A novel high-throughput *in vitro* and multivariate spike train analysis platform for drug neurotoxicity and method of action identification

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Abstract

Neurotoxicity produces significant compound attrition during drug discovery. Currently available *in vitro* assays cannot predict all toxicity mechanisms due to the failure of general cytotoxicity assays to predict sublethal target specific electrophysiological liabilities. Ion channel and receptor activity assays can be used to predict some seizure potential, but these only focus on specifically measured targets for prediction and may miss responses that rely on a combination of targets. For example, most evaluation of seizure inducing compounds occurs later in preclinical *in vivo* studies which have much higher costs. Therefore, the development of a high-throughput *in vitro* assay to screen compounds for electrophysiological liabilities would derisk compounds earlier at lower cost and greater reliability. Here we demonstrate the use of a 48-well Axion BioSystems microelectrode array (MEA) along with custom data analysis algorithms/Matlab scripts to screen for neurotoxic liabilities using spike data from cryogenically preserved rat cortical neurons (eCiphr®Neuro assay). We developed a panel of spike train descriptor statistics – for activity (firing rate), synchronization, and inter-spike and inter-burst organization – that distinguish between descriptor fingerprints of negative and positive control compounds. Evidence is presented that firing rate as an isolated descriptor fails to identify all liabilities but that a carefully chosen multivariate panel addresses this problem. Moreover, the specific compound descriptor fingerprints often identify a neurotoxic method of action (MOA) that may stratify compounds into subgroups within a broader general liability class, e.g. seizurogenic. We illustrate these methods by considering several examples within three classes of seizurogenic compounds: $GABA_A$ antagonists (e.g. gabazine), glycine receptor antagonists (e.g. strychnine), and NMDA receptor agonists (e.g. NMDA). All showed significant changes in one or more descriptor endpoints that were characteristic of their MOA. In contrast, negative controls (acetaminophen, ibuprofen, naproxen, and DMSO) showed no endpoint alteration. To further demonstrate the responsiveness of the cells in the assay, we tested domoic acid, a neurotoxin known to cause amnesia, and found that it completely blocked neural spikes at 10µM. In addition, GABA (GABA agonist), tetrodotoxin (sodium channel blocker) and tetraethylammonium (TEA) (potassium channel blocker) were also tested with similar effects. These results illustrate the power within the high-throughput rat cortical neuron MEA data for predicting compound induced neural toxicity.

Significance for drug discovery

Nervous system side effects comprise the fourth most common cause of drug attrition from pharmaceutical industry discovery pipelines

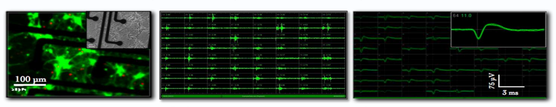
Phase	Preclinical	Phase I - III	Post-Approval
Rate of Attrition	12% to 14%	Up to 21%	2%

Table 1. Drug discovery attrition rate due to neurotoxic side effects
Frequency of drug discovery attrition due to neurotoxic side effects. Adapted from Redfern WS et al. SOT 2010 Poster 1081 provided by Tim Hammond

Among the most concerning side effects are drug-induced seizures, which are due to excessive and synchronous firing of cortical neurons and have been implicated in causing brain injury as well as increased incidence of mortality. Often, seizures can result in episodes of abnormal, convulsive motor activity. Based on the characteristics of this pathology, it's clear that the clinical manifestations of seizures are complex in origin and nature. Neural structure or function may be altered by many different mechanisms (receptor modulation, metabolic disruptors, etc.). Target-based approaches are not efficient for predictive toxicity screening because without prior knowledge of a chemical's mode of action thousands of different channels, receptors and proteins might have to be tested. Independent of the mechanism, these alterations induce a functional change that is recorded holistically by the MEA.

Experimental Methods and Data Acquisition

1. **Rat cortical neurons are thawed and plated on PEI/Laminin coated 48-well MEA plates with 16 channels per well**
2. **Cells are incubated at 37°C for 14 - 17 days with medium changes 3x a week.**
3. **Before the addition of compounds, a 15 minute baseline recording is acquired on the Axion Biosystems Maestro system.**
4. **Media is then spiked with drug and incubated for 60 minutes at 37°C . (Final DMSO concentration of 0.2%)**
5. **15 minutes of post-dose data are acquired.**
6. **Signal Processing (e.g. filtering and peak detection) conducted with Axion AxIS software - Spike time stamps and waveforms output as MATLAB data structures**



(A) Neurons are cultured on the microelectrode array.

(B) Spontaneous extracellular activity is recorded on each electrode

(C) Unit level action potentials on each channel are detected and quantified.

