Application of Microelectrode Array Approaches to Neurotoxicity Testing and Screening

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Abstract Neurotoxicity can be defined by the ability of a drug or chemical to alter the physiology, biochemistry, or structure of the nervous system in a manner that may negatively impact the health or function of the individual. Electrophysiological approaches have been utilized to study the mechanisms underlying neurotoxic actions of drugs and chemicals for over 50 years, and in more recent decades, high-throughput patch-clamp approaches have been utilized by the pharmaceutical industry for drug development. The use of microelectrode array recordings to study neural network electrophysiology is a relatively newer approach, with commercially available systems becoming available only in the early 2000s. However, MEAs have been rapidly adopted as a useful approach for neurotoxicity testing. In this chapter, I will review the use of MEA approaches as they have been applied to the field of neurotoxicity testing, especially as they have been applied to the need to screen large numbers of chemicals for neurotoxicity and developmental neurotoxicity. In addition, I will also identify challenges for the field that when addressed will improve the utility of MEA approaches for toxicity testing.

Keywords Developmental neurotoxicity · Neurotoxicity · Screening

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1 Introduction

The nervous system, and especially the developing nervous system, is uniquely sensitive to perturbation by a wide variety of natural toxins, drugs, and a wide range of environmental chemicals (metals, pesticides, solvents, etc.). The nervous system is unique from other organ systems (such as the liver, kidney, and lungs) due to the nature of its function; it must receive input from the environment, rapidly transmit that information over long distances, integrate information from multiple sources, store it and generate appropriate responses. This is accomplished through the rapid transition of biochemical to electrical signals (and vice versa) and through spatio-temporal patterns of electrical signals to encode and convey information within networks of interconnected neurons and to target tissues (e.g., smooth and skeletal muscle, endocrine glands).

Because of the complexity of the nervous system, there are a wide variety of approaches used to study how its function is perturbed. At the whole animal level, the fields of behavioral pharmacology and toxicology have been important to identifying how different toxicants alter function of the nervous system (Weiss and Laties 1975), as have the fields of neuroimaging and neuropathology. Neurochemical approaches have also been widely utilized to understand mechanisms underlying toxicant actions on the nervous system. However, because of the electrical excitability of the nervous system, neurophysiological approaches have been critical to both identifying and understanding which, and how, compounds alter nervous system function. At the whole animal level, neurophysiological approaches such as visual, auditory, and somatosensory evoked potential recordings (Otto et al. 1988; Boyes 1993, 1994) helped to identify the neurotoxicity of solvents and pesticides. By contrast, at the cellular and sub-cellular level, patch-clamp and sharp electrode recordings helped to identify the mechanisms by which metals disrupted neurotransmission at the neuromuscular junction and by which pyrethroids produced acute neurotoxicity by altering voltage-gated sodium channel kinetics in neurons (Shafer and Atchison 1995; Narahashi 2002).

Small networks of interconnected neurons are critical to nervous system function. These networks often exhibit synchronous and oscillatory behavior (Uhlhaas et al. 2009; Salinas and Sejnowski 2001), which when disrupted are associated with pathological disease, including schizophrenia, epilepsy, autism, and neurodegenerative diseases (Uhlhaas and Singer 2006). Because the field of microelectrode array recording evolved later than other electrophysiological approaches, much less is known about how neurotoxicants alter function at the level of neuronal networks. However, MEAs have a unique niche in the neurotoxicologist's toolbox, as they alone facilitate the evaluation of how chemicals alter the function of small networks of interconnected neurons. One way that this approach is contributing to our knowledge is by providing additional mechanistic information on the actions of compounds on neural networks. A second, and perhaps more important way that MEA approaches are impacting the field of neurotoxicity is in the screening of compounds for their potential to cause neurotoxicity or developmental neurotox-

icity. The lack of information on these endpoints for thousands of chemicals has resulted in an urgent need for rapid and economical approaches to address this data gap, which can in part be filled by MEA approaches. As such, the role of MEAs in neurotoxicity screening has been an area of considerable growth in the last decade and will be further addressed below.

In this chapter, I will present an overview of how neural networks cultured on MEAs have been used to address both mechanistic questions and screening approaches related to neurotoxicity testing. I will discuss some important methodological considerations of using MEAs for this purpose, as my experience has been that good methodology is critical to obtaining usable screening data. Finally, I will also present some challenges for the future, better utilization of the rich information in MEA recordings and better incorporation of neural networks derived from humans into neurotoxicity studies with MEAs.

2 Platforms and Methodological Considerations

Standard MEA formats usually consist of a grid of planar microelectrodes (typically 8–64 electrodes/MEA) that are 10–50 μm in diameter and are spaced from 150–300 μm apart, such that they will detect signals from separate portions of the network. These differ from the high-density MEAs, which can contain thousands of CMOS-based electrodes that are closely spaced (~20 μm) such that multiple points can be recorded from the same neuron. More information on these systems can be found in chapters "Large Scale, High-Resolution Microelectrode Arrays for Interrogation of Neurons and Networks" and "Active High-Density Electrode Arrays: Technology and Applications in Neuronal Cell Cultures" of this book. Although they have not yet been utilized in toxicological studies, they offer the opportunity to evaluate chemical effects on action-potential generation and propagation, and associate electrical changes with structural features at the level of the individual cell. However, since CMOS-based MEAs have not been widely utilized in neurotoxicological studies, the rest of this chapter will focus on the conventional format MEAs. In the last decade or so, MEA recording approaches have become much more available to the scientific community as MEA systems have been commercialized and software has been improved to facilitate the execution of experiments and analysis of the resultant data. The throughput of MEA systems has also increased, from single well systems with (typically) 60–64 electrodes/MEA chip to multi-well plate formats that may have as many as 96 wells each with 8 electrodes. Typically, an MEA system will consist of the following components: MEA chips or plates; amplifier, computer; data collection and analysis software. In terms of laboratory space, MEA systems have a small footprint (a few square feet) and are easy to accommodate. Detailed information on systems and requirements can be found on the websites of the manufacturers of MEA equipment, provided

