



Evaluation of multi-well microelectrode arrays for neurotoxicity screening using a chemical training set[☆]

Emma R. McConnell ^a, Maxine A. McClain ^{a,1}, James Ross ^a, William R. LeFew ^b, Timothy J. Shafer ^{b,*}

^a Axion Biosystems, Atlanta, GA, United States

^b Integrated Systems Toxicology Division, NHEERL, ORD, US Environmental Protection Agency, Research Triangle Park, NC, United States

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ABSTRACT

Microelectrode array (MEA) approaches have been proposed as a tool for detecting functional changes in electrically excitable cells, including neurons, exposed to drugs, chemicals or particles. However, conventional single well-MEA systems lack the throughput necessary for screening large numbers of uncharacterized compounds. Recently, multi-well MEA (mwMEA) formats have become available to address the need for increased throughput. The current experiments examined the effects of a training set of 30 chemicals on spontaneous activity in networks of cortical neurons grown on mwMEA plates. Each plate contained 12 wells with 64 microelectrodes/well, for a total of 768 channels. Of the 30 chemicals evaluated, 23 were known to alter neuronal function *in vivo* ("positives"), including 6 GABAergic and 3 glutamatergic antagonists/agonists, 4 pyrethrroids, 3 metals, 2 cholinesterase inhibitors, 2 nicotinic acetylcholine receptor agonists, valproic acid, verapamil, and fluoxetine. Seven compounds expected to have no effect on neuronal function were tested as "negatives" (glyphosate, acetaminophen, salicylic acid, paraquat, saccharin, D-sorbitol and amoxicillin). Following collection of 33 min of baseline activity, chemical effects (50 μM or highest soluble concentration) were recorded for 33 min. Twenty of the positives altered the mean network spike rate by more than the 14% threshold (two standard deviations from the mean for DMSO control). The three positives without effect were bifenthrin, nicotine and imidacloprid. None of the negative compounds caused a change in activity beyond the threshold. Based on these results, the mwMEA assay has both high sensitivity (87% identification of positive compounds) and specificity (100% identification of negative compounds). These experiments demonstrate the capacity of mwMEAs to screen compounds for neurotoxic effects mediated by a broad variety of mechanisms.

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1. Introduction

The National Academy of Sciences report on "Toxicity testing in the 21st century" highlighted the need for efficient *in vitro* methods to screen chemicals for their potential to cause toxicity (NRC, 2007). This report proposed that high-throughput/high content *in*

vitro screening (HTS/HCS) assays would facilitate hazard identification for thousands of chemicals for which toxicological information is lacking. Such screening approaches will need to link changes measured at the cellular or subcellular level to adverse effects through a toxicity pathway so that there will be confidence in predicting toxicological outcomes *in vivo*.

Many HTS/HCS endpoints assess changes in biochemical and/or cellular morphology markers, such as enzyme activity, receptor binding affinity, cell morphology (Radio and Mundy, 2008), or physiological endpoints, such as regulation of intracellular calcium (Pereira et al., 2009; Cao et al., 2010, 2011a; Liu et al., 2010), sodium (Cao et al., 2011b), membrane potential (Joesch et al., 2008) and ion channel function (Dunlop et al., 2008; Castle et al., 2009). When considering the problem of screening compounds for the potential to disrupt nervous system function, physiological assessment is crucial, as disruption of ion channels, receptors and other critical determinants of neuronal excitability are key events in the toxicity pathways of many known neurotoxicants. Disruption of neuronal excitability produces substantial and rapid disruption of nervous system physiology, and often precedes or

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¹ Corresponding author at: Integrated Systems Toxicology Division, MD-B105-03, U.S. Environmental Protection Agency, Research Triangle Park, NC 27711, United States. Tel.: +1 919 541 0647; fax: +1 919 541 4849.

E-mail address: Shafer.tim@epa.gov (T.J. Shafer).

¹ Current address: Seahorse Bioscience, Inc. 16 Esquire Road North Billerica, MA 01862.

occurs in the absence of other biochemical or morphological changes. Examples include insecticides, a variety of convulsants and metals, as well as a wide range of natural toxins. However, current *in vitro* assays based on biochemical and morphological changes are not optimized for detecting this type of toxicity. These assays do not incorporate measurement of the key events in the toxicity pathways of such neurotoxicants (e.g. changes in intracellular ion concentrations are secondary events), or are not amenable to collecting data at a rate which can capture the most rapid neurophysiological events, for example disruption of voltage-gated sodium channels and action potential generation by pyrethroid insecticides. By contrast, currently available electrophysiological approaches are not well designed for toxicity screening, as these assays typically consider only one potential target at a time (e.g. a particular ion channel) and often employ non-neuronal expression systems rather than neuronal tissue. Furthermore, neither biochemical nor HTS physiological (e.g. patch clamp) approaches consider chemical effects on neuronal network function. Many of these traditional assays were designed as targeted screens for pharmaceutical-lead compound development and lack the ability to detect a broad spectrum of different neurotoxicants. Thus, efficient screening assays that detect neurotoxic or neuroactive chemicals based on changes in function are lacking, particularly those that are sensitive to changes mediated by disruption of a variety of different toxicity pathways.

One physiological approach that addresses these limitations is *in vitro* microelectrode array (MEA) recording. Using MEAs, spontaneous and evoked activity in neuronal networks can be recorded from a variety of different neuronal preparations, including primary cultures, tissue slices and intact retinas. Neuronal activity in cultures grown on MEAs is sensitive to a variety of drugs and chemicals and responds to a broad spectrum of pharmaceutical compounds (Gross et al., 1997). As such, neuronal networks on MEAs are a potential method of assessing effects of many different pharmacological classes of drugs and chemicals on nervous system function. To date, assessment of chemical effects using MEAs has been primarily on a chemical-by-chemical basis to understand the toxicity of individual chemicals or chemical classes (Keefer et al., 2001; Gramowski et al., 2000; Gopal, 2003; for review, see Johnstone et al., 2010). Use of MEAs has been proposed as an *in vitro* neurotoxicity screening method (Johnstone et al., 2010) and a recent study demonstrated consistent reproducibility and reliability of MEA measurements across five laboratories (Novellino et al., 2011). One limitation of MEA approaches is that throughput of this methodology has been low and is limited by the MEA hardware. To date, only one publication has assessed the ability of MEAs to screen a set of twenty environmental chemicals (Defranchi et al., 2011). In this study, activity of chemicals was determined by conducting cumulative concentration-response assessments, which is time-consuming and limits the number of chemicals that can be assessed. A more efficient approach would be to screen compounds at a single concentration to identify those that are “hits” and then characterize concentration-responses if desired. However, experiments have not been conducted to assess this approach for its ability to detect neuroactive and neurotoxic compounds. Thus, the goal of the current experiments was to evaluate the ability of MEAs to detect neuroactive and neurotoxic chemical effects on the basis of testing a single concentration. To do so, these experiments examined the effects of a set of 30 environmental chemicals and drugs on spontaneous network activity in primary cortical cultures using a 768-channel, multi-well-MEA (mwMEA) system. The present study employed the concept of a “training set” of chemicals outlined by Crofton et al. (2011), wherein the chemicals selected for testing were well characterized in terms of their ability to alter (or not alter) neurophysiological endpoints. This allows for assessment of

mwMEAs to identify and prioritize compounds for additional testing.

