NeuroToxicology 44 (2014) 204–217

Contents lists available at ScienceDirect

NeuroToxicology

Multi-well microelectrode array recordings detect neuroactivity of ToxCast compounds☆

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ARTICLE INFO

Article history:
Received 30 April 2014
Accepted 23 June 2014
Available online 2 July 2014

Keywords:
Screening
ToxCast
Microelectrode arrays
Electrophysiology

ABSTRACT

Spontaneous activity in neuronal cultures on microelectrode arrays (MEAs) is sensitive to effects of drugs, chemicals, and particles. Multi-well MEA (mwMEA) systems have increased throughput of MEAs, enabling their use for chemical screening. The present experiments examined a subset of EPA's ToxCast compounds for effects on spontaneous neuronal activity in primary cortical cultures using 48-well MEA plates. A first cohort of 68 compounds was selected from the ToxCast Phase I and II libraries; 37 were positive in one or more of 20 individual ToxCast Novascreen assays related to ion channels (NVS_IC), with the remainder selected based on known neuroactivity. A second cohort of 25 compounds was then tested with 20 originating from the ToxCast Phase I and II libraries (not hits in NVS_IC assays) and 5 known negatives from commercial vendors. Baseline activity (1 h) was recorded prior to exposing the networks to compounds for 1 h, and the weighted mean firing rate (wMFR) was determined in the absence and presence of each compound. Compounds that altered activity by greater than the weighted change of DMSO-treated wells plus 2SD were considered "hits". Of the first set of 68 compounds, 54 altered wMFR by more than the threshold, while in the second set, 13/25 compounds were hits. MEAs detected 30 of 37 (81.1%) compounds that were hits in NVS_IC assays, as well as detected known neurotoxicants that were negative in NVS_IC assays, primarily pyrethroids and $GABA_A$ receptor antagonists. Conversely, wMFR of cortical neuronal networks on MEAs was insensitive to nicotinic compounds, as only one neonicotinoid was detected by MEAs; this accounts for the bulk of non-concordant compounds between MEA and NVS_IC assays. These data demonstrate that mwMEAs can be used to screen chemicals efficiently for potential neurotoxicity, and that the results are concordant with predictions from ToxCast NVS_IC assays for interactions with ion channels.

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1. Introduction

Since the release of the National Academy's report entitled *Toxicity Testing the the 21st Century* (NRC, 2007), there has been an increased emphasis on the development of high-throughput/high content screens for the purpose of predicting the toxicity of chemicals and/or screening and prioritization of chemicals for subsequent testing. Adverse outcome pathways (AOPs) are a concept central to the use of data from in vitro screens to predict toxicity (Ankley et al., 2010; Watanabe et al., 2011), and describe scientific evidence to support the sequence of events linking a molecular initiating event (MIE) to the adverse outcome in vivo. Toxicity pathways are a subset of AOPs and describe the sequence of normal physiological responses from the MIE to the alterations in tissue function, that when sufficiently perturbed by chemicals, will lead to adverse responses (NRC, 2007).

The ToxCast program at the U.S. Environmental Protection Agency (Dix et al., 2007; Judson et al., 2010) includes ~500 in vitro assays used to profile the bioactivity of environmental

☆ This work was conducted as part of a Cooperative Research and Development Agreement between the US EPA Office of Research and Development and Axion Biosystems (CRADA 644-11). Preparation of this document has been funded by the U.S. Environmental Protection Agency. This document has been reviewed by the National Health and Environmental Effects Research Laboratory and approved for publication. Approval does not signify that the contents reflect the views of the Agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

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http://dx.doi.org/10.1016/j.neuro.2014.06.012
0161-813X/Published by Elsevier Inc.

compounds. Assays in the ToxCast suite include those for nuclear receptors, G protein-coupled receptors and ion channels, among others (Judson et al., 2010) and are potential MIEs that may lead to carcinogenesis, endocrine disruption, heptatic, renal, neuro- or other toxicities. Although previous investigations have begun to build putative AOPs related to developmental toxicity (Chandler et al., 2011; Sipes et al., 2011; Kleinstreuer et al., 2011a), vascular development and (Kleinstreuer et al., 2011b, 2013) and endocrine disruption (Rotroff et al., 2013), other potential toxicity pathways linking ToxCast endpoints to adverse outcomes have yet to be investigated, including those linked to neurotoxicity.

Adverse outcome pathways linked to neurotoxicity have not yet been formally described, with the exception of excitotoxicity linked to kainate-type glutamate receptors (Watanabe et al., 2011). However, it is well established that disruption of ion channel function can be an initiating event that leads to neurotoxicity following exposure to a broad variety of compounds (Coats, 1990; Narahashi, 2002; Tomizawa and Casida, 2005). Disruption of ion channel function by chemicals including insecticides such as the pyrethroids, organochlorines, and other chemical classes often leads to alterations in cellular excitability that ultimately result in perturbation of the functioning networks and pathways critical to nervous system homeostasis, resulting in neurotoxic signs and symptoms.

The ToxCast assay suite contains twenty binding assays (Novascreen (NVS_IC) assays) that measure the interaction of compounds with different ion channels. However, interference with ligand binding does not always equate to disruption of channel function, and the type of disruption produced (agonist, antagonist, modulator) cannot always be determined on the basis of binding alone. Thus, prediction of toxicity solely on the basis of binding results is unreliable (Lü and An, 2008). By contrast, rapid and efficient assays that allow assessment of ion channel function also have limitations (Lü and An, 2008). For example, high-throughput patch-clamp assays often require that ion channel subunits be expressed in non-neuronal cell models. This may lead to differences in responsiveness compared to native neurons and for toxicity screening where the target is unknown, is inefficient because multiple ion channels must be individually examined. Such approaches also do not allow for the measurement of activity in functionally connected networks of neurons, a prerequisite for nervous system function.

Neuronal networks grown on microelectrode arrays (MEAs) have been proposed as a suitable approach for neurotoxicity screening that is sensitive to different classes of compounds (Johnstone et al., 2010), reproducible across laboratories (Novellino et al., 2011) and provides high sensitivity (correct identification of known positive compounds) and specificity (correct identification/rejection of known negative compounds) for training sets containing 20–30 compounds (DeFranchi et al., 2011; McConnell et al., 2012). Recently, it has been demonstrated that mwMEAs can provide sufficient throughput for compound screening while retaining the other desirable qualities of single well MEAs (McConnell et al., 2012). Thus, neuronal networks grown on mwMEAs provide a means to evaluate further the potential neurotoxicity of ToxCast compounds. However, previously tested training sets (DeFranchi et al., 2011; McConnell et al., 2012) have been limited in the compound space that was examined, as well as in the number of compounds from a particular class of compounds that were represented. Furthermore, these previous studies have not used compounds for which there are other screening data available, nor did they attempt to place results into the context of adverse outcome or toxicity pathways. Finally, while networks of cortical neurons grown on MEAs are sensitive to many classes of compounds, these initial studies (DeFranchi et al., 2011; McConnell et al., 2012) indicated that changes in firing rate of the network may be insensitive to compounds acting via the nicotinic acetylcholine receptor (nAChR). Thus, the "fit-for-purpose" of the assay needs further investigation in this regard.

The present studies were designed to test subsets of compounds from the ToxCast phase I and II inventories in primary cultures of cortical neuronal networks grown on mwMEAs. The compounds tested were selected based on their activity (or lack thereof) in NVS_IC ion channel assays in ToxCast, or their known neurotoxicity in vivo. This allowed for the testing of two separate but related hypotheses: (1) compounds testing positive in NVS_IC ion channel assays will alter network mean firing rate; and (2) compounds that alter firing rate will be neuroactive or neurotoxic in vivo. Because the NVS_IC assays consider only a subset of all potential MIEs that could alter network firing rate, it is not reasonable to expect that compounds that are negative in these assays will necessarily be without effects on network firing rate measured using MEAs. In total, 92 unique compounds were examined using MEAs (saccharin was included in both cohorts), including 6 inhibitors of acetylcholinesterase (AChE), 10 compounds active on $GABA_A$ receptors, 9 compounds that alter voltage-gated sodium channel (VGSC) function and 7 compounds known to act on nicotinic acetylcholine receptors. This dataset will provide additional information on the "fit-for-purpose" of cortical neuronal networks as a screening tool, and in combination with the ToxCast data, allow for the proposal of putative toxicity pathways that may contribute to neurotoxicity.

2. Materials and methods

2.1. Compounds

Eighty-eight ToxCast compounds (the five negatives in the second cohort were not from ToxCast) were evaluated in two separate cohorts. The first cohort contained 68 ToxCast compounds and included two negative control compounds: acetaminophen and saccharin (McConnell et al., 2012) without activity in vitro or in vivo. In this cohort, compounds were selected on the basis of (1) known neurotoxicity/activity (based on the authors' review of the literature) and/or (2) positive result in one of 20 Novascreen (NVS_IC) assays in ToxCast. The second cohort contained 20 ToxCast compounds as well as five additional negative controls: amoxicillin, glyphosate, saccharin, salicylic acid, and sorbitol. The saccharin used in the first cohort was sourced from ToxCast while in the second it was purchased directly from Sigma. The second cohort selected compounds that were not positive in ToxCast NVS_IC assays but were active in other ToxCast Assays. Additionally, some compounds in this set were selected because they belonged to the same class as compounds in the first cohort (e.g. conazoles and tetramethrin), which were active in MEAs in the first set. Compound name, CAS #, class, target and action (when known), NVS_IC hit count (the number of NVS_IC assays with a positive result), and molecular initiating event are listed in Table 1. The first cohort included several "failed" pharmaceutical compounds for which limited information is available; these are referred to as "pharma 1–6" in the present manuscript. The 88 ToxCast compounds were received in as 20 mM (unless otherwise noted in Table 1) stock solutions in dimethyl sulfoxide (DMSO). The five additional negative compounds included in the second set were obtained from Sigma (St. Louis, MO) and prepared as 50 mM stock solutions in DMSO (glyphosate was prepared in H_2O). DMSO (Sigma), pancreatin (Sigma), and EtOH (Pharmaco-Aapr, Brookfield, CT) were obtained from commercial vendors.

