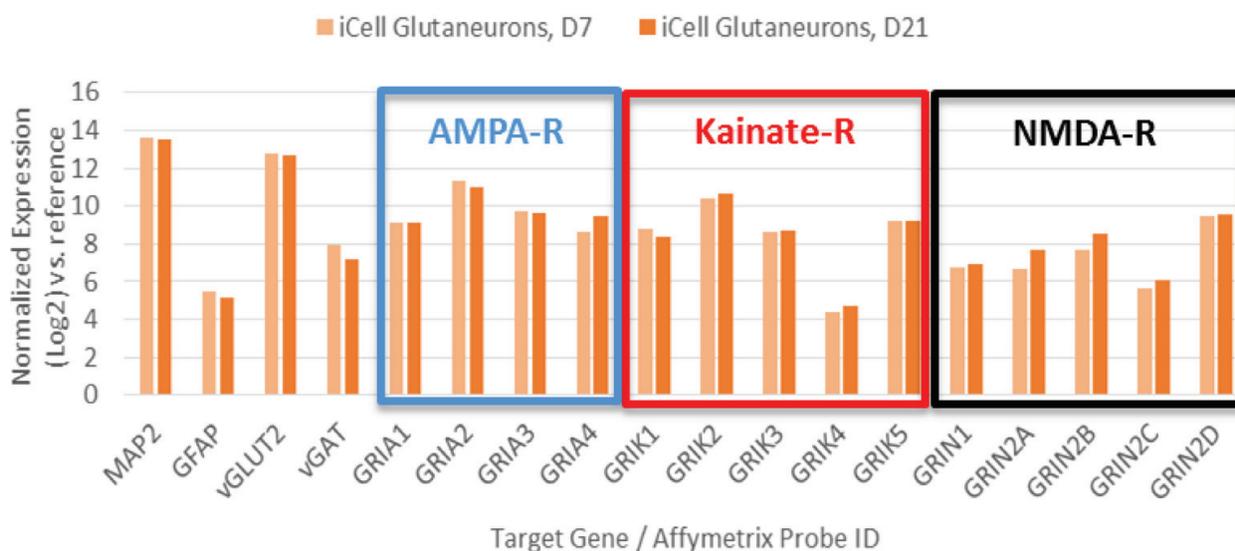


Figure 2. Targeted single-cell analysis to characterize gene transcription in untreated iCell[®] GlutaNeurons at DIV7 and DIV21. We found that most cells were positive for glutamatergic gene transcripts in the absence of differential expression at the two different time points. Abbreviations: GRIA—glutamate receptor, ionotropic, AMPA; GRIK—glutamate receptor, ionotropic, kainate; GRIN—glutamate receptor, ionotropic, NMDA.

humans. Therefore, initial GlutaNeuron assessments rely on paradigm compounds that have been previously well-characterized *in vivo* [8]. While they provide a way to model *in vitro* glutamate excitotoxicity, iPSC-derived GlutaNeurons do not enable the determine relationships between compound and brain segment-specific toxicological responses [9].

3. Glutamate excitotoxicity

There are three major classes of ionotropic glutamate receptors (iGluRs) and eight subtypes of metabotropic glutamate receptors (mGluRs) [10]. The iGluRs are predominantly located in postsynaptic sites and mediate rapid excitatory transmission, while mGluRs are expressed in both neurons and glial cells and mediate delayed transmission. The amino acid L-glutamate functions as an excitatory neurotransmitter that preferentially binds to iGluRs and mGluRs on postsynaptic neurons which follows depolarization by Na^+ , K^+ , and Ca^{2+} , resulting in an action potential [11]. Conversely, the inhibitory neurotransmitter gamma-aminobutyric acid (GABA) functions to result in hyperpolarization of postsynaptic neurons [12]. Glutamate is synthesized in neurons, while excessive glutamate is taken up from the synaptic cleft by glial cells (astrocytes) via excitatory amino acids transporters EAAT1 and EAAT2 [13, 14]. In astrocytes, glutamate is converted to glutamine by the enzyme glutamine synthase (GS). The glutamine is transported back into presynaptic terminals via the “neuronal” excitatory amino acids transporter EAAT3, where it is transformed by the glutaminase to yield glutamate that returns to vesicles via vesicular glutamate transporters (vGLUT1 and vGLUT2). This biochemical process is referred to as “the glutamate-glutamine cycle” [15]. Activation of mGluRs by glutamate leads to intracellular inositol trisphosphate (IP3) turnover, activation of ryanodine receptor channels in the endoplasmic reticulum (ER) membrane, and subsequent release of Ca^{2+} from the ER into the cytoplasmic space. Increased cytoplasmic Ca^{2+} can induce mitochondrial uptake of Ca^{2+} which, if excessive, may result in the production of reactive oxygen species (ROS) caused by prostaglandin/leukotriene formation and/or insufficient intracellular antioxidants/free radical scavengers; production of reactive nitrogen species (RNS) and/or production of apoptogenic factors; activation of calcium dependent phospholipases, endonucleases, and proteases; untoward mitochondrial alterations (loss of buffering capacity); progressive neuronal excitotoxicity; caspase activation; ATP depletion; and ultimately neuronal apoptosis, necrosis and/or death [16–18]. Glutamate and structurally related analogs of glutamate can interact with N-methyl D-aspartic acid receptors (NMDAR) under membrane depolarization conditions (removal of intrinsic Mg^{2+} channel block of relative Ca^{2+} -dependent responses), resulting in large amounts of Ca^{2+} influx which activates downstream signal transduction cascade to produce neurotoxic damage. These effects can be abrogated by antagonists. Additionally, binding of glutamate to α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors (AMPA) and/or kainate (KA) receptors (KAR) results in Na^+ influx, membrane depolarization and opening the voltage-dependent Ca^{2+} channels, signaling excitotoxicity. Conversely, minimal excitotoxicity may follow mitochondrial depolarization, leading to autophagy, a neuronal protective mechanism.

4. Kainate-induced rat status epilepticus model

In vitro platforms need to be coupled with *in vivo* models to establish translational relevance. For example, kainate acid (kainate or KA)-induced rat status epilepticus model (**Figure 3A**) is hallmarked by behavioral status epilepticus for up to 7 h following kainate injection coupled