2. Materials and methods

2.1. Chemicals

The sources, purities and vehicles used to dissolve test chemicals are listed in Table 1. Unless otherwise noted, all chemicals were prepared as 50 mM stock solutions in their respective solvents, stored at -20 °C in amber glass vials treated with Sigmacote and diluted 1:1000 into the media for a final concentration of 50 μM. The following considerations were important in the selection of these chemicals: (1) selection of a broad variety of different potential mechanisms of action (i.e. potential toxicity pathways), (2) previous data characterizing the ability (or lack thereof) of the drugs or chemicals to disrupt spontaneous network activity, (3) known ability to affect function of the nervous system, *in vivo* and (4) previous use of the compound as a negative control in neurotoxicity assays. For most chemicals, previously published data were used to establish their activity on MEAs, and references are provided in Table 1. Of the 30 compounds selected, 23 were known to alter neuronal function, while 7 are not expected to exhibit neurotoxicity *in vivo*. All other chemicals were obtained from commercial vendors and were reagent quality or better.

2.2. Multi-well MEA plates

Microelectrode array plates were composed of 12 wells with each well containing an array of 64 embedded nano-porous platinum electrodes (30 μm diameter, 200 μm center-to-center spacing), for a total of 768 channels (Axion Biosystems Inc., Atlanta, GA). To prepare the plates for culture, the mwMEAs were placed in a 55 °C oven overnight. After removal, each plate was submerged in ethanol (70%) for 1 h. After removing the ethanol and air-drying, each well was coated with a 50 μg/mL solution of poly-L-lysine (Sigma Chemical, St. Louis, MO). The solution was removed after 1 h, and the plates were dried and stored at 18 °C until the day of culture. Note: Since completing these studies, we have found that the plates can be sterilized by being submerged in ethanol for 5 min, air dried and placed in the 55 °C oven for 3–4 h (upside down with the lids on).

2.3. Cell culture on MEAs

Cells were prepared from Long-Evans rats on postnatal day 0–1. The pups were decapitated, and the neocortex was separated from the brain and placed in cortical buffer (127 mM NaCl, 5 mM KCl, 170 μM Na₂HPO₄, 205 μM KH₂PO₄, 5 mM glucose, 59 mM sucrose, 100 U/mL penicillin/streptomycin, pH 7.4). The cortex was minced finely with scissors and triturated until homogenous. Cells were then filtered through a 100 μm screen. The electrode field in each well was coated with laminin at the beginning of the culture by placing a 50 μL drop of laminin (Sigma) solution (1 mg/mL) in the center of the well. Upon completion of the dissection (~45 min), the laminin was removed by aspiration and 50 μL of media containing 2.5 × 10⁵ cells was placed directly over the electrode field within each well. Cells were left to adhere for 10–15 min before adding 1 mL of Neurobasal-A (NBA) media (500 mL Neurobasal-A (GIBCO), 14 g sucrose (Sigma), 1.25 mL glutamine (200 mM, GIBCO), 5 mL glutamate (2.5 mM, Sigma), 5 mL penicillin/streptomycin (Sigma), 50 mL FBS (GIBCO), pH 7.4). The day following the cell plating (day *in vitro* (DIV 1)) NBA media was replaced with NBA + B27 media (500 mL Neurobasal-A, 14 g sucrose, 1.25 mL glutamine (200 mM), 5 mL glutamate (2.5 mM),

Table 1

Details on the training set of chemicals used.

Chemical positives	Chemical class	CAS #	Vehicle	Purity (%)	Source ^c	Previous data in MEAs
Bicuculline	GABA _A antagonist	40709-69-1	DMSO/EtOH	≥90	Sigma	Gross et al. (1997)
Bifenthrin ^a	VGSC pesticide	82657-04-3	DMSO	89.0	Gift	Losa et al. (2009)
Carbaryl	AChE inhibitor	63-25-2	DMSO	99.8	Chem Service	Defranchi et al. (2011)
Chlorpyrifos oxon	AChE inhibitor	5598-15-2	DMSO	>98	Chem Service	Unpublished lab data
β-Cyfluthrin ^a	VGSC pesticide	68359-37-5	DMSO	99.2	Gift	Losa et al. (2009)
Deltamethrin	VGSC pesticide	52918-63-5	DMSO	99.5	Chem Service	Meyer et al. (2008)
Diazepam	GABA _A modulator	439-14-5	DMSO	>98	Sigma	Novellino et al. (2011)
Domoic acid ^b	Glutamate R antagonist	14277-97-5	H ₂ O	≥90	Sigma	Hogberg et al. (2011)
Fipronil	GABA _A antagonist	120068-37-3	DMSO	98.5	Chem Service	Defranchi et al. (2011)
Fluoxetine	SSRI	114247-09-5	DMSO	>98	Sigma	Novellino et al. (2011)
Imidacloprid	nAChR pesticide	138261-41-3	DMSO	99.5	Chem Service	–
Ketamine	NMDA R antagonist	33795-24-3	DMSO	>99	Sigma	Gross et al. (1995)
Lead ^b	Neurotoxic heavy metal	6080-56-4	H ₂ O	>98	Aldrich	–
L-Glutamate	Glutamate R agonist	19285-83-7	H ₂ O	>98	Sigma	Frega et al. (2011)
Lindane	GABA _A antagonist	58-89-9	DMSO	97	Aldrich	Unpublished lab data
Methylmercury ^b	Neurotoxic heavy metal	115-09-3	DMSO	93	Aldrich	van Vliet et al. (2007)
Muscimol	GABA _A agonist	18174-72-6	H ₂ O	≥98	Sigma	Novellino et al. (2011)
Nicotine	nAChR agonist	54-11-5	DMSO	>99	Sigma	Defranchi et al. (2011)
Permethrin	VGSC pesticide	52645-53-1	DMSO	55	Chem Service	Meyer et al. (2008)
RDX ^a	GABA _A antagonist	121-82-4	DMSO	>99.5	Gift	Williams et al. (2010)
Trimethyltin ^b	Neurotoxic heavy metal	56-24-6	H ₂ O	>95	ICN Biomedicals	Gramowski et al. (2000)
Valproic acid	Broad spectrum anticonvulsant	1069-66-5	DMSO	>98	Sigma	Gross et al. (1995)
Verapamil	VGSC blocker	152-11-4	DMSO	≥99.0	Sigma	Novellino et al. (2011)
Chemical negatives	Chemical class	CAS #	Vehicle	Purity (%)	Source ^c	Reference
Acetaminophen	Cox-2 inhibitor	103-90-2	DMSO	99	Sigma	Breier et al. (2008)
Amoxicillin	Antibiotic	26787-78-0	DMSO	N/A	Sigma	Breier et al. (2008)
Glyphosate	Herbicide	1071-83-6	H ₂ O	>99	Chem Service	Breier et al. (2008)
Paraquat	Herbicide	1910-42-5	H ₂ O	99.9	Sigma	Defranchi et al. (2011)
Saccharin	Sweetener	82385-42-0	DMSO	>99	Sigma	Breier et al. (2008)
Salicylic acid	Phytohormone	69-72-7	DMSO	≥99	Sigma	Defranchi et al. (2011)
d-Sorbitol	Sweetener	50-70-4	DMSO	>98	Sigma	Breier et al. (2008)

^a Bifenthrin and cyfluthrin were graciously provided by Kevin Crofton at the US Environmental Protection Agency and were from the same stocks as used by Wolansky et al. (2006). RDX was provided by Dr. Larry Williams of the US Army Public Health Command.