Table 1
Information on compounds tested using the MEA system.

Compound name	CAS#	Class	Target/action[c]	NVS_IC hits[d]	MIE[e]	Known neurotoxic[f]	% wMFR Δ	MEA outcome	%Activity in ToxCast[g]
1st Cohort									
Aldicarb	116-06-3	Carbamate	AChE inhibition (reversible)	0	–	Yes	−7.9	NEG	2
Bensulide	741-58-2	Organophosphate	AChE inhibition	1	5	Yes	−88.7	POS	16
Carbaryl	63-25-2	Carbamate	AChE inhibition (reversible)	0	–	Yes	−30.5	POS	6
Chlorpyrifos	2921-88-2	Organophosphate	AChE inhibition	1	5	Yes	−47.5	POS	9
Chlorpyrifos oxon	5598-15-2	Organophosphate	AChE inhibition	0	–	Yes	−63.6	POS	9
Abamectin	71751-41-2	Mectin	GABA-a agonist	4	3,5,6	Yes	−100	POS	18
Emamectin	155569-91-8	Mectin	GABA-a agonist	4	3,4,5	Yes	−100	POS	23
Milbemectin	NOCAS_34742	Mectin	GABA-a agonist	3	3,6	Yes	−99.9	POS	21
Chlordane	57-74-9	Organochlorine	GABA-a antagonist	0	–	Yes	66.9	POS	15
Dieldrin	60-57-1	Organochlorine	GABA-a antagonist	0	–	Yes	56.8	POS	7
Endosulfan	115-29-7	Organochlorine	GABA-a antagonist	0	–	Yes	18.9	POS	13
Fipronil	120068-37-3	Phenylpyrazole	GABA-a antagonist	0	–	Yes	−86.9	POS	8
Heptachlor epoxide	1024-57-3	Organochlorine	GABA-a antagonist	0	–	Yes	2.5	NEG	6
Lindane	58-89-9	Organochlorine	GABA-a antagonist	0	–	Yes	93.7	POS	2
Methoxychlor	72-43-5	Organochlorine	GABA-a antagonist	0	–	Yes	−72.1	POS	11
Acetamiprid	135410-20-7	Neonicotinoid	Nicotinic agonist	2	7	Yes	14	NEG	1
Clothianidin	210880-92-5	Neonicotinoid	Nicotinic agonist	2	7	Yes	3.9	NEG	1
Imidacloprid	138261-41-3	Neonicotinoid	Nicotinic agonist	2	7	Yes	−20.5	POS	1
Nicotine	54-11-5	Neonicotinoid	Nicotinic agonist	2	7	Yes	3.3	NEG	1
Thiacloprid	111988-49-9	Neonicotinoid	Nicotinic agonist	2	7	Yes	−2.5	NEG	2
Thiamethoxam	153719-23-4	Neonicotinoid	Nicotinic agonist	0	–	Yes	−11.8	NEG	1
Pharma 1	298198-52-4	Failed Pharma	Nicotinic agonist, partial alpha 7	4	4,7,8	Yes	0.3	NEG	4
5,5-Diphenylhydantoin	57-41-0		Na channel antagonist	0	–	Yes	−51.2	POS	1
Allethrin	584-79-2	Pyrethroid	Na channel modifier	1	5	Yes	−100	POS	14
Bifenthrin	82657-04-3	Pyrethroid	Na channel modifier	0	–	Yes	17.3	POS	4
Cyfluthrin	68359-37-5	Pyrethroid	Na channel modifier	0	–	Yes	−99.8	POS	4
Fenvalerate	51630-58-1	Pyrethroid	Na channel modifier	0	–	Yes	−73.6	POS	6
Permethrin	52645-53-1	Pyrethroid	Na channel modifier	0	–	Yes	16.4	NEG	5
Eugenol	97-53-0	Phenylpropene	Na channel antagonist	0	–	Yes	−43.1	POS	2
p,p′-DDT	50-29-3	Organochlorine	Na channel modifier	0	–	Yes	−27.7	POS	16
p,p′-DDD	72-54-8	Organochlorine	Breakdown product of DDT	0	–	–	−95.3	POS	15
p,p′-DDE	72-55-9	Organochlorine	Breakdown product of DDT	0	–	-	41.2	POS	14
Myclobutanil	88671-89-0	Conazole	Ergosterol biosynthesis inhibitor	1	4	–	−61.3	POS	5
Hexaconazole	79983-71-4	Conazole	Demethylation inhibitor	1	5	–	−90.7	POS	8
Propiconazole	60207-90-1	Conazole	Demethylation inhibitor	1	4	–	−92.6	POS	7
Tetraconazole	112281-77-3	Conazole	Demethylation inhibitor	3	3,4	–	−87.2	POS	10
Flusilazole	85509-19-9	Organosilicone fungicide	Ergosterol biosynthesis inhibitor	2	3,4	–	−82.8	POS	8
Imazalil	35554-44-0	Fungicide	Ergosterol biosynthesis inhibitor	1	4	–	−82.8	POS	12
1,2-Propylene glycol	57-55-6	Glycol	No intended target	0	–	–	−1.6	NEG	1
1,3-Diphenylguanidine	102-06-7	Plasticizer	No intended target	2	3,5	–	−96.9	POS	5
Saccharin	82385-42-0	Artificial sweetener	No intended target	NEG-Cont	–	–	−24.7	POS	1
Acetaminophen	103-90-2	Aniline analgesics	COX inhibitor	NEG-Cont	–	–	−9.7	NEG	1
Amiodarone[a]	19774-82-4	Antiarrhythmic	Adrenergic blocker	4	2,3,4	–	−69	POS	24
Cyazofamid	120116-88-3	Fungicide	Complex III Qi inhibitor	2	7	–	−63.7	POS	11
Dibutyl phthalate	84-74-2	Plasticizer	No intended target	0	–	–	−71.3	POS	6
Diphenhydramine	147-24-0	antihistamine	H1 receptor antagonist	2	3,4	Yes	−92.3	POS	7
Enadoline	124378-77-4	Failed pharma	Kappa-opioid agonist	0	–	Yes	−80.8	POS	1
Haloperidol	52-86-8	Antipsychotic	Dopamine Antagonist	3	3,4	Yes	−90.8	POS	12
Isotiazoline	26172-55-4	Isothiazolinones	Biocide	0	–	–	−17.3	POS	8
Maneb	12427-38-2	Herbicide (dithiocarbamate)	Dopamine β-hydroxylase inhibitor	0	–	Yes	9.1	NEG	13
Mepiquat	24307-26-4	plant growth regulator	Inhibits gibberellic acid synthesis	2	7	Yes	−7.3	NEG	1
Pentamidine[a]	140-64-7	Anti-microbial	Nuclear metabolism inhibitor	3	1,3,4	Yes	−96.1	POS	15
Reserpine	50-55-5	Antihypertensive	Monoamine transmitter depletion	3	3,4	Yes	−99.5	POS	13
Rotenone	83-79-4	Botanical insecticide	Electron transport inhibitor	0	–	Yes	−94.4	POS	11
Spiroxamine	118134-30-8	Fungicide	Fungal RNA polymerase inhibition	2	3,4	–	−40.7	POS	5
Thidiazuron	51707-55-2	Plant growth regulator	Plant growth regulator	2	5	–	−57.2	POS	3
Tributyltin	1461-22-9	Organometal	Multiple potential neuronal	4	2,3,4	Yes	−100	POS	27

Table 1 (*Continued*)

Compound name	CAS#	Class	Target/action[c]	NVS_IC hits[d]	MIE[e]	Known neurotoxic[f]	% wMFR Δ	MEA outcome	%Activity in ToxCast[g]
1st Cohort									
Volinanserin	139290-65-6		5-HT2A antagonist	3	3,4	Yes	−89	POS	6
Zamifenacin	127308-82-1		Muscarinic antagonist (m3)	3	3,4	Yes	−62.9	POS	17
Acrylamide	79-06-1			0	–	Yes	34.5	POS	1
Butachlor	23184-66-9	Herbicide		1	3	–	7.1	NEG	15
Diethyl butanedioate	123-25-1	flavoring/food additive		0	–	–	−20.4	POS	1
Difenzoquat	43222-48-6	Herbicide		2	3,4	Yes	−97.8	POS	7
Pharma 2	NOCAS_47377	Failed pharma		5	2,3,4,5,6	–	−78.7	POS	25
Pharma 3	349495-42-7	Failed pharma		0	–	–	−29.9	POS	4
Pharma 4	NOCAS_47362	Failed pharma		4	3,4,5	–	−78.5	POS	16
Pharma 5	478149-53-0	Failed pharma		3	4,7,8	–	18.8	POS	2
Pharma 6	NOCAS_47387	Failed pharma		4	2,3,4	–	−99.2	POS	18
2nd Cohort									
Cyproconazole	94361-06-5	Conazole		0	–	–	−39.4	POS	3
Difenoconazole	119446-68-3	Conazole		0	–	–	−95.3	POS	14
Diniconazole	83657-24-3	Conazole		0	–	–	−100	POS	12
Fenbuconazole	114369-43-6	Conazole		0	–	–	−100	POS	4
Fluconazole	86386-73-4	Conazole		0	–	–	−28	NEG	0
1H-1,2,4-Triazole	288-88-0	Conazole precursor/ synthesis		0	–	–	−25.6	NEG	1
Ziram	137-30-4	Dimethyldithiocarbamate		0	–	Yes	6	NEG	17
Mancozeb	2234562	Dithiocarbamate		0	–	–	−8.7	NEG	16
Lactofen	77501-63-4	Herbicide	Protoporphyrinogen oxidase inhibitor	0	–	–	−97.9	POS	7
17beta-Estradiol	50-28-2	Hormone	ER-Agonist	0	–	Yes	−37.6	NEG	11
17beta-Trenbolone	10161-33-8	Hormone	AR-Agonist	0	–	–	−65.7	POS	13
Prochloraz	67747-09-5	Imidazole fungicide?	AR-Antagonist	0	–	–	−100	POS	14
Fenthion	55-38-9	Organothiophosphate	AChE inhibition	0	–	Yes	−68.5	POS	5
Tetramethrin	7696-12-0	Pyrethroid	Na channel modifier	0	–	Yes	−90.1	POS	7
Piperonyl butoxide	51-03-6	Synergist	P450-inhibition	0	–	–	−51.8	POS	6
Butyl benzyl phthalate	85-68-7			0	–	–	−99.2	POS	7
Di(2-ethylhexyl) phthalate	117-81-7			0	–	–	0.3	NEG	4
Genistein	446-72-0		ER-agonist	0	–	Yes	−75.7	POS	14
Perfluorooctane sulfonic acid	1763-23-1			0	–	–	−10.1	NEG	15
Vinclozolin	50471-44-8		AR-antagonist	0	–	–	−83.6	POS	3
Amoxicillin[b]	26787-78-0			NEG-Cont	–	–	−4.9	NEG	NA
Glyphosate[b]	1071-83-6			NEG-Cont	–	–	3.6	NEG	NA
Saccharin[b]	82385-42-0			NEG-Cont	–	–	−6.5	NEG	1
Salicylic acid[b]	69-72-7			NEG-Cont	–	–	−1.6	NEG	4
Sorbitol[b]	50-7-4			NEG-Cont	–	–	−16.3	NEG	NA

