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Screening the ToxCast Phase II Libraries for Alterations in Network Function using Cortical Neurons Grown on Multi-well Microelectrode Array (mwMEA) Plates

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Abstract

Methods are needed for rapid screening of environmental compounds for neurotoxicity, particularly ones that assess function. To demonstrate the utility of microelectrode array (MEA)-based approaches as a rapid neurotoxicity screening tool, 1055 chemicals from EPA's Phase II ToxCast library were evaluated for effects on neural function and cell health. Primary cortical networks were grown on multi-well microelectrode array (mwMEA) plates. On day *in vitro* (DIV) 13, baseline activity (40 min) was recorded prior to exposure to each compound (40 μ M). Changes in spontaneous network activity (mean firing rate; MFR) and cell viability (lactate dehydrogenase (LDH) and CellTiter Blue (CTB)) were assessed within the same well following compound exposure. Following exposure, 326 compounds altered (increased or decreased) normalized MFR beyond the hit thresholds based on 2 $\times$ the median absolute deviation of DMSO-treated wells. Pharmaceuticals, pesticides, fungicides, chemical intermediates, and herbicides accounted for 86% of the hits. Further, changes in MFR occurred in the absence of cytotoxicity, as only 8 compounds decreased cell viability. ToxPrint chemotype analysis identified several structural domains significantly enriched with MEA actives relative to the total test set (e.g., biphenyls and alkyl phenols). The top 5 enriched ToxPrint chemotypes were represented in 26% of the MEA hits, whereas the top 11 ToxPrints were represented in 34% of MEA hits. These results demonstrate that large-scale functional screening using neural networks on MEAs can fill a critical gap in assessment of neurotoxicity potential in ToxCast assay results. Further, a data-mining approach identified ToxPrint chemotypes enriched in the MEA-hit subset, which define initial structure-activity relationship inferences, establish potential mechanistic associations to other ToxCast assay endpoints, and provide working hypotheses for future studies.

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INTRODUCTION

The U.S. Environmental Protection Agency's (EPA) ToxCast program was introduced in 2007, following the release of the National Research Council's report on *Toxicity Testing in the 21st Century* (NRC, 2007). The program's objective was to generate data using new high-throughput screening (HTS) and high-content screening (HCS) methods to facilitate assessing potential toxicity for large and diverse libraries of chemicals used in commerce and the environment, most of which currently lack adequate toxicity data for hazard prioritization and risk assessment. (Dix et al., 2007; Kavlock et al., 2012). The approach provides an alternative method to aid in the assessment of compound toxicity that is designed to keep pace with the expanding numbers of compounds to which humans and the ecosystem are potentially exposed, while avoiding the limitations associated with traditional animal testing methods (i.e., cost, time, throughput, etc; Judson et al., 2009). Further, integrating the information obtained from these HTS and HCS *in vitro* assays in an adverse outcome pathway (AOPs) framework facilitates models predictive of toxicity (Ankley et al., 2010; Watanabe et al., 2011; Valdivia et al. 2014).

The US EPA's ToxCast program currently includes a battery of over 800 assays covering a wide range of cellular responses and mapped to ~300 signaling pathways [<https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data>]. Whereas the number of assays within ToxCast is large, very few assays focus on endpoints specific to the nervous system and its development (Radio et al., 2015). In addition, AOPs specifically related to neurotoxicity currently are limited, representing an important knowledge gap (Bal-Price et al., 2015). The ToxCast chemical library currently undergoing screening includes over 3,800 unique compounds, of which the most complete assay coverage, to date, is available for approximately 1,100 compounds tested in the earliest Phases I and II of the program. Chemicals tested through Phase II of the ToxCast program included approximately 300 chemicals, mostly pesticides, which were screened previously in Phase I, and a larger, more structurally diverse set of approximately 770 unique chemicals that were chosen to represent the broader environmental exposure and toxicity chemical space (Richard et al., 2016). Compound categories represented within this ~1100 chemical portion of the Phase II test set examined in the present study include pesticides, pharmaceuticals, antimicrobials, flame retardants, food-additives, and "green" chemicals, among others. Several of these compound categories, specifically those classified as insecticides, are known to disrupt the function of ion channels, leading to neurotoxicity (Coats, 1990; Narahashi 2002; Tomizawa and Casida, 2005). Within the AOP framework, disruption of ion channel function and changes in cellular excitability may serve as AOP molecular initiating events (MIEs), leading to neurotoxicity following compound exposure.

Measuring changes in firing rates and patterns of extracellularly-recorded action potentials from neural networks grown *in vitro* on microelectrode arrays (MEAs) can be an efficient and accurate approach to screen compounds for neurotoxicity hazard (for review, see Johnstone *et al.*, 2010). Primary cortical cultures grown on multi-well MEAs (mwMEAs) exhibit spontaneous electrical spikes and groups of spikes (bursts) that are associated with neuronal action potentials (Nam and Wheeler 2011) and are sensitive to modulation by glutamatergic agonists and antagonists (Frega et al., 2012; Lantz et al., 2014). This

technique is reproducible across laboratories (Novellino et al., 2011; Vassallo et al., 2016) and sensitive to a broad range of chemicals. The procedure measures function rather than a specific biochemical or morphological endpoint, making it a viable option to assess compound effects on neural activity (Defranchi et al., 2011; McConnell et al. 2012; Valdivia et al., 2014). The rates and patterns of activity measured by mwMEAs are sensitive to disruption through a variety of pathways (Johnstone et al., 2010; Valdivia et al., 2014) and compound effects are concentration-dependent (Frega et al., 2012; Mack et al., 2014).

