In Vitro Neurotoxicity Testing Using Functional Human iPSC-Derived Neurons

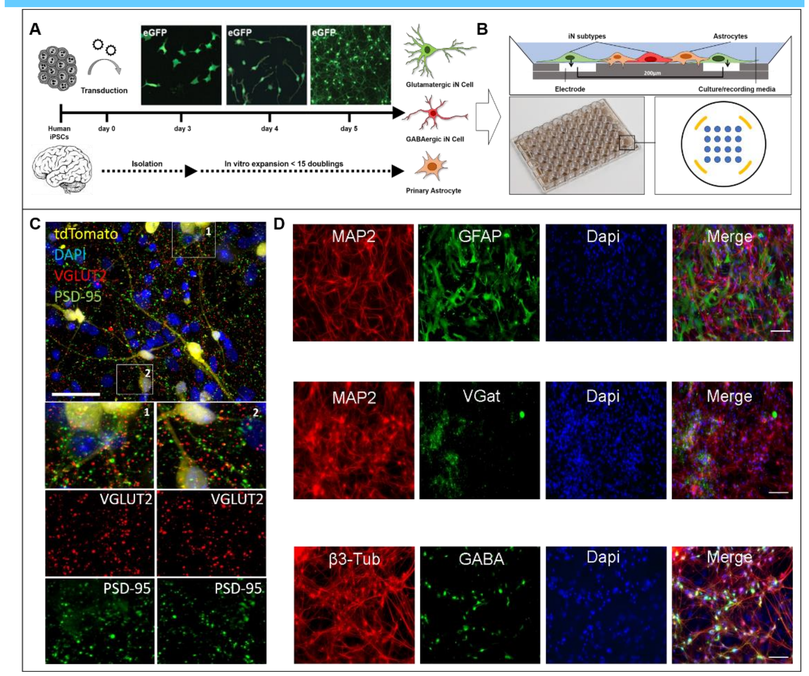
H Liu[1], L Saavedra[1], K Wallace[2], T Freudenrich[2], T Portmann[1], J Davila[1], TJ Shafer[2], M Wernig[3], and D Haag[1]

[1]NeuCyte Inc., San Carlos, CA, [2]ISTD, NHEERL, ORD, US EPA, [3]Institute for Stem Cell Biology and Regenerative Medicine, Dept of Pathology, Stanford University School of Medicine, Stanford, CA

1. Abstract

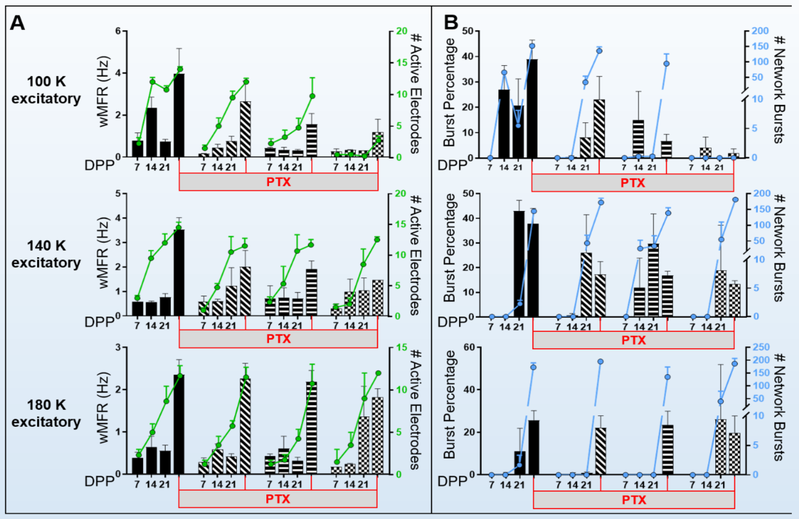
We here describe a new screening platform using highly functional neural cultures with defined cell ratios consisting of glutamatergic excitatory and GABAergic inhibitory neurons that were separately generated by direct conversion from human iPSCs (NeuCyte SynFire®), as well as primary human astroglial cells. Such neuron/glia co-cultures showed rapid development of pronounced neuronal activity and robust formation of synchronized network activity mediated by synaptic transmission. To evaluate the applicability of this new platform for neurotoxicity screening, we measured acute effects of neurotoxic test compounds (GABAA receptor antagonists, organotin, and pyrethroid insecticides) on network activity and compared responses to rat primary cortical cultures. Importantly, we observed largely corresponding dose-dependent alterations in firing rate, bursting and synchrony metrics in iPSC-derived human neuron/glia and rat primary cultures. These results demonstrate the utility of this direct-differentiated human model for functional neurotoxicity screening using MEAs. (This abstract does not reflect EPA Policy).