[a] Compounds were received as 20 mM stock solutions in DMSO with the following exceptions; the stock concentration was 12.1, 15.1, 16.6, 16.9, and 17.5 mM for Abamectin, Amiodarone, Pharma 6, Reserpine, and Pentamidine isethionate, respectively.
[b] Negative controls were tested at 50 μM.
[c] Target/action-molecular target for each compound identified by literature searches and the action on that target.
[d] NVS_IC hits. The number of hits in the 20 Novascreen (NVS_IC) ion channel assays considered for this study (see Supplemental Table 1 for further details).
[e] MIE – molecular initiating event. The type of ion channel acted upon by the compound in ToxCast. See Table 3 for additional information.
[f] The designation "Yes" indicates that the compound is neurotoxic as described in Section 2. The "–" symbol indicates that there is no determination regarding neurotoxicity.
[g] Percentage of all assays in ToxCast in which the compound was considered a "hit" or had activity.

2.2. Multi-well MEA plates

Microelectrode array plates (M768-KAP Kapton) were composed of 48 wells and 768 electrodes total. Each well contained an array of 16 embedded nano-textured gold platinum microelectrodes (~40–50 μm diameter, 350 μm center-to-center spacing), plus four integrated ground electrodes (Axion Biosystems Inc., Atlanta, Georgia).

2.3. Primary cortical culture on mwMEAs

All aspects of procedures involving laboratory animals were approved by the 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. One day before culture, the electrode surface in each well was coated with 0.05% polyethylenimine (Sigma) in HEPES (pH 8) and incubated at 37 °C for 1 h. Each well was then washed 3 times with 500 mL sterile water and allowed to air dry. Plates were stored at 4 °C until the day of culture. Primary cultures of cortical neurons were prepared from Long–Evans rats on postnatal day 0–1 (Mundy and Freudenrich, 2000). Briefly, the pups were decapitated and the neocortex was carefully separated from the brain. The neocortex was placed in a dish containing 3–5 mL/animal of cold cortical buffer (137 mM NaCl, 5 mM KCl, 170 μM Na_2HPO_4, 205 μM KH_2PO_4, 5 mM glucose, 59 mM sucrose, and 100 U/mL penicillin/0.1 mg/mL streptomycin). The cortex was minced with sterile surgical scissors and triturated until homogenous. To digest the tissue, the minced cortex was transferred into a 25 cm^2 flask with trypsin (1.0 g in 400 mL Cortical Buffer, Sigma) at 1 mL/animal. The flask was incubated in a water bath at 30 rpm and 37 °C for 4 min

and 45 s. Two milliliters of DNAase was added to the flask and again the solution was incubated at the same settings for 4 min and 45 s. To stop the digestion, warmed cortical medium (DMEM with GlutaMax, FBS, 10 mM HEPES, 100 U/mL penicillin/0.1 mg/mL streptomycin) was added to the flask. The subsequent cell suspension was transferred into 50 mL conical tubes and centrifuged at 300 × *g* (1800 rpm in the Beckman Allegra 6R) for 5 min at room temperature. The supernatant was discarded and the pellet was resuspended in 10 mL cortical media with 1 mL DNAase. The cells were then centrifuged for 5 min at 300 × *g* and the resulting pellet was re-suspended again in the manner described above without DNAase and filtered through a 100 μm pore Nitex filter into a sterile beaker to remove the meninges, debris, and large clumps of tissue. Prior to each culture, the electrodes of each well were coated with a 50 μL drop of laminin and incubated for 1 h. Excess laminin was removed and cells were seeded at 150 K cells/well in a 25 μL drop over the mwMEA's electrodes, then plates were placed in a humidified, 5% CO_2 incubator at 37 °C. After 1 h, and additional 450 μL of cortical media was added and cells were returned to the incubator. After 2 h, media was replaced with 500 μL NB/B27 media (500 mL Neurobasal-A Medium (1×), 10 mL B-27 Supplement (50×), 5 mL GlutaMax (100×), 5 mL Pen-Strep). Three days after culture cytosine arabinoside (5 μM) was added. Cells were fed with ½ media changes every 7 days.

Cultures prepared using this method have been extensively characterized and consist primarily of glutamatergic and GABAergic neurons and glial cells (Mundy and Freudenrich, 2000). Over a period of days in culture, the neurons extend axons and dendrites (Harrill et al., 2013) and form extensive networks with defined synapses (Harrill et al., 2011). Activity in the form of spontaneous spiking and bursting on MEAs also develops over the first two weeks in culture, and has been shown to be decreased by treatments that decrease neurite outgrowth (Robinette et al., 2011). This spontaneous activity is also sensitive to acute effects of a variety of neurotransmitter receptor agonists and antagonists, as well as compounds that modulate ion channel function (McConnell et al., 2012; Mack et al., 2014). As such, this is a suitable model system for the assessment of potential neurotoxicity pathways based on ToxCast assays.

2.4. Compound handling

Stock compound solutions were received in as 20 mM stock solutions in DMSO and kept in a Greiner round bottom 96-well plate at room temperature. The compound plate was sealed with Topseal-A 96 sealing tape and parafilm. These seals were removed prior to compound use and replaced afterwards. Each compound was diluted twice immediately prior to the experiment as follows. Compounds were transferred from their stock plate to a Costar 96-well dosing plate. The ToxCast compounds were then diluted 1:10 in NB/B27 media. The five compounds from Sigma were diluted 1:20 in media. For the second dilution, 10 μL of solution from the dosing plate was transferred to the 48-well MEA plate containing 500 μL of media, resulting in a final concentration of 40 μM (five additional negatives at 50 μM). Finally, 0.5 μL DMSO was added to one or more wells per 48-well MEA plate as a negative control at concentration of 0.5 μL/500 mL (0.1% by volume).

2.5. MEA recording

Acquisition of spontaneous network activity from cortical cultures utilized Axion Biosystems' Maestro 768-channel amplifier, Middle-Man data acquisition interface, and Axion Integrated Studios (AxIS) v1.5.2 (or later) software. Channels were sampled simultaneously with a gain of 1200× and a sampling rate of 12.5 kHz/channel. On day in vitro (DIV) 12 or 13 using the Maestro, spontaneous activity of neuronal cells on mwMEA plates was recorded and inspected to determine individual well usability. An electrode must have an average of ≥5 spikes/min to be considered active. Any wells that did not exhibit spontaneous activity levels of at least 10 active electrodes were deemed unusable and not treated with a compound in following experiments. Experiments were conducted on DIV 14 or 15. Any electrodes with rms-noise levels >10 μV were grounded prior to data recording. A butterworth band-pass filter (300–5000 Hz) was utilized along with a variable threshold spike detector (Biffi et al., 2010) set at 8× standard deviation of the rms noise on each channel during recordings. Three different file types were saved, a raw data file (*.raw file) that included all data, a spike counts file (*.csv file) that included the spikes per electrode with a 1 second bin time, and an alpha map (*.map file) that included spike timing and profile information. Baseline activity was recorded for 1 h; directly after baseline recording compounds were added to each well at a final concentration of 40 μM (five additional negatives at 50 μM), unless otherwise noted (see Table 1 for details). Each mwMEA also contained wells (at least one well/mwMEA plate) that received 0.1% DMSO. An hour of spontaneous activity was recorded following compound treatment and the same file types were collected as during the baseline recording period.

To generate concentration curves, first serial dilutions (mM; 2, 0.5, 0.15, 0.05, 0.015, 0.005, 0.0015) of the selected compounds were made on a dosing plate in NB/B27 media. 10 μL of solution was transferred from the dosing plate to the media (500 μL) in each well on the mwMEA plate for a final concentration (in μM) of 40, 10, 3, 1, 0.3, 0.1, and 0.03. Compounds were selected for concentration–response assessment based on several factors, including whether or not they were positive or negative in the single point screen and whether or not they were known or expected to have activity. Additional information for selection of specific compounds is provided in the figure legends.

After experimental data were collected, electrodes were cleaned by soaking with a solution of 1× phosphate buffered saline and pancreatin (~2.5 g/50 mL) overnight. MEA wells were then rinsed 3 times with deionized water and plates were sterilized in 70% ethanol for 5 min followed by baking in a 55 °C oven for 4–5 h upside down with the lid on. No plate was utilized more than 3 times.