^b For domoic acid, methylmercury, and trimethyltin a 10 μM concentration was used. For lead a 30 μM concentration was used.

^c Addresses: Sigma Aldrich Chemical Company, St. Louis, MO; Chem Service, West Chester, PA; ICN Biomedicals, Irvine, CA.

5 mL penicillin/streptomycin, 10 mL B-27 (GIBCO), pH 7.4). At DIV 4 NBA + B27 media was replaced with glutamate-free NBA + 10%, FBS media. The plates were checked at least once every 7 days for evidence of evaporation. If evaporation was apparent, media was removed from all wells and replaced with 1–1.5 mL fresh culture media.

2.4. MEA recording

Spontaneous network activity from cortical cultures grown on MEA plates was recorded using hardware and software from Axion Biosystems. The hardware consisted of the Maestro 768-channel amplifier, Middle-man data acquisition interface, and a personal computer. Data acquisition for the channels was managed by the software, Axion's Integrated Studio (AxIS 1.4.2). Channels were sampled simultaneously with a gain of 1200× and a sampling rate of 12.5 kHz/channel. On-line spike detection was done with the AxIS adaptive spike detector, a minor variant of the 'AdaBandFlt' detector method described in (Biffi et al., 2010). All recordings were conducted at 37 °C.

Between DIV 6 and 14 plates containing the cultures were placed into the Maestro and spontaneous activity of each well was evaluated to determine culture viability. Application of the test chemicals occurred between DIV 12 and 22, with a median of 14 DIV for all plates. Any wells that did not show spontaneous activity on the day planned for the experiment were not treated with a chemical. Any electrodes with rms-noise levels >10 μV were grounded. The experimental protocol for testing the chemical training set is summarized in Fig. 1 and described in detail below.

For recordings, a Butterworth band-pass filter (with a high-pass cutoff of 300 Hz and low-pass cutoff of 5000 Hz) was applied along with a variable threshold spike detector set at 8× standard

deviation of the rms-noise on each channel. Baseline recordings (33 min) were taken and data were saved to 3 different file types simultaneously; a raw data file (*.raw file) that included all data, a spike counts file (*.csv file) that included the spikes per electrode with a 1 s bin time, and an alpha map (*.map file) that included spike timing and profile information. Stock solutions of chemicals (1 μL) were added to each well by diluting the solution in 200 μL of media from the appropriate well, then returning the media and chemical to its well of origin. The particular chemical treatment that each well received was determined by a web-based randomization procedure (www.randomizer.org). Each plate also contained wells (one well each) that received only 1 μL dimethylsulfoxide (DMSO, Sigma) and bicuculline methiodide (50 μM BIC, Sigma), the latter of which was evaluated as an endpoint selective control which increased activity (Crofton et al., 2011). DMSO was used as a vehicle control even though some chemicals were dissolved in water; the final concentration of DMSO or water added to each well was 0.1% (1 μL/mL), which did not alter pH or ionic balance. Recordings (33 min) with the chemical treatment were taken, collecting the same file types as during the baseline. Finally, 1 μM tetrodotoxin (TTX) was added to each well and 10 min of activity was recorded. Addition of TTX served as an assay selective control that decreased activity. To confirm results, a small number of recordings in single well MEAs were conducted using hardware and software from Multichannel Systems (Reutlingen, Germany) as previously described (Meyer et al., 2008).

After experimental data were collected, wells were cleaned by soaking with 1 mL of a solution of 1× phosphate buffered saline (PBS) and ~1.5 g pancreatin for 5–12 h. Wells were rinsed 3× with deionized water and plates were placed in a 55 °C oven overnight. Plates were then submerged in ethanol (70%) for 1 h and coated in

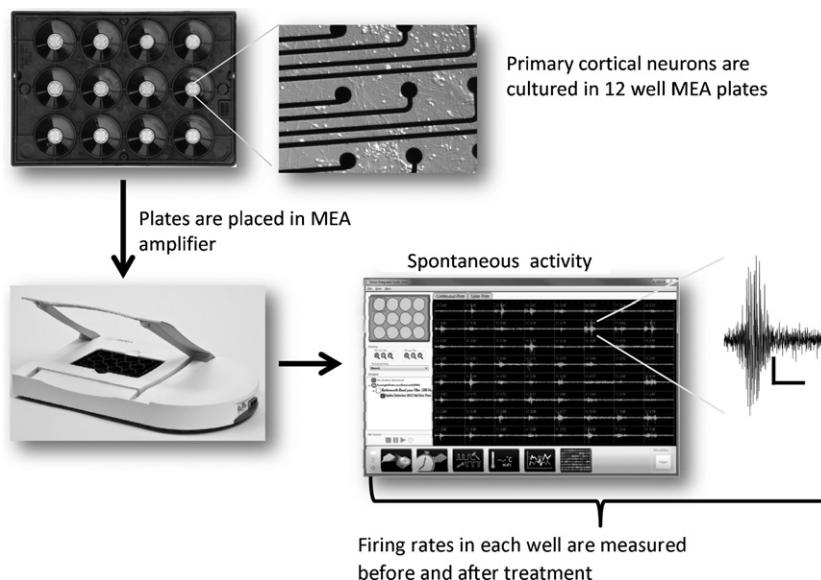


Fig. 1. Flow chart for examination of chemical training set effects on spontaneous activity in mwMEA plates. Primary cultures of cortical neurons are grown in 12 well MEA plates for 12–22 days *in vitro* (DIV). The mwMEA plate is shown in the upper left hand corner of the figure. The photomicrograph shows cortical cells growing on the electrodes. Each electrode in this inset is 30 μm in diameter. To conduct the experiment, the plate is placed in the Maestro amplifier which is connected to a PC. All filters and spike detectors are applied online as part of Axion's Integrated Studio (AxIS) software. An example screenshot showing the spontaneous activity in one well of the 12 well plate is illustrated, along with an inset showing an example of a burst of action potentials resulting from spontaneous activity. The scale bar is 50 ms and 20 μV . As indicated, for each experiment, 30 min of control activity was recorded, followed by 30 min in the presence of a training set chemical. Specific details are provided in Section 2.

poly-L-lysine for reuse. Each plate was used between 2 and 5 times. A check for delamination of the bisphenol-A complex¹ epoxy that insulates the electrodes was performed following the second or third use and before every additional use thereafter. For this, 70% ethanol (200 μL) was placed in the center of each well before adding 1 mL 1× PBS buffer and placing the plate in the Maestro system. Mean referencing (noise subtraction) was turned off and the baseline noise was examined. Delamination was the attributed cause if more than 50% of the electrodes had baseline activity of less than 6 μV , and such plates were not reused.

