Establishing an In Vitro Assay for Predicting Neurotoxicity Risks Using Functional Human iPSC-Derived Neurons

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1. Abstract

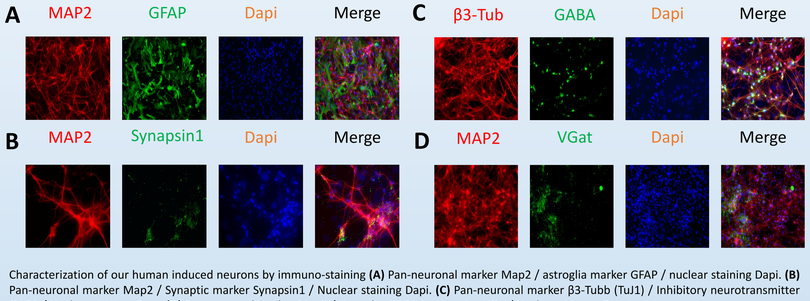
Human induced pluripotent stem cell (iPSC)-derived neural cultures show great promise in serving as an alternative to animal models and primary cultures for neurotoxicity testing. Here we present a human in vitro assay system for risk prediction of adverse effects of chemical compounds.

Based on direct reprogramming of iPSCs into highly functional neurons of defined subtypes (induced neurons, iNs) we developed a pure human neuronal/glial co-culture platform by combining defined ratios of glutamatergic and GABAergic iNs together with astroglial cells on multielectrode arrays (MEAs). These co-cultures showed robust neuronal activity, including synaptically driven spontaneous network synchronized burst firing (SBF) at 3-4 weeks after seeding. Using quantitative analysis of SBF parameters such as MF (Max Frequency), coefficient of variation (CV) of MF, CV of IMFI (Inter MF Interval) and following principal component analysis, our platform allowed the characterization of neurotoxicity effects of test compounds. By comparing the optimized SBF of each tested compound to the standard deviation (SD) of SBF for negative control compounds, we could predict adverse effects in a relative scale, such as low risk for lower than 1x SD, medium risk for 2x SD, and high risk for 3x SD. For drug development, this predictability can inform decision making on further compound optimization or dose selection.

As part of the HESI NeuTox Group, we tested a set of 12 compounds with clinically reported seizurogenic and other neurological adverse effects in patients, which were in part undetected in rodent-based preclinical testing for neurological safety assessment. Importantly, our human system was able to not only identify specific alterations in neuronal activity of all test compounds in a dose-dependent manner but to also determine seizure-like firing patterns. For instance, Chlorpromazine showed seizurogenic neurotoxicity in human at plasma level of 1.9 µM. Our system predicted its neurotoxicity as medium risk at 1.0 µM and high risk at 3.0 µM.

Hence, we report the development and proof of concept of a novel iPSC-derived neuronal/glial in vitro approach for the quantitative assessment of neurological liabilities of chemical compounds in a medium-throughput setting.

2. SynFire iN Cell Technology

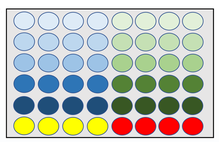


We combined our iN technology with human primary astroglial supporter cells on 48-well MEA plates to develop a pure human neural co-culture system consisting of glutamatergic excitatory neurons (140K cells/well), GABAergic inhibitory neurons (60K cells/well), and astrocytes (70K cells/well). Reproducible formation of spontaneous synchronized neuronal network activity could be detected 3-4 weeks after plating.

3. Experimental Design

After seeding, co-cultures were allowed to mature for 21-22 days. Spontaneous neuronal activity was assessed on an Axion Maestro system as follows: baseline activity was recorded for 15 min following an equilibration period of 20 min. Then, a set of 11 proconvulsant compounds, 2 negative controls, and compound solvent (DMSO 0.1%) were added to individual wells according to the following plate layout. Neuronal responses after dosing were recorded for 75 min.

A



DMSO 0.1%
Positive Control: BIC 3uM
Compound 1 [1-5 Doses]
Compound 2 [1-5 Doses]

A. Single Plate Assay (48-well MEA plate). Two compounds per plate, 5 concentrations, 4 replicates. DMSO and one positive control, 4 replicates each, were also included. **B. Test Compounds Table** showing the concentrations used per compound and their modes of action.