Table 1 Manufacturers of MEA equipment

Company	Format	Website
Axion Biosystems	Single and multi-well	www.axionbiosystems.com
MED64	Single and multi-well	www.med64.com
Multichannel Systems	Single and multi-well	www.multichannelsystems.com
Maxwell Biosystems	Single and multi-well (CMOS-based systems)	www.mxwbio.com/

in Table 1. Other laboratory requirements will be a cell culture facility including a laminar flow hood, incubator and associated equipment. One consideration of importance, especially for multi-well systems, is data storage space; a single 1 h raw recording from a 48 well plate on the Axion system can be as large as 50 GB. Thus, an active laboratory can easily produce several terabytes of data in a few months. Having adequate space to store and back up this data is crucial, especially if the data are to be used for regulatory purposes, where there may be specific requirements for data storage and handling.

Good quality MEA data, especially in higher-throughput systems, starts with good and consistent laboratory tissue culture techniques. In part, this is driven by having experienced hands preparing the plates and the cultures. However, even those less experienced with tissue culture techniques can prepare high quality cultures by following established protocols closely and consistently. There are several publications that address good cell culture practices related for toxicology studies (Pamies et al. 2017, 2018; Pamies and Hartung 2017; Eskes et al. 2017). Successful MEA recordings can be made from a wide variety of different tissue types, including primary cultures of rodent cortex (Xiang et al. 2007), hippocampus (Arnold et al. 2005), brain stem (Su and Jiang 2006), auditory cortex (Gopal and Gross 1996), and dorsal root ganglion (Newberry et al. 2016). Active cultures can be prepared from either fresh tissue, or from frozen cells, which gives researchers some options for tissue sources. There has been tremendous progress in the availability of human-derived tissues for MEA recordings in the past decade. Several different vendors now supply human embryonic or inducible pluripotent stem cell-derived models that result in neural networks that exhibit robust spiking, bursting, and coordinated bursting, similar to their rodent counterparts (Fig. 1). For these commercially available human models, the vendors often have worked out and provide detailed protocols for the use of their cells on different MEA systems. Following these protocols as written will result in successful recordings and make the best use of these cells, which are not inexpensive.

Another important methodological consideration is attention to detail during the execution of experiments. Network activity is sensitive to and influenced by temperature, pH, osmolarity, and physical disruptions. Most MEA systems have built-in temperature control, and some newer systems also have environmental controls for humidity and CO_2 that help to mitigate against evaporation of the media and pH changes that may occur over time with longer recordings. Mechanical

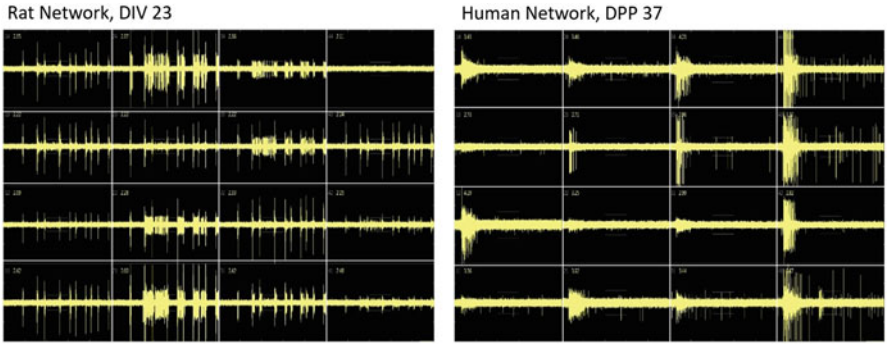

Fig. 1 Comparison of rodent vs human neural networks. Screenshots of spiking and bursting activity in a rat (left) and human (right) cortical network grown in 48 well plates for 23 and 37 days, respectively. Human networks of direct differentiated neurons (1.4×10^5 excitatory cells, 0.6×10^5 inhibitory cells), and glia (0.7×10^5) astroglial cells/well recorded at 37 days post-plating (DPP) show similar spiking and bursting patterns when compared to rat cells plated at 150,000 cells/well from post-natal day 0 cortex (as described in Valdivia et al. 2014) recorded at 23 days in vitro (DIV). Human cells provided by J. Davila and D. Haag, Stanford University

disturbances can temporarily change network activity, so typically a period of time (10–30 min) is needed for activity to stabilize again following transfer of cultures from the incubator into the amplifier or the addition of test compounds. Thus, each laboratory needs to establish the appropriate amount of time for both based on their experience, and dosing needs to be done carefully in order to minimize the disturbance.

With respect to evaluating the potential neurotoxicity of compounds using MEAs, it is important to consider whether network activity is being disrupted due to effects on the neurophysiology or concomitantly with alterations in cell health. This is important whether acute or longer term (e.g., developmental or delayed/"chronic") effects are being examined. Most single-well MEA formats are made from glass or other clear substrates, making morphological evaluation possible. Further, the low throughput of these formats is also amenable to the more time-consuming evaluation of morphological alterations on each network treated with a compound. The increased availability of multi-well format MEAs has complicated evaluation of cell health for two reasons; the number of networks to evaluate is dramatically increased and not all multi-well formats are transparent, which prevents visual and/or morphological assessments. Consider an experiment where triplicate measurements are made across three 48 well plates. Even if visual inspection is possible, there are 144 wells to inspect. Thus, other methods are required to examine cell health following treatment with potentially neurotoxic compounds. One can use "sister plates" to examine cell health in parallel with MEA experiments. However, this increases both time and materials required for tissue culture and maintenance of cells. Wallace et al. (2015) demonstrated that multiplexed measurements of network activity and cell viability could be made