2.5. Data analysis

The spike count files generated from recordings were used to calculate the number of active electrodes (AEs; defined as an electrode having an average of more than 5 spikes/min) in each well, the average per-well mean firing rate (MFR or spikes/min), and the standard deviation of the average per-well MFR. The first 3 min of each data file was removed in order to let the activity stabilize in the Maestro. Data from wells that contained fewer than 10 active electrodes were not included in analysis. Data from single electrodes were removed *post hoc* if changes in spike rate indicative of noise on that channel were observed; data from only 4 electrodes from 4 separate wells were removed for this reason.

The MFR was first calculated for both the baseline and treated cases for each electrode. The average per well MFR for the baseline and the treated condition were determined for every experiment. For each individual chemical, the MFR averages for each well was weighted by the corresponding total number of active electrodes using EXCEL's SUMPRODUCT function and then averaged across the sum of those electrodes. The resultant weighted mean firing rate (wMFR) averages for the baseline and treated cases were then compared to compute a percentage change in the treated case

relative to the baseline case. Additionally, a simple percentage change in the total number of active treated electrodes relative to the total number of active baseline electrodes was calculated in order to capture possible changes in the number of AEs.

2.6. Propidium iodide/Hoechst staining for cytotoxicity

The 30 chemicals studied were tested for cytotoxicity using the fluorescent, vital stain propidium iodide (PI) which binds to DNA only in cells with a compromised plasma membrane. Using the above cell culture method, 96 well plates were cultured with a density of 40,000 cells/well. At DIV 13 or 14 chemicals were added (at the same concentration as used in MEA testing) to each well and incubated at 37 °C for 30 min. Triton X100 (1% by volume) was included on one well of each plate as a positive control to induce cell death, and each plate also contained wells that had no chemicals added to serve as negative controls. After 30 min, media was removed and 100 μL of 5 μM PI (Invitrogen) in Locke's buffer was added to each well with wide orifice pipette tips that reduced the mechanical disturbance to the cultures. Plates were incubated at 37 °C for another 20 min before aspirating the PI. Then 100 μL of warm fixative containing 3 $\mu\text{g}/\text{mL}$ Hoechst 33342 was added to each well and incubated at room temperature for 20 min. Fixative was aspirated and wells were washed two times with 100 μL Ca/Mg-free PBS (Gibco) leaving the final wash in the wells. Plates were then sealed with optical plate tape and stored at 4 °C until images were collected.

Quantitative fluorescence imaging with a Cellomics ArrayScan V^TI (ThermoScientific, Pittsburgh, PA) was used to determine the number of PI positive cells in each well. Fluorescence images were obtained in two channels: valid cells were identified using Hoechst stain (Hoechst stains DNA in all cell nuclei) by imaging in channel 1 using an excitation wavelength of 365 ± 25 nm and an emission wavelength of 515 ± 10 nm. PI positive cells (PI stains DNA only in cells with compromised plasma membranes) were imaged in channel 2 using an excitation wavelength of 549 ± 4 nm and an emission wavelength of 600 ± 12 nm. Images were analyzed using the Cellomics Target Activation Bioapplication. A mask was created

¹ There is no free bisphenol A following reaction of the epoxy as it is not present in excess. Cells grown in mwMEA plates form neuronal networks comparable to those grown on glass.

around all Hoechst stained nuclei in channel 1 and used to identify cells in channel 2. Nuclei in channel 2 were considered positive for PI staining when the average fluorescent intensity was greater than $3\times$ background. The percentage of PI positive cells was calculated for each well, with a minimum of 300 cells counted/well. Each chemical was applied to two wells in two different cultures ($n = 4$). Values from the four trials were averaged to determine cytotoxicity.

3. Results

3.1. Baseline and control responses

Because multi-well MEA plates are a new technology, preliminary experiments were conducted to characterize the development of network activity on these plates in comparison to single-well MEAs. The ontogeny of activity, both in terms of numbers of active electrodes (AEs) as well as the MFR on those electrodes demonstrated a steady pattern of increases between DIV 5 and 12, with more stable activity thereafter (Fig. 2). This is comparable to previously published data using a single-well MEA system (Robinette et al., 2011). Next, a concentration-response curve was determined for the GABA_A receptor antagonist BIC. BIC induced a concentration-dependent increase in MFR, with an EC₅₀ value of 0.30 μM (Fig. 3), which is consistent with laboratory historical values of 0.41 μM (unpublished data) for this compound. Over the course of the experiments with the chemical training set compounds (see Table 1), a total of 158 wells were recorded from 10 different cortical culture preparations. Averaged over all of the baseline recordings, there were 27 ± 12.2 active electrodes/well (mean \pm s.d., $n = 158$) and 49 ± 27.3 spikes/min/well (mean \pm s.d., $n = 158$). BIC (50 μM) and tetrodotoxin (1 μM) were evaluated as endpoint selective controls (Crofton et al., 2011) that increase and decrease activity, respectively. As an endpoint-selective control compound, BIC increased MFR to $131 \pm 13\%$ of control values (mean \pm s.d., $n = 18$). However, in several cases (8), the overall network response to BIC was either no net change or a decrease in MFR. This is consistent with previous observations in our laboratory as well as previous reports of heterogeneous network responses to bicuculline (Sokal et al., 2000). If these 8 wells are removed, then the average increase in MFR was $165 \pm 13\%$ ($n = 10$). This is slightly lower than the laboratory historical value of 181% for BIC plus the GABA_B antagonist SCH50911 (Meyer et al., 2008). By contrast, responses to TTX were consistent, with activity in all treated wells ceasing completely within 1 min after application of this toxin. For those wells treated with DMSO, an overall increase $22 \pm 9.5\%$ (mean \pm s.d., $n = 18$) in unweighted MFR was observed. Neither DMSO nor BIC had a significant effect on the number of AEs, whereas by default there were no AEs in the presence of TTX.

3.2. Chemical training set responses

There are several different approaches that could be utilized to determine what magnitude of a change in activity would be considered a “hit” from a screening context and thus select a chemical for additional testing. In the present experiments, each well served as its own control, and the change in activity elicited by a treatment can be easily expressed as a percent of that control activity. Changes in mean firing rate have often been used as a metric for drug and chemical induced changes in network activity (for example: Gramowski et al., 2000; Gopal, 2003; Meyer et al., 2008; Shafer et al., 2008; Novellino et al., 2011). However, it is necessary to account for the potential variability seen in the number of AEs which can incorrectly value the MFR between and within experiments. To account for differences in numbers of active electrodes between treatment groups, the MFR was weighted for both the control and treated condition based on