B

Compounds	Solvent	Concentrations (uM)	Mode of Action	Seizurogenic
Picrotoxin (PTX)	DMSO	0.3, 1, 3, 10, 30	antagonist of $GABA_A$ receptors	Yes
Pentylenetetrazol (PTZ)	DMSO	10, 30, 100, 300, 1000	antagonist of $GABA_A$ receptors	Yes
Pilocarpine	DMSO	0.3, 1, 3, 10, 30	muscarinic receptor agonist	Yes
Linopirdine	DMSO	1, 3, 10, 30, 100	selective KCNQ channel blocker	Yes
4-Aminopyridine (4-AP)	DMSO	0.3, 1, 3, 10, 30	potassium channel blocker	Yes
Chlorpromazine	DMSO	0.1, 0.3, 1, 3, 10	D2 receptor antagonist	Yes
Amoxapine	DMSO	0.3, 1, 3, 10, 30	blocks dopamine receptors, and the reuptake of norepinephrine and serotonin	Yes
Phenytoin	DMSO	1, 3, 10, 30, 100	sodium channel blocker	No
Maprotiline	DMSO	0.1, 0.3, 1, 3, 10	inhibits reuptake of norepinephrine	Yes
Clozapine	DMSO	0.1, 0.3, 1, 3, 10	targets D2 receptors and an antagonist of 5-HT_{2A} receptor	Yes
Amoxicillin	DMSO	1, 3, 10, 30, 100	inhibitor of bacterial wall biosynthesis	No
Acetaminophen	DMSO	1, 3, 10, 30, 100	COX-1 and 2 inhibitor (prostaglandin production blocker)	No
Bicuculline (BIC)	DMSO	3	antagonist of $GABA_A$ receptors	Yes
DMSO	Vehicle	0.1%	-----	No

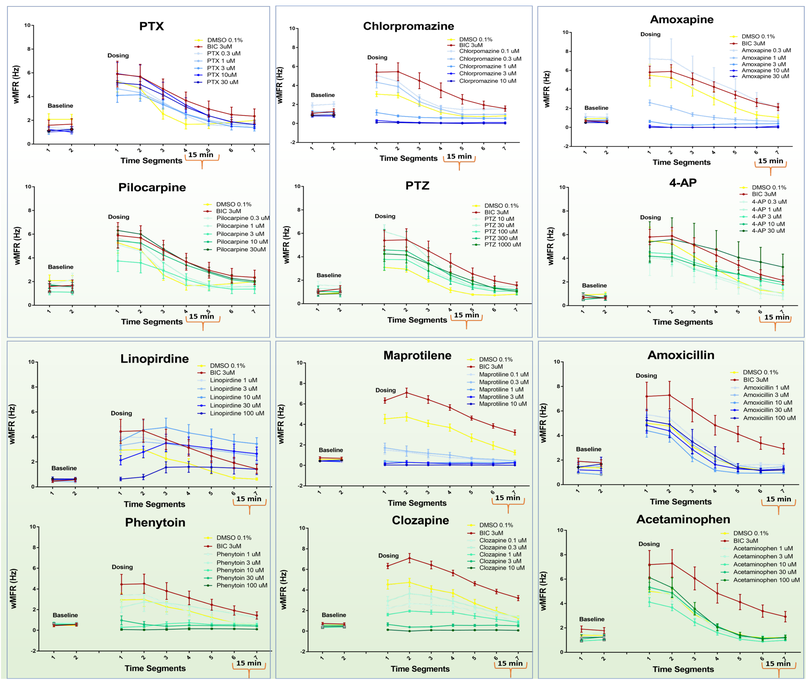
4. Human iN/glia Co-culture Characterization

Characterization of our human induced neurons by immuno-staining **(A)** Pan-neuronal marker Map2 / astroglia marker GFAP / nuclear staining Dapi. **(B)** Pan-neuronal marker Map2 / Synaptic marker Synapsin1 / Nuclear staining Dapi. **(C)** Pan-neuronal marker β3-Tubb (TuJ1) / Inhibitory neurotransmitter GABA / Nuclear staining Dapi. **(D)** Pan-neuronal marker Map2 / Vesicular GABA transporter VGat/ Nuclear staining Dapi.