The development of mwMEA platforms has increased throughput while maintaining high specificity and sensitivity, thereby making this technology ideal for the screening of compound sets to determine functional changes in neural networks (see, e.g., McConnell et al., 2012; Valdivia et al., 2014; Nicolas et al., 2014). Furthermore, the advent of higher-throughput MEA systems has allowed for the development of a multiplexed approach that permits in-well determination of compound effects on both neurophysiology and cellular viability (Wallace et al., 2015). Thus, this multiplexed approach provides assessment of both effects on neural network function and cell health within the same network, ensuring the ability to differentiate between compound-induced changes in neural activity and overall reductions in cell health in response to treatment with compounds. Recent studies using small (20–100) compound sets (Defranchi et al. 2011; McConnell et al., 2012; Valdivia et al., 2014; Nicholas et al., 2014) have demonstrated that changes in spontaneous neural network activity using mwMEAs can identify compounds that are neuroactive and/or neurotoxic with high sensitivity and specificity. However, this approach has not been applied to larger (>100) compound sets.

The present studies were designed to demonstrate the ability of neural networks grown in 48 well mwMEA plates to screen a large set of chemicals. To do so, a single-concentration screen (40 μ M) of compounds from the Phase 1 and Phase 2 subset of EPA's ToxCast Phase II library (~1100 chemicals) in primary cultures of cortical neural networks grown on mwMEA plates was conducted. In addition to determining compound effects on spontaneous neural firing rates, cell health was determined in-well by assessing lactate dehydrogenase (LDH) release and effects on mitochondrial activity using CellTiter Blue (CTB). Subsequent studies will assess the concentration-response relationships and determine the potency of ToxCast Phase II compounds found to be active in the mwMEAs following the single point concentration screen. By testing these compounds in this manner, an important data gap for neuroactivity of compounds in the ToxCast chemical space has been addressed. In addition, the present study examined analysis approaches that provide a basis for utilization of the data for a variety of purposes, including AOP development, prioritization of additional chemical sets, structure-activity relationships and read-across screening.

MATERIALS AND METHODS

Chemical Selection and Preparation

The ToxCast chemical set provided for the current study consisted of a total of 1101 plated samples, which included 1055 unique chemical substances. This set of chemicals comprised the major portion of chemicals submitted for the full battery of testing in Phase II of the

ToxCast program, and included the re-procured set of 293 unique compounds tested in Phase I (denoted ph1_v2), and a newly added set of 768 compounds (denoted ph2). Nine ph1_v2 compounds served as plating replicates along with 2 copies of 5 α -dihydrotestosterone, for a total of 56 samples included for internal quality control. Bicuculline (BIC) methiodide was subsequently added to test plates to serve as a positive MEA control; thus a total of 1056 compounds were screened. A complete current listing of compounds represented in the ToxCast chemical library, including annotation of the Phase II chemical inventory subsets screened in the present study, i.e., ph1_v2 and ph2, is available for download at <ftp://ftp.epa.gov/dsstoxftp> and is described in Richard *et al.*, 2016.

Stock solutions of each compound used in the present study (made to a target concentration of 20 mM in dimethyl sulfoxide, DMSO) were received from EPA's ToxCast Chemical Contractor (Evotec, South San Francisco, CA) in 50 μ L aliquots in sealed, round-bottom 96 well plates. Upon arrival, 96-well plates were wrapped in parafilm and stored at -20°C until use. Prior to each experiment, compounds were thawed and immediately diluted 1:10 in NB/B27 media in a v-bottom 96-well plate for dosing. To dose, 10 μ L from the dosing plate was transferred to the designated well on the 48-well mwMEA plate. Each well of the mwMEA plate contained 500 μ L of media, resulting in a final concentration of 40 μ M for each compound. DMSO (0.2 % by volume; n=3 wells/plate), GABA_A antagonist bicuculline (25 μ M BIC; n=2 wells/plate), and one well per mwMEA plate was reserved as a lysis control to determine total levels of lactate dehydrogenase (LDH) (see details below). To complete the single-point concentration screen of all compounds from the Phase II libraries, ~42 compounds were screened per week in triplicate over ~ 30 weeks.

Primary cortical culture on mwMEAs

Prior to plating rat primary cortical cultures, multi-well microelectrode (mwMEA) plates from Axion Biosystems Inc. (Atlanta, GA) were prepared for culture by coating with polyethyleneamine as previously described (Valdivia *et al.*, 2014). Each 48-well mwMEA plate consisted of a total of 768 nano-textured gold platinum microelectrodes (~40–50 μ m diameter, 350 μ m center-to-center spacing) with 16 electrodes/well plus four integrated ground electrodes (M768-KAP Kapton, Axion Biosystems Inc., Atlanta, Georgia).

All procedures involving laboratory animals were approved by the EPA National Health and Environmental Effects Research Laboratory's institutional laboratory animal health care and use committee and were in compliance with applicable federal guidelines for laboratory animal experimentation. Primary cortical neural cultures were prepared as previously described (Mundy and Freudenrich, 2000; Valdivia *et al.*, 2014) from Long-Evans pups on postnatal day 0–1, with minor modifications. Additional details can be found in the Supplementary Methods. Full media changes occurred on days *in vitro* (DIV) 5 and 9, while cells received a 1/2 media change on DIV 12 (24 h prior to experiment). The mixed primary culture used for the present experiments consists of glutamatergic and GABAergic neurons and glia (Mundy and Freudenrich, 2000). The neurons possess both axons and dendrites (Harrill *et al.*, 2013), form synapses (Harrill *et al.*, 2011), and exhibit spontaneous activity (spikes and bursts) that is blocked by tetrodotoxin (Wallace *et al.*, 2015) and is correlated to neurite length (Robinette *et al.*, 2011).

Multiplexed Screening Approach

The present experiments used a multiplexed approach to examine the effects of ToxCast library chemicals on spontaneous neural activity and cell health in primary cortical cultures. Effects on cell health were determined immediately following the conclusion of the electrophysiological recording, in the presence of the compound, using two commercially available assays: LDH and CTB (Wallace et al., 2015). A more detailed description of the viability assays is found in the Supplementary Methods.

