An effort to build an in vitro high-throughput screening for seizure liability based on calcium oscillation of human iPS-derived dopaminergic neurons with astrocyte

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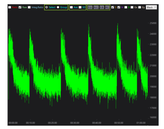
1. Introduction

Drug-induced seizure liabilities are typically tested in expensive and low throughput ex vivo rat hippocampal brain slices. An in vitro model based on micro-electrode arrays using human iPSC derived neurons has been suggested (https://doi.org/10.1093/toxsci/kfy029). There is a surge of interest in higher throughput methods, one of them being fluorescence measurement of calcium oscillations in neuronal cultures. However, progress has been limited by the long imaging acquisition time for an entire 96- or 384-well plate, and also by the heterogeneity of neurons derived from human iPSCs. With the advent of new camera technologies as well as availability of highly enriched, functionally mature human neurons derived from iPSCs, these limitations can now be overcome. Here we attempt to build a model to compare human iPSC derived neurons and astrocyte, with rat primary cells upon treatment of reference and control compounds.

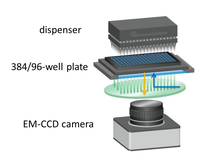
Here, we optimized the assay conditions for a calcium-sensitive fluorescent dye (Cal-520AM) to Ca2+ transients in respective neurons. The fluorescence measurements for a 384 plate were performed with 70 ms interval for 5 min at 37℃, in the high-throughput manner, using a camera-based kinetic plate reader, FDSS (Hamamatsu), which measures fluorescence signals of all 384 wells in a microplate simultaneously. The burst rates of neurons (Primary Rat neuron and Cellular Dynamics International iCell® Dopa neuron and iPS astrocyte) labeled with Cal-520AM, which were estimated from fluorescence signal. Then, we examined effects of 11 reference compounds including 4-AP, Picrotoxin on Ca2+ transient-waveforms to detect drug-induced waveforms changes and burst rate changes, etc. Such high-throughput fluorescence measurements of Ca2+ transient in primary or hiPSC derived neurons would be greatly useful for assessment of the seizure risk of compounds, particularly at the early stages of drug discovery..

FDSS/µCELL



200Hz Ca2+ oscillation data

Hamamatsu FDSS7000EX/µCELL: A Camera-based Kinetic Plate Reader



2. Material and Method

Compound	Function	Dose1	Dose2	Dose3	Dose4	Dose5
4-Aminopiridine(4-AP)	Seizurogenic Compounds – Potassium Channel Blocker	>0.3	1	>3	10	>30
Pentylenetetrazole(PTZ)	GABA antagonist,circulatory and respiratory	10	>30	100	>300	1000
Picrotoxin (PTX)	Toxin(GABA antagonist-Seizurogenic Compounds)	0.1	>0.3	1	>3	10
Linopirdine	acetylcholine releaser(CNS),release of acetylcholine and other neurotransmitters.	1	>3	10	>30	100
Pilocarpine	eye drop use(cholonergic)	>0.3	1	>3	10	>30
Strychnine	Glycine Receptor Antagonist-Seizurogenic Compounds)	>0.3	1	>3	10	>30
Amoxapine	antidepressant(CNS),inhibits 5-HT2 serotonergic receptors.	>0.3	1	>3	10	>30
Chlorpromazine(CPZ)	antipsychotic(CNS)	0.1	>0.3	1	>3	10
Phenytoin	anti-seizure	1	>3	10	>30	100
Amoxicillin	antibiotic	1	>3	10	>30	100
Acetaminophen	Negative compounds	1	>3	10	>30	100
DMSO	Vehicle	0.1	(-)	(-)	(-)	(-)

(µM)

Sample preparations

Rat primary neuron(E15), iCell® Dopa neuron and iPS Astrocytes (Cellular Dynamics International) were plated to the PEI-coated 384-wellplates (Corning) or Nanofiber plate(Nanofiber Solutions (Columbus, OH)). at 35,000 cells/well and incubated in 80 ul of the culture medium. On the day 14-21 after plating, the cells were loaded with fluorescent dyes for Ca2+ transient measurements.