2.6. Cytotoxicity

All of the compounds were examined for cytotoxicity using a fluorescent propidium iodide (PI) stain and a Hoescht counter stain, following the methods outlined in McConnell et al. (2012). The former stains DNA in cells with a compromised plasma membrane while the latter stains DNA in all cells. Cells were cultured on poly-L-lysine-coated Costar 96-well plates at 40,000 cells/well, using the methods described above. On DIV 14 or 15, plates that demonstrated uniform distributions of neurons (by visual inspection) were treated with compounds at the same concentration as in the single point screen (40 μM, unless otherwise noted). Each plate had at least one well of Triton X-100 (1% by volume) to establish a positive control. The treated plate was incubated at 37 °C for 1 h. After compound treatment, media was removed and 100 μL of 5 μM PI (Invitrogen P1304MP) in Locke's buffer was carefully added to the wells via wide orifice pipette tips to reduce the mechanical disturbance to the neurons. Plates were again incubated at 37° for 20 min before aspirating the PI working solution and 100 μL of warm fixative containing 3 μg/mL Hoechst 33342 was added to all wells. After 20 min at room temperature, the fixative was aspirated and wells were washed two times with 100 μL Ca/Mg-free PBS

(Gibco #70013-032). The final wash was left in the wells. Plates were then sealed with optical plate tape and stored at 4 °C until images were collected (within 14 days of fixation).

Fluorescence images were analyzed via the Cellomics Target Activation Bioapplication and a Cellomics ArrayScan VTI (Thermo-Scientific) which allows for image acquistion and analysis in up to 4 separate channels. Valid cells were identified using Hoescht stain by imaging in channel 1. The Hoechst stained nuclei identified in channel 1 were used to categorize cells in channel 2. PI positive cells were imaged in channel 2. Positive PI stained nuclei were identified when the average fluorescent intensity was greater than 3 times the background. The percentage of PI positive cells was calculated for each well, with a minimum of 300 cells counted per well. Each compound was tested in at least 4 wells from two different cultures and values from the four trials were averaged. To determine cytotoxicity, a threshold level was determined based on the percentage of PI positive cells in DMSO treated wells, plus 2 times the standard deviation of the DMSO treated wells. When the average value exceeded this threshold, the compound was classified as cytotoxic.

2.7. Data analysis

For all analyses, the well is considered an observation ('*n*'), as this was the unit of treatment. In all cases data were collected from a minimum of two culture preparations from separate animals on separate dates. The spike counts file (*.csv file) obtained from both baseline and treated recordings were used to calculate the number of active electrodes (>5 spikes/min) in each well and the average per-well mean firing rate (MFR or spikes/min). Wells that contained fewer than 10 active electrodes during the baseline recording were not included in analysis (~15% of wells). Wells were not removed if active electrodes decreased to less than 10 following treatment. Post-hoc analysis determined that the first 20 min of baseline recording was a period of activity stabilization and thus were omitted from analysis. Following this adjustment, the mean ± sd slope for firing rate vs time in control wells was −0.23 ± 0.77 spikes/min. Post-hoc evaluation of the stability of recordings indicated that 29 wells had an average change of greater than or equal to −1.5 or +1.2 spikes per minute during the baseline recording period and those wells were deemed unstable (approximately greater than 2SD of the mean) and excluded from further analyses. For the remaining wells, each well served as its own control and the percent change in activity resulting from compound treatment to that well was then calculated. To account for potential misrepresentation of the mean firing rate (MFR) due to differences in active electrodes, the MFR was weighted for both control and treated conditions. The MFR average for each well was weighted by the corresponding total number of active electrodes using Excel's Sumproduct function (McConnell et al., 2012). This was then averaged across the sum of those electrodes. The resultant weighted mean firing rate (wMFR) averages for the baseline and treatment were then compared, resulting in a percentage change in the treated case relative to the baseline case. An average change of the wMFR was determined for each compound across all plates. A hit window was established based on the effect of the compound vehicle, DMSO, on the weighted MFR (wMFR). The hit window for the single point screen was set at plus or minus the mean percent change caused by DMSO plus two standard deviations.

3. Results

3.1. Cohort 1

A total of 417 wells were recorded from across seven different cortical culture preparations during the first cohort's single point screen; 27 wells were not considered for further analysis because they did not meet the criterion for slope of control firing rate defined in the methods. The wMFR over all wells during the baseline period was 117.4 ± 26.1 (mean ± sd, $n = 390$), with each well having an average of 14.8 ± 1.6 active electrodes. The overall wMFR change induced by DMSO was a decrease of 14.4 ± 1.2% (mean ± SD, $n = 30$). Thus, the "hit" window established for the single point screen was ± 16.7%. In the single point screen, 54 of the 68 ToxCast compounds were found to alter the wMFR greater than the threshold. Of the active compounds, 47 caused a decrease in activity while only 7 (chlordane, dieldrin, endosulfan, bifenthrin, lindane, p,p′-DDE, and acrylamide) caused an increase in firing rate. Acetaminophen and saccharin (ToxCast) were evaluated as negative controls that should not increase or decrease activity past the threshold. Acetaminophen was negative as expected; and only caused a wMFR decrease of 9.7% ($n = 6$). By contrast, response to saccharin was positive in the single point screen, causing a 24.7% decrease in wMFR ($n = 7$).

Concentration–response curves were generated for nine compounds; eight of which were positive (imidacloprid, cyfluthrin, bifenthrin, chlordane, hexaconazole, tetraconazole, emamectin benzoate and "pharma 2") and one negative (thiacloprid,) in the single point screen. Results were concordant with unweighted MFR changes in the single point screen at 40 μM (Fig. 1), with the eight compounds that were positive in the single point screen causing concentration-dependent changes in MFR, and thiacloprid being without effect.

3.2. Cohort 2

The second cohort's single point screen consisted of 167 wells recorded from three different cortical cultures; 4 wells were not considered for further analysis because they did not meet the slope criterion for control firing rate. The wMFR over all wells during the baseline period was 101.0 ± 40.1 (mean ± sd, $n = 163$), with each well having an average of 14.7 ± 1.6 active electrodes. The second cohort hit threshold was established using the same method for the first cohort, using the average wMFR + 2SD for DMSO treated wells. The average wMFR change resulting from DMSO treatment was a decrease of 35.7 ± 1.7% ($n = 14$). Thus the "hit" window for the second cohort was ± 39.1% change from control. This is higher than for the first cohort as well as previously published studies (14.7%; McConnell et al., 2012). As there was no clear explanation for the higher result (e.g. clear outlier wells or plates, recordings containing noisy electrodes, etc.), this value was used. Of the 25 compounds in the second cohort, 13 were found to alter neuronal activity past the threshold wMFR, with all 13 hits decreasing the wMFR. With the exception of cyproconazole (−39.4% change in wMFR), all of the hits clearly altered wMFR well beyond the higher threshold in this cohort. Only three compounds (fluconazole, triazole and 17β-estradiol) had wMFR values that fell between the threshold value for the first and second cohorts. None of the five negative compounds in the second cohort (amoxicillin, glyphosate, saccharin, salicylic acid, and sorbitol) caused changes greater than the hit threshold for either cohort.

Concentration–response curves were generated for eight compounds; four of which were hits (genistein, vinclozolin, 17β-Trenbolone, and piperonyl butoxide) and four of which were negatives (fluconazole, 1H-1,2,4-triazole, 17β-estradiol, and macozeb) in the single point screen. Concentration–responses were concordant with unweighted MFR changes in the single point, screen expect for the change caused by fluconazole and genistein (Fig. 2). In the single point screen fluconazole was not a hit, but at the same concentration in the concentration response fluconazole increased MFR at the highest concentration tested (40 μM). Interestingly, effects of the other conazoles, both in single