MEA Recordings

Spontaneous activity in the cortical cultures was recorded using an Axion Biosystems Maestro 768 channel amplifier and Axion Integrated Studios (AxIS) v1.8 (or later) software. The amplifier recorded from all channels simultaneously (gain = 1200×; sampling rate = 12.5 KHz/channel). Recordings were conducted at 37°C and filtered with a Butterworth band-pass filter (300–5000 Hz), which removes slower local field potentials, leaving only fast potentials “spikes” associated with action potentials (Pine, 2006; Nam and Wheeler, 2011). On-line spike detection was performed with the AxIS adaptive spike detector, using a threshold of 8× the root mean square (rms) noise on each channel. Any electrode with rms noise levels greater than 5 µV was grounded (*i.e.*, no data were recorded). Once grounded, an electrode remained grounded for all subsequent treatments. Wells were deemed usable if on the day of the exposure 10 electrodes were active (defined as 5 spikes/min). On DIV 13, a minimum of three wells on three separate plates from one cortical culture preparation were treated with each compound at a single concentration (40 µM). Prior to recording baseline activity, each mwMEA plate was placed in the Maestro at 37°C and allowed to sit for 20 min while firing rates stabilized. Baseline activity (40 min) was recorded before the addition of each compound. An additional 40 min of spontaneous activity was recorded in the presence of each compound. Changes in mean firing rate (MFR) relative to baseline were assessed following compound treatment.

Assay Data Analysis

To assess compound effects on network firing rates, the MFR in each well was normalized according to the following formula: $nMFR = -1(MFR_T - MFR_B)/(0 - MFR_B)$, where $nMFR$ is the normalized mean firing rate, and MFR_T and MFR_B are the mean firing rates after treatment and baseline recordings, respectively. The median and median absolute deviation (MAD) of $nMFR$ for all wells treated with DMSO were determined, and treatments where the median $nMFR$ exceeded twice the MAD of DMSO treated wells, in either the up or down direction (*i.e.*, increase or decrease), were considered hits. A full listing of the 1056 unique chemical substances (1055 ToxCast compounds + bicuculline methiodide) tested in the current study is provided in Supplemental Table S1, along with the overall $nMFR$ hit assignment (up, down, hit, or no-hit) and with chemical annotations (including SMILES structures) provided by EPA’s Distributed Structure-Searchable Toxicity (DSSTox) database (accessible from EPA’s Chemistry Dashboard at <https://comptox.epa.gov/dashboard>).

CPCat Category Analysis

The Chemical Products Categories (CPCat) database contains assignments of use categories (e.g., pesticides, pharmaceuticals, cosmetics, etc.) and biological target activities (e.g., estrogen and androgen receptor binders, ERS1 and AR) for tens of thousands of chemicals in commerce and the environment (Dionisio et al., 2015). Compound target and use associations were programmatically compiled by cross-referencing CASRN to hundreds of chemical lists that were, in turn, tagged by association to various uses and aggregated within the EPA's ACToR database (Judson et al., 2009). Where data were available to annotate a CASRN record, a single primary use and biological target was assigned to each chemical within CPCat. The DSSTox CASRN list associated with the current mwMEA tested chemical list was used to query the CPCat database content within the iCSS ToxCast Dashboard (<http://actor.epa.gov/dashboard2/>, accessed 6/1/2016), and yielded a list of primary biological targets for approximately half of the mwMEA tested chemicals (539 total), and use categories for all compounds in the original tested chemical list (excluding bicuculline methiodide, which was subsequently assigned to use category "Pharmaceutical").

ToxPrint Chemotype Analysis

To explore possible chemical substructural feature (i.e., chemotype) enrichments in the MEA active set (i.e., hits) relative to the total tested set, the publicly available ToxPrint feature set (V2.0_r711; <https://toxprint.org/>) was employed along with the associated Chemotyper visualization application (<https://chemotyper.org/>), both developed by Altamira [Altamira, Columbus, OH] and Molecular Networks [Molecular Networks, Erlangen, GmbH] under contract from the US Food and Drug Administration (FDA) (Yang et al., 2015). A chemical structure-data (SD) file for the Phase II ToxCast chemicals screened in the current study (i.e., ph1_v2, ph2) was exported from the DSSTox database, and can be generated from the publicly available DSSTox TOXCST SD file (available from the DSSTox Data download page at <ftp://ftp.epa.gov/dsstoxftp>). The SD file was imported into the public Chemotyper, mapped to the public set of 729 ToxPrints, and exported as a binary [1,0] fingerprint file to support further analysis. Screenshots of search results displayed within the Chemotyper application were used to generate all pictorial representations of the ToxPrints and their superposition onto ToxCast chemicals.

ToxPrint chemotype (CT) enrichment statistics were evaluated in the MEA hit space (i.e., 318 active MEA chemicals with structures) relative to the full set (1028 MEA-tested chemicals with structures). The smaller number of CTs (318 active/1028 MEA-tested) is because not all substances could be assigned structures (for example mixtures, such as C10–21 sulfonic acids phenyl esters (DTXSID1047526) or milbemectin (DTXSID8034742)); bicuculline methiodide also was not included. Enrichment was based on a computed Odds Ratio (OR) for each CT according to the following formula:

$$OR = TPos \times Tneg / (Fpos \times Fneg) \quad \text{Eq. (1)}$$

Where Tpos=True Positive (chemical is MEA hit and contains CT), Tneg=True Negative (chemical is not MEA hit and does not contain CT), Fpos=False Positive (chemical is MEA hit and does not contain CT), Fneg, False Negative (chemical is not MEA hit and contains CT). Additionally, the Fischer's exact test (using the R package script, <https://www.r-project.org/>) was used to compute the p-value (pval) significance of the enrichments, which gives greater weight to enrichments within larger CT sets, i.e. containing larger numbers of chemicals. OR values >3 and pval 0.05 cutoffs were used to filter the CT results for significance and further examination. Similarly, the OR and pval significance of ToxPrint CT enrichments were previously computed (K. Connors, unpublished results) for the full suite of publicly available ToxCast assay results (the latter available at <https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data>). Consulting the precomputed ToxCast assay CT enrichment results (in each assay's hit space) for the same CTs found to be enriched in the mwMEA assay allowed for the identification of a subset of ToxCast assay endpoints that could be potentially mechanistically related to the mwMEA assay endpoint.