by using lactate dehydrogenase (LDH) and alamar blue (AB) assays immediately following recordings (detailed protocols are available in Brown et al. 2017). Caveats regarding any cell viability assays should be kept in mind. For example, the assays above reflect the health of all cells in the culture, whether neurons or glia. Thus, effects on a specific population might be blunted or missed, if for example a compound was only cytotoxic to neurons. Thus, the lack of a response in these assays does not necessarily indicate that a compound had no adverse effects on cell health. By contrast, a decreased signal also does not necessarily indicate cell death, especially following developmental or longer term exposures. For example, a compound might decrease glial proliferation, resulting in lower total LDH as well as reduced metabolic activity (AB), even though no cell death has occurred. Even with these caveats, having some information on cell health can be useful in screening, and more mechanistic assessments can always be conducted as a follow-up to hits (chemicals that alter network function).

The availability of multi-well MEA formats has made possible screening large numbers of compounds for potential effects on network activity and development of network activity. There are several experimental design issues that must be considered when conducting screening experiments using multi-well plates, including how many replicates are needed, whether to place those replicates on the same plate or different plates, and whether or not there are differences between wells along the edge of the plate compared to those in the interior. Each laboratory will have to determine empirically what works best in its hands. However, our experience with 48 well MEA plates has been that well-to-well variability is as high or higher than plate-to-plate variability, and that culture-to-culture variability is higher than either of these. This is likely due to our use of primary cultures as each culture is made from a different litter of animals every week. When screening for neurotoxicity or developmental neurotoxicity, we typically test compounds in triplicates across three different plates within the same culture (Fig. 2). This is a common approach for screening of compounds (Malo et al. 2006) as it reduces biological variability due to day-to-day and culture-to-culture differences. For higher throughput, a single high concentration of a compound can be screened to identify "hits" (e.g., Strickland et al. 2018). This allows more compounds to be tested on a plate, and hits can then be followed up with concentration-response characterization. A concern when screening with multi-well plates is that the microenvironment of the wells along the edge of the plate differs from the middle wells, giving rise to differences in cellular responses. Therefore, we analyzed historical data for potential differences between edge and interior wells, and did find that some parameters were statistically different. However, the mean differences between edge and interior wells were very small (Table 2) and were only detected as statistically different due to the large sample size. As such, they were not considered biologically relevant. However, to prevent all of the control data coming from edge wells, we commonly distribute our control wells on each plate between both edge and control wells (Fig. 2).

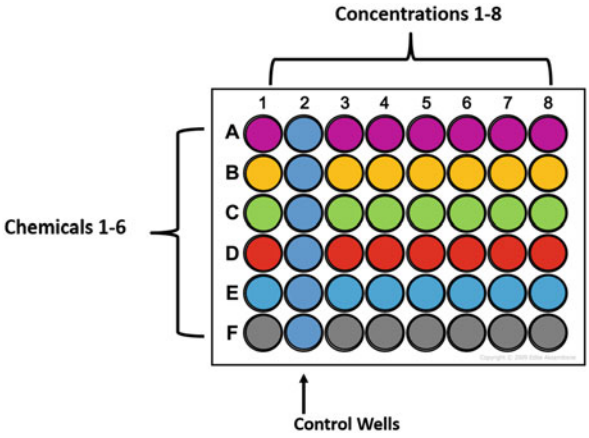

Fig. 2 Example plate layout for screening chemicals. A typical arrangement of chemicals on MEA plates for a screening experiment. Six chemicals are tested over seven different concentrations on triplicate plates. One row on each plate is used for each chemical (represented by different colors, while different concentrations (typically increasing) are arranged across columns 1–8). The blue wells represent control wells, which are always located so that both edge and interior wells are included. Each plate contains six control wells; if needed, some wells can be used for positive control (e.g., bicuculline or tetrodotoxin) treatments, or as control wells for viability assays (see Brown et al. 2017 for additional details)

Table 2 Comparison of edge and inner wells on 48 well MEA plates

Endpoint	*p*-value from paired *t*-test	Mean difference[a]	DF (# of averaged recordings −1)
Mean firing rate	NS	NA	199
Burst/min	NS	NA	199
% of spikes in burst	2.739×10^{-9}	−5.369	199
# active electrodes	0.02963	−0.28667	199
# actively bursting electrodes	0.01117	−0.352	199
# of network spikes	1.418×10^{-7}	20.18396	199
% spikes in network spike	0.02814	0.8132	199
r	0.003842	−0.01311	199

50 total plates (each with four DIVs (5, 7, 9, and 12) resulting in recordings from 200 edge well and 200 inner well values for paired *t*-test)

[a]Differences were calculated by subtracting the inner well values from the edge wells and taking the mean of the result. Mean (Edge well − Inner well)

3 Data and Data Analysis for MEA Recordings

As mentioned above and shown in Fig. 1, neural activity as measured by MEAs is complex and results in spatially and temporally rich patterns of activity. Initially, toxicological studies using MEAs focused primarily on the mean firing rate (MFR) of the network (although some studies examined multiple parameters). The focus

on MFR was driven by the fact that it is both a sensitive measure and the one that is easily extracted from the data, which facilitated rapid evaluation of whether or not a compound disrupted activity. However, other parameters can be extracted from the recordings that describe the characteristics of bursting, synchrony, oscillations of network activity and mutual information (Ball et al. 2017) contained in the network. These and other network parameters are described in Table 3. As discussed in Sect. 6, a more complete description of the pattern of network parameter disruption caused by a potential toxicant might be useful to identify the class of compound or predict in vivo activities (e.g., seizures).