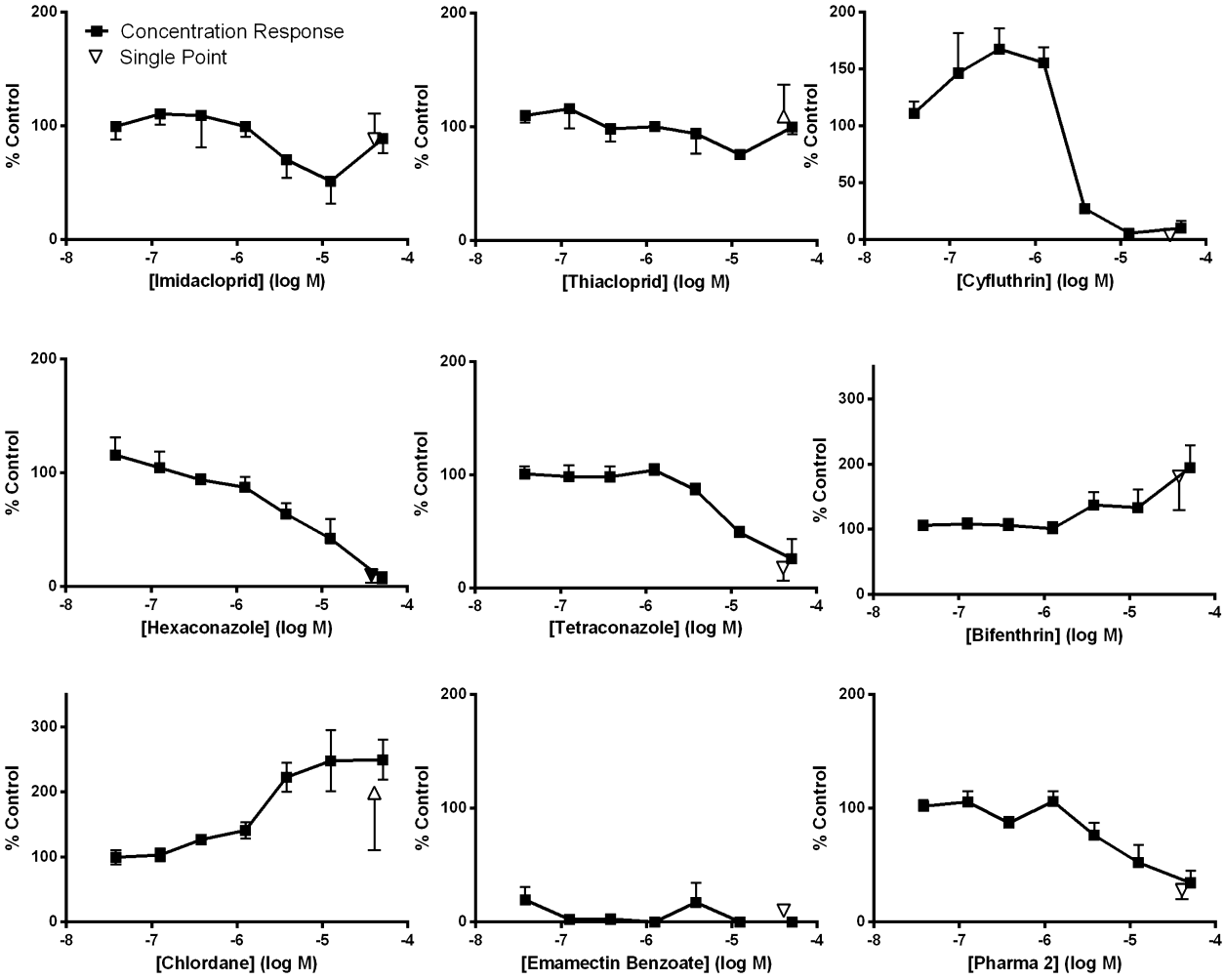

Fig. 1. Comparison of concentration–response and single point screen data for selected compounds in the first cohort. Nine concentration–response relationships were determined for a subset of compounds from the first set of 68 compounds. Effects on unweighted MFR in cortical networks are shown as percent change from 40 min baseline recording. Values are means ± SEM from at least 2 wells from separate cultures. Unweighted MFR changes from the single point screen (40 μM) are shown as open triangles, and are plotted slightly offset for clarity. Imidacloprid and thiacloprid were selected because they were positive and negative, respectively, in the single point screen, but were known to act on the nAChR. Bifenthrin, cyfluthrin, chlordane and emamectin were selected because they were positive in the single point screen and also known to interact with voltage-gated sodium channels (bifenthrin and cyfluthrin) and $GABA_A$ receptors (chlordane and emamectin). Hexaconazole and tetraconazole were selected because they were positive in the single point screen, yet not known to have a specific neuronal target. Finally, Pharma 2 was selected because it was a positive in the single point screen and was positive in 5 NVS_IC assays.

point screens as well as in the concentration–response studies (hexaconazole, tetraconazole) were to decrease MFR. For genistein, changes in MFR did not reach the same level as observed in the single point screen, and in fact were similar to those caused by 17β-estradiol, which was negative.

3.3. Cytotoxicity

The majority of the compounds did not affect cell viability after 1hr of exposure. The average percent of cells staining positive for PI from wells exposed to the negative control, DMSO, across all the plates used in the first cohort was 12 ± 10.9% (mean ± SD, $n = 14$). Based on the DMSO effect, a threshold of 33.8% (mean + 2SD) was set as a threshold for cytotoxicity. Triton X-100 (1% by volume), the positive control, caused 67 ± 11.8% (mean ± SD, $n = 14$) of the cells to stain positive for PI. In the first cohort only tributyltin chloride caused cytotoxicity with 48 ± 18.6% of the cells staining positive for PI (Fig. 3). In the second cohort, DMSO caused 6.0 ± 5.1% (mean ± SD, $n = 12$) of cells to stain PI positive and Triton X-100 (1% by volume) caused 69.9 ± 9.6% (mean ± SD, $n = 18$) of the cells to stain positive for PI. Thus a threshold of 14.7% PI positive cells was used to determine cytotoxicity; only ziram caused cytotoxicity, resulting in staining 34.9 ± 10.2% (mean ± SD, $n = 6$) of the cells positive for PI (Fig. 4). Diniconazole caused 15.6% of cells to stain positive for PI, and was just below the threshold for cytotoxicity.

3.4. Outcomes based on ToxCast hits

As noted in the methods, 20 ToxCast NVS_IC assays related to ion channels were used as a basis for selection of the compounds tested in MEAs. In the first cohort, 37 compounds were positive in at least one of the 20 NVS_IC assays, 13 compounds were positive in two NVS_IC assays, eight compounds were positive in three NVS_IC assays, and seven compounds were positive in 4 NVS_IC assays. "Pharma 2" had the greatest number of NVS_IC hits and was the only compound with five NVS_IC hits. There were six NVS_IC assays that did not detect any compounds as hits in ToxCast (Supplemental Table 1). All of the second cohort compounds were negative in these NVS_IC assays. Since concentration–response curves were not determined for all compounds, the comparisons that follow will be made based on results in the single point screen in the MEA assay.

Supplementary table related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neuro.2014.06.012.

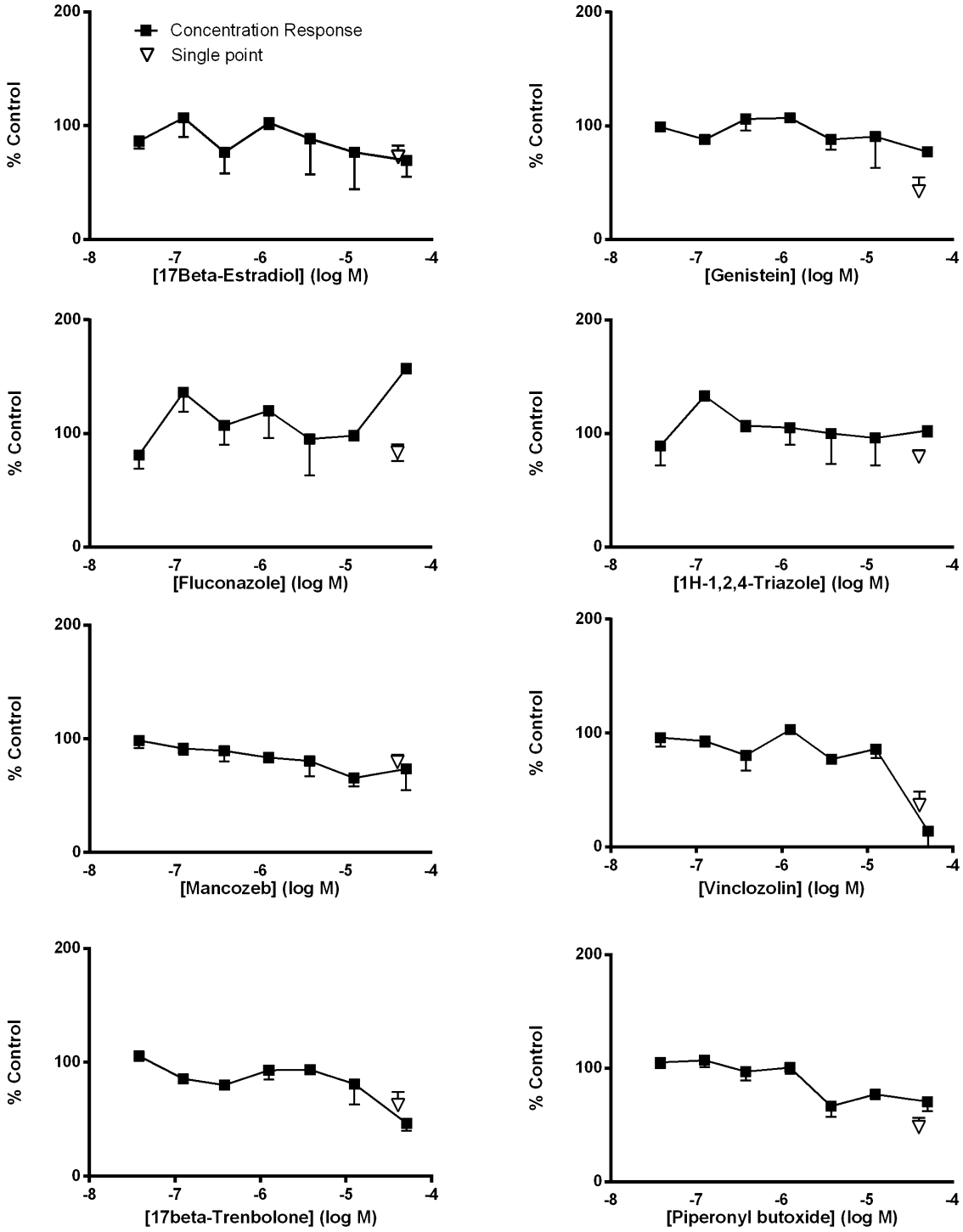

Fig. 2. Comparison of concentration–response and single point screen data for selected compounds in the second cohort. Eight concentration–response relationships were determined for a subset of compounds from the second cohort of compounds. Effects on MFR in cortical networks are shown as percent change from 40 min baseline recording. Values are means ± SEM from at least 2 wells from separate cultures. Unweighted MFR changes from the single point screen are shown as open triangles, and are plotted slightly offset for clarity. 17-β-estradiol and genistein were selected because they were both ER agonists and gave opposite results in the single point screen. Fluconazole and 1H-1,2,4-triazole were selected because they were negative in the single point screen and were a conazole or conazole metabolite, respectively. Mancozeb was selected due to its being highly active in other ToxCast assays (positive in 16% of assays) and because it was negative in the single point screen. Vinclozolin and 17-β-trenbolone were selected because they are an AR antagonist and agonist, respectively, and both were positive in the single point screen. Finally piperonyl butoxide was selected because it is a commonly used synergist with pyrethroids, and was positive in the single point screen.