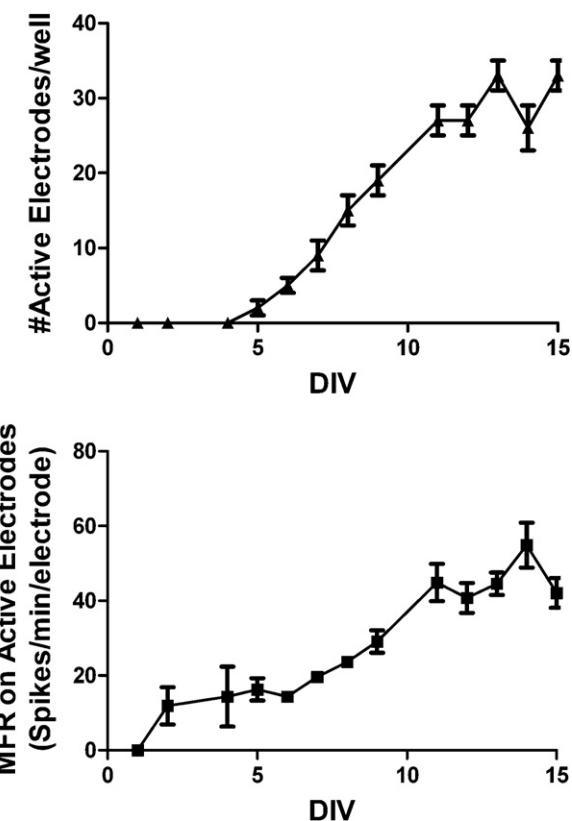


Fig. 2. Average number of active electrodes (AE)/well (bottom) and mean firing rate (MFR) on active electrodes as a function of days *in vitro* (DIV). There are a total of 64 potential electrodes in each well. The culture date is considered to be DIV 0. On the days indicated, 10 min of activity were recorded and the plate was returned to the incubator. Data are the means \pm SEMs of 12–29 wells from one to three 12 well MEA plates. Data are shown for up to 15 DIV, but similar results were obtained from plates through DIV 22.

the number of active electrodes. The mean percent change in wMFR induced by the vehicle control, DMSO, was an increase of $12.2 \pm 0.93\%$ (mean \pm s.d.) when a cross validation technique was used to determine the standard deviation of the weighted mean. Therefore, the detection threshold was set to 14%, which was the mean change induced by DMSO plus two standard deviations from this mean, regardless of the direction of the change. Although weighting normalizes the MFR based on the number of active electrodes, the wMFR does not reflect changes in the number of active electrodes. Thus, changes in the number of active electrodes were

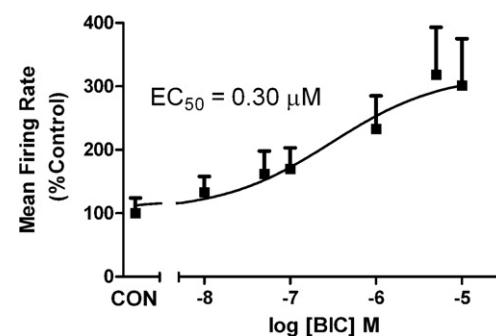


Fig. 3. Concentration–response relationship for bicuculline effects on MFR in cortical networks. A cumulative concentration curve was constructed by increasing the concentration of BIC in each well and recording for 10 min. Values shown are the means \pm SEM from 12 wells on a single plate. Based on previous concentration–response characterizations, higher concentrations of BIC (50 μM) cause similar increases in activity (data not shown).

Table 2Chemical training set effects on network activity (active electrodes and weighted mean firing rate) and viability.^a

	Active electrodes			Weighted mean firing rate (spikes/min)		
	Baseline	Treated	Mean % change	Baseline	Treated	Mean % change
<i>Positives</i>						
Bicuculline	23	22	-11.5	49.8	63.3	27.1
Bifenthrin	15	14	-5.2	57.6	60.6	5.2
Carbaryl	24	19	-21.1	36.5	23.4	-36.0
Chlorpyrifos oxon	28	25	-18.6	71.2	37.2	-47.8
Cyfluthrin	23	18	-29.1	33.4	47.2	41.4
Deltamethrin	29	19	-33.5	41.7	51.9	24.6
Diazepam	23	4	-80.0	33.2	27.0	-18.6
Domoic acid ^b	22	0	-100.0	79.4	0.0	-100.0
Fipronil	36	20	-37.5	37.7	19.9	-47.2
Fluoxetine	27	4	-87.9	45.4	10.4	-77.1
Imidacloprid	40	40	0.3	42.9	42.7	-0.3
Ketamine	34	2	-90.2	66.8	5.8	-91.3
Lead ^c	15	14	-13.8	44.4	58.5	31.5
L-Glutamate	31	26	-24.5	38.6	140.6	264.4
Lindane	27	28	6.5	54.8	84.0	53.1
Methylmercury ^b	27	22	-11.9	76.6	53.7	-29.9
Muscimol	33	0	-100.0	28.5	0.0	-100.0
Nicotine	33	33	0.7	52.9	59.9	13.3
Permethrin	20	18	-6.7	41.6	61.1	47.0
RDX	24	23	-3.3	63.8	104.8	64.2
Trimethyltin ^b	27	17	-34.8	52.7	32.5	-38.4
Valproic acid ^d	24	23	-5.2	38.2	47.1	23.4
Verapamil	33	12	-66.5	42.7	11.6	-72.9
<i>Negatives</i>						
Acetaminophen	34	34	-0.9	44.4	41.7	-6.1
Amoxicillin	31	31	-1.2	48.6	51.5	6.1
Glyphosate	27	26	-2.4	44.3	38.5	-13.1
Paraquat	23	23	-0.8	52.6	53.2	1.1
Saccharin	29	30	2.3	38.7	42.7	10.3
Salicylic acid	32	32	4.7	49.3	52.8	7.0
D-Sorbitol	25	27	9.4	50.7	49.4	-2.4
Vehicle	29	29	0.4	42.7	47.9	12.2

^a Data represent the mean over all experiments ($n=3$ –7, except bicuculline ($n=18$) and DMSO ($n=18$)). Chemicals were added to wells between DIV 14 and DIV 22. A chemical was considered a hit if the average percent change for wMFR was greater than 14% ($2\times$ the standard deviation from the mean percent change of wMFR for the DMSO), or if the number of AEs was changed by more than 20%.

^b Due to limited solubility, these chemicals were tested at 10 μ M.

^c Due to limited solubility, this chemical was tested at 30 μ M.

evaluated as a second criterion for a hit. DMSO treatment did not change the number of active electrodes. To provide some margin for biological variability, a 20% or greater change in the number of AEs was also considered a hit.

The overall number of AEs and wMFR responses for the chemical training set are presented in Fig. 4 and Table 2. Using the thresholds established above, none of the 7 negative chemicals reached the threshold values for either parameter. Of the 23 positive compounds, 20 caused a change in wMFR that exceeded the threshold value of 14%. Twelve chemicals caused a change in the number of AEs that exceeded the threshold of 20%, but this was a subset of the chemicals that were detected by changes in wMFR. Changes in wMFR included both increases and decreases (Fig. 5, supplemental Fig. 1), indicating that the MEAs could detect chemicals that altered spontaneous activity in either direction. Overall, 11 chemicals decreased and 9 chemicals increased wMFR. Interestingly, of the 9 chemicals that increased wMFR, 4 caused decreases in #AEs that reached threshold values. The three chemicals that were expected to cause changes in activity but did not were nicotine (13.3% change in wMFR), imidacloprid (0.3% change in wMFR), and bifenthrin (5.2% change in wMFR). Thus, using both criteria, none of the negative compounds were detected as a hit, while 20 of 23 positive compounds were considered hits. Therefore, cortical neurons grown in mwMEAs have a sensitivity (correct identification of positives) of 87%, a specificity (correct identification of negatives) of 100%, and a concordance (correct identification of all chemicals) of 90%.