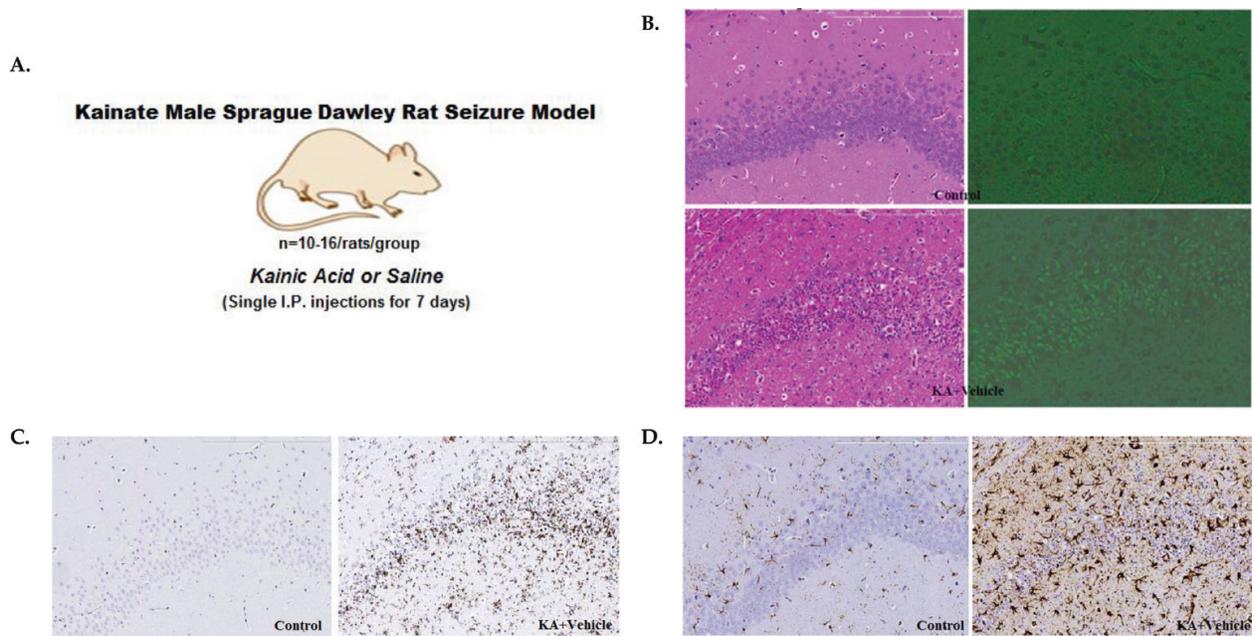


Figure 3. Hematoxylin and eosin staining and Fluoro-Jade B immunostaining in male Sprague Dawley rats that received once daily intraperitoneal (I.P.) injections of either vehicle (saline for injection USP, n = 10) or kainate (8 mg/kg/day, n = 16) for 7 consecutive days (A). In 9/16 KA-treated rats, mild to marked neuronal degeneration marked by increased Fluoro-Jade B immunolabeling when compared to controls (B), ED1/CD68 immunolabeling, indicating increased microglial activation (C) and GFAP immunolabeling, indicating increased astrogliosis (D) were identified in the hippocampus CA1-CA3 regions.

Kainate Related Brain Histopathology Findings (Day 8)					
Hippocampal Lesion	Normal	Minimal	Mild	Moderate	Marked
None	1/16	10/16	10/16	10/16	10/16
CA1	1/16	0/16	0/16	0/16	0/16
CA1, CA3	0/16	0/16	1/16	0/16	3/16
CA1, CA2, CA3	0/16	0/16	0/16	0/16	4/16

Lesion severity scoring scheme: Normal (0 affected cells); 1-Minimal (1-100 affected cells); 2-Mild (101-200 affected cells); 3-Moderate (201-300 affected cells); and 4-Marked (>300 affected cells).

Table 1. Summary of brain histopathology finding in male Sprague Dawley rats that received once daily intraperitoneal injections of kainate (8 mg/kg/day, n = 16) for 7 consecutive days. Brain tissues were unremarkable from the control group rats that received vehicle (saline for injection USP, n = 10) for 7 consecutive days (data not shown).

with brain lesions characterized by mild to marked neuronal degeneration (**Table 1, Figure 3B**), microglial activation [increase in number] (**Figure 3C**) and astrogliosis (**Figure 3D**) in the hippocampus CA1–CA3 regions.

5. Kainate-induced neuronal toxicity in-a-dish

Kainate-induced experimental seizure in animals replicate features of human temporal lobe epilepsy (TLE), while neuronal toxicity in-a-dish models [19, 20] are useful because direct exposure of cells to specific concentrations of compound results in the establishment of EC50 or IC50 concentrations. These cell-based models are usually dispersed in a monolayer bed, with or without an astrocytic layer beneath it. Generally, 18-day-old fetal neurons are commonly used, and even “mature” for several weeks in culture, these neurons are not comparable to adult neurons *in vivo* [21]. The excitatory iCell® GlutaNeurons were derived using iPSC technology. Spontaneous electrical activity, including mean firing rate, was measured via micro-electrode array (MEA) technology before and after reference compound application.

The human iCell® GlutaNeurons are differentiated from a master bank of stably iPSCs and provided as cryopreserved single-cell suspensions in 1.5 ml frozen cryovials (containing at least 5–6 million cells/vial) on dry ice. Upon receipt, the original vials were immediately transferred to liquid nitrogen tank storage until defrosted for cell culture according to the User’s Guide provided by the vendor. At the time of plating, the cells were thawed for exactly 2 min in a 37°C water bath. Complete BrainPhys medium comprised of the BrainPhys Neuronal Medium, iCell DopaNeurons Medium Supplement, iCell Nervous System Supplement, N-2 supplement, laminin, and penicillin-streptomycin. The Complete BrainPhys medium is serum free and has been specially formulated whereby it enables cell viability and function while limiting the proliferation of progenitor or non-neuronal cells. Cells were gently transferred to a 50 ml tube and 1 ml of the complete BrainPhys medium was added drop-wise and swirled gently to minimize osmotic shock while an additional 8 ml of BrainPhys medium was slowly added. The cell suspension was centrifuged at 400× g at room temperature for 5 min, carefully aspirate the supernatant, and resuspend the cell pellet in 2-3 ml of the complete BrainPhys medium after cell counting. The re-suspended cells (100 µl/well) were seeded at a density of 4×10^4 cells/well (12.5×10^4 cell/cm²) in a Corning 96-well white clear flat bottom polystyrene microplate (Corning, New York), or at a density of 8×10^4 cells/well in an Axion 48-well MEA plate (**Figure 4**).

Cells were maintained at 37°C (5% CO₂) in the complete BrainPhys medium as describe below. The cell culture plate well surfaces were freshly prepared with a base layer of Poly-L-ornithine solution (0.01%; Sigma Aldrich) and a top coating of Matrigel solution (0.028 mg/ml; Sigma-Aldrich), which are used to promote cell attachment, viability, and function. Spontaneous electrical activity was recorded as described previously [23]. In general, homogeneous neuronal populations produce spontaneous action potentials; therefore, mean firing rate (MFR) reflects the action potentials over time which can be used in part to quantify cell functionality. Multiple action potentials within a short time period represent neuronal burst. The cells were