MEA Reference Set

For the purposes of evaluating concordance of the current mwMEA results to previously published MEA results, and to serve as a literature reference set for future studies, a curated DSSTox structure file was created that consolidated results for chemicals for which MEA results from one or more labs had been reported previously. For the purposes of this exercise, MEA hit calls (Hit, Neg) that were generated by somewhat different experimental protocols as they evolved over time, were collapsed to the DSSTox chemical substance level and consolidated into a single table. DSSTox curation requires review, reconciliation, and accurate assignment of chemical names and CAS registry numbers (CASRN), which are then mapped to a unique chemical structure that is specific to salt, hydrate and stereo-specific forms, where appropriate (for further details, see <https://www.epa.gov/chemical-research/distributed-structure-searchable-toxicity-dsstox-database>). Curation of previously reported MEA outcomes resulted in some MEA results being mapped to slightly different chemical forms (e.g., Muscimol and Muscimol hydrobromide), and merging of some MEA results based on review of reported CASRN and names (e.g., fluoxetine and fluoxetine hydrochloride). The resulting MEA literature reference list (denoted MEA_Ref), consisting of source names and CASRN mapped to DSSTox identifiers for 146 unique substances, and annotated with references and curation notes, is provided in Supplemental Table S2.

RESULTS

Data were collected for a total of 4,218 wells from 90 mwMEA plates across 30 different cortical culture preparations. The average MFR over all the wells during baseline recordings was 113.11 ± 58.5 (mean $\pm$ sd) spikes/min with each well having an average of 14.87 ± 2.4 (mean $\pm$ sd) active electrodes. The overall change in response to DMSO was a decrease from a baseline of 121 ± 48 to 101 ± 42 (mean $\pm$ sd, n=81 wells) spikes/min; this decrease was similar to that observed by Valdivia et al., 2014. BIC caused an increase to $125 \pm 57\%$ (mean $\pm$ sd, n=54 wells) of baseline values. It should be noted that the values reported here have not been normalized so that they may be compared to previous work (McConnell et al., 2012; Valdivia et al., 2014).

Compound Effects on Mean Firing Rate

The distribution of the nMFR is presented in Figure 1 and is clearly skewed towards compounds that decreased nMFR. DMSO, the solvent control, decreased the median nMFR scores by 23.21%. Compound “hit” thresholds were determined using 2× median absolute deviation (MAD) of DMSO treated wells (MAD = 21.7; hit thresholds were –65 and +20). Overall, 308 compounds decreased nMFR below the lower hit threshold (“hits down”) and 18 increased nMFR above the upper hit threshold (“hits up”; Figure 2, top). Compounds were deemed cytotoxic if they induced greater than 20% increase in LDH release and/or decreased mitochondrial activity by more than 80%. Changes in nMFR occurred largely in the absence of cytotoxicity, as only 8 compounds decreased cellular viability following exposure. Compounds inducing cytotoxicity were: UK-337312, tributyltin chloride, tributyltin methacrylate, phenylmercuric acetate, 9-phenanthrol, gentian violet, mercuric chloride, and ketoconazole. All eight of these compounds decreased MFR beyond the threshold, and thus are considered to be “hits”, but are flagged as cytotoxic.

Evaluation of Assay Performance

The performance of the assay was evaluated using three different metrics. First, the concordance of results from replicate samples within the currently tested compound set was examined. Second, concordance was evaluated between the current results and those from Valdivia et al., 2014 for the compounds that were common between the two sets. This provides an intra-laboratory comparison. Finally, concordance between the results for compounds tested in both the present study and in previous studies included in the DSSTox MEA literature reference list, MEA_Ref, was evaluated.

There were 10 compounds included as replicates in the present compound test set, generating a total of 33 test results. The result for each sample occurrence of these compounds is presented in Supplemental Table S3. With respect to hit calls, 6/10 replicated compounds (chlorophene, bisphenol A, oryzalin, triadimenol, triclosan, and allethrin) demonstrated 100% concordance among all 20 sample replicates, with all 6 compounds labeled as “down” hits. For 3 of the 4 remaining compounds (5 α -dihydrotestosterone, azoxystrobin, and mancozeb), all 9 replicate nMFR values decreased, but only 4/9 of the replicates decreased nMFR greater than the hit threshold. Perfluorooctane sulfonate (PFOS) was the most inconsistent of the replicate compounds; 3/4 replicates increased nMFR (two increased greater than the hit threshold), whereas one replicate decreased nMFR slightly. Differences in the responses did not correlate to the lot number of the compounds in any case.

The current set of MEA hits were also compared to the results for the ToxCast compounds previously screened in a single lab by Valdivia et al., 2014. It should be noted that hits in the latter study were determined based on weighted MFR, so the method was slightly different from the current study. Despite these differences, there was 74% overall concordance in hit-calls between the two evaluations (Supplemental Table S2) for the 87 compounds that were common to both sets of data. In 8/23 cases where hit calls were not concordant, the overall changes in nMFR and weighted MFR were similar, but differences in where the hit

thresholds were determined based on DMSO-treated wells for each study resulted in results that were not concordant.

Extending the comparison of the current mwMEA results to the full overlapping set of 92 compounds in the MEA_Ref dataset of 146 compounds, which combined the results of several published studies from different laboratories, an overall concordance of 71% was computed. Of the 92 compounds in the overlapping set, the MEA_Ref consensus calls classified 76% (70/92) as hits, whereas the present study (mwMEA), identified 60% (55/92) as hits. This implies lower sensitivity but higher specificity of mwMEA results relative to previously published (MEA_Ref) results. Furthermore, the current mwMEA hit calls agreed with 49/70 (i.e., 70%) of the MEA_Ref hits, whereas a significantly larger fraction of the mwMEA hits, 49/55 (i.e., 89%), were confirmed as hits in the MEA_Ref dataset (Supplemental Table S2).