2. NeuCyte's Human iPSC-Derived Neural Co-Cultures



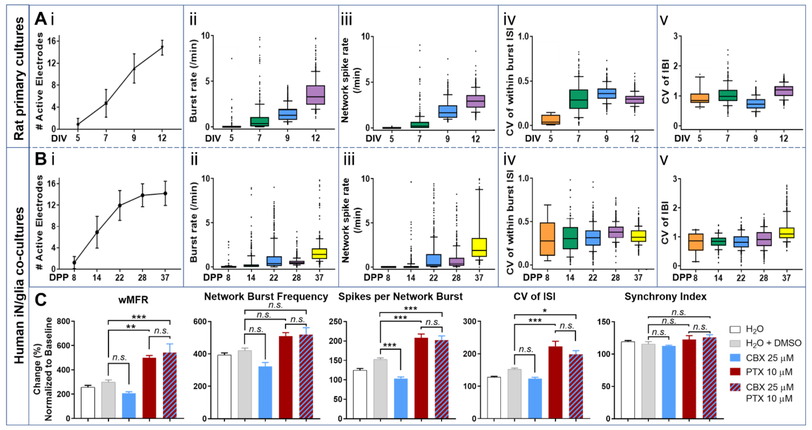
Pure human-derived neural co-cultures for neurotoxicity assessment. (A) Schematic workflow for direct conversion of iPSCs into excitatory or inhibitory neurons using lentiviral delivery of neurogenic transcription factors (upper panel). Primary astroglial cells are expanded in vitro (lower panel). (B) Schematic representation of induced neuron (iN)/glia co-cultures grown on 48-well MEA (multielectrode array) plates. (C) Mixed excitatory/inhibitory iN co-culture on astrocytes containing a small fraction of tdTomato-expressing iN cells. Proximity of the pre- and post-synaptic markers VGLUT2 and PSD-95 indicated formation of functional synapses. Boxes 1 and 2 show selected regions for higher magnification images below. (D) Characterization of human-derived neuron/glia co-cultures by immunofluorescence imaging. Upper panel: Pan-neuronal marker microtubule associated protein 2 (MAP2), astroglial marker glial fibrillary acidic protein (GFAP), and nuclear DAPI staining showed non-overlapping and homogenous distribution of neurons and astroglial cells. Middle panel: Pan-neuronal marker MAP2, vesicular GABA transporter (VGat), and nuclear DAPI staining showed presence of inhibitory cells as a fraction of co-cultured neurons. Lower panel: Pan-neuronal marker class III β-tubulin (TuJ1), inhibitory neurotransmitter GABA, and nuclear DAPI staining showed the presence of inhibitory cells as a fraction of cultured neurons. Scale bar = 50µm.

3. Optimizing Cell Ratios and Densities for Neural Network Activity Readouts



Comparison of different iN cell densities and glutamatergic excitatory to GABAergic inhibitory ratios based on the development of neural activity over time (7, 14, 21 DPP) and after network activation on MEAs. (A) Neuronal spiking (weighted mean firing rate, wMFR) and number of active electrodes showed a steady increase over time and an additional rise after network activation (PTX) for both 100K and 140K excitatory cell densities co-cultured with 30-40% inhibitory neurons. (B) At DPP 21, high percentages of spikes occurring within a burst (burst percentage) with the high numbers of total network bursts are observed for 140K excitatory cells densities co-cultured with 30-40%, 40-50%, and 50-60% inhibitory neurons. Out of these conditions, 140K excitatory with 30-40% inhibitory neurons showed the largest increased in coordinated network activity upon PTX treatment.