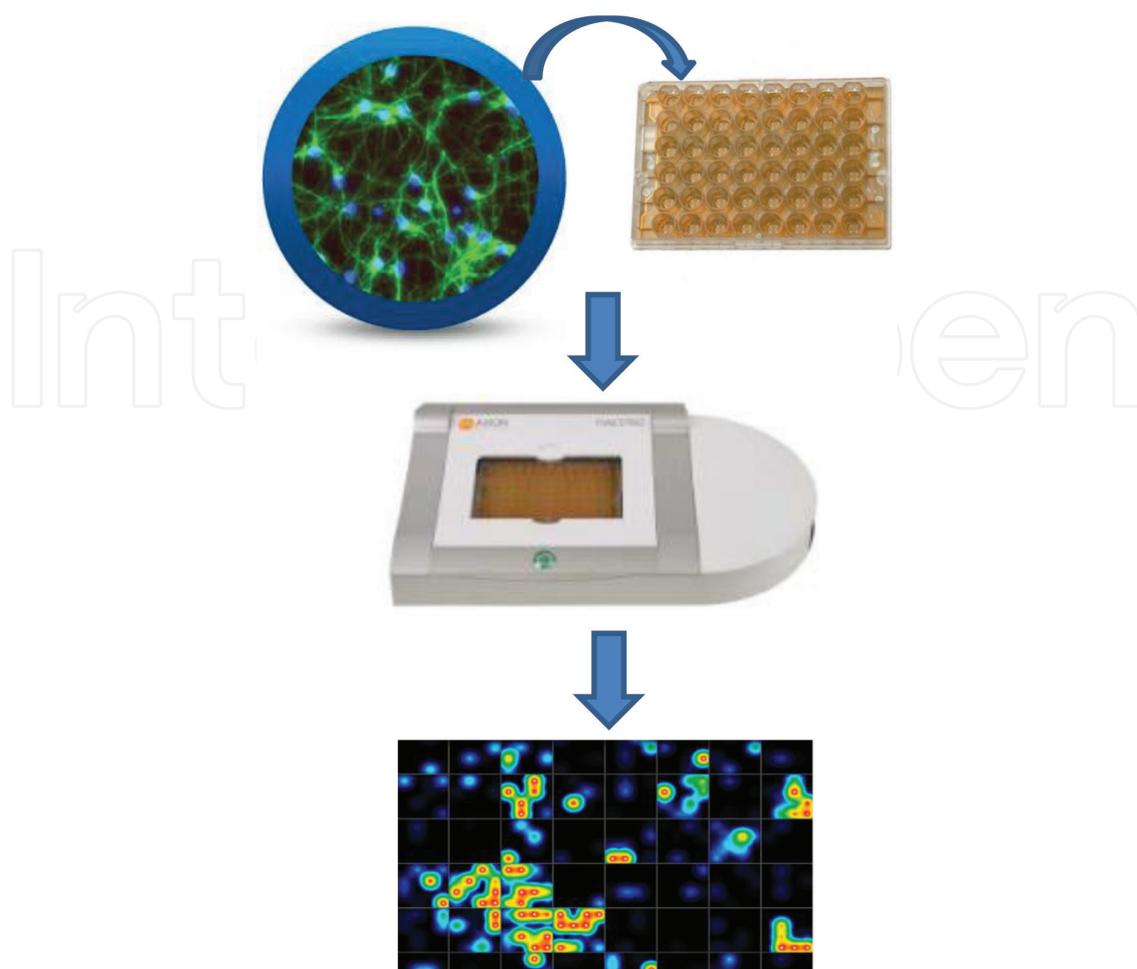


Figure 4. For example, the Axion classic 48-well MEA plates consist of wells with an array of 16 individually embedded, nanotextured gold microelectrodes with four integrated ground electrodes, yielding a total of 768 channels across the entire plate [22]. The culture plate temperature can be maintained at 37°C using an integrated heating system and temperature controller based on the low throughput maestro platform (Axion BioSystems, Inc.). The newer low throughput, maestro PRO and high throughput, Maestro APEX platforms from Axion BioSystems Inc. (not shown) may also be leveraged.

maintained at 37°C in an incubator with 5% CO₂ for several days *in vitro* (DIV) with exchange of 50% of the medium every 2 days before treatment and subsequent parameter measurements. Per our use, the iCell[®] GlutaNeurons were maintained in culture for up to 21 days in culture medium without appreciable loss of viability (data not shown).

We tested glutamate receptor agonists and antagonists iCell[®] GlutaNeurons at DIV9, using biochemical/functional assays to assess changes in cell viability, biomarker expression levels and synaptic activity to systematically evaluate concentration and time dependent glutamate excitotoxicity. Cells were treated with either vehicle (media or 0.1% DMSO), agonist (glutamate), kainate (positive control agonist) or antagonist (NMDA-R Antagonist, MK801; KA-R/AMPA-R Antagonist, NBQX; and FAAH Inhibitor, URB597) for up to 24 h, or pre-treated with glutamate receptor antagonists or FAAH inhibitor for 1 h followed by an agonist for up to 24 h. Changes in cell density were determined subjectively using routine bright field microscopic analysis (**Figure 5**). Concentration-dependent glutamate and kainate induced reductions

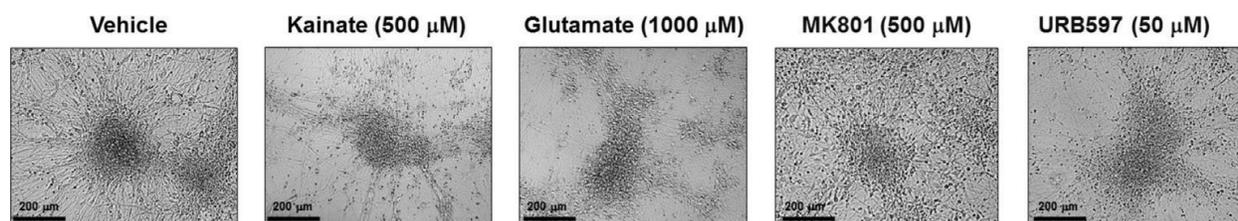


Figure 5. Changes in cell density of iCell® GlutaNeurons. Reductions in cell density were observed in GlutaNeuron-astrocyte co-cultures following exposure to kainate (B) when compared to vehicle (A). Phenotypic changes were apparent with glutamate only (C). Alone, no MK801- (D) or URB597- (E) related effects were observed. iCell® GlutaNeurons were imaged in brightfield using an EVOS XL digital inverted microscope.

in mean cell viability were assessed and compared at 30 min (acute effect; data not shown) and 24 h (lasting effect; **Figure 6**). For replicate wells for the same treatments, the mean values relative to concurrent controls were used to calculate the area under the concentration curve (AUC) in GraphPad Prism 7.0 software. After test compound treatment using the CellTiter-Glo® Luminescent Cell Viability Assay kit (Promega Corporation, Madison,). Briefly, the CellTiter-Glo® reagent added directly to cells culture medium results in cell lysis and generation of a luminescent signal (by luciferase reaction) proportional to the amount of ATP, which is directly proportional to the number of viable cells present in culture. In this study, ATP levels released from viable cells mitochondrial were measured in the 96-well plate format with

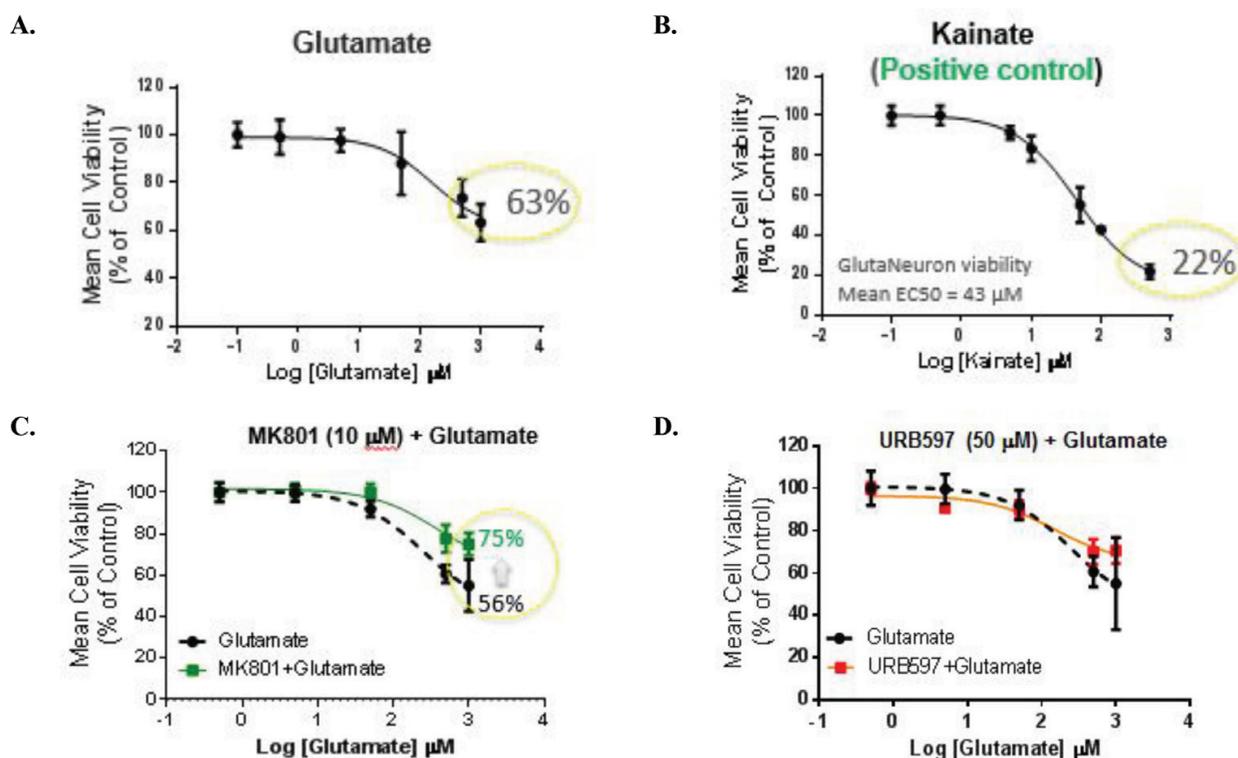


Figure 6. Mean cell viability of iCell® GlutaNeurons following exposure to glutamate (A, 63% loss) and kainate (B, 22% loss) for 24 h when compared to controls. MK801 (NMDA antagonist, 10 μ M = IC₅₀) partially blocked the glutamate-induced loss (~20%) of mean cell viability (C). FAAH inhibitor URB597, 50 mM) did not reduce the glutamate-induced effects (D).

approximately 4×10^4 or 8×10^4 cells/well 24 h after drug treatment using a microplate luminescence reader. All experiments were performed in triplicate wells for each condition (vehicle or per test compound concentration) and repeated at least three times. Blockade of KA-induced effects on mean cell viability by pre-treatment with different concentrations of NBQX was evident (data not shown).