5. Results

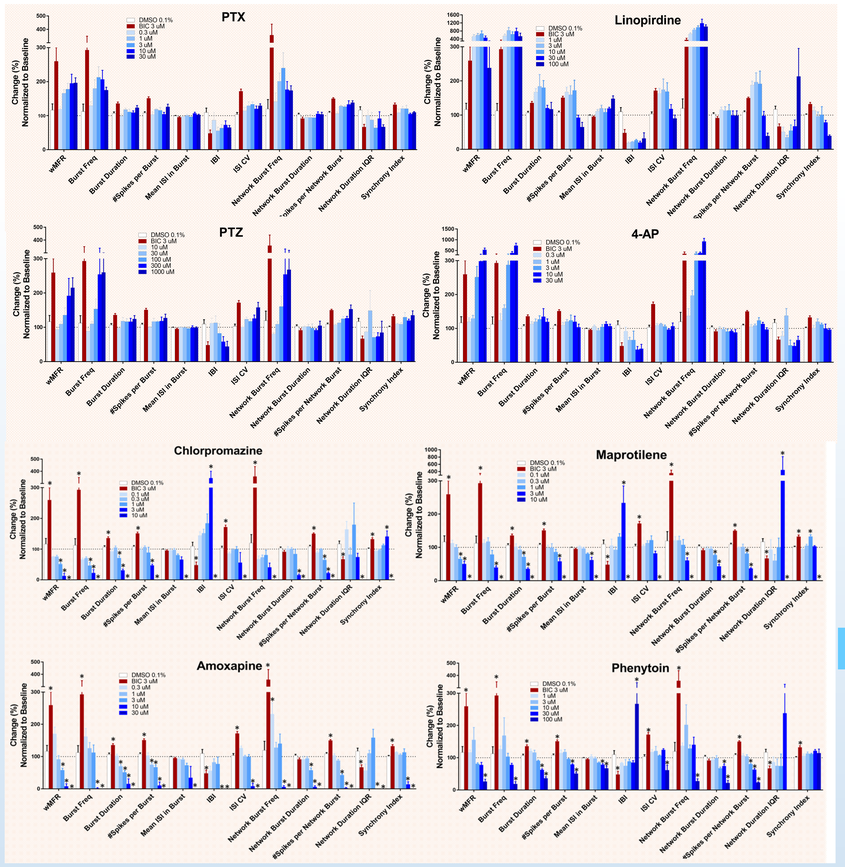
5A. Data Binning Analysis

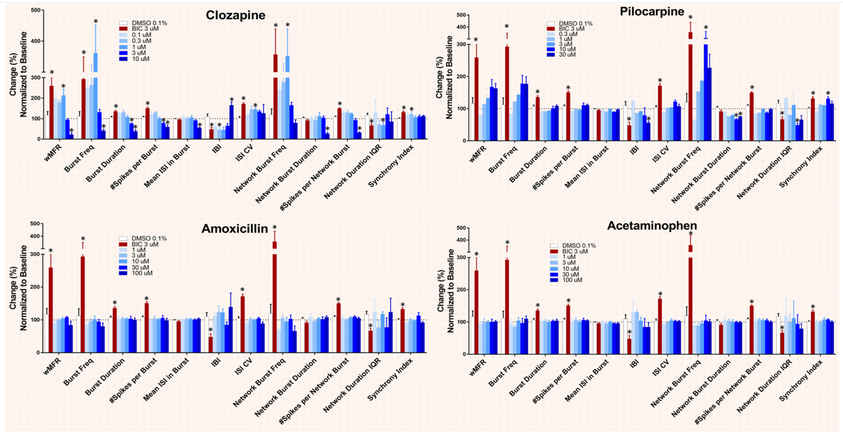
After processing baseline (15 min) and dosing (75 min) recordings using the AxIS software, neuronal activity measurements inform of weighted mean firing rates (wMFR) values were binned into 10 min segments across both baseline and dosing periods for each tested compound. This analysis allowed for detection of the time window in which the vehicle DMSO has equilibrated after dosing. A 15 min time period after equilibration was used for further analysis (normalization to baseline and comparisons to controls)..