Categories Enriched with Active Compounds

The CPCat database was used to determine what categories of compounds were hits in the present data. It should be noted that the categorization here is slightly different from legal definitions under the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA), wherein herbicides, fungicides, biocides and insecticides are all sub-categories of “Pesticides”. Largely mirroring the overall composition of the full chemical set tested in the mwMEA assay, a large majority, 86%, of the hits (Figure 2, middle) could be assigned to five compound “use categories”, i.e., Pharmaceuticals (87), Microbicides/Fungicides (67), Pesticides (56), Herbicides (36), and Chemical intermediates (33) based on the primary use of compounds in CPCat. Furthermore, three of these use category sets – Pharmaceuticals, Microbicides/Fungicides, and Pesticides – contained a much higher percentage of mwMEA hits than the overall MEA hit rate, 31%, observed in the full test set (Figure 3). Other use categories, including Solvents, Plasticizers, Food Flavor Fragrances, Chemical Intermediates and Surfactants, were found to contain a much lower percentage of hits than were observed in the full test set. The full list of CPCat use categories and biological targets for the MEA test set chemicals is provided in Supplemental Table S4.

Several of the compounds represented in Pesticides and Herbicides are either known or suspected to target the nervous system. All 56 of the Pesticide compounds were further classified for use as Insecticides. Thus, this use category included a number of compounds known to act on sodium ion channels (e.g., prallethrin, fenpropathrin, cypermethrin, allethrin, methoxychlor, and tetramethrin) or inhibit acetylcholinesterase activity (e.g., aldicarb, pirimicarb, oxamyl, thiodicarb, dimethoate). Compounds known to act on the GABA_A receptor (e.g., fipronil, aldrin, and lindane) were also among those represented in this category. Active compounds classified as Herbicides (37 total) included both dinitro aniline alkylates (e.g., prodiamine and flumetralin) and carbamates (e.g., chlorpropham, and tri-allate). A total of 67 compounds used as Microbicides/Fungicides were active. Compounds in this category included strobins (e.g., picoxystrobin, azoxystrobin) and conazoles (e.g., tebuconazole, tetraconazole, fenbuconazole).

Pharmaceuticals represented the largest use category within both the tested set of chemicals and within the MEA hit set, with a total of 87 compounds altering network function.

Compounds represented in this category included steroids (5 α -dihydrotestosterone, 17 α -estradiol, and mifepristone), as well as compounds known to target calcium (amiodarone hydrochloride) and sodium (MK-274) ion channels and opioid receptors (methadone hydrochloride and enadoline). Compounds acting on serotonergic (fabesetron hydrochloride, volinanserin, SB243213A, elzasonan, PD 0343701) and dopaminergic (haloperidol and chlorpromazine hydrochloride) processes were also active and altered network activity beyond threshold.

When examining compound targets, a total of 121 compounds altering neural network function beyond the hit thresholds could be assigned to targets (Figure 2, bottom), some of which have been shown to be sensitive in neuronal networks grown on MEAs. These targets included acetylcholinesterase (AChE), as well as GABA_A receptors (e.g., lindane, abamectin, fipronil), glutamate, and voltage-gated calcium and sodium channels (e.g., allethrin, tetramethrin). Further analysis identified several additional sensitive targets that have not been previously demonstrated in neural networks on MEAs, including estrogen receptor 1 (ESR1), microtubule disruptors, mitochondrial disruptors, sterol biosynthesis inhibitors and tachykinin receptor 1 (TACR1, or substance P receptor). The enrichment of hits in specific compound categories (e.g., Pesticides/Insecticides) and the observation that multiple hits are found within some chemical classes (e.g. pyrethroids, conazoles) warrants a more rigorous analysis of the MEA hit space.

ToxPrint Chemotype Enrichment Analysis

A cheminformatics data-mining approach using the publicly available ToxPrint structure feature set (referred to as chemotypes, or CTs) was used to explore more systematically possible enrichments of well-defined substructures in the MEA hit space. A statistically significant CT-MEA enrichment indicates that a structural feature (CT) occurs more often in the MEA hit space (i.e., MEA active subset) than in the overall tested space (i.e., all MEA tested chemicals). The top 11 ToxPrint CTs, meeting the statistical threshold criteria $OR \geq 3$ and $pval \leq 0.05$ for the 318 MEA hits (up or down) for which DSSTox structures could be assigned relative to the total tested set of 1028 structures, along with sensitivity and specificity metrics for constructing a standard confusion matrix, are provided in Supplemental Table S5.

These results are presented graphically in Figure 4, with the blue bars representing enrichments as percentages exceeding the 30% total hit rate within the full MEA tested structures set (i.e., 318/1028), the orange dots indicating the total number of chemicals in the CT subgroup (CT-Tot), and a sample of four ToxPrint CT images shown. Each enriched ToxPrint CT, in turn, is represented within a set of chemicals having a higher than expected fraction of hits, but is also represented to a smaller extent within the inactive chemical set. For a full listing of the 1028 MEA-tested chemicals with structures mapped to the top 11 CTs, see Supplemental Table S6. To illustrate how ToxPrint CT enrichments can be explored further within a data mining workflow, cases involving the 4 ToxPrint CT graphs in Figure 4 will be considered in more detail.

Figure 5 displays all chemical structures within the MEA tested set containing either CT479 (chain:aromaticAlkane_Ph-C1-Ph), on the left, or CT488 (chain:aromaticAlkene_Ph-

C2_acyclic_generic), on the right, with MEA hits indicated within green borders and non-hits within gray borders. The OR enrichment statistics for the CT479 and CT488 groups are 5.4 and 4.3, respectively. An overlapping set of 9 compounds containing both CT479 and CT488 is represented in the center box, with 8/9 hits corresponding to a much higher OR (18.3) than either CT group separately. Additionally, once this more highly enriched overlapping set is removed from the CT488 superset (i.e., CT488_minus_CT479), the remaining set of 14 compounds (7 hits, 7 inactives) is no longer significantly enriched with hits (OR=1.9), whereas the remaining compounds in the CT479_minus_CT488 set retains some significance (OR=4.0), but less than in the full CT479 set.