The Axion BioSystems Maestro MEA technology is a label-free platform that measures electrical activity of iCell® GlutaNeurons when cultured directly on MEA plates. Established algorithms within Axion Integrated Studios (AxIS) v1.9 software can detect and quantify several parameters to assess changes in neuronal functionality, excitability and connectivity. On DIV10 and DIV16, spontaneous network activity was recorded using Axion Biosystems Maestro 768 channel amplifier and AxIS) v1.9 software. The amplifier recorded from all channels simultaneously using a gain of 1200× and a sampling rate of 12.5 kHz/channel. After passing the signal through a Butterworth band-pass filter (300–5000 Hz) on-line spike detection (threshold = $8 \times$ rms noise on each channel) was done with the AxIS adaptive spike detector. All recordings were conducted at 37°C. Spontaneous network activity was recorded in the absence of compounds for 4 min (baseline), then compounds were added and a continuous 30 min (acute effects), or various time points (4 min/time points) up to 24 h (sub-chronic effects) of activity were recorded. Only wells with 10 or more active electrodes (>5 spikes/min) during the

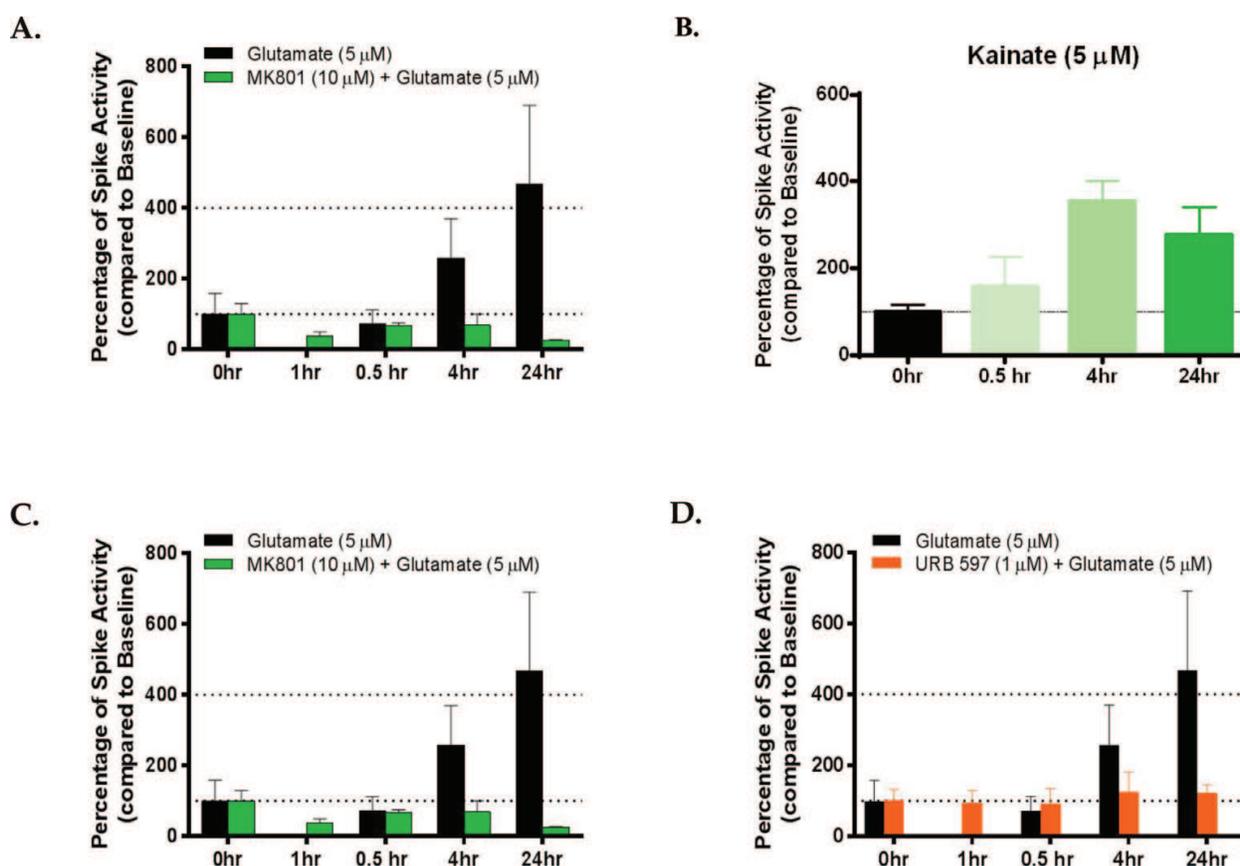


Figure 7. Kainate (A) and glutamate (B) induced changes in MFR in GlutaNeurons. MEA data showed that MK801 (10 μM) attenuated the glutamate (5 μM)-induced excitation at 4 and 24 h (C). Cells pre-exposed to URB597 reduced glutamate (5 μM)-induced excitation at 4 and 24 h in GlutaNeurons (D).

baseline recording period were used in the analysis. Stock solutions of compounds were made to $100\times$ the desired final concentration in $100\ \mu\text{l}$ media, then $2\ \mu\text{l}$ of stock solution was added to each well ($200\ \mu\text{l}$ media) to reach the final desired concentration. Dedicated wells were used on each 48 well plate for vehicle controls. At least three replicates of the viability assay and were performed. At least triplicated wells were measured in each replicate. Following addition of neat compounds, electric activity was recorded continuously for 30 min to determine the acute effect on neuronal activity, or at different time points up to 24 h to determine the sub-chronic effect on neuronal activity. Briefly, neurons synaptic activities (spontaneous or induced action potentials) recorded in 4 min intervals at -2 , 0 (baseline), 0.5, 2, 4 and 24 h post-treatment, and MFR was used a biomarker to quantify functional changes in GlutaNeurons. Between 0.5-24 h post treatment, pharmacologically relevant concentrations of kainate (**Figure 7A**) and Glutamate (**Figure 7B**) induced changes in MFR in GlutaNeurons. MEA data showed that MK801 ($10\ \mu\text{M}$) attenuated the Glutamate ($5\ \mu\text{M}$)-induced excitation at 4 and 24 h (**Figure 7C**). Cells pre-exposed to URB597 reduced Glutamate ($5\ \mu\text{M}$)-induced excitation at 4 and 24 h in GlutaNeurons (**Figure 7D**).

6. iPSC-derived neuronal models to enable early de-risking of inducible neurotoxicity

Drug development is extremely costly and challenging [24]; moreover, lack of confidence in translatability often leads to failure during clinical trials. Neurotoxicity caused by candidate

