In vitro assessment of drug-induced seizure liability using a multi-electrode array based rat cortical neuronal assay.

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Introduction

Central nervous system toxicities are a leading cause of pharmaceutical compound attrition. Drug-induced seizures, which are due to excessive and synchronous firing of cortical neurons, have been implicated in causing anoxic 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.

The "gold standard" of ex vivo pre-clinical seizure detection has been the rat hippocampal brain slice assay. This assay requires the use of mature rats, slicing of very delicate brain tissue and a complex system to record electrophysiological changes in neuronal firing. Studies have shown that while the brain slice retains the cellular architecture that allows for recording of evoked potentials, seizures can occur in different parts of the brain that are distant to the hippocampus. Several recent studies have highlighted the use of dissociated cortical neurons cultured on multi-electrode arrays (MEA) for the study of seizurogenic activity of compounds. The rat cortical neuronal assay, in particular, has been shown to develop network behavior and retain receptors and ion channels (GABA, NMDA, AMPA, NaV1.3, etc.) that have been implicated in seizure activity. Because the MEA is plate-based, there is the potential to simultaneously record from 12, 48 or up to 96 wells, thereby affording higher throughput than brain slice assays.

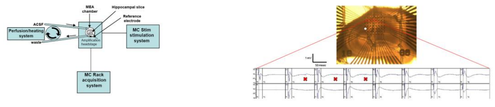
In this study, we sought to evaluate the use of both rat hippocampal brain slices and embryonic cortical neurons cultured on MEA plates to identify compounds that were observed to cause convulsive activity in vivo (rat/dog).

Methods

Rat Hippocampal Slice

Experiments were carried out with 3-4-week-old Sprague-Dawley rats without gender distinction (from Elevage Janvier, France). The brain was quickly removed after decapitation and soaked in ice-cold oxygenated buffer with the following composition (mM): KCl 2, NaH_2PO_4 1.2, $MgCl_2$ 7, $CaCl_2$ 0.5, $NaHCO_3$ 26, Glucose 11 and Saccharose 250. Hippocampal slices (350 and 400 µm) were cut with a McILWAIN tissue chopper or a vibratome and incubated at room temperature for at least 1 h in Artificial Cerebro-Spinal Fluid (ACSF) of the following composition (mM): NaCl 126, KCl 3.5, NaH_2PO_4 1.2, $MgCl_2$ 1.3, $CaCl_2$ 2, $NaHCO_3$ 25 and Glucose 11. All data were recorded with an MEA set-up from MultiChannel Systems (MCS, Reutlingen, Germany) composed of a 4-channel stimulus generator and a 60-channel amplifier headstage connected to a 60-channel A/D card.

CA1 neuron firing at various extracellular K^+, population spikes and area as well as 4-AP induced epileptiform discharges were recorded prior to and 30 minutes after compound perfusion. Temperature was maintained at 37°C.



Rat Cortical Neuronal Assay

Cryopreserved aliquots of rat cortical neurons (QBM Cell Science) were thawed and plated on 48-well microelectrode arrays precoated with 0.1% polyethyleneimine (Fluka) in a borate buffer solution and pretreated with a laminin (Sigma) solution (1mg/mL). After a centrifugation step, cells were resuspended in complete NB/B27 medium and a 25 µL aliquot containing 75,000 cells was seeded in the center of the well directly over the electrode grid. The plates were incubated for 2 hr at 37°C in a humidified environment to allow sufficient attachment before 300 µL of NB/B27 medium was added to each well. After a 2-day incubation period, an additional 200 µL of NB/B27 was added to achieve a final well volume of 500 µL. The cells were maintained in a humidified incubator at 37°C for 14 – 17 days with 60% media changes 3 times a week before experimental procedures were performed.

All recordings were obtained with Axion Biosystems Maestro microelectrode array, a 768-channel high throughput MEA platform, utilizing their 48-well plates configured with 16 electrodes per well. Prior to compound addition, a baseline of spontaneous activity was recorded at a sampling rate of 12500 Hz using the temperature regulated (37°C) Maestro system controlled by Axion's Integrated Studio (AxIS 1.8.1.5) software package. At a later time, the raw data file was played back using a Butterworth adaptive band-pass filter with a high-pass cutoff frequency of 300 Hz and low-pass frequency cutoff of 5000 Hz. A spike detector process using adaptive threshold crossing was also applied set to 5.5x standard deviation of the noise on each channel. The AxIS spike file was exported to MatLab (MathWorks) for spike train analysis and statistics and the alpha map file was exported to NeuroExplorer (Nex Technologies) for spike train visualizations and raster plots.