Typically, the ability of a compound to disrupt network activity is reported in one of two ways, alteration of activity beyond a preset threshold (e.g., McConnell et al. 2012; Valdivia et al. 2014; Strickland et al. 2018) or by reporting the potency of the compound for altering a particular parameter (e.g., Defranchi et al. 2011; Brown et al. 2016; Frank et al. 2017; Zwartsen et al. 2018). The former is typically employed when testing only a single concentration of a compound for effects on network activity. Typically, the change in one or more parameters is compared to the change resulting from treating sister wells with the vehicle used for dissolving the compounds, such as DMSO, ethanol, or water. Often, the test compound is not considered active (or a hit) unless the change it causes lies beyond one or (more typically) two times the change caused by the vehicle. The latter approach, in which potency is determined, can be used when networks are exposed to multiple concentrations of the same compound, either in a cumulative manner (the concentration of compound is increased in the same well and compared to pre-exposure values) or a "multi-well" approach, where each well receives one concentration of a compound and the effects are compared to pre-exposure values and/or wells treated with vehicle. In either case, potency is usually determined by calculating an EC_{50} (Effective Concentration that changes the response by 50%, compared to control) by fitting the data to a non-linear relationship such as a sigmoidal dose-response curve.

While the above approaches work for single timepoint measurements, they fail to take advantage of the ability of MEAs to make repeated measures of network function over time. However, analysis of such data is more complex, as the overall effects is a function of both time- and concentration-parameters. To address this issue, we have used the area under the curve, which captures both time and concentration-dependent effects (Fig. 3). We first calculate the trapezoidal area under the curve (AUC) for each concentration and timepoint, and then determine the EC_{50} values by fitting the AUC values at each concentration to a sigmoidal dose-response relationship.

4 Use of MEAs for Acute Neurotoxicity Screening

MEAs have been used to understand the actions of neuroactive and neurotoxic substances since they were introduced in the late 1990s. In 2010, Andrew Johnstone, myself, and others (Johnstone et al. 2010) reviewed the use of MEAs for this purpose

Table 3 Examples of different parameters of network function that can be evaluated by MEA recordings

Type of measure	Parameter	Description
General activity	Mean firing rate or mean spike rate	The mean firing (spiking) rate. Typically measured on each electrode, with the value the mean across all electrodes in the well and reported in spikes/min or in Hz
	Burst rate	The number of bursts per unit time; typically reported in bursts/min
	Number of active electrodes	Number of electrodes firing at or above a pre-defined spike rate
	Number of actively bursting electrodes	Number of electrodes with burst rates at or above a pre-defined burst rate
Bursting activity	Interspike Interval (ISI) within a burst	Time interval between spikes within a burst. Typically reported in milliseconds
	Percentage of spikes in burst	The number of spikes that occur within a burst as a percentage of the total spikes. Recorded at the electrode level but often reported as a well-wide average
	Mean burst duration	Mean duration of a burst. Typically reported in milliseconds (ms)
	Mean interburst interval (IBI)	Mean time interval between bursts. Typically reported in seconds (s)
Network connectivity	Number of network spikes (also called network bursts)	A network spike (or burst) is activity that co-occurs simultaneously across a set minimum number of electrodes (see Eytan and Marom 2006)
	Network spike peak	The number of electrodes active at peak of network spike
	Network spike duration (NSD)	The average duration (ms) of a network spike
	SD of network spike duration	Standard deviation of network spike duration. Describes how consistent the network spike is
	ISI in network spike	Mean interspike interval for spikes in network spikes
	Mean number of spikes in network spikes	Number of spikes in network spike
	% spikes in network spike	Number of spikes that occur within a network spike over the total number of spikes
	Mean correlation (r)	The average of all pairwise correlation between all electrodes
	Normalized mutual information	A measure of complexity and synchrony in a network that is robust with respect to changes in the size of the network (see Ball et al. 2017)

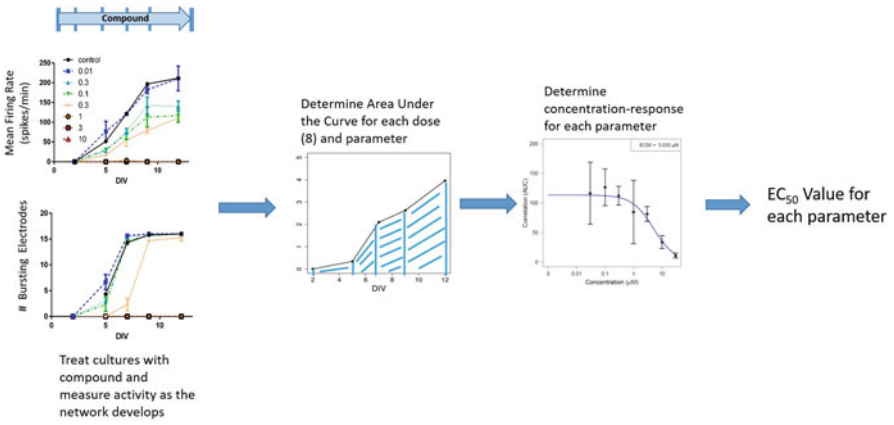

Fig. 3 Determining EC_{50} values based on Area Under the Curve (AUC) measurements. In the example above, networks were exposed to compound and activity was recorded on days in vitro (DIV) 5, 7, 9, and 12. On the far left, examples are shown for two parameters, mean firing rate and number of bursting electrodes, but the approach can be expanded to multiple parameters. In the middle panel, an example is shown illustrating how the trapezoidal area under the curve is determined for each concentration (8 in this case) and parameter. Once AUC values are determined for each parameter, they can be fit to sigmoidal concentration-response relationships (right panel)