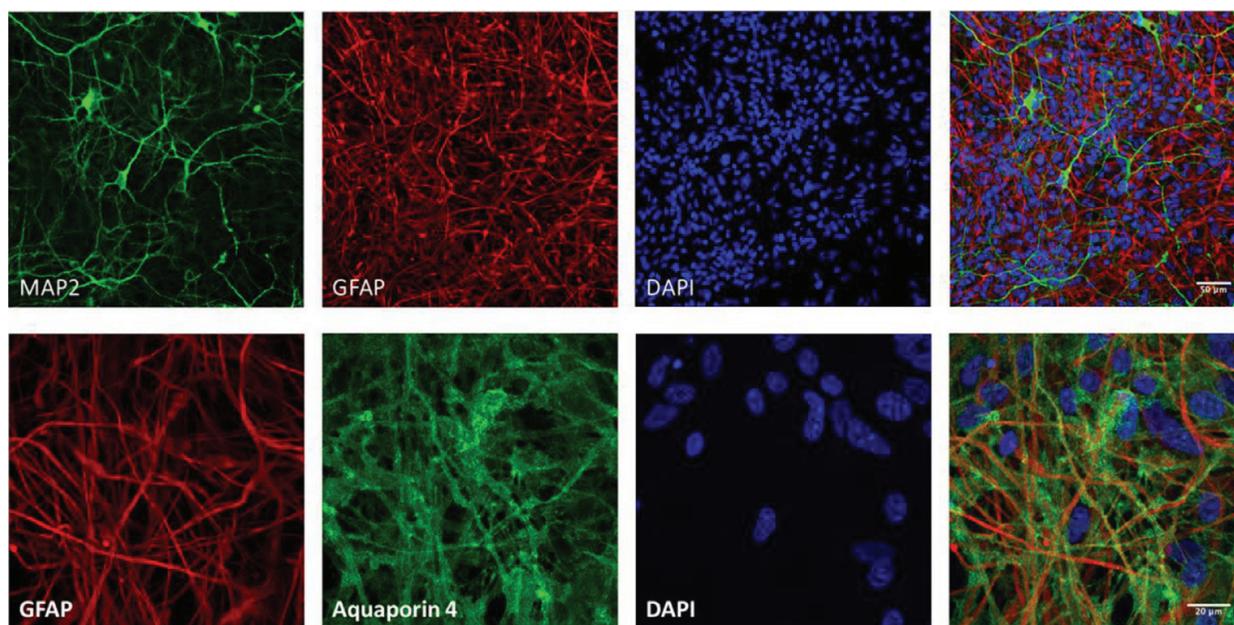


Figure 8. StemoniX's 2D human MicroBrain[®] model is comprised of human iPSC cells-derived cortical neurons and astrocytes that are amenable to glutamatergic/GABAergic modulation. This model shows typical identity and functional markers, including MAP2: Neuronal marker; GFAP: Astrocyte marker; Synapsin I: Neuronal functional (synapse) marker; Aquaporin4: Astrocyte functional marker and DAPI: Nuclear marker [29–30].

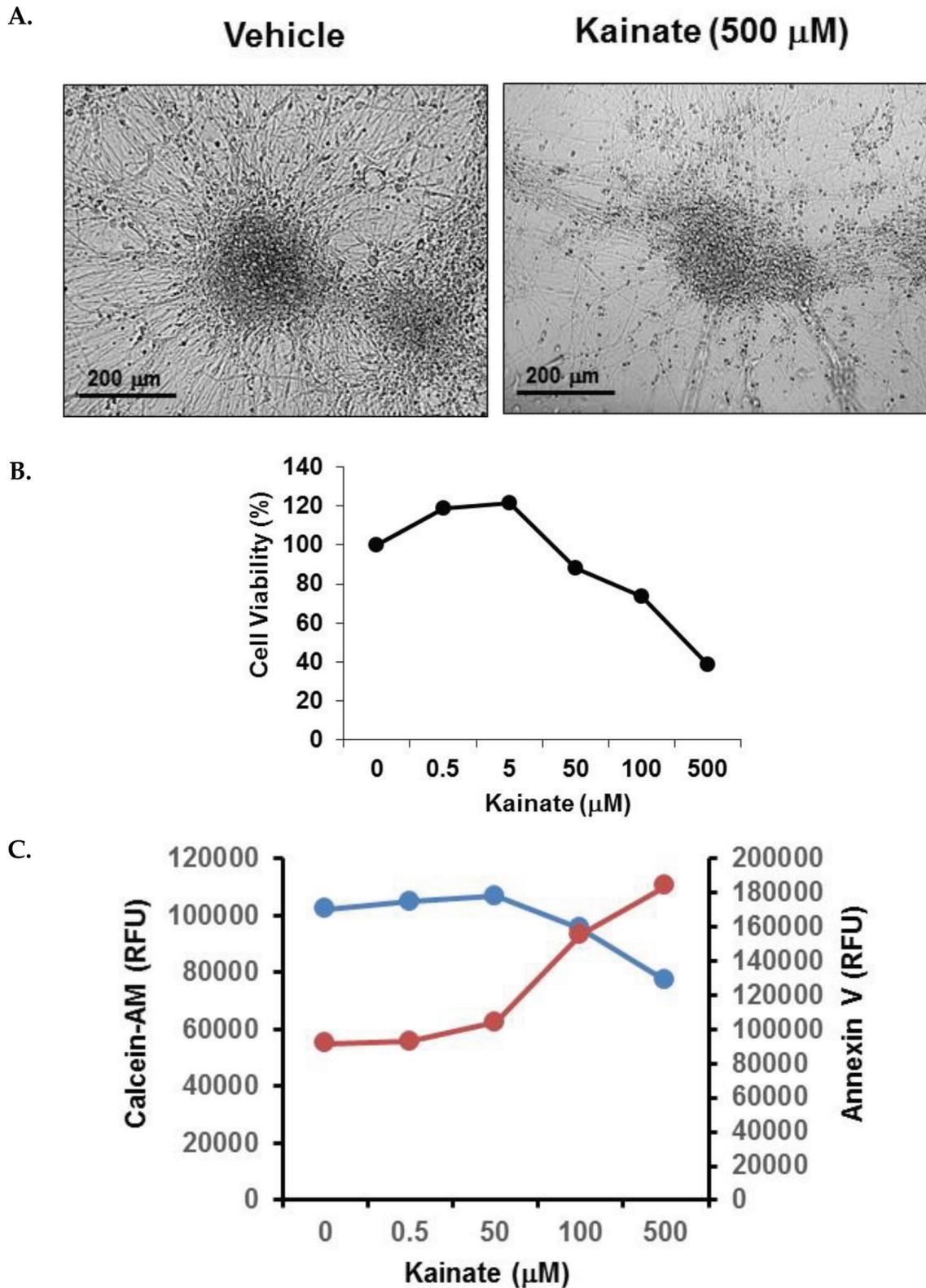


Figure 9. Changes in cell density of StemoniX's pre-plated, assay ready high throughput 2D human glutamatergic MicroBrain[®] platform (DIV33) imaged in bright field using an EVOS XL digital inverted microscope (A). Quantitative reductions in cell density were observed following 24-h exposure to kainate when compared to vehicle (1% DMSO) using a CellTiter-Glo[®] Luminescent Cell Viability Assay kit (B). Calcein-AM (blue line) and Annexin V (red line) assays were used to detect kainate-induced loss of cell viability and increased apoptosis, respectively (C).

drugs can lead to temporary or permanent harm to the central or peripheral nervous system. In case of glutamate excitotoxicity, excessive stimulation of the neurons occurs due to brain injury. Therefore, a major goal of the pharmaceutical industry has been to reduce late stage compound attrition due to neurotoxicity. Pharmaceutical companies, consortia (the Health and Environmental Sciences Institute) and regulatory authorities continue to establish more predictive models and biomarkers for early identification of neurotoxicity [25]. It is generally accepted, that iPSC-derived GlutaNeurons offer advantages over *in vivo* models: (1) isolated cells for glutamatergic signal transduction assessment in a dish; (2) low- and/or high-throughput screening at less cost and time than *in vivo* study conduct, although the MEA data interpretations may take several weeks; and (3) mechanistic assessment of neurotoxicity *in vitro* [26, 27]. Considering that candidate drugs may exhibit deleterious excitotoxic liabilities, iPSC derived GlutaNeurons support screening tools for early de-risking potentially translatable neurotoxicity. iCell® GlutaNeurons provide a relevant, excitatory neuronal model that enables researchers to study human neuronal network development and activity through interrogation and manipulation of relevant pathological pathways, thereby providing a new and valuable tool for drug discovery.