4. Neural Network Ontogeny and Synapse-Mediated Network Activity



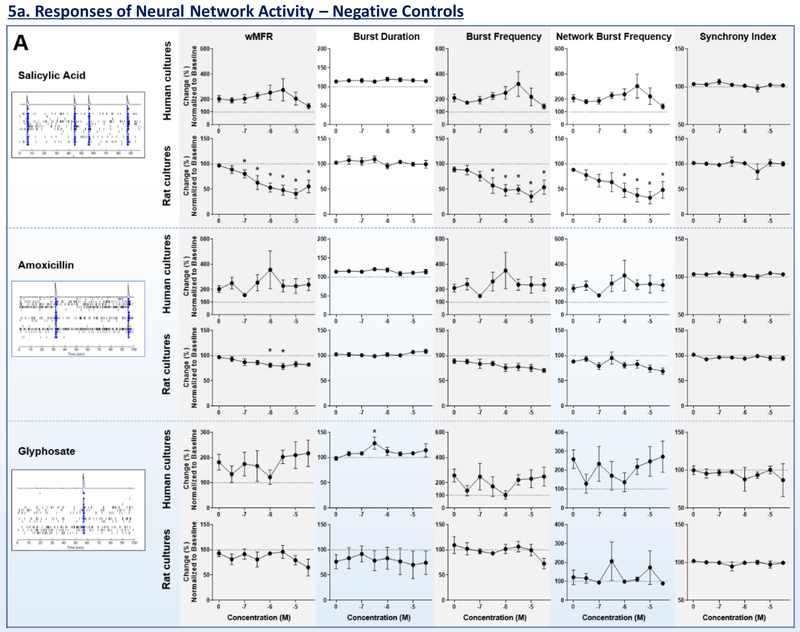
Characteristics of neural network development in rat primary cortical cultures and human iN/glia co-cultures. (A) MEA measurements of network ontogeny in rat primary cultures showed a steady increase of active electrodes, burst rate, and network burst rate (i-iii) for the first 12 days in vitro (DIV). The coefficient of variation (CV) of inter-spike intervals (ISI) occurring within bursts (iv) plateaued at around DIV 9. The CV of the inter-burst intervals (IBI) slightly increased over time and reached a maximum at DIV 12 (v). (B) MEA measurements of network ontogeny in human iN/glia co-cultures displayed an increasing number of active electrodes (i) along maturation until reaching a maximum at 28 days post plating (DPP). Burst rates and network burst rates (i, ii) showed a steady increase until DPP 37. The CV of within burst ISI (iv) appeared very heterogenous across wells and stabilized over time until DPP 37 at value of 0.33, similar to rodent cultures (Aiv). The CV of IBI (v) slightly increased during culture duration and reached a maximum at DPP37. (C) Coordination of neural network activity in human iN/glia co-cultures is independent of direct electrical coupling through gap junctions. Application of the gap junction blocker carbenoxolone (CBX) did not alter single neuronal spiking (wMFR) nor network burst frequency or synchrony. Treatment with PTX significantly increased single spiking and most network activity parameters (network burst frequency, spikes per network burst and CV of ISI). Co-application of CBX did not alter this evoked effect. Asterisks indicate significant mean differences with p-values (* 0.05, ** 0.01, and *** 0.005) determined by one-way AOVA (n=8) followed by Tukey's test for multiple testing.

5. Alteration of Neuronal Network Activity in Responses to Neurotoxic Chemicals in Human and Rodent Neural Cultures

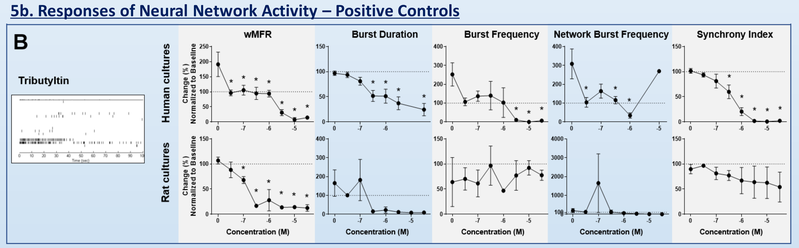
Table: Neurotoxic compounds for pilot study