and further proposed that MEAs could be a useful tool for screening larger numbers of compounds for potential neurotoxicity. As part of the review, we included a table that summarized the use of MEAs for toxicity studies, and therefore I will not present that same information here. Since then, neural networks grown on MEAs have been used to determine the activity of a large number of different types and classes of compounds, including agrochemicals (Alloisio et al. 2015), pyrethroid insecticides (Shafer et al. 2008; Meyer et al. 2008; Mohana Krishnan and Prakhya 2016; Baskar and Murthy 2018) and mixtures of pyrethroids (Scelfo et al. 2012; Johnstone et al. 2017), nanoparticles (Gramowski et al. 2010; Strickland et al. 2016a, b), tricresyl phosphate (Duarte et al. 2017), illicit drugs (Hondebrink et al. 2016), glufosinate (Lantz et al. 2014), antiepileptic drugs (Colombi et al. 2013), excitotoxicants (Frega et al. 2012), components of harmful algae (Alloisio et al. 2016), neuroactive toxins (Pancrazio et al. 2014; Kasteel and Westerink 2017), and metals (Dingemans et al. 2016; Huang et al. 2016).

In addition to these studies directed at understanding specific types of compounds, tremendous progress has been made in the last 8 years towards demonstrating that neural networks grown on MEAs are indeed useful for acute neurotoxicity screening. This has in part been driven by the availability of commercially available multi-well MEA formats that have substantially increased throughput of MEA testing as well as the publication of the report from the NRC on *Toxicity testing in the 21st Century* (NRC 2007), which called for increased development of predictive, in vitro approaches for toxicity hazard characterization.

Two of the earlier studies demonstrating proof-of-principle for using MEAs for neurotoxicity screening were published by Defranchi et al. (2011) and McConnell et al. (2012). In both cases, the authors selected a small number of compounds (20 and 30, respectively) that had well-established effects on nervous system function or lack thereof (negative controls). Neural networks grown on MEAs were shown to have high sensitivity (correct identification of active compounds), with these studies reporting that 77–87% of the neurotoxic/neuroactive substances altered mean firing rates of networks. Specificity (correct identification of non-neuroactive compounds) was also high, ranging from 86 to 100%. In an additional study, Nicolas and co-workers demonstrated 88% sensitivity of rat cortical cultures grown on MEAs to detect 15 known neuroactive compounds, including marine neurotoxins found in seafood (Nicolas et al. 2014). These initial studies indicate that neural networks grown on MEAs could be useful for screening compounds where potential for neurotoxicity had not yet been evaluated, and in the case of marine toxins, may be a less expensive, faster and more ethical approach than current animal-based approaches (Nicolas et al. 2014). There is also evidence that results across different laboratories and platforms are also quite replicable. Two different studies involving multiple laboratories have demonstrated consistency in results in response to neuroactive drugs (Novellino et al. 2011) as well as positive and negative control neurotoxicants (Vassallo et al. 2017). While only a small number of chemicals were tested in each of these studies, the collective high sensitivity and cross-laboratory reproducibility indicates that MEAs offer potential for neurotoxicity screening. Nevertheless, additional evaluation of the approach, including testing larger numbers of chemicals, was needed.

As a follow-up to the study by McConnell, my laboratory obtained 93 compounds from the ToxCast library (Richard et al. 2016) and tested these in primary cortical networks grown on MEAs. These compounds had all been tested in the ToxCast program, which examines the effects of compounds in a battery or over 800 assays. A sub-set of 20 ToxCast assays measure activity towards voltage- and ligand-gated ion channels. Again, MEAs were quite specific, detecting approximately 73% of compounds that were recognized to be neurotoxic/neuroactive. In addition, MEAs also detected classes of compounds that were not identified as active in the ToxCast ion channel assays, including GABAergic and pyrethroid compounds (Valdivia et al. 2014). Interestingly and importantly, this study also indicated that combining the MEA assay with ToxCast assays may improve screening for neurotoxicity overall, as the cortical culture used in this study appears to be relatively insensitive to nicotinic compounds (McConnell et al. 2012; Valdivia et al. 2014), which were well detected by alpha-bungarotoxin binding assays in ToxCast. Combining the MEA and ToxCast assays resulted in 85% sensitivity. Thus, MEAs also appear to be complimentary to other screening approaches, increasing their value as part of an integrated testing approach.

These initial studies provided the justification to screen the entire Phase I and II libraries of the ToxCast chemical space in rat primary cortical neurons grown on MEAs. The goal here was not to evaluate sensitivity or specificity of MEAs, but rather to demonstrate that they could be used to screen a large set

of chemicals (Crofton et al. 2011); the Phase I and II library consists of 1055 unique substances. Greater than 85% of the active compounds (326 total) fell into five broad chemical categories: pesticides, pharmaceuticals, chemical intermediates, microbiocides/fungicides and herbicides (Strickland et al. 2018). Since the chemical space covered by ToxCast includes broad categories such as fragrances, "green chemicals," food flavors and additives, and surfactants (Richard et al. 2016), these results indicated that compounds which disrupt network activity may be largely comprised of those that were specifically designed to be biologically active (e.g., pharmaceuticals, pesticides, etc.). Testing of additional compounds will help to confirm this observation. The entire set of compounds was screened at a single concentration in less than a year (Strickland et al. 2018). While this may not on the surface appear to be very remarkable, consider that this was done without the assistance of automation (plating or dosing robots, for example), and that the single largest limiting factor was the ability of our tissue culture facility to generate cultures. In this case, the use of fresh primary cultures, due to requirements for timed pregnant animals, reduced the number of cultures that could be made to one per week. Additional considerations related to screening are discussed below.