Figure 1. MEA measures spontaneous neural action potential

Spike Train Analysis

The objective is to create an analytic framework to extract from the spike time stamps a set of multivariate features (descriptors) to construct a pattern that provides a "fingerprint" of a particular class of chemical perturbation to be quantified by statistical analysis. Computations are done on a per channel basis but features must be related to an entire well. Spike train descriptors fall into four categories: activity, interspike interval (ISI) descriptors, burst organization, and inter-channel synchronization. Custom MATLAB scripts are developed to compute 40 descriptors which are reported at the well level by a statistical aggregation algorithm. The most useful of these features are retained for fingerprinting classes of compounds. Technical challenges included mitigating the effect of large variations in activity across channels and limiting the number of free parameters in the individual descriptor computations.

In the absence of any precise mathematical definition of "burst", several burst identification algorithms were studied and a customized version of the Poisson Surprise Method was selected to parse spike trains into bursts.

To calculate pair-wise synchronization between channels in a well, the method of Kreutz et al (ISI-distance metric) was identified as the most suitable for automated analysis of entire plates with many channels of varying activity level due to its lack of free parameters and need for binning in time. The median of the pairwise synchronizations was reported as the per well synchronization measure.

The ability to produce significant MOA-specific feature fingerprints will be illustrated by considering three classes of seizurogenic compounds

Selected Spike Train Descriptors

- Firing Rate - Number of spikes normalized by time of the recording.
- Burst Rate - Number of bursts normalized by time of the recording.
- Median number of Spikes in Burst.
- Coefficient of variation (CV) of the inter-spike intervals (ISI) - The difference in time between adjacent spikes in the channel spike train is computed (ISI). Their mean and standard deviation is computed to yield a coefficient of variation which is often interpreted as a measure of burstiness (Lei et al)..
- Median Burst Duration - Burst duration refers to the length of time that a burst lasts between the first and last spike in a particular burst.
- Normalized IQR Burst Duration - The Interquartile Range of the burst duration is normalized by the median of the burst duration, it is a nonparametric measure of variation in burst duration values. This is a measure of burst duration regularity. The smaller the normalized IQR, the more uniform the bursts.
- Median Interburst Interval -The interburst interval is the time between the trailing spike of each burst and the leading spike of the subsequent burst.
- Median ISI-distance - ISI-distance is a measure of pairwise channel synchronization (Kreuz et al)
- Normalized Median Absolute Deviation (MAD) Burst Spike Number – Indicator of statistical dispersion of the spikes in bursts.
- Median ISI/Mean ISI– Measure of spike organization within bursts. Increases as spike/burst organization deteriorates.

Representative Spike Train Raster Plot

We illustrate the structure of a typical baseline raster plot for a well compared to its structure following dosing with the $GABA_A$ antagonist picrotoxin. The qualitative visual differences in the dynamics of the spike train are quantified through computation of the spike train features.

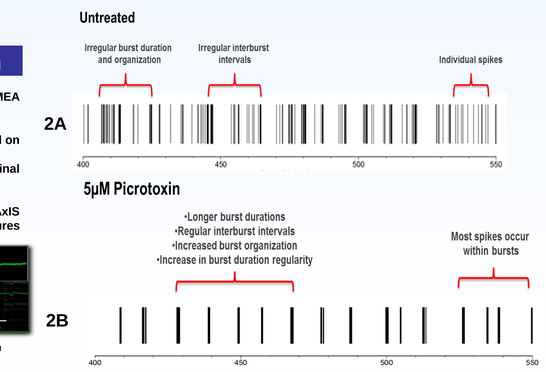
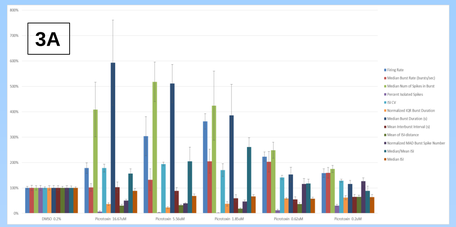
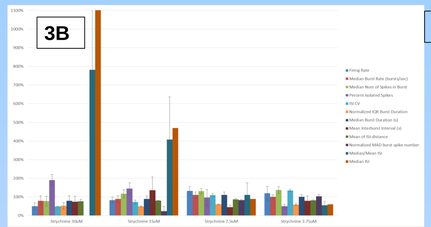


Figure 2A, B. Raster plots of spontaneous spike trains for untreated and 5µM picrotoxin treated rat cortical neurons. Plots are made with NeuroExplorer software.

MOA Discrimination of Seizurogenic Compounds





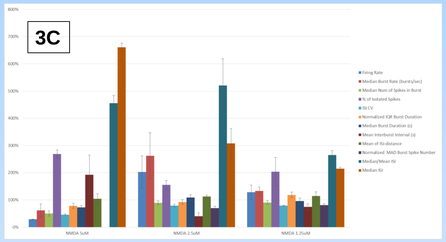


Figure 3 A, B, C: Bar graphs of the DMSO control normalized differential regulation (before and after dosing) of the selected spike train descriptors are presented for:

(A) picrotoxin ($GABA_A$ antagonist);
(B) strychnine (glycine receptor antagonist);
(C) NMDA (NMDA receptor agonist).