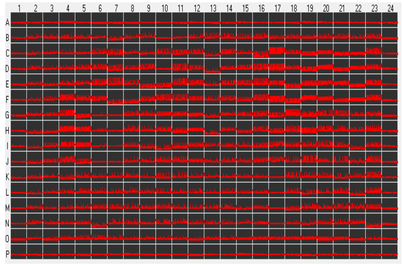
For measurements of Ca2+ transients, 50 µg of the dye (Cal-520 AM (AAT Bioquest)) was diluted in 45 µl DMSO and then in 20 ml of culture medium (CDI) containing 1.25 mM probenecid (Sigma-Aldrich) warmed at 37 ℃ (2 µM of dye final concentration). After the culture medium was removed, 72 ul of the dye solution was applied to the well, and incubated for 45 min at 37 ℃ in 5% CO2 . The measurements were performed with low Mg2+ buffer(0.1mM Mg2+). .

After one fluorescence measurement in the absence of test compounds (pre), 8 ul of each test compound (10 x final concentration) in PBS (-) was applied to the wells, and the cells were incubated for 10-45 min at 37 ℃ in 5% CO2. In this study, a library of pharmacologically active compounds (Office for University-Industry Collaboration, Osaka University, Japan) was used.

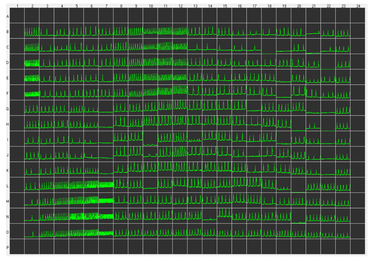
Fluorescence measurements and analysis

The fluorescence intensity changes of all wells in a microplate were taken simultaneously with every 70 ms for 5 min (4000 frames) at 37 ℃ using a kinetic plate reader, FDSS 7000EX or FDSS/uCELL (Hamamatsu). The intracellular Ca2+ concentration changes (calcium waveforms) were analyzed using the FDSS Waveform Analysis Software (Hamamatsu), which estimates peak rate, peak-to-peak time, peak width at 10%-90%, rising slope, falling slope, and more. All compounds were tested in N=4～5, 5 dose conditions. The result of each well was normalized with that of control (0.1 % DMSO).

3. FDSS Ca2+ oscillation data



FDSS Ca2+ oscillation image of Rat cortex neuron from 384 well Nanofiber plate



Ca2+ oscillation image of human iPSC-derived Dopa/Astrocytes co-cultured neuron (CDI) from 384well plate

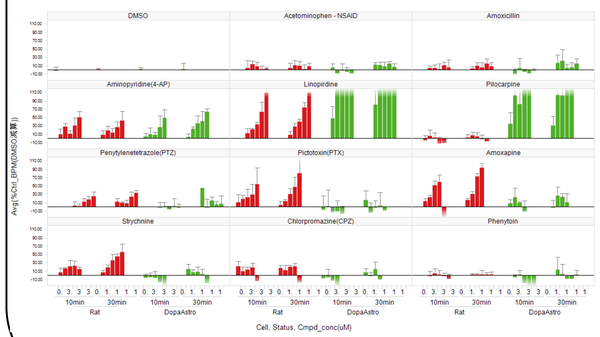
5. Result

Summary of effects of some reference compounds on Ca2+ transient-waveforms measured with fluorescent dyes in Rat Primary cortex neurons (E15) and human iPS-derived neurons.