Wells with no or sparse activity were eliminated from the experiment. After a 3 minute equilibration time, baseline recordings of approximately 15 minutes in length were obtained immediately before the addition of treatment compounds and controls. For analysis, electrodes with 100 or more spikes were determined to be "active" and only wells with 5 or more active electrodes were used in the final analysis. Following a 1 hr incubation at 37°C with compounds, another 15 minute recording was obtained after a 3 minute equilibration time. If a treated well fell below the activity threshold due to compound effect, only spike count was determined and reported. All other parameters were not calculated. The final DMSO concentration in all wells was 0.2 %, which has no significant effect on neuronal activity (data not shown).

Endpoints

The following nine endpoints were chosen to cover a range of changes elicited from different classes of compounds and pharmacological challenges as discovered during the development and validation of this assay.

➢Coefficient of variation (CV) of the interspike intervals (ISI) – The difference in time between adjacent spikes for spike trains in each channel was computed to obtain the inter-spike intervals. The mean and standard deviation of the ISI's for each channel was computed to yield a coefficient of variation. Changes in this endpoint are often interpreted as a measure of the change in the burstiness of the spike train.

➢Mean Burst Duration- The spike trains were parsed into bursts using a customized version of the Poisson Surprise (PS) burst identification method. Burst duration refers to the length of time that a burst lasts between the first and last spike in a particular burst.

➢Normalized Burst Duration IQR- The Interquartile Range of the burst duration was normalized by the median of the burst duration. It is a nonparametric measure of variation in burst duration values. This endpoint is a measure of burst duration regularity; the smaller the normalized IQR, the more uniform the bursts.

➢Number of Spikes in Burst – The spike trains were parsed into bursts using our customized version of the Poisson Surprise (PS) burst identification method. The number of spikes that occurred within a burst was calculated.

➢Interburst Interval- The spike trains were parsed into bursts using our customized version of the Poisson Surprise (PS) burst identification method. The interburst interval was calculated by determining the time between the trailing spike of each burst and the leading spike of the subsequent burst.

➢Interspike interval distance- ISI-distance was calculated by the Kreuz et al. method for spike train synchrony.

➢Median/Mean ISI - Measure of spike organization within bursts- increases as burst/spike organization deteriorates.

➢Burst Rate- Number of bursts normalized by time of the recording.

➢Firing rate- Number of spikes normalized by time of the recording.

Results

Control- validation

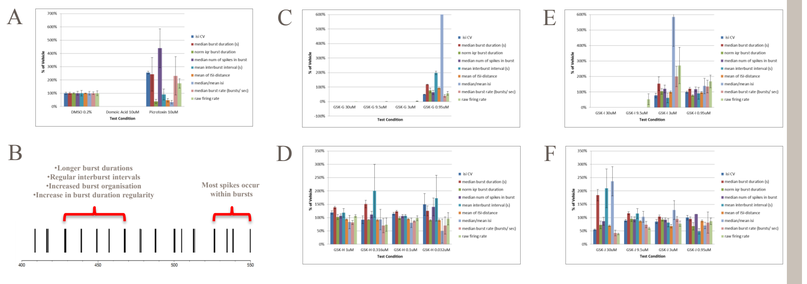


Figure 1: A) Picrotoxin (10µM), a GABAa antagonist, causes an increase in firing rate, as well as an increase in all synchrony related endpoints. Domoic acid (10µM) causes a cessation of firing. B) Raster plots of Picrotoxin-induced (5µM) changes in neuronal firing suggest increased organization of burst activity and synchrony in firing pattern. C-F) AMPA, apamin, NMDA and linopirdine were also studied to characterize the neuronal MEA platform.