3.3. Confirmation of results

Results for six chemicals were confirmed using a single-well MEA system. Sorbitol (50 μ M) treatment resulted in a wMFR change of 1.9% ($n=3$), nicotine treatment resulted in a wMFR change of 0.8% ($n=2$), and imidacloprid treatment produced a decrease in wMFR by 6.7% ($n=2$). By contrast, both verapamil and domoic acid inhibited wMFR by 100% ($n=2$) and fipronil treatment resulted in a 93.1% decrease in wMFR ($n=2$). These results confirm the multiwell results that sorbitol, imidacloprid, and nicotine had no effect, and verapamil, domoic acid, and fipronil decreased the wMFR beyond the threshold for a hit. Concentration-response curves were generated for nicotine, acetaminophen, deltamethrin and domoic acid in a single, separate experiment ($n=1$). The results (data not shown) were consistent with the single concentration screening results as well as other concentration-response data generated in the lab for these chemicals.

3.4. Cytotoxicity assay

Incorporation of information regarding cell viability can be informative for screening assays by helping to determine if effects are driven by cytotoxicity, rather than more specific disruption of cellular processes. The majority of the chemicals in the training set were without cytotoxic effects on the cortical cultures after a 30 min exposure (Fig. 6). Fluoxetine (50 μ M) caused the most cytotoxicity with $95 \pm 5\%$ of cells staining PI positive (mean \pm s.d.,

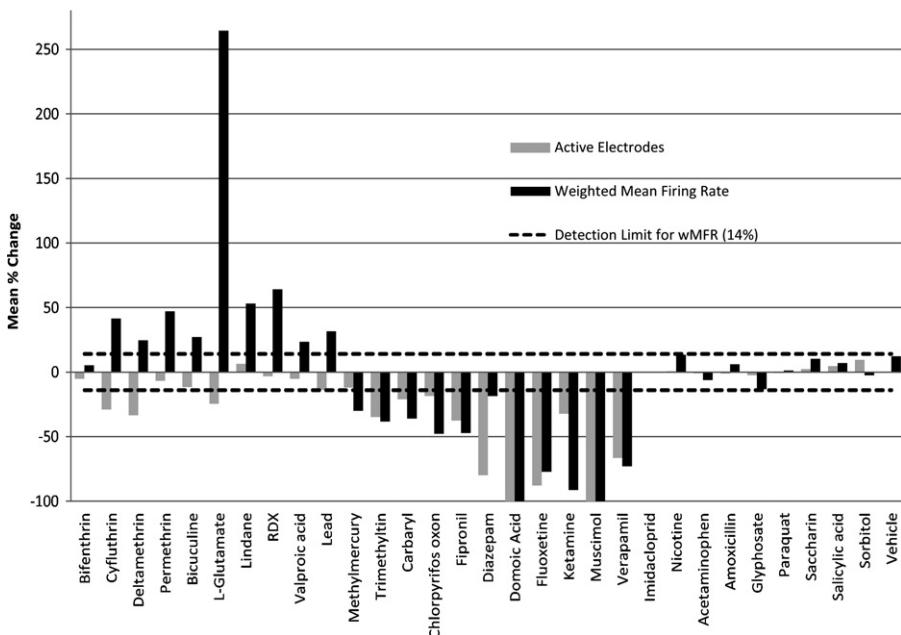


Fig. 4. Results from single concentration tests at 50 μ M or highest soluble concentration for chemical training set showing percent change ($n = 3\text{--}7$, except bicuculline ($n = 18$) and DMSO ($n = 18$)) in both weighted mean firing rate (wMFR) and active electrodes (AEs). Chemicals were applied to independent wells between 14 and 22 DIV and changes were calculated from control data from each individual well. The hit detection threshold line is for the wMFR and represents two standard deviations from the mean percent change for treatment with DMSO, the vehicle. 20 out of 23 neuroactive chemicals were detected as hits while all 7 negatives did not pass the detection threshold. Bifenthrin, nicotine, and imidacloprid were false negatives. Domoic acid, methylmercury, and trimethyltin were tested at 10 μ M, and lead was tested at 30 μ M.

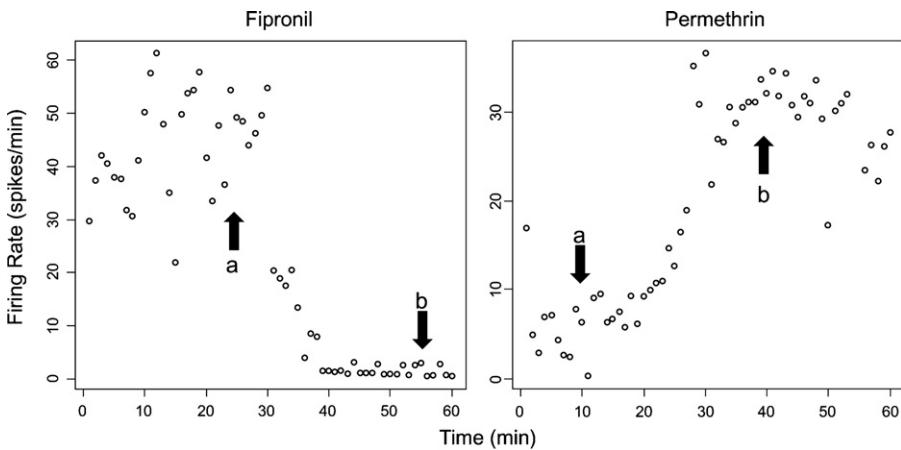


Fig. 5. Examples of training set chemicals that decrease (Left, fipronil) and increase (Right, permethrin) MFR in MEAs. An example experiment illustrates the average firing rate/min of the active electrodes in one well of a mwMEA plate. The first 30 min illustrates baseline activity in the absence of any treatment, while 31–60 min illustrate activity in the presence of permethrin and fipronil. These examples were constructed by combining two separate files, saved in the absence and presence of treatment, respectively, and the first 3 min of each of these files were removed as described in Section 2. In supplemental Fig. 1, raster plots are provided for each point labeled by the arrows (a and b), showing activity before and after the addition of chemical.

$n = 4$). Cytotoxicity has been reported in glioma and human neuroblastoma cells following a 24 hr exposure to fluoxetine at similar concentrations (Levkovitz et al., 2005). Fipronil (50 μ M) also consistently caused some cytotoxicity, with $34.1 \pm 8.2\%$ of cells staining positive for PI (mean \pm s.d., $n = 4$). Both methylmercury and lindane also caused some cytotoxicity, as $13.1 \pm 10.7\%$ and $47.1 \pm 40.0\%$ of cells stained positive for PI, respectively ($n = 4$).

