New iPSC-Based Neural *In Vitro* Approach for Seizure Liability Testing

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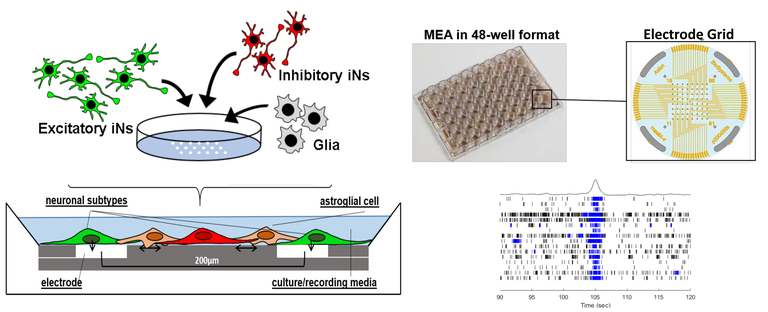
1. Abstract

Standard animal models have shown insufficiencies in predicting adverse effects of therapeutic compounds particularly on the CNS, mostly because of limited concordance with human neurotoxicity. The high attrition rate of new drugs in clinical studies is largely attributed to CNS-related safety failures unidentified in pre-clinical testing. One of the most common issues encountered during safety assessment over the past 5 years is the induction of seizures at relevant therapeutic concentrations. Moreover, $GABA_A$ receptors as primary mediators of inhibitory neurotransmission, represent a vulnerable target for neurotoxic environmental chemicals, many of which lead to seizures in humans. Therefore, new human *in vitro* assays are urgently needed for de-risking of new drugs and higher throughput testing of multitude of chemicals with daily human exposure. Based on direct reprogramming of induced pluripotent stem cells (iPSCs) into highly functional neurons of defined subtypes, we developed a pure human neuronal/glial co-culture platform for comprehensive electrophysiological measurements using multi-electrode arrays (MEAs). When combining defined ratios of glutamatergic to GABAergic neurons together with astroglial cells, robust neuronal activity, including synaptically driven spontaneous synchronized network bursting can be recorded at 3-4 weeks post seeding. Due to parallel acquisition of multiple parameters, our platform allows detailed characterization of neurotoxicity effects of test compounds. Here, we optimized our platform to specifically and quantitatively assess chemically-induced seizure-like activity in a semi high-throughput setting. As part of the HESI NeuTox Group, we tested a set of 11 compounds with clinically reported seizurogenic effects in patients, which partially remained undetected in rodent-based testing. Importantly, our human system was able to identify specific alterations in neuronal activity of all test compounds in a dose-dependent manner, and determine seizure-like firing patterns in the most potent subset. We also used our platform to test a set of environmental neurotoxicants with well-established effects on human $GABA_A$Rs, and successfully identified changes in activity indicative of seizurogenic effects over a large range of concentrations. Hence, we report the development and proof of concept of a novel iPSC-based neuronal/glial *in vitro* approach for the assessment of seizure liabilities of chemical compounds in a human-relevant cell context.

2. SynFire iPSC-Derived Neural (iN) Cell Technology



We combined our iN technology with human primary glial 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 can be detected 3-4 weeks after plating.

3. Experimental Design

After seeding, cultures were allowed to mature for 21-22 days. Spontaneous neuronal activity was assessed by using the Axion Maestro system as follows: baseline activity was recorded for 15 min, right after an equilibration period of 20 min. Then, a set of 11 proconvulsant compounds, 2 negative controls, and the vehicle in which all the test compounds were dissolved (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]

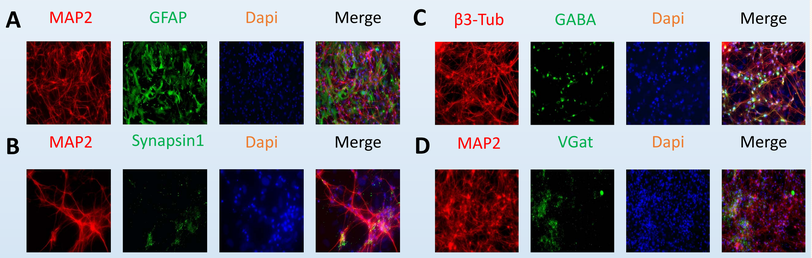
A. Single Plate Assay (48-well MEA plate). Two compounds per plate, 5 concentrations, 4 replicates. DMSO and one positive control, 4 replicates each, were also included.