Compound	CAS #	DTXS ID	Class	Effect	Solvent	Purity
Amoxicillin	26787-78-0	DTXSID303704	penicillin-class antibiotic	negative control	DMSO	≥90%
Salicylic acid	69-72-7	DTXSID7026368	non-steroidal anti-inflammatory drug	negative control	DMSO	≥99%
Glyphosate	38641-94-0	DTXSID0034649	organophosphorus herbicide	negative control	water	46 %
Bicuculline	485-49-4	DTXSID3042687	isoquinoline alkaloid	$GABA_A$ antagonists	DMSO/ethanol	≥99%
Picrotoxin	124-87-8	DTXSID7045605	convulsant alkaloid	$GABA_A$ antagonists	DMSO	98%
Lindane	58-89-9	DTXSID2020686	organochloride insecticide	$GABA_A$ antagonists	ethanol	99%
Dieldrin	60-57-1	DTXSID9020453	organochloride insecticide	$GABA_A$ antagonists	DMSO	≥95%
Permethrin (shown in section 6)	52645-53-1	DTXSID8022292	type I pyrethroid insecticide	modulation of Voltage-sensitive sodium channel (VSSCs) kinetics	DMSO/ethanol	≥91%
Deltamethrin (shown in sec. 6)	52918-63-5	DTXSID8020381	type II pyrethroid insecticide	prolonged modulation of VSSCs kinetics	DMSO/ethanol	≥98%
Cypermethrin (shown in sec. 6)	52315-07-8	DTXSID1023998	type II pyrethroid insecticide	prolonged modulation of VSSCs kinetics	DMSO/ethanol	≥98%
Esfenvalerate (shown in sec. 6)	66230-04-4	DTXSID4032667	Type I/II pyrethroid insecticide	intermediate modulation of VSSCs kinetics	DMSO/ethanol	98.5%
Tributyltin	56-36-0	DTXSID7043950	organotin biocide	oxidative stress	DMSO	96%

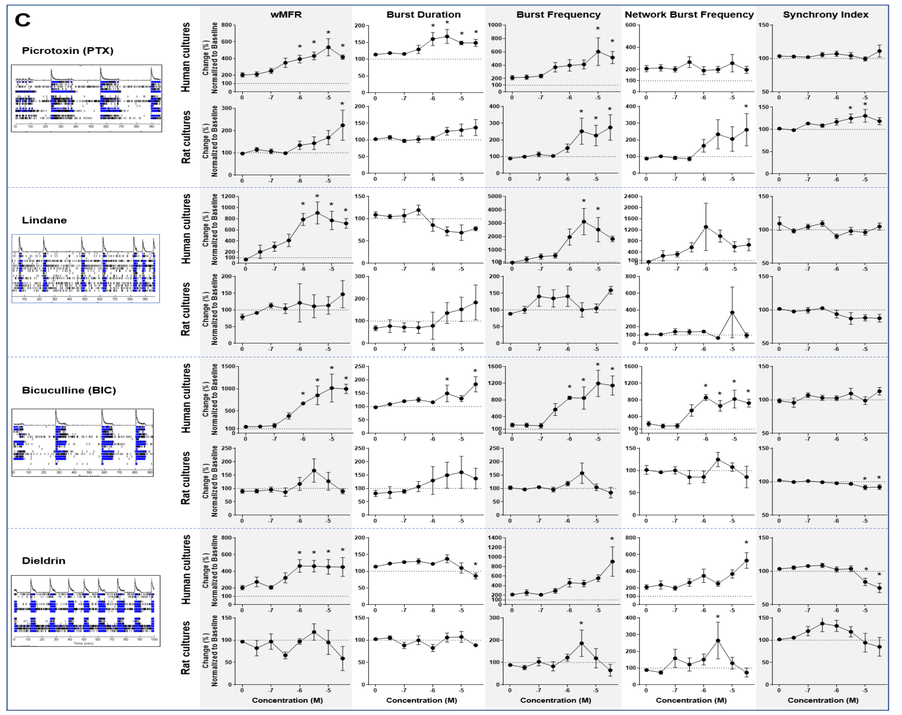
5a. Responses of Neural Network Activity – Negative Controls