4. Discussion

The present experiments demonstrate that spontaneous activity in cortical neurons grown on mwMEA plates have an ontogeny and pharmacological sensitivity similar to that observed on single-well MEAs. Furthermore, as demonstrated here, mwMEAs can provide a rapid and robust method of screening

compounds for potential neuroactivity. Under the thresholds selected for identification of a hit, only three of twenty-three compounds expected to alter activity were not identified as hits (false negatives), and none of the seven compounds expected not to alter activity were incorrectly identified as hits (false positives). Only four compounds caused measurable cytotoxicity, indicating that for most of the compounds tested, activity was selectively affected during the exposure. Thus, this assay has a sensitivity, specificity, and concordance of 87, 100 and 90%, respectively. These results indicate that a single concentration screening approach can be used to detect chemicals with the potential to alter neuronal function; such “hits” could then be confirmed with concentration-response assessments. Using such an approach in mwMEA plates can significantly increase the throughput of MEAs for neurotoxicity testing.

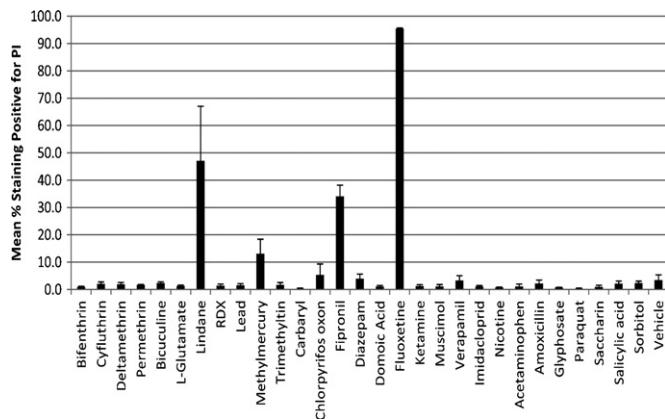


Fig. 6. Cytotoxicity assay was performed on the training set of chemicals. Cells were treated for 30 min with the chemical and stained with propidium iodide and Hoechst. Quantitative fluorescence imaging was used to determine the percent of total non-viable cells (e.g. propidium iodide positive). Some cytotoxicity was observed following treatment with fluoxetine, lindane, fipronil, and methylmercury. Valproic acid was not tested due to limited quantities of stock solution. Values are the means \pm SEMs for 4 observations in two separate cultures. Domoic acid, methylmercury, and trimethyltin were tested at 10 μ M, and lead was tested at 30 μ M.

The basic aspects (ontogeny, pharmacological sensitivity) of spontaneous activity of cortical neuronal networks remain consistent across a number of MEA platforms and laboratories (for review, see Johnstone et al., 2010). In the present experiments, the ontogeny of spontaneous activity was consistent with previous results (Robinette et al., 2011), as was the pharmacology. TTX, which blocks voltage-gated sodium channels, blocked all activity. Bicuculline-induced increases in activity occurred with an EC₅₀ value similar to that which had been historically observed in the laboratory. In some cases, BIC did not result in overall increases in network activity. This has been observed in our laboratory (unpublished data) and heterogeneous responses in other laboratories have also been reported (Sokal et al., 2000). Together with the previous characterizations of spontaneous activity in cortical networks, these results indicate that many aspects of network development and pharmacology are intrinsic to neuronal networks, and develop independently from the MEA platform.

The current study utilized concepts for assay development that were outlined by Crofton et al. (2010). Specifically, TTX and BIC were evaluated as assay-specific control compounds based on their known mechanisms of action. The network response to TTX was a consistent loss of network spiking activity, indicating that this compound is a good in-plate control for inhibition of activity. By contrast, responses to BIC were not consistent, even though the overall net effect of this compound was to increase activity and it was detected as a "hit" by the criteria used for these studies. However, by making some minor alterations in the tissue culture methods (addition of a step filtering cells prior to plating), increases in MFR following BIC (25 μ M) are more consistent (53/54 wells increased MFR; unpublished data). As such, evaluation of BIC as an in-plate control that increases MFR is ongoing.

To evaluate the ability of mwMEAs to screen for effects on neuronal network function, a training set of 23 compounds was selected and tested at a single concentration. The training set tested the ability of mwMEAs to detect alterations mediated by a wide variety of pharmacological and toxicological actions, and included compounds that were agonists or antagonists at GABAergic, glutamatergic, and nicotinic cholinergic receptors, voltage-gated sodium channels (pyrethrins) and acetylcholinesterase (chlorpyrifos oxon, carbamate), as well as other neuroactive/toxic compounds that are less specific (valproic acid, lead,

trimethyltin, methylmercury). Seven compounds were selected as negative controls; two of which (paraquat and salicylic acid) had been previously reported not to alter network activity (Defranchi et al., 2011) and five of which previously had been used as negative controls for developmental neurotoxicity assays (Breier et al., 2008; Radio et al., 2008).

MEAs provide high-content data in the form of spatial and temporal information regarding spontaneous network spiking and bursting activity. Thus, a number of metrics could have been considered in the decision to label a compound as a hit. However, in the context of screening large numbers of compounds, it is important to have a simple and rapid metric for hit selection. In the present study, two parameters were utilized to determine whether or not a compound was considered a hit in the assay: a >20% change in the number of AEs or a change in wMFR of 2 standard deviations beyond the mean change caused by the vehicle (DMSO). Both of these metrics can be quickly extracted from the raw data. The wMFR was clearly a more sensitive endpoint, as all 20 of the compounds considered hits altered this value, consistent with the widespread use of MFR in previous studies (for review, see Johnstone et al., 2010). When assaying large numbers of compounds, weighting the MFR by the number of AEs, as was done in the present studies, increases comparability across wells. Twelve compounds changed the number of AEs, but no compound changed only the number of AEs. Diazepam effects exceeded the threshold for wMFR by only a small amount (~18.6% change), but they also clearly exceeded the threshold value for number of AEs (~80% change). Because diazepam usually but not in every case, decreased activity to cessation, the change in MFR weighted against number of AEs is skewed. Thus, in cases like diazepam, it is useful to also consider the number of AEs as a metric to detect a hit.

For most of the compounds in the training set, the changes in activity were not associated with changes in cell viability, as only four compounds caused viability changes. Fluoxetine caused ~95% of the cells to be PI positive in the 30 min test period, while fipronil, lindane and methylmercury caused measurable, but not complete cytotoxicity. It is more difficult to determine the contribution of this cytotoxicity to the changes in activity observed with these compounds. While the PI assay does provide information about non-viable cells in the network, without additional counter-staining for neurons and glia, it is not possible to determine whether the cytotoxicity is occurring evenly across these two different cell types or if one is more vulnerable than the other. However, toxicity to either neurons or glia could influence activity, the former by direct effects (loss of firing neurons) and the latter by indirect effects (loss of glial support). In the context of screening large chemical sets, follow up of hits with characterization of the concentration-response for effects on activity as well as cell viability will be able to provide information regarding the specificity for effects on activity.