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 Cell Characterization

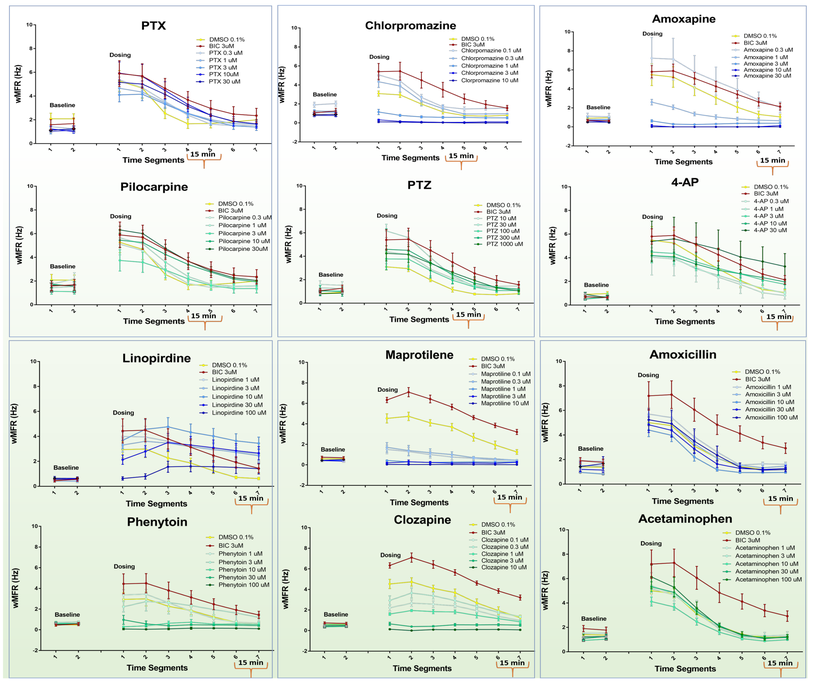


Characterization of our human induced neurons by immuno-staining **(A)** Pan-neuronal marker Map2 / Astroglia marker GFAP / Nuclear staining Dapi. **(B)** Pan-neuronal marker Map2 / Synaptic marker Synapsin1 / Nuclear staining Dapi. **(C)** Pan-neuronal marker β3-Tubb (TuJ1) / Inhibitory neuron $GABA_A$ receptor, α1 / Nuclear staining Dapi. **(D)** Pan-neuronal marker Map2 / Vesicular GABA transporter VGat/ Nuclear staining Dapi.

5. Results

5a. Data Binning Analysis

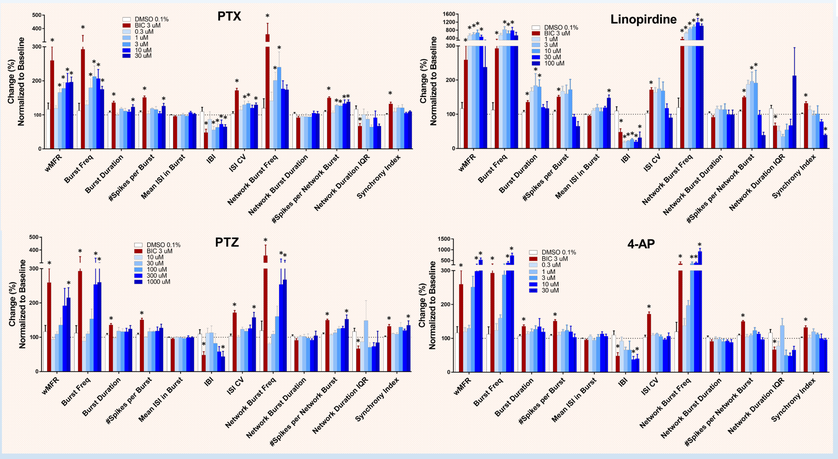
After processing the baseline (15 min) and the dosing (75 min) recordings using the AxIS software, the wMFR values were binned into 10 min segments across both baseline and dosing periods for each test compound. This analysis allows for detection of the time window in which the vehicle DMSO has equilibrated after dosing. That period (15 min) was used for compound normalization and comparisons against baseline.