Dose response is presented and each compound/dose combination is run in triplicate. Standard errors of the mean are shown in the plots. Panel A also includes the response of the DMSO control before and after dosing.

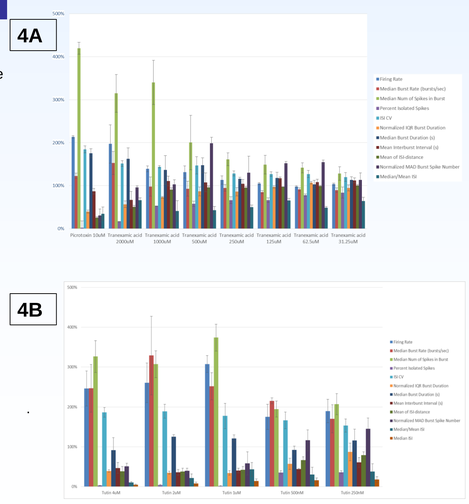
Control Compound	MOA	Firing Rate	Median Burst Rate (bursts/sec)	Median Num of Spikes in Burst	Percent Isolated Spikes	ISI CV	Normalized IQR Burst Duration	Median Burst Duration (s)	Mean Interburst Interval (s)	Mean of ISI-distance	Normalized MAD Burst Spike Number	Median/Mean ISI	Median ISI
Picrotoxin	GABAa antagonist	increase	increase	increase	decrease	increase	decrease	increase	decrease	decrease	decrease	decrease	decrease
Gabazine	GABAa antagonist	increase	increase	increase	decrease	increase	decrease	increase	increase	decrease	decrease	decrease	decrease
Bicuculline	GABAa antagonist	no change	increase	increase	decrease	increase	decrease	increase	increase	decrease	decrease	decrease	decrease

Control Compound	MOA	Firing Rate	Median Burst Rate (bursts/sec)	Median Num of Spikes in Burst	Percent Isolated Spikes	ISI CV	Normalized IQR Burst Duration	Median Burst Duration (s)	Mean Interburst Interval (s)	Mean of ISI-distance	Normalized MAD Burst Spike Number	Median/Mean ISI	Median ISI
Strychnine	Glycine receptor antagonist	decrease	decrease	decrease	increase	decrease	decrease	decrease	decrease	decrease	decrease	increase	increase
Brucine (>25µM)	Glycine receptor antagonist	decrease	decrease	decrease	increase	decrease	decrease	decrease	increase	decrease	decrease	increase	increase

Control Compound	MOA	Firing Rate	Median Burst Rate (bursts/sec)	Median Num of Spikes in Burst	Percent Isolated Spikes	ISI CV	Normalized IQR Burst Duration	Median Burst Duration (s)	Mean Interburst Interval (s)	Mean of ISI-distance	Normalized MAD Burst Spike Number	Median/Mean ISI	Median ISI
NMDA (2.5µM)	NMDA receptor agonist	increase	increase	no change	increase	no change	no change	no change	decrease	no change	decrease	increase	increase
Quinolinic Acid (100µM)	NMDA receptor agonist	increase	increase	no change	no change	decrease	no change	no change	decrease	no change	no change	increase	increase

Table 2. Comparison of the spike train descriptor fingerprint of seizurogenic compounds falling in to three different MOAs: $GABA_A$ antagonists, glycine receptor antagonists, and NMDA receptor agonists. Differential regulation of the predose versus postdose response of a descriptor is marked as increased , unchanged, or decreased. Fold changes were normalized by the corresponding response of the DMSO controls and assessed for statistical significance. Each MOA yields a distinct pattern.

Mixed Receptor Response



Frequently, the precise seizurogenic mechanisms of a compound are not well understood and/or are due to a combination of different receptor activation/inhibition. We consider two such cases.

FIGURE 4A:
Tranexamic acid (TXA) is a widely administered antifibrinolytic drug that reduces blood loss during surgery. TXA evokes seizures in both laboratory animals and patients but the underlying mechanisms are not clear. A recent investigation (Lecker et al) noted the structural similarity between TXA and glycine and pursued the hypothesis that TXA causes seizures due to inhibiting the activity of glycine receptors. Lecker et al presented supporting evidence with measurement of glycine activated currents in mouse neurons grown in a dissasociated cell culture. We used our MEA analysis platform to conduct a dose response study on TXA for doses ranging from 31.25 to 2000µM. At high doses, the TXA fingerprint closely resembles that of the $GABA_A$ antagonist picrotoxin. We hypothesize that although TXA might effect multiple receptors, $GABA_A$ antagonism is actually the dominant seizurogenic effect.