In total, there were 56 compounds tested in the MEAs which were not hits on the NVS_IC assays. Table 2 compares the results of the present experiments for the NVS_IC and MEA assays.

Of the 37 compounds which were NVS_IC hits, 30 were positive (81.1%) in the MEA single point screen and caused a change in neuronal activity beyond the set threshold; of the seven compounds that were hits in NVS_IC assays but were negative in MEAs, only butachlor and mepiquat were not a nicotinic agonist or a neonicotinoid insecticide. Conversely, of the 56 compounds expected to be negative from the NVS_IC assays, 19 were negative in the single point MEA screen. Of the negative controls, all five were negative in the second cohort and acetaminophen was

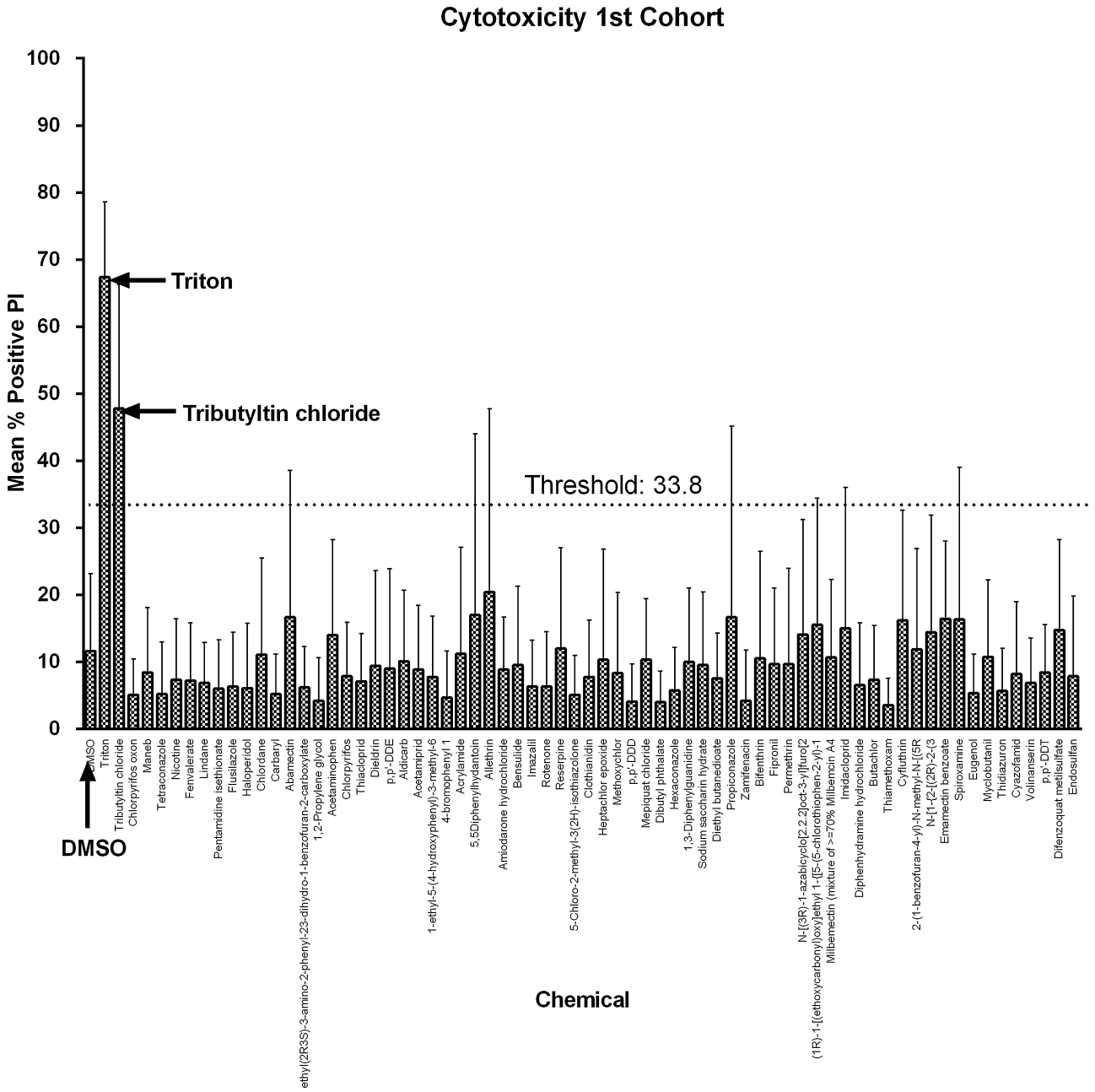

Fig. 3. Cytotoxicity of 1st cohort of compounds tested in the MEAs to cortical cultures. Primary cultures from rat cortex were plated in 96-well plates as described in Section 2. Cultures were exposed to test compounds at a concentration of 40 μM (unless otherwise noted in Table 1) for 1 h, then cell death was assessed using a propidium iodide assay as described in Section 2. The means and SD values are plotted and the dotted line indicates the mean + 2SD of the number of PI positive cells in DMSO treated wells (33.8%). Only tributyltin caused cytotoxicity that exceeded this threshold of PI positive cells.

negative in the first cohort. Saccharin, which was tested in both cohorts, was a hit in the first cohort but negative in the second cohort. It is noted that the sources of saccharin were different in each of these cohorts.

The NVS_IC assays were categorized into nine molecular initiating event groups based on the molecular target of the assay–glutamate receptors, potassium channel, calcium channel, sodium channel, $GABA_A$ receptor, glycine receptor, nAChRs, and serotonin receptors. Agreement information on NVS_IC MIE and MEA hits are provided in Table 3. This information can be used to demonstrate that putative toxicity pathways (Fig. 5) mediated by calcium channels, sodium channels and $GABA_A$ receptors are supported by the combination of information from NVS_IC and MEA assays. However, as there are several compounds (for example, the conazoles) that were positive in the MEA assay that were not hits in the ToxCast NVS_IC assays, it is clear that there are other MIEs that can have toxicity pathways leading to altered firing in neuronal networks.

Table 2
Agreement between NVS_IC expected hits and single point screen MEA hits.

	Positives	Negatives
Expected based on ToxCast NVS_IC	37	56
Observed in MEAs (based on wMFR)	67	26

3.5. Outcomes based on class

A number of the compounds tested in these cohorts are well recognized to disrupt function of receptors, channels or enzymes

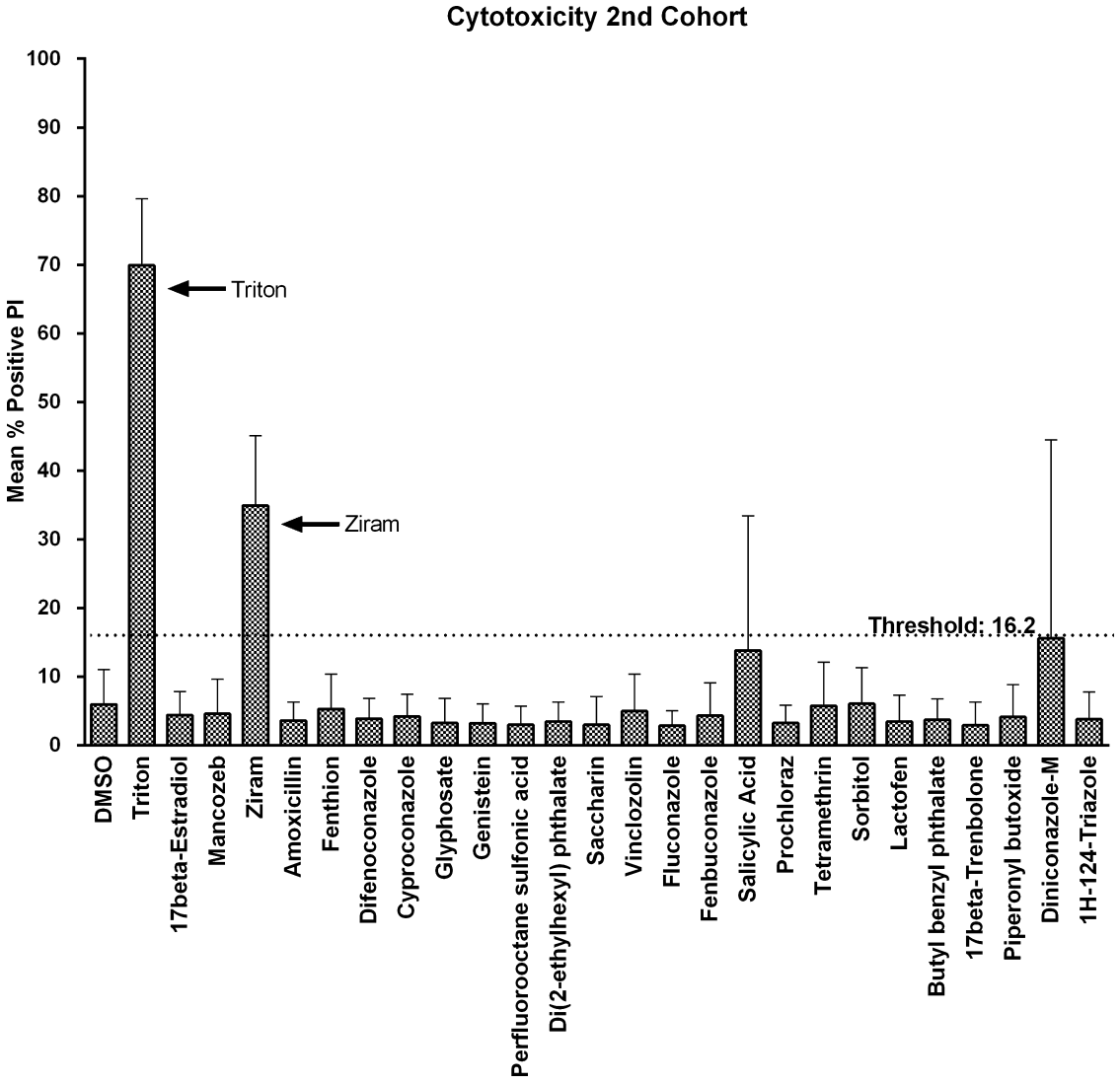

Fig. 4. Cytotoxicity of 2nd cohort of compounds tested in the MEAs to cortical cultures. Primary cultures from rat cortex were plated in 96 well plates as described in Section 2. Cultures were exposed to test compounds at a concentration of 40 μM (unless otherwise noted in Table 1) for 1 h, then cell death was assessed using a propidium iodide assay as described in Section 2. The means and SD values are plotted and the dotted line indicates the mean + 2SD of the number of PI positive cells in DMSO treated wells (16.2%). Only ziram caused cytotoxicity that exceeded this threshold of PI positive cells.

important to nervous system function, and in some cases there were multiple compounds with common nervous system targets; as such, these targets could also be considered as molecular initiating events in toxicity pathways leading to adverse outcomes.