If a large set of compounds were being screened using this assay, identified hits would be confirmed either by conducting a concentration-response curve or by testing the compound in a "2nd tier" assay. In addition, it is recognized that simply assessing changes in the wMFR and #AEs with MEAs does not provide information regarding mechanism of action. Thus, hit characterization could also include more detailed examination of other parameters of activity (bursting, synchrony burst structure, etc.). Application of approaches such as spike train analysis (Brown et al., 2004) and features selection (Schroeder et al., 2008) might also allow sorting of hits into different pharmacological classes (for review, see Johnstone et al., 2010). Since the majority of compounds in this training set have already been evaluated in MEAs, confirmation of the present results was limited to a subset of positive and negative compounds in a single-well system using a different MEA platform. The two compounds that were not hits

(sorbitol and nicotine) did not alter wMFR, while the three compounds that were hits (fipronil, domoic acid, verapamil) all changed wMFR by more than 90%. Of the 30 compounds in the present study, 16 of them have been tested in other laboratories using various MEA systems; for 14 of those 16 compounds, the results of the present study are consistent with the previous data from other laboratories; this includes not only detecting the ability of a chemical to cause a change, but also the direction of that change (e.g. increase or decrease in spontaneous activity). The two compounds from this group that did not replicate were nicotine which was without effect in the present study, and valproic acid, which increased activity above threshold, rather than decreasing it (Gross et al., 1995). However, the concentrations of valproate reported to decrease activity were 300–700 μM (Gross et al., 1995), much higher than tested in the present studies. These data indicate that assessment of drug and chemical effects on neuronal network function is a robust approach that is reproducible across laboratories and platforms and is consistent with a previous study focused on reproducibility of MEA results (Novellino et al., 2011).

The lack of nicotine effect was confirmed in the present study by testing it at a single concentration of 50 μM in another system, as well as testing the concentration–response to nicotine. Although the latter was only a single replication, it is in agreement with other unpublished data from this laboratory wherein nicotine concentrations up to 500 μM cause only a ~15–20% change in firing rates (unpublished data). Nicotine was identified as a neuroactive substance in MEAs by Defranchi et al. (2011) with increases in MFR in their study observed at 100 μM nicotine. Thus, the lack of effect of nicotine in the present study may be due to the concentration being too low to elicit an effect. However, the efficacy of nicotine on neuronal nicotinic acetylcholine receptors in primary hippocampal cultures is in the range of ~10–25 μM (Alkondon and Albuquerque, 1993), much lower than the concentrations used in either MEA study. The effects reported by Defranchi and co-workers may not have been mediated by nAChR, which was not tested in that study. Alternatively, it is possible that the culture model used here is not responsive to nicotinic modulation, and the lack of response to nicotine in the present study reflects this. Imidacloprid has a lower affinity for some subtypes of nAChR than nicotine (Tomizawa and Casida, 2005) and can function under some conditions as a partial agonist (Nagata et al., 1998). Given the lack of response to nicotine in the present study, it is not surprising that imidacloprid did not alter network activity.

Compared to the two nicotinic agonists, it is interesting that two cholinesterase inhibiting insecticides, chlorpyrifos oxon and carbaryl, caused clear effects in the present study. Carbaryl also produced complete inhibition of MFR in studies by Defranchi and colleagues. These results are consistent with previous reports of alterations in spontaneous activity of neural networks by cholinesterase inhibitors (Keefer et al., 2001). The lack of response to nicotine indicates that effects of cholinesterase inhibitors in the present study were not due to direct activation of nAChR or to activation secondary to acetylcholinesterase inhibition. One possible mechanism that may account for the effects of chlorpyrifos oxon and carbaryl is that they were mediated by muscarinic AChR secondary to inhibition of acetylcholinesterase. Carbachol, acting through muscarinic ACh receptors, has been reported to alter neuronal activity in MEAs (Tateno et al., 2005). Acetylcholinesterase-independent actions have been reported for these compounds, especially for chlorpyrifos oxon (for reviews, see Pope, 1999; Slotkin, 2004), including direct actions on muscarinic AChR, as well as other targets. Thus, an alternative explanation for the current results with these two compounds is that their effects were mediated via direct interaction with muscarinic AChR or

other non-cholinergic actions. Further study of the responsiveness of this culture model to cholinergic compounds, including agonists and antagonists of both nicotinic and muscarinic receptors, as well as characterization of the expression of cholinergic receptors, will help to clarify its utility to detect compounds that act via cholinergic pathways.

The third compound that was not detected as a hit was bifenthrin. Bifenthrin is a type I pyrethroid insecticide, which act via disruption of voltage-gated sodium channel gating kinetics (for review, see Soderlund et al., 2002). Three other pyrethroid compounds included in this study all caused effects that were greater than the hit thresholds. Two of these compounds (cyfluthrin and deltamethrin) are type II pyrethroids, which produce longer lasting modifications of sodium channel gating than do type I compounds. However, the type I compound permethrin also had effects in the present studies, so it is unclear why bifenthrin was without effect. In a recent study examining sodium and calcium flux in mouse cortical neurons, bifenthrin had low efficacy in modulation of both responses (Cao et al., 2011a,b). This lack of efficacy may underlie the response in the present study. However, under conditions where inhibitory GABAergic neurotransmission is blocked, bifenthrin reduced network firing rate in a concentration-dependent manner and has an IC₅₀ value in the nM range (Losa et al., 2009; manuscript in preparation). Thus, bifenthrin could be considered a false negative in the current study.

The need for *in vitro* assays to screen chemicals for their hazard potential is well recognized (NRC, 2007; Bal-Price et al., 2008; Andersen and Krewski, 2009; Boekelheide and Campion, 2010). The nervous system presents unique challenges with respect to screening assays because its functional output involves spatial and temporal patterns of electrical activity that are influenced by the phenotypes of various cells that comprise different neuronal pathways. For *in vitro* assays based on biochemical and morphological endpoints, it is often difficult or not possible to predict how changes in these endpoints may ultimately influence neuronal function. In addition, there are many different known toxicity pathways in the nervous system that will result in significant, and sometimes fatal, toxicity outcomes prior to or in the absence of changes in protein levels or gene expression. Neurophysiology has been utilized for many decades as a sensitive indicator of chemical neurotoxicity. However, many neurophysiological approaches lack the throughput necessary to be efficient screens for neuroactivity/toxicity. MEAs have been proposed as a viable approach for screening that has the ability to detect changes caused by many different classes of compounds (Johnstone et al., 2010; Novellino et al., 2011). However, single well MEA platforms widely utilized to date have limited throughput capability and thus are an inefficient approach for assaying large numbers of chemicals. The mwMEA plates utilized in the present experiments offer a significant increase in capacity for measurement of compound-induced alterations in neuronal network function. Furthermore, the design of the multi-well plate in compliance with ANSI specifications for multi-well tissue culture plates will facilitate automation, further increasing throughput. Future advancements, such as expected 48 and 96 well mwMEA plates, will result in higher throughput and offer the potential to develop neurotoxicity screening assays capable of screening greater numbers of chemicals.

Conflict of interest

Emma R. McConnell, Maxine A. McClain, James Ross: These authors work for Axion BioSystems, which makes multiwell microelectrode array hardware and software; William R. LeFew, Timothy J. Shafer: None declared.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.neuro.2012.05.001>.

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