5 Use of MEAs to Screen Compounds for Developmental Neurotoxicity

In the last 20–30 years, there have been world-wide reports of increasing rates of neurodevelopmental disorders (Grandjean and Landrigan 2006, 2014; Hertz-Picciotto et al. 2006; Karr 2012; Polańska et al. 2012) such as autism and attention-deficit hyperactivity disorder (ADHD). While increased diagnostic awareness does contribute to this, it is unlikely to completely account for these increases. There is concern that developmental exposure to environmental chemicals may contribute to the etiology of these diseases (Grandjean and Landrigan 2006, 2014). Testing chemicals for potential developmental neurotoxicity (DNT) is time-consuming, expensive, and animal-intensive (Crofton et al. 2012), and as a result, only slightly more than 100 (Makris et al. 2009) of the tens of thousands of compounds present in the environment (Judson et al. 2009) have been evaluated for DNT using formalized guideline studies. To address this data gap, considerable effort has been directed over the last decade towards development of rapid, cost-effective in vitro screens capable of testing large numbers of compounds for the potential to cause DNT. The proposed assays cover different biological processes important to development of the nervous system, such as proliferation and differentiation of neuroprogenitor cells, synapse and network formation, among others. Furthermore, a wide variety of approaches ranging from genomic/transcriptomic profiling, morphological assessment using high-content imaging, and behavioral assessments in alternative species (e.g., zebrafish) have been proposed. Recently, a comprehensive review and proposal for development of a tiered screening strategy for DNT testing

has been published (Bal-Price et al. 2018); assessment of compound effects on neural network development using MEA approaches is a critical component of this approach.

The use of MEAs for DNT screening offers three clear benefits to a battery of assays. First, and foremost, it provides for a functional assessment of compound effects on network development. Many of the other assays are based on structural changes or alterations in the expression of genomic or transcriptomic signals. A general feature of neurons grown on MEAs is a clear ontogeny of spontaneous activity reflecting network development (Fig. 4). Spontaneous network activity initially consists of sporadic, unorganized single action potential "spikes" that over time transitions to an organized network that exhibits synchronous bursting activity (Biffi et al. 2013; Charlesworth et al. 2015; Chiappalone et al. 2006; Cotterill et al. 2016; van Pelt et al. 2005; Wagenaar et al. 2006a). A second important feature of MEAs for DNT screening is that they are non-invasive, and thus allow for repeated measurement from the same network on multiple occasions during network development. Thus, effects of environmental compounds on neural network development can easily be evaluated in MEAs; most of the other assays that would comprise a DNT screening battery rely on assessment of compound effect at a single timepoint following exposure. Finally, network development is a more apical process than many of the other processes that are proposed in the battery (e.g., proliferation, neurite outgrowth). Thus, it incorporates aspects of neuronal differentiation and neurite outgrowth, synaptogenesis, interactions between neurons and glia, potentially making network formation assays using MEAs a more "broadband" endpoint that may be capable of catching compounds that other assays miss. However, this possibility cannot be explored fully until more chemicals have been tested across several assays in the proposed battery.

Two early studies established the proof-of-concept that MEAs could be utilized to screen compounds for potential developmental neurotoxicity. We demonstrated that the protein kinase C inhibitor bisindolylmaleimide (Bis-1) caused decreases in the firing and bursting rates of the networks following exposure during the first 2 weeks in vitro (Robinette et al. 2011). Furthermore, these changes in network development occurred at concentrations that decreased neurite outgrowth (Harrill et al. 2011). Our colleagues working at the European Commission's Joint Research Center showed that exposure to low concentrations of domoic acid during network development and maturation increased network activity and altered the pharmacological responsiveness of the network to bicuculline (Hogberg et al. 2011). An important limitation highlighted by these studies was that they relied on single-well MEA devices, and thus lacked the throughput necessary to be useful for screening purposes. This was addressed by the advent of multi-well MEA formats that appeared on the market around the time these studies were published. A second limitation of these studies was that while they included untreated controls, they evaluated only one compound each and did not include a "negative" control compound.

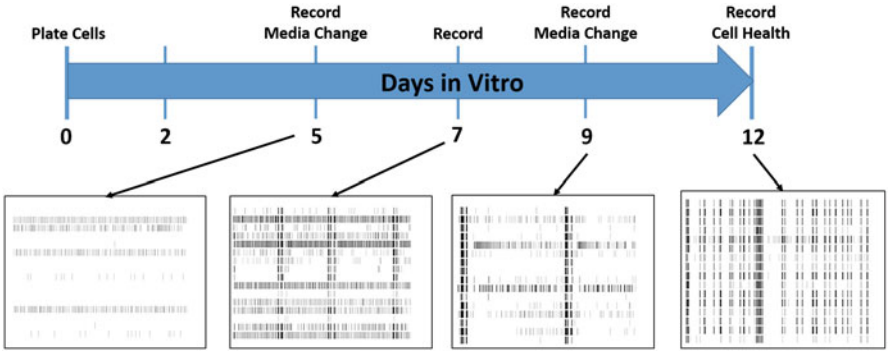

Fig. 4 Experimental design for the Network Formation Assay (NFA) using MEAs. To screen compound for the potential to cause developmental neurotoxicity, primary cortical cultures are treated with compounds 2 h after plating, so that the compound is present throughout development of the network. Recordings are made (15 min) on days in vitro (DIV) 5, 7, 9, and 12, and media is changed (with refresh of chemical) on DIVs 5 and 9. Following recording on DIV 12, cell health is assessed using lactate dehydrogenase and alamar blue assays. The raster plots illustrate network activity from untreated networks on DIVs 5, 7, 9, and 12. As the culture matures, activity increases across the electrodes in a well and becomes more organized