5B: Electrophysiological Responses of 12 Compounds for 12 MEA Parameters Recorded in Human SynFire iN/glia Co-cultures

For each MEA parameter, measurements from vehicle- or compound-treated wells were normalized to their respective baseline values. All parameters are expressed as percent change.





5C. Toxicity Prediction Using Optimized Synchronized Burst Firing (SBF) Signal

We have adopted a data processing method based on optimized SBF signals (Matsuda et al 2018, BBRC). MF (Max Frequency), CV of MF, and CV of IMFI (Inter MF Interval) of SBF were measured and used for burst analysis and principal component analysis (PCA)

SBF signal optimization

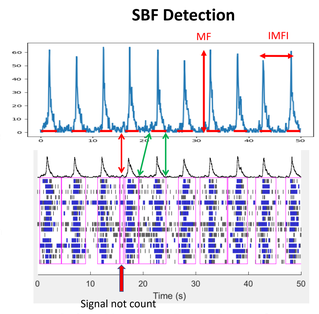
- Spikes with Inter-spike interval of 16 ms or less were considered to belong to the same SBF
- For SBF with number of spikes under 5 spikes/SBF, the data sets were eliminated to reduce noise
- SBFs were combined if the inter-SBF interval was under 240 ms
- The similar activities were counted as one SBF if the number of spikes in an SBF was over 100 spikes/SBF.

PCA analysis

- Measure SBF parameters MF (Max Frequency), CV of MF, CV of IMFI (Inter MF Interval) in 15 min segment
- Normalized to Baseline
- Performed PCA to obtain PC1 and PC2
- Computed mean of replicates
- Computed Euclidean distance between each testing compound and DMSO (solvent, negative control) in measurements of SDs

Toxicity threshold

Low Risk ≤ SD; Medium Risk: > SD but ≤ 2SD; High Risk > 2SD



SBF Detection shows more accurate network burst numbers and duration

5D. Highly Consistency Between in vitro Toxicity in iN/glia Co-cultures and in Human/Animal Neurotoxicity

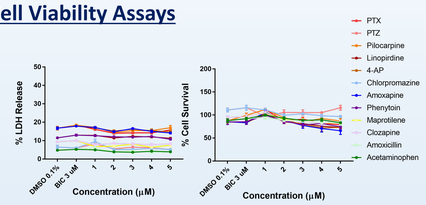
Compound	0.1uM	0.3uM	1uM	3uM	10uM	30uM	100uM	300uM	1000uM	Neurotoxicity Events
4-Aminopyridine			1.1							Psychosis
Acetaminophen									331	Nontoxic
Amoxapine			1.6							Seizurogenic
Amoxicillin								181		Psychiatric effects at high dose
Bicuculline*			2*							Seizurogenic, Convulsant
Chlorpromazine			1.9							Seizurogenic
Clozapine				3.1						Agranulocytosis, CNS side effects
Linopirdine*					10*					Seizurogenic in brain slice
Maprotilene			1.5							Seizures at high dose
Picrotoxin*		0.6*								Seizurogenic
Metrazol(PTZ)*									333*	Seizurogenic in mice at high dose
Phenytoin						79				Nystagmus and Sedation

Neurotoxicity scale by MEA measurement: Low Risk | Medium Risk | High Risk

Human/Animal toxic plasma concentration (µM): 333

* Animal data used

5E. Cell Viability Assays



% LDH Release and % Cell Survival per compound across concentrations. No significant differences when compared to vehicle DMSO.

6. Summary

- 12 test compounds proposed by HESI were evaluated by our neurotox assessment assay on hiPSC-derived SynFire iN/glia co-cultures. Compounds with neurotoxicity in humans and animals also showed in vitro toxicity on the NeuCyte platform. Our neurotox platform was capable of quantitatively predicting neurotoxic risk of tested compounds in a medium-throughput manner
- Our results indicated that a novel efficient neurotox assessment platform has been developed by combining NeuCyte's SynFire neurons with Axon MEA technology, which can provide an alternative way to evaluate neurotoxicity *in vitro*. We expect this new testing platform would significantly reduce the cost to assess chemical compound's neurotoxicity at early stage of drug discovery.