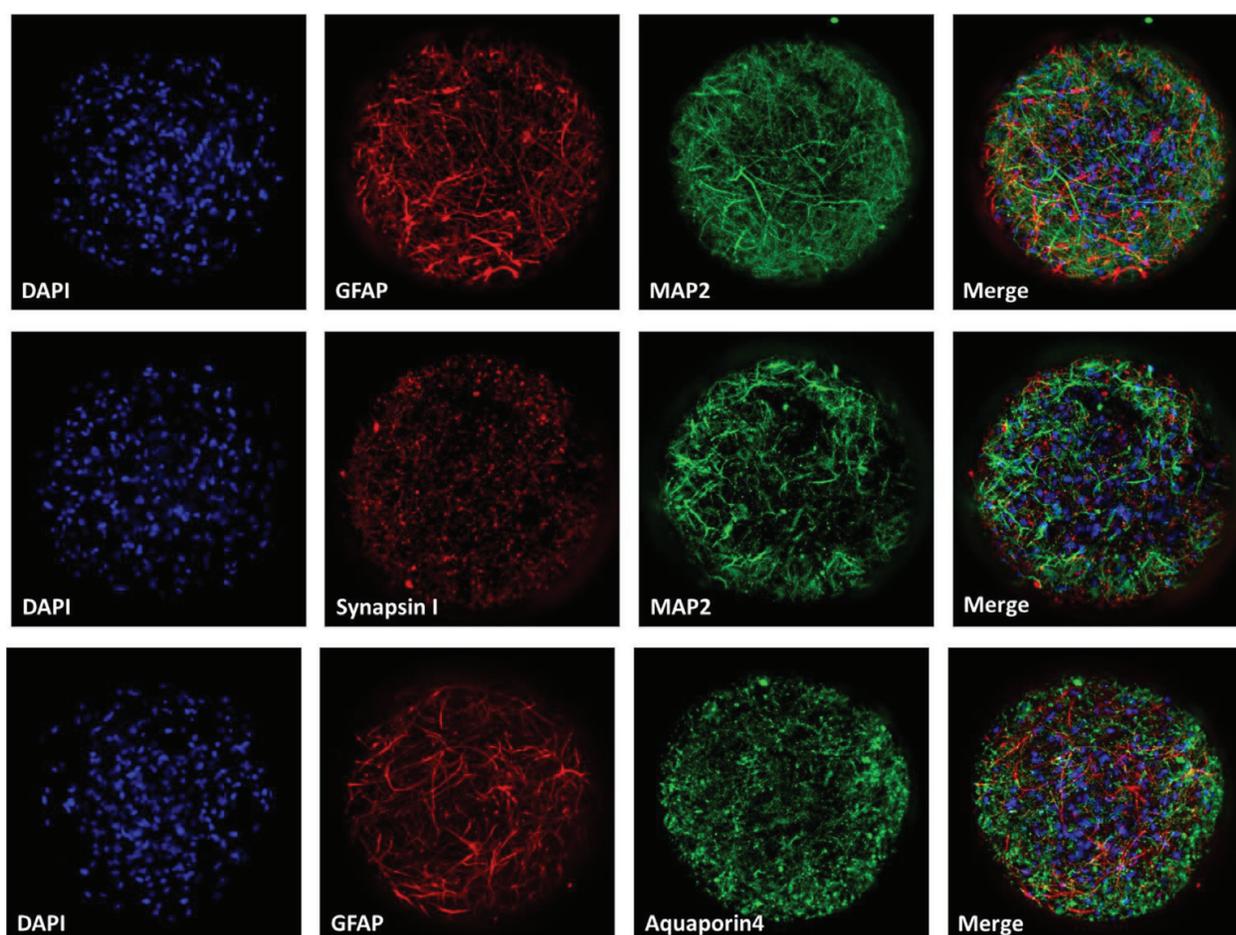


Figure 10. The StemoniX 3D Human MicroBrain® model contains a balanced population of cortical neurons and astrocytes which display typical identity and functional markers, including MAP2: Neuronal marker; GFAP: Astrocyte marker; Synapsin I: Neuronal functional (synapse) marker; Aquaporin4: Astrocyte functional marker and DAPI: Nuclear marker [30].

Previous studies revealed changes in brain organoid viability, proliferation, differentiation, and migration [28]. Newer human neural models try to replicate the brain complexity incorporating key features of the organ, such as the presence of a functional population of astrocytes. The MicroBrain® 2D model from StemoniX, (**Figure 8**) consists of a physiological relevant mixture of human iPSC cells-derived cortical neurons and astrocytes [29]. Tests using this system have the potential of better recapitulate the intricate interplay between different neural cells and physiological response observed *in vivo* (**Figure 9**).

Recently, 3D human brain organoids have been used to assess spontaneous and synchronized neuronal activity and glutamate excitotoxicity. One interesting model is that the MicroBrain 3D Assay Ready Plates® (StemoniX), which consists of human cortical neurons and astrocytes spheroids (**Figure 10**) in 384-well format [29], containing one spheroid per well. These neural brain organoids are highly homogenous in size at approximately 500 µm and show phenotypic markers of mature cellular (e.g., synaptic proteins and glutamate transporters). Quantifiable synchronized spontaneous calcium oscillations may be detected from this 3D MicroBrain platform using a kinetic, high-throughput Fluorometric Imaging Plate Reader (FLIPR®). High speed confocal imaging confirmed homogenous calcium oscillations at the cellular level, whereas MEA analysis demonstrated robust synchronous glutamatergic/GABAergic circuitry. Moreover, synchronized calcium oscillations may be utilized as a biomarker for neuronal network activity in 3D MicroBrains. Finally, the MicroBrain 3D platforms are offered as pre-plated cells delivered at room temperature, speeding up the turnover of results, dispensing the need for long-term culture in the lab before the assay.

7. Recent advances in the development and validation sensitive and specific biomarkers for drug-induced neurotoxicity using iPSC-derived neuronal models

As noted above, pharmaceutical companies, the Health and Environmental Sciences Institute consortia and regulatory authorities have committed resources intended for developing and validating sensitive and specific biomarkers for early detection of drug-induced neurotoxicity [25]. Recent studies have shown synchronized calcium oscillations as a useful biomarker for neuronal network activity in CDI's 2D GlutaNeurons using the Hamamatsu FDSS7000EX/µCELL (a camera-based kinetic plate reader) [31] (**Figure 11**) and StemoniX's 3D MicroBrains using FLIPR [30] when cultured in 384-well plates (**Figure 12**). Synchronous spontaneous (MicroBrain 3D) or 4-AP-induced (GlutaNeurons) calcium flux are evident in these *in vitro* platforms. Taken together, inducible calcium flux in these models appears as a value-added parameter to enable early discovery compound screening. Additional studies will reveal the variables between *in vivo* calcium changes in the brain and those noted using various neuronal on-a-dish models as well as the translational values for calcium flux to MEA data, when monitoring for changes in electrophysiological parameters related to neurotoxin exposures.

MicroRNAs (miRNAs) are small non-coding RNAs that regulate post-transcriptional gene expression in pathologic processes and can be found in plasma, serum and brain tissue, for example, circulating miRNA concentrations were differentially expressed in epilepsy patients