The availability of multi-well MEA formats has accelerated development of a network formation assay on MEAs that could be used for DNT screening. Brown and colleagues demonstrated that assay positive controls (Crofton et al. 2011) altered network formation as expected, and that the negative control compound acetaminophen was without effects (Brown et al. 2016). The general protocol for this assay is illustrated in Fig. 4. Following this, the approach was used to screen a set of 86 compounds which consisted of compounds where there was evidence in the literature that they caused developmental neurotoxicity in vivo, compounds that were putative "negative" compounds, and compounds with unknown effects on nervous system development in vivo (Frank et al. 2017). In Table 4, an analysis of the sensitivity and specificity of this data is presented, similar to that presented in Harrill et al. (2018) for data from high-content imaging assays. The network formation assay using MEAs has both high sensitivity (correct identification of in vivo DNT compounds) and specificity (correct identification of compounds without evidence of DNT in vivo). When results are filtered to include only those where the effect on the network activity parameter was at least threefold more potent than the effect on viability, the sensitivity of the assay decreases. However, consider that not all compounds that cause DNT in vivo will alter network formation, as well as the fact that sensitivity for this assay is higher than for any of the other assays evaluated in Harrill et al. (2018).

The ability of MEAs to make multiple assessments over time was utilized for a subsequent analysis of the data in Frank et al. (2018) that determined "tipping points" for chemical effects on network development. Tipping points represent the critical concentration above which perturbations in function can no longer

Table 4 Sensitivity and specificity of the network formation assay for developmental neurotoxicants

Measure	# compounds	Correct classification[a]	% correct	Correct selective classification[b]	% correct selective classification
Sensitivity	60	49	82	35	58
Specificity	23	21	91	21	91

Sensitivity = number of correctly identified compounds with evidence of in vivo DNT that had effects in the network formation assay in vitro
Specificity = number of correctly identified negative compounds; those that are without effects on DNT in vivo and were without effects in the network formation assay in vitro
[a]Considers effects on network activity endpoints and cell viability
[b]Considers only specific effects wherein there was at least a threefold difference between the EC_{50} value for network activity endpoints compared to the EC_{50} for viability

be compensated for using homeostatic mechanisms. Of the 64 compounds that altered some aspect of network activity in Frank et al. (2017), tipping points could be determined for 42 of them (Frank et al. 2018). Further, for a small number of compounds where sufficient data were available for estimation of in vivo concentrations, the tipping point could be related to in vivo levels that were associated with developmental neurotoxicity. Although the number of chemicals that have been assessed is small, the results indicate that effects of some of these compounds to disrupt network development are comparable to in vivo levels that are associated with developmental neurotoxicity.

6 Future Directions

While tremendous progress has been made in the last decade towards using MEAs for neurotoxicity and developmental neurotoxicity screening, there are several areas where improvements could be made that would increase the acceptance and utilization of MEA data for regulatory decision-making. The first area is to have larger numbers of chemicals tested by more laboratories, including chemicals in common across laboratories. While it might not seem like the most effective use of resources to re-test the same chemicals, it will provide the data needed to increase confidence that MEA data are replicable and reliable for screening and decision-making purposes. In addition, it will help to define the "fit-for-purpose" of the assay, by demonstrating classes of chemicals or particular pharmacological responses that may not be detected by MEA assays. For example, work related to whether or not neural networks on MEAs are capable of detecting nicotinic compounds has been inconsistent. Previous studies in my laboratory (McConnell et al. 2012; Valdivia et al. 2014) with nicotine and neonicotinoid insecticides (with the exceptions of clothianidan and thiamethoxam) indicated a lack of sensitivity to nicotinic compounds or a false negative response (e.g., due to dose selection). By contrast, reports from other laboratories indicate varying effects of nicotine on mean

firing rate (MFR) of cortical cultures on MEAs, including slight increases at 100 μM (Defranchi et al. 2011; Hondebrink et al. 2016), lack of statistically significant effects (≤500 μM; Mack et al. 2014) or significant inhibition (300–1000 μM; Hondebrink et al. 2016). Effects of the nAChR-selective antagonist mecamylamine were limited to changes in burst duration and the percentage of spikes occurring in a burst (Hammond et al. 2013). These parameters were not evaluated in the current or previous (Defranchi et al. 2011; McConnell et al. 2012; Valdivia et al. 2014; Hondebrink et al. 2016) studies. Thus, it may be that there is a better metric than MFR to detect nicotinic effects in neural networks.

Better use of the rich spatial and temporal data provided by MEA recordings is a second area where advancements could increase the acceptance and utilization of MEA data for regulatory decisions. More information on burst detection methodologies is available in the chapter by Cotterill and Eglen (this volume), so the focus here will be on how characterization of bursting and other metrics of network activity have been applied to toxicity assessments using MEAs. To date, the majority of studies of effects of neuroactive or neurotoxic compounds have focused primarily on their actions on the MFR of networks, despite the fact that many other parameters of activity regarding the spike train can be evaluated. In part, this is because the MFR has traditionally been a very sensitive metric and is easily extractable from the data. However, vendor supplied software for some systems now routinely analyze multiple aspects of network activity, facilitating examination of multiple endpoints, and scripts for such analyses are also increasingly freely available through sources such as GitHub. The small number of overall studies that consider multiple endpoints have universally demonstrated its added value. In 2014, Mack and co-workers demonstrated that a group of different classes of neurotoxicants could be separated by considering multiple bursting endpoints and conducting a principle components analysis of the data. This approach clearly separated $GABA_A$ antagonists from other classes of compounds (Mack et al. 2014). Using a similar multiparametric approach, Alloisio et al. (2015) were able to demonstrate different patterns of activity that separated 11 pesticides into four groups producing different phenotypic changes in activity (Alloisio et al. 2015). A more recent study (Bradley et al. 2018) has demonstrated that a group of 16 seizuragenic compounds could be distinguished and assigned to different groups based on 12 parameters of firing, bursting, and synchrony from MEA recordings while similar approaches were used by Bader et al. (2017) to characterize phenotypically different responses mediated by different $GABA_A$ and $GABA_B$ receptor-active pharmacological agents (Bader et al. 2017). Consideration of bursting characteristics as well as measures of network connectivity (e.g., correlated activity across electrodes) is also an efficient approach for identification of compounds that alter neural network development (Brown et al. 2016; Frank et al. 2017). Using random forest analysis, these studies demonstrated that as a network matures in vitro, network parameters besides MFR, such as mutual information (Ball et al. 2017), correlation (r), burst rate, and number of network spikes/bursts, become increasingly more important to correct identification of treatments that alter activity in neural networks grown on MEAs. From the standpoint of screening