Table 3
Comparisons between molecular initiating event (MIE) groups[a] and MEA single point screen.

	MIE	NVS_IC expected pos.	MEA Pos.	MEA Neg.
1	Glutamate receptor	1	1	0
2	Potassium channel	4	4	0
3	Calcium channel	20	19	1
4	Sodium channel	21	20	1
5	$GABA_A$ receptor	11	11	0
6	Glycine receptor	2	2	0
7	nAChR	9	3	6
8	Serotonin receptor	2	1	1

[a] The 20 Novascreen assays considered in the present experiments were grouped into eight different molecular initiating events (MIE; see Fig. 5 for details), and the number of compounds having positive NVS_IC results in each group (“NVS_IC expected pos.”) were compared to whether or not those same compounds were positive or negative in the MEA assay.

A comparison between the responses in the NVS_IC and MEA assays for these compounds is presented in Table 4. In general, there were 6 compounds known to cause AChE inhibition, 10 compounds known to interact with $GABA_A$ receptors, 7 nicotinic agonists, and 9 modifiers of VGSC. In the single point screens, 5/6 of the AChE inhibitors were hits. Aldicarb was the only AChE inhibitor that did not cause a change in the wMFR greater than the threshold. Similarly, 9/10 $GABA_A$ modifiers were hits, with heptachlor epoxide being the only compound that was not a hit

Table 4
Comparisons between responses in NVS_IC assays and MEAs for compounds with known nervous system targets.

Molecular target	NVS_IC assays	MEAs (wMFR)
Acetylcholinesterase (inhibitors)	2/6[a]	5/6
$GABA_A$ receptor	3/10[b]	9/10
Nicotinic ACh receptor	6/7	1/7
Voltage-gated sodium channel	1/9	8/9

[a] Does not include ToxCast assays for cholinesterase inhibition.
[b] No compounds (0/7) that inhibit $GABA_A$ receptors were detected as hits.

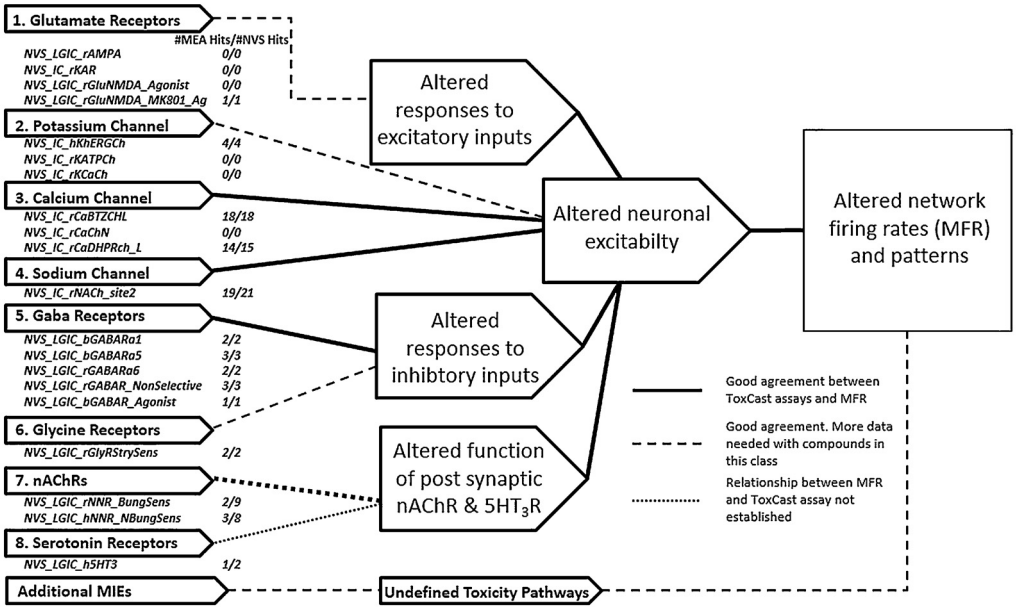

Fig. 5. Proposed Toxicity Pathways linking changes in ion channel function to altered network function on MEAs. Groups of potential molecular initiating events (MIEs) are listed on the left-hand side of the figure, numbered 1–9. Underneath each group heading is the list of ToxCast Novascreen (NVS_IC) assays that assess compound interactions with receptors relevant to that group. The Toxicity Pathways proceed to the right with Key Events (e.g. altered responses to excitatory inputs), ultimately leading to altered network firing rates and patterns that can be detected by the MEAs. Listed in smaller print below each MIE category are the ToxCast NVS_IC assays that assess that MIE, with the number of hits on that assay in the NVS_IC and MEA assays, respectively.

in the initial screen. For those compounds known to modify VGSC function, 8/9 were positive, with permethrin not causing greater than threshold level changes in activity. By contrast, only 1/7 nAChR agonists induced a greater than threshold level change in wMFR. Lastly, 7/9 conazoles tested were positive in the MEA assay.

3.6. Outcomes based on in vivo neurotoxicity

Based on evidence in the literature or re-registration eligibility documents from the U.S. Environmental Protection Agency, 48 of the ToxCast compounds examined over both cohorts were classified as "known neurotoxic" (Table 1; see supplemental Table 2 for further documentation). Thirty-five of these 48 compounds altered wMFR beyond the threshold value in their respective cohorts.

Supplementary table related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neuro.2014.06.012.

4. Discussion

The present results demonstrate that 30/37 compounds positive in ToxCast NVS_IC assays altered network function measured using MEAs (Table 3), supporting the hypothesis that compounds testing positive in ToxCast Novascreen assays for ion channels would also alter neuronal function in cortical networks. However, 36 of 56 compounds that were negative in ToxCast NVS_IC assays altered network function in the MEAs, indicating that negative results in these 20 assays does not correspond to a lack of neuroactivity in MEAs. However, 20 of these 36 compounds were classified here as "known neurotoxic" compounds, and include the pyrethroids, $GABA_A$ antagonists and cholinesterase inhibitors. Further, this latter result is not necessarily unexpected, as there are many potential molecular initiating events in the nervous system aside from those in the 20 NVS_IC assays considered here. With respect to the second hypothesis, the MEA assay detected 35/48 compounds known to be neurotoxic/neuroactive in vivo. Further inspection of the results revealed important aspects of both ToxCast NVS_IC assays and neuronal activity measured by MEAs that can inform interpretation of screening data from both ToxCast and MEAs. This includes evidence that each approach misses some specific, but different, classes of compounds, that the MEA assay exhibits >70% sensitivity for neuroactive compounds, and that NVS_IC and MEA assays combined exhibit greater than 85% sensitivity. These and other related topics are discussed in detail below.

4.1. Expected hits based on NVS_IC ToxCast assays and putative toxicity pathways

Considering the results of these experiments in the context of Toxicity Pathways (NAS 2007) is useful because it allows assessment of where there is good agreement between the putative MIEs identified here (Table 3) and key events in neuronal tissues such as altered network function. For three of these MIEs ($GABA_A$ receptors, voltage-gated calcium and sodium channels) compounds with positive results in NVS_IC assays also altered network function, For three other pathways (glutamate receptors, potassium channels and glycine receptors), agreement between NVS_IC assays and MEAs was good, but are based on only a limited number of compounds. Similarly, putative toxicity pathways linked to serotonin receptors also did not contain enough test compounds. By contrast, only 2/9 compounds that were positive in NVS_IC assays for nAChR disrupted wMFR in cortical networks. Of the 7 compounds that did not have effects on wMFR in MEAs, 5 were nicotine or neonicotinoid insecticides. Thiamethoxam, a neonicotinoid, was without effect in both the NVS_IC assays as well as MEAs. Mepaquat (DeFranchi et al., 2011), which was positive in the NVS_IC assay for nicotinic receptor binding but did not alter wMFR, along with nicotine and imidacloprid (McConnell et al., 2012; Mack et al., 2014) were negative in MEAs in previous studies. There is some evidence that the neurotoxicity of mepiquat involves nicotinic receptors (EFSA, 2008), thus its lack of effects in the MEA assay is consistent with the other nicotinic compounds. While it is possible that the lack of effects of neonicotinoid compounds in the MEAs, which have a lower affinity for mammalian than insect nicotinic receptors (Tomizawa and Casida,

2005), could be the "true" response, the lack of effect of nicotine prevents this conclusion from being made with any confidence and indicates that a limitation using wMFR in primary cortical cultures for screening may be a lack of sensitivity to nicotinic compounds. These results are also consistent with the presence of nicotinic receptors, but not cholinergic neurons in primary cortical culture used in these experiments (unpublished data), which suggests that cholinergic input into neuronal networks in this in vitro system may be lacking or have minimal influence on overall network activity.