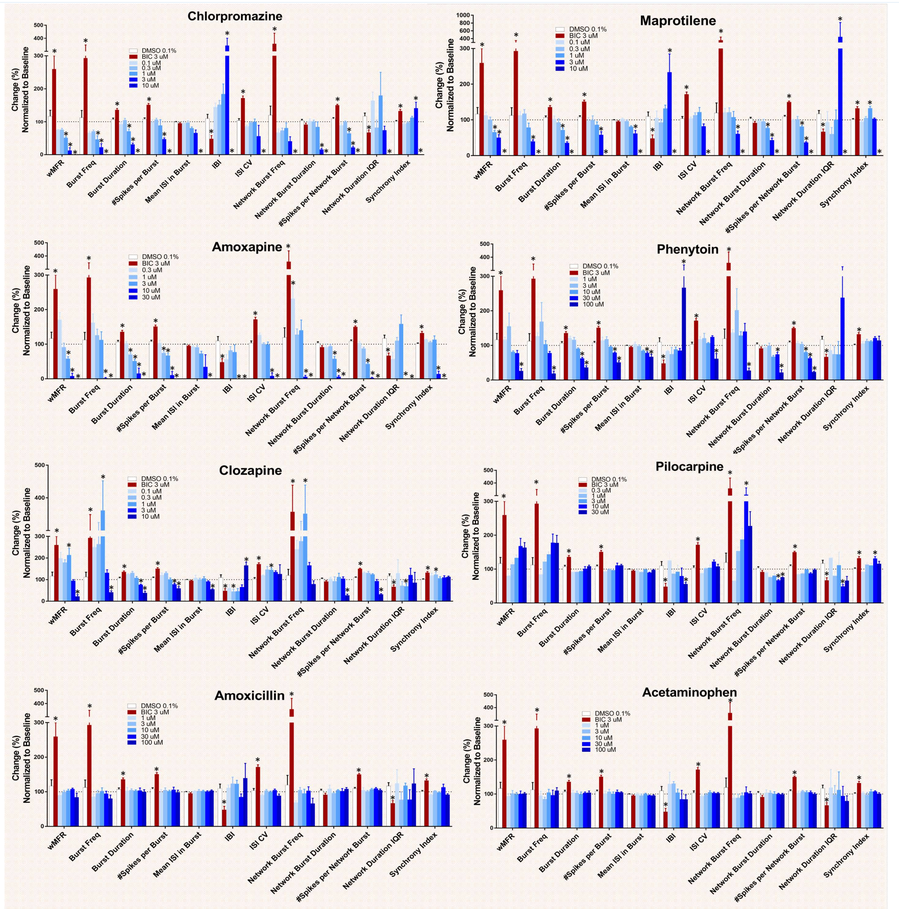


5B. Neural Responses to Test Compounds: Analysis by MEA Parameters

MEA Parameter	Description
Weighted mean firing rate (wMFR)	Total number of spikes divided by the total time of the analysis, and normalized against the number of active electrodes
Burst frequency	Total number of single-electrode bursts divided by the duration of the analysis
Burst duration	Average time from the first spike to last spike in a single-electrode burst
Number of spikes per burst	Average number of spikes in a single-electrode burst
Mean inter-spike-interval (ISI) within burst	Mean inter-spike interval (time between spikes) in a single electrode burst
Inter-burst-interval (IBI)	Average time between the start of single-electrode bursts (time between bursts)
ISI Coefficient of Variation	The coefficient of variation (standard deviation/mean) of the inter-spike interval, the time between spikes, for an electrode. This is a measure of spike regularity and bursting
Network burst frequency	Total number of network bursts divided by the duration of the analysis
Network burst duration	Average time from the first spike to last spike in a network burst
Number of spikes per network burst	Average number of spikes occurring in a network burst
Network Normalized Duration IQR	Interquartile range of network burst durations. This metric provides a measure of network burst duration regularity.
Synchrony Index	A unitless measure of synchrony between 0 and 1. Values closer to 1 indicate higher synchrony.