unknown compounds for potential neuroactivity/neurotoxicity, developing methods to characterize "fingerprints" would be exceptionally valuable, as the fingerprints of unknown compounds could be compared to a database of known compounds to classify an unknown into a particular mode of action (e.g., compound A resembles a pyrethroid, while compound B resembles an opioid). Such information could be used for a number of purposes, to choose between safer drugs/chemicals during the development process, to rank compounds within a particular class with respect to potency or to design focused in vivo studies that would require fewer animals. In the example of the compounds above, one might evaluate compound A for stereotypical signs of pyrethroid poisoning (choreoathetosis, salivation, hyperactivity, tremor), while compound B would be evaluated for sedation. However, more widespread use of multiparametric evaluation is needed, including the testing of many more chemicals from different classes of compounds.

The use of human-derived, rather than rodent, neurons is also important to advancing toxicity testing with MEAs. Chapter 6 (Narkilahti and co-workers) of this book is devoted to use of human models in MEAs, so the comments here will focus on the use of these models in toxicity testing and screening. To date, there have been small numbers of publications that have examined effects of neurotoxic compounds on network activity using neurons derived from human embryonic or inducible pluripotent stem (iPS) cells. In terms of ethical considerations, inducible pluripotent-derived neurons may be preferable and are becoming widely available through a number of vendors. In one of the earliest studies, sub-micromolar levels of methylmercury dramatically inhibited network activity in human embryonic stem cell-derived neural networks (Ylä-Outinen et al. 2010). More recently, iCell neurons from CDI were used to evaluate the effects of glutamate, GABA, endosulfan, and amphetamine on network activity (Tukker et al. 2016). While there were differences in the activity of the iCell neurons and rat primary cortical cultures, the former responded to these four treatments appropriately. Hondebrink et al. (2017) have recently characterized the actions of the psychoactive substance methoxetamine on network activity in both cortical (glutamatergic and gabaergic) and midbrain (dopaminerginic) iPS-derived neurons, with and without glia. In the presence of glia, the concentration-response was left-shifted compared to recordings in the absence of glia. In addition, the midbrain culture was less sensitive than the cortical culture to inhibition of activity by methoxetamine (Hondebrink et al. 2017). In one of the few studies where direct comparisons of neurotoxic effects have been made in human and rodent networks, the potency of the marine neurotoxin tetrodotoxin was equipotent in human and rodent networks (10 and 7 nM, respectively; Kasteel and Westerink 2017). Additional studies comparing concentration-response between human and rodent networks are needed to understand better species differences and to facilitate cross-species comparisons between in vitro and in vivo rodent data and in vitro human data that will allow extrapolation to in vivo exposures in humans. To date, the studies above have focused mostly on exposures following establishment of robust network activity. While it would be desirable to use human stem cell-derived networks for screening compounds for potential developmental neurotoxicity, this may be challenging. In general, neural networks derived from

human models tend to take longer (∼3–5 weeks) to develop robust, coordinated spiking and bursting activity that is typically observed in rodent cultures (2–3 weeks; Odawara et al. 2014). This more prolonged developmental profile is less useful for higher-throughput screening because an assay would be longer and require more time and resources to test a compound compared to rodent cultures. However, with currently available human models, it should be possible to confirm activity observed in rodent models, when such data are required.

A unique feature of the nervous system is its plasticity. At the whole animal level, this is exhibited in the form of learning and memory, while at the circuit level, processes such as long-term potentiation (LTP) and long-term depression (LTD) are possible mechanisms that may mediate some forms of learning and memory. Plasticity is also sensitive to disruption by chemical neurotoxicants (Gilbert 2000; Ogiue-Ikeda et al. 2008; Holahan and Smith 2015). LTP and LTD are easily measured in recordings from preparations such as hippocampal slices, and there are well-established protocols for inducing these that can be carried out by any competent laboratory in the world. Although numerous protocols to induce plasticity changes in dissociated neurons grown on MEAs have been published (Arnold et al. 2005; Chiappalone et al. 2008; Massobrio et al. 2015; Odawara et al. 2016), none has been established to date as a ubiquitous protocol (see Wagenaar et al. 2006b for further discussion). The establishment of a protocol for examining plasticity in dissociated neural networks should be a high priority among neurobiologists working in this area. Current methods that could be used to screen compounds for effects on plasticity are either hippocampal slices from rodents or non-mammalian preparations such as *C. elegans* or Drosophila. These models either lack throughput or relevance to humans, whereas a dissociated culture model using human neurons grown on MEAs (e.g., Odawara et al. 2016) could dramatically increase throughput and provide human relevance for testing of drugs that enhance or chemicals that perturb plasticity.

Summary Since their first uses in the late 1990s, MEA approaches have evolved from a niche neurophysiological application into commercially available, high-throughput and high-content platforms. During this time, their use to address neurotoxicological questions has increased steadily. At present, these platforms are being utilized to address mechanistic issues and screen compounds for neurotoxicity and developmental neurotoxicity. The more recent greater availability of human neural models has only served to increase both the possibilities and the relevance of this approach to toxicity testing, and the promises of future improvements mean that MEAs will be a relevant and well-utilized approach that will provide meaningful data to both scientists and regulators.

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