GSK- Compounds

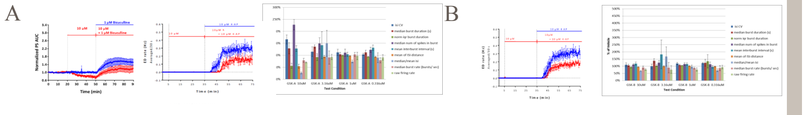
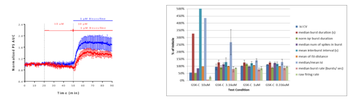


Figure 2: A) GSK-A, in vivo (canine), induced animal collapse, extreme whole body twitching/trembling, rapid breathing, salivation and/or vocalization 2 hours postdose at free Cmax ≈ 0.9µM-4µM. Changes in neuronal firing in brain slice did not support the behavioral observations up to 10µM, whereas MEA assay suggests increased burst activity and synchrony from 3.16µM-10µM. B) A compound from the same chemical series, GSK-B, did not produce neuro-behavioral observations in vivo (canine 4.2µM, rat 22µM free Cmax), and did not produce effects in brain slice or MEA.

C



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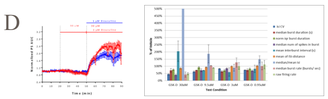
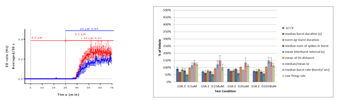


Figure 3: C) GSK-C, in vivo (canine), induced tonic convulsion after the first dose on Day 5. The male (≈ 1.18µM free) had multiple instances of tonic convulsion during the first 3 hours after dosing, while the female had one episode of convulsion 1 hour and 30 minutes after the first dose. Changes in neuronal firing in brain slice did not support the behavioral observations up to 10µM, whereas MEA suggest decreased firing, increased burst duration and disorganization within bursts at 10µM. D) GSK-D, in vivo (rat), induced convulsions, hunched posture, piloerection, tremors, prostration, rapid breathing and salivation at 3.5µM free Cmax. The brain slice assay produced increased bicuculine-dependent neuronal firing (30µM), while the MEA produced a decrease in burst organization and neuronal firing (30µM).

E



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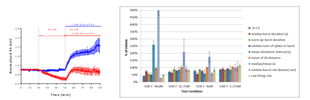

Figure 4: E) GSK-E, in vivo, was well tolerated at all doses up to 0.95µM free Cmax. Little to no concentration-dependent changes in firing were observed in the cortical neuronal assay up to 0.5µM, whereas the hippocampal brain slice suggesetd an increase in firing. F) GSK-F, in vivo (rat), induced severe clinical signs, including tonic/clonic convulsions, subdued behaviour, cold to touch, hunched posture, piloerection and excessive salivation at ≈ 3.4µM free Cmax. A decrease in burst organization with subsequent reduction in firing was observed at 4µM in the MEA assay, as compared to 40µM in the hippocampal brain slice.

Summary

- Two MEA electrophysiology platforms evaluated
  - Rat hippocampal brain slices
  - Rat cultured cortical neurons
- Initial studies suggest that cultured cortical neurons are more predictive of in vivo findings
  - Ability to classify compounds as pro/anti convulsant
  - Increased predictivity at relevant exposure

•For six tested GSK compounds

	Rat Hippocampal Brain Slice		Rat Cortical Neuronal Assay	
	Seizurogenic *in vivo*	**No Effect *in vivo***	**Seizurogenic *in vivo***	**No Effect *in vivo***
Seizurogenic			**4**	
Mixed Effects	**1**	**1**		
Anticonvulsant	**3**	**1**		
No Effect				**2**
	0 % Sensitivity **50% Specificity**		**100% Sensitivity** **100% Specificity**	

Conclusions

Rat cortical neuronal MEA assay was able to identify changes in neuronal firing caused by standards and proprietary compounds with known convulsant or seizurogenic properties. Because the output is composed of various indicators of firing activity and pattern, the assay provides enhanced granularity to compound-dependent effects on the CNS. In our limited experiment, the rat cortical neuronal MEA assay was more sensitive in identifying compounds that produced convulsive effects in vivo, in comparison to the rat hippocampal brain slice preparation, and should be considered when designing an in vitro safety pharmacology strategy.

Acknowledgements

All studies were conducted in accordance with the GSK Policy on the Care, Welfare and Treatment of Laboratory Animals and were reviewed the Institutional Animal Care and Use Committee either at GSK or by the ethical review process at the institution where the work was performed.

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