5b. Responses of Neural Network Activity – Positive Controls

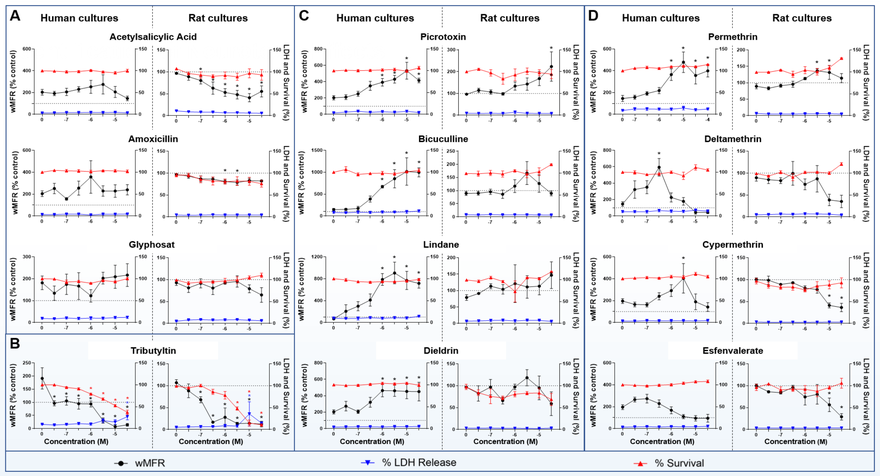


5c. Responses of Neural Network Activity – GABA Blockers



Alteration of neuronal network activity in human iN/glia co-cultures and rat primary cortical cultures in response to neurotoxic compound treatment on MEAs. Depicted activity parameters were selected to represent overall spike rates (wMFR) as well as bursting (burst duration, burst frequency) and network busting structures (network burst frequency, synchrony index). The left panels illustrate representative raster plots of dosed human iN/glia co-cultures. (A) Treatment with three non-neurotoxic negative control compounds (salicylic acid, amoxicillin, and glyphosate) did not change neural network activity in human iN/glia co-cultures except for an increase in burst duration at a single medium concentration of glyphosate. In rat cortical cultures, treatment with amoxicillin and glyphosate did not considerably alter network activity at any concentration. In contrast, salicylic acid showed a concentration-dependent decrease in spiking (wMFR) and bursting (burst frequency, network burst frequency). (B) Treatment with a neuro-cytotoxic control compound (tributyltin) impaired neuronal firing and network activity at medium to high concentrations in both human iN/glia co-cultures and rat cortical cultures. (C) Treatment with neurotoxicants that modulate $GABA_A$ receptor activity (picrotoxin (PTX), lindane, bicuculline, and dieldrin) showed an overall activating effect on neuronal spiking and network activity in both culture types with human iN/glia co-cultures exhibiting a generally higher sensitivity. In rat cortical cultures, dieldrin only showed a tendency for increased burst rates at a 3 µM concentration.

6. Functional and Structural Neurotoxicity



Separate detection of functional and structural neurotoxic effects of tested chemicals in human iN/glia co-cultures and rat primary cortical cultures. For ease of comparison neuronal spike rates (wMFR, black lines) are plotted together with cell viability measurements represented by cell survival (red lines) and LDH release (blue lines). (A) Treatment with non-neurotoxic negative control compounds (salicylic acid, amoxicillin, and glyphosate) did not impair cell viability in human or rat neural cultures. In human iN/glia cocultures, none of these compounds considerably altered neuronal activity whereas in rat cortical cultures, salicylic acid decreased activity in the medium to high concentration range (see Fig. 4A). (B) Treatment with a neuro-cytotoxic control compound (tributyltin) decreased cell viability and neural activity in a concentration-dependent manner in both human and rat neural cultures. (C) Treatment with neurotoxicants modulating $GABA_A$ receptor activity (PTX, bicuculline, lindane, and dieldrin) showed no significant effect on cell viability in human or rat neural cultures. In contrast, neuronal spiking was increased for all these chemicals at medium to high concentrations, except for dieldrin in rat cortical cultures. (D) Treatment with chemicals modulating VSSC kinetics (permethrin, deltamethrin, cypermethrin, and esfenvalerate) did not affect cell viability in human or rat neural cultures. In human iN/glia cocultures, permethrin, deltamethrin, and cypermethrin increased neuronal spiking either high or at medium concentrations, whereas esfenvalerate decreased spiking at higher doses. In rat cortical cultures, deltamethrin, cypermethrin, and esfenvalerate decreased neuronal spiking at high concentrations, whereas high doses of permethrin slightly increased spike rates. Asterisks indicate significant mean differences to vehicle control with a p-value ≤0.05 determined by one-way ANOVA (n=6) followed by Dunnett's multiple comparison test.

7. Conclusions

1. Neural network activity of human iPSC-derived iN/glia co-cultures on MEAs can identify neurotoxic effects of environmental chemicals
2. Distinct modes of action are reflected in specific changes of neural network activity
3. The compounds we tested demonstrated largely corresponding alterations of neural network activity in both human iPSC-derived neural cultures and rat primary cortical cultures (current standard at the EPA) in a dose-dependent manner
4. We observed somewhat higher sensitivity of human iPSC-derive neural cultures with the four GABA blockers