The MEA assay detected effects of 20/21 phase I and II compounds that were positive in the *NVS_IC_rNACH_site2* assay for voltage-gated sodium channels. Not among these 21 compounds were 6 pyrethroid insecticides and DDT, which modulate the kinetics of voltage-gated sodium channels (Narahashi, 2002). Of these 7 compounds, only allethrin was positive in a ToxCast NVS_IC assay, and the assay that detected it was a $GABA_A$ receptor assay, not the *NVS_IC_rNACH_site2* assay for voltage-gated sodium channels. However, 6/7 of these compounds were detected by the MEA assay; only permethrin was not positive in the MEA assay. Earlier studies with MEAs (Meyer et al., 2008; Scelfo et al., 2012; McConnell et al., 2012), have demonstrated effects of permethrin, and it is not clear why it was negative in the current study. Thus, MEAs perform well at detecting effects of broad classes of compounds acting on sodium channels, yet the *NVS_IC_rNACH_site2* assay in ToxCast performed poorly at detecting pyrethroids, not detecting any of these compounds in the Phase I or II library. The reason for the poor performance of this assay for the pyrethroids may lie in its endpoint, which is disruption of ^{3}H-batrachotoxin binding to site 2 of the rat VGSC (modified from Creveling et al., 1983). Pyrethroids do not bind directly to this site (Trainer et al., 1997), but allosterically enhance batrachotoxin binding (Brown et al., 1988). The Novascreen assay may not be sensitive to allosteric effects on the batrachotoxin binding site.

Four conazoles, as well as 2 fungicides (flusilazole and imazalil), were included in the first cohort due to effects on multiple MIEs in ToxCast assays; all 6 of these compounds were also positive in the MEA assay. As such, in the second cohort, 5 additional conazole fungicides, plus the 1,2,4-triazole moiety, all without effects on NVS_IC ion channel assays were included. In the second cohort, 4/5 of the conazoles were positive in the MEA assay, with fluconazole and 1,2,4-triazole having no effect. Other non-conazole fungicides which were both positive (cyazofamid, spiroxamine) and negative (prochloraz) in the NVS_IC assays were also positive in the MEA assay. These data indicate that some fungicides activate toxicity pathways impacting network function as measured by MEAs. However, fungicides differ from many of the other compounds tested here in two important aspects. First, the MIE or MIEs are not as clear, since even conazoles that did not activate NVS_IC ion channel assays impacted neuronal function in the MEAs. Perhaps this involves toxicity pathways linked to firing rate via disruptions of calcium homeostasis, as recent work by Heusinkveld et al. (2013) demonstrated that 6 fungicides (imazalil, flusilazole, fluconazole, tebuconazole, triadiamefon and cyproconazole) alter intracellular calcium homeostasis, and viability in PC12 cells. Only fluconazole, which was also inactive in the MEA assay, was without effects on calcium responses (Heusinkveld et al., 2013). Second, it is not clear if and how these compounds contribute to adverse outcome pathways, as many fungicides are not recognized to cause neurotoxicity. To this end, when 14 different conazole compounds were examined for effects on motor activity, including three common to those used here (diniconazole, hexaconazole and propriconazole), only triadimefon caused significant effects (Crofton, 1996). Although triadimefon was not tested in the current experiments, it does alter cortical network activity in MEAs (unpublished data). Whether the lack of adverse neurological outcomes with fungicides is due to pharmacokinetic factors (e.g. compounds rapidly metabolized) or other pharmacodynamic factors deserves further investigation, but is outside the scope of the current work.

One caveat regarding the consideration of the MEA results in terms of the MIEs/toxicity pathways proposed here is that non-specific effects of compounds cannot be discounted. Most compounds were tested at a concentration of 40 μM, at which effects of some of these compounds may not be limited to a specific receptor. Non-specific effects exist for many of the compounds used here and complete discussion is outside the scope of this manuscript. However, as an example, several of the cholinesterase inhibitors have also been reported to have effects on muscarinic acetylcholine receptors and other components of synaptic transmission (Pope et al., 2005), including voltage-gated calcium channels (Meijer et al., 2014). Thus, the toxicity pathways proposed here are at best putative toxicity pathways that need to be confirmed by supportive literature and additional experiments, including concentration–response assessment and confirmation that the direction of effect in MEAs is consistent with the known or demonstrated effect on an ion channel and its expression in excitatory and inhibitory neurons in the network. It should be noted, however, that toxicity pathways are based on biological relationships, not compound effects. Thus, compounds can act via multiple MIEs and toxicity pathways.

4.2. Detection of known neurotoxic compounds by MEAs; Sensitivity, specificity, and fit for purpose of the assay with primary cortical networks.

Overall, there were 48 compounds that are recognized to cause neurotoxicity in vivo (Supplemental Table 2). MEAs detected 72.9% (35/48) of these compounds. This is lower than previously reported for this laboratory using a smaller set of compounds (87%, McConnell et al., 2012), but similar to other reports (77%, DeFranchi et al., 2011). However, this is the largest number of compounds tested in MEAs to date and contained seven (eight, including mepiquat) compounds thought to act via nAChR, only 1 of which was active. If the assay's "fit-for-purpose" acknowledges that the assay in its current form is insensitive to this class of compounds, then the sensitivity rises to 85% (34/40). Similarly, if data from the MEAs and the NVS_IC assays are combined, then 41 of the 48 (85%) compounds are detected. The compounds not detected by either the MEA or NVS_IC assays (aldicarb, heptachlor, thiamethoxam, permethrin, maneb, ziram and 17β-estradiol) do not comprise any particular class, indicating that there is not a particular class of compounds that is not detected well by the combination of the two assays. It is difficult to compare the sensitivity of this assay to other published, in vitro assays for neurotoxicity testing (Gartlon et al., 2006; Hayess et al., 2013; Krug et al., 2013; Verstraelen et al., 2014; Wilson et al., 2014) because these assays have tested fewer compounds (6–40 compounds) and do not directly report the sensitivity of the assay.

Assessing specificity in the present study is more difficult, as the first set of compounds did not contain a sufficient number of negative control compounds to do so. In the second set of compounds, 0/5 negative control compounds were active. These data agree with previously reported specificity rates near 100% (DeFranchi et al., 2011; McConnell et al., 2012). As discussed above, the combined sensitivity of the NVS_IC and MEA assays approaches 85%, however, the effects of this combination on specificity are difficult to address, as only saccharin and acetaminophen are ToxCast compounds. Thus, there are insufficient data to assess specificity of the combined MEA/NVS_IC assay results for neurotoxicity. While not assessing specificity, there

were 13 compounds (including the negative controls) tested in the current study that were not hits in the NVS_IC or MEA assays and are not recognized to cause neurotoxicity in vivo (Supplemental Table 3). Three of these compounds are highly active in ToxCast assays, including mancozeb (active in 16% of ToxCast assays), butachlor (15%) and perfluorooctane sulfonic acid (PFOS; 15%). Although expected to be positive, ziram (17%) and maneb (13%) were also negative in the MEA assay. This indicates that the MEA assay is not simply responding to any compound that has high biological activity. Ziram was cytotoxic but not detected as a hit in the MEA assay. This may indicate that cytotoxicity per se does not necessarily alter wMFR, but may also be a discrepancy due to testing of cytotoxicity and activity in two different assays which employ different cell densities. Future compound test sets will need to include larger numbers of negative control compounds to assess better the specificity of MEAs for larger compound sets.

Supplementary table related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neuro.2014.06.012.

There were also 16 compounds that were positive in the MEAs, but were not positive in the NVS_IC assays, nor widely recognized to be neuroactive/neurotoxic (Supplemental Table 4). In general, sufficient data in the literature is lacking for all of these compounds to make definitive conclusions regarding the expected effects in vivo and/or in vitro. Thus, they cannot be neatly classified into categories such as "known neurotoxic" and are not known to have mechanisms of action relevant to the nervous system. For example, four of these compounds are conazole fungicides that, as discussed above, have been reported to alter calcium homeostasis in neurons in vitro. Thus, activity in MEAs may not be surprising, but there are no other in vitro data to further support this finding, nor are these compounds well-recognized neurotoxicants. There are several reasons why a compound (for example, the conazoles) may be active in the MEA assay, yet inactive in vivo. The in vitro MEA assay lacks significant metabolic capacity and a blood-brain barrier, both of which may prevent a compound from disrupting neuronal function following exposure in vivo. Another example of the difficulty in classifying these compounds are the three androgen receptor active compounds in this group. There were not sufficient data in the literature for any one of these three compounds to be confident that it should be neuroactive in vitro and/or neuroactive/neurotoxic in vivo. However, rapid actions of androgens in the nervous system, including actions on ion channels, are well described (Michels and Hoppe, 2008). Thus, their ability to alter wMFR in the MEAs may be a true reflection of an ability to alter neuronal function, but confirmation of this will require additional data. Similarly, more data are required for the other compounds in order to understand the MEA results in the context of both in vitro and in vivo neuroactivity/neurotoxicity.

Supplementary table and references related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neuro.2014.06.012.

In summary, primary neuronal cultures grown in mwMEAs have been characterized for screening chemicals for functional effects on the nervous system. The present results identified detection of nicotinic compounds by the MEA assay and detection of pyrethroids by NVS_IC assays in ToxCast as "blindspots" in compound space covered by these assays. Such information can be used to improve both the MEA and NVS_IC assays in ToxCast. This might be accomplished by modifying *NVS_IC_rNACH_site2* assay to better detect the pyrethroids, while detection of nicotinic compounds in the MEA assay may be improved by considering endpoints in addition to wMFR, or by utilizing different brain regions (e.g. hippocampus) or culture conditions. Overall, these data demonstrate the feasibility of screening compounds for neurotoxicity in a rapid and efficient manner using MEAs to as well as the value added of combining screening approaches that assess compound effects on different endpoints.

Conflict of interest statement

Two authors are employees of Axion Biosystems, which makes MEA devices. Other authors declare that they have no conflicts of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

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