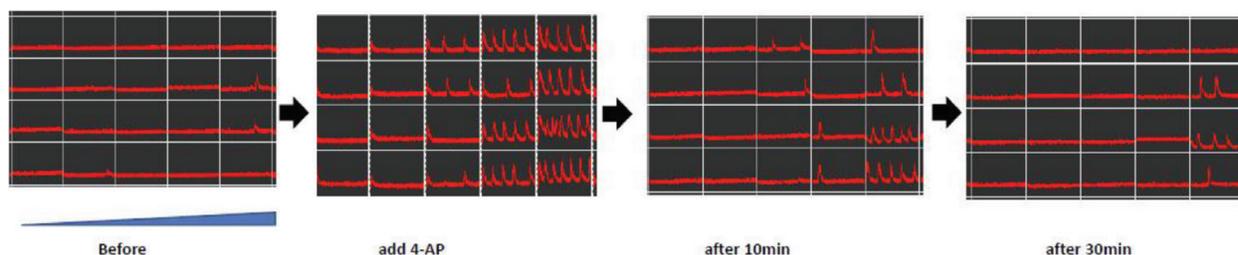
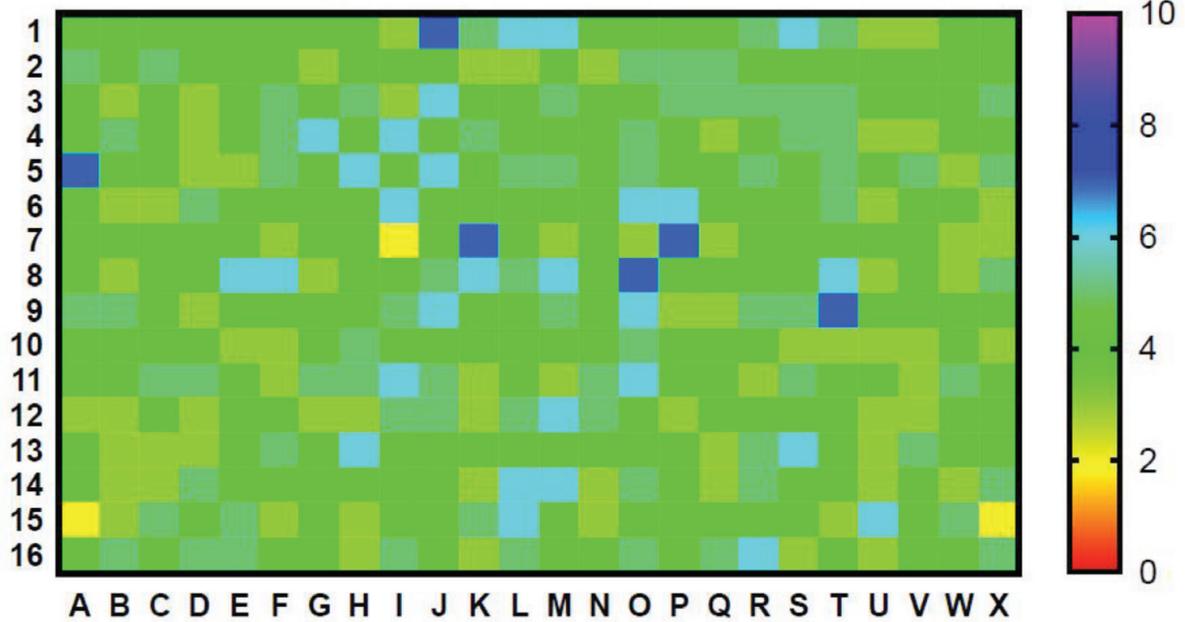


Figure 11. Representative detection of 4-aminopyridine (4-AP)-induced synchronization of calcium oscillations in CDI's 2D GlutaNeurons at DIV28 (post seeding). Interestingly, spontaneous basal synchronized calcium flux activities are not evident in CDI's 2D GlutaNeurons. N = 4 replicates. 5-point dose concentration response condition. Each well was normalized to concurrent control.

compared healthy controls [32]. Notably, significantly decreased expression level changes in serum hsa-miR-15a-5p showed sensitivity/specificity for diagnosis of epilepsy. To determine the utility of miRNAs in a rat model of acute experimental seizure, we evaluated miRNA expression level changes in serum as well as flash frozen cerebrum (cortex) and hippocampus from male Sprague Dawley rats that received the prototype compound, pentylenetetrazol, PTZ by single I.P. injection (**Figure 13**). In this study, single injection of PTZ at 60 mg/kg, I.P. was a subconvulsive dose, while single injection of PTZ at 80 mg/kg, I.P. induced decreased activity, straub tail, whole body tremor, heavy breathing, decreased body temperature (cold to touch), and seizures (irregular clonus of head and fore and hind limbs) by approximately 20 min post dosing. Serum glucose (a conventional seizure biomarker) was increased at the time of acute convulsive activity. Using the Abcam Fireplex™ Multiplex Rodent Discovery miRNA Toxicity Panel, significantly upregulated (35) or downregulated (26) miRNA expression level changes were detected from PTZ-treated rats with seizures versus controls without seizures; cerebral cortex revealed that miRNAs were significantly upregulated (1) or downregulated (5) in the PTZ-treated rats versus controls; and hippocampus revealed that miRNAs were significantly upregulated (8) or downregulated (3) in the PTZ-treated rats versus controls (data not shown). The miRNA profiles differed between cerebral cortex and hippocampus. As have been report for epilepsy patients, PTZ-induced acute seizures in rats were characterized by significantly decreased expression level changes in miR-15a-5p in cerebral cortex and serum (**Figure 12**). Taken together, circulating miR-15a-5p could be a minimally-invasive biomarker to enable monitoring for epilepsy in human patients [32] and PTZ-induced rat models of acute seizure. Additionally, retrospective analysis revealed 6 significantly upregulated miRNAs (mmu-mir-664-3p, mmu-mir-137-3p, mmu-mir-466i-5p, mmu-mir-204-5p, mmu-mir-221-3p, mmu-mir-29c-5p) and 3 significantly downregulated miRNAs (mmu-mir 324 5p, mmu-mir-140-5p, mmu-mir-328-3p) in male SD rats with seizures following once daily injections of the KA (8 mg/kg/day, i.p.) for 7 consecutive days versus control (vehicle: saline-treated) rats without seizures (author's unpublished data). These miRNAs showed promise as tissue-based exploratory biomarkers in rats with brain injury following KA-induced experimental seizures. Additionally, early data suggest that selected miRNAs detected from paraffin-embedded brain tissue slices may also correlate with profiles in hippocampal lesions (author's unpublished data).

A.

Heat Map of Calcium Flux Activity



B.

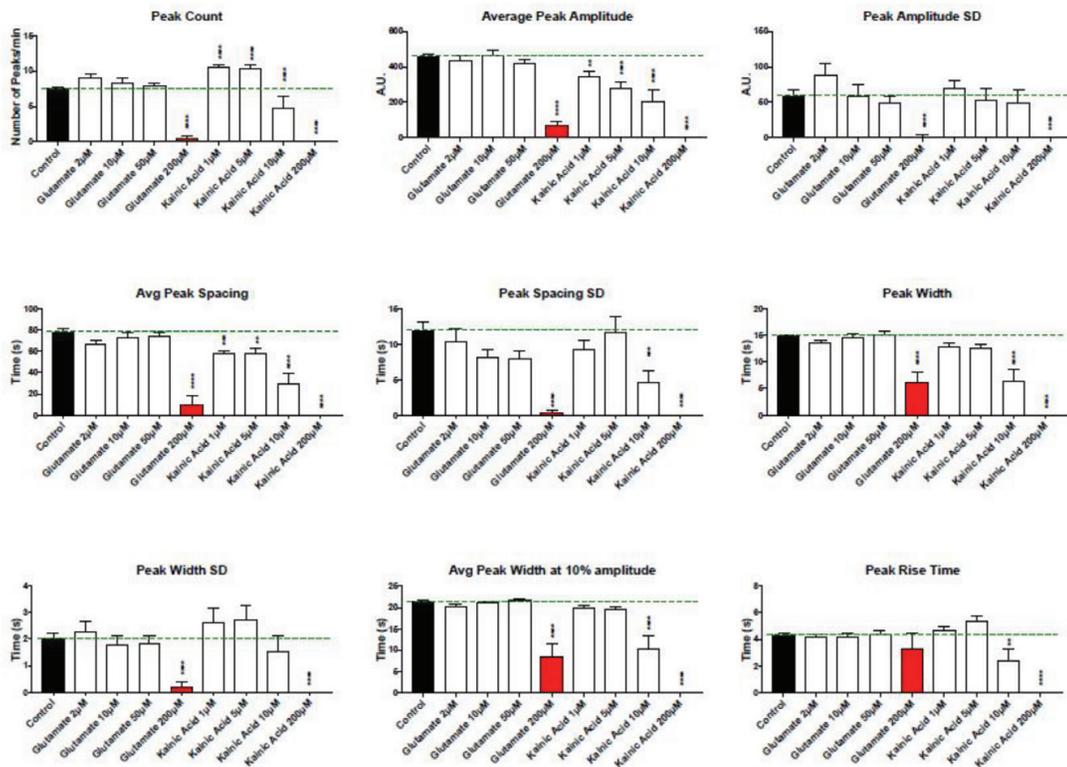


Figure 12. Representative heat map of spontaneous basal synchronized calcium flux activity in StemoniX's 3D Human MicroBrain® (A) and characterization of synchronous glutamatergic calcium flux (30 min) post glutamate, kainate or control treatment (B). N = 6 replicates. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ (when compared to concurrent control).