Figure 6 presents a second example illustrating how ToxPrints can be augmented with new rules to capture important chemical modifiers within a local CT domain. CT482 (chain:aromaticAlkane_Ph-C6) is an example of a ToxPrint set containing relatively few chemicals (5 total, 4 of which are hits), but with a relatively high odds ratio (OR=9.0). The related CT481 (chain:aromaticAlkane_Ph-C4) is present in all 5 CT482 compounds, as well as 2 additional inactive compounds; hence, it corresponds to a less significant overall enrichment (OR=3.0). However, upon closer inspection, all 4 MEA hits in the CT481 set are p-alkyl phenols, whereas all 3 inactives lack a hydroxyl group. Hence, augmenting CT481 with a rule stating that chemicals within the group are MEA active only if they contain a p-hydroxyl and inactive otherwise (i.e., CT481_R1) correctly classifies all 7 compounds.

Figure 7 shows the set of 6 tested chemicals containing the enriched CT665 (ring:hetero_[6]_N_O_1_4-oxazine_generic), 5 of which are MEA hits (green border). A list of 6 ToxCast assays found to be significantly enriched with hits in the CT665 chemotype subgroup (OR > 4) are listed to the right of the figure (see Supplemental Table S7). These include 3 G-protein coupled (opiate) receptor binding assays (NVS_GPCR) and 3 rat ion channel receptor binding assays (NVS_IC_r...), all targets that are considered mechanistically relevant to MEA activity. Further strengthening the association of the mwMEA assay results to these 6 ToxCast assays, CT479, representing a distinct chemical domain and a different set of chemicals, was also found to be significantly enriched in the same 6 ToxCast assays (OR>4). Additionally, 4 of the 5 active chemicals within the CT665 subgroup shown in Figure 7 were found to be hits in one or more of the rat ion channel receptor binding assays, further strengthening possible associations between the mwMEA assay and these ToxCast assay targets. Following a similar line of inquiry to this example, the MEA-enriched CT482 (chain:aromaticAlkane_Ph-C6) from the previous example in Figure 6 was found to be significantly enriched in 16 endocrine-related ToxCast assays (see Supplemental Table S8), most likely due to the known enrichment of phenols in activating estrogenic (ER) and androgenic (AR) targets.

DISCUSSION

The present study examined a total of 1055 unique compounds in EPA's ToxCast Phase II library to determine effects on spontaneous neural network activity and cell health in primary cortical cultures. These data provide the most comprehensive examination of compound effects on neural network function available to date, both in terms of biological compound space and the total number of compounds tested. In addition, these results expand

and build upon previous studies examining the effects of ToxCast compounds using small cohorts of compounds (~100) suspected or known to be neurotoxic (Valdivia et al., 2014). In the present experiments, a total of 326 compounds (~30%) altered MFR beyond one of two thresholds, with 18 compounds (including the positive control, bicuculline methiodide) increasing MFR and 308 compounds decreasing MFR following 40 min compound exposure at a single concentration (40 μ M). While further evaluation of concentration-response relationships for active compounds will provide information on potency and efficacy for comparison across ToxCast Assays, the present results are informative even without this information.

The US EPA's ToxCast program includes a wide range of cell-based assays covering a number of key signaling pathways. However, few assays are cell-based, functional assays that focus on endpoints specific to the nervous system and its development. The advent of higher throughput MEA platforms, in addition to the development of multiplexing protocols (Wallace et al., 2015) allowing for the collection of multiple in-well measurements from the same network, have made mwMEA systems a viable tool for efficiently screening large (>1,000) sets of compounds in a timely manner. In the present study, a total of 1102 samples (1101 ToxCast + bicuculline) were screened in triplicate in 48 well MEA plates in under a year, without the benefit of automation for any aspect of the assay workflow. This screening capability represents a significant advancement in this technology, which previously relied upon single well recordings.

Overall, results were reproducible when compared to previous ToxCast compound screening conducted in mwMEA plates (Valdivia et al., 2014). In the present study, the performance of the assay as assessed by several measures was consistent. Replicate samples within the plating set provided generally consistent results, even if in 4 cases the resulting hit calls were divergent due to the selection of hit cutoffs. When comparing compound effects in this study to previous studies, the level of concordance is similar to reports from other laboratories, wherein ~70–80% of neuroactive compounds were identified by neural networks on MEAs (Valdivia et al., 2014; Defranchi et al., 2012; McConnell et al., 2012). Two caveats should be noted with respect to the MEA reference set used for these comparisons. First, it does have some bias towards data generated from this laboratory, so in some respects, this analysis may be more representative of within-laboratory reproducibility. Second, only ~8 compounds (e.g. bicuculline, carbaryl, fluoxetine, domoic acid, deltamethrin, verapamil, chlorpyrifos oxon and fipronil) have been tested multiple times in multiple laboratories. Thus, without more rigorous testing of larger numbers of compounds by multiple laboratories, it is difficult to have high confidence in the outcome (Hit or Neg) for any given compound. However, two studies with very limited numbers of compounds (3–6) have demonstrated high reproducibility between multiple (4–6) laboratories (Novellino et al., 2012; Vassallo et al., 2016). Improvements in screening methods (i.e., collecting triplicate measurements on the same date) for large compound sets have reduced variability associated with handling of mwMEA plates and freeze/thaw of compounds (Malo et al., 2006, Wallace et al., 2015). However, additional improvements, including automated plating of cultures and automated dosing, could be used to increase further throughput for large compound sets, while decreasing variability and errors associated with the assay. For example, it could be that the most discordant of the 4 replicates of PFOS was the result of a pipetting or other

error that could be eliminated or reduced by increased automation of this assay. Finally, while not related to reproducibility of results, it should be noted that the metabolic competency of the cultures used in the present study is not well characterized and expected to be limited in comparison to liver; this may impact results compared to *in vivo* neurotoxicity when metabolism is important in the activation or de-activation of a compound.