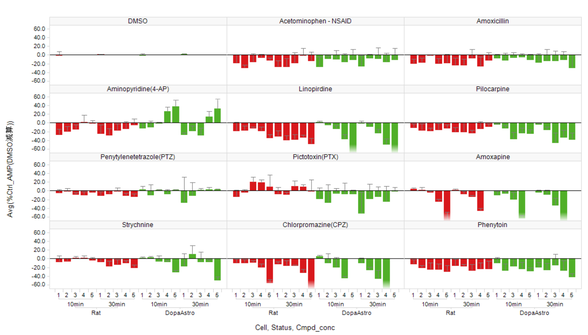
Compounds	Human clinical dose	Rat cortical neuron	Rat primary cortex neuron(0.1mM Mg2+)				CDI iPS Dopa/astrocytes co-cultured (0.1mM Mg2+)			
			BPM	AMP	AUC	Comments	BPM	AMP	AUC	Comments
4-Aminopiridine(4-AP)	seizure	burst coordination improved	↑ ↑ ↑			BPM over 25% up at 1uM	↑ ↑ ↑	↑ ↑ ↑	↑ ↑ ↑	AUC over 25% at 10uM
Pentylenetetrazole(PTZ)	seizure	activity increased/Excitatory	↑ ↑			BPM over 10% up at 100uM				no change
Picrotoxin (PTX)	seizure	burst coordination improved/Excitatory	↑ ↑	↑ ↑		BPM over 15% up at 0.3uM	↓			BPM >-10% Inhibitory(1uM)
Linopirdine	NA	NA	↑ ↑ ↑			BPM over 20% up at 3uM	↑ ↑ ↑	↓		BPM over 40% up at 1uM
Pilocarpine	seizure	no effect or activity slightly increased/Excitatory	↑		↑ ↑	AUC over 20% up at 10uM	↑ ↑ ↑	↓		BPM over 30% up at 0.3uM
Strychnine	seizure	no effect or activity reduced	↑ ↑ ↓		↓	BPM over 15% up at 1uM	↓	↓ ↓	↓ ↓	AMP >-30% Inhibitory(30uM)
Amoxapine	sedation	no effect or activity reduced	↑ ↑ ↓			BPM over 20% up at 1uM	↑ ↑ ↓	↓	↓	BPM over 5% Inhibitory(0.3uM)
Chlorpromazine(CPZ)	sedation	activity reduced/inhibitory	↑ ↑ ↓	↓		BPM >-10% Inhibitory (1uM)	↓ ↓	↓ ↓	↓ ↓	Burst stop at 10uM(10min)
Phenytoin	inhibitory	activity reduced	↓	↓		AMP >-10% Inhibitory (1uM)	↓ ↓	↓ ↓	↓ ↓	BPM >-15% Inhibitory (10uM)
Amoxicillin	NA	NA	↑	NC	NC	BPM over 10% up at 30uM	NC	NC	NC	no change
Acetaminophen	NA	NA	↑	NC	NC	no change	NC	NC	NC	no change
DMSO	NA	NA	NC	NC	NC	no change	NC	NC	NC	no change

4. Result

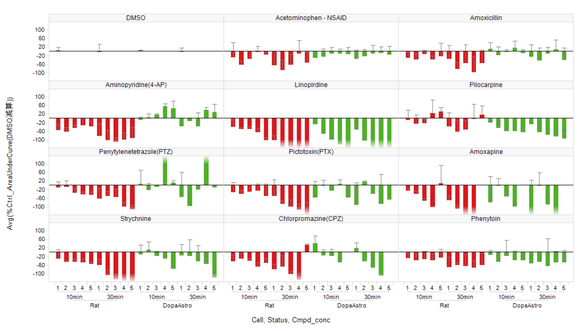
Spotfire analysis of FDSS drug-induced synchronization of Ca^{2+} oscillation data in Rat cortex neuron and human iPSC-derived Dopamine/Astrocytes co-cultured neurons (N=4～5, 5 dose, 384 well plate and using low Mg2+ buffer(0.1mM Mg2+)



Burst per 5 minutes (% DMSO Ctrl)



RFU Amplitude (%DMSO Ctrl)



RFU Area under curve (% DMSO Ctrl)

1. **We measured Ca2+ oscillations with a calcium-sensitive fluorescent dye (Cal-520AM) in rat primary neuron and human iPSC- derived Dopa and Astrocytes neurons in the high-throughput manner using a camera-based kinetic plate reader, FDSS.**
2. **We examined effects of the reference compounds and detected drug-induced FDSS parameter changes on Ca2+ oscillations-waveforms and are collecting more data in 200Hz. For critical analysis of the Ca2+ oscillations data, the high speed measurement might be very important assay parameter in FDSS.**
3. **High-throughput fluorescence measurements of Ca2+ transient in human iPSC-derived neurons would be greatly useful for assessment of seizure risk of compounds, particularly at the early stages of drug discovery.**

6. Acknowledgement

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