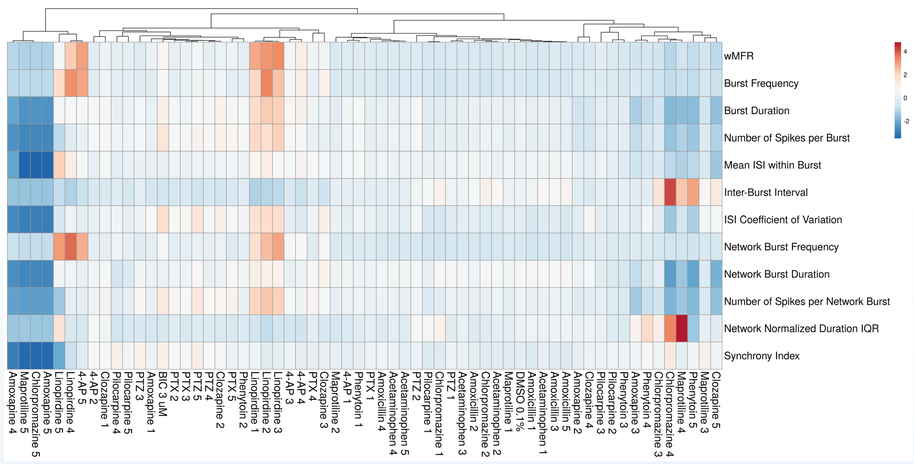
Standard parameters for assessment of neuronal network activity assessed in this study are depicted in the table. Recordings were performed on Axion's Maestro system using the AxIS software. Acquired raw data was processed using the following settings: Neural Broadband as Analog Mode setting, Butterworth filter (high pass 200 Hz, low pass 5KHz) as Digital Filter setting, Adaptive Threshold Crossing of 8 SD as Spike Detector setting, ISI Threshold as Burst Detector setting, and Envelope algorithm as Network Burst setting.





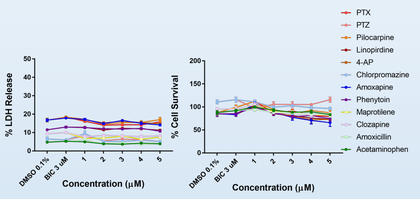
For each MEA parameter, measurements from vehicle- or compound-treated wells were normalized to their respective baseline values. All parameters are expressed as percent change. Significance for Bicuculine (+ Ctrl) relative to DMSO (Vehicle) was determined via Student's T-test (n = 4, *p<0.05). Significance for test compounds relative to DMSO (Vehicle) was determined via One-Way ANOVA (n = 4, *p<0.05).

5c. Clustering Analysis



Heat Map and Clustering Analysis: MEA Parameters (rows) are centered; unit variance scaling is applied to rows. Compounds in increasing doses (columns) are clustered using correlation distance and average linkage.

5d. Cell Viability Assays



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

6. Summary

- NeuCyte's SynFire neural cells allow for a fully defined human neural co-culture which is ideal for in vitro neurotox assessment.
- Upon exposure to the Hesi Neutox MEA group test compounds, our neural co-culture system show reproducible and robust dose dependent changes in spike rates, bursting and network firing parameters. These changes were not seen in the negative control compounds acetaminophen and amoxicillin.
- By focusing on a select set of network firing parameters we have begun to identify firing patterns resembling ictal discharges as they occur during status epilepticus.
- Clustering of the data allows for grouping of compounds by firing patterns.
- More seizuregenic and non-seizuregenic compounds with well defined modes of action need to be tested to further optimize our seizure prediction capacity.