Within the ToxCast Phase II libraries, compounds are subdivided into groups or categories that serve to represent their primary use. Unsurprisingly, four of the five largest active compound use categories detected in the MEAs were Pesticides, Pharmaceuticals, Fungicides, and Herbicides. In many cases, compounds within these four use categories are known to target key neural processes, receptors and/or ion channels. This was evident in the categories of targets documented to be associated with these chemicals, which included both ion channels (both sodium and calcium) and neural receptors (e.g., GABA, ESR1, TACR1, and AChE), as well as those known to disrupt mitochondria, microtubule formation, and compounds inhibiting sterol biosynthesis (see Figure 2). When assessing compound target sensitivity, the MEAs proved to be most sensitive in detecting compounds known to target ion channels (including 10/13 pyrethroid insecticides) and GABAergic input. In some cases, as with the previous study (Valdivia et al., 2014), this MEA assay often detected active compounds that were not hits in other assays present in the ToxCast battery. For example, Silve et al. (2015) reported that two compounds with known *in vivo* neurotoxicity, methidathion and endosulfan, lacked activity in the ToxCast *in vitro* assay endpoints for acetylcholinesterase inhibition and interactions with the GABA_A receptor, respectively, both of which are established molecular targets of these compounds (Silva et al., 2015). Both of these compounds were identified as active in the mwMEA assay.

Similar to previous MEA studies (Valdivia, et al. 2014), nicotine and neonicotinoid insecticides (with the exceptions of clothianidan and thiamethoxam) were not among identified hits. This may indicate an overall lack of sensitivity to nicotinic compounds. However, it has been reported in primary cortical cultures that expression of the $\alpha 7$ nicotinic ACh subunit is limited to the neural somata and initial apical dendrite, and that 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., 2012; McConnell et al., 2012; Valdivia et al., 2012) studies. A variety of parameters can be extracted from MEA data that describe neural network activity at the level of the individual unit (e.g., electrode) and/or network, and it may well be that other parameters are more sensitive to the effects of nicotinic compounds and would improve the domain of applicability of an MEA assay. In this respect, recent work has demonstrated that consideration of bursting characteristics as well as measures of network connectivity (e.g. correlated activity across electrodes) is most efficient for identification of compounds that alter neural network development (Brown et al., 2016; Frank et al, submitted). Thus, a more comprehensive analysis that includes these metrics, although more resource intensive and time-consuming, may improve identification of compounds that alter network function following acute exposure.

Interestingly, a total of 17 compounds known to target estrogen receptor 1 (ESR1) altered neural network function. To date, effects of estrogen receptor agonists/antagonists on neural network function on MEAs have not been reported in the published literature. Thus, this represents a novel finding. However, these results are consistent with the well-established “rapid actions” (not mediated by nuclear activation) of androgens (Hill et al., 2015) and estrogens (Kow and Pfaff, 2016) on a variety of ion channels relevant to neural network function, including, but not limited to, voltage-gated calcium and potassium channels and ionotropic GABA_A, NMDA and AMPA receptors. While most of these compounds are classified for use as pharmaceuticals (e.g., tamoxifen, 17 α -estradiol, and diethylstilbestrol), compound use categories for these estrogenic compounds targeting ESR1, including plastics (bisphenol A and bisphenol AF), flavones (daidzein), dyes (4-nonylphenol), and chemical intermediates (e.g., 4-(1,1,3,3-tetramethylbutyl)phenol), were also represented. Consistent with the use category analysis, the structure-based chemotype enrichment analysis independently identified a defined subgroup of alkyl phenols highly enriched with mwMEA hits; the same chemotype was found to be enriched in the active sets of 16 ToxCast assays targeting nuclear receptors, ER and AR, establishing a significant association of the assay endpoints in this local chemistry domain.

The chemotype enrichment approach employed in the current study offers a promising structure-based approach for mining and exploring ToxCast assay data. Such an analysis, based on a set of well-defined, viewable, publicly available chemical features, can yield quantifiable statistics and transferable results, and can provide a starting point for further structure-activity exploration and mechanistic hypothesis generation within local domains of chemistry. The set of 729 ToxPrint chemotypes employed in the present study were specifically designed for fingerprinting and structure-activity studies, and are able to detect a wide range of bond, atom, chain and ring types in diverse structures spanning the environmental exposure and safety assessment chemical landscape of interest to FDA and EPA (see Yang et al., 2015 and Richard et al., 2016). As such, they are well suited to profiling of ToxCast activity subsets, generating interpretable results, identifying subgroups of activity enrichments, establishing putative mechanistic linkages across multiple data streams (e.g., assays) constrained within well-defined local chemistry domains, and identifying novel, putative AOPs for further exploration. The preliminary analysis presented here, by way of several examples, demonstrates an intuitive data mining strategy that can yield actionable structure-activity hypotheses for informing follow-up testing, as well as putative mechanistic associations across assay targets in local chemistry domains, associations that would likely have been obscured by a less chemically supervised, more global correlation analysis.

In the current study, the top 5 most significantly enriched ToxPrint chemotypes were found to be present in 26% of the MEA hits, whereas a somewhat larger set of 11 chemotypes were present in 34% of the MEA hits determined in the single point screen. Furthermore, 2 of the top 5 enriched chemotypes were found to be jointly present in an overlapping set of biphenyls with a conjugated bridge carbon, and this overlapping set had a significantly higher enrichment statistic. Clearly, detecting patterns such as these are useful for refining structure-activity inferences, charting future validation studies, and can contribute knowledge to a local chemotype domain for informing read-across studies and safety

assessments. An automated workflow to facilitate this type of chemotype exploration is currently under development and will be needed to engage non-chemists more effectively. It is important to recognize that a major portion of the MEA hit space was not explored in this initial chemotype analysis. Considering additional chemical properties and features, or exploring combinations of chemotypes might be useful in accounting for a larger fraction of the hit space moving forward. Additionally, using chemotypes to define stable clusters of “similar” chemicals, independent of the particular dataset under study, would allow one to explore putative linkages across assays even if the tested chemical sets were not the same.