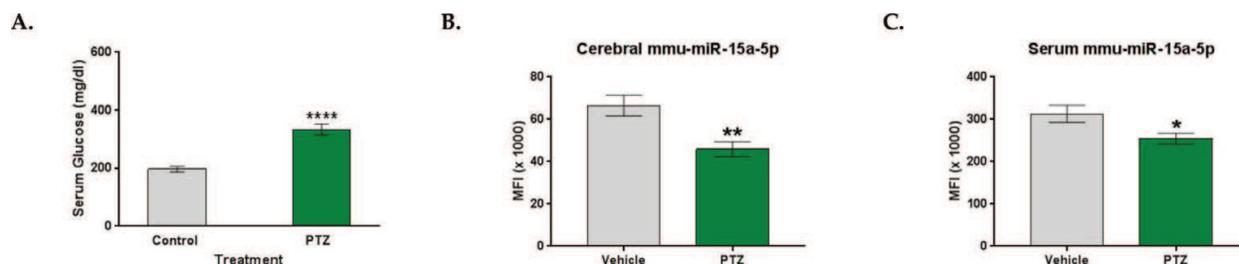


Figure 13. Male Sprague Dawley rats received a single I.P. injection of either vehicle (saline for injection USP, $n = 10$) or pentylenetetrazol (PTZ, 80 mg/kg, $n = 10$). In 10/10 PTZ-treated rats, decreased activity, straub tail, whole body tremor, heavy breathing, decreased body temperature (cold to touch), and seizures (irregular clonus of head and fore and hind limbs) were evident by approximately 20 min post dosing. Significant increases in serum glucose concentrations were detected in the PTZ-treated rats when compared to controls (A). Using the Abcam Fireplex™ Multiplex Rodent Discovery miRNA Toxicity Panel, PTZ-induced acute seizures in rats were characterized by significantly decreased expression level changes in miR-106b-5p in cerebral cortex (B) and serum (C). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (when compared to concurrent control).

When compared to those derived from animal models and/or animal-derived cell systems, miRNAs from iPSC-derived human neuronal models which may have greater potential for translation to human serum-based biomarkers. Interestingly, profiling of secreted miRNAs as novel biomarkers of neurodegeneration in CDI's iPSC-derived human neurons following acute (24 h) exposure to selected neurotoxicants has been reported [33]. Lessons learned from these studies included leveraging human iPSC-derived neurons at well-characterized stages of decline in morphology as indicated by high-content imaging analysis of neurite outgrowth parameters and cell viability (ATP release assay on replicate plates). Hence, CDI's human iPSC-derived neurons were treated at 2 h post-plating with 0.1–100 μM of neurotoxicants (including but not limited to bisindolylmaleimide I, colchicine, doxorubicin, paclitaxel and rotenone) or acetaminophen (negative control). Acute miRNA expression level changes were determined from conditioned cell culture medium, while adhered cells were analyzed using high-content imaging analysis. The low compounds concentrations had no/minimal effect on cell viability but with a significant decrease in neurite outgrowth, while the high compound concentrations induced significant decreases in both endpoints. No effect on any endpoint was observed with acetaminophen. Where relevant, neurotoxins that induced at least a 2-fold change in miRNA expression were potentially related to a decrease in neurite outgrowth, with further clustering based on association to cell viability, yielding: (1) no effect, (2) significantly decreased, or (3) independent of effect; indicating a relationship of miRNAs to progressive stages of neurodegeneration. While further investigations are warranted, the miRNAs profiles identified in cell culture medium of treated CDI's iPSC derived neurons may have greater translation to human serum-based biomarkers than those derived from either rodent cell lines and/or animal models of neurodegenerative disease(s). The usefulness of monitoring changes in miRNA profiles from 3D iPSC-derived neuronal models (e.g., Stemonix's 3D MicroBrains) has not been reported.

8. Summary

In vitro models support risk assessment in drug discovery and development. While, the extrapolation of toxicity data from animal models to humans is imperfect due to differences in anatomy and physiology, these traditional models are used because they represent whole-organism

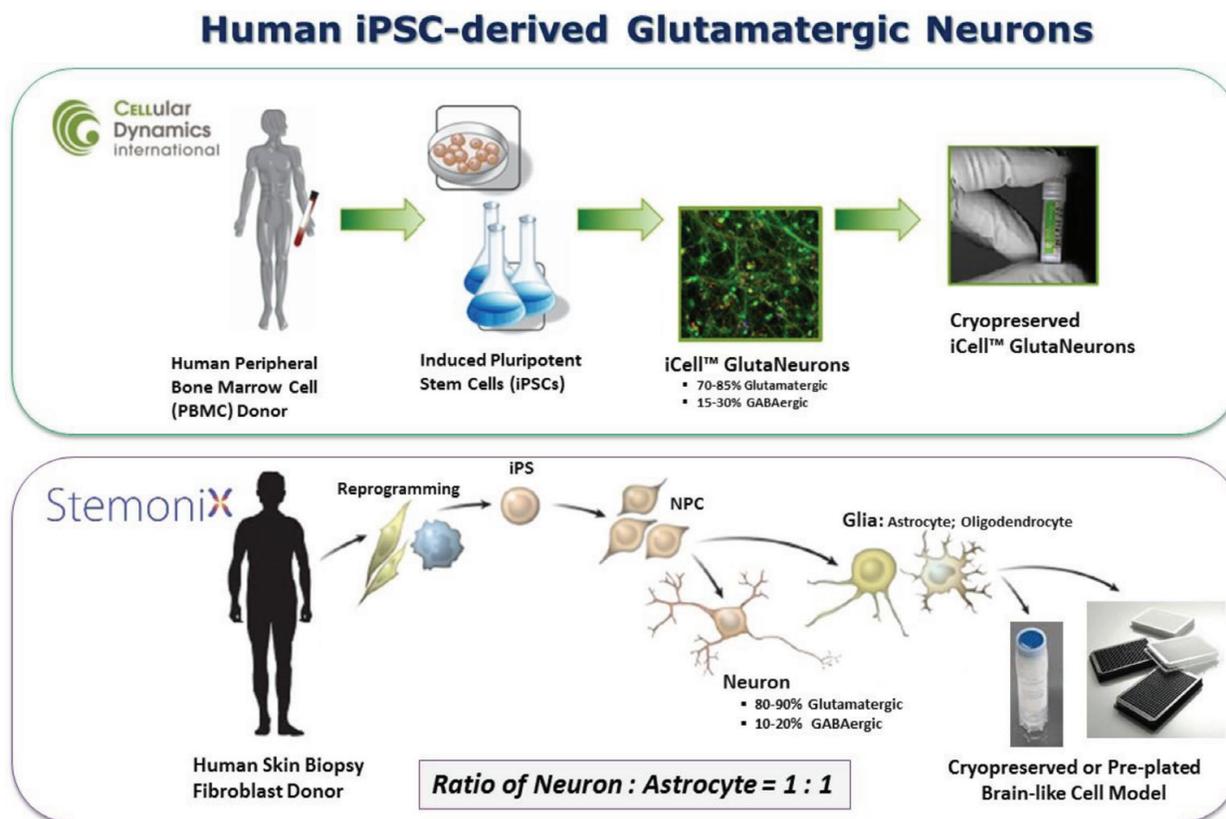


Figure 14. Adapted cartoons of the Human iCell® GlutaNeurons (Cellular Dynamics International) and Human MicroBrain® model which contains a balanced co-culture of cortical neurons and astrocytes (StemoniX).

biology that is not well replicated by *in vitro* methods. Increasing pressure to Replace, Reduce, and Refine the use of animal models in toxicity testing has propelled the use of *in vitro* systems to characterize the cellular and molecular mechanisms underlying biological changes associated with neurotoxicant exposure. The availability of iPSC-derived human GlutaNeuron cell models (Figure 14) provides relatively easy access to previously unattainable cell types from the human CNS system, which not only offer a unique source of neuronal cell types for candidate drug screening, but also provide new platforms to enable addressing the limitations of conventional animal models and primary cell cultures commonly used for mechanistic assessment of potentially translatable inducible excitotoxicity in drug discovery.

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