FIGURE 4B:
Tutin is a poisonous picrotoxane molecule compound isolated from the shrub *Coriaria ruscifolia*. Recently, its effects on glycine receptors (GlyR) expressed in HEK 293 cells using whole-cell patch-clamp techniques were studied (Fuentalba et al) to understand differences in action compared to picrotoxin and to evaluate its potential pharmacologic use. We used the MEA platform to investigate the possibility of a mixed receptor response for tutin exposure. We find that the $GABA_A$ antagonist signature of picrotoxin is also the dominant effect in the tutin dose-response analysis.

Raster Plots by MOA

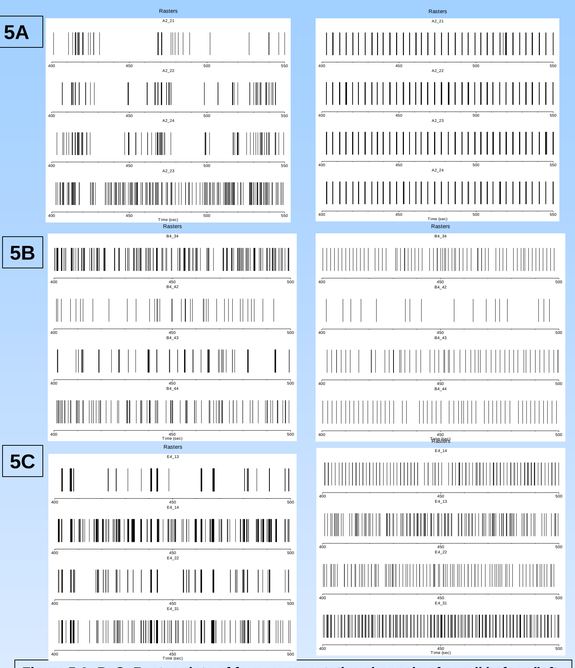


Figure 5 A, B, C: Raster plots of four representative channels of a well before (left panels) and after dosing (right panels) for A.) picrotoxin, B.) strychnine, and C.) NMDA. The time intervals are 100 seconds except for picrotoxin (150 s). Qualitative differences of these plots are quantified by the descriptors of figure 3.

Discussion and Conclusions

- **Rat cortical neurons plated on microelectrode arrays generate robust and spontaneous neural spike activity, organize into bursting patterns and are responsive to a wide range of neurotransmitters, pharmacological agonists, and antagonists.**
- **Spike activity or neural structure or function may be altered by many different mechanisms** (receptor modulation, metabolic disruptors, etc.). Independent of the mechanism, these alterations induce a functional change that is recorded by the MEA (Johnstone et al 2010).
- **eCiphr®Neuro is a validated MEA neurotox assay**. Negative controls (acetaminophen, ibuprofen, naproxen, and DMSO) showed no endpoint alteration. To further demonstrate the responsiveness of the cells in the assay, we tested domoic acid, a neurotoxin known to cause amnesia, and found that it completely blocked neural spikes at 10µM. In addition, GABA (GABA agonist), tetrodotoxin (sodium channel blocker) and tetraethylammonium (TEA) (potassium channel blocker) were also tested with similar effects (Bradley et al 2014)
- **Descriptors derived from spike activity may be used to generate "fingerprints" for distinct MOAs and this analysis is illustrated with the case study of distinguishing between three classes of seizure inducing compounds**.
- With a large enough set of well-annotated test compounds, our methodology could be generalized to prediction with Machine Learning techniques.
- Recent work suggests that *in vitro* MEA based approaches are more predictive than the *ex vivo* rat hippocampal brain slice assay (Chaudhary et al 2014)
- eCiphr®Neuro can be used for screening assays (ie. blocking of seizurogenic acitivity by anti-convulsives.

References

Lei, H. *et al*, PLoS ONE, (2011), Vol.6, Issue 8
Legendy, CR and Salcman, M, J. Neurophysiol., (1985), 53:926-939
Kreuz, T, *et al*, Journal of Neuroscience Methods, (2007) 165:151-161
Robinette BL *et al*, (2011) Front Neuroeng 4; Article 1
Novellino A *et al*, (2011) Front Neuroeng 4; Article 4
Johnstone AFM, *et al*, NeuroToxicology 31 (2010) 331–350
Chaudhary KW, *et al*, poster , Safety Pharmacology Society Conf., Oct. 2014
Bradley J, et al., poster Society of Toxicology Conf., Apr. 2014
Lecker I, et al, J.Clinical Investigation, 122(12) (2012)
Fuentealba J, et al. Neuropharmacology 60 (2012)