Another way in which chemotyping approaches, such as described here, could be employed in future studies, is to address the issue of possibly missing neuroactive compounds due to selective predetermined thresholds. This method allows for the comparison of compounds that were excluded based on thresholds to compounds that were selected as hits, to determine if they are structurally similar. The results could serve as a basis for selecting a compound to assess concentration responsiveness, e.g., if it is structurally similar to a set of active compounds, or is an inactive member of a significantly enriched chemotype subgroup (see, e.g., Figures 4–6). Furthermore, information obtained from chemotyping will also provide a useful link between our results and the results of the other assays included within ToxCast. Lastly, this chemotyping approach also may be utilized to prioritize testing of larger numbers of compounds in the MEA assay. If another large set of compounds were to be tested in this assay, one could examine that set of compounds for chemotypes enriched in activity on neural networks, and give priority to testing compounds containing enriched chemotypes over those lacking such structures.

Conclusions

The present results demonstrate the ability of an MEA-based screen examining neural network activity to identify potentially neuroactive compounds from a set of over 1000 compounds. To date, this is the largest set of compounds screened for neuroactivity using this approach. An analysis approach that links the results in this assay to those in other ToxCast assays based on structural features of active compounds yielded actionable inferences that can be used to inform mechanistic hypotheses and guide follow-up studies. As such, data from this assay are helping to fill an important biological niche (neurotoxicity) where there are few cell-based, functional assays in the ToxCast battery of assays.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Histogram of normalized mean firing rate values.

Changes in spontaneous neural network activity (mean firing rate; MFR) in primary cortical neurons grown on mwMEA plates was determined following exposure to 1056 unique chemicals from EPA's Phase II ToxCast libraries. All 1056 compounds were screened at a single concentration (40 μ M). DMSO and the GABA_A antagonist bicuculline (BIC) methiodide were included as controls on each mwMEA plate. To assess compound effects on network firing rates, the MFR in each well was normalized according to the following formula: $nMFR = -1(MFR_T - MFR_B) / (0 - MFR_B)$, where nMFR is the normalized mean firing rate, and MFR_T and MFR_B are the mean firing rate after treatment and baseline recordings, respectively. The mean and standard deviation of nMFR for all wells treated with DMSO was determined, and treatments where the median nMFR exceeded $2 \times$ the sd of DMSO treated wells were considered hits (noted as "thres down" and "thres up" on histogram). Following exposure, a total of 325 compounds altered MFR beyond threshold, with 308 compounds decreasing activity (red) and 17 compounds increasing (green) network activity.

All Compounds

Compound Categories

Compound Targets

Figure 2. Compounds designed to be biologically active comprise the majority of hits.

A total of 1055 unique compounds from EPA's ToxCast Phase 2 libraries were screened at a single concentration (40 μ M) in primary cortical cultures grown on mwMEA plates to determine acute (40 min) effects on neural network function. Top: A total of 325 compounds altered network firing rates (MFR) beyond threshold with 308 compounds decreasing activity. In addition to decreasing activity, 8 of these compounds were also cytotoxic. A total of 18 compounds (including bicuculline methiodide) increased MFR, while 730 compounds did not alter MFR beyond either threshold. Middle: Of the 326 compound identified as hits

in the mwMEA assay, 253 compounds (~78%) were categorized for use in five major categories within ToxCast. These categories were pesticides (52), pharmaceuticals (86), fungicides (48), herbicides (35), and chemical intermediates (32). Bottom: A total of 121 compounds (including bicuculline methiodide) out of the 326 total hits could be assigned to gene targets or modes of action (Supplemental Table 4). Shown here are any target genes/MOAs that contained 5 or more compounds, and the total number of compounds assigned to that category. Of the 205 compounds not represented, 87 did not have an assigned target gene/MOA in CPCat.

Figure 3.

Bar graph indicating percentage of MEA hits within each of 7 CPCat use categories, where green bars indicate enrichment of the use category chemicals in the MEA hit set, i.e. significantly exceeding the overall hit rate of 31%, and blue bars indicate depletion of the use category in the MEA hit set.

Figure 4.

Top 11 MEA hit-enriched ToxPrint chemotypes, plotting percentage (blue bar) of hits in each chemotype group relative to the total MEA hit rate of 30% ($x=0$), with the total number of chemicals in each ToxPrint chemotype set indicated by the orange ball; 4 sample ToxPrint graphs are shown in colored boxes to the right of the bar plot; grayed bonds in CT482, CT488, and CT479 represent aromatic systems and dashed bonds in CT665 are either aromatic or aliphatic.

Figure 5:

Two MEA-enriched ToxPrint chemotype sets are shown: Left; CT479, chain:aromaticAlkane_Ph-C1-Ph and Right; CT488, chain:aromaticAlkene_Ph-C2_acyclic_generic, with the green border indicating MEA hits and the grey border indicating MEA non-hits. Center box contains 9 chemicals (8 MEA hits, 1 non-hit) that share both CT479 and CT488 chemotypes with a higher enrichment statistic (OR=18) than either CT479 or CT488 alone. Furthermore, when the CT479_AND_CT488 overlap set is removed from the CT488 set, the remaining chemicals show no significant enrichment of MEA hits (OR = 1.9).

Figure 6:

Chemicals containing ToxPrint CT482 (chain:aromaticAlkane_Ph-C6) are nested within the less restricted CT481 (chain:aromaticAlkane_Ph-C4) chemotype set and yield a higher MEA hit odds ratio statistic (OR=9.0 vs 3.0) due to the larger percentage of MEA hits (green border) vs. MEA non-hits (grey border); however, when a rule (denoted R1) requiring a p-hydroxyl substitution for MEA activity is added to CT481, all 7 chemicals are correctly classified.

Figure 7:

All 6 MEA-tested chemicals containing CT665 (ring:hetero_[6]_N_O_1_4-oxazine_generic) are shown on left, 5 of which are MEA hits (green border). A sample of ToxCast assays found to also be significantly enriched with hits in both the CT665 and CT479 chemotype subgroups (OR > 4) are listed, including 3 G-protein coupled (opiate) receptor binding assays (NVS_GPCR) and 3 rat ion channel receptor binding assays (NVS_IC_r...), all of which could be potentially relevant to MEA activity. Upon further examination, 4 of the 5 MEA hits within the CT665 subgroup shown were found to have 1 or more activities in the rat ion channel receptor binding assays, strengthening possible associations among the assays.